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Sjögren's syndrome (SS) is a chronic autoimmune (AI) disease resulting from damage to the lacrimal and salivary glands, and it is primarily manifested by dry eyes and mouth. In about half of the affected persons, symptoms of involvement of other organs appear (joints, gastrointestinal tract, kidneys, nervous system), and the disease is often associated with other AI disorders (rheumatoid arthritis and systemic lupus erythematosus). Patients also have an increased risk of developing hematological malignancies. SS is mostly diagnosed between the fourth and sixth decade of life, and over 90% of the patients are women. The syndrome was described 90 years ago (in 1933) by a Swedish ophthalmologist Henrik Sjögren, in whose honor World Sjögren's Day is celebrated every year on July 23 (on the birthday of this great doctor). World SS Day was established to highlight the importance of recognizing this serious disease from which millions of people worldwide suffer.

Sjogrenov sindrom (SS) je hronična autoimunska (AI) bolest koja je posledica oštećenja suznih i pljuvačnih žlezda i primarno se manifestuje suvoćom očiju i usta. Kod oko polovine obolelih osoba javljaju se simptomi zahvaćenosti drugih organa (zglobova, gastrointestninalnog trakta, bubrega, nervnog sistema), a bolest je često povezana sa drugim AI poremećajima (reumatoidni artritis i sistemski eritemski lupus). Takođe, kod obolelih postoji i povećan rizik od razvoja hematoloških maligniteta. SS se uglavnom dijagnostikuje između četvrte i šeste decenije života, a preko 90% obolelih su žene. Sindrom je pre 90 godina (1933. godine) opisao švedski oftalmolog Henrik Sjogren, u čiju čast se dan obolelih od SS obeležava svake godine 23. jula (na dan rođenja tog velikog lekara). Svetski dan obolelih od SS ustanovljen je da bi se naznačila važnost prepoznavanja te ozbiljne bolesti od koje pati više miliona ljudi u svetu.

GENERAL REVIEW

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# Novel molecular classification of endometrial cancer – current and future clinical implications

## Nova molekulska klasifikacija karcinoma endometrijuma – sadašnje i buduće kliničke implikacije

**Aljoša Mandić*†, Gabriel-Stefan Nadj†, Nevena Stanulović*†, Slobodan Maričić*†, Bojana Gutić*†**

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**Key words:**
**biomarkers; chemotherapy, adjuvant; classification; drug therapy; endometrial neoplasms; histological techniques.**

**Ključne reči:**
**biomarkeri; lečenje lekovima, adjuvantno; klasifikacija; lečenje lekovima; materica, neoplazme; histološke tehnike.**

### Introduction

Endometrial cancer (EC) is the fourth most common female cancer in Serbia, with a peak incidence between 60 and 70 years of age. It is the most common gynecological disease in our country [1–3]. Conditions that drive excess estrogen production, such as obesity, metabolic syndrome, and estrogen therapy without progesterone, are thought to be the main causes of this disease [4–6]. Selective estrogen receptor modulators, drugs used for managing infertility, breast cancer, ovulatory dysfunction, and postmenopausal osteoporosis, multiply the risk of EC [7, 8]. Other conditions that lead to an increase in relative risk of EC are polycystic ovary syndrome, nulliparity, late menopause, Lynch syndrome, Cowden syndrome, and others [9, 10].

Surgery is the cornerstone for the initial management of EC. Most commonly, minimally invasive removal of the uterus, ovaries, and fallopian tubes, along with occasional sentinel lymph node mapping, provide the basis for adequate staging [11].

The major challenge for clinicians who care for patients with EC is distinguishing between those who can be treated with surgery alone and those in need of adjuvant therapy.

Current tools for risk stratification are insufficient in differentiating patients who are at risk for recurrent or metastatic disease. That is due to a subjective and, therefore, inconsistent histological categorization [12–15].

Data from The Cancer Genome Atlas (TCGA) has helped us gain a better understanding of EC. It is a diverse set of diseases, with genomic differences driving different treatment outcomes. New patient subsets have been defined, and new questions have emerged. The main question is which patients benefit the most from adjuvant treatment [16, 17]?

### Classification of EC – histological approach

EC may come in the form of multiple neoplasms with very different characteristics and clinical outcomes. Histopathological (HP) evaluation, along with grading and subtyping, has traditionally been the cornerstone of EC classification. Over 25 different tumors have been described – ranging from epithelial hyperplasia to mesenchymal neuroectodermal tumors [18].

The other vital histological characteristic is grade. ECs are graded using the Federation of Gynecology and Obstetrics (FIGO) classification system on a scale from 1 to 3, according to the relative proportions of the glandular and solid-tumor components [19].

Tumor grade has a massive impact on prognosis. Grade 1 and 2 tumors are considered low grade and are associated with a better prognosis compared to grade 3 tumors, which are considered high grade.

In 1983, Bokhman [20] defined two types of EC based on clinical and histological characteristics. Type 1 tumors are mostly estrogen-dependent, commonly endometrioid, and have a more favorable prognosis. On the other hand, type 2 tumors are more diverse and more aggressive, leading to a less favorable prognosis. This classification system was a big step in the quest for a better understanding of EC.

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As mentioned, the major challenge is identifying subsets of patients in need of adjuvant therapy. For patients with an advanced stage of EC, there is no dilemma – all such patients will benefit considerably from adjuvant therapy. However, for stage I of the disease, identifying where patients lie on the spectrum of risk for recurrent disease – low, intermediate, or high – is still a great challenge. This challenge has led to the development of multiple risk stratification systems [20–24] based on data from landmark clinical trials such as PORTEC-2 [24] and PORTEC-3 [25]. Sadly, none can reliably predict disease recurrence or lymph node involvement [12, 26]. The reason for these limitations is unclear, but presumably, it is due to the limitations of HP and clinical data. Interobserver variability is high even among expert pathologists. EC grade assignment is subject to significant variation. One-third of cases with high-grade EC lacks a diagnostic consensus on the exact histologic type [27–30]. This data indicates that a more precise risk stratification model is needed.

**Molecular classification of EC**

An enormous change in the way we see EC subgroups has been introduced by TCGA [16].

The use of genomics, transcriptomics, and proteomics identified four molecular subgroups and several (we show four) predictive biomarkers based on genetic characteristics.

*Ultramutated/DNA polymerase epsilon (POLE) mutated group*

Pathogenic variants of the DNA polymerase epsilon, catalytic subunit *(POLE)* gene comprise approximately 10% of all endometrioid EC. *POLE* encodes a catalytic subunit of DNA polymerase epsilon, which is responsible for maintaining fidelity during DNA replication [31]. Mutations in these proofreading domains cause increased replication errors and result in an ultramutated phenotype. These tumors have an exceptionally high frequency of somatic mutations and a high occurrence of tumor-infiltrating lymphocytes (TILs). Typical features of this group include a presentation at a relatively young age and early stage, high tumor grade with scattered tumor, and rich in TILs and/or peritumoral (Crohn's-like) lymphocytes. As shown in Figure 1 [32], this group has very favorable outcomes (> 96% five-year survival) despite common aggressive pathologic features – for instance, high-grade or present lymphovascular space invasion [33, 34]. With the pronounced presence of TILs in this group, immunotherapy (IT) with immune checkpoint inhibitors (CIs) such as nivolumab may be an option for these patients [35, 36].

*Hypermutated/microsatellite instability (MSI) group*

About a third of all ECs belong to this group, characterized by a dysfunction in the DNA mismatch repair (MMR) system involved in an MMR of DNA postreplication. These tumors are most commonly endometrioid ECs, along with some non-endometrioid subtypes such as clear cell ECs [37, 38]. These ECs are also characterized by the presence of TILs, which makes them good targets for CIs. Pembrolizumab was approved by the United States Food and Drug Administration (FDA) in 2017 for a subset of these patients with progressive disease. Notably, this is the first FDA tissue/site agnostic drug approval [39]. Multiple clinical trials are ongoing in this patient population, targeting PI3K/AKT/mTOR pathways. Drugs like temsirolimus have failed to produce a robust benefit, but these pathway mutations continue to be targets of ongoing clinical trials [40, 41].

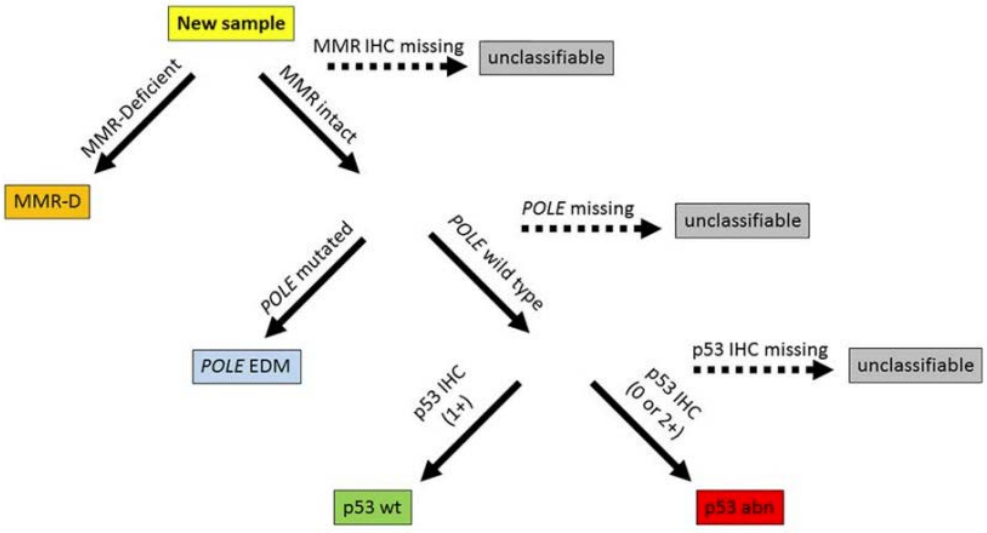


**Fig. 1 – Steps in molecular classification with Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE).**
**MMR – mismatch repair; MMRd – MMR-deficient; IHC – immunohistochemical; *POLE* – DNA polymerase epsilon; *POLE* EDM – *POLE* exonuclease domain mutations; p53wt – p53-wild type; p53abn – p53-abnormal. Figure taken from Talhouk et al. [32].**

*Copy number low group/microsatellite stable group*

A third group encompasses most of the low-grade (grade 1 and 2) ECs in the TCGA analysis. This group is characterized as genomically stable tumors with moderate mutational load ECs that are also not MMR deficient [16]. These are mostly endometrioid ECs, with good response rates to hormonal therapy due to the high presence of estrogen receptors and progesterone receptors.

*Copy number high/serous-like group*

This group includes mostly serous endometrial tumors and around a quarter of high-grade endometrioid tumors. These feature prominent somatic copy number alterations and often have *TP53* mutations (92% of cases) – similar to high-grade ovarian and basal-like breast carcinomas. Other amplified oncogenes are *MYC*, *ERBB2* (*HER2*), and *CCNE1*, all of which influence cell-cycle regulation [16, 17]. The prognosis was generally poor, and significantly worse progression-free survival (PFS) was noted compared to other groups, as shown in Figure 2. It has been well documented that tumor suppressor p53 leads to rapid tumor progression and invasion [42].

Molecular analysis of patient data from the landmark PORTEC-3 trial suggests that patients with p53 abnormalities have superior outcomes when treated with chemotherapy in addition to radiation, compared to radiation alone [42]. Trials are ongoing on therapeutic modalities, such as trastuzumab, that exploit molecular features of this subclass, such as *HER 2* [43].

