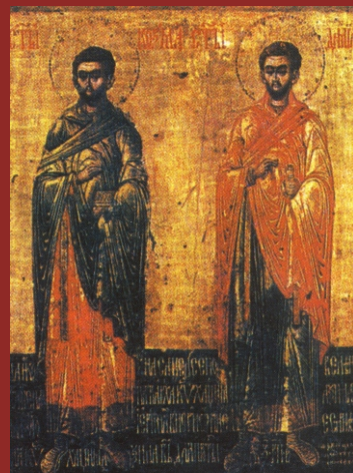


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IMMUNOMODULATORY EFFECTS OF TGF-B IN THE PATIENTS WITH IMMUNE THROMBOCYTOPENIA	5
<i>Miloš Kostić, Tanja Džopalić, Nikola Živković, Ana Cvetanović</i>	
THE ADVANTAGES OF ANTIBIOTIC PROPHYLAXIS IN NEUROSURGERY	14
<i>Predrag Milošević, Boban Jelenković, Nebojša Miljković, Goran Ivanov, Miroslav Popović</i>	
FUNCTIONAL IMPROVEMENT UP TO SIX MONTHS AFTER TOTAL HIP ARTHROPLASTY: MEASURED BY SELF-REPORTED QUESTIONNAIRE AND RANGE OF HIP FLEXION	20
<i>Mirjana Kocić, Marina Milenković, Marija Spalević, Aleksandra Krstović, Anita Stanković, Dragan Zlatanović</i>	
PREDICTIVE IMPORTANCE OF TUMOR BUDDING, LYMPHOVASCULAR AND PERINEURAL INVASION IN COLORECTAL CARCINOMA	28
<i>Tijana Denčić, Maja Jovičić-Milentijević, Aleksandar Petrović, Goran Radenković, Marko Jović, Sonja Šalinger-Martinović, Simona Stojanović</i>	
PREDICTIVE IMPORTANCE OF MORPHOMETRIC ANALYSIS OF TRIPLE-NEGATIVE BREAST CANCER	38
<i>Nikola Živković, Maja Jovičić-Milentijević, Ana Cvetanović, Miloš Kostić, Miodrag Djordjević, Dane Krtinić</i>	
CORRELATION BETWEEN DILATATION OF THE BILE DUCTS AND OXIDATIVE STRESS IN PATIENTS WITH CHOLEDOCHOLITHIASIS	47
<i>Zoran Damjanović, Milan Jovanović, Milan Radojković, Dušan Sokolović, Aleksandar Karanikolić, Nebojša Ignjatović, Nemanja Stepanović, Goran Damjanović, Marko Gmijović</i>	
THE CHARACTERISTICS OF HOSPITALIZED MEASLES AFFECTED CHILDREN DURING THE 2017-2018 EPIDEMIC IN THE JABLANICA DISTRICT	53
<i>Marija Stojiljković, Ivan Rančić, Milan Golubović, Milica Jakovljević, Mirjana Miljković</i>	
BONDING EFFICIENCY OF UNIVERSAL ADHESIVE TO DENTINE IN DIFFERENT ETCHING AND CURING MODES	60
<i>Milan Miljković, Stefan Dačić, Goran Radenković, Milica Jovanović</i>	
EMERGENCY CONDITIONS IN SURGERY	66
<i>Bobana Milojković, Dragan Mihajlović, Marija Dimitrijević, Boban Milojković, Jelena Ignjatović</i>	
"BRAIN PLASTICITY" AND STRESS	72
<i>Marina Kostić</i>	
THE EFFECT OF THE INFLAMMATORY REFLEX ON THE HEART	81
<i>Aleksandar Kamenov, Vladimir Stojiljković, Saša Živić, Marko Gmijović, Milan Lazarević, Mladjan Golubović, Dragan Milić</i>	
SELF-INFLICTED PENETRATING CARDIAC INJURY: TWO DIFFERENT SURGICAL APPROACHES	88
<i>Vladimir Stojiljković, Saša Živić, Dejan Perić, Aleksandar Kamenov, Nenad Paunović, Marko Gmijović, Dragan Milić</i>	
STIGMATIZATION OF PSYCHIATRIC PATIENTS AMONG STUDENTS IN SERBIA	93
<i>Gordana Nikolić, Maja Stanojković, Miroslav Krstić, Olivera Žikić, Nikola Stojanović, Suzana Tošić-Golubović, Maja Simonović, Jelena Kostić</i>	
ANTIOXIDANT ACTIVITY, TOTAL PHENOL AND TANNIN CONTENT OF DIFFERENT VARIETIES OF FLOURS	100
<i>Bojana Miladinović, Kristina Ilić, Dušica Stojanović, Milica Kostić, Milica Milutinović, Suzana Branković, Dušanka Kitić</i>	
PROPOLIS: CHEMICAL COMPOSITION, BIOLOGICAL AND PHARMACOLOGICAL ACTIVITY-A REVIEW	108
<i>Sanja Stojanović, Stevo J. Najman, Biljana Bogdanova-Popov, Svetozar S. Najman</i>	
CAVERNOUS HEMANGIOMA OF THE LIVER WITH EXTRAMEDULLARY HEMATOPOIESIS AND MALIGNANT CELLS EMBOLIZATION IN AN ELDERLY PATIENT: A CASE REPORT	114
<i>Marinko Paunović, Batrić Vukčević, Zoran Terzić, Milorad Magdelinić, Ljiljana Vučković, Janja Raonić, Vanja Balšić, Jelena Vučinić</i>	



"RESTLESS LEGS" SYNDROME - THE MOST COMMON DISORDER YOU HAVE NEVER HEARD OF: A CASE REPORT <i>Jelena Stamenović</i>	120
PATIENTS WITH DIFFERENT METASTATIC MALIGNANCIES AND ACUTE MYOCARDIAL INFARCTION WITH ST SEGMENT ELEVATION TREATED WITH PRIMARY PERCUTANEOUS CORONARY INTERVENTION <i>Sonja Šalinger-Martinović, Dragana Stanojević, Zorica Dimitrijević, Tomislav Kostić, Snežana Ćirić-Zdravković, Branislava Ivanović, Stefan Momčilović</i>	125
ERRATUM	132
 Secretariat	
GUIDELINES FOR PAPER SUBMISSION TO ACTA MEDICA MEDIANAE	137



IMUNOMODULACIJSKA SVOJSTVA TGF-B KOD BOLESNIKA SA IMUNSKOM TROMBOCITOPENIJOM	5
<i>Miloš Kostić, Tanja Džopalić, Nikola Živković, Ana Cvetanović</i>	
PREDNOSTI ANTIBIOTSKE PROFILAKSE U NEUROHIRURGIJI	14
<i>Predrag Milošević, Boban Jelenković, Nebojša Miljković, Goran Ivanov, Miroslav Popović</i>	
POBOLJŠANJE FUNKCIJE KUKA TOKOM PRVIH ŠEST MESECI NAKON TOTALNE ARTROPLASTIKE: MERENO NA OSNOVU UPITNIKA ZA SAMOPROCENU FUNKCIJE I OBIMA POKRETA FLEKSIJE KUKA	20
<i>Mirjana Kocić, Marina Milenković, Marija Spalević, Aleksandra Krstović, Anita Stanković, Dragan Zlatanović</i>	
PREDIKTIVNI ZNAČAJ TUMORSKOG PUPLJENJA, LIMFOVASKULARNE I PERINEURALNE INVAZIJE KOD KOLOREKTALNOG KARCINOMA	28
<i>Tijana Denčić, Maja Jovičić-Milentijević, Aleksandar Petrović, Goran Radenković, Marko Jović, Sonja Šalinger-Martinović, Simona Stojanović</i>	
PREDIKTIVNI ZNAČAJ MORFOMETRIJSKE ANALIZE TRIPL NEGATIVNOG KARCINOMA DOJKE	38
<i>Nikola Živković, Maja Jovičić-Milentijević, Ana Cvetanović, Miloš Kostić, Miodrag Đorđević, Dane Krtinić</i>	
POVEZANOST DILATACIJE ŽUČNIH PUTEVA I OKSIDATIVNOG STRESA KOD BOLESNIKA SA HOLEDOKOLITIJAZOM	47
<i>Zoran Damnjanović, Milan Jovanović, Milan Radojković, Dušan Sokolović, Aleksandar Karanikolić, Nebojša Ignjatović, Nemanja Stepanović, Goran Damnjanović, Marko Gmijović</i>	
KARAKTERISTIKE HOSPITALIZOVANE DECE OBOLELE OD MALIH BOGINJA TOKOM EPIDEMIJE OD 2017. DO 2018. GODINE U JABLANIČKOM OKRUGU	53
<i>Marija Stojiljković, Ivan Rančić, Milan Golubović, Milica Jakovljević, Mirjana Miljković</i>	
EFIKASNOST VEZIVANJA UNIVERZALNOG ADHEZIVA ZA DENTIN PRIMENOM RAZLIČITIH ADHEZIVNIH I SVETLOSNOPOLIMERIZUJUĆIH TEHNIKA	60
<i>Milan Miljković, Stefan Dačić, Goran Radenković, Milica Jovanović</i>	
URGENTNA STANJA U HIRURGIJI	66
<i>Bobana Milojković, Dragan Mihajlović, Marija Dimitrijević, Boban Milojković, Jelena Ignjatović</i>	
"PLASTIČNOST MOZGA" I STRES	72
<i>Marina Kostić</i>	
EFEKAT ANTIINFLAMATORNOG REFLEKSA NA SRCE	81
<i>Aleksandar Kamenov, Vladimir Stojiljković, Saša Živić, Marko Gmijović, Milan Lazarević, Mlađan Golubović, Dragan Milić</i>	
PENETRANTNA TRAUMA SRCA IZAZVANA SAMOPOVREĐIVANJEM – DVA RAZLIČITA HIRUŠKA PRISTUPA	88
<i>Vladimir Stojiljković, Saša Živić, Dejan Perić, Aleksandar Kamenov, Nenad Paunović, Marko Gmijović, Dragan Milić</i>	
STIGMATIZACIJA PSIHIJATRIJSKIH BOLESNIKA OD STRANE STUDENATA U SRBIJI	93
<i>Gordana Nikolić, Maja Stanojković, Miroslav Krstić, Olivera Žikić, Nikola Stojanović, Suzana Tošić-Golubović, Maja Simonović, Jelena Kostić</i>	
ANTIOKSIDATIVNA AKTIVNOST, SADRŽAJ UKUPNIH POLIFENOLA I TANINA RAZLIČITIH VRSTA BRAŠNA	100
<i>Bojana Miladinović, Kristina Ilić, Dušica Stojanović, Milica Kostić, Milica Milutinović, Suzana Branković, Dušanka Kitić</i>	
PROPOLIS: HEMIJSKI SASTAV, BIOLOŠKA I FARMAKOLOŠKA AKTIVNOST – PREGLED	108
<i>Sanja Stojanović, Stevo J. Najman, Biljana Bogdanova-Popov, Svetozar S. Najman</i>	
KAVERNOZNI HEMANGIOM JETRE SA EKSTRAMEDULARNOM HEMATOPOEZOM I EMBOLIZACIJOM MALIGNIM ČELIJAMA: PRIKAZ SLUČAJA	114
<i>Marinko Paunović, Batrić Vukčević, Zoran Terzić, Milorad Magdelinić, Ljiljana Vučković, Janja Raonić, Vanja Balšić, Jelena Vučinić</i>	



SINDROM "NEMIRNIH NOGU" – NAJČEŠĆI POREMEĆAJ ZA KOJI NIKADA NISTE ČULI: PRIKAZ SLUČAJA	120
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Jelena Stamenović

BOLESNICI SA RAZLIČITIM METASTATSKIM MALIGNITETIMA I AKUTNIM INFARKTOM MIOKARDA SA ELEVACIJOM ST SEGMENTA TRETIRANI PRIMARNOM PERKUTANOM KORONARNOM INTERVENCIJOM	125
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*Sonja Šalinger-Martinović, Dragana Stanojević, Zorica Dimitrijević, Tomislav Kostić,
Snežana Čirić-Zdravković, Branislava Ivanović, Stefan Momčilović*

ERRATUM	132
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Uredništvo

JEDINSTVENI KRITERIJUMI ZA OBJAVLJIVANJE NAUČNIH RADOVA U BIOMEDICINSKIM ČASOPISIMA	134
--	------------

PROPOZICIJE ZA PISANJE RADOVA U ACTA MEDICA MEDIANAE	136
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IMMUNOMODULATORY EFFECTS OF TGF- β IN THE PATIENTS WITH IMMUNE THROMBOCYTOPENIA

Miloš Kostić¹, Tanja Džopalić¹, Nikola Živković², Ana Cvetanović³

The novel concept of immune thrombocytopenia (ITP) pathogenesis is focused on CD4⁺ T cells, currently considered indispensable in stimulating B cells to produce anti-platelet antibodies. In this *in vitro* study, we have investigated CD4⁺ T cell profiles of ITP patients and immunomodulatory effects of TGF- β treatment. CD4⁺ T cells were isolated from peripheral blood mononuclear cells of healthy controls and ITP patients. After brief incubation, the levels of T helper signature cytokines and the number of T regulatory cells (Tregs) were determined. TGF- β immunomodulatory properties were analyzed by the alterations in cytokine production of IFN γ , IL-4, IL-10, IL-17, and IL-2 and Treg frequency, after six-day treatment. ITP patients had decreased level of IL-4 and IL-10, increased IL-17 level and augmented both IFN γ /IL-4 and IL-17/IL-10 ratio. Interestingly, in the six-day CD4⁺ T cell cultures without TGF- β , no statistically significant differences in cytokine levels between the control and ITP group were documented, except for IL-10 which was significantly lower. In ITP group treated with TGF- β , a significant increase of IL-10 as well as a decrease in IL-17/IL-10 ratio was detected, compared to TGF- β untreated ITP group. There was also an increase in the Treg frequency. The results of our study suggest that ITP patients have Th1 and Th17 biased cell-mediated immune response that can be corrected by enhanced TGF- β signaling. However, it appears that long-term culturing of the CD4⁺ T cells may not be a suitable experimental model for studying immunomodulating effects in ITP due to dynamic phenotype fluctuations of these cells in *ex vivo* conditions.

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Key words: immune thrombocytopenia, TGF- β , immunomodulation, IL-10, IL-17

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Introduction

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by the reduced platelet number ($< 100 \times 10^9/L$) due to their enhanced degradation and compromised production, which is often clinically manifested by the hemorrhagic diathesis (1). The majority of ITP patients have various anti-platelet antibodies, mainly directed against membrane bound glycoprotein complexes – GPIIb-IIIa and GPIb-IX (2), thereby the breakdown of B

cell self-tolerance was considered the central pathological event in the disease pathogenesis. Regarding the nature of auto-antigens and auto-antibodies in ITP, recently, it has become clear that B cells, in order to mount anti-platelet immune response, require the assistance of auto-reactive, platelet specific CD4⁺ T cells (3-5). Depending on the conditions of the microenvironment, naïve CD4⁺ T cells during initial antigen priming could adopt different polarization states, including Th1, Th2, Th17, Th22, Th9, T regulatory cells, and follicular T helper cells, each with specific cytokine profile and functional characteristics.

Th1 cells are defined as IFN γ and IL-2 producing subset of CD4⁺ T cells that express T-bet as a master regulator, while their differentiation programme is dependant on IL-12 signaling. Th2 cells, on the other hand, predominantly produce IL-4, IL-5, and IL-13, express GATA-3 as a master transcription factor and require IL-4 for complete differentiation. The equilibrium between Th1 and Th2 cells is perturbed in numerous immune mediated diseases and ITP is not an exception. Accordingly, ITP was classified as Th1 mediated disorder, based on the higher plasma IFN γ /IL-4 ratio, higher blood Th1/Th2 cell ratio and the ratio between the gene expression levels of lineage specific master trans-

cription factors - T-bet and GATA-3, in peripheral blood mononuclear cells (PBMCs) (6-10). Additionally, many of these parameters were found to be negatively correlated with platelet counts, suggesting pathological relevance of Th1/Th2 immune deviation in the ITP pathogenesis (9, 10).

Th17 cells represent a unique subset of CD4^+ effector T cells which in addition to IL-17, produce IL-21 and IL-22. Their differentiation pattern involves the activation of transcription factor ROR γ T induced by the combination of proinflammatory cytokines IL-6, IL-1, IL-23, and TGF- β . ITP patients have elevated plasma level of IL-17 and other Th17 associated cytokines - IL-1, IL-6, and IL-23 (11, 12), but also a higher level of ROR γ T expression in PBMCs and frequency of Th17 cells in the blood (12-14). In addition to the aberrant Th17 immune response, both numerical and functional abnormalities of T regulatory cells (Tregs) have been documented in ITP. Tregs are CD4^+ T cell subset expressing the transcription factor FoxP3, which can be induced by a combination of cytokines TGF- β and IL-2. In contrast to other CD4^+ T cell subsets, Tregs upon antigen recognition induce active immunosuppression by producing anti-inflammatory cytokines TGF- β and IL-10 or by direct cell to cell contact via inhibitory molecules (negative co-stimulators) CTLA-4 and PDL-1. Although decreased in number (15, 16), Tregs isolated from ITP patients also have compromised suppressive functions due to decreased production of TGF- β and IL-10, while serum/plasma levels were found to be decreased and positively correlated with the platelet counts (17-20).

One of the essential characteristics of both Th17 cells and Tregs is their plasticity, i.e. the ability to modify their phenotype and acquire the characteristics and functions of other CD4^+ T cell subsets. Although they perform completely opposite functions, Tregs and Th17 cells share a common differentiation pattern involving TGF- β ; however, the definite differentiation fate of activated CD4^+ T cells is determined by additional factors including the TGF- β concentration and different microenvironmental stimuli. Specifically, TGF- β alone, at higher concentrations, drives CD4^+ T cell differentiation towards

Tregs, but at lower concentrations and in the presence of IL-6 and IL-23, it favors Th17 cell differentiation (21). In ITP, Th17/Treg balance was found to be increased and in the positive correlation with disease activity (22). In accordance with mutual plasticity of these cell lineages, in the tumor micro-environment, Th17 cells were shown to trans-differentiate into Tregs and TGF- β involvement in this process was suggested (23). Given that the therapy of many autoimmune diseases, including ITP, is today largely based on non-specific immunosuppression, which entails numerous side effects, this finding could be of particular importance in the treatment of Th17-mediated autoimmune diseases as it opens the possibilities for modulating immune response in terms of Th17 cell conversion to Tregs.

Aim of the study

The objective of the present *in vitro* study was to investigate CD4^+ T cell profiles of ITP patients and immunomodulatory effects of TGF- β treatment.

Patients and Methods

Clinical and demographic characteristics of the study participants

The study included 6 subjects, median age 56 (32-69) years, both males and females, subdivided into two groups - a control and ITP group. The control group included 3 healthy volunteers, 2 males and 1 female with normal platelet counts ($> 150 \times 10^9/\text{L}$). The ITP group included 3 patients, 2 males and 1 female with verified ITP diagnosis according to the criteria established by the International Working Group (24). All the subjects were promptly informed about the relevant details concerning their participation in the study and gave written informed consents. This study was approved by the Ethical Committee of the Clinical Center Niš, Serbia under the identification number 16297/5. Detailed demographic and clinical characteristics of the study participants are shown in Table 1.

Table 1. Demographic and clinical characteristics of the study participants*

	ITP (n = 3) Mean (range)	Control (n = 3) Mean (range)
Gender (male/female)	2/1	2/1
Age (years)	67 (61-69)	39 (32-50)
Platelet counts ($\times 10^9/\text{L}$)	67 (20-110)	294(243-340)
Duration of the disease (years)	5 (1-10)	N/A
Phase of the disease	remission	N/A
Therapy	prednisone	N/A
Splenectomy	No	N/A

* N/A - Non Applicable

CD4⁺ T cell isolation and experimental protocols

Blood samples (10 mL) were collected by cubital venepuncture using EDTA as an anticoagulant. After sampling, the blood was diluted in RPMI-1640 medium (1:1) and PBMCs were isolated using centrifugation over a Ficoll-Hypaque density gradient (*Histopaque-1077*; Sigma-Aldrich, St. Louis, Missouri, USA). Upon PBMC isolation, CD4⁺ T cells were separated as a negative fraction by immunomagnetic sorting using EasySep™ Human CD4⁺ T Cell Isolation Kit (*Stemcell Technologies, Vancouver, Canada*) according to the manufacturer's instructions. The purity of isolated cells was verified by flow cytometry and more than 99% of isolated cells were CD4⁺ (*data not shown*). CD4⁺ T cells of the control and ITP group were seeded into 24-well plastic plate - 1×10^6 cells per well in 1.5 mL of RPMI-1640 medium supplemented with 2.05 mM L-glutamine, 25 mM HEPES (*Sigma-Aldrich, St. Louis, Missouri, USA*) and 10% inactivated fetal bovine serum - FBS (*Capricorn Scientific GmbH, Ebsdorfergrund, Germany*). In order to evaluate Th profile of the tested subjects, CD4⁺ T cells were incubated at 37°C in 5% CO₂ at 95% humidity for 24h, and then stimulated with phorbol myristate acetate - PMA (20 ng/mL) and ionomycin (100 ng/mL) (*Sigma-Aldrich, St. Louis, Missouri, USA*) 8h prior supernatant sampling. The remaining cells were used for Treg detection by flow cytometry. CD4⁺ T cells were also incubated with or without human recombinant TGF- β 1 (3 ng/mL) (*Merck KGaA, Darmstadt, Germany*) for 6 days in order to evaluate its immunomodulatory properties.

Sample analyses

In the collected cell supernatants, IFN γ , IL-4, IL-10, IL-17, and IL-2 levels were determined using commercial ELISA kits: Human IFN γ Quantikine ELISA Kit, Human IL-4 Quantikine ELISA Kit, Human IL-10 Quantikine ELISA Kit, Human IL-17 Quantikine ELISA Kit, and Human IL-2 Quantikine ELISA Kit (*all obtained from R&D Systems, Minneapolis, Minnesota, USA*) according to the manufacturer's instructions. The percentage of Tregs was estimated by flow cytometry. Firstly, cells were washed in stain buffer (*BD Biosciences, Franklin Lakes, New Jersey, USA*), and then permeabilized and fixed using Transcription Factor Phospho Buffer Set (*BD Biosciences, Franklin Lakes, New Jersey, USA*). To identify Tregs, anti-CD4FITC, anti-CD25APC, and anti-FoxP3PE (*all ob-*

tained from BD Biosciences, Franklin Lakes, New Jersey, USA) antibodies were applied. After washing, sample analyses were performed on BD LSRFortessa using BD FACSDiva™ software.

Statistical analyses

All statistical analyses were performed in the GraphPad software (GraphPad Software, LLC, San Diego, CA, USA). The results are presented as the average values \pm standard deviation (SD). The intergroup differences were established using the Student's t-test, and the p values less than 0.05 were considered statistically significant.

Results

T helper cell profile of ITP patients

T helper (Th) cell profile of ITP patients was assessed by the level of signature cytokines in the supernatants of CD4⁺ T cell cultures. After 24h incubation, a statistically significant decrease in the IL-4 and IL-10 was documented as well as an increase in IL-17 level. Both IFN γ /IL-4 and IL-17/IL-10 ratios were increased; however, there was no a significant difference in the frequency of Treg (Figure 1).

Immunomodulatory effects of TGF- β

Isolated CD4⁺ T cells from the control and ITP group have been cultured for 6 days with or without TGF- β . Interestingly, in the cultures without TGF- β , no statistically significant differences in cytokine levels between the control and ITP group were documented, except for IL-10 which was significantly lower in the ITP group (Figure 2).

In ITP group treated with TGF- β , a significant increase of IL-10 as well as a decrease in IL-17/IL-10 ratio was detected, compared to TGF- β untreated ITP group. There was also a marked increase in the frequency of Tregs (Figure 3).

However, after comparing cytokine profile of the ITP group treated with TGF- β and untreated control group, a significant decrease in IFN γ and IFN γ /IL-4 ratio as well as an increase in IL-10 production was found. The frequency of Tregs was significantly higher in TGF- β treated ITP group (Figure 4).

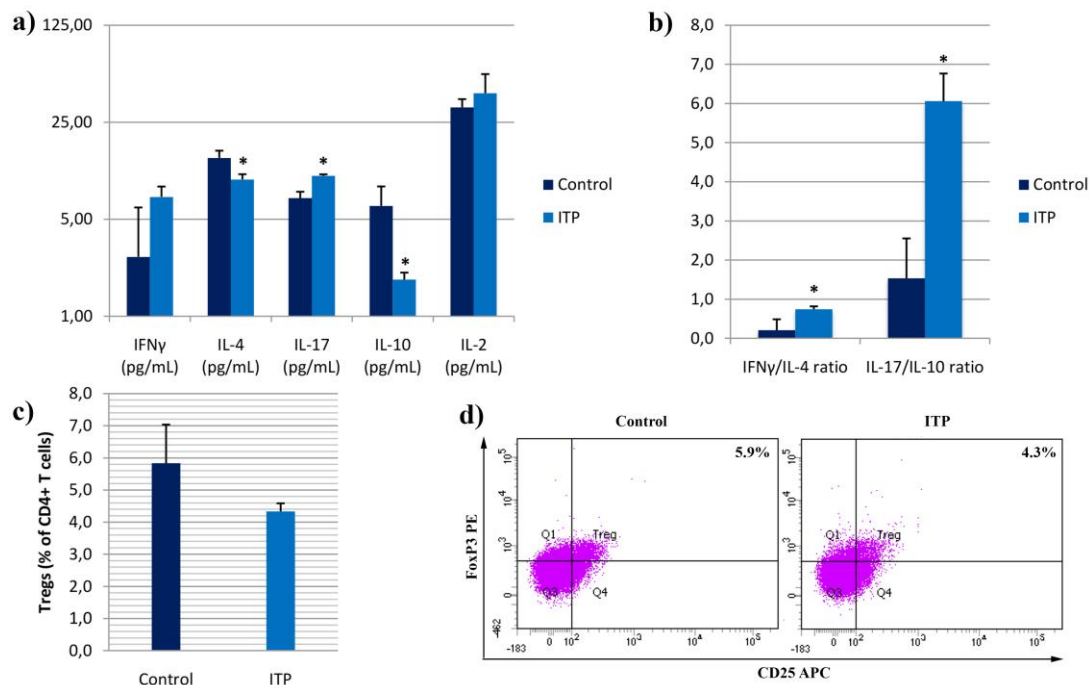


Figure 1. CD4⁺ T cell profile of ITP patients after 24h incubation†

†CD4⁺ T cells isolated from the control and ITP group were cultured for 24h and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th cell signature cytokines was determined; b) IFN γ /IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4⁺ T cells in one experiment. (*p < 0.05 versus control)

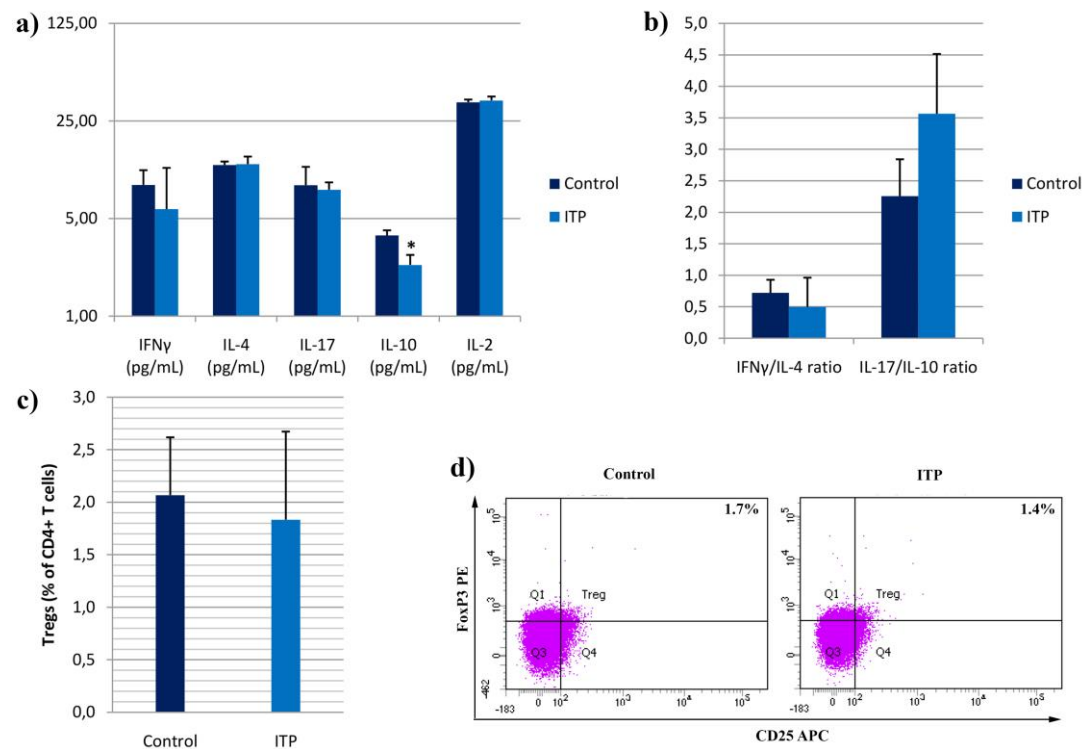


Figure 2. CD4⁺ T cell profile of ITP patients after 6-day incubation without TGF- β ‡

‡CD4⁺ T cells isolated from the control and ITP group were cultured without TGF- β for 6 days and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th signature cytokines was determined; b) IFN γ /IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4⁺ T cells in one experiment. (*p < 0.05 versus control)

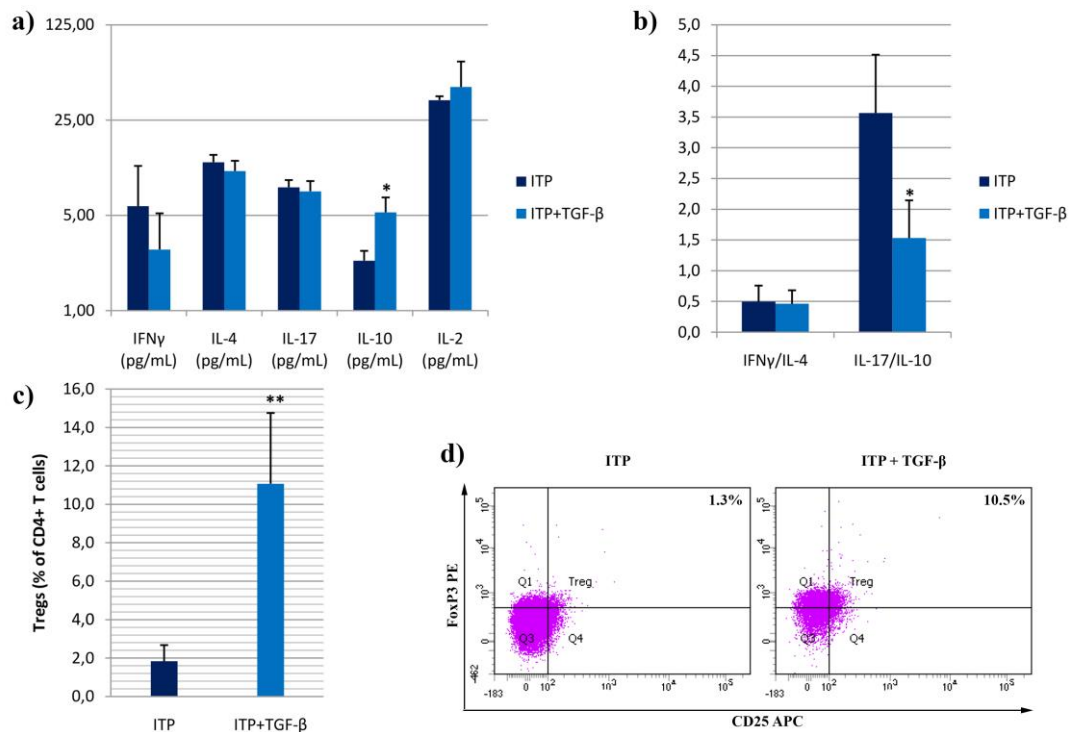


Figure 3. CD4⁺ T cell profile of ITP patients after 6-day incubation with TGF- β [◊]

[◊]CD4⁺ T cells isolated from the ITP group were cultured for 6 days with or without TGF- β (3 ng/mL) and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th signature cytokines was determined; b) IFN γ /IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4⁺ T cells in one experiment. (*p < 0.05; **p < 0.01 versus ITP)

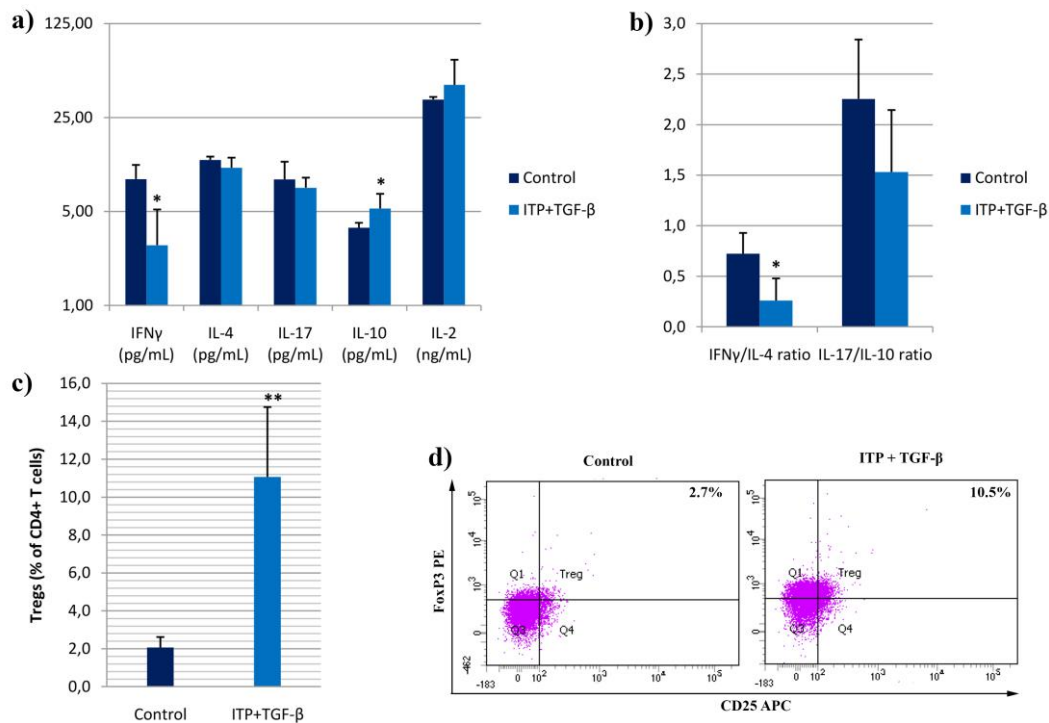


Figure 4. Immunomodulatory effects of TGF- β in ITP patients compared with healthy controls^Δ

^ΔCD4⁺ T cells isolated from the ITP patients treated with TGF- β (3 ng/mL) and untreated CD4⁺ T cells obtained from healthy controls were cultured for 6 days and stimulated with PMA and ionomycin 8h prior supernatant sampling. In collected supernatants: a) the level of Th signature cytokines was determined; b) IFN γ /IL-4 and IL-17/IL-10 ratio was calculated; c) in the remaining cells, Treg frequency was reported; d) representative flow cytometric analyses of CD4⁺ T cells in one experiment. (*p < 0.05; **p < 0.01 versus control)

Discussion

Currently, the abnormalities of CD4^+ T cell compartment in patients with ITP are well established (25). Accordingly, in our study, we have also observed Th1 and Th17 polarization of cellular immune response, based on higher cytokine ratio of $\text{IFN}\gamma/\text{IL-4}$ and $\text{IL-17}/\text{IL-10}$ in the cultures of isolated CD4^+ T cells, indicating disturbed balance of both Th1/Th2 and Th17/Treg axes. Similar observations, although using different methodological approaches, such as direct cytokine detection in patient's plasma and the identification of CD4^+ T cell subsets in the blood, were made by other authors (22, 26-28). Interestingly, in these studies, pulsed high-dose dexamethasone treatment was shown to correct reported Th1/Th2 and Th17/Treg immune deviations, further supporting their relevance in the disease pathogenesis (27, 28). Patients enrolled in our study were, however, in the remission induced by long-term prednisone therapy and it appeared that immunological pattern of the diseased remained unaltered. This finding might suggest that beneficial effects of prednisone treatment were achieved by non-selective immune suppression rather than the restoration of Th balance. Different non-specific acting mechanisms of prednisone have been previously proposed, including reduced phagocyte capacity to internalize antibody coated platelets (29), reduced anti-platelet antibody production due to lymphotoxic effect (30), and enhanced platelet production (31). Additionally, immunosuppressive effects of corticosteroids mainly result from the inhibition of proinflammatory transcription factors, particularly nuclear factor kappa B (NF- κ B) and activating protein-1 (AP-1), which are active during initial T cell priming, regardless of their future differentiation fate, but also in various other cells of both innate and acquired immunity, directly suggesting their non-selective mechanism of action (32).

One of the unexpected findings of our study was that, in contrast to the short-term (24h) cultures, in six-day cultures previously observed difference in the cytokine profile of CD4^+ T cells isolated from ITP patients and healthy controls did not persist, except for IL-10 level which was significantly decreased. Plausibly, the explanation of this effect could be associated with extreme plasticity of these cells, which differentiation state could be considered only in the context of microenvironment conditions. Butch et al. have also shown that peri-pheral blood T cell subsets do not retain an original pattern of cytokine production during extended culture periods under conventional non-polarizing conditions (33). Thus, the restoration of cytokine balance in ITP group could be a consequence of pro-longed *ex vivo* cell culturing, which do not mirror conditions present *in vivo*. Although, this experimental model has been used for investigating CD4^+ T cell profile of ITP patients (34-36), our results suggest that *in vivo* studies are required in order to adequately address this issue.

Recently, it has been shown that Th17 cells can trans-differentiate into T regulatory 1 (Tr1) cells – a specific subset of IL-10 producing T cells with suppressive functions, during a physiological immune

response in the presence of TGF- β 1 (37). Considering that we, as well as others (38), have observed a disturbed $\text{IL-17}/\text{IL-10}$ ratio in patients with ITP, TGF- β could be a promising immunomodulatory candidate in the restoration of immunological tolerance in ITP. Furthermore, in both adult and pediatric ITP patients, the plasma levels of TGF- β were found to be decreased, positively correlated with the platelet counts but negatively with the levels of pro-inflammatory $\text{IFN}\gamma$ and IL-17 (17, 18, 20). Indeed, in our study, TGF- β treatment induced a decrease of $\text{IL-17}/\text{IL-10}$ ratio, primarily due to enhanced production of IL-10. In order to investigate potential cellular source of enhanced IL-10 production, we have examined the frequency of Tregs which are also well established IL-10 producers, and shown a significant increase following TGF- β treatment. When compared with the untreated control group, the TGF- β treated ITP group had decreased $\text{IFN}\gamma/\text{IL-4}$ ratio as well as increased IL-10 production and Treg frequency suggesting that TGF- β induces polarization of cellular immune response toward more tolerogenic phenotype. Thus, enhancing TGF- β signaling in ITP patients could be a promising therapeutic strategy and some initial efforts in this direction have been made. Specifically, all-trans retinoic acid (ATRA) has been shown to be capable of promoting Treg development and their suppressive capacity, simultaneously inhibiting Th17 differentiation by enhancing TGF- β signaling (39-41). Furthermore, ATRA was able to restore macrophagal M1/M2 imbalance documented in ITP towards M2 phenotype, which development and function are also related to the TGF- β signaling (42, 43). In the study of Dai et al., ATRA therapy proved to be efficient in ITP treatment, considering significant increase of Treg percentage and IL-10 levels, which is in line with our results observed after TGF- β treatment. Unfortunately, in this study TGF- β levels were not evaluated (44).

Conclusion

Our data reconfirm deviant polarization of CD4^+ T cells in ITP patients and suggest that TGF- β could restore disturbed Th1/Th2 and Th17/Treg balance, indicating that enhancing TGF- β signaling could be a promising therapeutic strategy. However, the study also raises a concern about prolong CD4^+ T cell cultures as a model of studying the phenotype characteristics of these cells, considering their spontaneous phenotype fluctuations in *ex vivo* conditions.

Acknowledgments

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Declarations of interest

None.

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Originalni rad

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doi:10.5633/amm.2020.0201**IMUNOMODULACIJSKA SVOJSTVA TGF- β KOD BOLESNIKA SA
IMUNSKOM TROMBOCITOPENIJOM***Miloš Kostić¹, Tanja Džopalić¹, Nikola Živković², Ana Cvetanović³*¹Univerzitet u Nišu, Medicinski fakultet, Katedra za mikrobiologiju i imunologiju, Niš, Srbija²Univerzitet u Nišu, Medicinski fakultet, Katedra za patologiju, Niš, Srbija³Univerzitet u Nišu, Medicinski fakultet, Katedra za onkologiju, Niš, Srbija

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Novi koncept patogeneze imunske trombocitopenije (ITP) fokusiran je na CD4^+ T ćelije, koje se trenutno smatraju ključnim u stimulisanju B ćelija na produkciju antitrombocitnih antitela. U ovoj *in vitro* studiji istraživali smo profil CD4^+ T ćelija obolelih od ITP i imunomodulacijska svojstva TGF- β . CD4^+ T ćelije izolovane su iz mononuklearnih ćelija periferne krvi zdravih ispitanika i ispitanika obolelih od ITP. Nakon kratkotrajne inkubacije, određeni su nivoi osnovnih citokina T pomoćničkih ćelija, kao i broj T regulatornih ćelija (Treg). Imunomodulacijska svojstva TGF- β analizirana su praćenjem izmena u produkciji citokina IFN γ , IL-4, IL-10, IL-17 i IL-2, kao i učestalosti Treg, nakon šestodnevnog tretmana. Oboleli od ITP imali su smanjen nivo IL-4 i IL-10, povećan nivo IL-17, kao i povišen odnos IFN γ /IL-4 i IL-17/IL-10. Zanimljivo je to da u šestodnevnim CD4^+ T ćelijskim kulturama, bez TGF- β tretmana, nisu evidentirane statistički značajne razlike u nivou citokina između ispitanika kontrolne i ITP grupe, osim nivoa IL-10, koji je bio značajno niži. U ITP grupi ispitanika, nakon TGF- β tretmana, registrovano je značajno povećanje IL-10, kao i smanjenje odnosa IL-17/IL-10 u poređenju sa ispitanicima ITP grupe, koji nisu tretirani na ovaj način. Takođe, primećena je povećana učestalost Treg. Rezultati naše studije sugerišu na to da oboleli od ITP pokazuju aberantnu Th1 i Th17 polarizaciju celularnog imunskog odgovora, što se može korigovati stimulisanim TGF- β signalizacijom. Međutim, ispostavlja se da dugotrajna kultivacija CD4^+ T ćelija nije pogodan eksperimentalni model za proučavanje imunomodulatornih svojstava u ITP, usled dinamičkih fluktuacija fenotipa ovih ćelija u *ex vivo* uslovima.

*Acta Medica Medianae 2020;59(2):05-13.***Ključne reči:** imunska trombocitopenija; TGF- β ; imunomodulacija; IL-10, IL-17

THE ADVANTAGES OF ANTIBIOTIC PROPHYLAXIS IN NEUROSURGERY

Predrag Milošević¹, Boban Jelenković¹, Nebojša Miljković², Goran Ivanov²,
Miroslav Popović²

Prophylactic use of antibiotics in neurosurgery means the use of antibiotics in order to prevent infections of wounds and internal operative structures.

The research was conducted on 44 patients with L4-L5 and L5-S1 disc herniation operated at the Clinic of Neurosurgery in Niš during 2018. In 26 patients, antibiotic prophylaxis was used. In the remaining 18 patients, antibiotic prophylaxis was not applied and they were grouped into a control group. The average duration of the operation was 50 minutes. The antibiotic was used in a single dose first generation Cephalosporin, Cefazolin 1 g IV for patients with body weight less than 80 kg and 2 g for patients with body weight more than 80 kg.

We observed the occurrence of wound infections and internal operational structures, the occurrence of unwanted effects and financial aspects.

The goal of the work was to demonstrate that antibiotic prophylaxis is more favorable due to shorter time use of antibiotics, less side effects and overall financial benefit comparing to continuous antibiotic therapy.

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Key words: antibiotic prophylaxis, neurosurgery, wound infections

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Introduction

Infection means the penetration of microorganisms into the body, with all the consequences of their mutual reactions, with both actors trying to maintain it.

An important condition for the emergence of infection is that a certain number of pathogenic or conditionally pathogenic microorganisms come into contact with sensitive tissue and multiplies there.

Surgical infections always penetrate the tissue through the front door in which local defense inflammatory reaction of the tissue has primary role, and then it is quickly associated with the general reaction of the organism. Significant factors for the development of a surgical infection are: the amount of inoculated microorganisms, i.e., their reproduction and excretion properties, and the resistance of

the patient's organism to infection, which has been reduced in people with atherosclerotic blood vessels and chronic patients. Hospital infections are one of the leading causes of increased morbidity and mortality of hospitalized patients (1-5), malnutrition patients, hypoproteinemic and anemic, as well as those with malignant diseases.

The causative agents of the bacterial infection in operated neurosurgical patients usually come from the immediate environment, such as the patient's bacterial flora or in the presence of aerobic bacteria in the operating room during surgical work and those represent a high risk for the development of the infection. In neurosurgical patients, the infection occurs in 3-4% of cases (6, 7).

Antibiotic prophylaxis involves the use of antibiotics before the onset of infection, to prevent contamination of the operative wound by bacteria that represent a normal skin flora such as *S. aureus*, *S. epidermidis*, *P. acnes*, or contamination of pathogenic bacteria from the patient's environment during and after the procedure.

The use of antibiotics prophylactically aims to achieve sufficient concentration of bactericides in the tissue during intervention and for the first 3-4 hours postoperatively. Another way of using antibiotics prophylactically is to use them a few days before and after surgery and it is not justified because it represents a realistic modality for spreading resistance in a hospital setting (8).

The most common causes of infection at the Clinic of Neurosurgery in Niš are:

- *S. epidermidis*
- *S. aureus*
- *P. aeruginosa*
- *Citrobacter*
- *P. vulgaris*
- *P. mirabilis*
- *Acinetobacter*

Prophylaxis has been used in neurosurgery since 1925, before the age of antibiotics, when the antiseptic Hexamine was used. Harvey Cushing, a pioneer of neurosurgery, had a rate of postoperative infections of 1% using only water and soap. From Cushing's era, all neurosurgeons use aseptic techniques in all types of operations (9).

Materials and methods

The research was carried out at the Clinic of Neurosurgery KC Niš.

During this study, we examined the effects of antibiotic prophylaxis in patients treated for lumbar disc hernias. The study was performed in 44 patients with their consent. In 26 patients, antibiotic prophylaxis was used. For the remaining 18 patients antibiotic prophylaxis was not used and they were assigned to the control group. We followed the movement of laboratory parameters of inflammation: CRP, sedimentation and leukocytes before and after surgery in 44 patients.

The average duration of the surgery was 50 minutes. Antibiotic was used in a single dose first generation Cephalosporin - Cefazolin 1 g IV for patients with body weight less than 80 kg and 2 g for patients with body weight more than 80 kg.

Results

There was no statistically significant difference in wound infection depending on the use of antibiotic therapy ($\chi^2 = 1.076$; $p = 0.583$) (Table 1).

Table 1. Infections of wounds and deeper structures when antibiotic prophylaxis used

Infections	Wound infections	Infections of deeper structures	No infections	Total	χ^2	p
Antibiotic prophylaxis	1 (33.3%)	0 (0.0%)	25 (62.5%)	26 (59.1%)	1.076	0.583
Continuous antibiotic therapy	2 (67.3%)	1 (100.0%)	15 (37.5%)	18 (40.9%)		
Total	3 (100.0%)	1 (100.0%)	40 (100.0%)	44 (100.0%)		

A statistically significant difference in the distribution of side effects was determined depending on the use of antibiotic therapy ($\chi^2 = 15.634$; $p = 0.001$). A significantly higher number of patients

who had antibiotic prophylaxis were without side effects, while patients with continuous antibiotic therapy more often had nausea and vomiting, diarrhea and stomach pain (Table 2).

Table 2. Frequency of side effects

Side effects	Nausea and vomiting	Diarrhea	Pain in the stomach	No side effects	Number of patients	χ^2	p
Antibiotic prophylaxis	2 (33.3%)	1 (14.3)	0 (0.0%)	23 (82.1)	26 (59.1)	15.634	0.001
Continuous antibiotic therapy	4 (66.7%)	6 (85.7)	3 (100.0%)	5 (17.9)	18 (40.9)		
Total	6 (100.0%)	7 (100.0%)	3 (100.0%)	28 (100.0%)	44 (100.0)		

A statistically significant difference in funding was found, depending on the use of antibiotic

therapy ($\chi^2 = 14.123$; $p < 0.001$). Patients for whom funds of up to RSD 2000 were spent for

antibiotic therapy were more likely to have antibiotic prophylaxis, while those with over RSD 2000 spent,

more often had continuous antibiotic therapy and associated therapy (Table 3).

Table 3. Financial aspects

Funds	Up to 2000 dinars	More than 2000 dinars	Number of patients	χ^2	p
Antibiotic prophylaxis	26 (72.2%)	0 (0.0%)	26 (59.1%)	14.123	< 0.001
Continuous antibiotic therapy and associated therapy	10 (27.8%)	8 (100.0%)	18 (40.9%)		
Total	36 (100.0)	8 (100.0)	44 (100.0%)		

Discussion

Postoperative infections are the main source of morbidity and mortality of surgical patients. Prophylactic use of antibiotics is indicated in neurosurgical operations where the risk of infection is greater than the risk of using antibiotics.

In neurosurgery, there is generally a low risk of infection compared to other surgical branches, only 3-4%, due to surgical strategies, technological quality and progress (10, 11). Postoperative infections in neurosurgical patients can be dramatic, rapidly progressive forms of meningitis, epidural abscess, subdural empyema, brain abscess, ventriculitis, sepsis. Therefore, the prophylactic use of antibiotics has proven to be very useful (12-14).

However, some studies have shown that prophylactic use of antibiotics in neurosurgery is still controversial. In practice, there is a "struggle" between advances in aseptic procedures and the abuse of high-dose antibiotics of the broad spectrum as a "standard".

Our study showed that there was no statistically significant difference in wound infection depending on the use of antibiotic therapy ($\chi^2 = 1.076$; $p = 0.583$) (Table 1). According to the results of numerous studies (7, 16), antibiotic prophylaxis leads to a reduction in the incidence of operative site infections. In contrast, this association in some other studies has not been confirmed (17, 18). Surgical infection increases the length of hospital stay (19). Prophylaxis has the potential to shorten hospital stay. There are several randomized studies that monitor the duration of hospital stay as a measure of outcome. They have shown that prevention of wound infection is associated with a faster return to normal activity after being discharged from the hospital (20).

A statistically significant difference was found during our study in the distribution of side effects depending on the use of antibiotic therapy ($\chi^2 = 15.634$; $p = 0.001$) (Table 2). A significantly higher number of patients who had antibiotic prophylaxis were without side effects, while patients with continuous antibiotic therapy more often had nausea

and vomiting, diarrhea and stomach pain. Antibiotics can cause disturbance of bacterial equilibrium in the intestines and allow the development of strains of bacteria that cause gases in the intestines and lead to inflammation. Sometimes antibiotics significantly destroy the normal intestinal flora, so the *Clostridium difficile* bacteria can accumulate and multiply in the intestines. It illuminates a very harmful substance in the human intestines and leads to a much more serious form of inflammation of the intestines called Pseudomembranous colitis. Diarrhea occurs during or shortly after taking antibiotics. Pseudomembranous colitis may occur practically after the administration of any antibiotic, or most commonly after the increase of Ampicillin, Clindamycin, Cephalosporin of the third generation and Fluoroquinolone (21). Exposure to systemic antibiotics leads to a disorder of normal colon flora and an increase in sensitivity to colonization and toxin production by *Clostridium difficile* and increases the risk of symptomatic *Clostridium* by 2 to 16 times (22). The unreasonable consumption of antimicrobial drugs is harmful to the health of patients, it causes possible side effects, toxic reactions and interactions with other drugs and leads to increased resistance of microorganisms to antibiotics (23). Therefore, we concluded that antibiotic prophylaxis should be applied in neurosurgery due to shorter time use of antibiotics, less overall dose, and less frequent appearance of serious complications due to shorter use of antibiotics. Several published guidelines (24-28) say that for all clean and most pure-contaminated procedures (the main problem of bacterial contamination of the skin), Cefazolin is the first choice medication due to its external action on a gram-positive coccus, which mainly causes skin contamination.

Very few prospective randomized studies of surgical prophylaxis included economic evaluation within the research. During our research, a significant statistical difference was found in financial resources depending on the use of antibiotic therapy ($\chi^2 = 14.123$; $p < 0.001$). Antibiotic therapy was used for prophylactic purposes and reduced the length of patients' stay in the hospital and did not cost more than RSD 2000, while the continuous use

of antibiotics extended hospitalization and it costed financially more. For most surgeries, however, prophylaxis only reduces short-term morbidity. Surgical infection increases the length of stay in hospital (28). The additional length of stay depends on the type of surgery. Prophylaxis has the potential to shorten the stay in hospital. There is little direct evidence to suggest that, a few randomized studies of (29, 30) included the length of stay in hospital as a measure of outcome. This is the evidence that the prevention of wound infection is associated with a faster return to normal activity after being discharged from hospital.

Based on our research it is mandatory to provide optimum protection in the work by respecting the principle of asepsis, use prophylactic antibiotics after the patient's consent by giving a minimum dose adjusted for the risk of infection and the status

of the patient, before the beginning of operation and 24 hours after surgery.

It should be noted that the prophylactic use of antibiotics is different from continuous antibiotic therapy; the use of high-dose broad-spectrum antibiotics has long been used as a standard but should be avoided.

Conclusion

Antibiotic prophylaxis should be applied in neurosurgery for the following reasons:

- shorter time of use of antibiotics,
- less overall dose of used antibiotics,
- rare occurrence of severe complications of long-term use of antibiotics (enteric infection with *C. difficile*, development of resistance),
- economic benefit comparing to continuous antibiotic therapy.

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PREDNOSTI ANTIBIOTSKE PROFILAKSE U NEUROHIRURGIJI

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Profilaktička upotreba antibiotika u neurohirurgiji podrazumeva upotrebu antibiotika u cilju sprečavanja infekcija rana i unutrašnjih operativnih struktura.

Istraživanje je sprovedeno na Klinici za neurohirurgiju u Nišu tokom 2018. godine i obuhvatilo je 44 bolesnika kojima je operisana hernija diska L4-L5 i L5-S1. Kod 26 bolesnika primenjena je antibiotska profilaksa. Kod preostalih 18 bolesnika nije sprovedena antibiotska profilaksa i oni su raspoređeni u kontrolnu grupu. Prosečno trajanje operacije bilo je 50 minuta. Antibiotik se koristi u jednoj dozi, cefalosporin prve generacije – cefazolin 1 g IV za bolesnike sa telesnom težinom manjom od 80 kg i 2 g za bolesnike sa telesnom težinom većom od 80 kg.

Primetili smo pojavu infekcija rana i unutrašnjih operativnih struktura, pojavu neželjenih efekata i posmatrali smo finansijske aspekte.

Cilj rada bio je pokazati da je antibiotska profilaksa povoljnija zbog kraće upotrebe antibiotika, manje nuspojava i ukupne finansijske koristi, u poređenju sa kontinuiranom antibiotskom terapijom.

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Ključne reči: antibiotska profilaksa, neurohirurgija, infekcije rana

FUNCTIONAL IMPROVEMENT UP TO SIX MONTHS AFTER TOTAL HIP ARTHROPLASTY: MEASURED BY SELF-REPORTED QUESTIONNAIRE AND RANGE OF HIP FLEXION

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The aim of this study was to assess patients' self-reported hip function, as well as hip flexion and flexion contracture preoperatively, three and six months after total hip arthroplasty (THA). The secondary aim was to analyze the extent to which postoperative patients' self-reported hip function three and six months after THA correlates with preoperative patients' self-reported function, hip flexion and flexion contracture.

A longitudinal cohort study included 100 patients with the end stage of hip osteoarthritis who underwent THA at the Orthopedic Clinic from May 2015 to November 2016. The patients were assessed at three time points: preoperatively and at 3 and 6 months after THA. First, hip flexion and flexion contracture were measured. Then the patients completed the self-reported questionnaire for evaluation of hip function-Oxford hip score.

The results showed poor self-reported hip function, hip flexion and flexion contracture preoperatively. At 3 months follow-up, as well as at 6 months follow-up, self-reported hip function and flexion were significantly increased ($p < 0.001$) and hip flexion contracture was significantly decreased ($p < 0.001$) in relation to preoperative values. According to values of Spearman correlation coefficient, significant correlations were found between self-reported hip function at 3 months follow-up and preoperative function, hip flexion and flexion contracture, but all correlations disappeared by 6-month follow-up. The study indicates that poor preoperative hip function and hip flexion may slow down functional recovery after TAK, but do not compromise the outcome 6 months after THA.

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Key words: Total hip arthroplasty, self-reported functional outcome, hip flexion, hip flexion contracture

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Introduction

Total hip arthroplasty (THA) is a successful surgical treatment for people with end-stage hip osteoarthritis, when conservative treatment is no longer effective (1-4). It provides significant pain relief and improvement in function to a painful, degeneratively altered and reduced in motion hip joint (4-6). THA is considered to be the operation of the twentieth century, as it has become a turning

point in the treatment of elderly people suffering from degenerative hip joint disease, with very good long-term results (7). It has been applied for more than half a century and has been constantly improved during this period. The number of the operations has been steadily increasing over the past decades, and in future decades an exponential rise in the number of both primary and revision THA is expected, because of the ageing population (8). Thanks to the advancement in implant technology, surgical techniques and anesthesia indications for the THA are significantly expanded but the most common indication for THA is still primary degenerative hip joint disease (9). These operations are now performed in younger, more active people who would not have been candidates for THA in the previous period (10). Most patients after THA achieve a satisfactory functional recovery however, in a minority of patients functional limitations remain (4, 8).

The assessment of outcomes after THA can be done in several ways. In the first decades after the introduction of THA, the outcome was evaluated by the orthopedic surgeon on the basis of implant

survival rates, implant-related complications, radiographic positions of endoprosthetic components, range of hip motion etc. (5, 11, 12). Later, several numerical scores were introduced to quantify the outcome of arthroplasty, which made comparison of the outcomes possible. Among the most commonly used numerical scores was the Harris's hip score (HHS), where assessment of pain and function, as well as measurement of the range of the hip motion and the presence of hip contracture were done by a surgeon (13).

On evaluating the outcomes, it appeared that patients are less satisfied than surgeons (6, 12, 14). Because of the discrepancy between the points of view of patients and clinicians in the past decades, the evaluation of outcome after arthroplasty has been shifted to the use of patient reported outcome measures (PROMs) so as to see whether arthroplasty is successful from the patient's perspective (2, 4, 8, 11, 12, 15). To evaluate hip arthroplasty outcomes, several PROMs instruments have been developed containing questions related to pain and functional activities specific for the hip joint. Among the most commonly used PROMs after THA is the Oxford hip score (OHS) (16). The OHS was designed to evaluate the functional ability of patients with THA (16). The OHS has been used since its introduction in 1996, and has proven reliability and validity (5). Furthermore, the OHS is strongly correlated with the HHS before and after hip surgery (5). The OHS and HHS have some similar items regarding pain and function evaluation, but the OHS does not include measuring of hip range of motion and flexion contracture (6).

We were interested in investigating the improvement of the hip function up to 6 months after THA, both from the perspective of the patient and on the basis of objective parameters, such as the range of hip flexion and flexion contracture.

Aim

Therefore, the primary aim of this study was to assess patients' self-reported function as well as range of active hip flexion and hip flexion contracture preoperatively and three and six months after THA. The secondary aim was to analyze the extent to which postoperative patients' self-reported hip function three and six months after THA correlates with preoperative patients' self-reported function, range of hip flexion and flexion contracture.

Patients and methods

A longitudinal cohort study recruited 122 patients with the end stage of hip osteoarthritis admitted at the Orthopedic Clinic, Clinical Center Niš, from May 2015 to November 2016 to undergo THA. All the patients received post-acute inpatient rehabilitation at the Physical Medicine and Rehabilitation Clinic starting a few days after THA. The inclusion criteria for the study were: primary unilateral THA for OA and willingness to participate in the study. The exclusion criteria were: cognitive impairment

(Mini-Mental State Examination score < 24) (17), revision THA or the previous lower limb surgery, severe chronic diseases that limit physical functioning. After applying the inclusion and exclusion criteria, 100 patients were eligible for the study and written informed consents were obtained.

The sociodemographic and health data that included age, sex, BMI value, duration of the disease symptoms, as well as comorbidity were collected preoperatively through face-to-face interviews and from medical records. The patients' body height and weight measures were taken, and body mass index (BMI) was calculated and recorded in kg/m². Comorbidity was assessed by the Charlson Comorbidity Index (CCI). It includes 19 conditions, each of which was assigned - 1, 2, 3 or 6 points. CCI was expressed as a summative score range from 0 to 33, with higher score indicating the higher comorbidity burden (18).

Assessments

The patients were assessed at three time points: preoperatively (24-48 hours before the surgery) and at 3 and 6 months after the surgery. First, hip flexion range and flexion contracture were measured. Then the patients filled in the self-reported questionnaire for evaluation of hip function - OHS.

Self-reported function

The self-reported function of hip joint was estimated using the OHS, which is designed and validated as a self-reported joint-specific questionnaire for evaluation of the outcomes of THA. The OHS consists of 12 questions which refer to hip pain and function during the last 4 weeks. Each question is in the Likert scale valued from 0 to 4. An overall score is created by summing the responses to each of the 12 questions. The total score can range from 0 to 48, where the higher score represents the better function (16, 19).

Active hip flexion and flexion contracture

The range of active hip flexion was measured in degrees using a long-arm, full-circle handheld goniometer. The patient was placed in a supine position on an examination table with hips at neutral and knees in extension. The goniometer was centered on hip joint. Then the patient moved the hip to the maximum flexion and the degree of flexion was measured and recorded. While returning to the starting position of the full extension, the limited motion was measured and expressed as hip flexion contracture. The range of active hip flexion was measured and recorded in degrees according to the method suggested by the American Academy of Orthopedic Surgeons (20). The same investigator with more than ten years of experience measured ROM twice at all assessment time points with the same goniometer (made of flexible clear plastic) and the mean value was recorded.

Statistical analysis

The continuous variables were described by means \pm standard deviations and by medians. For the categorical variables, absolute numbers and percentages were given. The distributions of the continuous variables were assessed for normality by Kolmogorov-Smirnov test. The differences between independent groups were analyzed by an unpaired t-test in case of a normal distribution or by Mann-Whitney U-Test if the distribution of data was not normal. A chi-square test was used to compare proportions of categorical variables between groups. To compare cases of two related observations a Wilcoxon Signed-Ranks was used because the distribution of data was not normal. To analyze associations between continuous variables Spearman (r) correlation coefficient was used. P values less than 0.05 were considered significant. The calculations were carried out using the SPSS statistical package version 15.0.

Results

Baseline characteristics of patients

The study included 100 patients with end-stage hip osteoarthritis immediately prior to THA. The mean age of study patients at baseline was 64.1 ± 8.7 years and 55% were female. The average duration of symptomatic osteoarthritis in our patients was 5.9 ± 3.9 years and according to the WHO classification they were overweight with the mean BMI of 27.5 ± 3.9 . Because of the role of comorbidity in recovery patients with THA, we calculated CCI and the mean was 0.5 ± 0.8 . Regarding examined parameters before surgery: the mean OHS was 13.9 ± 5.5 , the mean hip flexion was 66.0 ± 18.7 degrees and the mean hip flexion contracture was 6.2 ± 4.9 .

Out of 100 patients who started the study, 92% attended the 3-month postoperative follow-up and this declined to an 81% attendance rate by the 6-month follow-up. The comparison of the baseline characteristics of the patients that completed the study and did not attend to the 6-month follow-up is presented in Table 1.

No significant differences were found in any of the baseline characteristics between the patients who completed the study and those who dropped out from the study (Table 1).

Table 1. Baseline characteristics of patients who completed the study versus patients who dropped out

	Patients (n = 100)				
Characteristics of patients	Completed the study (n = 81)		Dropped out of the study (n = 19)		<i>p</i>
Age (years)	64.02 ± 8.73 (65.00)		64.53 ± 8.57 (64.00)		0.8216
Gender					
Male	37	45.68%	8	42.11%	0.7792
Female	44	54.32%	11	57.89%	
BMI (kg/m²)	27.17 ± 3.77 (26.70)		28.89 ± 4.28 (28.10)		0.0976
Symptoms duration (years)	5.56 ± 3.51 (5.00)		7.16 ± 5.28 (6.00)		0.1208
CCI (0-33 score)	0.58 ± 0.86 (0.00)		0.21 ± 0.42 (0.00)		0.1136
Hip flexion (degrees)	65.19 ± 18.58 (70.00)		69.47 ± 19.14 (70.00)		0.4708
Hip flexion contracture (degrees)	5.99 ± 4.36 (5.00)		6.84 ± 6.71 (5.00)		0.9533
OHS (0-48 score)	14.06 ± 5.54 (13.00)		13.42 ± 5.23 (14.00)		0.8121

Continues variables are given as means \pm SD (medians); categorical variables are given as frequencies (percentages); BMI, Body mass index; CCI Charlson Comorbidity Index (higher score indicating the higher comorbidity burden); ROM, range of motion; OHS, Oxford hip score (higher score representing the better function)

Examined parameters up to six months after THA

Table 2 shows the values of the examined parameters before surgery, at 3 and 6 months postoperatively, for the patients who participated in the whole of the study. At 3 months, as well as at 6 months postoperatively in our cohort of patients all the three examined parameters significantly improved in relation to preoperative values at the highest level of significance ($p < 0.001$). Self-reported function had significantly increased from 14.06 (SD

5.54) preoperatively to 31.91 (SD 3.84) at 3 months after surgery ($p < 0.001$) and to 42.15 (SD 3.87) at 6 months after surgery ($p < 0.001$). The range of hip flexion movement had significantly increased from 65.19 (SD 18.58) preoperatively to 80.62 (SD 9.66) at 3 months after surgery ($p < 0.001$) and to 93.58 (SD 7.76) at 6 months after surgery ($p < 0.001$). The mean degree of hip flexion contracture was significantly decreased from 5.99 (SD 4.36) preoperatively to 2.78 (SD 3.45) at 3 months after surgery ($p < 0.001$) and to 1.91 (SD 2.91) at 6 months after surgery ($p < 0.001$) (Table 2).

Table 2. Examined parameters preoperatively, at 3 and 6 months follow-up for THA patients who completed the study

Examined parameters	Assessment time		
	Preoperatively	3 months follow-up (n = 92)	6 months follow-up (n = 81)
OHS	14.06 ± 5.54 (13.00)	31.91 ± 3.84 (32.00)***	42.15 ± 3.87 (43.00)***
Hip flexion (degrees)	65.19 ± 18.58 (70.00)	80.62 ± 9.66 (80.00)***	93.58 ± 7.76 (95.00)***
Hip flexion contracture (degrees)	5.99 ± 4.36 (5.00)	2.78 ± 3.45 (0.00)***	1.91 ± 2.91 (0.00)***

Continues variables are given as means±SD (medians); OHS, Oxford hip score (higher score representing the better function); Wilcoxon Signed-Ranks (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$)

Correlation between examined preoperative parameters and post-operative OHS

The correlation between the examined preoperative parameters and the post-operative OHS is shown in Table 3. The significant correlations were found between the 3 months post-operative OHS and all the examined preoperative parameters. There was a positive correlation with the preopera-

tive OHS (Spearman's $r = 0.30$; $P = 0.0038$) and the preoperative hip flexion (Spearman's $r = 0.27$; $P = 0.0095$), but a negative correlation with the preoperative hip flexion contracture (Spearman's $r = -0.25$; $P = 0.0146$). No significant correlation of the 6 months post-operative OHS value with any of the examined preoperative parameters was found (Table 3).

Table 3. Correlation between preoperative OHS, hip flexion and flexion contracture with postoperative OHS

Examined parameters preoperatively	OHS 3 months follow-up			OHS 6 months follow-up		
OHS	0.30	(0.0038)	***	0.19	(0.0894)	
Hip flexion	0.27	(0.0095)	**	0.11	(0.3458)	
Hip flexion contracture	-0.25	(0.0146)	*	-0.15	(0.1841)	

OHS, Oxford hip score; Spearman's (* - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$)

Discussion

Our results showed significant improvement of all the three examined parameters (self-rated hip function, hip flexion and flexion contracture) at the follow-up 3 and 6 months after THA in comparison to their preoperative values.

In order to evaluate hip function from the patients' perspective in the present study the OHS was used as a PROMs instrument (16, 19). The mean preoperative OHS was very low with the value of 14 points which represents a very poor hip function according to Kalairajah et al. (21). In contrast to our findings, as well as in other studies, the preoperative OHS was higher than in our study with the range between 16.4 and 19.7 (3, 4, 8, 11, 12, 15).

As expected, at 3 and 6-month check-ups after THA, we found statistically significant improvement of OHS ($p < 0,001$). Three months post-operatively the mean OHS value was approximately 32 points, and six months post-operatively it was approximately 42 points. According to Kalairajah et al., the achieved OHS value of 42 points after six months is considered as an excellent outcome (21). Also, such OHS value indicates the satisfactory hip function according to Judge et al. whose study shows that the threshold for OHS for high satisfaction 6 months following THA was 35 points (15). Our findings are consistent with other authors' findings who also found significant improvement of OHS 3 and 6 months after THA as compared to pre-operative values (5, 11, 12). In the study of Heiberg et al. that included 1523 patients with primary THA who completed the six-month post-operative OHS Questionnaire, the mean OHS was 38.8 (15) which was almost identical to the mean OHS of 39 points found by Kjærgaard et al. in 100 patients 6 months after THA (11). Other studies that examined patients up to 12 months after THA found substantial OHS improvement 12 months after THA as compared to the preoperative values (3, 4, 5, 6, 8) while some authors used another OHS scale consisting of 12-60 points, with minimum score of 12 points representing the best hip function (5, 6). Based on the evaluation data from a regional joint arthroplasty registry for 3203 THA patients, Hamilton et al. found that the mean OHS 12 months post-operatively was 38.6 (3). In 619 patients, Arden et al. found the mean OHS higher with the value of 43 points on an average 12 months after THA (8). The identical OHS of 43 points one year after surgery was shown by the multicentric study conducted in seven hospitals across England and Scotland, which included 1375 patients with 1431 primary THA for osteoarthritis. This study followed the patients 5 years after THA and demonstrated that the mean value of OHS did not change between 1 year after surgery and 5 years after surgery (4).

In 2016, in the systematic review, Hofstede et al. showed that although patients with worse pre-operative function had a greater improvement, they did not achieve the postoperative level of those with higher preoperative function (1). In contrast to their findings, in our study, the patients had poor pre-operative score, but 6 months after THA they

achieved excellent hip function. In the present study, the same or even slightly better than in other studies, postoperative OHS 3 and 6 months after surgery can be attributed to the fact that all the patients in our study after the acute rehabilitation at the Orthopedic Clinic received 21 days long post-acute rehabilitation at the Physical medicine and rehabilitation Clinic.

The hip range of motion, in evaluation of the outcomes after THA, has a disputable value (22). In his score for hip function evaluation (HHS), Harris assigned only 5 out of total of 100 points to the overall hip range of motion, considering that what is necessary for hip function is relatively low range of motion in comparison to what is available by the anatomy configuration of a normal hip joint (13). As opposed to Harris, Davis et al., showed that the range of hip motion is more significant than what was considered before. They analyzed the range of hip motion in 1383 patients (1517 hips) with primary THA and determined that postoperative range of hip motion is in strong correlation with hip the function in the sense that the higher the range of motion the better is the hip function (22). The required range of hip motion for different activities of daily life varies considerably. Walking on a flat surface, as the most common DLA, does not require high hip flexion movement. Some daily life activities, such as tying the shoes, catching items from the floor and moving from standing to sitting, require high hip flexion movement (minimum 95 °C)(23).

For the evaluation of outcomes after THA, we used active hip range of flexion and degree of flexion contracture of hip. We decided to measure only the range of hip flexion, not the total range of hip movement, because it has been proved that the range of hip flexion correlates strongly with the total range of movement in all three planes (24). In the present study, it was found that the preoperative average active hip flexion was 65 degrees and the hip flexion rate 6 degrees. The mean preoperative hip flexion in our study was lower than in studies of other authors (2, 5), where the mean preoperative flexion was 81 degree. Our patients had a pre-operatively higher degree of hip flexion contracture compared to Heiberg et al. (2). A possible explanation for an average lower preoperative OHS, a lower degree of hip flexion and a higher degree of flexion contracture in our study may be that in Serbia patients usually postpone surgery, and functional parameters are disturbed in a higher degree.

In the present study, a statistically significant increase in hip flexion was found 3 months after the THA when the average flexion was approximately 81 degrees, and 6 months after THA when the average F was approximately 94 degrees. Also, a statistically significant reduction in hip flexion was found to be 2.78 ± 3.45 3 months after the THA, and 6 months after the THA it was 1.91 ± 2.91 . Increasing the range of movement is important because it is associated with a better hip function (22). Reduction of flexion contraction is also significant because the hip flexion contracture after THA causes pain in the hip joint and lower part of the back and walking disorders (25). Other authors found a higher hip flexion than we did in our study 3 months after surgery (2,

5). Explanation for a lower range of movement 3 months after surgery in our patients can be found in the lower degree of preoperative hip flexion. Röder et al. in a retrospective study that included data from approximately 13,000 THA, found that a lower preoperative hip movement also led to a minor improvement in mobility after THA (26). However, at the control examination, six months after surgery, our patients achieved a relatively satisfactory hip flexion of an average of 94 degrees, which is found approximately similar to findings of other authors (2, 5). In the study of Kawai et al. which involved 120 patients with a primary THA, the mean hip flexion was 91.9 at 3 months post-THA, and 92.6 at 6 months post-THA (5). In the study of Heiberg et al. which included 88 patients with a primary THA, the mean hip flexion 3 months after surgery was 84, and 12 months after surgery 94 degrees. A statistically significant increase in hip flexion compared to preoperative values was achieved at the 12-month follow-up after surgery (2). They had a 6-week exercise program starting 3 months postoperatively (which can be taken as an explanation for the significant improvement shown 3 months after THA). It is important to note that comparisons of hip flexion are often difficult to perform, since in many studies, the way in which the hip flexion is measured is not precisely explained (in which position the patient was initially set and whether it is an active or passive range of movement).

Regarding the correlation of postoperative self-reported function 3 months after surgery and the examined preoperative parameters, a positive correlation was found with preoperative function ($p < 0.01$) and hip flexion ($p < 0.01$), and negative correlation with hip flexion contracture ($p < 0.05$). However, 6 months after THA, no correlation of postoperative function was found with any of the examined preoperative parameters. It shows that the lower preoperative OHS values and lower hip flexion and higher degree of hip flexion contraction are in correlation with the lower level of self reported hip function 3 months after THA, but not in correlation with the hip function 6 months after surgery, thus they are not expected to be correlated with the function 6 months after surgery. The results

obtained could indicate that patients with better preoperative parameters recover faster after surgery. It does not mean that the patients with worse preoperative parameters are not good candidates for THA, but the recovery in such patients requires longer period of time. Our findings are partly in accordance with findings of some other authors. Several studies have found, like our study, a correlation of the postoperative function with a preoperative function 3 months after surgery (4, 5), but in contrast to our study, in these studies a correlation of the preoperative function and postoperative function was found not only after 3 but also after 6 and 12 months (4, 5). In contrast to the present study, Kawai et al. did not find a correlation between preoperative flexion and the postoperative self-reported function either 3, or 6, or 12 months after THA (5). Further longitudinal studies with larger sample size are required in order to provide more information about the correlation of poor preoperative function and hip range of motion, and postoperative functional outcomes.

Conclusion

We found poor self-reported hip function, hip flexion and flexion contracture immediately before THA. Our results showed significant improvement of all the three examined parameters (self-rated function, hip flexion and hip flexion contracture) from the preoperative values to the control values at 3, as well as at 6 month check-up after THA. A positive correlation was found between self-reported function 3 months after THA with preoperative function ($p < 0.01$) and hip flexion ($p < 0.01$), as well as a negative correlation with hip flexion contracture ($p < 0.05$), but all correlations disappeared within 6 months postoperatively (for all the parameters). The study indicates that in a cohort of patients undergoing THA, operating on patients with poor hip function, low degree of range of hip flexion and high degree of hip flexion contracture may slow down functional recovery, but not compromise the outcome 6 months after THA.

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Originalni rad

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doi:10.5633/amm.2020.0203**POBOLJŠANJE FUNKCIJE KUKA TOKOM PRVIH ŠEST MESECI NAKON
TOTALNE ARTROPLASTIKE: MERENO NA OSNOVU UPITNIKA ZA
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Cilj ove studije bila je procena funkcije kuka iz perspektive bolesnika, kao i merenje obima pokreta fleksije kuka i fleksione kontrakture preoperativno, tri meseca i šest meseci nakon totalne artroplastike kuka (TAK). Sekundarni cilj bila je analiza korelacije stepena postoperativne funkcije kuka, tri meseca i šest meseci nakon TAK, sa preoperativnom funkcijom, obimom pokreta fleksije i stepenom fleksione kontrakture kuka.

Longitudinalnom kohortnom studijom obuhvaćeno je 100 bolesnika sa uznapredovalim osteoartritisom kuka, kod kojih je urađena TAK na Ortopedskoj klinici, od maja 2015. do novembra 2016. godine. Bolesnici su procenjivani u tri vremenska perioda: postoperativno, 3 meseca i 6 meseci nakon TAK. Prvo su mereni obim pokreta fleksije i fleksiona kontraktura. Zatim su bolesnici popunjavali Oksford upitnik za samoprocenu funkcije kuka.

Rezultati su pokazali da je preoperativno funkcija kuka, iz perspektive bolesnika bila loša, kao što su loše bile i fleksija kuka i fleksiona kontraktura. Na kontrolnim pregledima 3 meseca i 6 meseci nakon TAK, postoperativno funkcija kuka i obim pokreta fleksije značajno su povećani ($r < 0,001$), a fleksiona kontraktura kuka značajno je smanjena ($r < 0,001$) u odnosu na preoperativne vrednosti. Prema vrednostima Spearmanovog koeficijenta korelacije, na kontronom pregledu 3 meseca nakon TAK utvrđene su značajne korelacije postoperativne funkcije kuka sa preoperativnom funkcijom kuka, fleksijom kuka i fleksionom kontrakturom, ali sve su korelacije nestale na kontrolnom pregledu 6 meseci nakon operacije. Studija pokazuje da preoperativno loša funkcija kuka i fleksija kuka mogu usporiti postoperativni funkcionalni oporavak, ali ne ugrožavaju ishod 6 meseci nakon TAK.

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Ključne reči: totalna artroplastika kuka, funkcionalni ishod iz perspektive bolesnika, fleksija kuka, fleksiona kontraktura kuka

PREDICTIVE IMPORTANCE OF TUMOR BUDDING, LYMPHOVASCULAR AND PERINEURAL INVASION IN COLORECTAL CARCINOMA

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Colorectal cancers comprise a heterogeneous group of malignant intestinal tumors that have various mechanisms of onset, based on different combinations of genetic and epigenetic alterations. Many authors suggest that the TNM staging most accurately determines the prognosis of each case of colorectal cancer, however other parameters that indicate the aggressive behavior of the tumor are required as well. Such parameters involve perineural invasion, lymphovascular invasion, and tumor budding. The aim of this paper was to examine association between perineural invasion, lymphovascular invasion, and tumor budding with the tumor stage in colorectal cancers. The study included histopathology cases of 142 large bowel cancers removed at the Surgical Clinic in Niš during the period of one year (2016). The tumor stages were determined based on the TNM classification recommended by AJCC. Perineural invasion, lymphovascular invasion, and tumor budding were relatively common finding: 20.4, 40.1, 44.4 percent of examined cases, respectively. Univariate logistic regression analysis revealed a statistically significant correlation between high grade tumor budding, lymphovascular and perineural invasion and the advanced stage of tumor disease. The evaluation method, according to the International Tumor Budding Consensus Conference (ITBCC), should be used to evaluate/define biological aggressiveness of a tumor, and may represent the basis of a routinely used staging system in patients with colorectal cancer.

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Key words: colorectal carcinoma, tumor budding, lymphovascular invasion, perineural invasion

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Introduction

Colorectal cancers are one of the commonest causes of disease and death in the world, especially in developed countries (1, 2). They are more frequent in men than in women, developing after the

age of 50, and more than 70% of carcinomas occur after the age of 60. Colorectal carcinomas account for 70% of all malignancies that occur in the gastrointestinal tract (3). They comprise a heterogeneous group of tumors that may develop based on several alternative genetic pathways, each involving different combinations of genetic and epigenetic changes (4). They are a consequence of chromosomal instability, microsatellite instability, and epigenetic mechanisms, and thereby, different responses to applied therapies. The most common histological type is conventional adenocarcinoma more than 90% originating from epithelial cells of the colorectal mucosa, whereas other histological types are infrequent. Most often they are moderately differentiated more than 70% and localized in the rectum and sigmoid colon. Large bowel cancers infiltrate the intestinal wall and spread onto regional lymph nodes, and most commonly, metastasizing hematogenously to the liver (5). Patients diagnosed with stage I of the disease have a 97% probability of five-year survival, whereas in stages II, III and IV the probability decreases to 78%, 64%, and 11%, respectively (6). Due to great advancements in the application of neoadjuvant immunotherapy, chemotherapy, and surgery, patients have a prolonged relapse-free

period, and permanent remission is achieved in some patients (7–9). In addition to the stage, and histopathology differentiation, there are other prognostic factors that indicate the aggressive behavior of the tumor (10), such characteristics involve tumor budding, lymphovascular invasion and perineural invasion.

Lymphovascular invasion indicates the presence of tumor emboli in lymph and blood vessels. Differentiating venous and lymphatic invasion is important as they have different clinical implications. Presence of lymphatic invasion has been shown to correlate well with lymph node metastasis (11–13), while venous invasion is associated with the potential for visceral metastases occurrence (14, 15).

The most widely used definition of PNI is broad, including invasion of tumor cells in, around and through the nerves. Namely, with the presence of tumor cells within any of the 3 layers of the nerve sheath (epineurium, perineurium and endoneurium) or juxtaposition of tumor foci outside of the nerve with involvement of 33% of the nerve's circumference (16). Some authors report PNI only when tumor cells are observed inside the perineurial layer (17).

Tumor buds signify the presence of individual or smaller groups of cancer cells, less than four cells on the invasive tumor front, and represent an independent indicator of poor prognosis (18–20). It has been observed that in two same-stage cancers, the one with tumor budding will exhibit more aggressive behavior (21). It is thought to be a manifestation of epithelial-mesenchymal transition. This transition is characterized by a series of cell alterations such as loss of cell adhesion molecules, increased production of extracellular matrix components, cytoskeletal alteration and ability to degrade basement membrane resulting in a phenotype with increased migratory capacity and invasive phenotype (22). Routine reporting is now advocated for extension, outlined by the International Tumor Budding Consensus Conference (ITBCC), with recommendations for the assessment and reporting of tumor budding in colorectal carcinoma (20).

The Aim of the Paper

The aim of the paper was to determine preference and degree of the association between high grade tumor budding, lymphovascular and perineural invasion with tumor stage in colorectal carcinomas.

Materials and Methods

The study included histopathology cases of 142 colorectal cancer patients which were undergoing surgical removal of the tumor at the Surgical Clinic of the Clinical Center Niš during the period of one year (2016). Surgically resected samples of large bowel with the tumor were fixed in aqueous 4% formaldehyde solution (10% formalin solution), and routinely processed to the paraffin blocks. Paraffin sections were stained by hematoxylin and

eosin (HE) method. Data used for this research were obtained from the histopathology report archives of the Pathology Center (Clinical Center Niš), included: anatomic localization of the tumor, tumor size, lymph node status, and metastasis stage (TNM), histological type of tumor, histological grade, presence or absence of peritumoral lymphovascular and perineural invasion. Determination of the disease stage as well as histopathology classification of the tumors were performed on the recommendation of AJCC (American Joint Committee on Cancer) (23). The grading system for colorectal cancer is based on the percentage of cancer gland formation. Well differentiated adenocarcinoma exhibits gland formation in more than 95% of the tumor, moderately differentiated in 50% to 95% of the tumor, and poorly differentiated adenocarcinoma exhibits less than 50% gland formation. Poorly differentiated adenocarcinoma accounts for 5% to 10% of all cases and are associated with a greater incidence of adverse outcome. Tumor buddings defined as single tumor cells or cluster of less than four cells at the invasive front of tumor. The quantification of tumor budding at the invasive front of tumor has been assessed on HE stained slides at magnification $\times 100$, thereby identifying fields with the highest density of tumor budding which are then analyzed at magnification $\times 200$. The specimen with the highest number of tumor budding is considered a hotspot (in a field measuring 0.785 mm^2). A two-tier system was used: low grade (0–9 tumor buds); and high grade (≥ 10).

Statistical Analysis

Interrelationships between tumor location and clinicopathological characteristics were analyzed using Chi square (χ^2) test. The univariate Cox proportional hazards regression using to calculate hazard ratios and 95% confidence intervals. Variables found to be statistically significant ($p < 0.05$). Analyses were performed using SPSS software version 24.

Results

The study consisted of 142 patients aged 67.4 ± 10.0 years, with age ranging from 22 to 88 years. Male subjects were predominant (62.7%), and their number was statistically significantly higher than the number of female subjects ($p < 0.05$) (Table 1).

Colorectal cancers were most commonly localized in the rectum (35.2%), which is statistically a more frequent localization compared to the sigmoid colon and other less represented localizations ($p < 0.001$). The most prevalent histological type conventional adenocarcinoma was present in 116 patients (81.7%) and was statistically far more present than other histological types ($p < 0.001$). The histological grade G2 was reported in 111 patients (78.2%) and was statistically more common than the histological grade G3 ($p < 0.01$). The depth of invasion T3 was present in 96 patients (67.6%) and was statistically more frequent than other depth of

invasion categories individually ($p < 0.001$). Metastases to regional lymph nodes were present in just over half of patients – 72 (50.7%), and compared to the total number of patients they averaged 2.6 ± 4.7 , with very high standard deviation given that the number of metastases to regional lymph nodes

ranged from 0 to 27. Stage III was the most common stage and was reported in 72 patients (50.7%), which was statistically more frequent compared to other stages individually. Clinical and pathological features are shown in Table 2.

Table 1. Demographic characteristics of subjects

Gender	n	%	Range
Female	53	37.3	
Male	89	62.7*	
Age	67.4	± 10.0	(22–88)

* $p < 0.05$

Table 2. Clinicopathological characteristics of the tumours

Localization	n	%	
Caecum	22	15.5	
Ascending colon	15	10.6	
Transverse colon	8	5.6	
Descending colon	6	4.2	
Sigmoid colon	41	28.9	
Rectum	50	35.2	***
Histological type			
Adenocarcinoma Conventional	116	81.7	***
Mucinous Adenocarcinoma	21	14.8	
Mixed Adenoneuroendocrine carcinoma	2	1.4	
Signet ring cell carcinoma	3	2.1	
Histological grade			
G2	111	78.2	**
G3	31	21.8	
Depth of invasion			
T1	1	0.7	
T2	24	16.9	
T3	96	67.6	***
T4a	14	9.9	
T4b	7	4.9	
Metastases to regional lymph nodes	72	50.7	
Average number of metastases to regional lymph nodes ^a	2.6	± 4.7	
TNM stage			
I	18	12.7	
II	45	31.7	
III	72	50.7	***
IV	7	4.9	

** $p < 0.01$, *** $p < 0.001$ (χ^2 test)

Lymphovascular invasion is the presence of single tumor cells or small cluster in a space lined by endothelial cells and/or containing erythrocytes surrounding the tumor cells. Vascular and lymphatic invasion in colorectal cancer (Figure 1 and 2).

Perineural invasion was detected in all neural

components of nerve fascicles, usually in form of single cancer glands, or less often as groups or single cancer cells (Figure 3).

Tumor buds were detected as the presence of single cells or small clusters of cancer cells at the invasive front of a tumor (Figure 4).