***Predictive biomarkers – L1 Cell Adhesion Molecule***

L1 Cell Adhesion Molecule (L1CAM) is a transmembrane protein first identified on postmitotic mice neurons by M. Schachner in 1984. These immunoglobulins are thought to drive invasion and metastasis by promoting aggressive cell behavior. L1CAM overexpression has been reported in various malignancies, while Zeimet et al. [44] were the first to report that ECs positive for this biomarker have worse outcomes.

Positive L1CAM was a powerful driver of unfavorable outcomes in low-grade and early-stage ECs – the 5-year disease-specific survival rate dropped from 100% to 71% for L1CAM-positive patients [45].

## Incorporation of molecular characteristics into everyday clinical usage

The TCGA classification is impractical in a clinical setting due to considerable cost and time requirements driven by genome sequencing. That has prompted research teams to develop pragmatic molecular classification systems that can be performed on standard HP samples. These serve as surrogates for the diagnosis of the four molecular subtypes described by the TCGA classification. Molecular classification systems have been developed by two groups in Vancouver and the Netherlands. The Dutch team retrospectively analyzed bio-banks from the PORTEC-1 and -2 (postoperative radiation therapy for endometrial carcinoma) trials, identified four molecular subgroups, and validated their prognostic value [46]. The Canadian team developed a tool for molecular

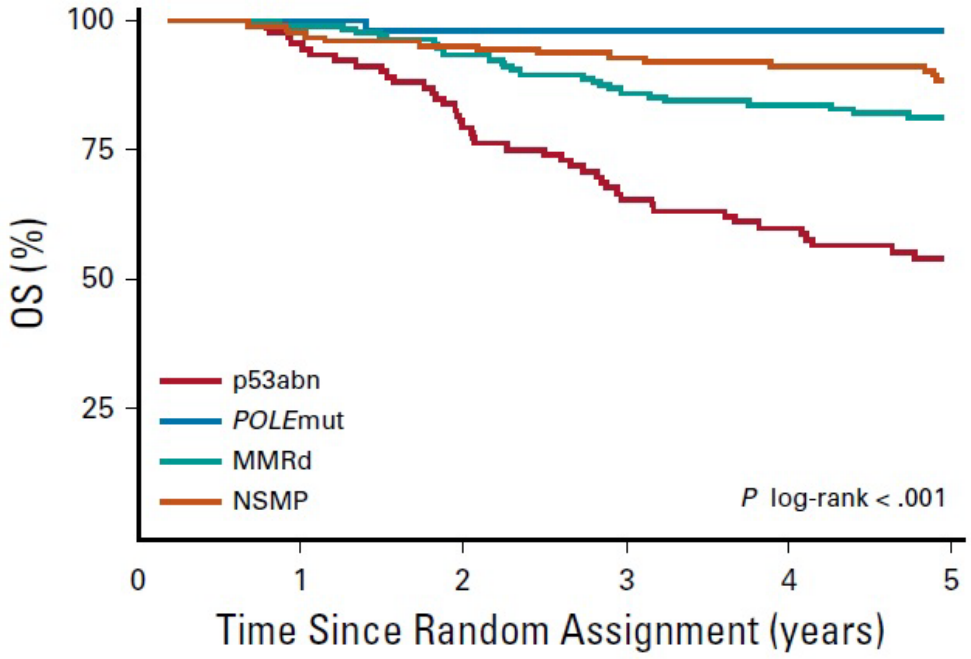


**Fig. 2 – Kaplan-Meier survival curve for 5-year OS in patients with p53abn EC (54.0%), *POLE*mut EC (98.0%), MMRd (81.3%), or NSMP EC (88.5%).**
**OS – overall survival; EC – endometrial cancer; *POLE* – DNA polymerase epsilon; MMRd – mismatch repair deficient; NSMP – no specific molecular profile; p53abn – p53-abnormal; *POLE*mut – *POLE*-mutated. Figure taken from León-Castillo et al. [42].**

classification named Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE). It was developed in agreement with strict National Academy of Medicine guidelines for biomarker tests based on omics [32]. These groups use three immunohistochemical (IHC) stains and sequencing for POLE exonuclease domain mutations (POLE EDMs) as surrogate markers corresponding to TCGA molecular subtypes. The results were four molecular subtypes, as shown in Figure 1. They were termed *p53* wild type (corresponding to TCGA copy-number – CN low), *p53* abnormal (corresponding to CN high), MMR defective (corresponding to MSI-H), and *POLE* EDM (corresponding to *POLE*-mutated group).

This simpler molecular classification system is advantageous when compared to the complexity of the TCGA classification. As mentioned, it works on standard formalin-fixed HP samples. With IHC stains for *p53* and MMR being readily available, the barrier to clinical implementation remains only *POLE* hotspot sequencing.

### Future research and implementation – PORTEC-4a

PORTEC-4a is a randomized trial that aims to compare rates of vaginal recurrence in women with high-intermediate risk EC. The control arm received standard adjuvant treatment with vaginal brachytherapy. The experimental arm received observation, vaginal brachytherapy, or external pelvic beam radiotherapy after surgery based on a patient-specific molecular-integrated risk profile [47].

The rate of vaginal recurrence was chosen as the primary outcome. Added metrics such as adverse events (AEs), patient-reported symptoms and quality of life (QoL), pelvic and distant recurrence, and healthcare costs related to cancer treatment also need to be studied.

### Utility in planning surgical treatment

Tissue samples gathered *via* endometrial curetting or pipelle biopsy will hopefully soon be available for molecular testing. Information obtained from these samples will most likely have an impact on surgical treatment and intraoperative decision management.

A hysterectomy and bilateral adnexectomy may suffice for patients burdened by *POLE* mutations, while more aggressive surgical treatment and lymph node dissection may be more suitable for *p53*-aberrant tumors.

### Utility in guiding adjuvant treatment decisions

The area that will be most impacted by the adoption of molecular classification is the adjuvant treatment of ECs. Variables such as stage, histological type, grade, depth of invasion, and others are currently used to guide surgical management and adjuvant treatment decisions. As mentioned, these variables do not sufficiently predict patient outcomes. Molecular classification effectively identifies different diseases that all belong in the landscape of EC, and clinical practice is moving toward treating them as such. These four subtypes differ concerning histogenesis, risk factors, hereditary susceptibility syndromes, molecular abnormalities, response to treatment, and outcomes. For instance, there is an interest in de-escalating treatment for early-stage *POLE*-mutated EC. The first randomized clinical trial for the use of molecular characterization as an integral component of guiding adjuvant treatment decisions for patients with stage I–II high-intermediate risk EC is PORTEC-4a [47]. The clinical effectiveness of the integrated pathological/molecular risk profile will be prospectively measured.

### Utility in guiding treatment decisions for progressive disease

In recent years, numerous novel therapeutic options for progressive EC have emerged – pembrolizumab, lenvatinib, bevacizumab, and others.

Bevacizumab, in combination with carboplatin/paclitaxel, is being evaluated for advanced EC in phase 2 trials such as GOG-86P and MITO END-2 [48, 49]. Based on National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology (NCCN guidelines®), bevacizumab is offered in the advanced disease setting in combination with platinum-based treatments or as a single agent after progression [50].

Patient selection for IT options is crucial as not all patients will benefit. For example, around 90% of ECs express PD-L1, which can serve as a biomarker for some immune CIs in certain tumor types [51].

There are multiple CIs that have demonstrated efficacy as monotherapy in the setting of advanced EC that had progressed on or after platinum-based chemotherapy. Studies such as KEYNOTE-028, KEYNOTE-158, GARNET, and others have evaluated the place of CIs in certain molecular subsets of ECs and have reported durable responses [52–54].

Another therapeutic strategy is to add immune CIs to other ITs, targeted agents, or chemotherapy. The hypothesis asserts that targeted therapies may alter the immune system, leading to better effectiveness of IT [55].

Lenvatinib combined with pembrolizumab was recently granted accelerated FDA approval, based on the KEYNOTE-146 trial, for previously treated advanced EC that is not MSI-H or deficient MMR (MMR-d). Dose reductions were noted in 53% of cases, and dosing was interrupted in 74% of patients. Both the reduction and the interruption of the doses occurred due to treatment AEs. Given the toxicity of this regimen, the comorbidities and toxicities of prior regimens should be taken into account in patient selection [56].

Multiple trials continue to explore the combination of IT with chemotherapy – phase 3 RUBY trial and phase 3 AtTEnd trial. These trials evaluate carboplatin/paclitaxel in combination with dostarlimab or atezolizumab in the setting of advanced and/or recurrent disease.

Another area of interest is the combination of CIs with PARP inhibitors. Trials such as olaparib/durvalumab DOMEC and rucaparib/atezolizumab/bevacizumab (ENDOBARR) investigate these combinations in patient populations with recurrent or metastatic ECs.

**Conclusion**

Our understanding of EC has been changed fundamentally by genomics. Depending on institutional resources, implementation of the molecular-based classification will vary, but it may well prove to be cost-effective because unnecessary or ineffective adjuvant treatment can be avoided. Ongoing research efforts are focused on identifying additional prognostically relevant biomarkers and optimally integrating the molecular risk profile with conventional clinicopathological variables to treat patients best.

Molecular classification will become the basis for adjuvant therapy directed at molecular subgroups and provide the framework for new trial designs that will explore the effectiveness of targeted agents and combination approaches. Ongoing clinical trials will hopefully result in better clinical decisions, thus leading to improved survival and QoL for patients.

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# Correlation between serum quantitative HBsAg and HBV DNA levels in chronic hepatitis B patients

## Korelacija između nivoa serumskog kvantitativnog HBsAg i HBV DNK kod bolesnika sa hroničnim hepatitisom B

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**Abstract**

**Background/Aim.** Quantitative hepatitis B virus (HBV) surface antigen (qHBsAg) has become increasingly widespread in the last few years in both diagnostic and therapeutic protocols for HBV infection. Numerous studies have proposed it as a surrogate marker for covalently closed circular DNA (cccDNA). The aim of the study was to determine the correlation between qHBsAg and HBV DNA viremia in untreated patients. **Methods.** The study included 112 untreated patients diagnosed with chronic HBV infection. Demographic and other data from medical records and laboratory analyses, taken as part of routine chronic HBV infection diagnosis with the determination of qHBsAg and HBV DNA viremia, were recorded for all patients. **Results.** The average age of the patients included in the study was 48.27 ± 15.14 years; males (58%) were more represented. qHBsAg levels had a high-intensity positive correlation with HBV DNA viremia. The concentration of qHBsAg, HBV DNA viremia, and the concentrations of alanine aminotransferase and aspartate aminotransferase showed statistically significantly higher values in HBV e antigen (HBeAg)-positive than in HBeAg-negative patients. **Conclusion.** Our study showed that qHBsAg has a high-intensity positive correlation with HBV DNA viremia. The use of qHBsAg is essential for determining the phase of chronic HBV infection, assessment of the success and length of treatment, as well as for safe discontinuation of antiviral therapy with a lower risk of relapse.