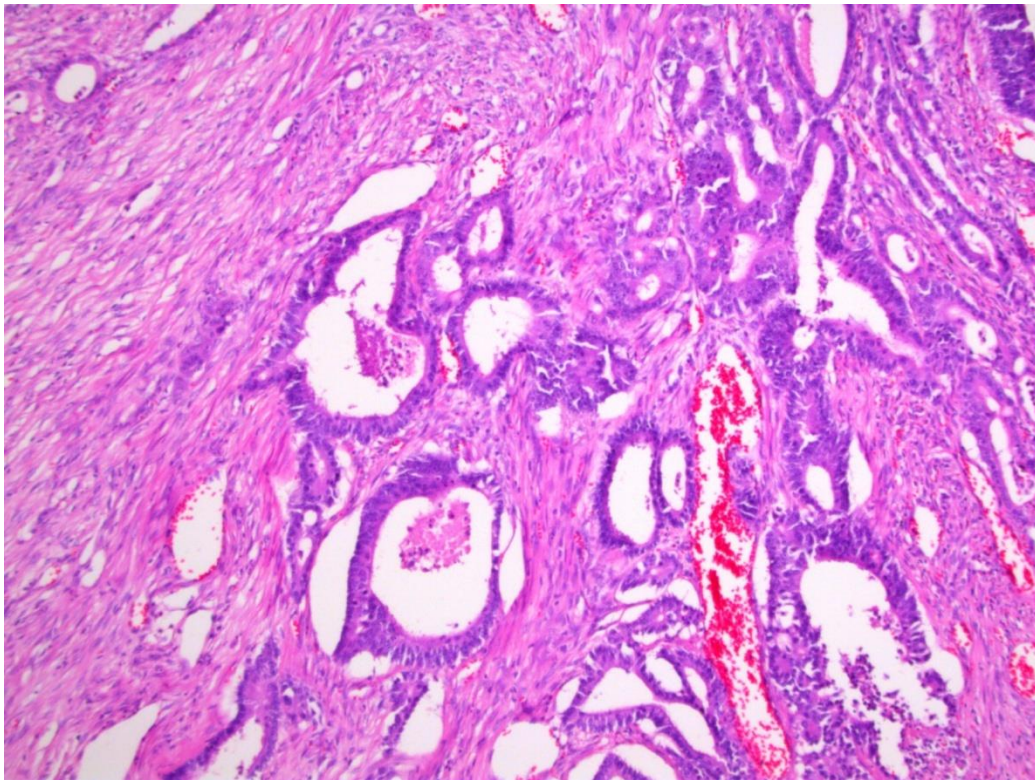


Figure 1. Intravascular invasion. Presence of cancer cell cluster in lumen of small vein. (HE, x200)

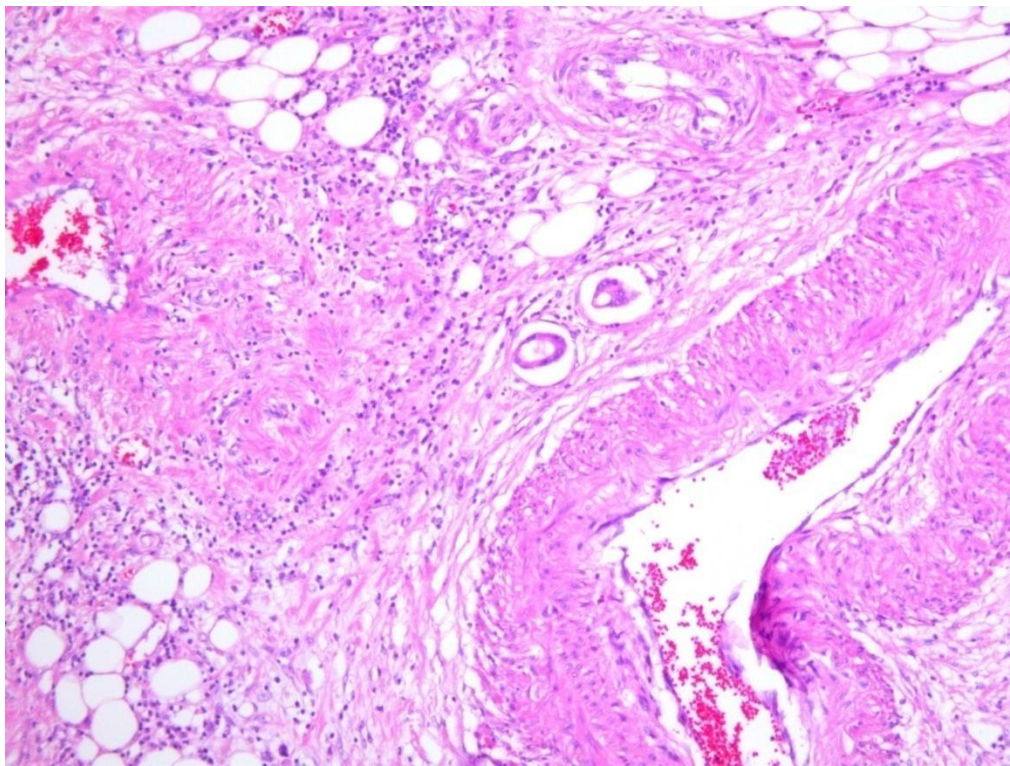


Figure 2. Cancer invasion of lymph vessels. Lumina of small lymphatic vessels occupied with cancer cell clusters. (HE, x200)

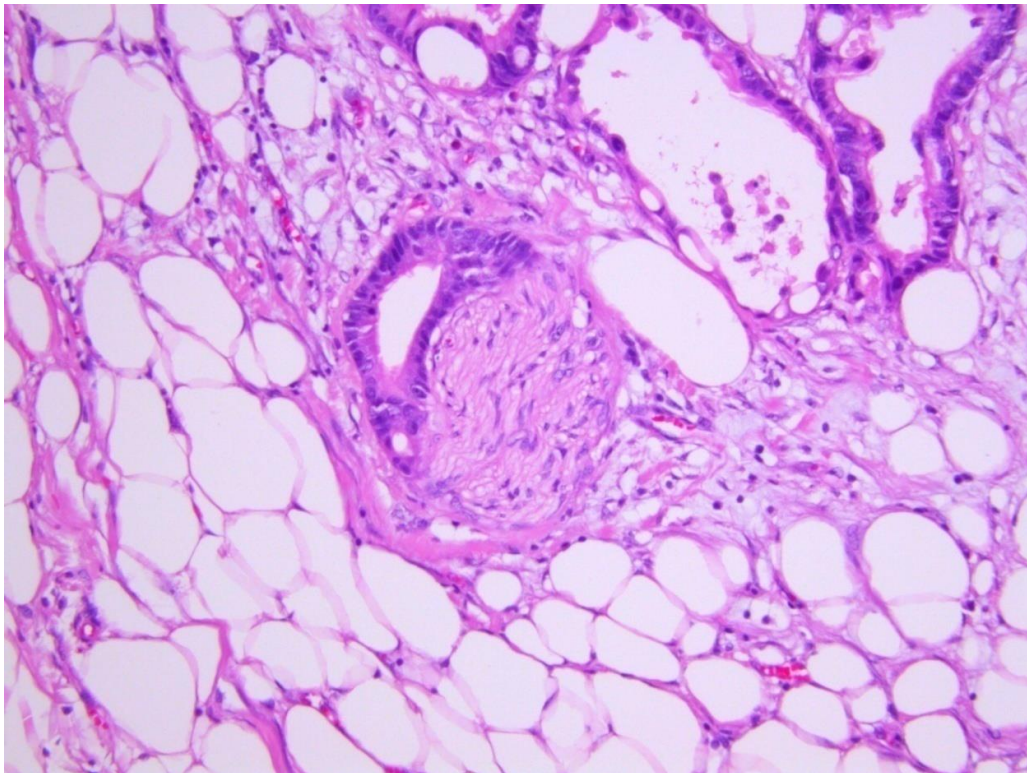


Figure 3. The most usually finding of PN was the juxtaosition of cancer glands to perineurium of nerve fascicles. (HE, x200)

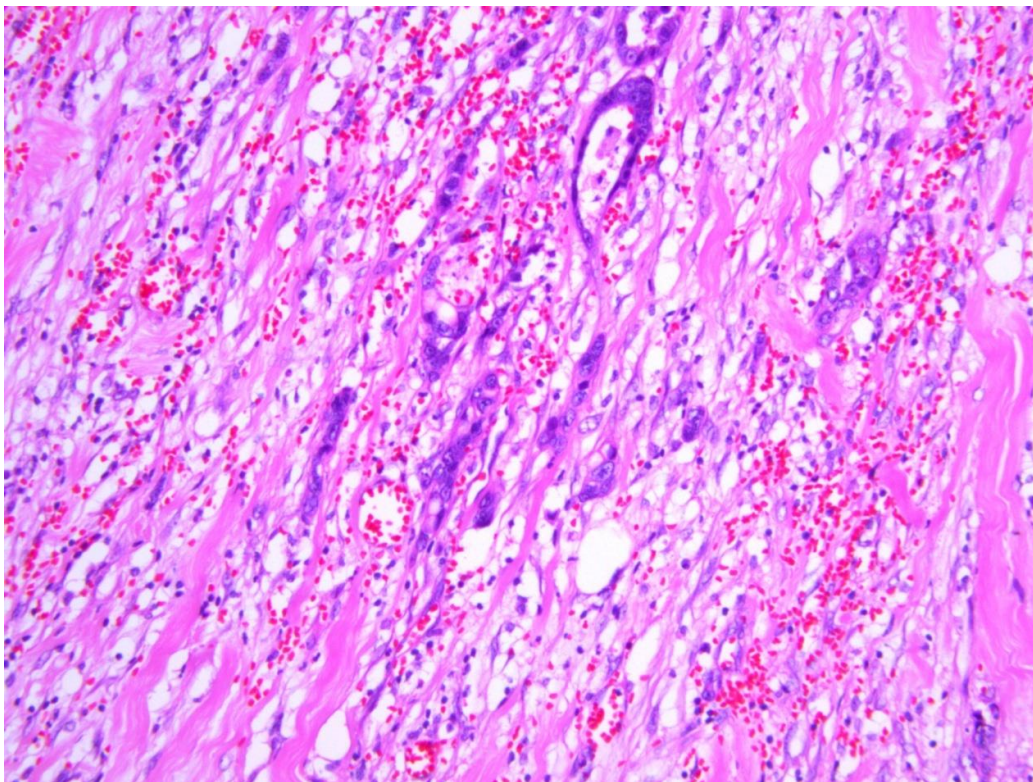


Figure 4. High grade tumor budding. Cancer cell strands, small clusters or single cells, extending from tumor periphery toward inflammatory reactive surrounding. (HE, x200).

Perineural invasion was found in 29 patients (20.4%). It was statistically considerably less common compared to lymphovascular invasion, reported in 57 patients (40.2%), or high grade tumor budding, reported in 63 patients ($p < 0.001$). The presence of lymphovascular invasion, perineural invasion and high grade tumour budding are shown in Table 3.

The univariate logistic regression analysis found that the odds ratio for the presence and absence of lymphovascular invasion showed a 13.8-fold increase in stage III or higher (5.6 to 34.2; $p < 0.001$). The relationship between lymphovascular invasion and TNM advanced stage are shown in Table 4.

The univariate logistic regression analysis found that the odds ratio for the presence and absence of perineural invasion showed a 6.8-fold increase in stage III or higher (2.2 to 20.9; $p < 0.001$). The relationship between perineural invasion and TNM advanced stage are shown in Table 5.

The univariate logistic regression analysis found that the odds ratio for low and high-grade tumor budding showed a 2.3-fold increase in stage III or higher (1.1 to 4.5; $p < 0.05$). The relationship between tumor budding and TNM advanced stage are shown in Table 6.

Table 3. The presence of lymphovascular invasion, perineural invasion, and high grade tumor budding

Lymphovascular invasion	57	40.2%	***
Perineural invasion	29	20.4%	
High grade tumor budding	63	44.4%	***

*** $p < 0.001$ (χ^2 test)

Table 4. The assessment of the stage on the presence of lymphovascular invasion – results of the univariate logistic regression analysis

Factor	OR	Limits 95%		p
		Lower	Upper	
Stage \geq III	13.8	5.6	34.2	< 0.001

Table 5. The assessment of the stage on the presence of perineural invasion – results of the univariate logistic regression analysis

Factor	OR	Limits 95%		p
		Lower	Upper	
Stage \geq III	6.8	2.2	20.9	0.0008

Table 6. The assessment of the stage on the presence of tumor budding – results of the univariate logistic regression analysis

Factor	OR	Limits 95%		p
		Lower	Upper	
Stage \geq III	2.3	1.1	4.5	0.019

Discussion

Colorectal carcinoma is one of the leading health problems in the world (1, 2). It occurs in elderly people after the age of 60 and is more common in men than in women. In our study, the mean age of patients was 67.4 ± 10.0 years, with age ranging from 22 to 88 years. The overall specimen is dominated by male patients (62.7%), who statistically significantly outnumbered female patients ($p < 0.05$). The largest number of these cancers was developed in the wall of the rectum, which is consistent with literature data (3).

Conventional adenocarcinomas were diagnosed in 81.7% of patients, whereas mucinous adenocarcinoma was reported in about 14.8% of patients, which is in accordance with the literature data (24, 25). Mucinous adenocarcinoma is a distinct subtype and is characterized by abundant mucinous components that comprise at least 50% of the tumor volume (26). In our study, 78.2% of colorectal carcinomas were of histology grade G2, and 21.8% of histology grade G3. The largest number of colorectal carcinomas was in stage III and II, 50.7% and 31.7%, respectively, whereas 4.9% of carcinomas were in stage IV. Stage I carcinomas amounted to only 12.7%, indicating that early diagnosis and screening for colorectal carcinoma were inadequate.

Tumor budding has been found to be an independent adverse prognostic factor in CRC and is a strong predictor of lymph node involvement, venous and lymphatic invasion, local recurrence, metastases and poor disease free survival (27), and in addition to lymphovascular and perineural invasion, it is a factor indicating more aggressive tumor behavior (21, 28, 29, 30). It has been observed that in two same-stage carcinomas, the one with tumor budding will exhibit more aggressive behavior (30). After the examination of our research material, high grade tumor budding was found in 63 patients (44.4%). Furthermore, a statistically significant correlation was discovered between high grade tumor budding and higher stages of colorectal carcinoma (31). However, recognition of tumor budding on H&E may occasionally present some challenges because of the presence of reactive stromal cells or histiocytes surrounding the invasive front of tumor and cytokeratin immunostaining can be performed to confirm the impression of tumor buds (32).

Lymphovascular invasion is defined as the presence of single cancer cells or cancer cell clusters within an endothelial lined channel surrounding smooth muscle or elastic lamina (33). The reporting of vascular invasion is highly variable and under-reported, due to the interobserver variability (34), with the incidence of venous invasion reported between 11% and 89.5% (15). Lymphovascular invasion is one of the significant factors indicating the biological aggressiveness of tumors (11–13, 33, 35, 36). In our study, lymphovascular invasion was present in 57 patients (40.1%) and showed statistical

significance in relation to the advanced stage of the disease.

Perineural invasion (PNI) is a continuous and multistep process of interaction developing between nerve structures and cancer cells (37, 38). Nerve cells and tumor cells can interact directly or through the opening and closing of the signal transduction pathways and/or the recognition and response of the ligands and receptors. Schwann cells mediate perineural invasion. Dedifferentiated Schwann cells come into direct contact with cancer cells. This direct contact results in the extension of protrusions from the cancer cells. Schwann cells intercalate between cancer cells, thereby promoting cancer dispersal from the tumor and migration toward the neural fascicles. Perineural invasion is an indicator of poor prognosis (39, 40). A recent large meta-analysis has shown that perineural invasion represents an independent prognostic factor and that stage II tumor patients with perineural invasion have poorer survival than stage III tumor patients without perineural invasion (24). PNI is associated with other pathological markers of poor prognosis such as lymphovascular invasion, poor differentiation and tumor budding (41). Our study confirmed perineural invasion in 29 patients (20.4%) and a statistically significant association with higher stages of the disease (16, 42).

Authors suggest that the TNM staging most accurately determines the prognosis of colorectal carcinoma (43), we find that additional diagnostic parameters, such as tumor budding, lymphovascular and perineural invasion are in strong correlation with advanced tumor stages. Tumor budding should be included in routine diagnostics, for a more accurate therapy and better patient follow-up.

Conclusion

In evaluated histopathology cases of colorectal cancers, lymphovascular invasion and tumor budding were statistically significantly often findings, than perineural invasion. The univariate logistic regression analysis revealed a statistically significant correlation between tumor budding, lymphovascular and perineural invasion and higher tumor stages in colorectal carcinoma. The ITBCC evaluation method should be used to assess tumor budding and may form the basis of a new staging system in patient with colorectal cancer.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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Originalni rad

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doi:10.5633/amm.2020.0204**PREDIKTIVNI ZNAČAJ TUMORSKOG PUPLJENJA, LIMFOVASKULARNE I PERINEURALNE INVAZIJE KOD KOLOREKTALNOG KARCINOMA***Tijana Denčić^{1,5}, Maja Jovičić-Milentijević^{1,5}, Aleksandar Petrović², Goran Radenković², Marko Jović², Sonja Šalinger-Martinović^{3,6}, Simona Stojanović⁴*¹Univerzitet u Nišu, Medicinski fakultet, Katedra za patologiju, Niš, Srbija²Univerzitet u Nišu, Medicinski fakultet, Katedra za histologiju i embriologiju, Niš, Srbija³Univerzitet u Nišu, Medicinski fakultet, Katedra za internu medicinu i zdravstvenu negu, Niš, Srbija⁴Univerzitet u Nišu, Medicinski fakultet, Katedra za oralnu hirurgiju, Niš, Srbija⁵Klinički centar Niš, Centar za patologiju i patološku anatomiju, Niš, Srbija⁶Klinički centar Niš, Klinika za kardiovaskularne bolesti, Niš, Srbija*Kontakt:* Tijana V. Denčić

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Kolorektalni karcinomi predstavljaju jedan od čestih uzroka oboljevanja i smrtnog ishoda u svetu. Na globalnom nivou, nalazi se na šestom mestu kod muškaraca i na četvrtom mestu kod žena. Kolorektalni karcinomi predstavljaju heterogenu grupu tumora, koji mogu da nastanu na osnovu nekoliko alternativnih genetskih puteva, od kojih svaki uključuje različite kombinacije genetskih i epigenetskih promena. Mnogi autori navode to da TNM stadijum najpreciznije određuje prognozu kolorektalnog karcinoma, međutim, potrebni su i drugi parametri. Osim TNM stadijuma, postoje i drugi prognostički faktori koji ukazuju na agresivno ponašanje tumora. Takvi parametri su limfovaskularna invazija, perineuralna invazija i tumorsko pupljenje. Obrađena su 142 bolesnika sa kolorektalnim karcinomom, koja su operisana na Hirurškoj klinici u Nišu. Cilj rada je da se utvrdi da li postoji povezanost tumorskog pupljenja, limfovaskularne invazije i perineuralne invazije, u odnosu na stadijum tumorske bolesti kod kolorektalnih karcinoma. Stadijum tumorske bolesti određen je na osnovu TNM klasifikacije preporučene od strane WHO i AJCC. U ovom radu, univarijantnom logističkom regresionom analizom, nađena je statistički značajna povezanost tumorskog pupljenja, limfovaskularne i perineuralne invazije sa uznapređovalim stadijumom tumorske bolesti.

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Ključne reči: kolorektalni karcinom, tumorsko pupljenje, limfovaskularna invazija, perineuralna invazija

PREDICTIVE IMPORTANCE OF MORPHOMETRIC ANALYSIS OF TRIPLE-NEGATIVE BREAST CANCER

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Triple-negative breast cancers denote malignant epithelial tumors showing complete hormonal independence with negative HER2 expression. Histologically, in most cases these are high-grade tumors, showing fields of central necrosis, lymphocytic infiltration, and fibrosis. The aim of the study was to examine morphometric parameters related to nuclear size depending on the type of carcinoma, as well as tumor proliferation. The entire research was conducted at the Center for Pathology and Pathological Anatomy, Clinical Center Niš. Sixty-four biopsy samples of triple-negative breast cancers were analysed, including 40 ductal, 6 lobular, 6 medullary, 4 ductulolobular, 4 metaplastic, 2 adenoid cystic and 2 apocrine carcinomas. The morphometric analysis was performed in the software package "ImageJ" version 1.52a. The statistical analysis of data was done in the software package SPSS 15.0. By comparing the values of the studied morphometric parameters, statistically significantly higher parameter values for Area, Perim and Feret were found in the group of medullary carcinomas, as well as the parameters for Integrated Optical Density. The value of integrated optical density was also very high in the ductal carcinoma group, but with no statistically significant differences due to high standard deviation. Metaplastic carcinoma showed the highest proliferative activity. Numerous similar studies have been trying to identify a specific marker of these carcinomas, which is still a challenge due to its aggressiveness. These are high-grade tumors with a broad spectrum of polymorphisms, usually with an overlapping morphological presentation, therefore, additional analyses are required in order to set adequate diagnosis.

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Key words: triple-negative breast cancer, immunohistochemistry, morphometry

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Introduction

Breast cancer is characterised by a wide range of morphological characteristics, unpredictable biological behaviour, various forms of presentation, sometimes unclear clinical evolution and generally

good response to therapy. According to the data of the American Cancer Society (ACS), as many as 25% of all cancers detected in women are breast cancers, accounting for about 1.5 million newly discovered cases on a global level, per annum. So far, the disease has been considered a disease of the modern world, but nearly 50% of cancers, with a mortality rate of around 58%, are detected in underdeveloped and developing countries. Given the insidious clinical course, most of them are detected in late stages of development when therapeutic options are limited, which results in low survival rates. In recent years, however, especially in developed countries, there has been a slight decline in the mortality rate owing to the coordinated work of the health service at all levels of health care and the acceptance of breast cancer as one of the key global health problems of today (1).

Considering the heterogeneity of breast cancer as a disease, major subtypes and classifications can be based on histopathological features, tumor grade, stage, receptor status, and gene expression (2).

Depending on whether there is a puncture of the basal membrane or not, all carcinomas can be divided into noninvasive (ductal and lobular carci-

noma in situ) and invasive where, due to the membrane puncture, we encounter stromal infiltration with the possibility of metastasis (invasive ductal carcinoma, invasive lobular carcinoma, medullary, colloid or mucinous, tubular carcinoma, etc.).

The histologic grading of breast cancer is nowadays commonly done by using the so-called Nottingham modification (Elston-Ellis) of the Bloom-Richardson grading system, in which the degree of tubule formation, nuclear polymorphism, and mitotic activity are taken into account for the assessment. Based on these criteria, both nuclear and histological, all carcinomas can be divided into 3 grades as well (Grade 1, Score 3 and 4), which deviate from normal cells the least, moderately (Grade 2, Score 5, 6 and 7) and poorly (Grade 3, Score 8 and 9) differentiated, which show the highest degree of polymorphism. Tumor grading is important not only because it can provide prognostic information, but because it is also a factor which decides on the therapeutic approach (3, 4).

First of all, from a clinical point of view, the TNM classification is very important. It is based on the assessment of tumor size and the degree of its invasion of surrounding breast tissue (T), the degree of involvement of regional lymph nodes (N), and the presence or absence of distant metastases (M), providing information that enables determining the stage of a tumor disease. When T, N and M are determined, the corresponding stage 0, I, II, III or IV is assigned (5, 6).

Local-regional recurrence of the disease has been described as ipsilateral, in the breast itself after lumpectomy, on the chest wall after mastectomy, or recurrent in the ipsilateral axilla, or supraclavicular lymph nodes (much less frequently in infraclavicular and intramammary lymph nodes). About 10-15% of patients with stage I and II tumor disease will develop recurrent disease after breast-conserving surgery and radiation therapy, while 10-20% of patients with stage I-IIIa will develop recurrent chest disease after mastectomy. Several factors are related to the biological behaviour of breast cancer, as well as to local-regional recurrence. They most commonly include lymphovascular invasion, younger patients, tumor growth rate, tumor margin resection status (tumor distance from the nearest resection margin), positive nodal status, high tumor grade, extensive in situ component, tumor multifocality and multicentricity, hormone-independent tumors, poor response to adjuvant therapy (7-10).

Defining biological characteristics of breast cancer can be facilitated by the characterization of different histologic types of tumors, understanding of the disease prognosis and the systemic therapy plan. The immunohistochemical analysis of protein expression in carcinoma cells is nowadays routinely used. Considering a wide range of immunohistochemical markers, ER, PR, HER2, and Ki67 have certainly seen the greatest use in prognosis and prediction.

Tumors that show expression of estrogen and progesterone receptors are referred to as hormone-dependent tumors. A positive immunohistochemical

reaction is used to interpret nuclear expression. About 80% of all breast tumors are ER+, and about 65% are PR+ (11). The importance of determining these receptors is seen in the choice of therapy, which, in case of their expression, specifically targets these antigens, i.e. hormone therapy, but also that tumors that are hormone-independent are very often of higher grade, with a significantly worse prognosis. Numerous genetic studies have identified a certain association between the overexpression of the HER2 oncogene which belongs to the group of epidermal growth factors and its product in the form of a transmembrane protein, and high-grade tumors that exhibit expansive and aggressive growth. Its overexpression occurs in 15-20% of breast cancer cases (12, 13). Numerous clinical studies have shown that tumors labelled as HER2+ tumors are high-grade tumors of high metastatic potential and poor prognosis. Ki67 is another marker that, if high, indicates a worse prognosis, and a high degree of malignant cell division. Considering these biological markers, that is, the presence or absence of ER and PR receptors, as well as HER2 overexpression, the following subtypes have been identified:

1. Luminal A (ER+ and/or PR+, HER2-, low Ki-67 proliferative index level). These tumors are lower-grade tumors (1 and 2), generally showing a slow growth tendency, indolent flow, and are less aggressive than other subtypes. They are characterised by the best prognosis and the best response to therapy. This group includes ductal invasive (no special type-NST), classic lobular invasive, tubular, cribriform, mucinous and neuroendocrine tumor. A cut-off value of 20% is used to estimate the height of the Ki67 proliferative index.

2. Luminal B (ER+ and/or PR+, HER2-/++ and high Ki-67 proliferative index level). This tumor group includes higher-grade carcinomas (2 and 3), with more aggressive and faster growth rates than Luminal A, with more frequent metastases in regional lymph nodes, as well as with more frequent recurrences.

3. HER2 positive non-luminal type (ER-, PR- and HER2+). Although these are highly aggressive tumors (mostly grade 3) with a high incidence of distant metastases, targeted Trastuzumab therapy has made significant progress in the therapeutic approach.

4. Triple-negative (ER-, PR-, HER2-). Despite their simple definition, from a morphological, genetic and clinical point of view, they are the most heterogeneous category of breast cancer. The most common is invasive ductal carcinoma (NST), followed by metaplastic carcinoma, carcinoma with medullary characteristics (medullary carcinoma), carcinoma with apocrine characteristics (apocrine carcinoma), secretory carcinoma and adenoid cystic carcinoma. Gene expression profiling has enabled the division of this group of tumors into different, prognostically essential subgroups: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR). BL1 breast cancer is most commonly invasive ductal NST with a

high Ki67 proliferative index and basal cytokeratin gene expression (CK5/6, CK14, CK17). BL2 tumors are also most commonly manifested as invasive ductal carcinomas of NST with basal cytokeratin expression, TP63, and growth factor signalling (EGF and IGFR1 pathways). The immunomodulatory subtype overlaps with carcinoma with medullary features containing rich lymphocytic infiltrate. Mesenchymal and mesenchymal stem-like subtypes are clinically and morphologically presented as metaplastic carcinoma characterized by both epithelial and mesenchymal differentiation. These are tumors enriched with genes important for the cell cycle and signal pathways of the growth factor. The LAR subtype is presented as carcinoma with apocrine differentiation which correlates with a molecular apocrine type. This carcinoma is characterized by luminal cytokeratin expression as well as the expression of androgen receptors and mRNA, which further explains their hormonal dependence (7, 14-19).

The aim

The aim of the study was to examine morphometric parameters of triple-negative breast cancers, related to nuclear size depending on the type of carcinoma, as well as tumor proliferation.

Materials and methods

The entire study was conducted at the Center of Pathology and Pathological Anatomy, Clinical Center Niš. The analysed material included tissue obtained by surgery and biopsy procedure of breast tumors from the Clinic of Surgery of the Clinical Center in Niš. All analysed patients were female. After the intervention, the tissue was fixed in 10% formalin for at least 24 hours. The microscopic analysis of the obtained preparations, stained with the standard hematoxylin-eosin method, set basic diagnostics. At the same time, a representative sample was determined, i.e. tumor field area with minimal necrosis fields and inflammatory infiltrate on which immunohistochemical staining was performed. Considering the high polymorphism of breast cancer, another of the parameters for the selection of the sample was the most polymorphic image on the histopathological preparation. Healthy breast tissue served as a positive, internal control. Ductal epithelial cells showed positive ER and PR expression, i.e. negative HER2 expression.

Sixty-four biopsy samples of triple-negative breast cancers were analysed, including 40 ductal, 6 lobular, 6 medullary, 4 ductulolobular, 4 metaplastic, 2 adenoid cystic and 2 apocrine carcinomas.

Immunohistochemical analysis

Within the standard analysis of steroid receptors, as well as HER2 oncoproteins, a group of triple-negative cancers was separated. The expression of Ki67, a marker of tumor proliferation, was analysed.

Immunohistochemical staining was performed on samples up to 5 µm of thickness obtained from

paraffin blocks. The representative sample was taken for analysis and the following antibodies were used: anti-ER (EP1, ready to use; DAKO, Glostrup, Denmark), anti-PgR (PgR636, ready to use; DAKO, Glostrup, Denmark), anti-HER-2 (HerceptTest™, DAKO, Glostrup, Denmark) and anti-Ki67 (MiB-1, ready to use; DAKO, Glostrup, Denmark).

Nuclear staining for estrogen and progesterone is considered a positive reaction. Adequate scoring was performed, relative to the intensity (0-3) and area (0-5) of the expression in the tumor tissue. Overall, a score of 8 suggested complete, high hormonal dependence, whereas a score of 0 suggested there was no reaction. Our samples were negative.

The Ki67 index was determined by analysing and counting positive nuclei on 10 visible fields, at magnification x40. The index was expressed as a percentage, as the ratio of positive tumor cells in relation to negative, unstained cells.

Morphometric analysis

The morphometric analysis was performed in the software package "ImageJ" version 1.52a (public domain software, Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA). Color microphotographs were obtained using a high-resolution digital camera (Nikon, DS-Fi1, Tokyo, Japan) connected to the microscope (Nikon, ECLIPSE 50i, Tokyo, Japan). After that, the image was transferred to a compatible computer and nuclear parameters were analysed using the package. The 8-bit image was manually processed, after calibration, using a computer mouse. 100 randomly selected tumor-cell nuclei of non-overlapping cells were analysed at magnification x40. The following parameters were analysed: Area, Perimeter, Feret diameter, all related to nuclear size, and Integrated Optical Density (IOD).

Statistical Data Processing

Continuous parameters were represented by mean values, standard deviations (SD) and medians. The normality of the distribution of continuous variables was determined using the Shapiro-Wilk test. Since the preconditions for using the analysis of variance (ANOVA) were not met, a comparison of the values of continuous variables between all groups was done individually using the Student's t-test of independent samples for normal distributions and the Mann-Whitney test for continuous parameters whose distributions deviated from normal.

Results

The study was conducted on samples of 64 carcinomas, and the histological presentation of triple-negative carcinomas is shown in Figure 1.

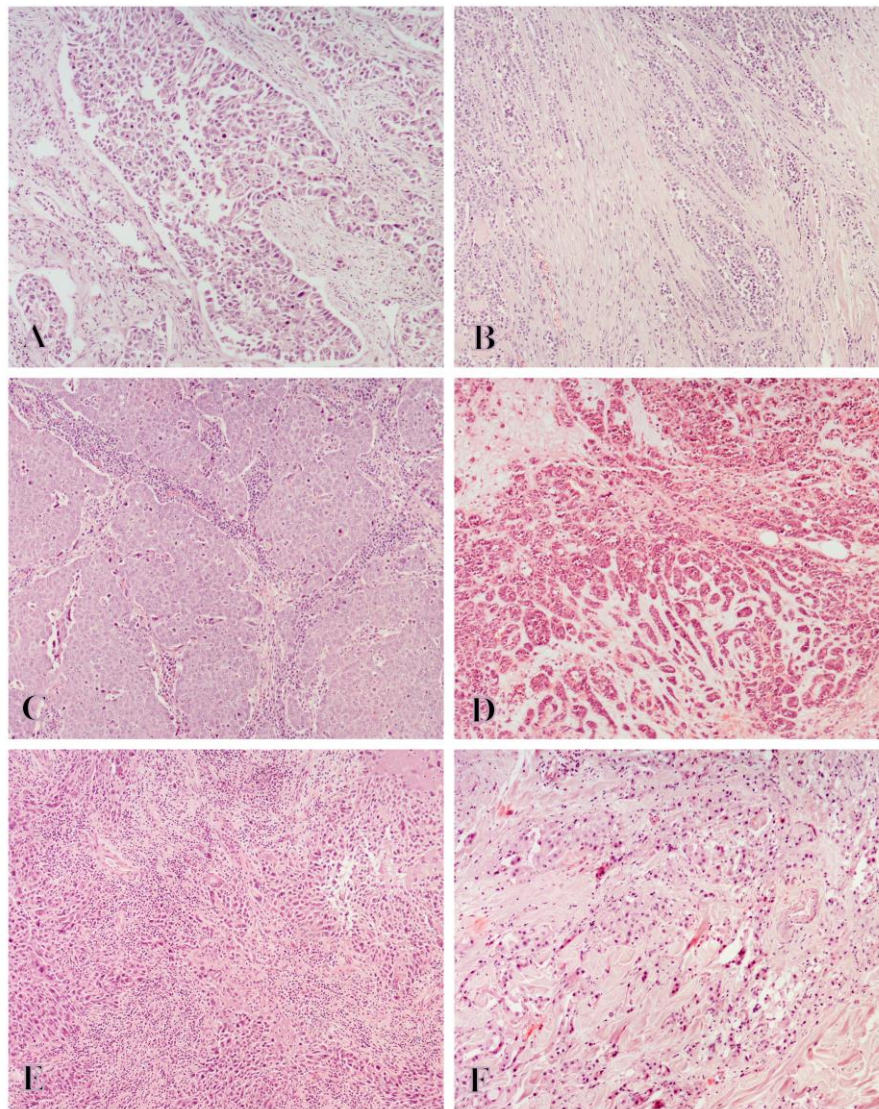


Figure 1. Six subtypes of triple-negative breast carcinoma (H&E; magnification X200);

- A – Ductal carcinoma (NST);
- B – Lobular carcinoma;
- C – Medullary-like carcinoma;
- D – Adenoid cystic carcinoma;
- E – Metaplastic carcinoma;
- F – Apocrine carcinoma.

The highest value of the parameter for nuclear size (Area) was reported in medullary-like carcinoma, statistically significantly higher than in lobular carcinoma ($p < 0.01$) and ductulolobular carcinoma ($p < 0.05$). In ductulolobular carcinoma, Area was also statistically significantly higher than in lobular carcinoma ($p < 0.05$) (Table 1).

The highest value of Perimeter was also reported in medullary-like carcinoma, statistically significantly higher than in lobular carcinoma ($p < 0.01$) and ductulolobular carcinoma ($p < 0.01$). Perimeter in ductulolobular carcinoma was also statistically significantly higher compared to metaplastic carcinoma ($p < 0.05$) (Table 2).

Furthermore, the highest values of Feret diameter were also reported in medullary carcinoma. They were statistically significantly higher compared to the values in lobular carcinoma and metaplastic carcinoma individually ($p < 0.01$) (Table 3).

If we exclude apocrine carcinoma, which was present only in two cases, the next in value is ductal carcinoma NST, which has high standard deviation, followed by medullary carcinoma with low standard deviation and a value of integrated optical density statistically significantly higher than that of lobular carcinoma ($p < 0.05$) (Table 4).

Table 1. Nuclear size (Area) values by carcinoma type

Ductal carcinoma NST	99.21 ± 43.66	(95.32)	
Lobular carcinoma	49.67 ± 14.56	(48.41)	
Ductulolobular carcinoma	95.40 ± 16.67	(95.40)	
Medullary-like carcinoma	98.05 ± 5.87	(97.95)	b** c*
Adenoid cystic carcinoma	68.84 ±	.	
Metaplastic carcinoma	65.74 ± 10.41	(65.74)	
Apocrine carcinoma	84.23 ±	.	

Data are shown as mean ± SD (Me)

* p < 0.05, ** p < 0.01, b - vs Carcinoma lobulare, c - vs Carcinoma ductulolobulare

Table 2. Nuclear perimeter values by carcinoma type

Ductal carcinoma NST	36.17 ± 7.97	(36.14)	
Lobular carcinoma	26.36 ± 3.59	(26.28)	
Ductulolobular carcinoma	36.21 ± 3.13	(36.21)	
Medullary-like carcinoma	36.84 ± 1.24	(37.05)	b**
Adenoid cystic carcinoma	30.62 ±	.	
Metaplastic carcinoma	30.31 ± 2.07	(30.31)	f*
Apocrine carcinoma	34.17 ±	.	

Data are shown as mean ± SD (Me)

* p < 0.05, ** p < 0.01, b - vs Carcinoma lobulare, f - vs Carcinoma ductulolobulare

Table 3. Feret diameter values by carcinoma type

Ductal carcinoma NST	13.16 ± 2.90	(12.92)	
Lobular carcinoma	9.60 ± 1.36	(12.97)	
Ductalolobular carcinoma	12.90 ± 0.88	(12.90)	
Medullary-like carcinoma	13.50 ± 0.33	(13.52)	bf**
Adenoid cystic carcinoma	11.10 ±	.	
Metaplastic carcinoma	11.19 ± 0.42	(11.19)	
Apocrine carcinoma	12.62 ±	.	

Data are shown as mean ± SD (Me)

* p < 0.05, ** p < 0.01, b - vs Carcinoma lobulare, f - vs Carcinoma metaplasticum

Table 4. Integrated optical density values by carcinoma type

Ductal carcinoma NST	34.46 ± 12.90	(32.65)	
Lobular carcinoma	18.93 ± 5.77	(16.48)	
Ductalolobular carcinoma	30.83 ± 3.46	(30.83)	
Medullary-like carcinoma	32.43 ± 2.72	(32.38)	b*
Adenoid cystic carcinoma	25.76 ±	.	
Metaplastic carcinoma	25.70 ± 2.07	(25.70)	
Apocrine carcinoma	32.90 ±	.	

Data are shown as mean ± SD (Me)

* p < 0.05, b - vs Carcinoma lobulare

The highest value of the proliferative Ki67 index was found in metaplastic carcinoma and it was statistically significantly higher than the ones in lobular carcinoma ($p < 0.05$) and ductulolobular carcinoma ($p < 0.01$) (Table 5).

The highest value of the parameter for tumor size was recorded in ductulolobular carcinoma. However, due to the use of different statistical tests

conditioned by the normality of distribution, only the value in ductulolobular carcinoma was found to be statistically significantly higher than that in metaplastic carcinoma ($p < 0.05$) (Table 6).

We excluded apocrine and adenoid cystic carcinoma, which were present only in two cases each, and therefore could not be compared to other carcinomas.

Table 5. Proliferative Ki67 index values by carcinoma type

Ductal carcinoma NST	48.00 ± 11.92	(48.50)	
Lobular carcinoma	44.00 ± 3.61	(45.00)	
Ductalolobular carcinoma	50.00 ± 2.83	(50.00)	
Medullary-like carcinoma	45.33 ± 9.50	(45.00)	
Adenoid cystic carcinoma	40.00 ± .	.	
Metaplastic carcinoma	51.50 ± 2.12	(51.50)	b**c*
Apocrine carcinoma	44.00 ± .	.	

Data are shown as mean ± SD (Me)

* $p < 0.05$, b - vs Carcinoma lobulare, c - vs Carcinoma ductulolobulare

Table 6. Tumor size (pT) by carcinoma type

Ductal carcinoma NST	2.40 ± 1.05	(2.00)	
Lobular carcinoma	3.00 ± 1.00	(3.00)	
Ductalolobular carcinoma	3.00 ± 1.41	(3.00)	e*
Medullary-like carcinoma	2.33 ± 0.58	(2.00)	
Adenoid cystic carcinoma	2.00 ± .	.	
Metaplastic carcinoma	1.00 ± .	.	
Apocrine carcinoma	4.00 ± .	.	

Data are given as mean ± SD (Me)

* $p < 0.05$, e - vs Carcinoma metaplasticum

Discussion

Triple-negative breast cancers denote malignant epithelial tumors showing complete hormonal independence with negative HER2 expression (0, 1+). They were first defined in 2005 and represent 10-15% of all diagnosed carcinomas (20, 21). About 75% of triple-negative cancers express basal markers CK 5/6, CK 14, CK 17, vimentin, cadherin, calponin, S100, p63 (22, 23). Those tumors that are not characterized by the presence of basal antigens but belong to the triple-negative carcinoma subtype generally have better prognosis. Such cases were found in the metaplastic carcinoma group and the adenoid cystic breast carcinoma group. There are numerous features of triple-negative breast cancers that are not found in any other type or subtype. Histologically, in most cases these are high-grade tumors, showing fields of central necrosis, lympho-

cytic infiltration, and fibrosis. In this type of tumors, there is often a discrepancy between the tumor size and the presence of metastases, either regional or distant, where even the smallest tumors were detected at stages when regional lymph nodal involvement was already present. In comparison to other types, more frequent metastases occurred at sites atypical for breast cancer such as visceral organs and soft tissues, unlike luminal tumors that most often metastasize to bone tissue, and brain metastases are identified as one of the most common. Carcinomas that occur more frequently in younger, premenopausal women are very often associated with BRCA1 and BRCA2 mutations, overweight women, and African-American women (24). These tumors are chemosensitive, therefore, chemotherapy is an optional method. Compared to other types, a relapse between 1 and 3 years after the diagnosis is much more common, thus the five-year

survival rate for these carcinomas is very low. Therefore, these tumors are most often poorly differentiated with a high degree of proliferation and a very high metastatic potential (25).

Recent studies have been based on the detection and identification of genetic mutations we encounter in these highly aggressive carcinomas. Lehmann et al. isolated six subtypes of triple-negative breast cancer: basal-like 1 and basal-like 2 (BL1 and BL2), immunomodulatory (IM), mesenchymal, mesenchymal stem-like (MSL) and luminal androgen-like subtype (LAR) (17). The BL1 subtype includes the mutation of genes responsible for the cell cycle and reparation of DNA molecules, whereas the BL2 is characterized by the increased expression of epidermal growth factors. The IM subtype includes genes responsible for the regulation of immune processes, whereas mesenchymal and MSL types include genes responsible for cellular motility and differentiation. The LAR subtype is characterized by an expressed androgen receptor. In the BL1 and BL2 subtypes, mutations of BRCA1 and BRCA2 genes are much more common than in other subtypes (26). After thorough analyses and convincing evidence obtained by examining contributions of immune and mesenchymal cells, Lehmann et al. Revised the classification into 4 subtypes - BL1, BL2, mesenchymal subtype and LAR, and proposed additional testing to confirm with confidence the IM and MSL subtypes as such (27). Burstein et al. classified triple-negative carcinoma into LAR, mesenchymal, basal-like immunosuppressed and basal-like immune activated (28).

Changes in the appearance, structure, and size of the nucleus are some of the basic characteristics of a neoplastic cell (29). Our study was based on this from the start: to examine the morphometric parameters in different types of invasive breast cancers. In everyday clinical practice, a pathologist encounters a challenge, and sometimes a problem in determining the type of tumor, given that they are dealing with a polymorphic histological image. Certain studies have addressed the morphometric analysis and highlighted the importance and applicability in the diagnosis of breast cancer (30-32). The most commonly analysed morphometric parameter is nuclear size (Area). Statistically significant differences in the medullary carcinoma group were obtained for morphometric parameters related to nuclear size. It was also observed that a statistically significant difference was present in the ductulobular carcinoma group with respect to lobular carcinoma. Having analysed nuclear perimeter, a significant difference between ductulobular carcinomas and metaplastic carcinoma was observed. The obtained results are considered to be of great

use in everyday work, in the differentiation of different types of carcinomas. The integrated optical density was the highest in the ductal carcinoma group, but due to high standard deviation there were no statistically significant differences. A statistically significant difference for this morphometric parameter was observed in the group of medullary carcinomas compared to the group of lobular carcinomas. In addition, these morphometric parameters have prognostic significance. In their study, Abdalla et al. specifically highlighted the size of the nucleus in tumor cells. This study suggested nuclear size of $71 \mu\text{m}^2$ a criterion for the classification of tumors into two groups. All tumors with values less than the above can be classified in the group of carcinomas with better prognosis and a longer survival interval. In contrast, the group of tumors whose nuclear size is larger than the criterion above are considered carcinomas with worse prognosis (30, 33).

Metaplastic carcinoma showed the highest proliferative Ki67 activity.

Conclusion

A myriad of similar studies have attempted to identify a specific marker of these carcinomas, which is still a challenge due to their high aggressiveness. These are high-grade tumors with a broad spectrum of polymorphisms, quite often with an overlapping morphological presentation, therefore, additional analyses are required to set adequate diagnosis, as well as further therapeutic procedures. Using current knowledge, the therapy is based on surgical techniques, radiation and chemotherapy, or combinations of both. Regular mammographic examinations are today the most significant measure in preventing not only this type but all types of carcinomas, as well as constant education of women regarding self-examinations and numerous risk factors.

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PREDIKTIVNI ZNAČAJ MORFOMETRIJSKE ANALIZE TRIPL NEGATIVNOG KARCINOMA DOJKE

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Tripl negativni karcinomi dojke predstavljaju maligne epitelne tumore koji pokazuju kompletnu hormonsku nezavisnost od negativne HER2 ekspresije. Histološki, u najvećem broju slučajeva, reč je o tumorima visokog gradusa, koji pokazuju polja centralne nekroze, limfocitnu infiltraciju i fibrozu. Cilj istraživanja zasniva se na ispitivanju morfometrijskih parametara, koji se odnose na veličinu jedra u zavisnosti od tipa karcinoma, kao i na proliferaciju tumora. Celokupno istraživanje sprovedeno je u Centru za patologiju i patološku anatomiju Kliničkog centra Niš. Analizirano je 64 biopsijska uzorka trostruko negativnih karcinoma dojke, i to 40 duktalnih, 6 lobularnih, 6 medularnih, 4 duktulolobularna, 4 metaplastična, 2 adeno- idno cistična i 2 apokrina karcinoma. Morfometrijska analiza vršena je u softverskom paketu „ImageJ” verzija 1.52a. Statistička analiza podataka rađena je u programskom paketu SPSS 15.0. Poređenjem vrednosti ispitivanih morfometrijskih parametara ustanovljene su statistički značajno više vrednosti parametara za površinu, obim i Feretov dijametar uzoraka grupe medularnih karcinoma, kao i parametra za Integrisanu optičku gustinu uzoraka iste grupe. Integrirana optička gustina bila je izrazito visoka i kod uzoraka u grupi duktalnih karcinoma, ali bez statistički značajne razlike zbog visoke standardne devijacije. Najveću proliferativnu aktivnost imao je metaplastični karcinom. Brojna istraživanja, slična ovom, pokušavaju da identifikuju specifičan marker ovih karcinoma, koji zbog velike agresivnosti i danas predstavlja izazov. Reč je o tumorima širokog spektra polimorfizma, visokog gradusa, sa veoma često preklapajućom morfološkom prezentacijom, pa su dodatne analize neophodne u cilju postavljanja adekvatne dijagnoze.

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Ključne reči: tripl negativni karcinom dojke, imunohistohemija, morfometrija

CORRELATION BETWEEN DILATATION OF THE BILE DUCTS AND OXIDATIVE STRESS IN PATIENTS WITH CHOLEDOCHOLITHIASIS

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The aim of this study was to examine the relationship of inflammatory parameters, lipid peroxidation and oxidative modification of proteins with ultrasound findings of biliary tract in patients with choledocholithiasis. The study included 70 subjects divided into two groups: the choledocholithiasis group (CHDL)-40 patients with obstructive jaundice caused by choledocholithiasis and the control group-30 healthy individuals. All the patients were anamnesticallly and clinically observed at the Internal Department of Military Hospital in Niš, Serbia.

The values of MDA were not significantly correlated with the degree of dilatation of intrahepatic and extrahepatic bile ducts, while the values of carbonyl groups showed a significant correlation with the degree of dilatation of intrahepatic bile ducts ($p < 0.05$). Dilatation of bile ducts was not significantly associated with inflammation and lipid peroxidation, however, oxidative modification of the protein showed a significant positive correlation with dilatation of the intrahepatic bile ducts.

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Key words: oxidative stress, choledocholithiasis, ultrasound, biliary ducts

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Introduction

Procedures used in the morphological diagnosis of extrahepatic caused obstructive jaundice are: ultrasonography (US), computed tomography (CT), magnetic resonance (NMR) and magnetic cholangiography (MRCP). Direct visualization of the biliary tract, either with cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC) is still the gold standard in the presentation of the biliary tract (1). Ultrasound as the cheapest, safest

and most sensitive technique for displaying hepatobiliary-pancreatic system represents an ideal screening method. This method is valid for discovering the cause of extrahepatic cholestasis in a wide variety of etiological cause. Extrahepatic obstruction suggests a dilatation of extrahepatic bile ducts, but displaying normal extrahepatic bile ducts cannot totally exclude the presence of obstruction (2).

Inflammation, cholestasis and oxidative stress are following disorders of choledocholithiasis (3-5). Previous studies in patients with choledocholithiasis showed a positive correlation between the number of monocytes and the activities of aspartate transaminase (AST), hyperbilirubinemia, and fibrinogen and albumin and alanine transaminase (ALT), while a negative correlation was observed between albumin and alkaline phosphatase (AP) (6). Significant positive correlation between the total, direct and indirect bilirubin with oxidative modification of proteins was proven, whereas neutrophils and the levels of total, direct and indirect bilirubin evaluation showed a significant positive correlation with lipid peroxidation (7, 8).

There are no literature data that show the correlation of the parameters of inflammation and oxidative stress with ultrasound findings of biliary tract in patients with choledocholithiasis. Therefore, the aim of this study was to examine the correlation of inflammatory parameters, lipid peroxidation and oxidative modification of proteins with ultrasound

findings of biliary tract in patients with choledocholithiasis.

Patients and methods

The study included 70 subjects divided into two groups: the choledocholithiasis group (CHDL)-40 patients with obstructive jaundice caused by choledocholithiasis and the control group-30 healthy individuals.

The patients with extrahepatic cholestasis due to mechanical obstruction caused by choledocholithiasis were included in the study. The obstruction of biliary ducts caused by other factors was not considered.

The diagnosis of obstructive icterus was made according to anamnestic data, clinical features, and biochemical and ultrasound examination of biliary ducts. For the ultrasound examination of biliary ducts in the supine position a Sono et Medison Co. Ltd. ultrasound was used. Based on ultrasound, examination was performed to evaluate the level of bile duct dilatation in two categories: dilatation of extrahepatic bile duct (DEBD) and dilatation of intrahepatic bile ducts (DIBD).

All the patients were anamnestically and clinically observed at the Internal Department of Military Hospital in Niš, Serbia. Basic biochemical indicators and parameters of oxidative stress were determined in Biochemical Laboratory of Military Hospital in Niš and the Laboratory of the Biochemistry Institute of the Faculty of Medicine in Niš.

All the patients with choledocholithiasis were tested in the first three days since the occurrence of cholestasis syndrome and before surgery or endoscopic retrograde cholangiopancreatography (ERCP) with papillotomy.

Participants of both groups did not differ in gender and age structure. Out of the total number of studied subjects, 37 (53%) were men and 33 (47%) women. The average age of the patients was 58.8 ± 15.9 years.

Biochemical analysis

Inflammatory and cholestatic parameters: activity of γ -glutamyltransferase (γ -GT), alkaline phosphatase, level of total, direct and indirect bilirubin, aspartate aminotransferase, alanine aminotransferase, albumin (Alb), fibrinogen (Fib), C-reactive protein (CRP), leukocytes (Leu), lymphocytes (Ly), monocytes (Mo) and granulocytes (Gr). The previously mentioned biochemical parameters were determined by the ready tests produced by Elitech Company, on the biochemical analyzer BTS-370 (Biosystem).

Assessment of oxidative protein modification products in form of carbonyl groups (CG), was made by using a colorimetric reaction with 2,4-dinitrophenylhydrazine (2,4 DNPH) and TCA (9). Assessment of carbonyl groups in amino acid residue is an im-

portant marker of oxidative protein modification. Concentration of carbonyl groups was expressed in $\mu\text{mol/g}$ plasma protein.

The intensity of lipid peroxidation in the plasma was measured spectrophotometrically, and based on the thiobarbituric response products as described by Ohkawa et al. (10). Malondialdehyde (MDA - lipid peroxidation end product) concentration was expressed as $\mu\text{mol/l}$, using the MDA molecular absorbance coefficient ($1.56 \times 10^{-5} \text{ mol cm}^{-1}$).

Statistical analysis

The data were analyzed by means of the commercially available statistic software package (SPSS® for Windows, v. 9.0, Chicago, USA) using the Student's t-test and Chi-square test. The results were presented as means \pm SD. Statistical significance was set to $p < 0.05$. To demonstrate the correlation between the parameters of inflammation and oxidative stress with ultrasound findings of the biliary tract in patients with choledocholithiasis Pearson's correlation coefficient was used (r).

Results

The results of laboratory parameters in control group and patients with choledocholithiasis are shown in Table 1.

Statistically significant difference of albumin ($p < 0.001$) and higher values of fibrinogen ($p < 0.05$) and CRP ($p < 0.001$) were in the group of patients with choledocholithiasis compared to the control group. Higher values of leukocytes ($p < 0.01$) and granulocytes ($p < 0.001$), with a decrease in the number of lymphocytes ($p < 0.001$), and monocytes ($p < 0.001$) were found in the group of patients with choledocholithiasis compared to the control group. Intensity of oxidative protein modification was followed by a change in the levels of carbonyl groups, which were statistically significantly higher in patients with choledocholithiasis ($p < 0.001$) compared to the control group of patients. The values of MDA were significantly increased ($p < 0.001$) in patients with extrahepatic cholestasis caused by choledocholithiasis, compared to the control group.

Correlation between indicators of inflammation and oxidative stress with ultrasound findings of the biliary tract in patients with choledocholithiasis is shown in Table 2.

In patients with choledocholithiasis, dilatation of intra and extrahepatic bile ducts did not significantly correlate with the systemic indicators of inflammation. The values of MDA were not significantly correlated with the degree of dilatation of intrahepatic and extrahepatic bile ducts, while the values of carbonyl groups showed a significant correlation with the degree of dilatation of intrahepatic bile ducts ($p < 0.05$).

Table 1. The results of laboratory parameters in the control group and patients with choledocholithiasis

Parameter	Control	CHDL
Albumin (g/l)	46.1 ± 4.3	36.7 ± 6.6***
Fibrinogen (g/l)	3.5 ± 1.1	5.1 ± 1.2*
C-reactive protein (mg/dl)	4.7 ± 1.3	11.2 ± 7.1***
Leukocytes (G/l)	6.1 ± 1.4	9.9 ± 6.3**
Lymphocytes (%)	28.8 ± 9.4	15.3 ± 8.2***
Monocytes (%)	8.5 ± 3.0	5.2 ± 3.9***
Granulocytes (%)	62.2 ± 8.9	79.4 ± 10.6***
MDA (μmol/l)	21.6 ± 2.0	51.2 ± 8.6***
Carbonyl groups (μmol/g protein)	5.7 ± 1.8	8.8 ± 3.0***

*p < 0.05; **p < 0.01; ***p < 0.001 compared to the controls

Table 2. Correlation between indicators of inflammation and oxidative stress, and ultrasound findings of biliary tract in patients with choledocholithiasis

Parameter	DIBD	DEBD
Albumin (g/l)	0.124	0.107
Fibrinogen (g/l)	-0.239	-0.247
C-reactive protein (mg/dl)	0.162	-0.041
Leukocytes (G/l)	-0.353	0.072
Lymphocytes (%)	0.176	-0.011
Monocytes (%)	0.165	-0.017
Granulocytes (%)	-0.201	0.008
MDA (μmol/l)	-0.15	0.01
Carbonyl groups (μmol/g protein)	0.4*	0.11

*p < 0.05

Discussion

In patients with obstructive jaundice, ultrasonography is the first and widely accepted method for displaying dilated intrahepatic bile ducts. Ultrasonography is very reliable in detecting the level of obstruction, and in most cases is able to identify the reason of obstruction (11). Recent years have developed new methods in the review of patients with diseases of the gall bladder and bile ducts. One of them is intraoperative ultrasonography, which can clearly see the invasion of the gallbladder and associated blood vessels in the case of tumors, and define the difference between intraluminal and extra

luminal tumors (12). Lipid peroxidation of unsaturated fatty acids associated with free radicals is adverse and negative process that leads to the damage of lipid membranes of cells. It may disrupt the regulation of membrane fluidity and permeability of the plasma membrane of hepatocytes (13). Lipid peroxidation products such as hydro peroxides of unsaturated fatty acids, short chain hydrocarbons and aldehydes (MDA), possess a pronounced cytotoxic effect (14). MDA toxicity is based on its ability to act as a mutagenic agent in a cell (15).

Oxidative modification of the protein and amino acids by free radicals is caused by the reaction of the side chains of amino acid residues of their

breaking away, or the fragmentation of the protein in the conversion of higher molecular form (16). The degree and type of oxidative damage, and therefore the consequences, depend on the affinity of free radicals to certain classes of biomolecules. In particular, the oxidations of sensitive amino acids are methionine and cysteine. As the largest oxidation number of mechanisms leading to the formation of carbonyl group, and this group is most often used as a marker of oxidative damage to proteins (17).

Due to these characteristics of oxidative stress markers, the objective of this study was to determine the correlation of MDA and carbonyl groups with ultrasound findings of dilatation of the bile ducts. The fact is that we have developed many methods for determining these parameters of oxidative stress, which does not require special and expensive equipment and can be implemented in standard-equipped laboratories, and the determination of their serum levels could be part of routine biochemical diagnostic algorithm in patients with choledocholithiasis (18).

Results of the research showed that the dilatation of intrahepatic and extrahepatic bile ducts did not significantly correlate with the systemic indicators of inflammation in the blood plasma. Also, the results of our study did not show a clear correlation between the values of the parameters of oxidative stress, and degree of dilatation of the biliary tree. Higher values ($p < 0.05$) of carbonyl groups in pa-

tients with dilatation of intrahepatic bile ducts were statistically significant. Possible explanation of this finding is persistent cholestasis in these patients, which led to the complete dilatation of the biliary tree and the consequent damage or oxidative stress of hepatocytes, which resulted in the increased production of carbonyl groups.

Dilatation of only extrahepatic bile ducts in the initial stage of biliary obstruction is likely still not enough for functional impairment of the liver parenchyma in the direction of oxidative stress. Also, because of its specificity, determining MDA and carbonyl groups could be useful in detecting and determining the intensity of eventually present cholangitis in patients with choledocholithiasis. This finding points to the importance of the earliest possible resolution of cholestasis in order to prevent parenchymal damage or justification for timely preventive hepatoprotective therapy.

Conclusion

In patients with choledocholithiasis, ultrasound diagnosis of dilatation of bile ducts was not significantly associated with inflammation and lipid peroxidation, however, oxidative modification of the protein showed a significant positive correlation with dilatation of the intrahepatic bile ducts.

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doi:10.5633/amm.2020.0206**POVEZANOST DILATACIJE ŽUČNIH PUTEVA I OKSIDATIVNOG STRESA
KOD BOLESNIKA SA HOLEDOLITIJAZOM**

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Cilj ovog istraživanja bilo je ispitati povezanost inflamatornih parametara lipidne peroksidacije i oksidativne modifikacije proteina sa ultrazvučnim nalazom bilijarnog trakta kod bolesnika sa holedoholitijazom. Istraživanje je obuhvatilo 70 ispitanika podeljenih u dve grupe: grupa bolesnika sa holedoholitijazom (CHDL) – 40 bolesnika sa opstruktivnom žuti-
com, izazvanom holedoholitijazom; i kontrola grupa – 30 zdravih dobrovoljaca. Svi bolesnici su anamnestički i klinički sagledani na Internom odeljenju Vojne Bolnice u Nišu (Srbija). Vrednost MDA nisu bile značajno povezane sa stepenom dilatacije intrahepatičnih i ekstra-
hepatičnih žučnih puteva, dok su vrednosti karbonilnih grupa pokazale značajnu korelaciju sa stepenom dilatacije intrahepatičnih puteva ($p < 0,05$). Dilatacija žučnih puteva nije bila značajno povezana sa inflamacijom i lipidnom peroksidacijom, dok je oksidativna modifikacija proteina pokazala značajnu pozitivnu povezanost sa dilatacijom intrahepatičnih žučnih puteva.

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Ključne reči: oksidativni stres, holedoholitijaza, ultrazvuk, žučni kanali

THE CHARACTERISTICS OF HOSPITALIZED MEASLES AFFECTED CHILDREN DURING THE 2017-2018 EPIDEMIC IN THE JABLANICA DISTRICT

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Despite the availability of safe, effective and inexpensive vaccine since the 1960s, in 2018, more than 140.000 people worldwide died from measles. The aim of this study was to determine socio-epidemiological and clinical characteristics among hospitalized, measles-infected children during the epidemic from December 2017 to July 2018 in the Jablanica District and to estimate the influence of socio-epidemiological factors on the severity of disease. This cross-sectional study involved 55 measles-infected, hospitalized children at the Pediatric Department of the General Hospital of Leskovac. Data were collected with an original questionnaire and analyzed using descriptive statistics methods. The influence of factors on the severity of disease among infected children with and without complications was examined using χ^2 and t-test. $P < 0.05$ was considered statistically significant.

During the measles epidemic in the Jablanica District in 2017-2018, 110 children were affected, aged 0-19 years. Out of a total measles affected children in the Jablanica District only 9 (8.18%) were vaccinated, of which 5 (4.54%) were hospitalized. A total of 55 measles-infected, hospitalized children aged 22.85 ± 23.94 months were analyzed. The most infected children, 25 (45.45%), were aged 0-12 months. The infected children were mostly living in poor conditions, 34 (61.81%). Pneumonia was the most common complication, 24 (51.88%). There were no lethal outcomes. Severe complications were more frequent in younger children ($p < 0.05$), in children who lived in poor conditions ($p < 0.05$) and in those who had infection during the winter ($p < 0.05$).

The development and implementation of strategies to achieve high coverage for measles vaccination and revaccination are necessary for measles elimination.

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Key words: measles, epidemic, socio-epidemiological factors, complications, children

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Introduction

Measles is a highly contagious, infective and preventable disease. Despite the availability of safe, effective and inexpensive vaccine since the 1960s, in 2018, more than 140.000 people died from measles

in the world (1). European Region achieved its highest ever estimated coverage for the second dose of measles vaccination in 2017 (90%). Nevertheless, there were countries with measles outbreaks which have experienced a range of challenges in recent years including:

- a) a decline or stagnation in overall routine immunization coverage in some cases,
- b) sub-optimal coverage at sub-national level or among some marginalized groups and
- c) immunity gaps in older populations.

Most cases of infection are occurring in unvaccinated or incompletely vaccinated individuals (2). In the WHO European Region during 2017, 25.863 measles cases were reported, while in 2018, 82.596 measles cases were reported. (3).

From 2017 to 2019, the measles epidemic outbreak happened in Serbia with 5.798 measles cases and 15 measles related deaths (4). Those were the first deaths caused by measles in Serbia after 20 years. The most cases of measles affected

were recorded in the Belgrade City, Nišava, Pčinja, Raška and the Jablanica Districts (4).

Symptoms of measles usually develop 7-10 days after exposure to infected person. Initially symptoms are fever ($> 38^{\circ}\text{C}$), cough, coryzal symptoms, conjunctivitis and Koplik's spots. After three to five days, maculopapular rash starts usually on the face and spreads to the rest of the body (5, 6). Common complications include pneumonia, croup, diarrhea, otitis media, conjunctivitis, mouth ulcers, and uncommon complications include encephalitis, myocarditis, pneumothorax, appendicitis. Long term measles complications are blindness, subacute sclerosing panencephalitis, chronic lung disease, malnutrition, growth impairment and recurrent infections (5, 7). Although measles infection is preventable, it can be a fatal and life-threatening infection.

The aim

The aim of this study was to determine clinical and socio-epidemiological characteristics among measles infected, hospitalized children during epidemic from October 2017 to July 2018 in the Jablanica District, and to estimate the influence of socio-epidemiological factors on the severity of the disease.

Methods and patients

This cross-sectional study involved 55 measles affected children hospitalized in the Pediatric Department of General Hospital in Leskovac during the outbreak of measles in the Jablanica District, from October 2017 to July 2018. The criteria for hospitalization were the presence of serious complications and parental/guardian consent for hospital treatment. Children with measles without complications who were living in poor socio-economic and hygiene conditions without the possibility of isolation and adequate care were also treated at the hospital. Prior to admission to the Pediatric Department, case history was taken and clinical examination was performed. Parents were additionally asked about socio-economic status, living conditions, contact with infected person, affected members in the family, vaccination status of the child and reasons for non-vaccination. Immunization card was requested. In cases of confirmed contact with the measles affected and with typical clinical manifestation (generalized maculopapular rash, fever above 38°C , cough, coryzal syndrome and conjunctivitis), the case was diagnosed as measles. In cases where there was no information about contact with the measles affected, but with typical clinical measles manifestation, serological confirmation was performed. It was performed at the Institute of Virology, Vaccines and Sera "Torlak" in Belgrade. Detection of specific IgM antibodies in serum confirmed the diagnosis of measles.

Measles complications were established using anamnestic data and physical examination (diarrhea, laryngitis, bronchitis, purulent conjunctivitis and

stomatitis). Otitis media was confirmed by an otoscopic exam. Pneumonia was confirmed by a chest radiograph with the presence of a pulmonary infiltrate. Central nervous system was considered to be affected if there was lethargy, irritability, headache, febrile seizures, disorientation or other neurological deficits.

According to the WHO Child Growth Standards (8), weight-for-age was classified as malnourished, well nourished, overweight and obese.

Data were collected with an original questionnaire that was specially created for this research and analyzed using descriptive statistics methods: frequency, percentage, means \pm standard deviation, depending on the type and variable distribution. Influence of social and epidemic factors on the severity of disease among the affected children with and without complications was examined using χ^2 and t-test for categorical and continuous dependent variables, respectively. A value of $p < 0.05$ was considered statistically significant.

Results

During the measles epidemic in the Jablanica District 2017-2018, 110 children were affected, age between 0-19 years. Eighty-nine (80.9%) affected children were referred to the Pediatric Department of General Hospital in Leskovac. Fifty-five (50%) children were hospitalized. In 11 (10%) measles affected children hospitalization was indicated due to complications, but the parents refused the hospital treatment. Out of a total measles affected children in the Jablanica District only 9 (8.18%) were vaccinated, of which 5 (4.54%) were hospitalized.

A total of 55 measles affected, hospitalized children aged 22.85 ± 23.94 months were analyzed. The most affected children were between 0-12 months (45.45%). Nearly one-quarter of the children (23.63%) were malnourished. There were 90.9% unvaccinated children. Reasons for non-vaccination were children under one year of age, 25 (45.45%), delayed routine immunization, 7 (12.72%), insufficient health education of parents about the vaccination and measles prevention, 16 (29.09%), migration/divorce of parents, 2 (3.63%). Most of the affected children lived in poor living conditions (61.81%), in multi-member families with some members also infected (54.54%). Infection was common in winter months (65.45%) (Table 1).

In the clinical presentation, typical signs (fever, cough, coryzal symptoms, conjunctivitis and maculopapular rash) were present in almost all infected children. Complications occurred in 78.1% of infected, hospitalized children. The most frequent complications were respiratory (55.81% pneumonia, 20.93% croup and 20.93% bronchitis). Diarrhea was present in 34.83% of cases. No encephalitis was present. Only one case of febrile convulsions was reported. There was no lethal outcome. Treatment outcome was favorable for all children (Table 2).

Table 1. Demographic, socio-epidemiological characteristics and outcome of treatment in hospitalized, measles affected children

Variable	Value
Total number of hospitalized children, n (%)	55 (100%)
Males/females, n (%)	27/28 (49.1/50.9)
Age (months), X \pm SD	22.85 \pm 23.94
Age categories (months), n (%)	
0-12	25 (45.45%)
13-24	15 (27.27%)
25-48	8 (14.54%)
> 48	7 (12.72%)
Roma children, n (%)	43 (78.1%)
BMI-SD, X \pm SD	-0.603 \pm 1.62
BMI-SD, WHO categories, n (%)	
malnourished	13 (23.63%)
normal	41 (74.54%)
overweight	1 (1.81%)
Vaccine status, n (%)	
unvaccinated	50 (90.90%)
vaccinated	5 (9.09%)
Reasons for non-vaccination, n (%)	
children under 1 year	25 (45.45%)
delayed routine immunization	7 (12.72%)
insufficient health education of parents	16 (29.09%)
migration/divorce of parents	2 (3.63%)
Poor living conditions, n (%)	34 (61.81%)
Infected during winter months, n (%)	36 (65.45%)
Affected member of family, n (%)	30 (54.54 %)

BMI – body mass index; WHO – World Health Organization; X– mean value; SD – standard deviation

Table 2. Clinical manifestation, complications and outcome of treatment in measles affected, hospitalized children

Variable	Value
Clinical signs, n (%)	
fever	55 (100%)
cough	51 (92.72 %)
coryzal symptoms	55 (100%)
conjunctivitis	54 (96.36%)
maculopapular rash	55 (100%)
typical	47 (85.45%)
atypical	8 (14.54%)
Complications of measles	43 (78.1%)
Pneumonia	24 (55.81%)
Bronchitis	9 (20.93%)
Croup	9 (20.93%)
Otitis media	1 (2.32%)
Diarrhea	15 (34.83%)
Febrile seizures	1 (2.32%)
Purulent conjunctivitis	22 (51.16%)
Mouth ulcers	9 (20.93%)
Duration of hospitalization, X \pm SD	5.65 \pm 2.67
Outcome of treatment, n (%)	
cured	54 (98.18%)
transported in tertiary institution	1 (1.81%)
lethal outcome	0 (0.0%)

X– mean value; SD – standard deviation

Young age (19.93 ± 19.31 vs. 33.33 ± 35.18), poor living conditions (74.41% vs. 33.3%) and infection during winter months (74.41% vs. 33.3%) were statistically significant ($p < 0.05$) in children with measles with complications. In both groups, there were more than half (58.33% vs. 53.48%) infected family members ($p > 0.05$). Gender structure, nourishment (-0.67 ± 1.66 vs. 0.33 ± 1.50), vaccina-

tion status (16.66% vs. 6.9%), duration of fever (6.48 ± 2.27 vs. 6.25 ± 2.05) and duration of rash (6.5 ± 1.67 vs. 7.23 ± 1.81) were not significantly different between children with complications and without complications ($p > 0.05$). Complications significantly extended the duration of hospitalization (3 ± 1.12 vs. 6.39 ± 2.50) ($p < 0.05$) (Table 3).

Table 3. Characteristics of measles affected children with and without complications

Variable	Without complications n = 12	With complications n = 43	p-value
Males, n (%)	6 (50%)	21 (48.83%)	NS
Age (months), $X \pm SD$	33.33 ± 35.18	19.93 ± 19.31	< 0.05
BMI-SD, $X \pm SD$	-0.67 ± 1.66	-0.33 ± 1.50	NS
Vaccinated, n (%)	2 (16.66%)	3 (6.97%)	NS
Poor living conditions, n (%)	4 (33.3%)	32 (74.41%)	< 0.05
Affected member of family, n (%)	7 (58.33%)	23 (53.48%)	NS
Infected during the winter months	4 (33.3%)	32 (74.41%)	< 0.05
Duration of fever, $X \pm SD$	6.48 ± 2.27	6.25 ± 2.05	NS
Duration of rash, $X \pm SD$	6.5 ± 1.67	7.23 ± 1.81	NS
Duration of hospitalization, $X \pm SD$	3 ± 1.12	6.39 ± 2.50	< 0.05

BMI – body mass index; X – mean value; SD – standard deviation

Discussion

In this cross sectional study we analyzed demographic, socio-epidemiological and clinical characteristics in all measles affected, hospitalized children and compared them among children with measles with and without complications. A similar outbreak of measles occurred in 2011 in Leskovac, which started in the Roma community and spread to the general population (10). An average age of measles affected in Europe, during the measles outbreak in 2017 and 2018 has progressively increased, from 10 years in 2009 to 17 years in 2019. In the period of 2016-2019, the average annual rates were highest in infants, up to 44 times higher than in the other age groups. Almost half (45%) of all measles deaths were reported in infants (11). Most measles-related deaths are caused by complications associated with the disease. Serious complications are more common in children under the age of 5, or adults over the age of 30 (12). Unvaccinated young children are at the highest risk of measles and its complications, including death. In Serbia, the most affected age groups were under 5 years and over 30 years, that are the groups with the highest risk for developing complications (4). The most hospitalized, affected children were aged between 0-12 months (45.45%). There were 50 (91%) under 5 years of age. During the measles outbreak in 2011 in Leskovac, mean age of hospitalized, infected children in our Department was 11.5 months (2-17 months) (13). This data showed that the children under 5 years of age, particularly infants, were the vulnerable groups with the high risk for measles complications in epidemics in the Jablanica District. Also, the age of affected

children was increased which means that the number of vaccinated children at 12 months was decreased.

MMR vaccine is absolutely safe, effective and inexpensive vaccine which protects 90% of the recipients. Children should get two doses of MMR vaccine, starting with the first dose at 12 months of age, and the second dose at 7 years. In the Jablanica District there were 91.82% measles affected unvaccinated children. In the European Region during the measles outbreak of 2017-2018, 87% of measles affected were unvaccinated (14), and in Serbia 94% of measles affected were unvaccinated (4). Coverage of the MMR vaccine in Serbia during 2018 was below 95% in 50% of the regions. The lowest coverage of revaccination was recorded in the Jablanica District (84.4%), which can be a major cause for the periodical outbreaks of the measles in the Jablanica District (15). Only 5 (4.54%) vaccinated children were hospitalized with moderate, not severe complications. Insufficient health education of parents about preventive measures and vaccination was the major reason for non-vaccination among measles affected hospitalized children (29.09%) in this research. Anti-vaccine campaign contributes to the low MMR vaccine coverage, and it is identified by the World Health Organization as one of the top ten global health threats in 2019 (16).

Measles can be a deadly disease if it comes to developing complications. Risk factors for a severe, complicated form of measles are the young age (particularly under 1 year), malnutrition, non-vaccination, living in poor conditions, immunodeficiency (malignancy, HIV, immunosuppressive therapy), vitamin A deficiency (5, 6, 17, 18, 19).

Living in socially and culturally separate communities, in overcrowded and poor conditions, with a

close contact between the members, especially during winter months is a risk factor for the severe measles form (6, 20). This phenomenon is most likely the result of the intense and longer exposure to the virus, which occurs in winter months. The household characteristics (socioeconomic, sociocultural and culture of health) are in interaction with other factors (e.g. malnutrition, hypoproteinemia, vitamin A and D deficiency, nonimmunized children) which can increase risk for measles complications.

Almost all measles affected children had typical clinical manifestation with fever (100%), cough (92.72%), coryzal symptoms (100%), conjunctivitis (96.36%) and maculopapular rash (100%). The most common complications were respiratory (pneumonia 55.81%, bronchitis 20.93% and croup 20.93%) and diarrhea (34.83%). Pneumonia is the most common, serious complication of measles, which occurs in 16-77% of hospitalized children (21, 22). In all measles-associated deaths, pneumonia is the cause of 60% (23). In the measles epidemic that occurred in Serbia during 2017-2018, 383 cases developed neurological or pulmonary complications. Nine people died of pulmonary measles complications, including two children aged four and two (14). Diarrhea with dehydration is the second common complication, most frequently among measles affected in Asia (21, 24). Encephalitis is a very dangerous complication with a high mortality rate. In some studies, the incidence of encephalitis was 7.8% (22). The mortality rate is 10-15% and 25% of patients had permanent neurological sequelae (25). In comparison to other studies which included measles affected children, this study showed similar occurrence for pneumonia, and low occurrence for otitis media, febrile seizures and encephalitis (21, 22, 23, 24, 25). In the literature data, mortality is significantly associated with infancy, unvaccinated status and encephalitis (22). In this study, there were no

lethal outcomes. Complications significantly extended the duration of hospitalization. Measles is a disease that can be prevented with inexpensive vaccine. Medical treatment and treatment of complications are more expensive than prevention (26). Measles infection has an adverse effect on the quality of life in affected children. Absence from school and a loss of income for parents who stay at home to care for them are a negative consequence of the infection (27). Isolation of measles affected children in hospitals, exposure of medical professionals to infection and intrahospital infection control were also problems in our Pediatric Department.

Conclusion

The result showed that the affected children were younger, unvaccinated, infected during the winter, living in poor conditions, with family members also affected, and with typical clinical presentation and complications. Severe complications were more frequent in younger children, in children who lived in poor conditions and who had an infection during the winter. Pneumonia was the most common complication. There were no lethal outcomes. Complications significantly extended the duration of hospitalization.

Immunization is the only effective preventive measure against acquiring measles. Therefore, it is necessary to strengthen the immunization programs, in every district of the country, to ensure $\geq 95\%$ 2-dose MMR coverage. Vaccination delay should be minimized in children. For susceptible adults, it is necessary to provide additional measles vaccination. Educational campaigns for the improvement of acceptance and timely vaccination with MMR vaccine among doctors and the general population are crucial.

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doi:10.5633/amm.2020.0207**KARAKTERISTIKE HOSPITALIZOVANE DECE OBOLELE OD MALIH BOGINJA TOKOM EPIDEMIJE OD 2017. DO 2018. GODINE U JABLANIČKOM OKRUGU***Marija Stojiljković^{1,2}, Ivan Rančić², Milan Golubović^{2,3}, Milica Jakovljević^{2,3},
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Upkos postojanju bezbedne, efikasne i jeftine vakcine još od 1960. godine, u 2018. godini u svetu je umrlo 140 000 ljudi zbog malih boginja. Cilj ovog rada je da ispita kliničke i socio-epidemiološke karakteristike hospitalizovane dece obolele od malih boginja, tokom epidemije od 2017. do 2018. godine u Jablaničkom okrugu i da utvrdi uticaj socio-epidemioloških faktora na ozbiljnost oboljenja. Radi se o studiji preseka, kojom je obuhvaćeno 55 obolele, hospitalizovane dece na Odeljenju pedijatrije Opšte bolnice Leskovac. Podaci su prikupljeni originalnim upitnikom i analizirani upotrebom deskriptivnih statističkih metoda. Uticaj ispitivanih faktora na težinu bolesti među obolelom decom, sa komplikacijama i bez njih, testiran je upotrebom χ^2 i t-testa. Statistički značajnim smatrana je vrednost $p < 0,05$. Tokom epidemije malih boginja u Jablaničkom okrugu, ukupno je obolelo 110 dece, od novorođenčadi do dece uzrasta do 19 godina. Od obolelih MMR vakcinu je primilo njih 9 (8,18%), od kojih je 5 (4,54%) bilo bolnički lečeno. Ukupno je hospitalizovano 55 dece, koja su dalje analizirana. Najviše hospitalizovanih bilo je u uzrastu od novorođenčadi do dece stare 12 meseci – njih 25 (45,45%). Ozbiljne komplikacije bile su češće među decom mlađom od 12 meseci ($p < 0,05$), decom koja su živela u siromašnim uslovima ($p < 0,05$) i decom koja su obolela tokom zimskih meseci ($p < 0,05$). Najčešća komplikacija bila je pneumonija – 24 slučaja (55,81%). Nije bilo letalnih ishoda.

Razvoj i implementacija strategija za postizanje visokog ishoda lečenja, što obuhvata vakcinacije i revakcinacije MMR vakcinom, neophodni su za prevenciju i eradicaciju malih boginja.

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Ključne reči: male boginje, epidemija, socio-epidemiološki faktori, komplikacije, deca

BONDING EFFICIENCY OF UNIVERSAL ADHESIVE TO DENTINE IN DIFFERENT ETCHING AND CURING MODES

Milan Miljković¹, Stefan Dačić², Goran Radenković³, Milica Jovanović⁴

Adhesive bond strength and polymerization shrinkage are influenced by many different factors, including etching mode and light properties of a curing source. Different etching and curing techniques have been developed in an attempt to reduce polymerization shrinkage and stress development, thereby increasing the bond strength at the composite/dentine interface. The purpose of this study was to evaluate the influence of total-etch and self-etch mode as well as continuous and soft-start light curing mode on micro-tensile bond strength of bulkfill composite to dentine using universal adhesive. According to the etching and lightcuring mode applied, teeth were randomly divided into four different groups (n = 15): TC (total-etch, continuous mode), TS (total-etch, soft-start), SC (soft-start, continuous mode) and SS (self-etch, soft-start). Cylindric composite build-ups were made with 3M FiltekBulkfill Posterior. Specimens were subjected to micro tensile bond strength test in a universal testing machine at a crosshead speed of 0.5 mm/min. The failure force was recorded in Newton (N) and calculated in MPa as a ratio of Newton to the surface of the bonded area. Data were analyzed using two-way ANOVA and Students t-test. Micro tensile bond strength was not affected by the lightcuring mode since no significant statistical difference was determined between the continuous and soft-start groups, irrespective of the etching mode used. On the other hand, micro tensile bond strength was highly influenced by the etching mode since significant statistical difference was determined between the total-etch and self-etch groups, irrespective of the lightcuring mode.

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Key words: bond strength, universal adhesive, total-etch, self-etch, soft-start

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Introduction

Strong adhesive bond between the filling and cavity walls is mandatory for long-lasting composite restorations. To achieve a strong adhesive bond, proper clinical technique and tooth surface preparation is required (1). The quality of adhesive bond is usually determined by measuring micro tensile or shear bond strength to enamel or dentine. Achieving optimal adhesive bond to dentine is more complicated when compared to enamel, due to the

presence of smear layer and histological differences between the two substrates (2).

In order to improve their clinical efficacy, the composition of dental adhesive systems is under constant development and new products are continuously introduced to the market. As a result, several generations of bonding agents are now available in the dental market with different number of bottles and their application technique (3). Multi-step total-etch adhesives are still considered to be the gold standard in achieving a strong bond despite of their time-consuming and technique-sensitive application. On the other hand, self-etch adhesives have greatly simplified the adhesive application process since they do not require a separate acid etching step, rinsing and drying. These systems have gained a lot of popularity in the last few years because they are extremely easy to use and time-saving (4).

The most recently introduced adhesive system to the dental market is "universal" or "multi-mode" adhesive which can be applied in total-etch, self-etch, or selective-etch mode, as well as on different materials. The goal of this new adhesive system is to further simplify the bonding procedure and clinicians' decision making when considering bonding agents and adhesive techniques (5).

However, universal adhesives have raised a lot of controversy regarding their bond strength and clinical performance. Manufacturers claim that optimal bond strength can be achieved regardless of the chosen application technique. The exact composition of new adhesives is never fully disclosed and information about the rate of shrinkage and hardness of adhesives after polymerization is very limited (6).

The purpose of this study was to evaluate the influence of different etching and light curing modes on the micro-tensile bond strength of universal adhesive to dentine. The following null hypotheses were tested in this study:

(1) etching mode will have no significant impact on micro tensile bond strength and there would be no significant difference between the total-etch and self-etch mode;

(2) light curing mode will have no significant impact on micro tensile bond strength and there would be no significant difference between the continuous and soft-start mode;

(3) etching and light curing mode interaction will have no significant impact on micro tensile bond strength.

Materials and methods

Sixty non-carious human teeth, extracted for periodontal, prosthetic and orthodontic purposes, were used in order to achieve the goal of this study. After extraction, the teeth were purified from blood and periodontal tissue detritus with a hard brush and stored in physiological saline at room temperature. In order to expose a flat dentine surface, teeth were sectioned in mesio-distal direction, parallel to their longitudinal axis, using low-speed diamond saw with water cooling. The exposed areas of dentine were then polished with silicone discs of different fineness (coarse, medium, fine). The smear layer from the exposed surface of dentine was removed by brush-

ing with a soft brush under running water for 60s. Samples were then transferred to an ultrasonic bath at 25 °C for 45 minutes. In order to mimic the humid environment in the oral cavity, prepared samples were stored in distilled water at room temperature, before use. The obtained dentine plates were then deposited into cylindric molds and fixed with super-hard stone, class IV (Zhermack Elite Stone).

The teeth were randomly divided into four different groups ($n = 15$) based on the etching and light curing mode applied: TC (total-etch, continuous mode), TS (total-etch, soft-start mode), SC (self-etch, continuous mode) and SS (self-etch, soft-start mode). In groups TC and TS, the exposed surface of dentine was etched according to the manufacturer's instructions with 37% phosphoric acid for 15s. The acid was thoroughly rinsed with water-spray for 10s and air-dried for 5s. 3M Universal Single Bond was then applied by micro brush for 5s and mildly air-dried for 5s until a uniform glass-like dentine surface was obtained. The adhesive was then light cured for 10s in the continuous or soft-start mode according to their respective groups, using Woodpecker Led-G lightcuring device. In groups SC and SS, the etching step was excluded, so the 3M Universal Single Bond was applied directly to the prepared dentine surface the same way as before. Cylindric composite build-ups were made with 3M FiltekBulkfill Posterior, using a plastic mold, 4mm in diameter and 4mm in height. Bulkfill composite was carefully packed in one layer and light cured in either continuous (1200 mW/cm² for 40s) or soft-start mode (400 mW/cm² for 10s + 1200 mW/cm² for 30s) according to their respective groups. The distance between the tip of the light curing device and the surface of the specimens was minimal. Plastic molds were then removed and samples were stored in a water bath at 37 °C before use. The materials used in this study are shown in Table 1.

Table 1. List of products used

Product	Manufacturer	Description
3M Universal Single Bond	3M ESPE (USA)	Universal adhesive
3M Etchant Gel	3M ESPE (USA)	37% Phosphoric acid
3M FiltekBulkfill Posterior	3M ESPE (USA)	Composite material
Zhermack Elite Stone	Zhermack (Germany)	Class IV stone

After 24 hours, specimens were subjected to micro tensile bond strength test in a universal testing machine (Wagezelle Load Cell, Hottinger Baldwin Messtechnik, Germany), at a crosshead speed of 0.5 mm/min. Specimens were secured in a metal base so that the bonded cylinder base was parallel to the shear force direction. The failure force was recorded in Newton (N) and calculated in MPa as a ratio of Newton to surface of the bonded area.

Statistical analysis was performed with SPSS soft-ware (version 23.0). Descriptive statistics, including the mean, standard deviation, minimum and maximum values were calculated for all groups.

Kolmogorov-Smirnov test was applied to confirm the normal distribution of the results. Two-way ANOVA (etching and light curing mode) and Students t-test were applied to determine whether significant differences in debond values existed

among the groups. Significance for all statistical tests was predetermined at $p < 0.05$.

Results

Descriptive statistics of the microtensile bond strength of different groups is illustrated in Table 2. The highest average bond strength value was related to TC group and the lowest bond strength value was recorded in the SS group. Kolmogorov-Smirnov test found normal data distributions ($p > 0.05$). Two-way ANOVA (Table 3) indicated that there were statistically significant differences for the variable etching mode ($p = 0.003$), but failed to

identify statistically significant differences for the variable light curing mode ($p = 0.406$) and any interaction between variables ($p = 0.829$). Students t-test identified significant statistical difference between the groups that have been conditioned with acid and the untreated groups, in general (Table 4). Higher bond strength was obtained in groups TC and TS compared to SC and SS ($p < 0.05$). No significant statistical difference was determined between the continuous and soft-start groups, in general (Table 5). Higher bond strength was obtained in groups TC and SC compared to TS and SS ($p < 0.05$).

Table 2. Descriptive statistics (in MPa) of microtensile bond strengths of the 4 subgroups tested

	Mean \pm SD	Min	Max
TC	28.2 \pm 7.2	18.3	41
TS	26.6 \pm 6.6	17.9	37.9
SC	23.1 \pm 4	18.7	31
SS	22.2 \pm 5.1	14.2	30.5

TC (total-etch,continuous); SC (self-etch,continuous);
TS (total-etch,soft-start); SS (self-etch,soft-start)

Table 3. Two-way analysis of variance

	Sum of Squares	Df	Mean Squares	F	Sig
Etching mode	332.762	1	332.762	9.644	0.003*
Curing mode	24.193	1	24.193	0.701	0.406
Interaction	1.633	1	1.633	0.047	0.829

*Statistical significance at the level of 5%. Df-degrees of freedom.

Table 4. Statistical comparison between the total-etch and self-etch groups

	Mean \pm SD	t	p
TC & TS	27.4 \pm 6.8	0.009*	0.05
SC & SS	22.7 \pm 4.5		

*Statistical significance at the level of 5%.

Table 5. Statistical comparison between the continuous and soft-start groups

	Mean \pm SD	t	p
TC & SC	25.7 \pm 6.3	0.955	0.05
TS & SS	24.4 \pm 6.2		

Discussion

Bond strength depends on a number of different factors, such as etching mode, light curing mode, composite resin type, filling technique, cavity shape and size. Previous research suggest that etching mode has the greatest impact on micro tensile bond strength (7). Curing mode may also significantly affect bond strength by regulating the degree of polymerization shrinkage which can be transferred to the bonding interface in the form of debonding tensile forces. Soft-start is a modified, two-step light curing mode which uses an initial low-light intensity to minimize the stress generated during polymerization, followed by a higher light intensity to provide proper mechanical properties (8). It has been previously reported that by slowing down the conversion in initial phase, negative effects of polymerization shrinkage and stress development on adhesive bond strength can be reduced at least to some extent (9). Thus light intensity and soft-start mode also affect the resin monomer penetrating the dentin collagen fiber network in forming the hybrid layer, thereby influencing the dentin bond performance (10).

The first null hypothesis of this study was rejected. Two-way ANOVA showed that the micro tensile bond strength is highly technique-dependent. Student's t-test also confirmed that there was a significant difference between the total-etch and self-etch groups, irrespective of the curing mode. Significantly higher bond strength was determined in TC and TS groups probably due to the formation of porosities in the dentine surface and penetration of resin into the porosities. Our findings are in line with those of previous studies conducted by Pouyanfaret al. which indicate that cavity treatment with phosphoric acid significantly increases the microretentive surface and enables the formation of a thick hybrid layer (5-50µm) (11). On the other hand, self-etch adhesive systems cause partial and superficial demineralization of the smear layer and dentin because of the presence of weaker acidic primers. Since demineralization depth depends on the pH of the acid monomer, hybrid layer formed in self-etch mode is much thinner (5-8µm) (12). In a study conducted by Manfroi et al. SEM analysis revealed apparent morphological differences in the bond interface between the total-etch and self-etch adhesive systems. It can be concluded that although eliminating the etch and rinse step may simplify the process of adhesive

application, it does not necessarily result in higher clinical success rate (13).

The second null hypothesis of this study was accepted. Two-way ANOVA showed that the micro tensile bond strength is not light-dependent. Student's t-test confirmed there was no significant difference between the continuous and soft-start groups, irrespective of the etching mode. Results of previous studies researching modified light curing protocols are contradictory, so opinions of the scientific community on the matter are divided (14). Also, there is still no agreement about the ideal light intensity in the initial phase of soft-start mode (15). Too high or too low initial intensity cannot achieve a good curing effect, so we used initial light intensity of 400mW/cm² in our experiment. In theory, soft-start should provide significant benefits to bond strength by reducing the degree of polymerization shrinkage and stress development (16). However, the results of our in vitro study revealed no significant influence of soft-start mode on bond strength. Moreover, higher values of micro tensile bond strength were determined when continuous mode was used. Thus, the benefits of soft-start light curing still remain questionable and further research is required to determine the relationship between light transmission and adhesive bond strength (17).

The third null hypothesis of this study was accepted. Interaction between the etching and curing mode had no significant impact on micro tensile bond strength. Important limitation of this study was that no thermal cycling or artificial aging was performed to better simulate clinical conditions.

Conclusion

According to the methodology employed and based on the obtained results, it can be concluded that micro tensile bond strength of universal adhesive to dentine was significantly affected by the etching mode. Higher bond strength was determined in total-etch mode, irrespective of the curing mode applied. Micro tensile bond strength of universal adhesive was not affected by the curing mode.

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doi:10.5633/amm.2020.0208**EFIKASNOST VEZIVANJA UNIVERZALNOG ADHEZIVA
ZA DENTIN PRIMENOM RAZLIČITIH ADHEZIVNIH I
SVETLOSNOPOLIMERIZUJUĆIH TEHNIKA***Milan Miljković¹, Stefan Dačić², Goran Radenković³, Milica Jovanović⁴*¹Univerzitet u Nišu, Medicinski fakultet, Istraživački centar za biomedicinu, Niš, Srbija²Klinika za stomatologiju, Odeljenje za bolesti zuba i endodonciju, Niš, Srbija³Univerzitet u Nišu, Mašinski fakultet, Laboratorija za ispitivanje materijala i mašina, Niš, Srbija⁴Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Odeljenje za stomatološku protetiku, Kragujevac, Srbija

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Na jačinu adhezivne veze i polimerizacionu kontrakciju utiču mnogi različiti faktori, uključujući tehniku nagrizanja i svetlosna svojstva izvora za polimerizaciju. Vremenom su razvijene različite tehnike adhezije i svetlosne polimerizacije, u cilju redukcije polimerizacione kontrakcije i razvoja stresa, čime se povećava jačina adhezivne veze na spoju kompozit – dentin.

Cilj ove studije bila je procena uticaja totalnonagrizajuće i samonagrizajuće tehnike, kao i kontinuiranog i rastućeg svetlosnopolimerizujućeg režima na jačinu adhezivne veze kompozita za dentin, primenom univerzalnog adhezivnog sistema.

Na osnovu primenjene tehnike nagrizanja i svetlosnopolimerizujućeg režima, uzorci su nasumično podeljeni u 4 grupe (n = 15): TC (totalnonagrizanje, kontinuirani režim), TS (totalnonagrizanje, rastući režim), SC (samonagrizanje, kontinuirani režim) i SS (samonagrizanje, rastući režim).

Cilindrične kompozitne nadogradnje napravljene su od 3M FiltekBulkfill Posterior. Uzorci su podvrgnuti testiranju jačine adhezivne veze mikrozatezanjem u univerzalnoj mašini za testiranje, pri brzini pomeranja od 0,5 mm/min. Sila pucanja detektovana je u Njutnima (N) i preračunata u megapaskale (MPa), kao odnos Njutna prema površini bonding regije. Podaci su analizirani testom Two-way ANOVA i Studentovim t-testom. Režim svetlosne polimerizacije nije uticao na jačinu adhezivne veze, pošto nije utvrđena statistički značajna razlika između kontinuiranih i rastućih grupa, nezavisno od primenjene tehnike nagrizanja. Sa druge strane, jačina adhezivne veze u velikoj meri zavisi od tehnike nagrizanja, pošto je utvrđena statistički značajna razlika između totalnonagrizajućih i samonagrizajućih grupa, nezavisno od režima svetlosne polimerizacije.

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Ključne reči: čvrstoća vezivanja, univerzalno lepilo, totalnonagrizanje, samonagrizanje, meko pokretanje

EMERGENCY CONDITIONS IN SURGERY

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Jelena Ignjatović³*

All acute conditions in surgery are clinically presented as acute abdomen. The aim of this study was to identify the most common causes of emergency conditions in surgery.

We included 165 patients operated in the Emergency Centre, Clinical Center Niš for intra-abdominal perforation with a final diagnosis of diffuse peritonitis and bleeding. There were 92 (56%) women and 73 (44%) men.

Appendicular perforation was the cause of peritonitis in 29 (27.9%) patients, gastric perforation in 24 (23.1%) patients, jejunum perforation in 5 (4.8%) patients, and ileum perforation in 7 (6.7%) patients. Large bowel perforation was the cause of peritonitis in 20 (19.2%) patients, rectum perforation in 6 (5.8%) patients and perforation of gall bladder in 13 (12.5%) patients. Gastric ulcer bleeding was found in 24 (39.3%) patients, duodenal ulcer in 18 (29.5%) patients, 11 (18.1%) patients had splenic injury and bleeding, in 5 (8.2%) patients liver bleeding occurred, and in 3 (4.9%) patients, the bleeding was caused by esophageal varices. The overall mortality was 20.6% (n = 34). Due to the consequences of septic condition (SC) 14 (41.2%) patients died, and 20 (58.8%) died of complications of gastrointestinal bleeding (GIB).

The morbidity and mortality rates of emergency conditions are still unacceptably high.

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Key words: acute abdomen, peritonitis, bleeding

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Introduction

All acute conditions in surgery are clinically presented as acute abdomen. Acute abdomen includes all abdominal conditions and diseases that require immediate surgical intervention.

Differential diagnostics should pay attention to the conditions that resemble an acute abdomen and they are not as well as pseudo-acute abdomen. It is most often acute pancreatitis, abdominal angina or a consequence of electrolyte disturbances (1). Timely and correct diagnosis is crucial, which includes a detailed anamnesis, clinical examination and appropriate diagnostic procedures. Timely diagnosis is the key to successful treatment (1).

Acute generalised peritonitis is an urgent condition and is known as one of the leading causes of death in non-traumatized patients despite advances in diagnosis and surgical treatment with mortality rate ranging from 10 to 32 % (1, 2).

It is well known that generalised peritonitis is the most important surgical condition in general surgical practice. The most common cause is perforation in the gastrointestinal tract.

The abdomen seems to be Pandora's Box because the surprises are the rule rather than the exception. The most important signs to make the adequate diagnosis are diffuse pain and rigidity of the abdomen with the presence of pneumoperitoneum on X-ray of the abdomen, which means gas under diaphragm (1, 3).