**Key words:**
**biomarkers; drug therapy; hepatitis b; hepatitis b, e antigens; hepatitis b surface antigens; hepatitis b virus.**

**Apstrakt**

**Uvod/Cilj.** Kvantitativni površinski antigen hepatitis B (qHBsAg) virusa je poslednjih nekoliko godina sve aktuelniji u dijagnostičkim i terapijskim protokolima hronične infekcije hepatitis B virusom (HBV). Prema mnogobrojnim studijama, predložen je kao surogat marker za cirkularnu kovalentno vezanu DNK (cccDNK). Cilj rada bio je da se ispita korelacija između qHBsAg i viremije HBV DNK kod nelečenih bolesnika. **Metode.** Istraživanjem su obuhvaćena 112 nelečena bolesnika sa dijagnozom hronične infekcije HBV. Zabeleženi su demografski i ostali podaci svih bolesnika iz medicinskih kartona, kao i rezultati rutinskih laboratorijskih analiza uz određivanje qHBsAg i viremije HBV DNK. **Rezultati.** Prosečna starost bolesnika obuhvaćenih istraživanjem bila je 48,27 ± 15,14 godina, a muški pol je bio zastupljeniji (58%). Nivoi qHBsAg su bili u pozitivnoj korelaciji visokog inteziteta sa viremijom HBV DNK. Koncentracija qHBsAg, viremija HBV DNK, kao i koncentracije alanin aminotransferaze i aspartat aminotransferaze pokazale su statistički značajno više vrednosti kod HBV e antigen (HbeAg)-pozitivnih nego kod HBeAg-negativnih bolesnika. **Zaključak.** Ovim istraživanjem je pokazano da je qHBsAg u pozitivnoj korelaciji visokog inteziteta sa viremijom HBV DNK. Upotreba qHBsAg je bitna za određivanje faze hronične HBV infekcije, procenu uspeha i dužine trajanja terapije, kao i za bezbedniji prekid antivirusne terapije sa manjim rizikom od relapsa bolesti.

**Ključne reči:**
**biomarkeri; lečenje lekovima; hepatitis b; hepatitis b, e antigeni; hepatitis b, površinski antigeni; hepatitis b, virus.**

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## Introduction

Despite a successful vaccination program, chronic hepatitis B (HB) virus (HBV) infection with repercussions in the form of hepatocellular carcinoma (HCC) and liver cirrhosis is still a global health concern [1–9]. According to the new nomenclature by the European Association for the Study of Liver (EASL) in 2017, there are five well-defined phases of chronic HBV infection: phase one being the HBV e antigen (HBeAg)-positive chronic HBV infection, phase two – HBeAg-positive chronic HB, phase three – HBeAg-negative chronic HBV infection, phase four – HBeAg-negative chronic HB, and phase five is an HBsAg-negative phase [1, 2]. Two classes of antiviral agents have been developed for treating chronic HBV infection: pegylated interferon alpha 2a (Peg-IFN-α-2a) and nucleoside and nucleotide analogs (NAs). Peg-IFN-α-2a is an antiviral and immunomodulatory agent that has a greater effect on the decrease of covalently closed circular DNA (cccDNA) quantity in the liver compared to NAs, which have a more pronounced antiviral effect. NAs inhibit reverse transcriptase and thus suppress viral replication but have weak to no effect on cccDNA [2]. Depending on the phase of infection, one of these classes of drugs or their combination is used [1]. Complete virus eradication is impossible due to the persistent cccDNA formed in the infected hepatocyte nucleus, which is the most resistant part of the viral DNA. Viral cccDNA is a mini-chromosome and is the primary site of chronic HBV infection [10, 11]. In the last few years, new diagnostic markers, such as quantitative HBV surface antigen (qHBsAg), HBV core-related antigen (HBcrAg), and HBV RNA, have been investigated to provide better insights into the chronic HBV infection phase, antiviral therapy effectiveness evaluation, HBsAg loss, discontinuation of antiviral therapy and possible relapse [12]. qHBsAg as a surrogate marker of cccDNA has recently become an additional focus of interest in monitoring the natural course of chronic HBV infection and the effect of antiviral therapy [13]. The level of qHBsAg reflects the level of free subviral particles, and although complementary to HBV DNA viremia, the obtained values show a lower degree of fluctuation over time [14]. Current therapeutic options lead to viral replication suppression, not virus eradication, and after antiviral therapy discontinuation, the disease may reactivate. Therefore, more and more research focuses on the question of when it is safe to discontinue NAs [12]. The main aim of our research was to examine the level and correlation of serum qHBsAg and HBV DNA viremia in untreated patients.

## Methods

The retrospective study included 112 untreated patients with chronic HBV infection, treated at the Clinic for Infectious Diseases, University Clinical Center of Vojvodina in Novi Sad, Republic of Serbia, from 2019 to 2020. The study was carried out following the ethical principles of good medical practice.

The inclusion criteria were age over 18 years and HBsAg-positive status longer than one year. The exclusion criteria were anti-hepatitis C virus seropositivity, human immunodeficiency virus infection, diagnosed autoimmune diseases, decompensated liver cirrhosis, and HCC.

Patients with chronic HBV infection were treated with standard diagnostic algorithms that included the determination of alanine aminotransferases (ALT) and aspartate aminotransferase (AST), quantitative HBsAg, HBeAg, and polymerase chain reaction (PCR) HBV DNA. The determination of HBV concentration by real-time PCR method was done on an Abbott m2000rt device with automatic isolation of HBV DNA on Abbott m2000sp. Elecsys HBsAg II Quant II assay (Roche Diagnostics, Germany) kit was used to determine qHBsAg on the Cobas e 411 device, which detects values from 5 to 13,000 IU/mL with a 1: 100 dilution; values below the detection level are marked as < 5 IU/mL, and values above as > 13,000 IU/mL. For qHBsAg values greater than 13,000 IU/mL, additional manual dilution was performed to get the final value.

Patients were divided into HBeAg-positive and HBeAg-negative based on their HBeAg status.

Statistical data processing was done in the SPSS program V20.0. The correlations of qHBsAg level with patient age, HBV DNA viremia, and aminotransferase values were examined by Spearman rank correlation. Values of $p < 0.05$ were considered statistically significant.

## Results

The mean age of the total of 112 patients included in the study was 48.27 ± 15.14 years (range 18–82 years). The distribution by gender showed a higher representation of males, 65 (58%), than females, 47 (42%). Elevated ALT levels, above upper limits of normal (ULN), and their average value, as well as elevated AST levels and their average value, are shown in Table 1. The mean qHBsAg value was 514 IU/mL (minimum-maximum, 5–1,666 IU/mL), and the mean HBV DNA viremia was 489 IU/mL (minimum-maximum, 25–4,190 IU/mL). The study participant demographic characteristics and biochemical parameters are shown in Table 1.

The correlation between qHBsAg levels and patient age, HBV DNA viremia, and aminotransferase levels was analyzed (Table 2). The results of this study show a statistically significant positive high-intensity correlation between qHBsAg and HBV DNA viremia. A statistically significant positive correlation was also observed between qHBsAg and ALT levels, although of mild to moderate intensity. No statistically significant correlation was observed between qHBsAg and AST levels. In addition, a statistically significant negative correlation between qHBsAg and the patient's age was shown. The observed correlation with age is moderate. The abovementioned results are shown in Table 2.

Data obtained in correlation analyses between qHBsAg and HBV DNA viremia, ALT concentrations and patient age are depicted in Figures 1, 2 and 3, respectively.

Since qHBsAg levels and HBV DNA viremia values decrease significantly with the patient's age, the relationship

**Table 1**

**Demographic characteristics and biochemical parameters of untreated patients (n = 112)**

| Parameter | Values |
|---|---|
| Gender, n (%) | |
| male | 65 (58) |
| female | 47 (42) |
| Age (years), mean ± SD | 48.27 ± 15.14 |
| HBeAg, n (%) | |
| positive | 18 (16.1) |
| negative | 94 (83.9) |
| AST (U/L), average (min-max) | 25.0 (21–35.7) |
| AST above ULN, n (%) | 19 (17.6) |
| ALT (U/L), n (%) | 26 (18–44.7) |
| ALT above ULN, n (%) | 25 (23.1) |
| qHBsAg (IU/mL), average (min-max) | 514 (5–1,666) |
| HBV DNA viremia (IU/mL), average (min-max) | 489 (25–4,190) |

**HBeAg – hepatitis B e antigen; AST – aspartate aminotransferase; ULN – upper limit of normal; ALT – alanine aminotransferase; qHBsAg – quantitative hepatitis B surface antigen; n – number; SD – standard deviation; min – minimum; max – maximum.**
**Note: Normal ranges for ALT and AST are 5–50 U/L and 1–31 U/L, respectively.**

**Table 2**

**Results of correlation analyses**

| Parameter | Age | HBV infection duration | qHBsAg | PCR HBV DNA | AST | ALT |
|---|---|---|---|---|---|---|
| Age | | | | | | |
| Coeff. ρ | 1.000 | 0.086 | -0.429** | -0.267** | -0.129 | -0.248** |
| Sig. (2-tailed) | / | 0.376 | 0.000 | 0.005 | 0.182 | 0.010 |
| n | 112 | 108 | 112 | 111 | 108 | 108 |
| qHBsAg | | | | | | |
| Coeff. ρ | -0.429** | -0.220* | 1.000 | 0.605** | 0.127 | 0.258** |
| Sig. (2-tailed) | 0.000 | 0.022 | / | 0.000 | 0.192 | 0.007 |
| n | 112 | 108 | 112 | 111 | 108 | 108 |
| PCR HBV DNA | | | | | | |
| Coeff. ρ | -0.267** | -0.233* | 0.605** | 1.000 | 0.322** | 0.395** |
| Sig. (2-tailed) | 0.005 | 0.016 | 0.000 | / | 0.001 | 0.000 |
| n | 111 | 107 | 111 | 111 | 107 | 107 |
| AST | | | | | | |
| Coeff. ρ | -0.129 | -0.129 | 0.127 | 0.322** | 1.000 | 0.743** |
| Sig. (2-tailed) | 0.182 | 0.183 | 0.192 | 0.001 | / | 0.000 |
| n | 108 | 108 | 108 | 107 | 108 | 108 |
| ALT | | | | | | |
| Coeff. ρ | -0.248** | -0.120 | 0.258** | 0.395** | 0.743** | 1.000 |
| Sig. (2-tailed) | 0.010 | 0.215 | 0.007 | 0.000 | 0.000 | / |
| n | 108 | 108 | 108 | 107 | 108 | 108 |

**Correlation is significant at the: *0.05 level (2-tailed); **0.01 level (2-tailed).**
**qHBsAg – quantitative hepatitis B surface antigen; PCR – polymerase chain reaction; HBV – hepatitis B virus; AST – aspartate aminotransferase; ALT – alanine aminotransferase.**
**n – number of patients.**

between qHBsAg and HBV DNA viremia was examined when the effect of the patient's age was excluded. The results of the partial correlation analysis showed that, even when the influence of the patient's age was excluded, qHBsAg and HBV DNA viremia continued to be statistically significantly positively correlated in low to moderate intensity ($p < 0.001$, $\rho = 0.393$). The correlation between qHBsAg and ALT was statistically significant ($p = 0.002$), positive, of low to moderate intensity ($\rho = 0.302$), even when the patient's age influence was removed.