Pain is the main reason that brings patients to the Emergency Center. Visceral pain is usually diffuse and not easy for localizing. Somatic pain is usually well localized, intermittent or constant and described as aching, gnawing and throbbing (1, 2). Acute gastrointestinal bleeding due to the dramatic clinical picture and the need for urgent therapeutic and diagnostic procedures have priority in hospital treatment and represents the reason for 1.5% of all emergency hospitalizations today.

The most important thing for emergency conditions is making an accurate diagnosis through the clinical examination, good history and adequate

diagnostic investigation (1). Acute abdomen is a condition that must be treated by many specialists (4).

The aim

The aim of this study was to identify the most common causes in emergency conditions in surgery.

Material and method

The study included 165 patients operated in the Emergency centre - Clinical Center Niš from 2017. to 2019 for an intra-abdominal perforation with a final diagnosis of diffuse peritonitis and patients operated for bleeding. Diffuse peritonitis is

defined as any intra-abdominal infection that extends beyond the transverse mesocolon (2).

Results

The study involved 165 patients operated on for acute abdomen. There were 92 (56%) women and 73 (44%) men (Figure 1).

Appendicular perforation was the cause of peritonitis in 29 (27.9%) patients, gastric perforation in 24 (23.1%) patients, jejunum perforation in 5 (4.8%) patients, and ileum perforation in 7 (6.7%) patients. Large bowel perforation was the cause of peritonitis in 20 (19.2%) patients, rectum perforation in 6 (5.8%) patients and perforation of gall bladder in 13 (12.5%) patients (Table 1).

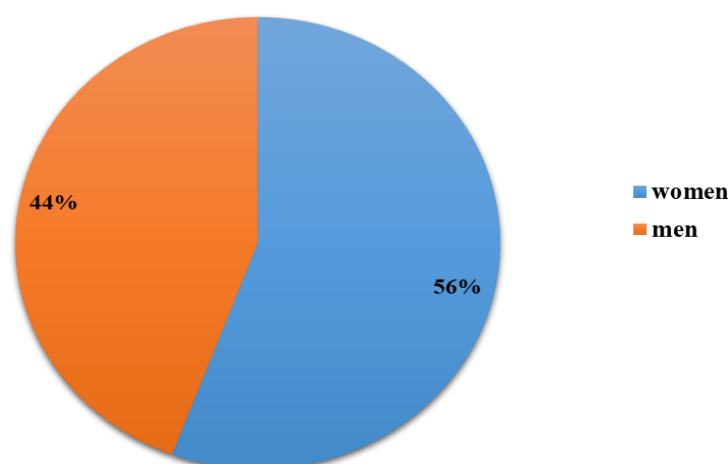


Figure 1. Distribution of patients operated of acute abdomen in relation to gender

Table 1. Distribution by causes of peritonitis

Causes of peritonitis	n	n (%)
Appendicular perforation	29	27.9
Gastric perforation	24	23.1
Jejunum perforation	5	4.8
Ileum perforation	7	6.7
Bowel perforation	20	19.2
Rectum perforation	6	5.8
Perforation of gall bladder	13	12.5

The cause of acute abdomen was perforation in 104 (63%) patients and in 61 (37%) it was intra-abdominal bleeding (Figure 2).

Gastric ulcer bleeding was found in 24 (39.3%) patients, duodenal ulcer in 18 (29.5%) pa-

tients, 11 (18.1%) patients had splenic injury and bleeding, in 5 (8.2%) patients liver bleeding occurred, and in 3 (4.9%) patients, the bleeding was caused by esophageal varices (Table 2).

The overall mortality was 20.6% (n = 34). Due to the consequences of septic condition (SC), 14 (41.2%) patients died and 20 (58.8%) patients

died of complications of gastrointestinal bleeding (GIB) (Figure 3).

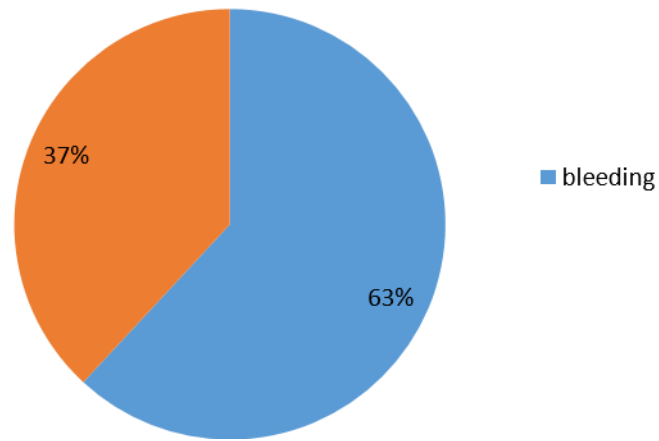


Figure 2. Distribution by causes of emergency condition

Table 2. Distribution by causes of intra-abdominal bleeding

Causes of intra-abdominal bleeding	n (%)	n (%)
Bleeding gastric ulcer	24	39.3
Duodenal ulcer	18	29.5
Splenic injury and bleeding	11	18.1
Liver bleeding	5	8.2
Esophageal varices	3	4.9

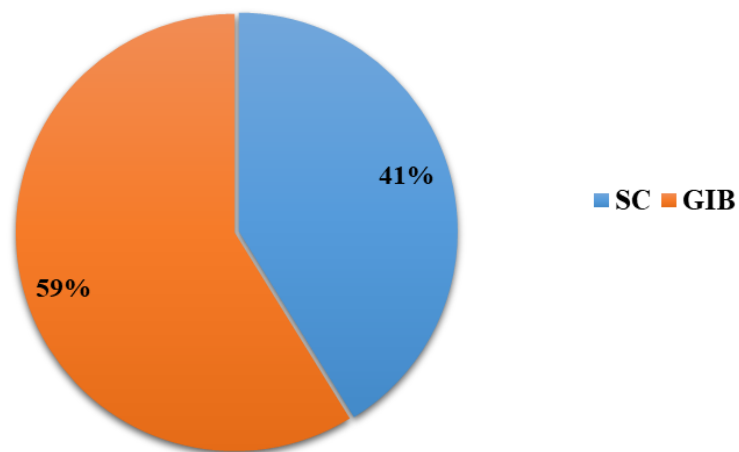


Figure 3. The overall mortality of patients

Discussion

Acute abdomen is a syndrome that occurs as a consequence of a pathological process in the intra-abdominal organs and leads to deadly complications as it progresses. It can be caused by illness or trauma and requires a rapid response. Abdominal pain can be confused with extra abdominal and thoracic conditions such as acute myocardial infarction and pneumonia (4, 5, 6).

Peritonitis can be defined in several ways.

Primary peritonitis is an infection of the peritoneal cavity that occurs in patients with ascites fluid and who do not have other intra-abdominal diseases or are not associated with them. The most common and most dangerous form of peritonitis is secondary peritonitis. It occurs due to spontaneous perforation of intra-abdominal organs, intestinal ischemia or after operations. Tertiary peritonitis is a recurrent infection in the abdomen occurring in patients after primary or secondary peritonitis (6). Sudden and sharp abdominal pain is the most significant sign of an acute abdomen. It takes necessary knowledge and skills to make a correct diagnosis. The localization of pain is important in determining various diagnoses, but sometimes the pain can also be projected or moved, which often leads to an error (5).

The localization of pain in the upper right quadrant is specific for acute cholecystitis or liver diseases. Pain in the epigastrium, then in the lower right quadrant, followed by fever and vomiting, indicates acute appendicitis. The existence of a gynecological or urological disease should also be ruled out. Ulcer disease is localized in the epigastrium or in the lower abdomen. It can occur before or after a meal, which determines whether it originates from the stomach or duodenum. Renal colic is accompanied by pain that is projected towards the genitals. The duration of the pain also determines the type of disease, whether the pain is short-lived and strong or lasts. The pain that occurs with perforations intensifies over time and spreads to the entire abdomen (4, 6, 11).

Colic is connected with many diseases of abdomen and it is the first sign. The pathophysiology of pain is thought to be smooth muscle contraction proximal to a partial or complete obstruction. The localization of colic usually helps to diagnose and find the reason of pain. The absence of colic can be found in several diseases, but rarely.

In case a patient experiences abdominal pain, fever, vomiting, fainting, or signs of blood loss, acute surgical disease should be suspected. A high mortality and morbidity rate has been observed in cases of acute abdomen in which there are other associated diseases.

Conditions in patients with abdominal pain that are suggestive of surgical or emergent conditions are fever, protracted vomiting, syncope or pre-syncope, and signs of gastrointestinal blood loss (5).

The mortality and morbidity rates are very high when the patient happens to have an associated disease in addition to the acute abdomen.

Acute abdomen resulting from intestinal ischemia and necrosis has a poor prognosis (4, 6).

One of the most common causes of acute abdomen is acute appendicitis that requires emergency surgery to reduce postoperative complications, and directly affects the reduction of mortality, which is approximately 1% in these patients (7, 12). Making a timely diagnosis is the most important thing in this condition.

In mechanical or paralytic ileus, ischemic necrosis of the intestinal wall occurs with bacteria penetrating the abdominal cavity causing peritonitis. Rupture of hollow organs such as perforation of gastroduodenal ulcer, rupture of colon cancer, inflamed diverticulum can cause peritonitis. These complications lead to secondary peritonitis where the primary pathological process is located in the abdomen itself.

The operation is urgent, when the abscess is evacuated, the damaged part of the intestine is resected, and a protective colostomy is performed. On rare occasions, an anastomosis is performed in the first act, due to the greater possibility of dehiscence of the anastomosis postoperatively (8).

A number of patients with perforations due to gastric cancer are usually treated by resective procedures. Jejunoileal perforations are not often common as a cause of peritonitis. Small intestinal perforations can be caused by intestinal necrosis or some trauma. Bleeding from the upper parts of the digestive system is one of the most common emergencies.

The incidence of infections after elective operations on the gastrointestinal tract ranges approximately 20–25% of patients with peritonitis (9, 10, 13).

To summarize, the conditions that require emergency operations generally are associated with increased morbidity and mortality, especially in old people (9, 10). As far as the mortality in our study is concerned, it is similar to the rates in the majority of published reports.

Conclusion

All the previous facts suggest that diffuse peritonitis and bleeding are the most common and most dangerous conditions in surgery. A good clinical assessment is still crucial for the diagnosis.

It is especially important to pay attention to peritonitis caused by organ perforation. Beyond any doubt, the morbidity and mortality rates of emergency conditions are still very high and it is necessary to make the right diagnosis in time and do adequate surgery as soon as possible in order to improve the outcome.

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URGENTNA STANJA U HIRURGIJI

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Sva akutna stanja u hirurgiji klinički se prezentuju kao akutni abdomen. Cilj ove studije je identifikovanje najčešćih uzroka hitnih hirurških stanja.

Uključeno je 165 bolesnika operisanih zbog intraabdominalne perforacije, čija je konačna dijagnoza bila peritonitis. Bolesnici su operisani zbog krvarenja, u Urgentnom centru Kliničkog centra Niš, od toga 92 (56%) žene i 73 (44%) muškarca.

Kod 29 (27,9%) bolesnika uzrok peritonitisa je perforacija apendiksa, kod 24 (23,1%) bolesnika perforacija želuca, kod 5 (4,8%) perforacija jejunuma, kod 7 (6,7%) perforacija ileuma. Kod 20 (19,2%) bolesnika uzrok peritonitisa je perforacija debelog creva, kod 6 (5,8%) perforacija rektuma i perforacija žučne kese kod 13 (12,5%) bolesnika. Kod 24 (39,3%) bolesnika utvrđeno je krvarenje želudačnog ulkusa, kod 18 (29,5%) krvarenje iz duodenuma, 11 (18,1%) bolesnika imalo je povredu i krvarenje slezine, kod 5 (8,2%) bolesnika javilo se krvarenje jetre i kod 3 (4,9%) bolesnika razlog krvarenja bili su varikoziteti jednjaka. Ukupna smrtnost bila je 20,6% (n = 34). Od posledica septičkog stanja umrlo je 14 (41,2%) bolesnika, a 20 (58,8%) usled komplikacija gastrointestinalnog krvarenja.

Stope morbiditeta i smrtnosti u urgentnim stanjima još uvek su neprihvatljivo visoke.

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Ključne reči: akutni abdomen, peritonitis, krvarenje

"BRAIN PLASTICITY" AND STRESS

Marina Kostić

The analysis of stress consequences, regarding its quality and intensity, is being done in a wide range, enveloping a spectrum of psycho-neuro-endocrinological, i.e., organic, cognitive, emotional and behavioral organism's response to the impact of stress. All the categories of organism's response occur due to an imbalance among the stressor on one side, and organism's resources available in the fight for overcoming stress, on the other. If psychological stability is not optimal, the disorder of organism's homeostasis will occur, physiological stability will be disturbed, psychosomatic symptoms and illnesses will appear as a result of ill health caused by stress, when "the target" can be any organ, system of organs or more systems in an organism. Human brain – the organ that differentiates us from all the other living beings by its complexity, monitors the whole organism as well as functions in all the fundamental fields: physical, physiological, psychological and cognitive, is considered to impact the response of the immune system to illness, and partly to a person's response to a medical treatment.

The aim of this paper was the analysis of the impact of stress on the brain as well as the consequences of stress, with the accent on the ability of brain known as "plasticity", and the possibilities of regeneration of damaged brain cells.

The paper describes the case of 36 year old patient, who, as a consequence of long lasting, chronic, intense stress, was diagnosed focal lesions in the white mass, during her first MRI scan of endocranium (in 2013). The patient was withdrawn from the stressful environment, and with the help of psychiatrist her psycho-social rehabilitation treatment started. By the check-ups in the following years, during medical monitoring, (2014, 2016) NAD reports of her MRI scan were found, taken on the same scanner and checked by the qualified radiologists.

The before mentioned medical case study supports the theory of brain plasticity, whereas it has to be pointed out that the findings about the embryonic development of the brain is of vital importance for understanding its ability to reorganize in response to outer stimuli, especially in the case of long lasting chronic stress or post traumatic states.

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Key words: stress, brain, brain plasticity, regeneration

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Introduction: brain plasticity and stress

Central nervous system (CNS) has a complex, hierarchical organization whose parts have specifically determined "ingerences" over different psycho-motor and other functions of the organism, wherefrom it is to organize and reorganize itself in different ways under the influence of experience,

sensory stimulation, traumas... Thus, not all the brain parts can be easily modified and altered by experience, meaning that, not all the brain parts are equally plastic. Brain plasticity implies changes on the genetic and synaptic level, as well as on biological, chemical and physical levels, and it is worth mentioning that brain changes reflect themselves on the behavior as well. Environmental factors, personal experiences, and stress especially, lead to changes that will manifest in behaviour and might be evidence that a process of new learning has begun that will result in them. Schafer (1) settled *the neurophysiologic hypothesis of stress caused behavior* based on the study of decortified animal's activities, that points out that consequential behavior under stress is directly related to sub cortical processes. Neurological damage caused, for example, by trauma or a stroke, when weakened functions controlled by the damaged part of the brain must be re-learned, is also a "trigger" for new learning. It does not mean that any learning will lead to brain changes but that those will be forms

manifested in behavior and everyday functioning of the organism.

Kolb and Gibb (2) state that after a brain injury, plastic changes happen, enabling recovery; these authors state three possibilities in that context – changes in organization of remaining circuits of the brain, the development of new circuits, including formation of new synapses and creation of new neural cells and glial cells that might replace some neural cells. Some changes in the morphology of neurons happen independently one from another, occasionally in opposite directions or some changes are interpreted as surrogate for others. Authors Robinson and Kolb (3) give an example: big behavioral changes occur under the influence of psychoactive drugs, but neural changes are localized in majority of cases on pre-frontal cortex and nucleus accumbens. Plasticity depends on the age. The logical assumption is that developing brain is more susceptible to change and more sensitive to influences of experience compared to the brain of adults and elderly people. However, it should be pointed out that not all the plasticities are equally good and useful. Although it is widely accepted that plastic brain changes improve motor and cognitive functions, they can in certain percentage of cases hinder some behavior. In this context, changes caused by medications, psychomotor stimuli are taken into consideration, as well as plasticity seen through the prism of pathological point of view (epilepsy, schizophrenia, dementia, etc.).

According to one of the sub-divisions (4) three main forms of plasticity are: synaptic plasticity, neurogenesis and functional compensational processing. Neurogenesis, related to creation and development of new neurons in the brain, is the source of many scientific disputes, research polemics and scepticism. Thesis on neuron production in the brain of adults imposes the question whether the neurons which die are replaced by new neurons.

Brain is a key organ in the process of stress: it determines what organism will experience as stress, it determines how the organism will deal with the stressors, it changes itself functionally and structurally – as a result of stress, simultaneously controlling physiological, psychological, cognitive, emotional and behavioral reactions of the organism that are a response to stress. That two-way signalization, e.i., communication of the brain and the body is being done through short-term mechanisms that are adaptive (allostasis), and long-term that are maladaptive (allostatic load), which means that brain plasticity is the base for adjustment to stressful circumstances (5).

Neural brain circuits are responsible for behavioral and allodynamic system responses to stressors including hippocampus and amygdale, limbic brain structures, that send processed "signals" of experience to lower vegetative areas of the brain (hypothalamus and brainstem) and higher cortical areas as pre-frontal cortex (PFC); thus hippocampus, amygdale and PFC are considered coordinators between the behavior and allodynamic systems in

reacting and confronting the outer and inner stimuli that pose a threat for organism's homeostasis; they play crucial role in cognition, emotions and impulse control, help the inter-pretation of stimuli, based on experience as threatening or otherwise influential (5).

Brain tissue samples of patients suffering from depression show that frontal cortex and hippocampus have glia cell loss and reduction in size of neuron body but not neuron loss (6). Although the reduction of prefrontal area occurs, scans show the increase of functional activity in the same area (6). It should be pointed out that prefrontal area and its functions with a healthy brain are susceptible to changes under influence of experience. The authors state a representative example of medical students whose cognitive flexibility changes and functional connectivity reduces due to exam stress, which is shown by MRI scan during the test, and all the symptoms disappear during the school break (6). The comparative analysis on rats points out that chronic stress leads to a reduction of cognitive flexibility and shrinking of the dendrites in PFC (6). It is interesting that the same stressors can have different impact on hippocampus and amygdale (5).

Prefrontal cortex is important for work memory and mechanisms of self-regulation and targeted behaviors, while structural and functional plasticity in this region is changed under the influence of behavioral experiences modifying neural circuits, thus directly impacting the function of the brain. Functions of dorsolateral PFC are important in the context of consideration of stress sensitivity, aging, and have a key role in the brain disorder development. As well as many other regions of the brain amygdale and PFC contain adrenal steroid receptors (6) and it is crucial to research the vulnerability and plasticity of PFC during the life cycle, since the results in this field will be of huge benefit in prevention, maintenance and enhancing of mental and physical health.

The aim of the paper

The aim of the paper was the analysis of the impact stress might have on brain and its consequences, with the emphasis on the feature of the brain known as "plasticity", as well as possibilities of regeneration of the damaged brain cells.

Case study

Personal anamnesis

Patient NN, 36 years old, a college graduate, permanently employed, divorced, one child, right handed, mother tongue Serbian. She has been living in Nis for 18 years. In her primary family she was the older child, her parents divorced after 27 years of marriage, when she was 28; family anamnesis burdened with malignant diseases: her father died of colon cancer when he was 56, and her four years younger brother died of acute lymphoblast leukemia in 2011 when he was 28, which was an extremely

traumatic event for the patient, additionally burdened with subsequent marital problems.

The person in question is of very high aspirations and ambitions, which she could not realize during the marriage, especially during the last few years – which she explains by the loss of confidence during the time, "adrift and under the will of mentally stronger spouse", resulting in very frequent sense of depression, despair, dissatisfaction, neuromuscular tension, very frequent headaches and other psycho-somatic problems. During that period she has been treated for viral pericarditis, after that for stomach ulcer together with the colon damage; after her brother's death she has visited a psychiatrist periodically with the aim of psychological strengthening and improving the problem facing skills; the prescribed therapy by sedative (Bromazepam 1.5 mg) and antidepressant (Trittico retard 150 mg) a minimal dose (1/3), has among other things, solved her problem with sleeping and insomnia.

She had tonsils and appendix removed by operation. Right handed.

Examinations done and diagnostic results

The first hospitalization was in February 2016 in the daily hospital for neurology because of the difficulties with the right eye as well as subjective feeling of uneasiness in the sense of pressure in the right eye, without any changes in the sharpness of sight. The symptoms mentioned have lasted for approximately two weeks before coming to the clinic, but the patient has not taken them too seriously, considering them temporary, although they have hindered her in doing her daily activities, especially reading and writing, and very frequently they have been followed by bodily instability and weakness. During the seven-day-stay at the daily hospital for neurology, she was given infusion (NaCl with aminophylline), injections of OHB 12; and the analyses and research done led to the following conclusions:

- Psychological finding on admission implied preserved vigilance and orientation without visible psycho-pathological symptoms; somatic findings within normal limits, with neurological status. An eye exam showed no pathological signs, except indicated visus correction (Myopia, VOS = -0.25, VOD = -0.5) dating from, according to the patient, previous 10 years ago. Lab results on admission were NAD. It should be pointed out that lab results and tests were NAD, all parameters within limited values, during all MRI scans and hospitalization.

- The first set EP (VEP, AEP, SSEP) done in 2013 was NAD; the last test (2016) was without any significant asymmetry on all modalities, NAD.

- Neuropsychological testing showed exceptional actual level of intelligence, with far better achievements in relation to the testing performed two years earlier. MMPI questionnaire showed a profile with minimum rise in depression scales as the fall of willful impulses.

- The conclusion from MRI brain scan, third in the period of three years, showed initial reductive

parenchyma changes on convexity parietally bilaterally, and other endocranium structures NAD.

Based on the anamnestic data, somatic and neurological findings clinical features presented *possible retrobulbar neuritis of the right eye*, and the control neuroimaging (brain MRI scan) and neurophysiologic diagnostics did not show the existence of structural lesions and functional disturbances of the brain parenchyma.

Within the context of this paper related to the brain plasticity, it was crucial to do a comparative analysis of MRI endocranium scans that were, except for the last one in 2016, abovementioned, done annually, in 2013 and 2014.

After MRI scan done in 2013 and the conclusion which indicated that lesions were in the form of demyelization, on the neurological level (EP scans, neuropsychological testing, etc.) it was clarified that there was no potential possibility of *multiple sclerosis*. However, in relation to the newest, abovementioned neuropsychological testing, the conclusion drawn at that moment was that the actual level of intelligence matched higher to above average level of intelligence, without quantitative determiners of attention disorder, but with evident significant flow of extreme anxiety, that resulted from willing avoidance of focusing on the stimuli. The recommendation was to see a psychiatrist and retest in 6 months, which was done a year later. It should be pointed out that MRI scans of the head were done on the same scanner in the Clinical Centre Niš.

2013 scan

On the day of 01.11.2013, T1W and T2W/FLAIR axial, T1W and T2W sagittal and T2W coronary tomograms through the brain were done. The scan showed two discrete T2W/FLAIR hyper-signal changes, by the atrium of the right lateral chamber as well as in the frontal sub cortex on the same side. A change of the same characteristics existed on the left hemisphere of the cerebellum, without lesions manifested in the brainstem and medulla oblongata. All the other structures tested were without pathological changes, new substances, or significant inflammatory manifestations. The conclusion of the radiologist was, therefore, that there were several focal lesions in the white mass that might have had the form of demyelization, with the recommendation of further control (Figure 1).

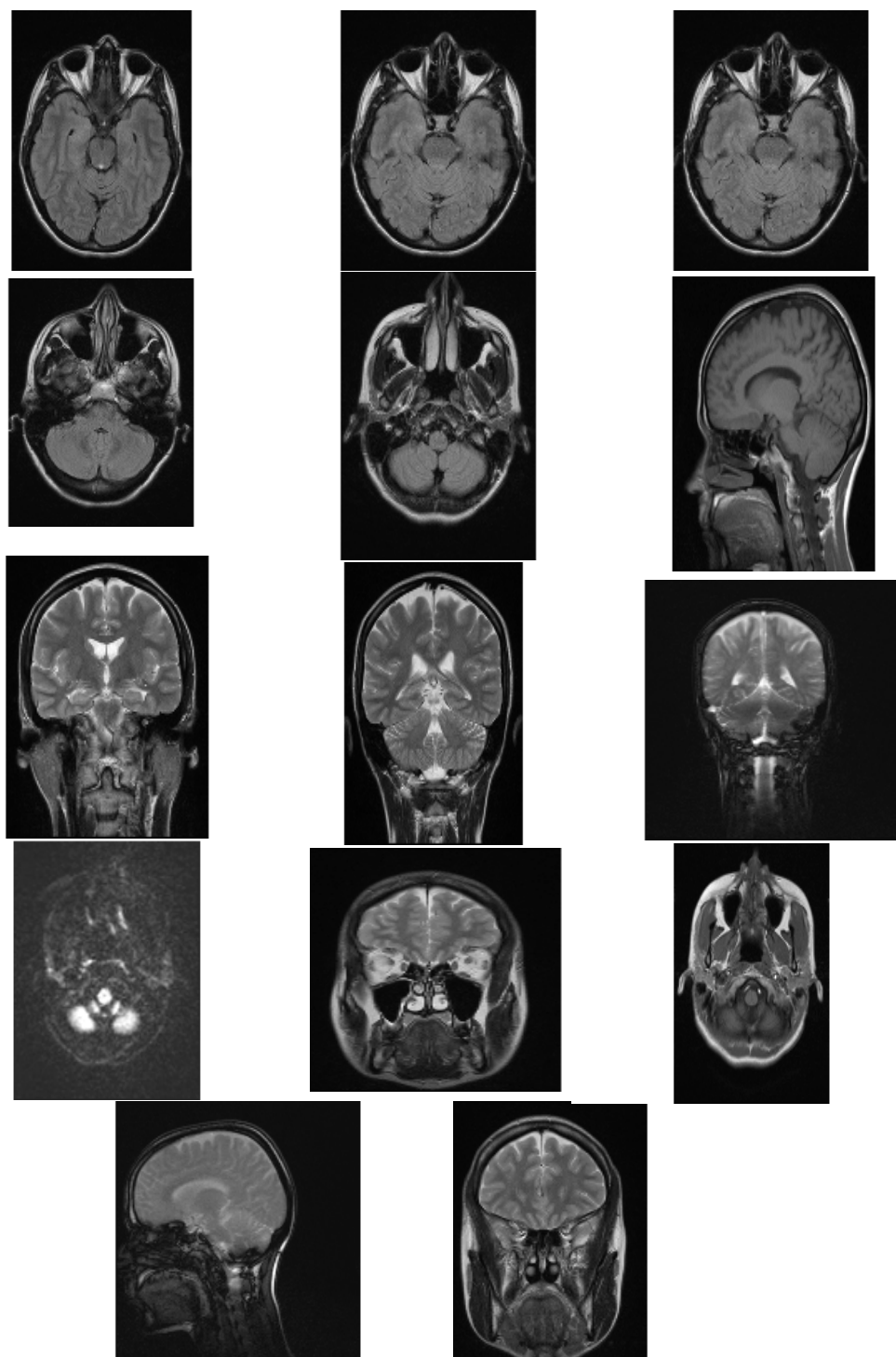


Figure 1. MRI neuroimaging in axial, sagittal and coronary plane

2014 scan

On the day of 17.10.2014, T1W and T2W/FLAIR axial, T1W and T2W sagittal and T2W coronary tomograms through the brain were done,

and compared to the previous above described MRI scan from 2013. In the frontal sub cortex on the right side there were two discrete T2W/FLAIR hypersignal changes, matching widened perivascular spaces. There were no lesions of brainstem, medulla

oblongata and cerebellum. All the other structures tested were without pathological changes, new substances, or significant inflammatory manifestations.

The conclusion of the radiologist that time was NAD (Figure 2).

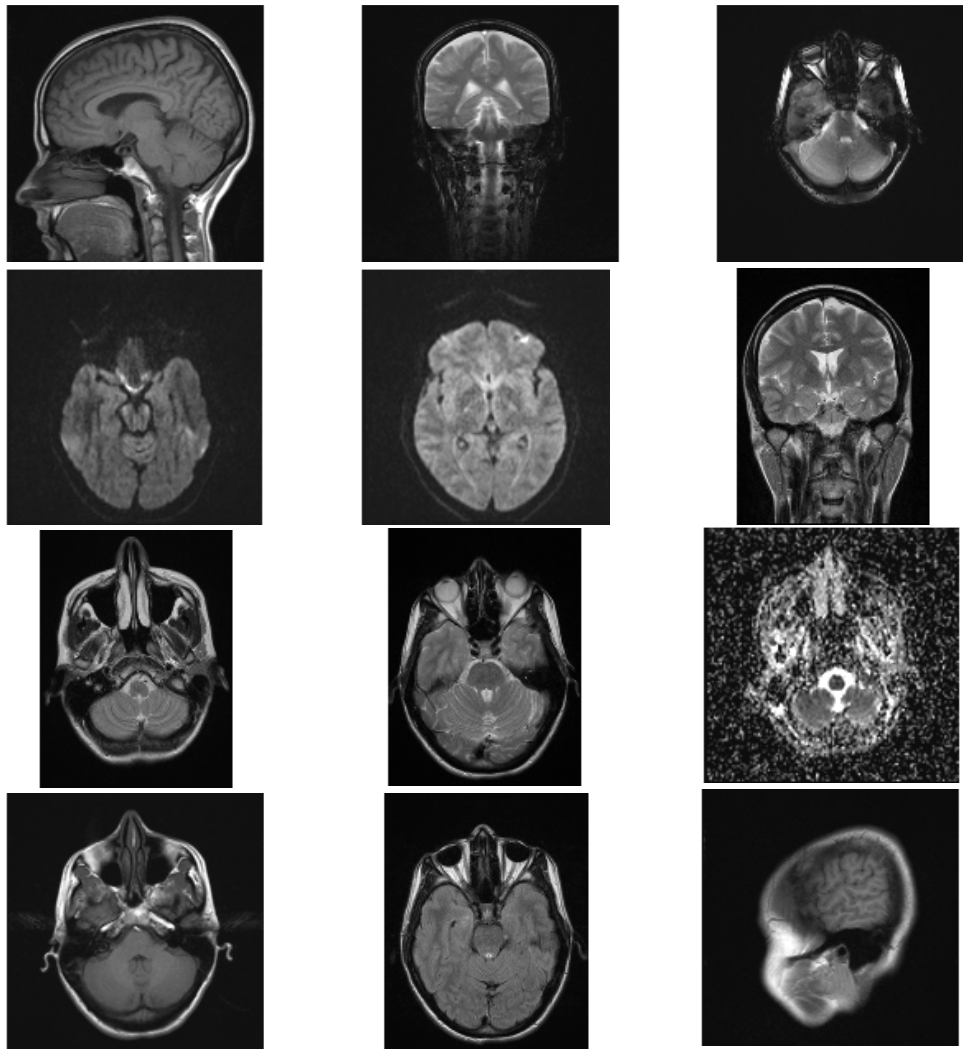


Figure 2. MRI neuroimaging in axial, sagittal and coronary plane

2016 scan

On the day of 17.02.2016 MRI scan of endocranium was done by standard procedure and it showed symmetrically wider peripheral CSF spaces; brain parenchyma of the proper morphology and signal intensity without focal lesions, no pathological

changes or signs of inflammation. Mediosagittal structures, ventricular system, brainstem and cerebellum of the proper content, structure and size. The conclusion – the initial reductive parenchyma changes in the convexity parietally bilaterally, with other endocranium structures NAD (Figure 3).

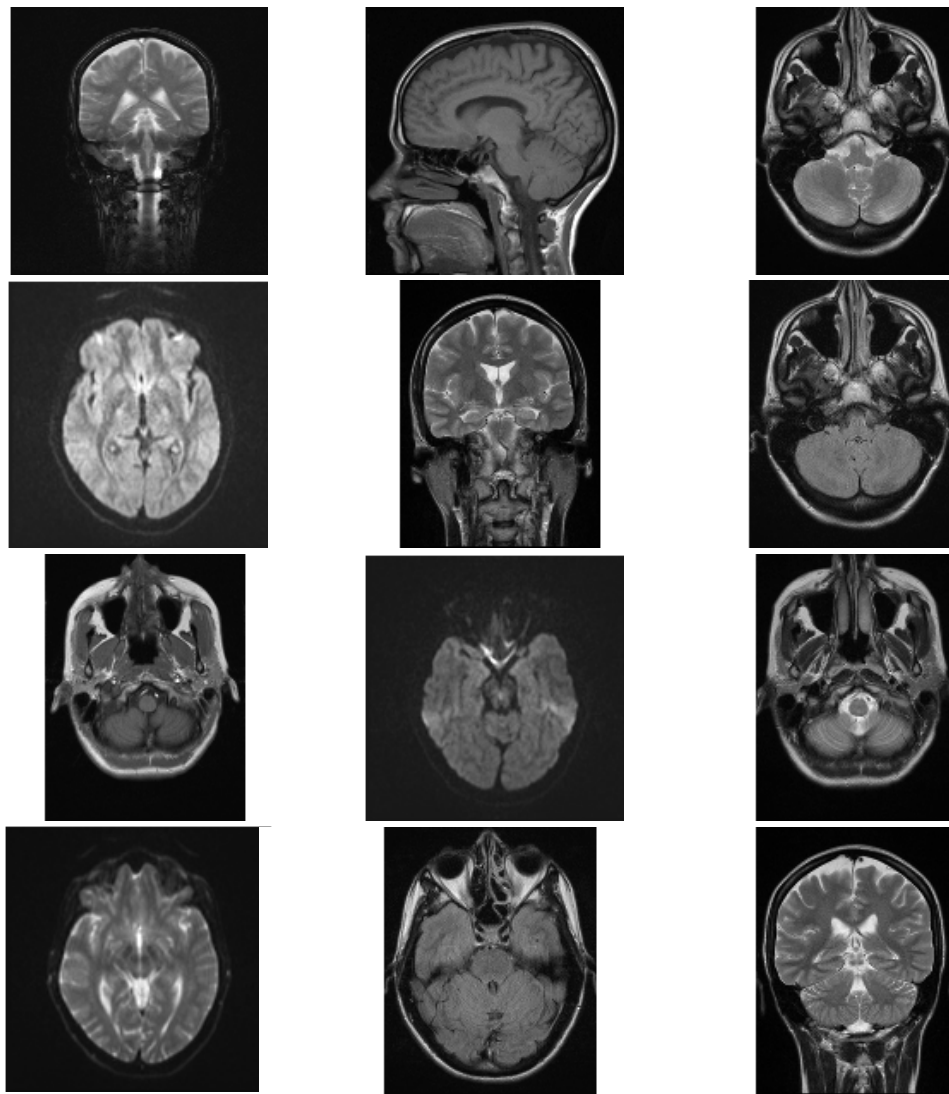


Figure 3. MRI neuroimaging in axial, sagittal and coronary plane

Discussion

Having in mind the plasticities described in the literature (synaptic plasticity, neurogenesis and compensatory plasticity) and their characteristics, the theoretical basis of the analysis of this topic and the results of contemporary medical research which go in its favour and support it with examples, the case of the patient Jane Doe (36 years old) can also be put in the abovementioned complex frame of scientific discussions on brain function and its role in coordination between the impact of the stressors on one hand and organism responses on the other.

It has been concluded that levels of plasticity are different in various ages and that interactions between different types of plasticities caused by activity, i.e., surrounding stimuli and the age of the patient are of crucial importance for understanding the decline of the level of intellectual and mental capacities the person faces as a consequence of the

effects of those surrounding stimuli in certain parts of the brain. The consequences can be degenerative processes or the processes of compensation of "endangered" neurons. Neurons have their extensions and form complex neural networks using them, and each damage means its "loosening", after which the network will naturally tend to regenerate or reorganize, that is evident in this case study. Timely rehabilitation establishes proper neural circuits and function of the damaged areas of the CNS. With aging, the plasticity is weakened and decreased, but still exists – not only during the normal functioning period but also in the pathological states; it is considered that the building, reorganization and regeneration of the connections between neurons, that will make up for "disintegrated" neurons, is of the core importance (7). There is a qualitative difference of the brain changes occurring as a consequence of the completely same experience but at different stages of life. Considering the fact that the

factors plasticity depends on are gender differences, aging and mood disorders (6), "accumulated" stress experiences can lead to brain neuron damage and the reduction of the plasticity, and in this paper it is documented that by timely pharmacological and psychological actions, intervention, strengthening as well as isolating from stressful environment, might lead to regeneration and recovery of the individual. Brain processes, i.e., plasticity depend on surrounding stimuli as well as internal environment by the organism itself, thus dictating allostasis that is the core of its homeostasis. Allostatic mechanisms are crucial in sudden stressful situations, but their impact is prolonged, they influence negatively both mental and physical health primarily because of the negative effects to the brain plasticity. Both mechanisms are connected to the concept of expectations and evaluation of the stressor, prediction, care and anxiety and cognitive preparation (5). Cognitive preparation has, in this case, in the long run, brought about positive effects in the sense of stressor evaluation and adaptation of the patient which resulted in the recovery on every level of functioning. At this point, it should be emphasized that not all stressful situations are negative; some forms of mild stress are necessary for normal, successful development of the individual (8).

What is the relationship between the brain plasticity and the duration of surrounding stimuli? Some changes occur under the influence of the stimuli for a short period of time, some are influenced by stimuli for a longer period of time, whereas other neurons have not undergone any other change or the course of changes has been reversed. Plasticity refers to all the ways the brain changes, including the changes followed by the damage of the functions and changes in behavior. Authors Kolb and Gibb (2) consider that changes in the brain can be seen in many levels of analysis, and result in changes in behavior, and that there are many ways for them to be explored, from global measuring of brain activity to *in vivo* scan. Thus, when it comes to changes in behavior, the patient was completely aware of them but often felt personal "helplessness" to resist them due to locus control disorder and disturbances which became frequent in the area of cognitive functions: memory, attention, concentration, sometimes even physical orientation. Plastic changes initiated by experience do not occur with equal probability in all parts of the brain, but depending on the traumatic influences, the occurrence of synaptic changes in different brain hemispheres has to be analyzed. Reaction of the organism to stress is always the same – adjustment. However, when it comes to plasticity it should be pointed out that plasticity is the basis of adjustment in stressful circumstances but it can also be the base for pathophysiological reactions of the organism to stressful circumstances, with direct influence on a person's mental and physical health (5), as in the case analyzed. During the period of two and a half years of monitoring of the patient's health, besides the before mentioned comparison of medical findings, it is of high importance to point out that, during the monitoring, the patient, after the divorce (the beginning of the monitoring period), at her own initiative, with support and

encouragement of the psychologist and the psychiatrist, as well as support found in professional medical literature, positive psychology and professional literature, has in many ways changed the way of thinking, style and way of life, and has carried out changes and her own self actualization in all the spheres of life (hobby, professional training, promotions at work, further education, sport and recreation...).

Extreme stressful experiences have functionally relevant effects on the dendrites and the number of neurons, and it is not only the influence on cognitive functions but also on neuro-endocrinal, autonomous functions, emotional regulation, cognitive processes, and patterns of behavior (5). Through the prism of this paper, along with the comparative analysis of MRI scans, there are significant differences in the results of neuropsychological tests (18.12.2013. and 18.02.2016.) which show significant improvement of general intellectual capacities – all the parameters (global, verbal and non-verbal IQ) and actual level that initially implied high to above average level, and afterwards to – above average intelligence. Although there were not any quantitative markers of attention disorder, mnestic data initially implied influence of highly expressed testable and general anxiety which resulted in willing avoidance of focusing on a certain type of stimuli. The fact that the patient at the very beginning of the monitoring was aware of the drastic decline of her intellectual capacities and abilities, anxiety that prevented her from daily functioning and mnestic disturbances, is intriguing – which she has blamed on long-term effect of the intense, chronic stress. Changing her life circumstances, surroundings, introducing positive stimuli and willing and intentional isolation from the stressful life context and the influence of stressful stimuli of any kind, positive changes occurred which have been noted on organic and neuro psychological level of functioning, which can be subsumed under the effects of brain characteristic that is the topic of this paper – its plasticity. It resulted in the improvement of general health, psychological stability, and readiness to take responsibility and face problems.

It can be concluded that the experience shapes the brain plasticity, but it raises the question whether it is possible to influence it by introducing focused system intervention, and whether there are ways for keeping elasticity and plasticity of pre-frontal part in spite of aging. Many research and studies confirm the thesis by their empirical findings: it is possible to intervene and change "the architecture" of the brain, improve the cognitive functions and self regulatory behavior. As an example, *cognitive training* is a good way of stimulating and activating of brain plasticity since it provides systematic drill necessary for setting up of new and strengthening of the existing neural connections in the neural network. The mature brain has greater ability for plasticity than thought before, which turns out to be very significant point for planning of behavioral and pharmacological treatment in the future.

Conclusion

Isolation from stressful life context and influence of stressful stimuli, positive changes in the functioning and achievement of personal ambitions and aspirations, that is, general satisfaction with life and self satisfaction are important factors which can influence on overcoming the consequences of long term, chronic stress the person has been exposed to, as well as its gradual elimination. The brain can be considered as an organ whose key role is in the process which defines what the organism will regard as stress, and the way the organism will handle the stressors regarding the adaptation and restoration of its homeostasis and allostasis. The before mentioned case study gives us the right to conclude that the brain is the organ susceptible to functional and structural changes as a result of stress, as well as confirming the fact that the same organ, at the same time controls physiological, psychological, cognitive, emotional and behavioral reactions of the organism as a response to stress. Based on all above mentioned, we can say that brain's plasticity explains the evident resulting changes seen on patient's MRI scans of endocranium, as well as significant improvements in achievements noticed

during the neuropsychological tests. This and similar cases, are pre condition for the progress of neuroscience and set up the task and obligation for the experts to use present findings and new discoveries on the development of the nervous system to better understand certain illnesses and their origin, beginning, and treatment.

Possible weak points of the research

Due to complexity of the brain and influence of many factors, both endogenous and exogenous, it should be pointed out that these brain changes can be caused by other factors, not only stressful ones, considering the fact that the patient has had serious viral et as well as positive family burden on leukemic diseases. In that way, one cannot exclude the possibility that, besides the psychological stressors some organic CNS diseases were in action as well. We should also have in mind that the MRI scan results were not analyzed by the same radiologist each time, but by three different radiologists, inter subjective compatibility must be considered too, with respect to interpersonal differences in observation, findings and interpretation of the scan results.

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doi:10.5633/amm.2020.0210**"PLASTIČNOST MOZGA" I STRES***Marina Kostić*Univerzitet u Nišu, Filozofski fakultet, Katedra za psihologiju, Niš, Srbija
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Analiza posledica stresa, s obzirom na njegov kvalitet i intenzitet, vrši se u širokom okviru, koji obuhvata spektar psihoneuroendokrinoloških, odnosno organskih, kognitivnih, emotivnih i bihevioralnih odgovora organizma na delovanje stresora. Sve kategorije odgovora organizma nastaju zbog disbalansa između stresora, sa jedne strane, i resursa organizma raspoloživih u borbi za prevladavanje stresa. Ako psihološka stabilnost nije na optimalnom nivou, doći će do poremećaja homeostaze organizma, narušavanja fiziološke stabilnosti i javljanja psihosomatskih simptoma i bolesti kao posledice stresom narušenog zdravstvenog stanja osobe, kada "meta" može biti bilo koji organ, sistem organa ili više sistema u organizmu. Ljudski mozak, organ koji nas svojom složenosti razlikuje od svih ostalih živih bića, ima nadzor nad celim organizmom i svim funkcijama, na svim fundamentalnim poljima: fizičkom, fiziološkom, psihološkom i kognitivnom, smatra se da utiče na odgovor imunološkog sistema na bolest, pa delimično i na odgovor pojedinca na lečenje.

Cilj rada je analiza uticaja koje stres može imati na mozak i posledica stresa, sa akcentom na svojstvo mozga poznato kao "plastičnost", kao i mogućnosti regeneracije oštećenih ćelija mozga.

U radu je prikazan slučaj bolesnice stare 36 godina, kojoj su, kao posledica uticaja višegodišnjeg, hroničnog i intenzivnog stresa prvim urađenim MR pregledom endokranijuma (2013. godine) konstatovane fokalne lezije u beloј masi. Bolesnica je povučena iz stresogenog okruženja i uključivanjem psihijatra otpočet je psihosocijalni rehabilitacioni tretman. Kontrolnim pregledima, koji su narednih godina usledili tokom medicinskog praćenja (od 2014. do 2016.), konstatovani su uredni MR nalazi pregleda glave, snimljeni na istom aparatu i pregledani od strane kvalifikovanih radiologa.

Navedeni prikaz bolesnice govori u prilog potvrdi teorije o "plastičnosti mozga", pri čemu treba naglasiti da su saznanja o embrionalnom razvoju mozga od osnovne važnosti za razumevanje njegove sposobnosti reorganizacije u odgovoru na spoljašnje nadražaje, naročito ako se govori o dugogodišnjem, hroničnom stresu ili o periodu posle povrede.

*Acta Medica Medianae 2020;59(2):72-80.***Ključne reči:** stres, mozak, "plastičnost mozga", regeneracija

THE EFFECT OF THE INFLAMMATORY REFLEX ON THE HEART

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The native immune system is the main component of defense against pathogen, injury and trauma. Vagal fibers contain sensory and motor components controlling different functions such as heart frequency and digestion. There are lots of reasons this nervous based anti-inflammatory pathway is useful, the cholinergic anti-inflammatory pathway, which is fast, separate and localized in tissue where the injury originated and can induce humoral systemic anti-inflammatory response which has a number of usefull effects on a number of heart diseases.

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Key words: heart disease, inflammatory reflex, vagal stimulation

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Introduction

Inflammation is a local, protective response to a microbial invasion or injury. This response must be harmonic and precisely regulated, therefore a response too strong or too weak will lead to morbidity and a shortened lifespan. Based on multiple scientific discoveries, the immune system is not viewed upon as autonomic, the vision of a selfregulating immune system is a long crushed paradigm. Experimental data show that the nervous system regulates immune functions and inflammation. The two-way communication between the brain and the immunological system is the underlying mechanism of inflammation control and immune system checkup. The discovery that the cholinergic neurons inhibit acute inflammation expands our knowledge and understanding about the modulation of immune response by the nervous system. The immune system is modulated by reflexes which adjust its center of balance which can be up or downregulated. The activity of the efferent vagal nerve maintains the

homeostasis by limiting the pro-inflammatory response within the healthy and protective and non-toxic range.

Survival is impossible without the everwatching sentries against pathogen and injury. Native immune system is the autopilot on this craft. When facing assault it unwillingly creates a separate, localized inflammatory response to prevent any unnecessary damage (1). That response is mediated by macrophages, monocytes and other immune cells which release cytokines, interleukins and other mediators of inflammation (2). The volume of this response is of vital importance, for insufficient response leads to immunodeficiency which can lead to infection or cancer (2). On the other side of the spectre, an exaggerated response of TNF- α and other pro-inflammatory mediators leads to systemic inflammation implicating serious pathological complications such as sepsis, Chron's disease, RA, MS (2, 3), atherosclerosis, diabetes, Alzheimer, cerebral and myocardial ischemic diseases. If the inflammatory response spreads to the circulatory system, as is the case in septic shock, meningitis and heavy trauma, the response can be more lethal than the initial stimulus. Inflammation control is conveyed by two main mechanisms: native immune system's self-control and immunoregulatory influence deriving from the brain (1).

Activated immune cells release anti-inflammatory cytokines, IL-10, IL-4, TGF- β and soluble TNF receptors which play a counter-regulating role in inflammation (2). Hypothalamic-pituitary-adrenal axis, which controls the release of glucocorticoids is a neuroendocrine immunomodulatory mechanism and originates from the brain (4). The initial cerebral response to inflammation is mediated by autonomous nervous system pathway activation (1). The afferent vagal fibers send a quick signal to the brain to start sending immunomodulatory responses in

the early phase of inflammation. Novel studies imply that the efferent vagal stimulus inhibits pro-inflammatory cytokine release and regulates inflammation, similar to the effects it has on the heart and gastrointestinal function (1). This efferent vagal function is noted as the cholinergic anti-inflammatory path (5). Homeostasis and health are reinstituted when inflammation is limited by anti-inflammatory responses which reoccur, are quick and reversible, localized and adaptive to changes in the sensory nervous signals and integrated in the nervous system. Recent discoveries have identified the basic nervous path which follows and regulates the anti-inflammatory response in reflex. The inflammatory sensory signal activates the anti-inflammatory response which is both quick and involuntary and acts as a countermeasure to prevent the inflammatory mediators into the circulatory system. The nervous system integrates the inflammatory response, gathers information on the invasive events in the organism, mobilizes defense and creates memory to enhance survival (1). Both cholinergic anti-inflammatory mechanisms inhibiting the activation of macrophage and cytokine release and vagal stimulation, electrical and pharmacological, prevent inflammation and cytokine release, which is clinically relevant in treating diseases.

The native immune system is the main component of defense against pathogen, injury and trauma. The host recognizes molecular patterns related to the pathogen receptors like the Toll like receptors (TLR) (10), so in case of infection a lipopolysaccharide, part of the gram negative bacteria or an endotoxin activate the TLR4 receptors found on the macrophage, dendritic and other immune cells. Receptor interaction with the ligand induces intracellular pathways, leading to cytokine and other pro-inflammatory mediators' release (2). Tissue damage and trauma can, in absence of infection, activate the native immune response. Novel work show that the necrotic tissue damage leads to heat-shock proteins release (HSP) and oxidized lipoprotein which in turn activate the immune cells via TLR (2, 8). Pro-inflammatory mediators induce vasodilatation, attract leukocyte and increase vascular permeability. The pro-inflammatory response is dampened by the anti-inflammatory mediators release (IL-10, IL-4...). Polyamine spermine released locally can suppress the pro-inflammatory response (9). Macrophage and dendritic cells, as antigen presenting cells, bind the native immune system with the lymphocyte T and B induction thereby laying foundation for creating specific immune response (2).

TNF- α is essential in the pro-inflammatory response, it is both necessary and sufficient as a mediator of local and systemic inflammation. The local rise in TNF- α causes the main clinical signs of inflammation (heat, swelling, pain and ruddiness). If released uncontrolled, it can lead from local to systemic and chronic inflammation leading to tissue damage and death. Many diseases (RA, ischemic-reperfusion damage, sepsis) are flagged as inflammatory because of the crucial contribution of patho-

logical complications of inflammation (2). With transition of local to systemic inflammation, the rise in TNF- α levels is toxic and mediates microvascular coagulation and capillary leak syndrome eventually leading to lethal shock (10). TNF sends its own and other proinflammatory mediators' synthesis into a spiral. Auto regulatory inflammation is normally characterized by a drop in TNF- α activity. Small quantities of TNF- α can contribute to the host defenses by limiting the spreading of pathogen into the circulation, enhancing coagulation around pathogen and stimulating the healing of damaged tissue. In a typical, successful inflammatory response, duration and quantity of released TNF- α are limited, its useful and protective properties overrule and its actions are local.

Vagal role in neuroimmunomodulation

Vagal fibers contain sensory and motor components controlling different functions such as heart frequency and digestion. It was only later discovered that besides controlling these classical physiological functions it also prevents TNF- α , HMBG1, IL-1 and other pro-inflammatory cytokines from immune cells (1). Afferent vagal fibers play a role in immune signal transmission to the brain during inflammation and efferent fibers lead to inflammation suppression. Both types of fibers make the cerebrally integrated reflex mechanism controlling inflammation timing (1). Sympathetic immunoregulatory response is also a component of the efferent part of the inflammatory reflex.

Afferent arch of the inflammatory reflex

The central nervous system takes in its immune signals both through humoral and nervous pathways. Blalock originally suggested that the immune system works as a "sixth sense", discovering microbial invasion and producing molecules to translate this information to the brain (12). TNF and other immune mediators can get to the brain centers in the circumventricular region deprived of the blood-brain barrier. And so, the dorsal vagal complex containing the sensory solitary tract nucleus, area postrema and dorsal vagal motor nucleus all respond to TNF- α blood level spikes and platos by changes in motor vagal activity. This humoral communication pathway between the immune and the nervous system is involved in the development of fever, anorexia and hypothalamic-pituitary responses to infection and injury, and also other behavioral disease manifestations. The most studied afferent pathway is the humoral one, through the hypothalamic-pituitary adrenal (HPA) axis, via circulating cytokines released from the peripheral leucocytes and then bonded to central cytokine receptors (13).

There is supposedly more than one possible way of signal conductance. One is active transport of cytokine through the BBB, the other being less likely through the cerebral vascular endothelia and thus inducing creation of secondary messengers such as prostaglandins and nitrous oxide. *In vitro* studies

have shown IL-1 β , IL-6 and TNF- α inducing changes in cerebral endothelial cells electric resistance. Alternatively, there is a possibility that soluble signal mediators, such as immune cytokines may get to the brain through the circumventricular organs with an incomplete BBB, enabling exchange of high molecular peptides between the blood and the brain. Experiments using highly neurotoxic mono-sodium-glutamate (MSG) resulting in lesions in the level of the hypothalamic arched nucleus (ARCN) (14, 15, 16). Lesions followed by a decrease in number of neurons and their degeneration in the area of ARCN leading to a derangement of the hypothalamic-pituitary-adrenal-thymus axis (HPATA) (14, 16) as well as hypothalamic-pituitary-thyroid axis (HPTA) (15). The neurons of the central ventral organs (CVO) are connected to the hypothalamic regions and nuclei of the neural stem, but the expression of the c-FOS protein is noted in the farther regions of the brain. During iv treatment with LPS a spike in number of c-FOS protein positive cells in the main group of noradrenergic cells (A1, A2, A6) in the paraventricular (PVN) (17), supraoptic (SO) hypothalamic nuclei and in the limbic structures such as trough strial terminal nucleus, central amygdaloidal nucleus and the ventral septal area is registered. CVO contains many hormones, neurotransmitters and transmitter-like factors such as ACTH, acetylcholine, serotonin, dopamine as well as a high concentration of adequate receptors. This system of neurons can reach the farthest parts of the central nervous system including the strictures of the limbic frontal brain. It can be concluded that CVO plays a crucial role in translating cytokine signals into nervous activity. Neurons express type I receptors for IL-1 (IL1-RI), which is the main receptor for mediating IL-1 type cell response. During the bonding process between IL-1 and IL1-RI, the receptor forms a heterodimer with an accessory peptide IL-1R. This complex bonds with a primary myeloid differentiation response protein 88 (MYD88, an adapting TLR protein) and recruits signal proteins which in turn activate MAPK (mitogen activated protein kinase) and nuclear factor- κ B (NF- κ B) (18). NF- κ B activation in the neurons is entwined in management of pro-inflammatory gene transcription (19). Inside the paraventricular nucleus IL-1 stimulates neuron depolarization by activating non-selective COX dependent cationic conduction (20). This confirms the paradigm that the presence of IL-1 in the brain during injury or infection activates the nervous circuits to mediate a local and systemic pathophysiological response. Besides IL-1, which plays a central role in the inflammatory reflex sensory arch activation, other endogenous and exogenous factors when joined with inflammation can activate the inflammatory reflex. Infections molecular products activate macrophage, monocyte and other early response cells. All of these molecules interact with TLR and finally lead to NF- κ B activation and an increased release of pro-inflammatory cytokines (21). Endogenous molecular products released from damaged cells during sterile injury or ischemia, even in absence of pathogen, include cytokines, HMGB1, HSP, hyaluronic fragments,

ATP, uric acid, heparin-sulphate and other molecular patterns related to injury (DAMP) (22).

Electrophysiological studies show that vagal stimulation can be achieved by TNF, other cytokines, mechanoreceptors, chemoreceptors, temperature and osmolality sensors which can all be activated inside the inflammation locus. Somatic sensory nervous signal inside the central nervous system is organized in a somatotropic fashion, like so that the sensory entry signal from a separate peripheral focal point is precisely localized in the ascending fibers and the brain.

Cholinergic anti-inflammatory pathway – efferent inflammation reflex pathway

Our understanding of basic inflammatory regulation mechanisms has evolved with the discovery of a nervous parasympathetic macrophage inhibition mechanism (5). It is named the "cholinergic anti-inflammatory pathway" for having acetylcholine as the primary parasympathetic neurotransmitter. It inhibits the release of TNF from LPS-treated macrophage (5). It should be underlined that acetylcholine only inhibits expression of TNF proteins in the macrophage, not the TNF induction of TNF mRNA, therefore, the inhibition is on a posttranscriptional level. Vagal stimulation significantly downregulates TNF, IL-1, IL-6 and IL-8 production, but not the anti-inflammatory cytokines production (IL-10, TGF- β). *In vivo* studies show that efferent cholinergic vagal neurons play a crucial role in controlling TNF levels and other immune responses. Macrophages that are exposed to acetylcholine get successfully deactivated. All vital organs are innervated by the vagal nerve, including those with a reticuloendothelial system (liver, lungs, spleen, kidneys and GIT) (23). Direct electric stimulation of the cholinergic efferent anti-inflammatory pathway protects the organism, significantly inhibits cytokine production by the innate immune cells in the mentioned organs and other tissues innervated by the vagal nerve (3, 24) and can downregulate the TNF serum concentration in endotoxemia. Limiting the release of potentially toxic mediators produced by the innate immune cells, the cholinergic anti-inflammatory pathway protects the organism from organ damage and death during endotoxemia, sepsis, hemorrhagic shock, colitis, arthritis, ileus, pancreatitis and other syndromes with abnormal cytokines release. The level of protection achieved by electrical vagal stimulation depends mostly on the used voltage, and goes hand in hand with heart rhythm stabilization during the hypotensive phase induced by reperfusion. Vagotomy is followed by increased TNF levels in the serum in response to endotoxin treatment (5). It has a way of sensitizing an organism to lethal effects of endotoxins. Vagal nerve has a tonic effect of suppression of TNF release. The effect is an analog to the tonic inhibitory influence it has on the heart rhythm. With the lack of vagal stimulation on the heart, the frequency rises, also the pro-inflammatory molecules production increases as does the organ damage during times of endotoxin exposure

(25). The net effect of the innate immune system to infection and injury is a reflection of the efferent inflammatory pathway reflex activity.

Vagal inflammation suppression characteristics

Efferent vagal stimulation is classically characterised by heart frequency lowering, induction of gastric motility, arteriolar dilatation and pupil narrowing. Inhibition of the inflammatory response can be added to this list. There are lots of reasons this nervous based anti-inflammatory pathway is useful. A diffuse anti-inflammatory network including glucocorticoids, anti-inflammatory cytokines and other humoral mediators is slow, divided, unintegrated and depends on the concentration gradients. On the other side of the spectrum is the cholinergic anti-inflammatory pathway, which is fast, separate and localized in tissue where the injury originated and can induce humoral systemic anti-inflammatory response, this is possible because vagal activity can be translated to the medullar reticular formation, LC and the hypothalamus, leading to increased ACTH release from the frontal pituitary lobe.

Compared to the speed of the regular, biological inflammatory response (hours and days), the nervous signaling is closer to lightning. This regulatory property is useful in critical stages of disease. After a short refractory period, in the absence of the next nervous signal, appropriate cells can retrieve function.

Immune homeostasis reflex control

The inflammatory reflex affects the magnitude and the volume of native immune system's response. During the state of inactivity, the inflammatory reflex contributes to the balance of the immune response on the molecular products of infection, injury or ischemia. Vagal response maintains homeostasis by limiting the pro-inflammatory response in the optimal range and the damaged tissue in a state of mild stimulation. Patients with inflammatory diseases have functionally elevated states of balance during inactivity, producing high levels of pro-inflammatory mediators and a momentarily reduced heart frequency variability rate, which is an index of reduced vagal activity. Healthy individuals have a lowered state of equilibrium of that comes with an elevated index of vagal activity and a lowered pro-inflammatory cytokines release response. Physical exercise, controlled breathing, fish oil consumption in daily diet and relaxation have a stimulating effect on vagal activity and suppress pro-inflammatory cytokine release (29).

Therapeutic implications

A question arises, does activating the inflammatory reflex cause immunosuppression? Experiments on animal models show that targeted therapy that boosts the inflammatory reflex activity actually normalizes the native immune response without its discontinuance or immunosuppression onset. Electric

or pharmacological stimulation of the inflammatory reflex sets the equilibrium plane inside the protective range instead of completely inhibiting innate immune response. Electric vagal stimulation or $\alpha 7nACh$ agonist administration reduces pro-inflammatory cytokine volume production by 50-75% without eliminating their activity (24, 26).

Besides electrically, it is possible to achieve vagal stimulation pharmacologically, small molecules can activate the inflammatory reflex by starting signals in the proximal components of the CNS. One such molecule is the CNI-1493, a tetravalent guanil hydrazone, described as an inhibitor of macrophage activation and TNF release. This substance inhibits TNF synthesis and inflammatory response both locally and systemically in animal model, and reduces Crohn's disease clinical symptoms significantly in small studies (29).

Specific anti-inflammatory response is achieved by acetylsalicylic acid administration, but not with dexamethasone. Amiodarone, an antiarrhythmic medication used to treat and prevent a number of types of irregular heartbeats, including ventricular tachycardia, ventricular fibrillation, and wide complex tachycardia, as well as atrial fibrillation and paroxysmal supraventricular tachycardia, is identified as a TNF synthesis inhibitor inside the monocytes *in vitro*, and also a potent vagal activity stimulator (30). Systemic administration of non-steroidal anti-inflammatory medicines, aspirin, indomethacin and ibuprofen increases vagal stimulation.

It is not noted that this pathway's activation causes immunosuppression because maximal suppression reduces pro-inflammatory cytokine level from toxic to normal. This concept is deemed to be a possible treatment for many inflammatory diseases, including infections, ischemia-reperfusion syndrome and injury.

There are a number of studies, both with on animal model and in human subjects with heart failure that have shown promising results.

Effectiveness of chronic electrical vagal stimulation has been assessed in three animal models of heart failure. Rats subjected to a large anterior myocardial infarction (MI) leading to heart failure were randomized to vagal and shamstimulated groups 14 days post-MI. The vagus was stimulated for 10 sec/min, with an intensity adjusted to reduce heart rate by 20-30 bpm (5- 10%). Rats randomized to vagal stimulation showed significant improvement in left ventricular (LV) hemodynamics and decreased mortality from 50% to 14% at 140 days (30).

In an established canine model of intracoronary microembolization-induced heart failure, vagal stimulation significantly improved LV function compared to sham operated animals (31).

In the same animal model, therapy with vagus nerve electrical stimulation combined with beta-blockade improved left ventricular systolic function beyond that seen with beta-blockade alone. More recently, the effects of chronic vagal stimulation in a canine rapid pacing model of heart failure (HF) were presented (32).

These dogs were subjected to 8 weeks of high-rate ventricular pacing with concomitant VNS in the active group and no stimulation in the control group. At the end of the 12-week study period, vagally stimulated animals had significantly lower LV end-diastolic and end-systolic volumes and higher LV ejection fraction. This result was obtained in the absence of any heart rate effect provided by vagal stimulation since both groups were subjected to the

same constant ventricular pacing. The effect of VNS on chronic pressure-overload hypertrophy, which is similar to heart failure with preserved ejection fraction (HFpEF), was evaluated in a guinea pig study (33).

Chronic low-amplitude VNS abolished adverse LV remodeling (end-systolic and end-diastolic volume changes) induced by chronic aortic constriction.

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EFEKAT ANTIINFLAMATORNOG REFLEKSA NA SRCE

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Nativni imuni sistem je glavna komponenta odbrane od patogena, povreda i trauma. Vagalna vlakna sadrže senzorne i motorne komponente, koje kontrolišu funkcije, kao što su srčana frekvenca i varenje. Ovaj antiinflamatorni put bitan je iz više razloga – brz je, funkcioniše separatno od imunog sistema i lokalizovan je na inicijalno povređeno tkivo i može indukovati humoralni sistemski antiinflamatorni odgovor, sa više pozitivnih efekata po srce.

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Ključne reči: srčane bolesti, upalni refleks, vagalna stimulacija

SELF-INFLICTED PENETRATING CARDIAC INJURY: TWO DIFFERENT SURGICAL APPROACHES

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Penetrating cardiac injuries represent a great challenge for the surgeon because of their lethality. It is estimated that more than 90% of mortality happens before the patient reaches the hospital and survival after stab wounds to the heart is about 50% as opposed to 11% after gunshot wounds.

A 58-year-old hemodynamically unstable male was brought to the ED with two stab wounds to the left chest. Two surgical approaches were applied to treat the injury. First, urgent left antero-lateral thoracotomy with left ventricle suture repair was performed. Few hours later, due to massive bleeding and hemodynamic instability, left ventricle repair through a median sternotomy with extracorporeal circulation and cardiac arrest was performed.

Heart injuries can be treated with two surgical approaches. Left thoracotomy can be feasibly applied in emergency department but median sternotomy provides better access to the heart and all important cardiovascular structures.

Prompt and early diagnosis associated with a well-trained surgical team can be crucial in the management of life-threatening cardiac injuries.

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Key words: penetrating, heart, injury

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Introduction

Penetrating cardiac injuries (PCI) are common in military conflicts but incidence amongst civilians has risen worldwide. Those injuries are associated with violence, industrial accidents and suicide attempts. According to the available data, patients with a PCI have a 90% mortality rate before reaching the hospital and survival after stab wounds to the heart is about 50% as opposed to 11% for gunshot wounds (1, 2, 3). Cardiac anatomy determines the injury pattern with the right ventricle most frequently affected, followed by the left ventricle, right atrium, and left atrium. The right ventricle composes the majority of the anterior surface of the heart and thus is the most vulnerable to penetrating

injuries. The left atrium is the least likely injured because of its small size and well-protected posterior location (4, 5).

Historically, on July 10, 1893, Dr. Daniel Hale Williams, a surgeon from Chicago, successfully operated on a 24-year-old man who had been stabbed in the heart during a fight. The wound in the right ventricle was not bleeding, so Williams did not place a stitch through the heart wound. He did, however, stitch close the hole in the pericardium. This operation is probably the first successful surgery involving a documented stab wound to the heart. Dr. Ludwig Rehn, a surgeon in Frankfurt, Germany, performed what many consider the first successful heart operation. On September 7, 1896, he operated a 22-year-old man who was stabbed in the heart. In Serbia (Valjevo), Dr. Jovan Mijušković performed first successful open-heart surgery.

On April 7, 1928 he operated on fifteen-year-old Dragomir Mitrović, inadvertently wounded in a small-caliber handgun.

Case report

A 58-year-old male was referred to our hospital Emergency Department at 07.00 AM for self-inflicted, two stab wounds of the left chest. At his arrival, patient was hemodynamically unstable with immeasurable blood pressure, heart rate of 120 and respiratory frequency of 30. During physical

examination, the presence of two stab wounds in the 4th intercostal space over the midclavicular line, on the left hemithorax, were noticed. A thoracic X-ray showed the presence of a left hydropneumothorax. The patient was brought to the operating room and the chest tube was inserted at posterior axillary line in the 5th intercostal space. In a few seconds about 2000 ml of blood drained. An urgent left antero-lateral thoracotomy was performed. After opening the pericardium, penetrating injury of the left ventricle lateral wall was observed. Hemostasis was achieved by placement of two "U" sutures (Prolene 3-0). The patient was transferred to the Intensive Care Unit.

Because of massive bleeding and hemodynamic instability, the patient was brought to the operating room again at 11.00 AM. This time, median sternotomy was performed. Two bleeding sites were noticed: one on the left ventricle lateral wall, which was treated during the first operation, and the other one on the left ventricle anterior wall (Figure 1). Both lesions were treated with pledget enforced, "U" sutures (Prolene 3-0), on a beating heart and subsequent fibrin sealant application (Beriplast® P Combi Set, CSL Behring). Hemostasis was achieved, two chest tubes were inserted and the patient was transferred to the Intensive Care Unit.

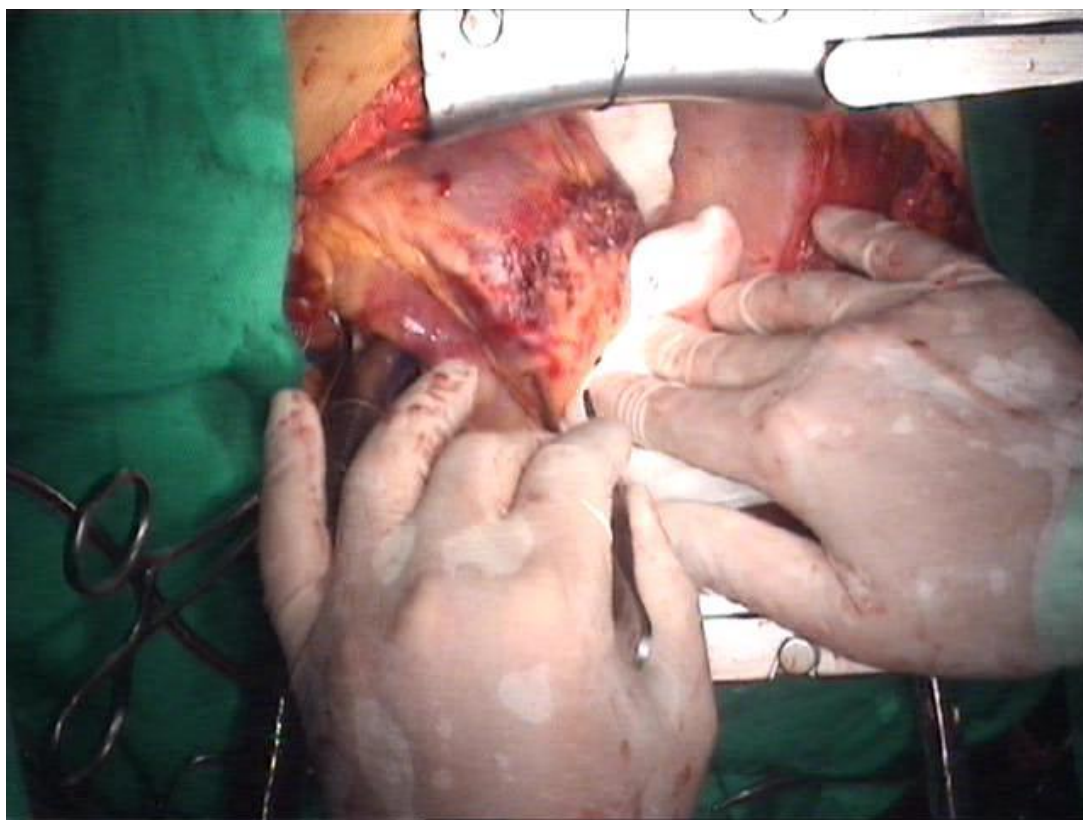


Figure 1. Two penetrating injuries on anterolateral wall of the left ventricle

Around 03.00 PM, suddenly, massive bleeding of about 700 ml of blood occurred again. The patient was immediately transferred to the operating room. After resternotomy, massive bleeding from previously treated lesions was noticed. This time, it was decided to repair left ventricle penetrating injuries using extracorporeal circulation and cardioplegic arrest. After heparin administration, arterial and venous cannulation was performed, aorta was clamped and 1150 ml of cold crystalloid cardioplegia solution was administered. After the heart stopped, all previously posted sutures were removed. Both lesions were treated with the same approach. In the first

layer, we used single "U" sutures (Prolene 3-0), enforced with 5 cm long teflon tapes on both sides of the lesions (Figure 2). In the second layer, teflon tapes were sutured with continuous over-over stitch (Prolene 3-0) (Figure 3). Hemostasis was achieved. The aortic cross clamp time was 34 minutes and extracorporeal circulation time was 79 minutes.

Postoperatively, the patient received 4 doses of resuspended red cells, 3 doses of fresh frozen plasma, 10 doses of cryoprecipitate and 20 doses of platelets. Postoperative care evolved without complications; the patient was discharged on the twelfth day.

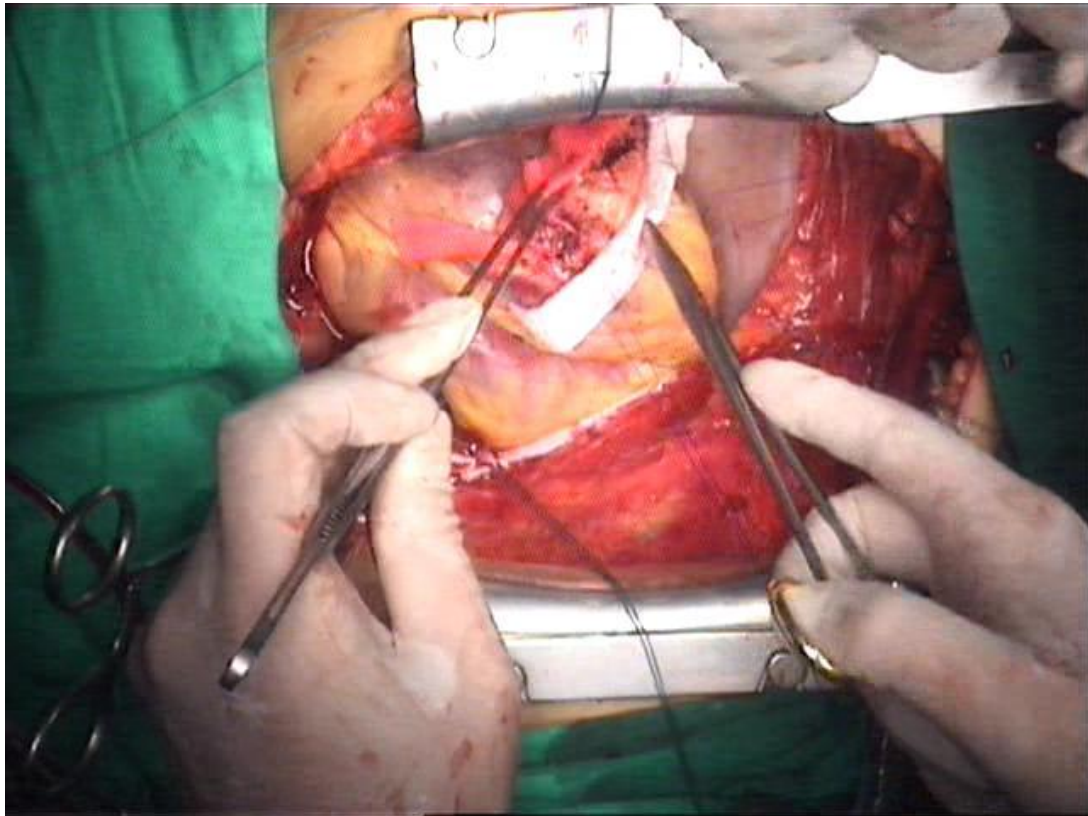


Figure 2. Left ventricle repair. First layer.

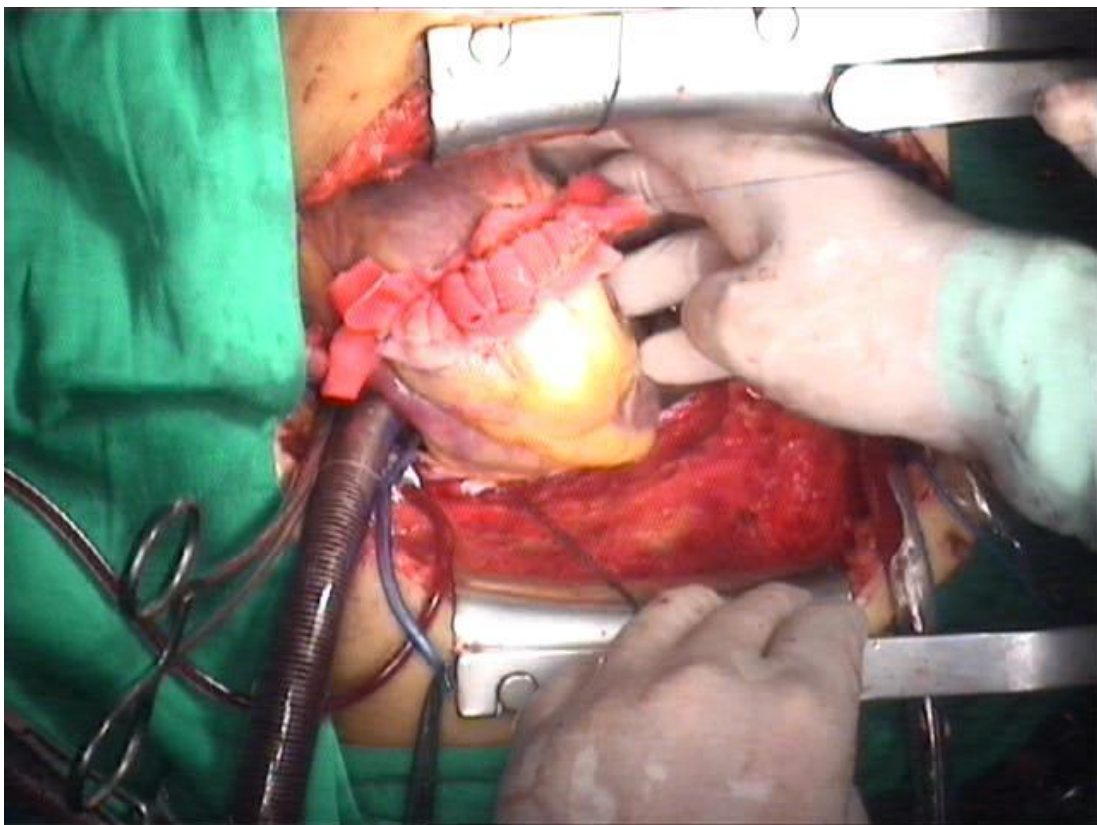


Figure 3. Left ventricle repair. Second layer.

Discussion

There are two commonly involved mechanisms in PCI: gunshot wounds (GSW) or wounds with a sharp object (WSO), with a survival rate of GSW victims of 11% versus 50% for WSO victims. In our patient's case, his injury was self-inflicted using a nail (WSO). Early diagnosis can be crucial in the management of life-threatening cardiac injuries. Subxiphoid window and noninvasive studies, such as echocardiography, remain the gold standard for diagnosis. Echocardiography has become a standard in the care of stable patients, becoming the first option in the emergency room.

An unstable patient, with tamponade or hemodynamic instability, must be taken to the operating room to perform an emergency thoracotomy or a median sternotomy.

Surgical approach could be fundamental to control the bleeding. According to the available literature, the advantages of median sternotomy approach are effective and extensive vision of the heart, great vessels, other structures in the mediastinum and to both pleural cavities and also allowing cannulation for a cardiopulmonary bypass. On the other hand, left anterolateral thoracotomy provides

rapid access to the right and left ventricles, facilitates access to posterior structures such as the esophagus, descending aorta, or left hilum and this is usual approach for emergency room thoracotomy. Nevertheless, the choice of surgical approach also depends on the surgeon's experience, the expected injuries according to the probable trajectory of the wounds, and the evidence of associated lesions (6, 7).

In our case, we were forced to apply both approaches to the same patient. The reason for this is the fact that the first surgery was performed by a team of thoracic surgeons and the other two operations by a team of cardiac surgeons. This confirms that the experience of the surgeon plays a very important role in the choice of surgical approach for PCI.

Conclusion

Prompt and early diagnosis associated with a well-trained surgical team can be crucial in the management of life-threatening cardiac injuries. The experience of the surgeon plays a very important role in the choice of surgical approach for PCI.

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doi:10.5633/amm.2020.0212**PENETRANTNA TRAUMA SRCA IZAZVANA SAMOPOVREĐIVANJEM –
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Penetrantne povrede srca predstavljaju veliki izazov za hirurge, zbog visoke stope smrtnosti. Smatra se da preko 90% smrtnih ishoda nastupi pre dolaska bolesnika u bolnicu. Preživljavanje nakon ubodnih povreda srca je oko 50%, dok je kod povreda izazvanih vatrenim oružijem oko 11%.

U Urgentni centar primljen je pedesetosmogodišnji muškarac u hemodinamski nestabilnom stanju, sa dve ubodne rane u predelu leve strane grudnog koša. U cilju zbrinjavanja povreda, primenjena su dva hiruška pristupa. Prvo je urađena urgentna levostrana anterolateralna torakotomija sa suturom leve komore. Nekoliko časova kasnije, zbog obimnog krvarenja i hemodinamske nestabilnosti, urađena je rekonstrukcija leve komore, pristupom kroz medijalnu sternotomiju, uz pomoć vantelesnog krvotoka i na zaustavljenom srcu.

Postoje dva osnovna hiruška pristupa za zbrinjavanje penetrantnih povreda srca. Levostrana anterolateralna torakotomija omogućava brz pristup u jedinicama urgentnog zbrinjavanja, ali medijalna sternotomija obezbeđuje bolji pristup srcu i ostalim važnim kardiovaskularnim strukturama.

Brza i rana dijagnostika, udružena sa dobro obučanim hiruškim timom, od presudnog je značaja u zbrinjavanju smrtonosnih povreda srca.

*Acta Medica Medianae 2020;59(2):88-92.****Ključne reči:*** penetrantna povreda, srce, povreda

STIGMATIZATION OF PSYCHIATRIC PATIENTS AMONG STUDENTS IN SERBIA

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Social psychiatry emphasizes the inclusion of mentally ill persons in family and social life. The negative attitude towards them is still an obstacle for achieving the best treatment results and their optimal quality of life. Young educated people could be an important community factor in the fight against stigmatization of psychiatric patients. The aim of the study was to determine the difference in attitudes among Serbian students attending different faculties, as well as to find out the correlation between students' attitudes and their demographic characteristics. The study involved 408 Serbian university students. The respondents were divided into three groups to determine the differences in attitudes between groups with different educational levels. We have used the Attitude Questionnaire and Demographic Questionnaire for this purpose. Medical students who had passed their exam in psychiatry, those who experienced psychological and psychiatric problems themselves, and those with a mentally ill family member had a positive attitude towards the mentally ill. Future health workers and the family members of mentally ill individuals are most important in the attempts to decrease stigmatization of psychiatric patients.

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Key words: stigmatization, students, psychiatric patients

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Introduction

In the contemporary complex human society, one in four people will experience some mental health issue throughout his life. Psychiatric illnesses have always been present, in primitive, as well as in highly civilized communities all over the world. In old times, the mentally ill were considered possessed by either divine or evil forces. These were the first attempts at understanding human psychology, its alterations, as well as the stigmatization of mentally ill (1).

In more recent past, psychoanalytic and biologically oriented scientists made a great contribution to understanding and treating mental illnesses. While the knowledge has accumulated, the attitudes towards psychiatric patients have also improved. Nevertheless, the society tends to mark these persons as incurable and even health professionals stigmatize them as undesirable (2). Stigma in psychiatry is defined as "a set of negative attitudes and beliefs that motivate public opinion to fear, avoid, refute and discriminate against people suffering from mental illness" (3). Some studies have confirmed the presence of more pessimistic views on the outcome of mental illnesses by mental health professionals than by general population (4). In the research by Corrigan, stigmatization in mental health institutions contributed to low self-esteem, frustration, isolation and worse outcome of psychiatric patients, especially in the domains of housing, employment, education (5, 6). There is a negative correlation between the experience of stigma and successful recovery in mental disorders. Many of these patients give up their treatments when exposed to stigmatization (7). Even some physicians do not diagnose or treat psychiatric patients, wrongly assessing their complaints as hallucinations or hypochondriacal preoccupations.

Considering these problems, we wanted to investigate the presence of stigma towards psychiatric patients in the student population in Niš.

Further, we wanted to find out if there are any differences in attitudes towards mental illnesses between students of medicine and students from the Faculty of Philosophy, their demographic characteristics, and educational or personal experiences with psychiatric diseases.

Methods and subjects

Our investigation involved 408 subjects, 290 females and 118 males, all students from Niš. They were divided into three groups of 136 respondents. The first group (group A) consisted of students of medicine who passed their examination in psychiatry (V and VI study year). The second group (group B) consisted of medical students who had not attended the classes in clinical psychiatry (I, II, III, IV study years). The third group (group C) consisted of students from the Faculty of Philosophy, Departments of Psychology, Pedagogy and Social Science. All participants gave their written consent for the study and the study was approved by the Ethics Committees of these Faculties. The survey was conducted in late 2018 and early 2019.

A sociodemographic questionnaire was used to collect the respondents data: gender, type of faculty they attended, study year, whether they were living with or without their families, their previous experience of psychological issues or psychiatric diagnoses, whether they ever sought psychological/psychiatric help up to that time, presence of psychiatric patients in their own families, and presence of mental health professionals in their families.

In order to evaluate the attitude towards persons with mental disorders, the Attitude questionnaire containing 18 items related to the attitude towards psychiatric patients was used. The questionnaire was previously constructed and validated in a research performed at the Faculty of Philosophy in Niš. All the answers were rated on the Likert scale from 1 to 5 as follows:

- 1 = completely incorrect;
- 2 = mostly incorrect;
- 3 = I am not sure;
- 4 = mostly true;
- 5 = completely true.

The higher obtained score meant a more negative attitude, except for the items 1, 6, 7, 17, 18, which were coded reversely, for the purpose of statistical analysis. The Attitude questionnaire score ranged from 18 to 90, and the scale reliability was estimated by Cronbach's alpha = 0.75.

Results

In our sample of 408 subjects, 71% were females. Demographic data indicated that 80% of students lived with their families, and that over a half of them had never had any psychological problems. Of those who experienced some mental issues, less than 20% had sought professional help. About one-third of the respondents had a relative who sought psychiatric help, and only 7% of the respondents had a family member involved in the profession related to mental health care (Table 1).

Table 1. Demographic data of the sample

ITEMS	GROUP A N (%)		GROUP B N (%)		GROUP C N (%)		TOTAL N (%)	
	YES	NO	YES	NO	YES	NO	YES	NO
Living alone	30 (22.05)	106 (77.94)	28 (20.58)	108 (79.41)	23 (16.91)	113 (83.08)	81 (19.85)	327 (80.14)
Psychologi issues*	74 (54.41)	62 (45.58)	53 (38.97)	83 (61.02)	70 (51.47)	66 (48.52)	197 (48.28)	211 (51.57)
Seeking help*	29 (21.32)	107 (78.67)	21 (15.44)	115 (84.55)	27 (19.85)	109 (80.14)	77 (18.12)	331 (81.12)
Psychological issues of family member*	42 (30.88)	94 (69.11)	42 (30.88)	94 (69.11)	45 (33.08)	91 (66.91)	129 (31.61)	279 (68.38)
Mental health worker as family member	8 (5.88)	128 (94.11)	12 (8.82)	124 (91.17)	7 (5.14)	129 (94.85)	27 (6.61)	381 (93.38)
Gender	Female 101 (74.26)	Male 35 (25.73)	Female 98 (72.05)	Male 38 (27.94)	Female 91 (66.91)	Male 45 (33.08)	Female 290 (71.07)	Male 118 (28.92)

Group A - Students of medicine who passed exam in psychiatry;

Group B - Students of medicine who have not attended classes of clinical psychiatry;

Group C - Students from Faculty of Philosophy;

*Demographic data with statistical significance relation to positive attitude towards psychiatric patients.

Comparing the attitude scores of the Attitude questionnaire between the groups, we found that there was a statistically significant difference in the overall attitude towards mentally ill persons ($p < 0.001$) (Table 2).

The level of statistical significance was reached in nine items related to the knowledge about mental illnesses, socializing with the affected

people and having a psychiatric patient in one's own family. The analysis indicated less stigmatization in a group of medical students who had passed the psychiatry exam compared to other two groups (0.001). A more positive attitude was present in group A, while there were no differences between group B and group C (40.85 ± 6.978 ; 44.85 ± 7.335 ; 44.52 ± 7.522).

Table 2. Difference in attitude between the studied groups

ITEMS	GROUP A	M \pm SD GROUP B	GROUP C	P
1. I would hang out with a person with diagnosed mental illness.*	2.24 \pm 1.036	2.35 \pm 0.923	2.53 \pm 0.910	0.046
2. I'm afraid to communicate with a mentally ill person *	1.80 \pm 0.995	2.18 \pm 1.032	2.38 \pm 1.040	< 0.001
3. Mentally ill persons are dangerous.*	2.39 \pm 0.800	2.67 \pm 0.911	2.56 \pm 0.876	0.028
4. Psychiatric patients are noticeable and recognizable in the society.	2.71 \pm 1.135	2.71 \pm 1.025	2.85 \pm 1.003	0.491
5. People are generally afraid of mental patients.	4.20 \pm 0.581	4.10 \pm 0.676	4.08 \pm 0.058	0.243
6. The media should not spread prejudice and a negative attitude towards mental illness.*	1.35 \pm 0.715	3.74 \pm 1.699	1.46 \pm 0.729	< 0.001
7. I would not mind that my partner is a mentally ill.	3.63 \pm 1.141	3.81 \pm 1.099	3.70 \pm 1.104	0.420
8. I do not even like to talk about mental illness.*	1.51 \pm 0.861	1.79 \pm 1.105	2.19 \pm 1.183	< 0.001
9. I do not want to know anything about mental illness.*	1.09 \pm 0.354	1.10 \pm 0.295	1.30 \pm 0.648	< 0.001
10. I know nothing about mental illness.*	1.17 \pm 0.480	1.61 \pm 0.845	1.99 \pm 0.977	< 0.001
11. I recognize a lot of people who are mentally ill.	2.75 \pm 1.031	2.74 \pm 1.116	2.59 \pm 1.007	0.360
12. The people have every right to rejects the mentally ill.	1.63 \pm 0.860	1.84 \pm 0.968	1.82 \pm 0.888	0.104
13. The one who suffers from mental illness cannot adapt to the life of healthy people.	2.36 \pm 0.866	2.42 \pm 0.993	2.41 \pm 1.036	0.862
14. I'm afraid I could go crazy.	1.93 \pm 1.059	2.13 \pm 1.256	2.03 \pm 1.205	0.380
15. The behavior of a mentally ill differs significantly from the behavior of healthy people.	3.01 \pm 0.915	3.00 \pm 1.033	3.06 \pm 0.964	0.863
16. I Would be ashamed of my family member's mental illness.*	2.01 \pm 1.075	1.75 \pm 0.925	2.04 \pm 1.108	0.038
17. It is difficult to distinguish a healthy and mentally ill person.	3.30 \pm 0.969	3.35 \pm 1.002	3.19 \pm 1.022	0.660
18. I try to find out more about the nature of mental illness.*	1.71 \pm 0.818	1.95 \pm 0.976	2.35 \pm 1.131	< 0.001
TOTAL SCORE	40.85 \pm 6.978	44.85 \pm 7.335	44.52 \pm 7.522	< 0.001

Group A - Students of medicine who passed the exam in psychiatry;

Group B - Students of medicine who have not attended classes of clinical psychiatry;

Group C - Students from other faculties;

AVR - Arithmetic mean;

SD - Standard deviation;

p- ANOVA level of statistical significance;

*Items which significantly differ between groups.

Linear correlation of the sample established a correlation between some demographic characteristics and attitude towards psychiatric patients. A positive attitude towards the mentally ill was seen in students who had experienced some psychological issues themselves ($p < 0.002$), those who asked for professional help ($p < 0.004$), and those who had

a mentally ill relative. A negative attitude was identified in those who denied experience of psychological/psychiatric symptoms, those who never asked for psychological support/help and did not have any family member with psychological or psychiatric issues (Table 3).

Table 3. Relation between negative attitude towards mentally ill and demographic characteristics of the sample

ITEMS	R ²	p
GENDER	0.052	0.294
Do you live alone?	-0.094	0.058
Have you ever had psychological/psychiatric issues?*	0.149	0.002
Did you ever ask for psychological/psychiatric help?*	0.143	0.004
Did any member of your family ever ask for psychological/psychiatric help?*	0.193	< 0.001
Is someone in your family professional health worker?	-0.001	0.978

R² - Pearson's correlation;

p - level of statistical significance;

*demographic data related to the attitude

Discussion

Psychiatric patients are still marginalized in the society and even the domain of medical care is not an exception. Their quality of functioning in everyday life does not depend solely on their illness, but also on social integration, which often depends on the presence of stigmatization. Despite modern treatment potentials, patients facing the stigma have limited access to many possibilities in their life. Stigmatization of psychiatric patients is present even among their family members, friends and mental health professionals. In medical settings, mentally disturbed patients are considered different and less adherent to their therapy (8).

Exploring possible stigmatization attitudes among the students of medicine, psychology, social science and pedagogy is important in early detection of the issues that could possibly influence future professionals in the field of mental health. This is essential for determining possible targets for anti-stigma interventions, since the opinions and behaviors of young people are highly modifiable (9). The students in our sample with basic knowledge of psychiatry had a more positive view on the mentally ill than other respondents. They were all young, healthy people, but those who experienced some psychological issues usually did not seek professional help. This could be an indicator of stigma and shame. The literature data suggests the importance of early interventions in psychological crisis, in order to overcome emotional distress (7). Avoidance of dealing with issues with the help of professionals, could lead to unhealthy behavior or psychopathological reactions of young people affecting their mental health. Risk health behaviors are often the way of relieving stress and could be an introduction to more serious medical conditions.

Less stigma towards the people with psychiatric issues was present in those students who had a family member with diagnosis of a mental disorder and who had not a mental health professional in the family. Such a positive attitude could be explained by their being better informed about the capability of mentally ill persons to recover and integrate into society. The available literature also suggests that personal relatedness to a mentally disturbed person is the way to understand, support and accept all such people as equal members of society (9).