By comparing the levels of qHBsAg in HBeAg-positive and HBeAg-negative patients, we obtained the results shown in Table 3. Eighteen (16.1%) patients were HBeAg-positive, and they were younger [mean age was 47 years (21–59)], 94 (83.9%) patients were HBeAg-negative, and they were older [mean age was 49 years (39–61)], but no statistical significance was determined ($p = 0.358$; $p > 0.05$). The concentration of qHBsAg, HBV DNA viremia, and the concentrations of ALT and AST were statistically significantly higher in the HBeAg-positive group than in the

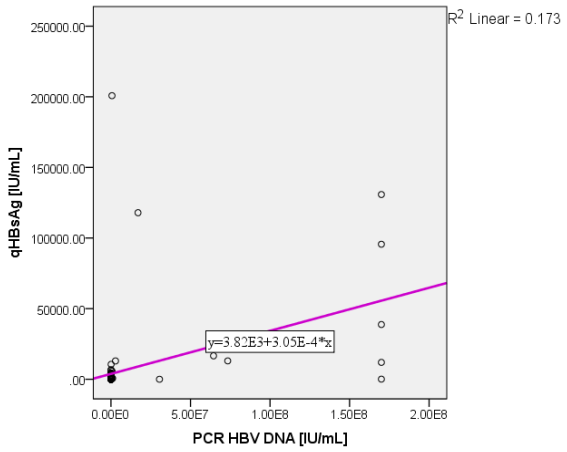


**Fig. 1 – Correlation between quantitative hepatitis B virus (HBV) surface antigen (qHBsAg) and HBV DNA viremia. PCR – polymerase chain reaction.**

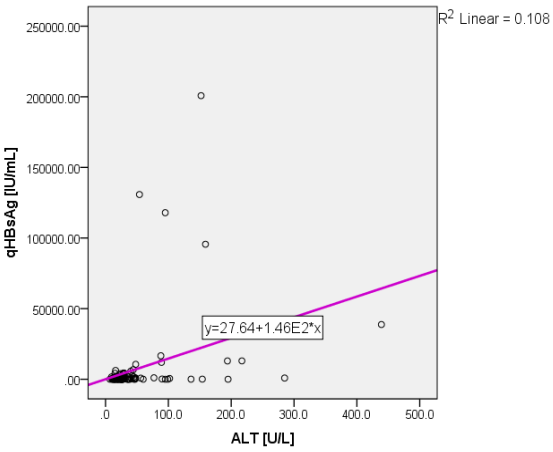


**Fig. 2 – Correlation between quantitative hepatitis B virus surface antigen (qHBsAg) and alanine aminotransferase (ALT).**

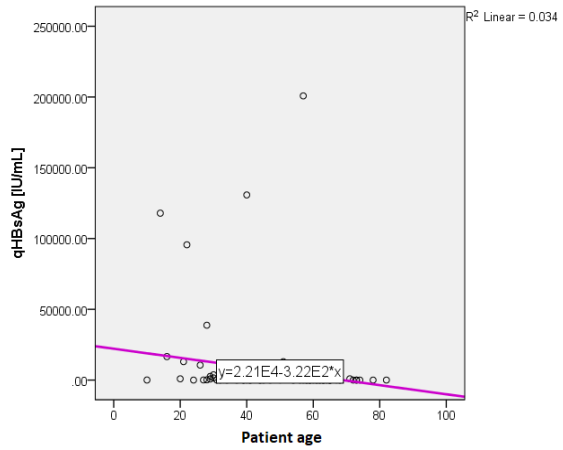


**Fig. 3 – Correlation between quantitative hepatitis B virus surface antigen (qHBsAg) and patient age.**

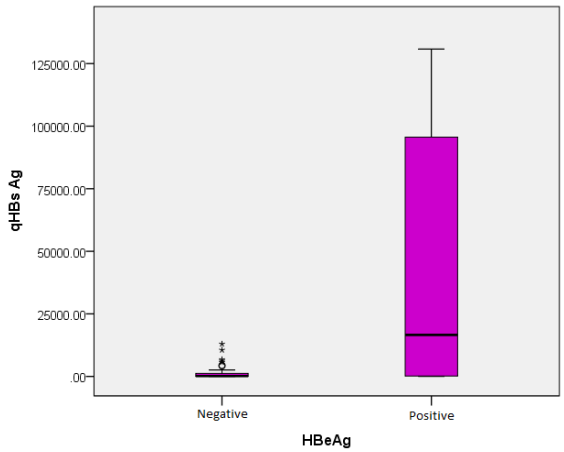


**Fig. 4 – Comparison of quantitative hepatitis B virus (HBV) surface antigen (qHBsAg) levels in HBV e antigen (HBeAg)-positive and HBeAg-negative patients.**

**Table 3**

**Demographic characteristics, biochemical findings, and values of qHBsAg levels in HBV e antigen (HbeAg)-positive and HBeAg-negative untreated patients**

| Parameter | HBeAg-positive, n = 18 | HBeAg-negative, n = 94 | *p*-value |
|---|---|---|---|
| Age (years) | 47 (21–59) | 49 (39–61) | 0.358 |
| ALT (U/L) | 95 (55–156) | 25 (17–34) | < 0.001 |
| AST(U/L) | 62 (38–81) | 24 (21–28) | < 0.001 |
| qHBsAg (IU/mL) | 910.7 (47.9–52,960.0) | 401.5 (5.0–1,426.7) | 0.032 |
| PCR HBV DNA (IU/mL) | 9,000,000.0 (19,980.0–170,000,000.0) | 264.0 (20.0–1,825.0) | < 0.001 |

**For abbreviations, see Table 2.**
**All values are expressed as average (minimum–maximum).**

HBeAg-negative group, while no statistical significance was observed for age.

Figure 4 shows a comparison of qHBsAg levels in HBeAg-positive and HBeAg-negative patients.

## Discussion

The study included 112 untreated patients diagnosed with chronic HBV infection. The mean age of patients was 48.27 ± 15.14 years, and males (58%) were more represented than females (42%). Similar data were obtained in the study by other authors [15–17].

According to the EASL guideline (2017), there are five stages of chronic HBV infection [1]. However, using the available data, the stage of the infection was difficult to recognize. It is well known that qHBsAg levels are influenced by the stage of HBV infection and that qHBsAg levels were significantly higher in HBeAg-positive than in HBeAg-

negative patients [18–20]. HBeAg is a viral protein present in the replicative stage of chronic HBV infection. It occurs at the same time or immediately after HBsAg and is present in serum for seven days after infection. In acute hepatitis, it usually disappears within two weeks to two months; persistence longer than ten weeks indicates chronic infection and may be present for many years, indicating HBV replication and, therefore, the patient's infectivity. Moreover, higher qHBsAg and HBV DNA viremia in HBeAg-positive patients indicate active HBV replication [21, 22]. The patients included in our study were predominantly HBeAg-negative, meaning that they were diagnosed in the later stage of the disease with advanced chronic HBV infection. This data speaks of the need for additional education of physicians in terms of screening the population for HBV infection at an early stage when the antiviral therapy success likelihood would be higher. The first clinical studies in 2004 by Degushi et al. [23] and Chan et al. [24] confirmed a positive correlation between qHBsAg and HBV DNA viremia and that qHBsAg levels were significantly higher in HBeAg-positive than in HBeAg-negative patients. The observed concentrations of qHBsAg and PCR HBV DNA in the studied cohort were statistically significantly higher in the HBeAg-positive group compared to HBeAg-negative patients ($p = 0.032$, $p < 0.001$). Similar results were obtained by Zoulim et al. [25].

Our sample results show a statistically significant positive high-intensity correlation between qHBsAg and HBV DNA viremia. In the Zhu and Zhang [26] study, as in the study by Jaroszewicz et al. [27], a positive correlation was observed between qHBsAg levels and HBV DNA viremia ($r = 0.657$, $p < 0.05$ and $r = 0.79$, $p < 0.01$). Different results were obtained in some other studies. No statistically significant correlation was found between qHBsAg levels and HBV DNA viremia ($r = 0.53$, $p = 0.606$) in the studies by Ganji et al. [28] and Mahdavi et al. [29] ($r = 0.231$, $p = 0.656$). Interestingly, in the global population of chronic HB patients, qHBsAg strongly correlates with HBV DNA viremia; the correlation is only weak in HBeAg-negative patients. Levels of qHBsAg are generally correlated with viremia. However, in low replicative states, as in inactive HBV infection, qHBsAg levels remain higher than serum HBV DNA levels, probably reflecting HBsAg secretion from integrated HBV DNA. Another possible reason is the reduced immune control of the host during HBsAg production as opposed to viral replication. The qHBsAg level provides additional information about the range of affected hepatocytes and the patient's immune control over the disease [30]. Our research supports this, noting a statistically significant positive correlation between qHBsAg and ALT levels as a marker of hepatocyte necrosis. Having in mind that ALT can only be found in the cytosol of hepatocytes, ALT levels are a much better indicator of hepatocellular damage compared to AST levels since AST can be found in the cytosol and mitochondria of skeletal and heart muscle, brain, kidneys, pancreas, lungs, erythrocytes, and leucocytes [19]. Furthermore, ALT and AST concentrations show statistically significantly higher values in the HBeAg-positive group than in the HBeAg-negative group ($p < 0.01$) as a result of a higher level of HBV replication in HBeAg-positive patients. In addition, a statistically significant negative correlation between qHBsAg and the patient's age was also shown. The observed correlation with age is moderate. The obtained results are in line with the study by Togo et al. [31], which indicates that during the natural course of infection, the qHBsAg level decreases with the advancement of the patient's age and chronic HBV infection duration.

## Conclusion

Our study showed that qHBsAg has a high-intensity positive correlation with HBV DNA viremia. The use of qHBsAg is important for determining the phase of chronic HBV infection, assessment of the success and length of treatment, as well as for safe discontinuation of antiviral therapy with a lower risk of relapse. However, further studies are needed to implement qHBsAg in existing therapeutic guidelines.

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# Prevalence of various forms of peripheral neuropathy in patients with systemic connective tissue diseases: a clinical and electrophysiological study

## Učestalost različitih formi neuropatija kod obolelih od sistemskih bolesti vezivnog tkiva: klinička i elektrofiziološka studija

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**Abstract**

**Background/Aim.** Peripheral neuropathy (PN) in systemic connective tissue diseases (SCTDs) represents the apparent disease complications or initial manifestations of clinically undiagnosed conditions. The aim of the study was to identify neuropathies (Ns) and their prevalence, point out the diagnostic significance of some electrophysiological (EP) parameters in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc), and establish their association with disease activity (DA) and disease duration (DDu). **Methods.** A prospective study was conducted at the Rheumatology Clinic of the Institute for Treatment and Rehabilitation "Niška Banja" over a three-year period. The study included 157 patients in total, of whom 61 had RA, 40 had SLE, and 56 had SSc. The following parameters were analyzed: age, gender, DDu, course of the disease, and DA index. Moreover, clinical, rheumatological, and neurological examinations, as well as neurology tests, nerve conduction studies (NCS), and laboratory analyses, were also conducted. **Results.** In the studied population, we were able to identify various forms of Ns (in 28.7% of patients) by NCS. In all three groups, the most prevalent type of Ns was axonal (23.6%), sensorimotor (18.5%), and polyneuropathy (23.6%). There was a significant association between DA and the occurrence of Ns ($p < 0.001$) in the total population. The most important EP parameter was the sensory nerve action potential amplitude of *nervus peroneus superficialis* [in 70 (44.6%) patients] and *nervus suralis* [in 35 (22.3%) patients], and compound muscle action potential amplitude of *nervus peroneus* [in 32 (20.4%) patients]. DDu in all three groups was longer in the population of patients with Ns. **Conclusion.** Ns are most common in patients with longer DDu and higher DA. The EP method is important in detecting Ns, especially in the early detection of subclinical forms of Ns and the prevention of disease complications.