Our assumption that education was important in building the right attitude towards the mentally ill, with or without any personal experience with psychological issues, was confirmed. The results indicated that medical students who had passed their exam in clinical psychiatry had a more positive attitude, while other medical students as well as students from other faculties had a more negative attitude towards psychiatric patients. This is in line with the findings of Zaninotto et al. from 2018, where male students of psychology in higher study years have had a more positive attitude and opinion about mentally ill patients (10). The investigators support the idea of "open doors" in psychiatric wards which would hopefully reduce social isolation of these patients. In their study, no other demographic variables were in correlation to attitude, but some personality traits, such as Agreeableness and Openness were positively associated with empathy towards psychiatric patients. We did not investigate personality types in our sample. However, our experience in clinical practice and team work also confirms the assumption that certain personality traits are more favorable in that regard for mental health workers. Future medical workers should be sensitive to persons with psychological issues and capable of accepting them as any other patients and treat them

with equal care as those without emotional disturbance. We could well expect that medical professionals had more profound knowledge of psychiatry and a more positive attitude towards mental illnesses. Nevertheless, some studies have demonstrated negative opinions and behaviors towards psychiatric patients in doctors, nurses, and psychologists, not different from that in the general population (11). Such an observation emphasizes the importance of education and practical experience in dealing with psychiatric patients, which could improve their being accepted as clients.

In our subjects, a positive attitude was encountered in those who had knowledge about psychiatry and some kind of first-hand experience with psychological/psychiatric issues, (whether personally experienced or seen in a family member). The result is similar to the findings of Yamaguchi et al., who have reported that the most important and effective way for reducing mental health related stigma are social contacts with the mentally ill. The contacts could be indirect or direct, as through professional training in psychiatric units (12).

Videotaped cases of some psychological issues or psychiatric syndromes could be an acceptable way to introduce college students to mental illnesses. This first step may decrease social distance and help future mental health workers to understand and build up a favorable opinion about psychiatric disorders. Direct contacts of students with patients, supervised by ward psychiatrists and psychologists, are the best way to get a realistic picture about patients' way of thinking, emotional expression and behavior.

Our results indicated that some factors were correlated to better attitudes towards people with mental health problems.

The limitation of the study was a limited representativeness of the sample, which involved students of medicine and social science, who had already chosen to be educated in the profession oriented towards helping people. Therefore, the results cannot be generalized to involve the general population as well. We did not explore other parameters, such as personality characteristics, which could also play a role in the formation of attitudes towards psychiatric patients. In studying the greatest obstacles to the inclusion of psychiatric patients into society, future work in this field should include other people of different ages and education levels.

Conclusion

In the studied student population in Serbia, formal education and personal experience of psychological issues are associated with a more positive attitude towards mental illnesses. Social interaction with ill persons could decrease stigma, especially if mental problems are encountered in one's family on a day-to-day basis. Educational programs should point out the problem of stigmatization, helping future professionals in field of medicine and social sciences to deal with all the patients without any prejudice.

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doi:10.5633/amm.2020.0213**STIGMATIZACIJA PSIHIJATRIJSKIH BOLESNIKA OD STRANE
STUDENATA U SRBIJI***Gordana Nikolić¹, Maja Stanojković², Miroslav Krstić³, Olivera Žikić¹, Nikola Stojanović⁴,
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Socijalna psihijatrija ističe značaj uključivanja mentalno obolelih osoba u porodični i socijalni život. Negativan stav prema ovim osobama predstavlja i dalje prepreku za postizanje najboljeg mogućeg tretmana i poboljšanja kvaliteta života. Mladi, edukovani ljudi mogli bi biti značajni činilac zajednice u borbi protiv stigmatizacije psihijatrijskih bolesnika. Cilj studije bio je da odredi razlike u stavovima srpskih studenata, koji pohađaju različite fakultete, kao i da otkrije povezanost njihovih stavova sa demografskim karakteristikama. Istraživanje je obuhvatilo 408 studenata srpskih univerziteta. Ispitanici su bili podeljeni u tri grupe, radi utvrđivanja razlike u stavovima između studenata različitih edukacijskih grupa. Koristili smo upitnik o stavovima i upitnik o demografskim karakteristikama. Studenti medicine, koji su položili ispit iz psihijatrije i oni koji su i sami iskusili psihološke i psihijatrijske probleme i oni koji su imali mentalno obolele članove porodice, imali su pozitivan stav prema mentalno obolelim osobama. Budući zdravstveni radnici i članovi porodice mentalno obolelih značajni su za smanjivanje stigmatizacije psihijatrijskih bolesnika.

*Acta Medica Medianae 2020;59(2):93-99.***Ključne reči:** *stigmatizacija, studenti, psihijatrijski bolesnici*

ANTIOXIDANT ACTIVITY, TOTAL PHENOL AND TANNIN CONTENT OF DIFFERENT VARIETIES OF FLOURS

Bojana Miladinović¹, Kristina Ilić², Dušica Stojanović¹, Milica Kostić¹, Milica Milutinović¹,
Suzana Branković³, Dušanka Kitić¹

Cereals are considered one of the most commonly used foods due to their properties. They are the main source of carbohydrates, mainly starch. In addition to carbohydrates, cereals also contain proteins, unsaturated fatty acids, minerals, B-group vitamins and vitamin E. This composition refers to the unprocessed form of cereals - whole grain cereals. They also contain phenolic compounds, such as phenolic acid, flavonoids, stilbene, coumarins and tannins. These are plants secondary metabolites and have a significant role in the prevention of many diseases due to their antioxidant properties.

The aim of this research was to determine the content of total polyphenols and tannins in wheat (type 500), maize, rye and whole wheat flour extracts, as well as the antioxidant activity of the samples. The highest content of total polyphenols was determined in whole wheat flour (6.38 ± 0.759 mg GAE/g), while the lowest amount was determined in wheat (type 500) flour (2.29 ± 0.32 mg GAE/g). Whole wheat flour extract showed the highest antioxidant activity ($IC_{50} = 5.63 \pm 1.331$ mg/ml), while wheat flour (type 500) extract showed the lowest antioxidant activity ($IC_{50} = 26.39 \pm 2.132$ mg/ml). Based on the results obtained, it can be concluded that whole grain cereals contain a large amount of polyphenols, which indicates a significant antioxidant activity. Due to their many properties, which have effect on improving health, cereals form the base of the pyramid of nutrition, as a model of healthy eating.

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Key words: wheat, maize, rye and whole wheat flour, polyphenols, antioxidant activity

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Introduction

Cereals are annual plants of grass family (Gramineae) whose seeds or grains are used for human and animal consumption and as an unprocessed material in the food industry. Cereals and cereal products are essential sources of energy, carbohydrates, proteins and fiber, and also contain various micronutrients such as vitamin E, thiamine, riboflavin, magnesium and zinc. According to Food and Agriculture Organization of United Nations (FAO),

wheat, corn and rice account for 60% of the world's energy intake. They are inexpensive to produce, easy to store and transport and do not deteriorate quickly if stored in a dry place (1, 2).

Cereal grain consists of three basic anatomical parts that differ in function, structure, composition, and all other characteristics. The parts of the grain are: endosperm, germ and bran. The structure of the grain, or its anatomical parts, depends on the cellular structure and chemical composition.

The outer shell of cereal grain - the pericarp, is rich in pentosans, cellulose and minerals. The aleurone layer anatomically belongs to the endosperm and it is removed along with the pericarp in the process of grinding. Aleurone is rich in proteins, lipids, pentosans, minerals and the vitamin B complex. Starch is the main compound of the endosperm. The embryo and scutellum form the germ and contain high contents of protein, lipids, vitamin E, sugar (sucrose and raffinose) and minerals (3).

The most important part in the processing of cereals is milling, whereby different types of flours and semolina are obtained. The by-products are bran and flour for animal feed, as well as impurities separated in the preparation process. Flour and semolina come from the endosperm, while animal flour

and bran originate from the husk and aleurone layer (4).

The milling products of cereals are the basic materials in the baking industry, which produce a various range of baked goods. The most important and used product is bread. The quality of both the dough and the end product depends on the properties of the flour. Composition and properties of flour vary depending on the type of cereals, climatic conditions, applied agro-technical measures, milling methods and storage (4).

Chemical composition of wheat flour

The chemical composition of the flour depends on the chemical composition of the grain and the type of flour, while the chemical composition of the grain depends on the type and variety of wheat, soil, climate, weather and agro-technical conditions during the growing season (4).

Water is very important factor in the sustainability of flour. With increasing moisture content, the absorption capacity of the flour decreases and thus the bread yield. The water content of the flour is limited by regulations and in our country is at most 15%.

The sugars found in wheat flour are: glucose, fructose, raffinose, maltose, sucrose, melobiose, glucofructose. The amount of sugars, especially maltose, significantly increases in the grain during germination. The total sugar content of wheat flour is about 1%.

Starch makes around 70% of the flour content. Wheat starch grains are round and size from 5 to 50 μ m. The properties of the dough are determined by the starch content in the flour, the size of the starch grains and the degree of their damage. Smaller and damaged starch grains can absorb more water. Starch consists of two macromolecular components: amylose (linear polymer) and amylopectin (branched polymer) (4).

Ballast or dietary fiber is very important from a nutritional aspect, although it is not absorbed in the human body. Dietary fiber present in wheat are cellulose, hemicellulose, lignin, pentosans (3).

Flour lipids are mainly composed of triglycerides of unsaturated fatty acids. Their presence can cause the rancidity of flour. Lipoproteins and glucolipids are also present. The fats are concentrated in the germ, while the rest of the grain contains small amounts. White flour has 1.15-1.8% fat content and whole grain flour 3.5-4.4%.

Flour proteins are albumins, globulins, gliadins and prolamins. They are directly responsible for the formation of the dough structure by the formation of gluten (4).

Mineral substances of flour are phosphorus, calcium, magnesium, chlorine, potassium. Other minerals are represented in traces. Vitamins are present in flour in very small amounts. Most of them are the B group vitamins (thiamin, riboflavin, niacin, pyridoxine), vitamin E, vitamin A (3, 5).

Whole wheat grain is a rich source of phytochemicals, compounds that participate in or occur in

the primary and secondary metabolism of plants. The most important metabolites are phenolic compounds, carotenoids, tocopherols, alkylresorcinols, benzoxazinoids, phytosterols and lignans (6).

Among the phytochemicals found in cereals, phenolic compounds have great attention because of their antioxidant properties. They range from 200 to 1200 mg/g dry matter (7). Phenolic compounds include phenolic acids, flavonoids, stilbene, coumarins, lignans and tannins. Phenolic acids and flavonoids are most commonly found in cereals (8). The most prevalent phenolic acid in wheat is ferulic acid (> 90%). Other derivatives of cinnamic acids (p-coumaric, caffeic) and benzoic derivatives (p-hydroxybenzoic, vanillic, gallic, protocatechuic and syringic acid) are also found in wheat flour, but in smaller quantities (9).

Qualitative and quantitative content of phenolic compounds depends on the genotype of wheat, factors of the environment in which it is grown as well as ways of processing and milling of the wheat. The content of phenolic compounds decreases significantly with the increase in the degree of refinement of flour. This is due to the specific distribution of the compounds in the cereal grain. Most phenolic compounds (> 90%) are esterified with cell wall components of the aleurone layer and pericarp, which are discarded during milling (9).

Wheat flour (type 500)

Type 500 or white flour is the most produced flour used for making bread and bakery products. It is obtained by grinding the central parts of the endosperm. It also has peripheral parts, which gives it a slightly darker color, increased content of minerals, proteins, fats and vitamins compared to flour type 400 (5).

Whole wheat flour

Whole wheat flour contains all the parts of the wheat grain makes it exceptional in nutritional properties. This type of flour contains starch, proteins, dietary fiber and essential fatty and amino acids, as well as vitamin E. Due to its composition, whole wheat flour is considered to be one of the most complete nutritional foods that are most commonly consumed in the form of bread or other bakery products. The product made of this flour have acceptable sensory characteristics (5).

Corn flour

Corn flour is flour from whole (integral) grains and therefore commercially prepared food based on corn flour is considered whole grain foods.

Corn (*Zea mays* L.) originates in the Western Hemisphere and, together with wheat and rice, is the most widely grown grain. Due to its high productivity, it is an economically very important cereal, an important and inexpensive energy source for animal nutrition, as well as a source of starch and sugars used in food industry (3).

Corn is a tall plant that belongs to the *Poaceae* family. Grain is located on the cob, and each consists of the seed coat - pericarp, rich in cellulose; aleurone layer - rich in proteins and minerals; endosperm - rich in starch, which is covered with protein matrix and is a source of nutrients; and germs, from which a new plant develops. Corn has the largest grain of all cereals (10).

There are hundreds of different varieties that are distinguished by different composition. They have different endosperm composition as well as protein content. They may also vary in the color of the pericarp, with some being typically yellow, while others may be white, red or blue (11, 12).

Some varieties are characterized by high protein content, while others by high lipid content. High lipid content hybrids are used in the diet of domestic animals as they provide high energy intake. However, they are not used in the production of flour, because of the increased risk of lipid oxidation, which reduces the shelf life of these products (11, 12).

The chemical composition of corn, its starch content, as well as the ratio of amylose and amylopectin, the protein and amino acid composition of the protein, to a large extent depend on the hybrid, and in recent times, from the genetic modification. A typical corn kernel consists on average of 70-75% starch, 8-10% protein, 4-5% lipids, 1-3% sugar (sucrose, glucose, maltose, fructose, galactose, cellobiose, ribose, mannose and xylose) and 1-4% ash, and is rich in phytosterols. The content of all compounds are genetically controlled and vary between varieties (13).

Proteins are found mostly in the corn endosperm and composition of corn proteins determines the quality of corn. The essential amino acids found in corn are tryptophan, threonine, leucine, isoleucine, lysine, valine, phenylalanine and tyrosine. The proteins found in the germ are different composition than those found in the endosperm, which is of great nutritional importance in grain processing (13).

The highest oil content is in the germ. It is predominant in unsaturated acids (86%) compared to saturated (14%) which makes corn oil highly valued. Refined corn oil contains linoleic acid 54-60%, oleic acid 25-31% and 2% of linolenic acid. Corn lipids are very stable due to their high content of natural antioxidants (14).

Corn contains provitamin A (carotenoids) and vitamin E, especially γ -tocopherol concentration of 94.1 mg/100 g. Vitamins such as thiamine, riboflavin, and nicotinic acid are found most in the aleurone layer (15, 16).

The percentage of ash in corn is about 1.3. Minerals found in corn are phosphorus, sodium, potassium, calcium, magnesium and zinc (17).

Corn grains contain polyphenols (tannins, saponins, quercetin), phenolic acids (gallic, chlorogenic, caffeic, hydroxycinnamic, ferulic), flavonoids (anthocyanins, catechins), carotenoids (zeaxanthin, α - and β -carotene), phytosterols (sitosterol, stigmasterol and campesterol) and other phytochemicals

that have shown a beneficial effect on human health (18, 19).

Rye flour

Rye is the youngest cereal compared to other cereals. Cultivated rye (*Secale cereale* L.) originates from the wild rye of the Mediterranean region - *Secale montanum*. Rye is genetically close to wheat and barley. After wheat, rye is the most important in the production of bread.

The outer layers of the grain are rich in vitamins, minerals and phytates. Endosperm is rich in starch and proteins, and germs in lipids and proteins. Compared to wheat, rye contains less starch and protein, and more dietary fiber and free sugars. Rye can be successfully used in functional food formulations due to its high fiber, vitamin and mineral content. Rye forms gluten so it cannot be used in the diet of celiac patients (3).

The total lipid content of rye is low, 1.5-2%, and does not differ from wheat and barley grains. Linoleic acid is the most represented fatty acid in rye grains (55.6%). Rye also contains higher amounts of other lipids than other cereals, followed by palmitic (16.5%), oleic (15.6%), linoleic (10.4%), eicosenoic (1.3%) and stearic acid (0.6%) (10).

Sitosterol is the predominant phytosterol in rye (49-64%). Other desmethylsterols present in significant amounts are campesterol, stigmasterol, stigmastadienol, as well as saturated sitostanol and campestanol. Total sterol content of rye is higher compared to wheat (10).

Rye grains contain 3.2% of total soluble sugars, the most common being sucrose (1.9%), raffinose (0.4%), fructose (0.1%) and glucose (0.08%) (3).

Rye is the rich source of phytochemicals such as phenolic acids, lignans and alkylresorcinols. There are also found phenolamides, large and diverse group of secondary metabolites which are formed by conjugation of phenolic compounds with polyamides such as spermidine, agmatine and putrescine (20).

Benzoxazinoids contain nitrogen and also are secondary metabolites. The best known benzoxazinoides are 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). All these compounds have been extensively investigated due to their potential positive health effects (20).

The aim

The aim of this study was to determine the content of total polyphenols (TP) and total tannins (TT) in wheat (type 500), maize, rye and whole wheat flour samples, as well as their antioxidant activity. It was also examined whether there was a statistically significant difference in the content of these compounds in the flour samples as well as in the antioxidant capacity.

Materials and methods

Material

Wheat flour (type 500) was purchased in the supermarket and samples of corn, rye and whole wheat flour were purchased from a local manufacturer.

All flour samples were prepared according to the method of Sedai (21). Briefly, a sample of 10 g of flour was extracted with 100 ml of 96% ethanol. The extraction was carried out by stirring for 1 h at room temperature. The solution was filtered through filter paper (Whatman, UK). The procedure was repeated twice, each with 100 ml of solvent. The filtered solutions (3 x 100 ml) were combined and evaporated to dryness with a vacuum evaporator. The dry extract was then redissolved in 96% ethanol to a volume of 10 ml. The solutions obtained were used to determine total polyphenols and tannins and antioxidant activity.

Total polyphenols and total tannins

Total polyphenol content was determined using Folin-Ciocalteu method (22). Total phenolics were expressed as galic acid equivalents (GAE) (mg GAE/g juice or extract).

Total tannins were determined using the same Folin-Ciocalteu procedure after removal of tannins by their adsorption on insoluble binding agent (polyvinylpyrrolidone, PVPP) (22). The results were expressed as galic acid equivalents (mg GAE/g).

Antioxidant activity

This spectrophotometric test measures the antioxidant activity of the flour samples to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (23). Various concentrations of extracts of (4 ml), were mixed with 1 ml ethanol DPPH solution (0.05 mM)

each with vigorous shaking. The control was mixture of solvent and DPPH solution. The absorbance was measured after 30 minutes in the dark at room temperature on UV-VIS spectrophotometer (Evolution 60 Thermo scientific) at 550 nm, as well as controls using the solvent as a blank. The inhibition of DPPH radicals in the presence of the test sample was calculated by the formula and expressed as a percentage (%):

$$\text{DPPH radical inhibition (\%)} = (A_k - A_u) / (A_k) * 100\%$$

where A_k presents the absorbance of the control reagent and A_u the absorbance of the sample. IC_{50} value presents the concentration of sample that inhibits 50% of the initial concentration of DPPH free radicals. IC_{50} value was calculated from the concentrations/% inhibition curve. The antioxidant activity was compared to the known antioxidant, ascorbic acid.

Statistical analysis

Statistical analysis was performed using SPSS software version 20. The results were processed using one-factor analysis of variance (ANOVA) and the Tukey test determined whether there was a statistically significant difference between samples with a significance level of 0.05.

Results

Total polyphenols and tannins

The content of total polyphenols and tannins are determined by calibration curve, which represents the ratio of increasing concentrations of gallic acid and the obtained absorbance values.

The content of TP and TT in wheat, corn, rye and integral wheat flour extracts are shown in Table 1 in mg GAE/g.

Table 1. Content of total polyphenols and tannins (mg GAE/g) in wheat, rye, corn and whole wheat flours

Flour	Total polyphenols (mg GAE/g)	Total tannins (mg GAE/g)
Wheat	2.29 ± 0.32a	0.99 ± 0.074a
Rye	2.6 ± 0.408a	1.46 ± 0.491a
Corn	5.88 ± 0.71b	4.27 ± 0.632a
Whole wheat	6.38 ± 0.759b	5.7 ± 1.547a

a, b Different letters indicate a statistically significant difference ($p < 0.05$) between the samples

The highest content of TP had whole wheat flour extract (6.38 ± 0.759 mg GAE/g) while the lowest had wheat flour extract (2.29 ± 0.32 mg GAE/g). The second by the abundance in TP was

corn flour extract (5.88 ± 0.71 mg GAE/g). Statistical analysis revealed that there was no statistically significant difference between the content of total polyphenols of whole wheat flour and corn

flour. There was a statistically significant difference between wheat flour type 500 and rye flour ($p < 0.05$).

The highest content of TT had whole wheat flour extract (5.7 ± 1.547 mg GAE/g) and the lowest wheat flour extract (0.99 ± 0.074 mg GAE/g). There was no statistically significant difference in tannin content between samples ($p < 0.05$).

Antioxidant activity

The antioxidant activity of wheat, rye, corn and wheat whole wheat extracts was determined in the DPPH system. The results are shown in Table 2 and are expressed as inhibitory concentrations (IC_{50} values). Statistically significant difference (95%) was found between whole wheat flour and other varieties of tested flour.

Table 2. Antioxidant activity of wheat, rye, corn and whole wheat flours

Flour	IC_{50} (mg/ml)
Wheat	$26.39 \pm 2.132a$
Rye	$18.08 \pm 4.112a$
Corn	$17.79 \pm 0.491a$
Whole wheat	$5.63 \pm 1.331b$

The results show the mean values of the three measurements \pm standard deviation
a, b: Different letters indicate statistically significant difference ($p < 0.05$) in
antioxidant activity between samples

Discussion

Phenolic compounds, such as flavonoids, phenolic acids and tannins, express various biological activities, including anti-inflammatory, anticancer and antiatherosclerotic activity. These activities may be related to their antioxidant capacity (24).

Most of the phenols in the cereal grain are located in the cell wall where they are complexed with polysaccharides and lignans. Grinding and processing of cereals reduce their content significantly.

All samples showed a significant amount of total polyphenols and tannins and antioxidant activity.

The study conducted by Mazzoncini et al. (2014) found that the content of phenolic compounds decrease with increased refinement of flour. The content of total polyphenols and tannins was 4-7 times higher in the bran sample compared to white flour, as well as the antioxidant activity of the same sample (9). The results of this study cannot be fully compared to ours due to the different units ($\mu\text{mol GAE}/100$ g extract), but the wheat flour sample (type 500) also had the lowest total polyphenol content of all whole grain flours (whole wheat, corn and rye flour), which is consistent with our results.

Sedej and associates (2010) also found that the highest amount of total polyphenols was in the whole wheat flour sample and the lowest in the wheat flour type 500 ($137.2 \mu\text{g GAE/g}$ and $37.1 \mu\text{g GAE/g}$) (21). Compared to theirs, our value for the wheat flour (2.29 ± 0.32 mg GAE/g) and whole wheat flour (6.38 ± 0.759 mg GAE/g) are signifi-

cantly higher. These differences may be due to the different varieties of wheat, the degree of processing, the agrochemical conditions of production, and the storage of the samples.

Ragae et al. (2006) determined the content of total polyphenols in wheat, rye, barley, millet and sorghum (25). Several studies have shown that 80% methanol is the effective solvent for the extraction of phenol and other polar substances from cereals, so the samples were extracted with this solvent. The total polyphenol content of the rye flour sample is $1026 \pm 16.9 \mu\text{g GAE/g}$. Our samples were extracted with 96% ethanol and the content of total polyphenols is twice as high (2.6 ± 0.408 mg GAE/g), indicating the importance of selecting the right solvent for the extraction.

De la Parra et al. (2007) determined the content of total polyphenols in 5 different corn varieties - white, yellow, blue, red and high-carotenoid corn (26). Samples were prepared by extraction with 80% ethanol. The smallest amount of total polyphenols was found in the red corn sample (243.8 ± 4.6 mg GAE/100 g dry extract) and the highest amount corn with high carotenoid content (320.1 ± 7.6 mg GAE/100 g dry corn extract). The white corn sample had twice smaller amount of total polyphenols (260.7 ± 6.1 mg GAE/100 g dry extract) compared to our sample (5.88 ± 0.71 mg GAE/g) obtained by extraction with 96% ethanol. As polyphenolic compounds have different solubilities depending on the polarity of the solvent, different amounts of total polyphenols can be expected.

The ability of an extract to remove free radicals depends on its concentration and is expressed as IC_{50} values. This is the sample concentration required to reduce the initial DPPH radical concen-

tration by 50% in experimental conditions. Therefore, a lower IC_{50} value indicates higher antioxidant activity.

This test demonstrated the antioxidant potential of all flour extracts, and especially the activity of integral wheat flour extract ($IC_{50} = 5.63 \pm 1.331$ mg/ml), which was expected due to the highest content of total polyphenols and tannins in this sample.

The lowest antioxidant activity was demonstrated by the wheat flour sample (type 500), whose IC_{50} value was 26.39 ± 2.132 mg/ml. Low antioxidant activity was expected, since this sample had the lowest content of total polyphenols.

There are not so many data about flours antioxidative activity. Corn flour, although having approximately the same amount of total polyphenols as whole wheat flour, exhibits slightly lower antioxidant activity ($IC_{50} = 17.79 \pm 0.491$ mg/ml). Adom et al. (2002) showed that corn had the highest antioxidant activity (181.4 ± 0.86 μ mol equivalent of ascorbic acid/g grain) compared to wheat, rice and oats (27). The results of our study cannot fully be compared to theirs because of the different ways of expression of antioxidant activity.

Conclusion

Based on the obtained results, the whole wheat flour extract exhibits the best antioxidant activity, while the lowest shows wheat flour type 500. The content of total polyphenols and tannins is highest in the whole wheat flour sample and the lowest in the wheat flour sample.

Cereals are a source of phenolic compounds and the beneficial effects on the body will be manifested if flour and whole grain cereal products are consumed.

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Originalni rad

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doi:10.5633/amm.2020.0214**ANTIOKSIDATIVNA AKTIVNOST, SADRŽAJ UKUPNIH POLIFENOLA I TANINA RAZLIČITIH VRSTA BRAŠNA***Bojana Miladinović¹, Kristina Ilić², Dušica Stojanović¹, Milica Kostić¹, Milica Milutinović¹, Suzana Branković³, Dušanka Kitić¹*¹Univerzitet u Nišu, Medicinski fakultet, Katedra za farmaciju, Niš, Srbija²Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija³Univerzitet u Nišu, Medicinski fakultet, Katedra za fiziologiju, Niš, Srbija

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Žitarice se zbog svojih osobina smatraju jednim od najčešće upotrebljivanih namirnica. One predstavljaju glavni izvor ugljenih hidrata, od kojih u žitaricama najviše ima skroba. Pored ugljenih hidrata, žitarice sadrže i proteine, nezasićene masne kiseline, minerale, vitamine B grupe i vitamin E. Ovaj sastav se odnosi na neprerađen oblik žitarica, tj. na integralne žitarice. Žitarice takođe sadrže fenolna jedinjenja, kao što su fenolne kiseline, flavonoidi, stilbeni, kumarini i tanini. To su sekundarni metaboliti biljaka, koji, zbog svojih antioksidativnih sposobnosti, imaju značajnu ulogu u prevenciji mnogih bolesti.

Cilj ovog rada bilo je određivanje sadržaja ukupnih polifenola i tanina u uzorcima pšeničnog (tip 500), kukuruznog, raženog brašna i integralnog pšeničnog brašna, kao i određivanje njihove antioksidativne aktivnosti. Najveći sadržaj ukupnih polifenola određen je u integralnom pšeničnom brašnu (6,38 mg GAE/g \pm 0,759 mg GAE/g), dok je najmanja količina određena u pšeničnom (tip 500) brašnu (2,29 mg GAE/g \pm 0,32 mg GAE/g).

Najbolju antioksidativnu aktivnost pokazao je ekstrakt integralnog pšeničnog brašna (IC_{50} = 5,63 mg/ml \pm 1,331 mg/ml), dok je najslabiju antioksidativnu aktivnost pokazao ekstrakt pšeničnog brašna (tip 500) (IC_{50} = 26,39 mg/ml \pm 2,132 mg/ml). Na osnovu dobijenih rezultata može se zaključiti da brašna od celih zrna žitarica sadrže veliku količinu polifenola, što upućuje na značajnu antioksidativnu aktivnost istih. Zbog svojih mnogobrojnih svojstava, koja utiču na poboljšanje zdravlja, žitarice čine temelj piramide ishrane, kao modela pravilnog načina ishrane.

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Ključne reči: pšenično brašno, kukuruzno brašno, raženo brašno i integralno brašno, polifenoli, antioksidativna aktivnost

PROPOLIS: CHEMICAL COMPOSITION, BIOLOGICAL AND PHARMACOLOGICAL ACTIVITY – A REVIEW

Sanja Stojanović¹, Stevo J. Najman¹, Biljana Bogdanova-Popov², Svetozar S. Najman³

Propolis is a honey bee product that is well known from ancient times for its beneficial effects on human health and has been widely used in traditional medicine. Chemical composition of propolis is very complex and rich which is why propolis has many biological activities, but is dependent on the geographical origin and plant sources from which bees collect the material to produce propolis. Despite great diversity of its chemical composition, propolis has many biological and pharmacological activities and is widely used in medicine, dentistry and pharmacy. In this review, beneficial effects of propolis through its biological and pharmacological activities are discussed.

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Key words: propolis, chemical composition, biological activity, pharmacological activity

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Introduction

Propolis, also called bee glue, is a natural resinous compound that is produced by honey bees. Honey bees collect the resins and other substances from various plant species, bring them back to the colony where they process it and utilize as propolis in the hives for a number of purposes. Not all species of the genus *Apis* (honey bee) produce and use propolis at the same degree (1-3). Among them, species *Apis mellifera* produces and utilizes propolis more than others (1-3). Both feral as well domesticated colonies use propolis, in tree cavities or commercial hives, for the same purposes such as

covering the holes and sealing the cracks in the nest, narrowing the hive entrance creating a smooth surface for comb attachment, as an "embalming" substance and for the protection (1, 2, 4). In addition to its role as a building material, propolis acts as physical, chemical and biological protector for the bees since it prevents uncontrolled airflow, acts as waterproofing wall against moisture, maintains constant humidity inside the hive, stabilizes the indoor temperature in the hive, creates protection against invaders, parasites and predators, acts as an antiseptic, prevents and inhibits the growth of pathogen microorganisms in the tree cavities and hives (1, 2, 4, 5). The word "propolis" originated from Ancient Greek word "pro" (which means "before" or in this context "in front of" or "at the entrance to") and the word "polis" (which means "the city" or "community" or in this context this may refer to the hive) (1, 2, 4, 6). This meaning of the word "propolis" is tightly connected to and refers to the main role of propolis as a substance that bees use to build the nest or the hive and its entrance which will serve for the protection and defense.

Learning from the bees and how they use propolis and for what purposes, people have been using propolis since ancient times. There are records suggesting the use of propolis by ancient Egyptians, Persians, Romans and Greeks for various purposes (2, 6, 7). Although the propolis was mainly used as a remedy for medical purposes since ancient times (5), it was also used for other purposes as well. There are records that it was used as a constituent of violin varnish by famous Stradivari, Amati and others (8). Similar to the use of propolis as "embalming" substance by bees, Egyptians used propolis as an anti-putrefactive agent to embalm

their dead (9). The Greeks used propolis as the primary ingredient of *polyanthus*, perfume which combined propolis, olibanum, styrax, and aromatic herbs (2, 8). As for medical purposes, it has been recorded that propolis was used as an antiseptic, cicatrizant and healing agent first by Greek and Roman physicians (9). The first data on the use of propolis for wound healing date back to the time of the Hippocrates who used propolis to cure wounds and ulcers, both external and internal (2). It is also known that Incas used propolis as an antipyretic agent while British pharmacopoeias (from the 17th century) listed propolis as an official drug (9). The later use of propolis was mainly focused on its antibacterial properties (9).

So far, more than 300 chemical compounds of propolis have been identified and this number is still growing. The chemical composition of propolis varies greatly depending on the geographical and botanical origin, i.e. climate factors, plant resources and collecting seasons (5, 10). The specificity of the local flora is the major determinant of the chemical composition of propolis and subsequent biological and pharmacological activity. Due to its complexity and variable composition, there is a need for standardization of propolis in order to use it as a commercial product for medical purposes (5). Raw propolis is rarely used directly for the treatment although grinded propolis in the form of powder is used as an addition to the honey and other food formulations (8). Prior to the formulation of different preparations and their analysis, extraction of biologically active substances from propolis is generally carried out using water or different organic solvents. Extraction method as well as used solvents may significantly influence biological and pharmacological activities of propolis. Despite the variations in chemical composition, all propolis preparations exert wide variety of unique biological activities that are the characteristics of all propolis types, regardless of its origin, extraction method and composition. Propolis exhibits many biological and pharmacological activities and is widely used in various fields of medicine, dentistry and pharmacy. Propolis application potentials are increasing significantly.

Chemical composition

Propolis is mainly composed of the following components: resins and vegetable balsam (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds including sugars, amino acids, vitamins, and minerals (5%) (10-14). Except resins and waxes, the main groups of chemical compounds found to be present in propolis are: phenols (e.g., flavonoids, polyphenols, phenolic acids and other phenolic compounds) and their esters, terpenes and terpenoids, steroids, aromatic acids, aromatic esters, aldehydes, alcohols, sugars, sugar alcohols and acids, amino acids, vitamins, fatty acids, hydrocarbons, mineral elements and alcohols (12, 14-16). Major group of phenolic compounds presented in propolis is flavonoid group which contribute greatly to the biological and pharmacological activities of propolis. According to the chemical structure, flavonoids presented in propolis are classi-

fied into: flavones, flavonols, flavanones, flavanols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones, flavans, isoflavans and neoflavonoids (12). Flavonoid group in propolis includes mainly the following substances: chrysin, pinocembrin, apigenin, rutin, luteolin, galangin, kaempferol, myricetin, catechin, naringenin, quercetin, tectochrysin, pinostrobin, acacetin and others (4, 13, 14, 17-19). Another important group of compounds found in propolis are phenolic acids, among which the most often are ferulic, cinnamic, caffeic, benzoic, salicylic and p-cumaric acids. In propolis other phenolic compounds (e.g., artemillin C) have been found as well as stilbenes and stilbene derivative resveratrol (12, 14, 17). Volatile compounds such as terpenes and terpenoids are reported to be present in propolis in only 10% but greatly contribute to the biological and pharmacological activities of propolis and are responsible for its characteristic fragrance (12). Among them, terpineol, camphor, geraniol, nerol and farnesol have been identified so far (4, 13, 18, 19). It has been noticed that the presence of different types of terpenoids in propolis varies depending on the geographical origin of propolis (4, 13, 18, 19). Hydrocarbons such as alkanes, alkenes, alkadienes, monoesters, diesters, aromatic esters, fatty acids and steroids have been identified so far in many different types of propolis (12). Some enzymes like succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and acid phosphatase are also found in propolis (14, 20). Minerals (macro- and microelements) which have been found in various propolis samples are Ca, K, Na, Mg, Mn, Fe, Si, Zn, Se, Cu, Ni, Al, B, Ba, Cr and Sr (4, 13, 14, 18-20). The trace element profiles of propolis can be used for identification of different propolis samples based on their location. Some toxic elements such as As, Cd, Hg and Pb have also been found in propolis (12, 21) especially in propolis samples collected from industrial and polluted areas which is why elements' analyses of raw propolis can be used as an indicator of environmental pollution. Propolis contains some important vitamins such as B complex vitamins, vitamins C and E (4, 13, 14, 18-20). Most of the data on chemical composition of propolis have been obtained by analysis of different propolis extracts. For propolis dissolution and extraction of biologically active compounds, several different solvents can be used: water, alcohols (e.g. ethanol and methanol), glycols (e.g. propylene glycol), oils (e.g. olive oil) and in considerably less extent, and only for analytical purposes, other organic solvents such as hexane, acetone, dichloromethane and chloroform (19). It is also very common to use a mixture of aforementioned solvents especially water-ethanol mixtures (8). The most suitable extraction systems are hydroalcoholic solutions since propolis represents a mixture of polar and nonpolar compounds (22). Also, due to the lipophilic characteristic of some compounds and low solubility in water, commercial propolis formulations are often ethanol - or glycol-based (22). Despite the great variety of chemical compounds found in different propolis samples, there are some general groups of chemicals responsible for so called "basic" biological activities of propolis regardless of the specific compounds.

Biological and pharmacological activity

Propolis and its extracts have numerous applications for the treatment of various diseases due to its great spectrum of biological activities. It has been found that propolis and its constituents possess antimicrobial (antibacterial, antiviral, antifungal), antioxidant, anti-inflammatory, immunomodulatory and anti-proliferative activities (6, 13, 14). Also, propolis and its components may stimulate the synthesis of extracellular matrix components (such as collagen), proliferation and migration of fibroblasts, differentiation of cells (6) and to possess both anti-apoptotic activity against normal cells while in the same time acts pro-apoptotic to some cancer cell lines. The activity of propolis depends on the chemical composition which is related to its geographical origin (19). Some biological activities of propolis are unique regardless of the propolis type and its origin (e.g. antimicrobial and antioxidant), while some specific biological functions depend on the presence of some biologically active compounds which is related to the botanical origin of propolis as well as to the extraction method that is used. Propolis, as a significant antimicrobial bee product, acts both against Gram-positive and Gram-negative bacteria, as well as aerobic and anaerobic bacteria with slightly higher activity against Gram-positive bacteria (6, 23). Propolis exerts its antibacterial effect on two levels: by direct action on the microorganisms and by stimulation of the immune system which results in activation of the natural defense mechanisms of the organism (23).

Due to its rich chemical profile and wide variety of biological activities, propolis may have great pharmacological and therapeutical applications and can be used as natural antiseptic, anti-inflammatory, antioxidant, antimicrobial (antibacterial, antiviral, antifungal), anticancer, immunomodulatory and wound healing agent in various preparations and as a part or in addition to some medications for the treatment of wide variety of conditions and disorders (6, 13, 14). So far, the following pharmacological properties of propolis have been reported: antiulcer, antacid, antihistaminergic, anti-inflammatory, antitumor, anesthetic properties, hepatoprotective, nephroprotective, cardioprotective, neuroprotective and pancreatoprotective activities (13, 14, 24-27). Furthermore, recent studies have revealed that propolis has hypoglycemic activity which may have positive impact on diabetic complications. It also modulates the metabolism of blood lipid levels which leads to a decrease in lipid peroxidation and scavenges the free radicals (27). The use of propolis has been proposed in some patents where propolis is suggested for the treatment of dental diseases, as an adjuvant in anti-cancer treatment, the use in cosmetic products, as an anti-inflammatory agent and as natural antibiotic (28). Hepatoprotective effect of propolis has been reported in many models of experimentally-induced hepatic injury in rats (16). Cardioprotective effect of Malaysian propolis against experimentally-induced ischemia in rats has been attributed to its anti-lipoperoxidative and antioxidant effects (26). Authors of this study suggested that this effect was probably achieved via two pathways,

through direct radical-scavenging activities of propolis against ISO-induced oxidative stress and that propolis contributed to endogenous antioxidant enzyme activity via inhibition of lipid peroxidation (26). The protective effect of propolis against UVA-induced apoptosis of human keratinocyte HaCaT cells was observed and it was suggested that this effect might be related to the reduction of ROS generation by UVA-irradiation (29). Protective effects of propolis extract against ethylene glycol induced hepatotoxicity and nephrotoxicity has been reported, and it has been shown that treatment with propolis extract normalized the level of magnesium, sodium, potassium, chloride and creatinine, alleviated urinary protein excretion and ameliorated the deterioration of liver and kidney function caused by ethylene glycol, which candidates propolis for potential treatment and prevention of urinary calculus, crystalluria and proteinuria (30). Nephroprotective effect of propolis has also been shown against significant cisplatin caused damage of kidney tissue in dose-dependent manner (31). Propolis was proposed as an effective candidate for prevention or treatment of oxidative stress and neuroinflammation in neurological diseases since it has been reported that propolis and its flavonoid constituents exert neuroprotective properties in studies *in vitro* and *in vivo* through their antioxidant, anti-inflammatory, and immunomodulatory activities (25). Protective effects of propolis on the liver of diabetic rats have been reported and attributed to the suppression of oxidative stress, inflammation and apoptosis of hepatocytes, while in the same time increasing the proliferation of hepatocytes which altogether improves liver function in diabetic state (32). Synergistic protective effect of Malaysian propolis with metformin as a drug used in antihyperglycemic medication was observed in streptozotocin-induced diabetic rats (32) suggesting propolis as a promising complementary therapy in type 2 diabetes mellitus (T2DM). When the effects of Iranian propolis extract on glucose metabolism, lipid profile, insulin resistance, renal and liver function as well as inflammatory biomarkers were examined in patients with T2DM, it was shown that propolis has beneficial effects on reducing post prandial blood glucose, serum insulin, insulin resistance and inflammatory cytokines, while elevating HDL-C concentrations and prevent liver and renal dysfunction in patients with T2DM (27). Protective effects of ethanolic propolis extract on cardiovascular system and hepatorenal functions has been reported and attributed to the antihyperlipidemic and antioxidant activity of propolis which favors the traditional use of propolis in hyperlipidemic disorders as an adjuvant agent in the form of food supplement (33). Anticancer activity of propolis, alone or as an adjuvant therapy, has been shown against brain, head and neck, skin, breast, liver, pancreas, kidney, bladder, prostate, colon and blood cancers in various *in vitro* and *in vivo* studies (34). Due to the good bioavailability by the oral route and immunomodulatory activity, propolis is reported to be a good candidate for an adjuvant anticancer therapy (35). Caffeic acid phenethyl ester (CAPE) is a very potent biologically active substance isolated from propolis which, among others, has

potent anticancer activity. It was shown that CAPE inhibited the proliferation and metastasis of nasopharyngeal carcinoma cells (NPC), enhanced the radiosensitivity of NPC cells and was synergistic in its action with chemotherapy and radiotherapy (36). Inhibition of the growth of breast cancer stem cells by CAPE has also been reported (37). Based on immunomodulatory effects of propolis which are shown to be achieved through the NO pathway and the effects on pro-inflammatory cytokines, some authors suggested that propolis may be a potential therapeutic candidate for modulation of inflammation in celiac disease (38). The use of propolis for the treatment of gynecological disorders has been reported due to its antimicrobial activities, especially against *Candida* species, as well as its anti-inflammatory activity (14). Propolis is proposed as an alternative treatment for chronic vaginitis, for an improvement in vaginal well-being, for recurrent vulvovaginal candidiasis and other gynecological disorders (14).

Conclusion

Propolis shows a great variety regarding composition which depends on its origin and plant sources from which is collected and produced. Different chemical composition may determine the specific biological activity of propolis, however, despite the variety in chemical composition, the biological and pharmacological activities of propolis are inevitable in many different examined model systems. The field of application of propolis is large and still growing, but given the variations in its composition that may influence its activity, it is necessary to carry out both detailed testing of each propolis preparation related to the origin as well as standardization of the preparation to the biologically active compounds that are mainly presented in propolis.

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PROPOLIS: HEMIJSKI SASTAV, BIOLOŠKA I FARMAKOLOŠKA AKTIVNOST – PREGLED

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Propolis je pčelinji proizvod koji je od davnina poznat po svojim povoljnim efektima na zdravlje ljudi i koji je u širokoj upotrebi u tradicionalnoj medicini. Hemijski sastav propolisa je veoma složen zbog čega propolis poseduje mnoge biološke aktivnosti, ali zavisi od geografskog porekla i biljnih izvora iz kojih pčele sakupljaju materijal za proizvodnju propolisa. Uprkos velikoj raznovrsnosti svog hemijskog sastava, propolis poseduje mnoge biološke i farmakološke aktivnosti i u širokoj je upotrebi u medicini, stomatologiji i farmaciji. U ovom pregledu razmatrani su povoljni efekti propolisa kroz njegove biološke i farmakološke aktivnosti.

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Ključne reči: propolis, hemijski sastav, biološka aktivnost, farmakološka aktivnost

CAVERNOUS HEMANGIOMA OF THE LIVER WITH EXTRAMEDULLARY HEMATOPOIESIS AND MALIGNANT CELLS EMBOLIZATION IN AN ELDERLY PATIENT: A CASE REPORT

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Cavernous hemangioma is the most common benign tumor of the liver (the incidence ranging from 1.4% to 52%). The etiology of this tumor is unclear, with several hypotheses based on the potential congenital source of the lesions. Extramedullary hematopoiesis is a process of blood cell production outside the bone marrow, usually occurring due to hematologic disease or bone marrow infiltration of other causes. We present a case of 78 year old male with suspected malignant disease of gallbladder, as well as a liver cavernous hemangioma with extramedullary hematopoiesis and malignant cell embolization (endothelial cells positive for CD31 and CD34; and malignant cells positive for pancytokeratin and CDX2). The etiology of extramedullary hematopoiesis in vascular lesions is discussed.

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Key words: cavernous extramedullary, hemangioma, hematopoiesis, malignant

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Introduction

Vascular lesions of the liver may present as cavernous hemangioma (CH), infantile hemangioendothelioma, epitheloid hemangioendothelioma, and angiosarcoma. CH is the most common benign tumor of the liver, with an incidence ranging from 1.4% to 52%; usually occurring in old age and in women (1). Autopsy material shows an incidence of 7%. CH structure consists of vascular spaces encased with fibrous tissue and a single layer of endothelial cells. The tumor is usually demarcated from the surrounding tissue. The term "gigantic CH" is reserved for tumors larger than 5 centimeters, often

containing foci of bleeding, thrombosis, hyalinization, fibrosis and calcification (2).

CH is usually asymptomatic and diagnosed incidentally. The indications for surgical treatment are the presence of abdominal symptoms, spontaneous/traumatic rupture, rapid growth, Kasabach-Meritt syndrome as well as an unclear preoperative diagnosis (3). Four types of surgical procedures are performed: liver resection, enucleation, ligation of the hepatic arterial blood vessel and liver transplantation; with the first two being the most common (4). Liver hemangioma etiology is still unclear, with a hypothesis related to the possible congenital source of the lesions (5).

Extramedullary hematopoiesis (EMH) represents the production of blood cells outside the bone marrow. In adults, extramedullary hematopoiesis is a pathological condition usually occurring due to a bone marrow malignant infiltration or fibrosis (6). It is suspected that EMH is triggered by the secretion of the granulocyte colony-stimulating factor (G-CSF) (7). There are several reports of liver hemangiomas harboring EMH foci, without a clear description of the etiological mechanism (8).

This paper contains a description of an interesting case presenting with a liver CH harboring EMH and malignant cells emboli.

Case report

A 78 year old male patient presented with spontaneous subcutaneous hematomas on the lower extremities, fatigue, and weight loss. His previous

medical history included a nephrectomy 12 years ago (due to a renal cell carcinoma), mechanical aortic valve replacement 5 years ago, hypothyreosis, hypertension, atrial fibrillation and a liver tumor (diagnosed 8 years ago incidentally and regarded as a liver hemangioma based on the ultrasonographic characteristics, measuring 95 x 40 mm, without changes in size or structure during the past several years).

On examination, the patient was pale and diaphoretic, with a normal clinical examination of the thorax and the abdomen. The complete blood count and the laboratory findings were all in the normal range. Chest X-ray showed no pathological findings. Abdominal ultrasonography showed the previously described liver tumor in the left lobe; irregular density of the gallbladder wall (suggestive of malignant infiltration) with multiple intraluminal stones (maximum size 32 mm). Computerized tomography of the abdomen showed the previously described changes, several lesions in the tenth and eleventh thoracic vertebrae, as well as the fourth lumbar vertebra and the base of the sacrum suggestive of metastatic deposits. No other pathological findings were found.

A decision was made to perform a laparoscopic biopsy of the aforementioned liver tumor due

to clinical suspicion of malignant disease. Exploration of the abdominal cavity showed the gallbladder infiltrating the liver and the greater omentum. A subcapsular fragment of the liver tissue adjacent to the tumor (size 12 x 9 x 8mm) was sent to the histopathological analysis. The tissue showed irregular, wide vascular spaces with uniform endothelial cells and connective tissue, with erythrocytes and hematopoietic cells (Figure 1). Several vascular spaces showed atypical (oval or cubic) cells, with an increased nuclear-cytoplasmic ratio, hyperchromatic nuclei, pathological mitosis, organized in solid or cribriform groups (Figure 2). Immunohistochemistry CD31 and CD34 stain showed a layer of endothelial cells covering the vascular spaces. The atypical cells were positive for pancytokeratin (Figure 3), CDX2, and negative for vimentin, CK7, CK20, HepPar1, CD31, CD34. The conclusion was that the tumor was a liver CH with elements of EMH and malignant cells emboli of uncertain origin.

The patient's clinical condition worsened daily. He was hospitalized for blood transfusion two months after the biopsy due to severe sideropenic anemia. During the hospitalization, the patient went into cardiac arrest and passed away after an unsuccessful attempt at cardiopulmonary resuscitation.

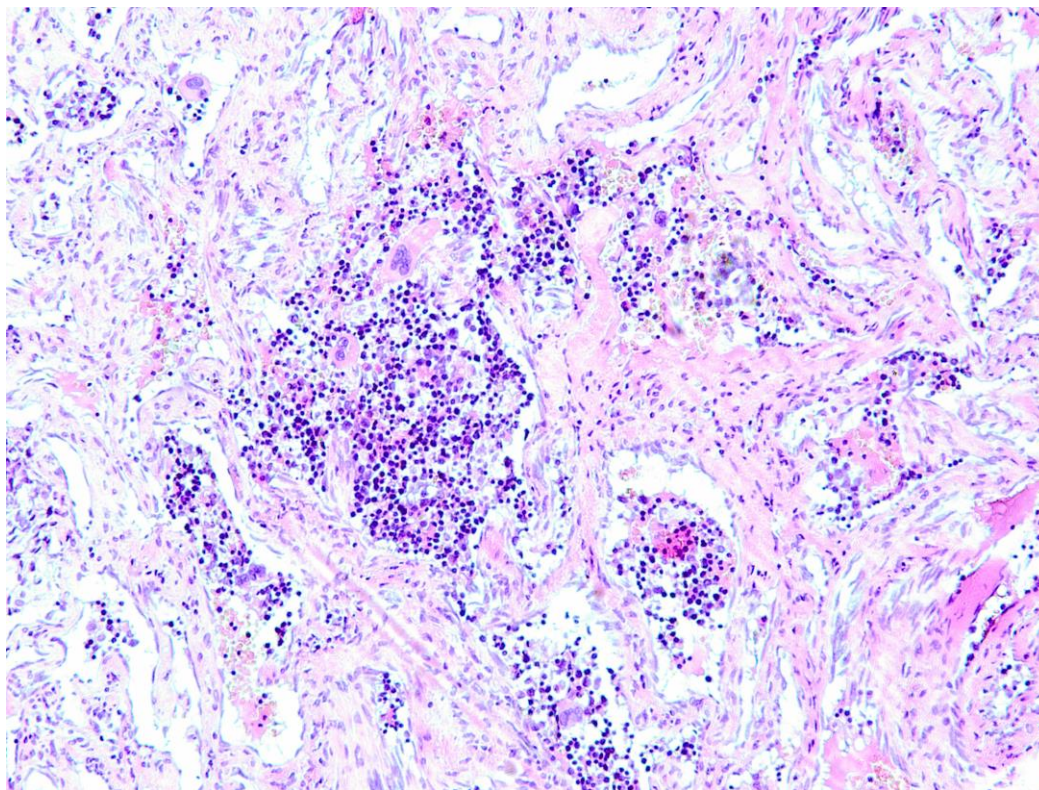


Figure 1. Extramedullary hematopoiesis in a liver hemangioma; hematoxylin-eosin stain, x10

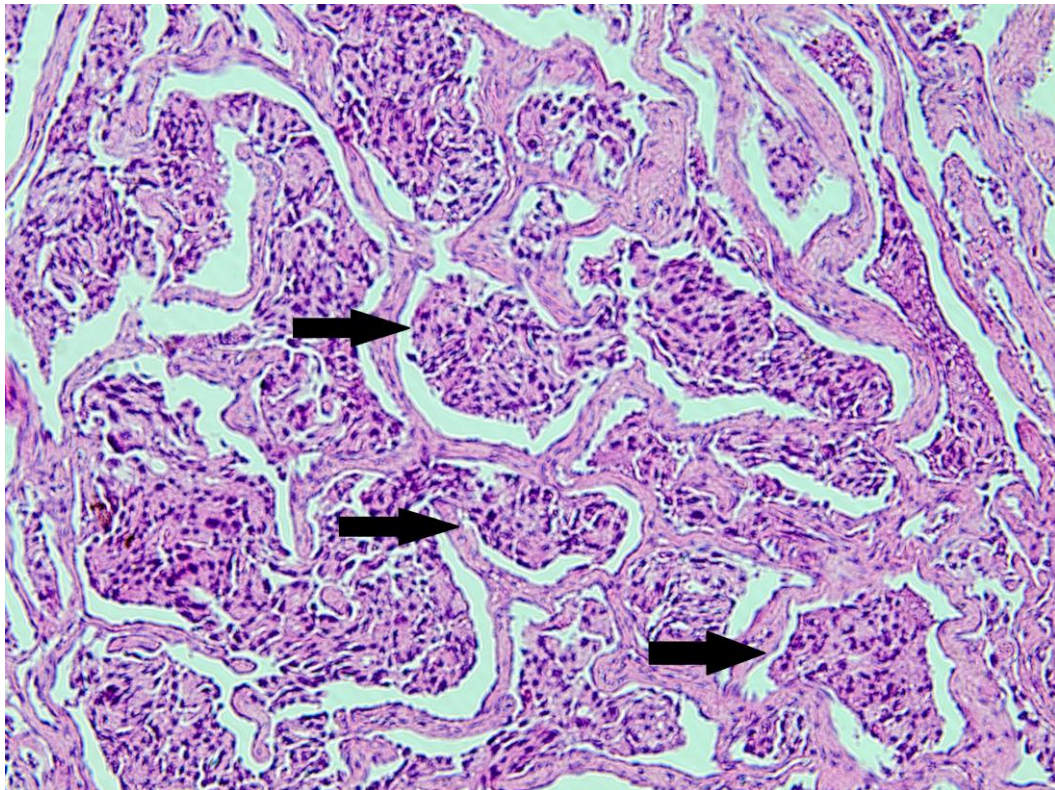


Figure 2. Clusters of tumor cells in a liver hemangioma (indicated by arrows); hematoxylin-eosin stain, x10

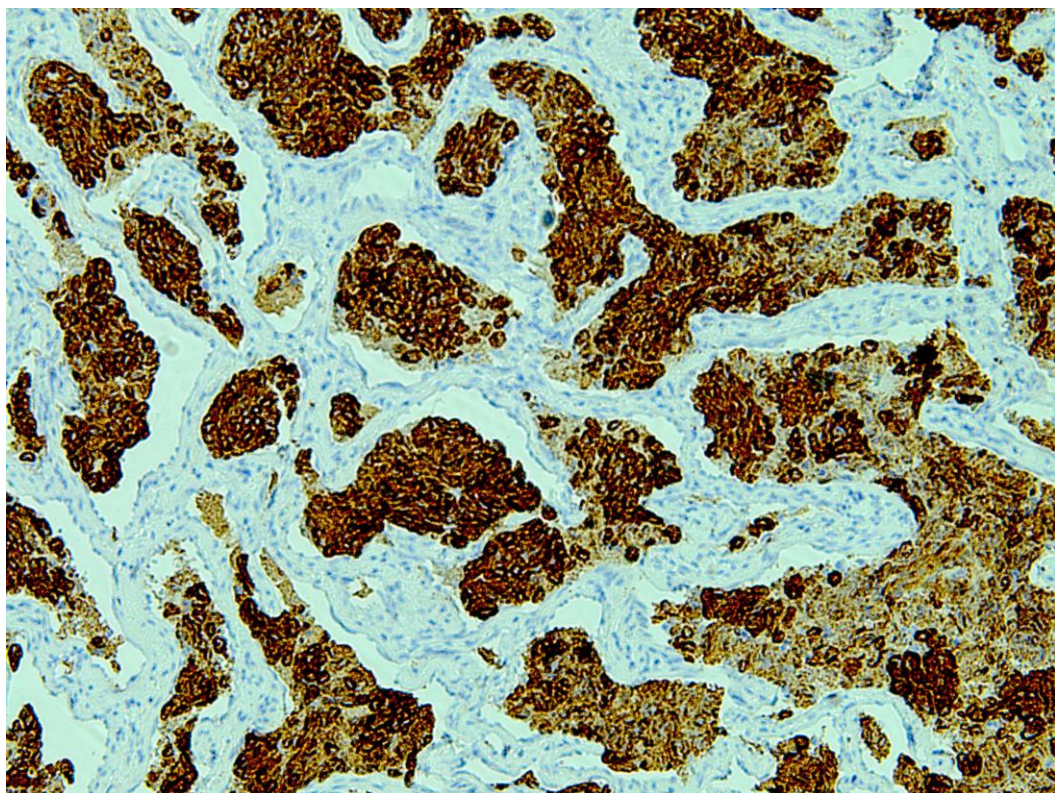


Figure 3. Tumor cells expressing pancytokeratin positivity, x10

Discussion

The case presented herein shows an interesting histopathologic finding in a patient suffering from malignant disease, most likely gallbladder carcinoma. His clinical condition after the laparoscopic biopsy of the liver tumor did not allow for gallbladder surgery to be performed; therefore, a final pathological diagnosis was not reached. The liver hemangioma, which likely existed before the onset of malignant disease, harbored malignant cells as well as hematopoietic cells. It is plausible that the malignant cells in the hemangioma originated from the carcinoma in the gallbladder wall. The histological image of vascular spaces in the liver tumor resembled CH, a lesion which is by itself benign. Liver vascular lesions may present a challenge for the pathologist, regarding the similarity with angiosarcoma. The histological appearance of an anastomosing hemangioma, a sinusoidal pattern of tightly packed capillary channels, may resemble an angiosarcoma-like image (8). However, anastomosing hemangiomas are sharply demarcated – they do not infiltrate the surrounding liver tissue. This differentiates them from hepatic small vessel neoplasm – a tumor showing an infiltrative growth pattern, but without the cytologic atypia characteristic for angiosarcoma (9).

It is interesting to consider whether EMH was triggered by malignant infiltration or it was present in the CH before the invasion by malignant cells. In order for EMH to occur, multiple systemic (bone marrow failure, excessive hematopoietic stimulation) and local (tissue inflammation and repair, abnormal cytokine secretion) factors are needed (10). In a literature review performed by Bao et al., multiple potential EMH triggers are identified in addition to G-CSF: chemotherapeutic agents (e.g. doxorubicin), cytokines or paracrine factors as well as pernicious anemia (11). The original explanation of EMH was based on the probability of the vascular tissue to provide support for the hematopoietic stem cells, similar to their interaction with the endosteum. It is considered that mesenchymal cells (such as non-myelinating Schwann cells, nesting-expressing cells, and CAR (CXCL12-abundant reticular) cells) support hematopoietic stem cells (12). In addition to the hematologic conditions associated with EMH (thalassemia, sickle cell anemia, hereditary spherocytosis, myeloid neoplasms, and myelodysplastic syndrome), liver EMH may occur in hepatoblastoma, he-

patic adenoma and hepatocellular carcinoma (13). The effect of CAR cells (and their expression of CXCL12, otherwise known as the stromal-cell derived factor 1 (SDF1)) in EMH-positive spleen is in the entrapment of the hematopoietic precursor stem cells (12). There are descriptions of EMH occurring in infantile hemangioendothelioma (14). In a series of 157 cases of pyogenic granuloma (lobular capillary hemangioma), Waraasawapati et al. described EMH in 10-8% of cases (15). Pinczewski and Papadimitriou stress the role of VEGF in EMH associated with epithelioid hemangioendothelioma (16).

Infantile hemangiomas occur in an adequate environment of cytokine secretion, mainly matrix metalloproteinases (MMP) and vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF-B1) as well as the basic fibroblastic growth factor (b-FGF). They arise from hematopoietic progenitor cells (17). Sequeira-Lopez et al. showed that blood cell progenitors might have a common origin with the blood vessels in the embryo, during the process of chemo-vasculogenesis (18).

Conclusion

This case report illustrates how a benign liver lesion may contain malignant cells and EMH. The diagnostic workup was not completed due to the patient's condition. The endoscopic evaluation of the upper and lower gastrointestinal tract as well as cholecystectomy should have been performed in order to identify the primary tumor. The pan cytokeratin positivity is indicative of an epithelial tumor, while CDX2 expression exhibited by the malignant cells suggests that the primary tumor is localized in the gastrointestinal tract. The negativity of CD31 suggests that the tumor origin is not vascular.

The CK7-/CK20- pattern is suggestive of hepatocellular carcinoma, germ cell tumor, seminoma, embryonal carcinoma, and other types of tumors, while most bile duct carcinomas are CK7+/CK20+. Owing to this, the gastrointestinal origin can be assumed with certain doubt. Future research should aim at explaining the role of VEGF and other cytokines as well as the process of chemovasculogenesis in the occurrence of EMH in hemangiomas (whether EMH arises simultaneously with hemangioma tissue or it sub-sequently settles in the tumor). Furthermore, the potential susceptibility of vascular lesions to harboring EMH should be examined.

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Prikaz bolesnika

UDC: 616.36-006.3:616.41
doi:10.5633/amm.2020.0216**KAVERNOZNI HEMANGIOM JETRE SA EKSTRAMEDULARNOM
HEMATOPOEZOM I EMBOLIZACIJOM MALIGNIM ČELIJAMA:
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Kavernozni hemangiom jetre je najčešći benigni tumor jetre (sa incidencijom u rasponu od 1,4% do 52%). Etiologija tumora je nejasna, sa nekoliko hipoteza koje se baziraju na mogućem kongenitalnom uzroku lezije. Ekstramedularna hematopoeza je proces produkcije krvnih ćelija van koštne srži, najčešće usled hematoloških bolesti ili fibroze koštne srži. Prezentujemo slučaj muškarca starog 78 godina sa sumnjom na malignitet žučne kese, kao i sa kavernoznim hemangiomom jetre sa ekstramedularnom hematopoezom i embolizacijom malignim ćelijama (endotelne ćelije pozitivne na CD31 i CD34; maligne ćelije pozitivne na pan-citokeratin i CDX2). Diskutovana je etiologija ekstramedularne hematopoeze u vaskularnim lezijama.

*Acta Medica Medianae 2020;59(2):114-119.***Ključne reči:** kavernozni hemangiom, ekstramedularna hematopoeza, malignitet

"RESTLESS LEGS" SYNDROME - THE MOST COMMON DISORDER YOU HAVE NEVER HEARD OF: A CASE REPORT

Jelena Stamenović

Restless legs syndrome (RLS) is a chronic neurological disorder and a clearly defined pathological condition characterized by four necessary and sufficient clinical symptoms, which are at the same time essential diagnostic criteria. RLS is clinically defined by the presence of an irresistible need for legs to move, with or without the feeling of paraesthesia, worsening of symptoms during rest and improvement with activity, and the onset or worsening of subjective problems in the evening or at the night.

The onset of symptoms is most common in the fifth and sixth decade of life, with a prevalence of about 10% in the general population of Europe and North America. Women are more likely to get the disorder than men, in a 2:1 ratio.

A 48-year-old patient turned to a neurologist because of her feelings of "tingling, burning, and uneasiness" in her legs, which were present for 6-7 months. The neurological finding was within the framework of physiological, non-pathological focal expression. After testing, pramipexole therapy was prescribed, which caused a significant reduction in the symptoms.

Patients use variable semantic phrases to describe their symptoms, but everyone has an irresistible need for movement. Due to the heterogeneity of subjective symptoms, sensory and motor symptoms of RLS were often attributed to other illnesses, and many patients remained undiagnosed and undetected. RLS is associated with a significant reduction in quality of life, comparable to that seen in chronic diseases such as diabetes and depression. Significant unrecognized and inadequate treatment of RLS indicates that better education about this disorder is needed.

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Key words: *restless legs syndrome, polysomnography, dopaminergic agonists*

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Introduction

The main clinical feature, or symptom of RLS or Willis-Ekbom's disease, is an unpleasant and continuous urgency for moving the leg during rest. During movement, symptoms stop, but re-occur at rest. This sensorymotor disorder significantly affects the quality of life, often leading to sleep disorders.

Past epidemiological studies have shown that the prevalence of RLS in Europe and North America is from 5% to 10% of adults in the general population. Regardless of such representation, RLS is still

not recognized enough in general medical practice. Very common symptoms are attributed to polyneuropathy, peripheral circulatory disorders or neurotic disorders (1).

Diagnostic criteria involve the presence of an unbearable urgency to move legs, which is accompanied by unpleasant sensations in the legs. Symptoms worsen during rest, most often in the evening and during the night. During movement, the symptoms are partially or completely reduced (2).

In the differential diagnostic procedure polyneuropathy, peripheral circulation disorders in the legs, as well as akathisia and other psychogenic disorders should be excluded. This means that the examination of a patient with RLS must be complex and comprehensive, using modern diagnostic methods.

The RLS therapy may be pharmacological and non-pharmacological, depending on the clinical presentation of the symptoms. The pharmacological approach involves the use of iron supplements, carbidopa/levodopa, benzodiazepine sedation, hypnotics, α 2 δ calcium channel ligands, dopamine agonist (ropinirole, pramipexole, and rotigotine). A non-pharmacological approach implies the application of an appropriate hygienic-dietary regime with the

correction of daily activities and regular physical exercises.

Case Report

A 48-year-old patient, female, was sent to the neurologist because of her feelings of "tingling, burning, and uneasiness" in her legs. The problems were present for 6-7 months, with a gradual, almost imperceptible beginning. The patient could not indicate the exact date of the onset of these symptoms. Initially, the symptoms appeared mainly in the evening hours during the break at home and lasted very briefly, for several minutes with spontaneous reduction. After a few months, the feeling of uneasiness in the legs accompanied by the tightening of the feet and the lower part of legs occurred during the afternoon hours and lasted longer than 10 to 30 minutes. The problems were reduced only after getting up from the sitting position and shorter walking in the room. At that time, the patient contacted a general practitioner who prescribed benzodiazepines, whose daily dose was gradually increased to 15 mg (5 mg tablets three times daily). After a short improvement, the symptoms recurred again, and the use of benzodiazepine was discontinued.

The patient contacted a neurologist at a time when daytime activities, especially in the afternoon, were significantly damaged. The neurological finding was within the framework of physiological, non-pathological focal expression.

In the differential diagnostic procedure, basic laboratory analyzes and differential blood counts were made, with a proper finding. EMNG of the upper and lower extremities, ultrasound examination of blood vessels of the leg and MR of endocranium did not show pathological changes.

Per exclusionem, she was diagnosed with RLS, as all four clinical diagnostic criteria were met.

After examination, pramipexole therapy was prescribed, which caused a significant reduction in the symptoms. The initial dose was 0.25 mg of pramipexole daily, in the evening. After three months the dose was increased to 0.50 mg of pramipexole in the evening, due to the recurrence of problems that were very mild and short duration, mainly in the evening or during a longer afternoon rest. After increasing the therapeutic dose of pramipexole, there was a complete reduction in the symptoms. The next control neurological examination was scheduled in three months. If these problems occurred again, a gradual increase in therapeutic doses of pramipexole was planned.

Discussion

Since the 1960s, the diagnostic criteria of the RLS have undergone several revisions and improvements, including the earliest, informal Ekblom's criteria from 1960. Based on the previous diagnostic criteria, the four basic diagnostic criteria of RLS published by the International Group for the Study of Restless Legs Syndrome (IRLSSG) in 2003

emphasized the importance of the urge to move legs in the diagnosis of RLS (2).

Diagnostic criteria of the International Restless Legs Syndrome Group (IRLSSG) are:

1. Foot movement, usually accompanied or caused by an unpleasant and uncomfortable feeling in the legs. Sometimes the urge for movement is present without the discomfort of legs, and sometimes the hands or other parts of the body may be involved.

2. An irresistible need for movement or unpleasant sensations begins or deteriorates during a period of rest or inactivity, such as sitting or lying down.

3. Unpleasant sensations or movements are partially or completely reduced during walking or stretching, at least as long as this activity lasts.

4. Movement or unpleasant feelings are more pronounced in the evening or at night than during the day, or occur only in the evening or at night. When the symptoms are significantly incapacitating, aggravation during the night does not have to be more pronounced in relation to daytime symptoms, but must be previously present.

A differential diagnostic procedure is important to exclude other medical or behavioral conditions accompanied by cramps in the legs or legs movements.

Support Criteria:

1. Family anamnesis for RLS
2. Positive therapeutic response to the use of dopaminergic drugs
3. Polysomnographic examination of periodic movements of limbs during wakefulness or sleeping (1).

RLS is thought to be a complicated sensorimotor syndrome that may be associated with changes in sensorimotor integration. The mechanism of such changes is still not clear. The aim of the study, conducted by Lin and associates in 2018, was to investigate the sensorimotor integration in patients with RLS through transcranial magnetic stimulation of motor evoked potentials (TMS-MEPs), preceded by peripheral electrical stimulation. The results of the study indicate increased motor cortical excitability of the leg and impaired sensorimotor integration in people with RLS. This disorder is thought to occur at the cortical level (3).

Recent literature suggests that the pathophysiology of RLS includes impaired cortical sensorimotor integration and the presence of hyperexcitability of the cortical-striatal-thalamic-cortical network, with the consequent hyperexcitability of spinal motor neurons. Intramuscular injection of botulinum toxin type A, in addition to muscle relaxation which causes inhibition of acetylcholine, significantly reduces the discharge of muscle spindles. There is evidence that intramuscularly injected botulinum toxins can reach the spinal cord from the site of injection and directly affect the spinal cord neurons. A study by Mittal and associates in 2018 showed that botulinum toxin type A can reduce the severity of symptoms within six weeks. The quality of life of

RLS patients can be improved up to six weeks after injection, and pain and discomfort are reduced over four weeks (4).

The genetic contribution to the development of RLS remains poorly explained. In 2007, the first large-scale genome wide association study identified three genomic regions associated with RLS. MEIS1, BTBD9 and MAP2K5/SKOR1 are genes located within these loci and their association with RLS was subsequently confirmed in a number of follow up genome wide association studies. Catoire et al. (2018) published the results of a study that directly link MEIS1 and SKOR1, two significantly associated genes with RLS and also prioritized SKOR1 over MAP2K5 in the RLS associated intergenic region of MAP2K5/SKOR1 (5).

RLS therapy is primarily pharmacological and non-pharmacological treatment has limited impact. Harrison et al. (2018) conducted a study that examined the effects of exercise for relaxation and stress relief in people with RLS. Physical exercises for stretching and fatigue of the lower extremities were performed at weekly intervals, with an estimate at the end of each interval. No significant differences were observed between the group that had physical exercises and control groups in the sixth week. Significant improvements have been recorded over time in all groups (6).

In a differential diagnostic procedure, it is important to distinguish RLS from other conditions and diseases that may have similar symptoms. Disorders of venous circulation are often accompanied by pain and cramps in the legs, but they do not have a circadian rhythm like RLS symptoms. Also, venous circulatory disorders appear with the changes in the skin, without improving with the use of dopaminergic therapy. Akathisia associated with the use of neuroleptics is characterized by the feeling of internal anxiety and the inability to rest during the day. In akathisia, movements occur on different parts of the body, without the subsequent feeling of relief. The symptoms of polyneuropathy that are characterized by painful burning in the extremities should be different from the symptoms of the RLS. Lumbosacral radiculopathy mainly has unilateral

sensory paraesthesias followed by painful sensitivity in the lower part of the spine and along the leg (1).

Pharmacological treatment involves dopaminergic medication (pramipexole, ropinirole and rotigotine) as first-line therapy. The therapeutic use of pregabalin is also gaining in importance. Opioids have shown efficacy in the treatment of RLS symptoms, but their adverse effects and the possibility of abuse significantly limit their use. Other pharmacological approaches include the addition of iron supplements in anemic patients, the use of some anticonvulsants and benzodiazepines. A very common comorbid disorder in RLS is depression. But treatment of depression should be cautious as selective serotonin reuptake inhibitors and tricyclic antidepressants may adversely affect the symptoms of RLS. The use of bupropion was recommended because no adverse effects were observed in its use (2). In a study carried out by Roshi et al. (2018), a statistically significant improvement in the activity of the daily living, with clonazepam compared to nortriptyline was demonstrated. Nortriptyline significantly improved patients' mood during eight weeks of administration (7).

After completing the differential diagnostic procedure and setting the exact diagnosis of RLS, treatment should be initiated using the dopamine agonist at the lowest possible dose, most often in the evening. If depressive symptomatology is present, antidepressant therapy may also be included. An adequate hygienic and diet regime, as well as adequate physical activity, is very important.

Conclusion

RLS is a relatively common disorder in clinical practice, but it is still insufficiently recognized by the doctors. Considering that it significantly affects daily activities, it is important to apply adequate therapy that is available. It is necessary to pay more attention to the symptoms and clinical presentation of this syndrome in order to provide better quality of life for the patients.

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Prikaz bolesnika

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doi:10.5633/amm.2020.0217**SINDROM "NEMIRNIH NOGU" – NAJČEŠĆI POREMEĆAJ ZA KOJI
NIKADA NISTE ČULI: PRIKAZ SLUČAJA***Jelena Stamenović*

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Sindrom "nemirnih nogu" (RLS) je hronični neurološki poremećaj i jasno definisano patološko stanje, koje karakterišu četiri neophodna i dovoljna klinička simptoma, koji su ujedno i osnovni dijagnostički kriterijumi. RLS je klinički definisan prisustvom neodoljive potrebe za pokretanjem nogu, sa osećajem ili bez osećaja parestezija, pogoršanjem simptoma tokom odmora i poboljšanjem tokom aktivnosti, kao i početkom ili pogoršanjem subjektivnih tegoba uveče ili tokom noći. Početak simptoma je najčešći u petoj i šestoj deceniji života, sa prevalencijom od oko 10% u opštoj populaciji Evrope i Severne Amerike. Žene oboljevaju češće od muškaraca, u odnosu 2 : 1.

Četrdesetogodišnja bolesnica obratila se neurologu zbog osećaja "trnjenja, peckanja i nelagodnosti" u nogama, koji su prisutni 6 - 7 meseci. Neurološki nalaz bio je bez patološkog fokalnog ispoljavanja. Nakon ispitivanja, propisana je terapija pramipeksolom, što je dovelo do značajne redukcije tegoba.

Bolesnici koriste varijabilne semantičke fraze za opisivanje simptoma, ali svima je zajednička neizdrživa potreba za kretanjem. Zbog heterogenosti subjektivnih tegoba, senzomotorni simptomi RLS-a često se pripisuju drugim bolestima, a mnogi bolesnici ostali su nedijagnostikovani. RLS je povezan sa značajnim smanjenjem kvaliteta života, uporedivim sa onim kod hroničnih bolesti, kao što su dijabetes i depresija. Značajno neprepoznavanje i neadekvatan tretman RLS-a pokazuju da je potrebna bolja edukacija o ovom poremećaju.

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PATIENTS WITH DIFFERENT METASTATIC MALIGNANCIES AND ACUTE MYOCARDIAL INFARCTION WITH ST SEGMENT ELEVATION TREATED WITH PRIMARY PERCUTANEOUS CORONARY INTERVENTION

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Cardiovascular diseases and malignancies are still the most potential causes of lethal event. These diseases very often co-exist in elderly population and arise from the same risk factors such as tobacco use. Improvement in malignancy treatment prolongs life, but may be harmful, due to hemo and radiotherapy regimen. Many chemotherapeutic agents are associated with angina and myocardial infarction, while radiotherapy is associated with coronary artery disease through direct endothelial injury. In addition, the prothrombotic and inflammatory status in metastatic malignancies promote the development of atherosclerotic plaques and thrombus formation in coronary arteries. In this paper, we present two cases of patients with different metastatic malignancies with acute myocardial infarction with ST segment elevation treated with primary percutaneous coronary intervention (pPCI) and with different short-term outcome.

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Key words: malignancy, myocardial infarction, PCI, outcome

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Introduction

As it is already known, cardiovascular diseases, along with the cancer, are the most common reason for high morbidity and mortality, widely spread around the world (1). Primary diagnosis and adequate treatment of patients with metastatic malignancies, suffering also from cardiac diseases, increase the rate of their survival (2). Patients on intensive oncologic therapy usually suffer also from coronary artery disease (CAD) due to the presence of the same risk factors (age, smoking tobacco, etc.) for both groups of diseases and application of che-

motherapy and/or radiation that can influence early atherosclerosis (3). In such patients, PCI is of great significance in treatment, especially in cases of metastatic malignancies.

In this paper, we report two patients with ST-elevation myocardial infarction (STEMI) and metastatic malignancies who were on different chemotherapy regimens and underwent primary PCI in our cath lab.

Case presentations

Case 1

A man, 50 years old, with nausea and abdominal pain, was admitted to hospital in May 2017. Physical examination revealed abdomen larger than normal and palpable tumorous formation. Huge retroperitoneal tumor was detected on MSCT scan, spreading from left inguinal region to lien, pressing urine bladder, stomach and intestines. A sample of a tissue was taken out by laparotomy, and subsequent pathohistological examination showed that the patient has atypical lipomatous tumor (ALT)/well differentiated liposarcoma (WLS). This was a clear indication for chemotherapy, and the patient was put on ifosfamidic protocol. Another MSCT scan in August 2017 revealed metastatic formations in cerebral regions, both frontal and temporal, and in the cerebellar region. Therefore, the second chemotherapy protocol was introduced in September 2017.

A month later, in October 2017, patient complained to chest pain, with propagation to shoulders and back, and therefore was referred to primary PCI center. ECG on admission showed ST-segment elevation in leads II, III, a VF and ST-segment depression in leads V3-V6, with R-wave rising. These findings indicated infero-posterior STEMI and the patient received a loading dose of Clopidogrel (600 mg), Aspirin (300 mg), and Heparin (7000 IV). Due

to the presence of oversized and tough abdomen covering the groins, the only possible approach in this case was trans-radial approach. Culprit lesion was detected in the distal right coronary artery (RCA), with significant lesions in the mid and proximal portion of the RCA, along with some non-significant lesions in the left anterior descending artery (LAD), and the left circumflex branch (LCX) (Figure 1 and 2).

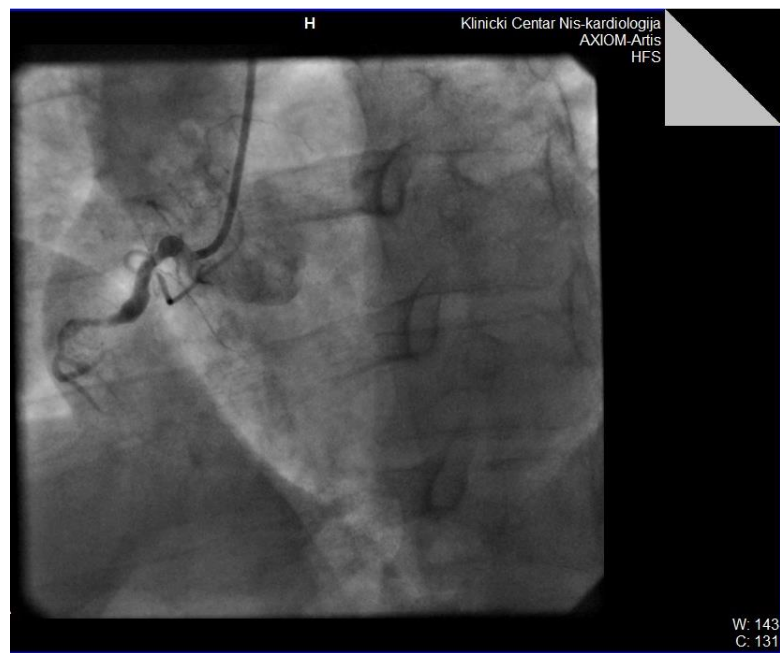


Figure 1. Right coronary artery with culprit lesion in the distal and significant lesions in the mid and proximal part



Figure 2. Non-significant lesions in LAD and LCX

Bare metal stents (BMS) were implanted in the distal, medial, and proximal RCA (Figure 3). During the next 5 days of in-hospital treatment with DAPT, LMWH, BB, ACEI, and statin, the patient was asymptomatic, and was discharged at his own request. Six hours after discharge from the hospital, the patient was readmitted due to the chest pain. ECG showed ST-elevation in inferior leads and in V4R lead, so the angiography procedure was re-

peated. It revealed stent thrombosis (Figure 4), but it appeared impossible for the operator to place intermediate coronary wire through in-stent occlusion. In a situation that GP2b3a inhibitors were not available, the only possibility was to administer systemic thrombolysis (TPA). Unfortunately, the patient felt into a shock and comma, and died after only 6 hours.



Figure 3. RCA after BMS implantation



Figure 4. Stent thrombosis in RCA

Case 2

A woman, age 58, was admitted to hospital due to severe cough, fatigue, and weight loss, in November 2017. Radiological and MSCT examination revealed a tumor formation in the lungs and

liver. Performed pathohistological examination revealed adenocarcinoma bronchogenes with hepatic and pulmonary metastasis. Consequently, the first chemotherapy regimen with paclitaxel and carboplatin was prescribed.

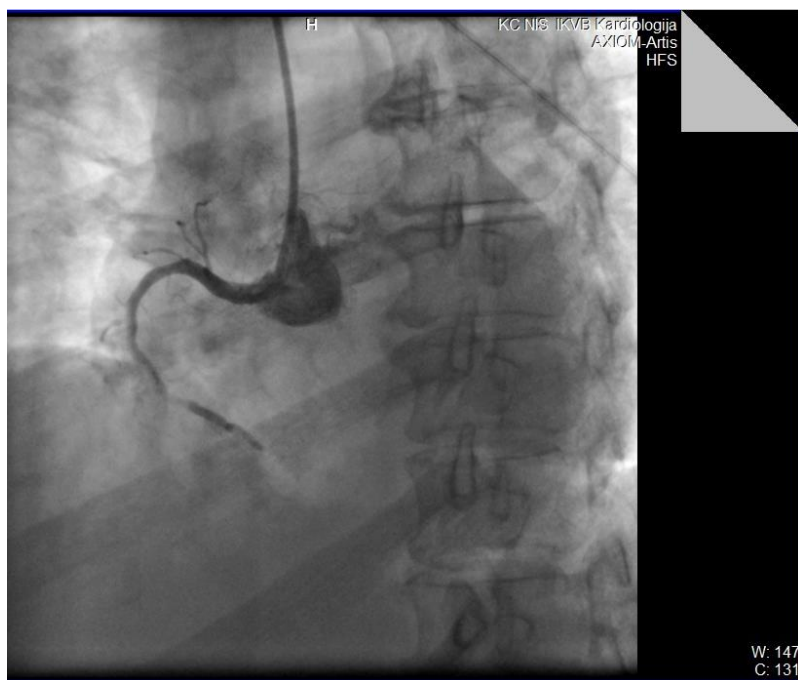


Figure 5. Culprit lesion in distal RCA

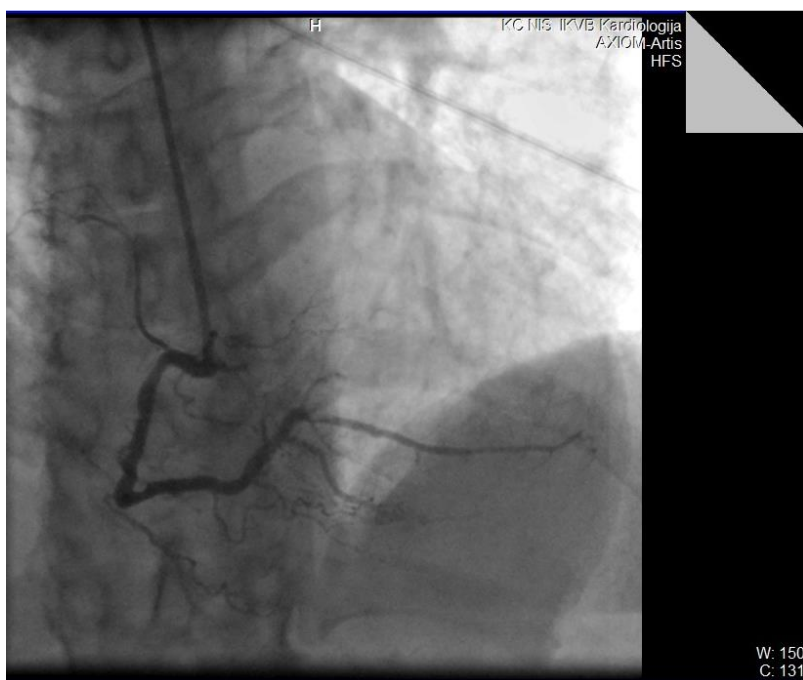


Figure 6. RCA after stent implantation

Two weeks after the first admission, she came to hospital again. While receiving chemotherapy, the patient felt chest pain and nausea, but the physicians assumed that described symptoms were related to cytostatic drugs application. The next day, she was referred to primary PCI center. ECG on admission showed ST segment elevation in leads II, III, and a VF and ST segment depression in leads V3-V6, with high R-wave, while echocardiography revealed akinesia of the inferior and hypokinesia of the posterior wall of the left ventricle. Culprit lesion in the distal RCA was reached by transradial approach, and BMS was implanted (Figure 5 and 6). The patient was hemodynamically stable during hospitalization, and was discharged after 5 days. Dual antiplatelet therapy was prescribed.

Discussion

Patients suffering from cancer are on increased risk of CAD and ACS. Chemotherapy and radiation seriously affect coronary arteries, which are the factors that, in association with an increased risk of thrombosis, greatly increase the risk of cardiovascular morbidity with lethal outcome. Conservative treatment of these patients is associated with low survival rate and the best possible therapeutic option for those with good survival prognosis is definitely PCI treatment (1).

An increased risk of ACS in patients suffering from cancer, and those on anti-cancer therapy, is based on abnormal coagulation that is associated with malignancy or antineoplastic therapy, that both increase the risk of thrombosis causing STEMI (4).

Liposarcoma can be very different in type and shape (well/poor differentiated, myxoid/round, pleomorphic), and all of them respond to some extent to chemotherapy, but this response is still not documented very well (5, 6). Trabectedin and eribulin have been in use in USA since 2015/2016, in cases of inoperable and/or metastatic liposarcoma and leiomyosarcoma, in patients that have already undergone anthracycline-based treatment (7, 8). Our first patient received the synthetic analog of cyclophosphamide ifosfamide. Nevertheless, it was recently shown that some alkylating agents, very similar to cyclophosphamide, such as cisplatin and ifosfamide, may be the cause of heart failure because of some pathological effects, including myocardial ischemia (9).

The patient (Case 2) suffered from bronchogenic adenocarcinoma and she started the chemotherapy with paclitaxel and carboplatin. The patients using paclitaxel rarely develop myocardial infarction (10). Carboplatin, as a derivative of cisplatin, is widely accepted in clinical praxis for its ability to reduce non-hematologic toxicity (11). However, cisplatin is proved to be associated with acute coronary thrombosis, in several vascular areas at the same time (12, 13). Cisplatin cause endothelial damage, and influences thromboxane production, platelet activation and aggregation (12, 14, 15). Patients with malignancy, who are treated with platinum-based drugs, have up to 7 time higher long-term risk of CAD and MI (16, 17).

Both of our patients were smokers, and both suffered metastatic malignancies, with no other risk factors for the development of CAD. According to Swedish Cancer Registry, that started to collect data in 2002, the risk for patients with metastasis to develop CHD was increased significantly between 2002 and 2008 (SIR 1.46; 95% CI 1.28–1.85), but the situation was not the same in patients without metastasis (SIR 0.99; 95% CI 0.93–1.05) (18). This increased risk for patients to develop CHD is due to different sites and types of cancer they suffer from. Inflammation is seen as one of the possible reasons because of its relation to atherosclerosis and hemostatic activation, while the relation between inflammation and hemostatic activation caused by cancer and CHD is also possible (19–21). Association of metastatic malignancy with CHD risk has already been observed in clinical practice, in the form of increased inflammation and hemostatic activation in patients with metastases.

In order to avoid ischemic complications caused by excessive coagulation, that is a result of lower fibrinolysis rate and influence of coagulation factors related to malignancy, antithrombotic treatment is an absolute necessity. PCI is seen to be a treatment of choice in cases when cancer is advancing rapidly, or in metastatic situations, when survival rate is expected to be low, i.e., 1 year or less (22). Unfortunately, there are only a few studies that are dealing with the issue of malignancy and PCI treatment (23).

In cases when PCI is a method used to treat CAD in patients with malignancy, both bare BMS and drug-eluting stents (DES) can be used for implantation in coronary arteries, although DES is preferred because of its lower in-stent thrombosis rate. In such PCI procedures, it must be also taken into consideration that hyper-coagulation in conjunction with some special effects in treatment of hematopoietic cells can cause bleeding (24).

Accessing the coronary artery with culprit lesion requires special attention in order to avoid bleeding at site. Complications that might occur are retroperitoneal hemorrhage (RPH), pseudo-aneurysm, artery-venous fistula, excessive bleeding (in thrombocytopenic patients), local infections, and delayed epithelium recovery after application of vascular closure device. In order to access the lesion properly, the procedure must be carefully planned (25).

SCAI recently published a consensus on special considerations for the patients with malignancy who are indicated for the cath lab, recommending a multi-aspect approach for patients with ACS (17). According to these considerations, the type of malignancy and survival prognosis must be taken into account when deciding to undergo PCI. This is of great importance in cases of gastrointestinal cancers because of the higher probability of bleeding in patients receiving antiplatelet and anticoagulation therapy. Also, there is a risk of anemia, a prediction of heart failure and lethal outcome in patients with STEMI, and thrombocytopenia which is followed by the increased bleeding risk (1, 17).

Conclusion

Patients suffering from cancer associated with STEMI are very challenging for all physicians, especially cardiologists. It is well known that the same loading DAPT, culprit lesion, and percutaneous procedure in different cases could have various outcomes along with possible complications. Malignancy

and CAD are interrelated, but the exact type of malignancy and its metastasis is of great importance in assessing different survival rate for different patients.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Prikaz bolesnika

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**BOLESNICI SA RAZLIČITIM METASTATSKIM MALIGNITETIMA I
 AKUTNIM INFARKTOM MIOKARDA SA ELEVACIJOM ST SEGMENTA
 TRETIRANI PRIMARNOM PERKUTANOM KORONARNOM
 INTERVENCIJOM**

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Kardiovaskularne bolesti i maligniteti predstavljaju, i dalje, najčešće potencijalne uzroke smrtnog ishoda. Ove bolesti vrlo često koegzistiraju u starijoj populaciji i proizilaze iz istih faktora rizika, kao što je konzumiranje duvana. Napredak u tretmanu maligniteta produžava životni vek, ali isto tako hemioterapija i radioterapija mogu dovesti do raznih oštećenja na nivou organizma. Primena mnogih hemoterapeutika udružena je sa razvojem angine pektoris i infarkta miokarda, dok je radioterapija udružena sa razvojem koronarne bolesti, preko direktnog oštećenja endotela. Pored toga, protrombotski i inflamatorni status u malignim bolestima podstiču razvoj aterosklerotskih plakova i formiranje tromba u koronarnim arterijama. U ovom radu prezentovali smo dva bolesnika sa različitim metastatskim malignitetima i akutnim infarktom miokarda sa ST elevacijom, koji su bili lečeni primarnom perkutanom koronarnom intervencijom (pPCI) i imali različite kratkoročne kliničke ishode.

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Ključne reči: malignitet, infarkt miokarda, PCI, ishod

**GENERAL, EPIDEMIOLOGICAL PARAMETERS AND IMMUNIZATION COVERAGE
OF CHILDREN SUFFERING FROM MORBILLI IN CENTRAL
KOSOVO AND METOHIJA**

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In the paper titled "GENERAL, EPIDEMIOLOGICAL PARAMETERS AND IMMUNIZATION COVERAGE OF CHILDREN SUFFERING FROM MORBILLI IN CENTRAL KOSOVO AND METOHIJA" by Vanja Ničković, Aleksandar Ranković, Ljiljana Šulović, Snežana Danić-Filipović, Snežana Marković-Jovanović, Zorica Vujnović-Živković, Jadranka Mitić, Hristina Kocić, Ilija Kocić, Marko Ristić, published in AMM journal in 2019, number 58 (4), there occurred a technical error on the page 32-41, with published wrong list of authors for the paper. We hereby apologize to the authors and readers. With authors' approval, we are now publishing the correct summary.

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OPŠTI I EPIDEMIOLOŠKI PARAMETRI I IMUNIZACIJA DECE OBOLELE OD MORBILA NA CENTRALNOM KOSOVU I METOHIJI

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U radu "OPŠTI I EPIDEMIOLOŠKI PARAMETRI I IMUNIZACIJA DECE OBOLELE OD MORBILA NA CENTRALNOM KOSOVU I METOHIJI" autora Vanja Ničković, Aleksandar Ranković, Ljiljana Šulović, Snežana Danić-Filipović, Snežana Marković-Jovanović, Zorica Vujnović-Živković, Jadranka Mitić, Hristina Kocić, Ilija Kocić, Marko Ristić, objavljenom u časopisu AMM za 2019. godinu broj 58 (4) na stranama od 32-41, došlo je do tehničke greške, pri kojoj je odštampan pogrešan spisak autora za ovaj rad. Ovom prilikom se izvinjavamo autorima i čitaocima. Uz saglasnost autora, u ovom broju objavljujemo ispravan spisak autora.

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