**Key words:**
**arthritis, rheumatoid; autoimmune diseases; connective tissue; diagnosis; lupus erythematosus, systemic; peripheral nervous system diseases; scleroderma, systemic.**

**Apstrakt**

**Uvod/Cilj.** Periferna neuropatija (PN) u sistemskim bolestima vezivnog tkiva (SBVT) predstavlja jasnu komplikaciju bolesti ili inicijalnu manifestaciju klinički nedijagnostikovane bolesti. Cilj rada bio je da se odrede tipovi i procenat zastupljenosti neuropatija (N), ukaže na dijagnostički značaj pojedinih elektrofizioloških (EF) parametara kod bolesnika sa reumatoidnim artritisom (RA), sistemskim eritemskim lupusom (SLE) i sistemskom sklerozom (SSc) i utvrdi njihova povezanost sa aktivnošću bolesti (AB) i dužinom trajanja bolesti (DTB). **Metode.** Istraživanje je obavljeno u formi prospektivne studije na Klinici za reumatologiju Instituta za lečenje i rehabilitaciju „Niška Banja" u trajanju od tri godine. U istraživanje je bilo uključeno ukupno 157 bolesnika, od kojh je 61 imalo RA, 40 je imalo SLE i 56 je bilo sa SSc. Analizirani su sledeći parametri: godine života, pol, DTB, tok bolesti i indeks AB. Obavljeni su i klinički, reumatološki i neurološki pregledi, neurološki testovi, ispitivanje sprovodljivosti nervnih vlakana (SNV) i laboratorijske analize. **Rezultati.** U ispitivanoj populaciji, ispitivanjem SNV registrovani su različiti oblici N (kod 28,7% bolesnika). U sve tri grupe, najčešći tipovi N bile su aksonska (23,6%), senzomotorna (18,5%) i polineuropatija (23,6%). Utvrđena je statistički značajna povezanost AB i pojave N ($p < 0,001$) u ukupnoj populaciji. Najznačajniji EF parametri bili su

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amplitude senzitivnih neurograma *nervus peroneus superficialis*-a [kod 70 (44,6%) bolesnika] i *nervus suralis*-a [kod 35 (22,3%) bolesnika] i motorna amplituda *nervus peroneus*-a [kod 32 (20,4%) bolesnika]. DTB u sve tri grupe bila je veća u grupi bolesnika sa N. **Zaključak.** Kod bolesnika sa većom DTB i većom AB, N su najčešće. Elektrofiziološka metoda važna je u detekciji N, naročito u ranom otkrivanju subkliničkih formi N i prevenciji komplikacija bolesti.

**Ključne reči:**
**artritis, reumatoidni; autoimunske bolesti; vezivno tkivo; dijagnoza; lupus, eritematozni, sistemski; živci, periferni, bolesti; sklerodermija, sistemska.**

## Introduction

Systemic connective tissue diseases (SCTDs) represent a heterogeneous group of autoimmune diseases that can affect all body systems, including the central nervous system (CNS) and peripheral nervous system (PNS) [1–3]. Neurogenic inflammation, autoantibodies-mediated changes, ischemia of the vascular wall, and metabolic mechanisms are believed to contribute to peripheral neuropathy (PN) in connective tissue disease (CTD). Earlier investigations have confirmed the correlation between disease activity (DA) and the degree of neuropathy (N) in small groups of patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), while some other studies demonstrated low DA scores in patients with neurological damage [4–5].

Concerning the number of nerves whose roots are affected, we distinguish between small fiber N and large fiber N. The diagnosis of large fiber N is made based on clinical and nerve conduction study (NCS) criteria, which is not possible in the case of small fiber N diagnosed by quantitative sensory testing (QST) and skin biopsy [6–8]. PN is more common, but most studies are focused on CNS; therefore, PN has been less frequently identified, possibly because it represents the apparent disease complications or initial manifestations of clinically undiagnosed conditions [9–11].

PN can be divided into different categories. Based on the number of damaged nerves, PN is divided into mononeuropathy, mononeuritis multiplex (MNM), and polyneuropathy (PoN). Depending on the damage to the nerve structures, it is divided into axonopathy, myelinopathy, and ganglionopathy or neuronopathy. Based on the function of the damaged nerves, there is autonomic, motor, and sensory N. Based on the anatomical site of the lesion, there is radiculopathy and plexopathy [12–14]. Neuropathies (Ns) are divided into length-dependent and non-length-dependent. Length-dependent Ns occur in a distal "stocking and glove" distribution, and non-length-dependent patterns affect the face, torso, and proximal extremities [7].

Taking into account the fact that studies conducted so far investigated PNS disorder in SLE and RA but not in systemic sclerosis (SSc), as well as their connection with the activity and duration of the disease, the aim of our study was to identify N types and their prevalence using neurological examination and NCS in all three clinical groups of patients and establish their association with DA and disease duration (DDu). Furthermore, we tried to establish the diagnostic importance of certain electrophysiological (EP) parameters related to DA and DDu, which would be relevant in the early detection of Ns as the complications of SCTDs.

## Methods

The investigation was performed as a prospective study from September 2017 to February 2020 at the Rheumatology Clinic of the Institute for Treatment and Rehabilitation "Niška Banja", Serbia. A total of 189 patients were asked to participate, and 157 were enrolled in the study. There were 61 patients with RA (54 females and 7 males), 40 patients with SLE (39 females and 1 male), and 56 patients with SSc (50 females and 6 males). All of these patients were diagnosed in the previously mentioned healthcare institution and were regularly followed up as outpatients by their rheumatologists. Informed consent was obtained from all the study participants. The approval of the Ethics Committee (No. 80921, from July 5, 2017) of the Institute for Treatment and Rehabilitation "Niška Banja" was also obtained. The enrolled patients fulfilled the classification criteria of the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) for SLE, RA, and SSc [15–17]. The patients with some other autoimmune rheumatic disease, non-differentiated or mixed systemic disease, acute or chronic disease of other organs, as well as those with Ns of another etiology (hypothyreosis, diabetic Ns, metabolic causes, infectious causes, uremia, traumatic Ns, iatrogenic Ns, alcohol abuse, paraneoplastic syndrome, hypovitaminosis B1, B12, and E) were excluded from our investigated group of patients. The following parameters were analyzed: age, gender, DDu, course of the disease, and DA; clinical, rheumatological, and neurological examinations were performed, as well as neurology tests and NCS assessment, and laboratory analyses. Neurological impairment was graded using the Neuropathy Impairment Score (NIS) = Neurologic Disability Score (NDS). Using this scoring system, 24 muscle groups were evaluated (cranial and muscles of the upper and lower limb), as well as muscle reflexes in five muscle groups and sensibility (touch-pressure, vibration, joint position, pinprick). Muscle strength scores were graded as follows: 0 = normal strength; 1 = 25% weak; 2 = 50% weak; 3 = 75% weak; 4 = paralysis. Reflexes and sensations are scored as follows: 0 = normal; 1 = decreased; 2 = absent [18–20]. NIS score was graded from 0 to 244, where a higher score denoted greater impairment [19, 21–23]. EP studies complemented the clinical assessment of the patients. N was assessed as the clinical form in patients with N on neurological examination, which was subsequently confirmed by NCS, and subclinical when it was not evidenced by neurological examination but was confirmed by NCS instead.

*Nerve conduction study*

The form and degree of Ns were determined by NCS of peripheral nerves using a 4-channel *Neurowerk* electromyoneurography (EMNG) system at the Institute for Treatment and Rehabilitation "Niška Banja". The compound muscle action potential (CMAP) was measured bilaterally in the median, ulnar, tibial, peroneal, medial plantar, and lateral plantar nerves, applying supramaximal percutaneous nerve stimulation. Sensory nerve action potential (SNAP) was measured in the median, ulnar, sural, and superficial peroneal nerves. Sensory nerve conduction was assessed antidromically. The median nerve CMAP was stimulated at the wrist, elbow, and axilla and registered above the *musculus* (*m.*) *abductor policis brevis*. The ulnar nerve CMAP was stimulated at the wrist, below and above the elbow and axilla, and registered above the *m. abductor digiti minimi.* The peroneal nerve CMAP was stimulated at the ankle, below and above the fibular head, and registered above the *m. extensor digitorum brevis*. The tibial nerve CMAP was stimulated posteriorly to the medial malleolus and proximally at the popliteal fossa and registered above the *m. flexor hallucis brevis*. Distal latency, amplitude, duration, velocity, F-wave latency, conduction blocks, and temporal dispersion were all measured. Skin temperature was measured at the dorsum of the foot using a digital surface thermometer, and the temperature ranged from 30 °C to 32 °C. A surface stimulating and recording electrode was used. Based on the clinical findings and NCS, all Ns were divided into the following groups: sensory, sensorimotor, axonal, axonal-demyelinating, demyelinating, MNM, distal symmetrical PoNs which did not fulfill the criteria for chronic inflammatory demyelinating PoN (CIDP) and MNM, and compressive Ns (syndromes of the carpal and tarsal tunnel). Electrodiagnostic examinations were performed using the standardized methodology according to the American Association of Neuromuscular and Electrodiagnostic Medicine (AANEM) [24, 25]. The results were compared with the reference values used in our institution.

*Laboratory analyses*

The laboratory and serological markers included parameters that are part of the index of the DA. Within the Disease Activity Score (DAS) 28 scale (DAS-28), erythrocyte sedimentation rate (ESR) was performed; within the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scale, complement, anti-dsDNA antibodies, the presence of protein in the urine, urine sediment, and platelet and leukocyte levels were determined; within the European Scleroderma Trials and Research (EUSTAR) activity index, sedimentation, C-reactive protein, antinuclear antibodies (ANA), and Anti-Scl 70 antibodies were analyzed [26–28].

*Disease activity index*

In patients with RA, an appropriate DA index (DAS-28 ESR score) was used to monitor the DA. Based on this score, DA in RA patients was classified as follows: DAS-28 < 2.6, remission; DAS-28 2.6–3.1, low activity; DAS-28 3.2–5.1, moderate activity; DAS-28 ≥ 5.2, high activity [26]. In all SLE patients, the SLEDAI was used, i.e., the Modified SLEDAI – 2000. The score was calculated as follows: 0–5, low activity; 6–12, moderate activity; and 13–20, high activity [27]. In SSc patients, a revised EUSTAR activity index was used to assess DA. According to this index, SSc patients were classified into two groups: < 2.5 inactive/moderately active disease; ≥ 2.5 active/very active disease [28].

*Statistical data processing*

The data were presented as arithmetic mean plus standard deviation (SD), i.e., in the form of absolute and relative numbers. The comparison of continuous values between the two groups was made using the *t*-test or Mann-Whitney *U* test. The comparison of categorical variables was performed using the Chi-squared or Fisher's test of exact probability. The hypothesis was tested with the statistical significance cut-off value set at $p < 0.05$. Statistical data processing was performed using the SPSS 20.0 software package.

**Results**

Sixty-one patients with RA, 40 patients with SLE, and 56 patients with SSc were included in the study. The mean age of the population was 55.2 years (± 10.8 SD) [minimum (min) – maximum (max): 33–82 years]. The average DDu was 13.7 ± 9.1 years (min 4 months; max 43 years). Females comprised 88.5% of RA patients, 97.5% of SLE patients, and 89.3% of SSc patients. There were no differences in DDu ($p = 0.249$), gender ($p = 0.180$), or NIS total score ($p = 0.587$) between the studied groups (Table 1). There was no correlation between biochemical and serological markers performed within the scales of DA with N (data are not shown).

In the study population, acute N was registered in 1 (2.2%) patient, subacute in 3 (6.7%) patients, and chronic in 41 (91.1%) patients. In our study, the group of patients with RA, SLE, and SSc was mainly affected by length-dependent PoN and was registered in 38 (84.4%) patients. Non-length-dependent N was recorded in a smaller percentage and was registered in 7 (15.6%) patients. There were no patients in our group with clinical signs of cranial nerve damage.

NCS findings did not differ significantly between the studied groups. Pathological findings were observed in 23.3% of RA patients, 35.0% of SLE patients, and 30.4% of SSc patients. In the studied population, we were able to identify various forms of Ns in 28.7% of patients with NCS. The prevalence of N forms was non-significantly different among groups ($p > 0.05$). In the studied population, 14.6% of examinees had a clinical form of N, while 14.0% had a subclinical form of N. The prevalence of clinical and subclinical forms did not differ significantly between the investigated groups ($p = 0.538$, $p = 0.734$) (Table 2).

Increased age was registered in all three groups (RA, SLE, and SSc), in which N was present in relation to patients without N.

**Table 1**

**Demographic and clinical characteristics of the studied groups of patients**

| Characteristics | Groups | | | *p*-value |
|---|---|---|---|---|
| | RA | SLE | SSc | |
| Age, years | 60.7 ± 10.6[a] | 49.9 ± 9.0 | 58.9 ± 10.4[a] | < 0.001[1] |
| Age at diagnosis, years | 46.8 ± 11.9[a] | 34.7 ± 10.5 | 47.7 ± 12.7[a] | < 0.001[1] |
| Disease duration, years | 13.9 ± 9.4 | 15.2 ± 9.1 | 14.6 ± 22.7 | 0.249 [1] |
| Gender | | | | |
| male | 7 (11.7) | 1 (2.5) | 6 (10.7) | 0.180[2] |
| female | 54 (88.5) | 39 (97.5) | 50 (89.3) | |
| NIS total score | 2.4 ± 6.5 | 4.2 ± 9.5 | 2.4 ± 6.9 | 0.587[3] |

**NIS – Neuropathy Impairment Score; SLE – systemic lupus erythematosus; RA – rheumatoid arthritis; SSc – systemic sclerosis. All results are shown as mean ± standard deviation, except gender which is shown as number (percentage).**

**[1] *t*-test; [2] Chi-squared test; [3] Mann-Whitney *U* test; [a] vs. SLE $p < 0.05$.**

**Table 2**

**Type of neuropathy based on nerve conduction study and clinical findings related to the studied groups**

| Type of neuropathy | Groups | | | | $p^1$-value |
|---|---|---|---|---|---|
| | Total | RA | SLE | SSc | |
| Type 1 | | | | | |
| normal finding | 112 (71.3) | 47 (77.0) | 26 (65.0) | 39 (69.6) | |
| sensory | 16 (10.29) | 6 (9.8) | 4 (10.0) | 6 (10.7) | 0.651 |
| sensorimotor | 29 (18.5) | 8 (13.3) | 10 (25.0) | 11 (19.6) | |
| Type 2 | | | | | |
| normal finding | 112 (71.3) | 47 (77.0) | 26 (65.0) | 39 (69.6) | |
| axonal | 37 (23.6) | 12 (19.7) | 10 (25.0) | 15 (26.8) | 0.122 |
| axonal-demyelinating | 5 (3.2) | 2 (3.3) | 1 (2.5) | 2 (3.6) | |
| demyelinating | 3 (1.9) | 0 (0.0) | 3 (7.5) | 0 (0.0) | |
| Type 3 | | | | | |
| normal finding | 112 (71.3) | 47 (77.0) | 26 (65.0) | 39 (69.6) | 0.398 |
| distal symmetrical polyneuropathy | 37 (23.6) | 11 (18.0) | 10 (25.0) | 16 (28.6) | 0.394 |
| mononeuritis multiplex | 5 (3.2) | 2 (3.3) | 3 (7.5) | 0 (0.0) | 0.093 |
| carpal tunnel syndrome | 16 (10.2) | 6 (9.8) | 4 (10.0) | 6 (10.7) | 0.987 |
| tarsal tunnel syndrome | 1 (0.6) | 0 (0.0) | 1 (2.5) | 0 (0.0) | 0.255 |
| CIDP | 1 (0.6) | 0 (0.0) | 1 (2.5) | 0 (0.0) | 0.255 |
| Neuropathy in the total population | 45 (28.7) | 14 (23.3) | 14 (35. 0) | 17 (30.4) | 0.428 |
| clinical form of neuropathy | 23 (14.6) | 8 (13.1) | 8 (20.0) | 7 (12.5) | 0.538 |
| subclinical form of neuropathy | 22 (14.0) | 6 (9.8) | 6 (15.0) | 10 (17.9) | 0.734 |

**CIDP – chronic inflammatory demyelinating polyneuropathy. For abbreviations of other terms, see Table 1. The results are expressed as numbers (percentages). [1] Chi-squared test.**
**Type 1 neuropathy – division of neuropathies based on the involvement of sensory or motor fibers; Type 2 neuropathy – pathophysiological division of neuropathies based on axonal or myelin damage; Type 3 neuropathy – patterns of peripheral neuropathy.**

DDu in all three groups was longer in the population of patients with N, but the difference was insignificant. There was a statistically significant association between DA and the occurrence of N in all three groups of our patients ($p < 0.001$). The highest DA and association with N were encountered in the group of RA patients (64.3%) and the lowest in the group of SLE patients (35.7%) (Table 3). DDu was significantly higher with a reduced SNAP amplitude of nervus (*n.) peroneus superficialis* ($p = 0.029$), CMAP amplitude of *n. peroneus* ($p = 0.029$) and SNAP amplitude of *n. suralis* ($p = 0.011$) (Table 4). A reduced SNAP amplitude of *n. peroneus superficialis* was not significantly associated with DA ($p = 0.307$). In contrast to that, a reduced CMAP amplitude of *n. peroneus* and SNAP amplitude of *n. suralis* was significantly associated with DA ($p = 0.001$). DA was high in 51.7% of patients with a reduced CMAP amplitude of *n. peroneus* and in 41.4% of patients with a reduced SNAP amplitude of *n. suralis* (Table 5). A high DA associated with N was found in 18% of patients and in only 11% of those without N (Figure 1).

The most important diagnostic EP parameters were amplitude and latency (but predominantly amplitude) of SNAP of *n. peroneus superficialis* (70 patients – 44.6%), SNAP of *n. suralis* (35 patients – 22.3%), and CMAP of *n. peroneus* (32 patients – 20.4%).

**Table 3**

**Association of neuropathy and SCTDs characteristics**

| Disease characteristics | With neuropathy | Without neuropathy | *p*-value |
|---|---|---|---|
| RA | | | |
| Age, years (mean ± SD) | 67.4 ± 7.3 | 58.6 ± 10.7 | 0.006[1] |
| Disease duration, years (mean ± SD) | 15.8 ± 11.8 | 13.3 ± 8.7 | 0.889[3] |
| DAS-28, n (%) | | | |
| remission | 0 (0.0) | 2 (4.3) | |
| low activity | 0 (0.0) | 10 (21.3) | < 0.001[2] |
| moderate activity | 5 (35.7) | 34 (72.3) | |
| high activity | 9 (64.3) | 1 (2.1) | |
| SLE | | | |
| Age, years (mean ± SD) | 55.3 ± 6.5 | 47.0 ± 8.9 | 0.004[1] |
| Disease duration, years (mean ± SD) | 16.6 ± 12.5 | 14.5 ± 6.8 | 0.726[3] |
| SLEDAI, n (%) | | | |
| low activity | 0 (0.0) | 9 (34.6) | |
| moderate activity | 9 (64.3) | 14 (53.8) | 0.005[2] |
| high activity | 5 (35.7) | 3 (11.5) | |
| SSc | | | |
| Age, years (mean ± SD) | 64.5 ± 5.4 | 56.5 ± 11.2 | 0.001[1] |
| Disease duration, years (mean ± SD) | 15.1 ± 11.2 | 10.7 ± 9.1 | 0.141[3] |
| Revised EUSTAR activity index, n (%) | | | |
| low activity | 0 (0.0) | 20 (51.3) | |
| moderate activity | 14 (82.4) | 13 (33.3) | 0.001[2] |
| active/very active | 3 (17.6) | 6 (15.4) | |
| Total population disease activity, n (%) | | | |
| remission | 0 (0.0) | 2 (1.8) | |
| low activity | 0 (0.0) | 39 (34.8) | < 0.001[2] |
| moderate activity | 27 (60.0) | 60 (53.6) | |
| high activity | 18 (40.0) | 11 (9.8) | |

**SCTDs – Systemic connective tissue diseases; DAS-28 – Disease Activity Score 28; SLEDAI – Systemic Lupus Erythematosus Disease Activity Index; EUSTAR – European Scleroderma Trials and Research. For abbreviations of other terms, see Table 1.**
**[1] *t*-test; [2] Chi-squared test; [3] Mann-Whitney *U* test.**

**Table 4**

**Disease duration related to the reduced CMAP and SNAP amplitude of *nervus peroneus, nervus suralis,* and *nervus peroneus superficialis***

| Nerve | Disease duration (years) |
|---|---|
| *Nervus peroneus superficialis* | |
| reduced amplitude (SNAP) | 15.0 ± 10.3 |
| normal amplitude (SNAP) | 11.4 ± 8.1 |
| *p*[1] | 0.029 |
| *Nervus peroneus* | |
| reduced amplitude (CMAP) | 16.4 ± 10.8 |
| normal amplitude (CMAP) | 12.5 ± 9.0 |
| *p*[1] | 0.029 |
| *Nervus suralis* | |
| reduced amplitude (SNAP) | 18.1 ± 11.5 |
| normal amplitude(SNAP) | 12.5 ± 8.8 |
| *p*[1] | 0.011 |

**CMAP – compound muscle action potential; SNAP – sensory nerve action potential. Results are expressed as mean ± standard deviation.**

**[1] Mann-Whitney *U* test.**

**Table 5**

**Disease activity related to reduced CMAP and SNAP amplitude of *nervus peroneus*, *nervus suralis*, and *nervus peroneus superficialis***

| Nerve | Disease activity, n (%) | | | *p*-value[1] |
|---|---|---|---|---|
| | Low | Moderate | High | |
| *Nervus peroneus superficialis* | | | | |
| reduced amplitude (SNAP) | 29 (70.7) | 52 (59.8) | 21 (72.4) | 0.307 |
| normal amplitude (SNAP) | 12 (29.3) | 35 (40.2) | 8 (27.6) | |
| *Nervus peroneus* | | | | |
| reduced amplitude (CMAP) | 5 (12.2) | 28 (32.2) | 15 (51.7) | 0.001 |
| normal amplitude (CMAP) | 36 (87.8) | 59 (67.8) | 14 (48.3) | |
| *Nervus suralis* | | | | |
| reduced amplitude (SNAP) | 2 (4.9) | 20 (23.0) | 12 (41.4) | 0.001 |
| normal amplitude (SNAP) | 39 (95.1) | 67 (77.0) | 17 (58.6) | |

**For abbreviations, see Table 4. [1] Chi-squared test.**

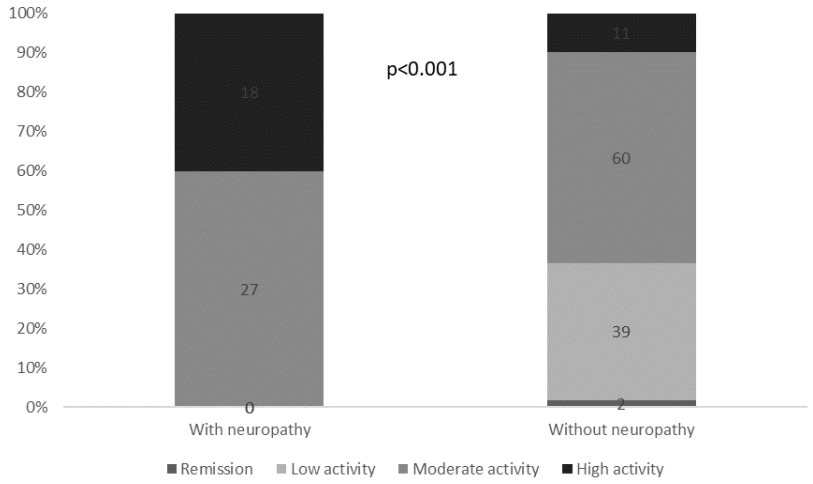


**Fig. 1 – Disease activity related to neuropathy.**

## Discussion

PNS disorders in SCTDs are various forms of N with insufficiently elucidated pathogenesis. It has been proposed that not only antibody titer and DA are involved but also the infarctions of *vasa nervorum* of epineural arteries, which result in axonal degeneration [29–31]. The available literature data point to a large number of studies aimed to describe CNS disorders in SCTDs [1, 32]. In contrast, the studies aimed to examine the diagnostic significance of NCS parameters in these diseases are relatively scarce, and these have dealt mainly with SLE and RA. However, the prevalence of Ns, NCS findings, and DA scores were not examined simultaneously among all three disease groups (SLE, RA, and SSc). In a study of 4,924 SLE patients, the prevalence of PoN was 73 out of 4,924 (1.5%) patients and higher in patients with an active form of the disease. In a group of 1,827 SLE patients, different types of PNS damage were investigated, and the connection of peripheral Ns with the DA was determined. In a retrospective study of 1,224 patients with SLE, the prevalence of PNS damage was 6.9% and was correlated with a high SLEDAI score. PN is an underdiagnosed complication in CTD and a particular challenge for rheumatologists and neurologists [4, 10, 14, 33]. The same applies to the diagnostic relevance of particular EP parameters, which was the aim of our study.

Our results showed that in the total studied population, the prevalence of N differed among the groups – 30.4% in SSc, 23.3% in RA, and 35.0% in SLE, respectively. The most common type of PN in the total population was axonal distal symmetrical PoN in 23.6% of patients, followed by sensorimotor N in 18.5% of patients while demyelinating N was much less commonly identified in 1.9% of patients. MNM N type was detected in 3.2%, carpal tunnel syndrome in 10.2%, sensory N in 10.2%, and tarsal tunnel syndrome in 0.6% of patients, while there was 1 (0.6%) patient with CIDP in the SLE group. Concerning systemic diseases, axonal N was present in 19.7% of RA patients, 25.0% of patients in the SLE group, and 26.8% of patients in the SSc group. The distal symmetrical PoN, which was more of an axonal type and did not fulfill the criteria for MNM [34], was found in 23.6% of the total studied population. In the paper by Olney [31], distal axonal Ns were most prevalent in SLE and SSc. Our results are similar to those. Aneja et al. [35] investigated a group of 66 patients with RA, with and without clinical manifestations of N, in whom NCS confirmed N in 37.8% of patients and demonstrated a high prevalence of subclinical N. Similar results were obtained by Canesi et

al. [36], Biswas et al. [37], and Lanzillo et al. [38]. In our study, there were 14.6% clinical and 14% subclinical disease forms. Subclinical disease was more prevalent in SSc and SLE patients. Studies have confirmed that patients may have EP signs of N in the absence of any clinical signs of peripheral nerve involvement, which underlines the importance of this method in the early detection of subclinical disease.

In the study by Toledano et al. [39] conducted on 524 patients with SLE, it was shown that PN was found in 93 (17.7%) patients. This percentage is lower compared to our results. Nevertheless, our cohort of patients had a longer DDu compared to the above group. Sensorimotor axonal PoN was the most common form, which was shown in our study as well. Similar results were reported by Florica et al. [9] in their retrospective study of 1,533 patients with SLE, out of which 14% had PN. The patients with N also had a high DA score (SLEDAI). PN most commonly affected the lower extremities, predominantly *n. peroneus* and *n. suralis*. Our study showed that the most severe changes affected *n. suralis* and *n. peroneus*, but also *n. peroneus superficialis*. Saigal et al. [40] reported 50 patients with SLE, out of whom N was electrophysiologically found in 36%. These authors reported that only SLEDAI was increased in patients with N. The underlying mechanism by which high DA influences the development of N has not been sufficiently studied so far. According to our results, there was a statistically significant association between DA and the prevalence of N, as well as between DA and EP parameters (amplitude of motor *n. peroneus* and *n. suralis*). Similar results were published by Mohamed et al. [41].

Various forms of Ns are also encountered in RA as a result of PNS damage. In the study by El-Hewala et al. [42], a group of 50 patients with RA was studied. Regardless of the DA, EP findings demonstrated N in 78% of patients, out of which 48% had compressive (entrapment) Ns, while the remaining 30% had symmetrical PoN with axonal degeneration. Our results showed that entrapment Ns were much less prevalent compared to other forms of Ns (in 6 patients – 9.8%). Several interesting studies reported a higher percentage of subclinical N forms confirmed by NCS and demonstrated a correlation of DDu and DA with N in RA [43], as was established in our study as well. Several studies could not demonstrate any association of N with DDu [44, 45].

SSc is a relatively rare systemic disease compared to SLE and RA. The correlation between N, EP parameters, and DA in SSc has been investigated in the smallest subset of studies, and this was one of the objectives of our study. In the study by Paik et al. [46], in a group of 60 patients with SSc, the PN was registered in 17 (28%) patients based on the Total Neuropathy Score (TNS) and EP changes in five patients with neuropathic symptoms and five patients without neuropathic symptoms. Our study showed that in SSc patients as well, distal symmetrical axonal N was the most common form. Our NCS demonstrated N in 30.4% of patients. There were 39 patients with a limited disease form in our study, while diffuse disease was found in 17 patients. Raja et al. [47] determined the prevalence of large fiber PN in the group of 60 patients diagnosed with SSc, and their results showed that 22 (36.7%) patients had PN, which is in correlation with the results of our study.

Since axonal N was seen at a higher percentage in the studied population, for which low amplitudes were specific electrophysiologically, our aim was to investigate the measure of the impact of DA and DDu on the reduction of amplitude given that it was the most important parameter in the initial N stages in autoimmune diseases. Some papers in the available literature show that amplitude changes are usually seen with *n. suralis* and motor *n. peroneus*, which agrees with our results. Our study demonstrated that DDu was significantly prolonged in the presence of reduced amplitude of motor *n. peroneus*, *n. peroneus superficialis*, and *n. suralis*. Decreased CMAP amplitude of *n. peroneus* and reduced SNAP amplitude of *n. suralis* correlated with DA and were the most significant neurophysiological parameter. Some studies show that peripheral Ns and neuropathic pain are more common in the elderly healthy population. For instance, Hanewinckel et al. [48] examined the prevalence of N in the elderly and middle-aged in a group of 1,310 participants and registered it in 5.5% of subjects. Most PoN were idiopathic and more common in men. They concluded that age was a risk factor for N. Similar results are published by Mello et al. [49], who applied NCS in older healthy individuals and concluded that abnormal tests were present in the elderly population. Our research also included elderly patients with a longer duration of systemic disease in whom we proved N by NCS testing, using normative values of EP parameters of our laboratory for elderly patients.

In our group of patients with RA, SLE, and SSc, large fiber N was registered in 45 (28.7%) patients, while in 25 (15.9%) patients, symptoms of tingling and burning were registered predominantly in the distal segments of the extremities, which was not confirmed by neurological examination and NCS. Therefore, there is a possibility that these patients have small fiber Ns that cannot be confirmed by neurological tests and NCS but can be confirmed by QST and skin biopsy, which would be important to conduct in future research.

## Conclusion

Ns are more commonly encountered in SLE, RA, and SSc patients with prolonged DDu and higher DA. The most common N type in these diseases is axonal sensorimotor PoN. As evidenced by NCS, subclinical disease forms are also common, which suggests that the method is important in the early detection of peripheral Ns. The most significant EP parameter is the CMAP amplitude of *n. peroneus*, SNAP amplitude of *n. suralis* and *n. peroneus superficialis*, which was significantly reduced in prolonged and highly active disease. The results of a small number of studies from the literature available to us show that there are patients with PoN without clinical signs (subclinical form) in the mentioned systemic diseases, so our future research will be focused on the application of NCS in the early phase of the underlying disease in correlation with SLEDAI with the aim of timely application of the adequate therapy for PoN.

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# Myeloid-derived suppressor-like cells – does their frequency change in patients with different stages of CRC?

## Ćelije nalik supresorskim ćelijama mijeloidnog porekla – da li se njihov broj menja kod bolesnika u različitim stadijumima kolorektalnog karcinoma?

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**Abstract**

**Background/Aim**. Colorectal cancer (CRC) is one of the most common cancers in the population, often leading to lethal outcomes. Myeloid-derived suppressor cells (MDSCs) belong to a heterogeneous group of immature cells thought to have an immunosuppressive effect that may aid in tumor development and spreading. The aim of this study was to analyze the frequency and significance of MDSC-like cells at different stages in patients with CRC. **Methods.** Peripheral blood (PB) samples of 83 patients at different stages of the disease and 12 healthy subjects (control group) were analyzed. MDSC-like cells were identified and enumerated in the PB samples of the participants based on the immunophenotypic characteristics of the cells. **Results.** A statistically significant increase in the absolute and relative number of polymorphonuclear (PMN) MDSC (PMN-MDSC)-like cells was observed in the PB of all the patients with CRC, compared to the healthy control group ($p < 0.0001$). No significant increase was observed in monocytic MDSC (M-MDSC)-like cells when they were analyzed without CRC stage stratification ($p > 0.05$). When the relative and absolute numbers of PMN-MDSC-like cells were analyzed in relation to the stages of CRC disease (TNM classification), a statistically significant difference was observed between the control group and patients in stages III and IV of the disease ($p = 0.0005$ vs. $p = 0.0003$ and $p < 0.0001$ vs. $p < 0.0001$, respectively). There was, as well, a significant difference when the numbers of PMN-MDSC-like cells in patients in stages I and II were compared to numbers in patients in stage IV of the CRC ($p = 0.0161$ vs. $p < 0.0001$ and $p = 0.0065$ vs. $p < 0.0001$, respectively). A statistically significant difference in the relative and absolute number of M-MDSC-like cells was observed only between patients in stages II and IV of the disease ($p = 0.0014$ and $p = 0.0002$, respectively). The highest number of MDSC-like cells was observed in stage IV of the disease according to the TNM classification. A positive correlation between the presence of these cells and the number of organs affected by metastatic changes was observed ($p < 0.0001$ for the relative and absolute number of PMN-MDSC-like cells and $p = 0.003$ and $p = 0.0004$ for the relative and absolute number of M-MDSC-like cells). **Conclusion.** CRC patients had a statistically significant increase in PMN-MDSC-like cells compared to healthy controls. The increase in absolute and relative numbers of these cells mostly follows the growth and progression of CRC, while a statistically significant difference in the number of M-MDSC-like cells is observed only between stages II and IV of the disease. The absolute and relative numbers of both subtypes of MDSC-like cells significantly correlate with the number of organs affected by CRC metastases.

**Key words:**
**colorectal neoplasms; myeloid-derived suppressor cells; neoplasm metastasis; neoplasm staging.**

**Apstrakt**

**Uvod/Cilj.** Kolorektalni karcinom (KRK) je jedan od najčešćih karcinoma u populaciji, koji često dovodi i do smrtnog ishoda. Supresorske ćelije mijeloidnog porekla (SĆMP) pripadaju heterogenoj grupi nezrelih ćelija, za koje se smatra da imaju imunosupresivni efekat, koji može da pomogne razvoju i širenju tumora. Cilj rada bio je da se analizira učestalost i značaj ćelija nalik SĆMP kod bolesnika u različitim stadijumima KRK. **Metode.** Analizirani su uzorci periferne krvi (PK) 83 bolesnika u različitim stadijumima bolesti i 12 zdravih ispitanika koji su činili kontrolnu grupu. U uzorcima PK su, na osnovu imunofenotipskih obeležja, identifikovane ćelije nalik SĆMP i određen je njihov broj. **Rezultati.** Utvrđen je statistički značajan porast apsolutnog i relativnog broja ćelija nalik

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polimorfonuklearnim (PMN) SĆMP (PMN-SĆMP) u PK svih bolesnika sa KRK, u odnosu na kontrolnu grupu ($p < 0,0001$). Kada nije vršeno poređenje prema stadijumima KRK, nije uočen statistički značajan porast broja ćelija nalik monocitnim SĆMP (M-SĆMP) ($p > 0,05$). Kada su analizirane relativne i apsolutne brojnosti ćelija nalik PMN-SĆMP u odnosu na stadijume bolesti KRK (TNM klasifikacija), utvrđena je statistički značajna razlika između kontrolne grupe i bolesnika u III i IV stadijumu bolesti ($p = 0,0005$ vs. $p = 0,0003$ i $p < 0,0001$ vs. $P < 0,0001$, redom). Takođe, nađena je statistički značajna razlika brojnosti ćelija nalik PMN-SĆMP poređenjem bolesnika u I i II stadijumu bolesti, u odnosu na brojnost tih ćelija kod bolesnika u IV stadijumu KRK ($p = 0,0161$ vs. $P < 0,0001$ i $p = 0,0065$ vs. $p < 0,0001$, redom). Statistički značajna razlika u relativnom i apsolutnom broju ćelija nalik M-SĆMP uočena je samo između bolesnika u II i IV stadijumu bolesti ($p = 0,0014$ i $p = 0,0002$, redom). Najveći broj ćelija nalik SĆMP uočen je u IV stadijumu bolesti, prema TNM klasifikaciji. Uočena je pozitivna korelacija između prisustva tih ćelija i broja organa koji su zahvaćeni metastatskim promenama ($p < 0,0001$ za relativni i apsolutni broj ćelija nalik PMN-SĆMP i $p = 0,003$, $p = 0,0004$ za relativni i apsolutni broj ćelija nalik M-SĆMP). **Zaključak.** Oboleli od KRK imali su statistički značajan porast broja ćelija nalik PMN-SĆMP u odnosu na zdrave ispitanike. Porast apsolutnih i relativnih vrednosti broja ovih ćelija većim delom prati rast i napredovanje KRK, dok je statistički značajna razlika broja ćelija nalik M-SĆMP uočena samo između II i IV stadijuma bolesti. Apsolutni i relativni broj oba podtipa ćelija sličnih SĆMP značajno koreliše sa brojem organa zahvaćenih metastazama u KRK.

**Ključne reči:**
**kolorektalne neoplazme; kostna srž, ćelije, supresorske; neoplazme, metastaze; neoplazme, određivanje stadijuma.**

## Introduction

Colorectal cancer (CRC) is one of the most common cancers in the population after breast and lung cancer. CRC ranks second in mortality from malignant diseases [1]. It usually occurs sporadically and less frequently as a consequence of inflammation and hereditary diseases. Prevention of CRC, early diagnosis, as well as modern therapy, can significantly reduce the occurrence and improve the successful treatment of this tumor. The therapy for the advanced stages of the disease is still insufficient. Recently, the attention of scientists has been focused not only on malignant CRC cells but also on cells with a pronounced immunosuppressive effect, which can facilitate the progression of tumors with their presence. Important cells with such an effect are myeloid derived suppressor cells (MDSCs) that derive from the bone marrow and are also present in healthy individuals in a small percentage. The increase in the number of MDSCs is not a feature of exclusively malignant diseases, and they can be elevated in many other pathological conditions such as inflammatory diseases, trauma, graft vs. host disease, as well as in some non-pathological conditions (pregnancy, obesity, aging) [2–4]. Increased production of these cells occurs under the influence of a strong impulse, which leads to increased myelopoiesis [5]. In addition to bone marrow myelopoiesis, the increase in the number of MDSCs is aided by extramedullary hematopoiesis, as well as the plasticity of myeloid cells [6]. Similar to many other examples in practice, the identification and testing of these cells was first done on mice in the 1970s, and after several years these cells were also found in humans. MDSCs were officially named in 2007, and their phenotyping was proposed in 2016. Today, three types of these cells are known. Polymorphonuclear (PMN) MDSCs – PMN-MDSCs – are the most common MDSCs (comprising three-quarters of total MDSCs), morphologically similar to neutrophils and defined as $CD14^-CD15^+CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ or $CD11b^+CD14^-CD66b^+$ [7]. The recently discovered lectin-like oxidized low-density lipoprotein (LDL) receptor 1 (LOX-1) as a marker of PMN-MDSCs in humans has facilitated the differentiation of these cells from neutrophils without the use of a gradient separation [8]. Monocytic MDSCs (M-MDSCs) are morphologically similar to monocytes and are defined as $CD14^+CD15^-CD11b^+CD33^+HLA\text{-}DR^-/Lin^-$ or $CD14^+CD15^+CD11b^+CD33^+HLA\text{-}DR^-/Lin^-$. There is another smaller group of early-stage MDSCs (es-MDSCs) that lacks markers for both monocyte and granulocyte populations and whose phenotype is $Lin^-$ (CD3, CD14, CD15, CD19, CD56)/$HLA\text{-}DR^-/CD33^+$ and contain immature progenitor and precursor cells [9, 10]. In addition to phenotypic determination, the molecular and functional definition has been used to confirm MDSCs. An important feature of these cells, unlike mature neutrophils and monocytes, is immunosuppression. It primarily affects T-cells, natural killer (NK) cells, and regulatory T-cells; the mechanisms by which suppression occurs include arginine, cysteine metabolism, oxidative stress, activation and regulation of other regulatory or suppressive cells, and macrophage activity. Furthermore, a difference in the mechanism of action of PMN-MDSCs and M-MDSCs was observed [11, 12].

In our study, we tried to determine whether there is a difference in the incidence of MDSC-like cells in healthy and CRC patients, as well as whether there are statistically significant changes in the incidence of MDSC-like cells at different stages of the disease, including a subdivision of patients in stage IV according to the number of metastatic affected organs.

## Methods

### *Patients and healthy controls*

The study included 83 patients diagnosed with CRC in different stages of disease according to the last, 8th Tumor, Nodus, Metastasis/American Joint Committee of Cancer (TNM/AJCC) classification, and 12 healthy controls. The study protocol was approved by the Ethics Committee of the

Military Medical Academy (MMA) in Belgrade, Serbia (from March 10, 2016) and every patient provided a signed consent form. None of the participants underwent chemotherapy or irradiation therapy, or some other immunosuppressive therapy prior to sampling. A blood sample was taken from the patients at the Clinic of Gastroenterology and Hepatology, MMA. They were then monitored from June 2016 until January 2018. Sample processing was performed at the Institute for Medical Research, MMA.

*Samples*

In the study, 3 mL of venous blood was sampled from the patients with CRC and participants from the control group. Immediately after sampling, erythrocytes were removed by lysis (EDTA, $NH_4Cl$, $KHCO_3$) for 20 min with constant stirring. Double washing of nucleated cells in culture medium (RPMI 1640) with 5% normal human serum was then performed with subsequent centrifugation and resuspension. To separate peripheral blood mononuclear cells (PBMC) for comparative analysis, we applied LSM 1077 lymphocyte separation medium. Separation was performed by centrifugation at 1,200 × g for 20 min. The interlayer was separated and washed twice in a culture medium. The number of cells was determined manually in the Neubauer chamber and automatically on the Beckman Coulter AcT blood cell counter. The cells were then resuspended at a concentration of $1 \times 10^6$ cells per 100 µL suspension for further staining.

*Immunophenotyping of cells*

We used the following monoclonal human antibodies to perform cell immunophenotyping: CD15-FITC and PECy7; CD33-PE and PECy7; CD45-ECD, HLA-DR PECy5, CD14-PEC-y7, CD16-FITC and PECy7; CD11b-PE, CD10-PECy7, CD3-FITC, CD19-FITC and CD56-FITC (Beckman Coulter, USA). Stained cells were then analyzed on a Beckman Coulter FC 500 flow cytofluorimeter using CXP analytical software. Upon completing the procedure, we determined the relative and absolute number of PMN-MDSC-like cells and M-MDSC-like cells in all study participants. MDSC-like cells were phenotypically defined as $Lin^-(CD3^-/CD19^-/CD56^-)$ /$HLA\text{-}DR^{-/low}CD11b^+$ cells. PMN-MDSC and M-MDSC-like subtypes were determined based on the expression of CD14 and CD15. PMN-MDSC-like cells were defined as $CD14^-$ $CD15^+$ and M-MDSC-like cells were defined as $CD14^+CD15^-$. Further differentiation of PMN-MDSC-like cells was done using CD10 and CD16 markers. The gating strategy for the detection of MDSCs in study participants was based on the previous work by Stanojević et al. [13].

*Statistical analysis*

All statistical analyses were performed in GraphPad Prism 9.0.2. The Kolmogorov-Smirnov, D'Agostino-Pearson, and Shapiro-Wilk tests were used to determine whether the data followed a normal, Gaussian distribution. In our study, we used a *t*-test, Chi-square test, and Mann-Whitney *U* test.

**Results**

*Immunophenotypic characteristics of PMN-MDSC-like cells and M-MDSC-like cells*

PMN-MDSC-like cells and M-MDSC-like cell subsets were identified according to the expression of CD15 and CD14, respectively, within the $HLA\text{-}DR^{-/low}CD11b^+$ $CD33^{low}Lin^-$ population in 83 patients in different stages of CRC according to the AJCC classification and in 12 healthy controls. Detection of PMN-MDSC-like cells and M-MDSC-like cells is shown in Figures 1 and 2, respectively. Briefly, for both major MDSC-like cell subpopulations – PMN-MDSCs and M-MDSCs – the initial gate was set on CD45 (pan-leukocyte antigen) positive cells ($CD45^+$ gate, Figures 1A and 2A, respectively) in order to allow final expression of the percentages of MDSC-like cells relative to all leukocytes present in the peripheral blood sample, taking the $CD45^+$ gate as the "parental gate". In the next step, $CD45^+$ events were plotted on the HLA-DR vs. CD11b dot plot, where the $HLA\text{-}DR^{-/low}$ and $CD11b^+$ events were selected for further evaluation (Figures 1B and 2B). For detection of PMN-MDSC-like cells, the next step included the assessment of the $CD11b^+HLA\text{-}DR^{-/low}$ events for CD16 molecule expression on CD16 vs. SS log dot plot (side scatter logarithmic) where the events with lower CD16 as well as lower SS log, compared to mature granulocytes (confirmed by CD10 expression on mature granulocytes in a different test tube combination: CD16FITC/CD11bPE/CD45ECD/HLA-DR PeCy5/CD10PECy7) (Figure 1E and J), were gated and colored black for further tracking (Figure 1C). Finally, the PMN origin of $CD45^+Lin^-CD33^+CD11b^+HLA\text{-}DR^{neg/low}$ $CD16^{low}$ cells was confirmed by strong expression of CD15 on these cells (Figure 1D). The assessment of Lineage cocktail (CD3FITC/CD19FITC/CD56FITC) negativity and CD33 positivity was performed in different test tube combinations (LinFITC/CD11bPE/CD45ECD/HLA-DRPECy5/CD16PECy7 and CD16FITC/CD11bPE/CD45ECD/HLA-DRPECy5/CD33PECy7, respectively, not shown). The same gating strategy in a healthy donor sample is shown in Figure 1 F–J.

For the detection of M-MDSC-like cells, the $CD45^+CD11b^+HLA\text{-}DR^{-/low}$ events were plotted on the CD14 vs. SS log dot plot in order to confirm their monocyte origin, gated and colored black for further tracking (Figure 2C). A further distinction from PMN cells, in addition to a clearly lower SS signal on CD45 vs. SS log dot plot, was achieved by demonstration of CD15 negative measurement (Figure 2D). The overlay histogram (Figure 2E) shows clearly negative HLA-DR expression in selected monocytoid cells compared to lymphocytes, which are used as an internal test control. The assessment of Lineage cocktail (CD3FITC/CD19FITC/CD56FITC) negativity and CD33 positivity was performed in different test tube combinations based on the CD14 positive events (LinFITC/CD11bPE/CD45ECD/HLA-DRPECy5/CD14PECy7 and LinFITC/CD33PE/CD45ECD/HLA-DRPECy5/CD14 PECy7, respectively, not shown). The same gating strategy in a healthy donor sample is shown in Figure 2 F–J.

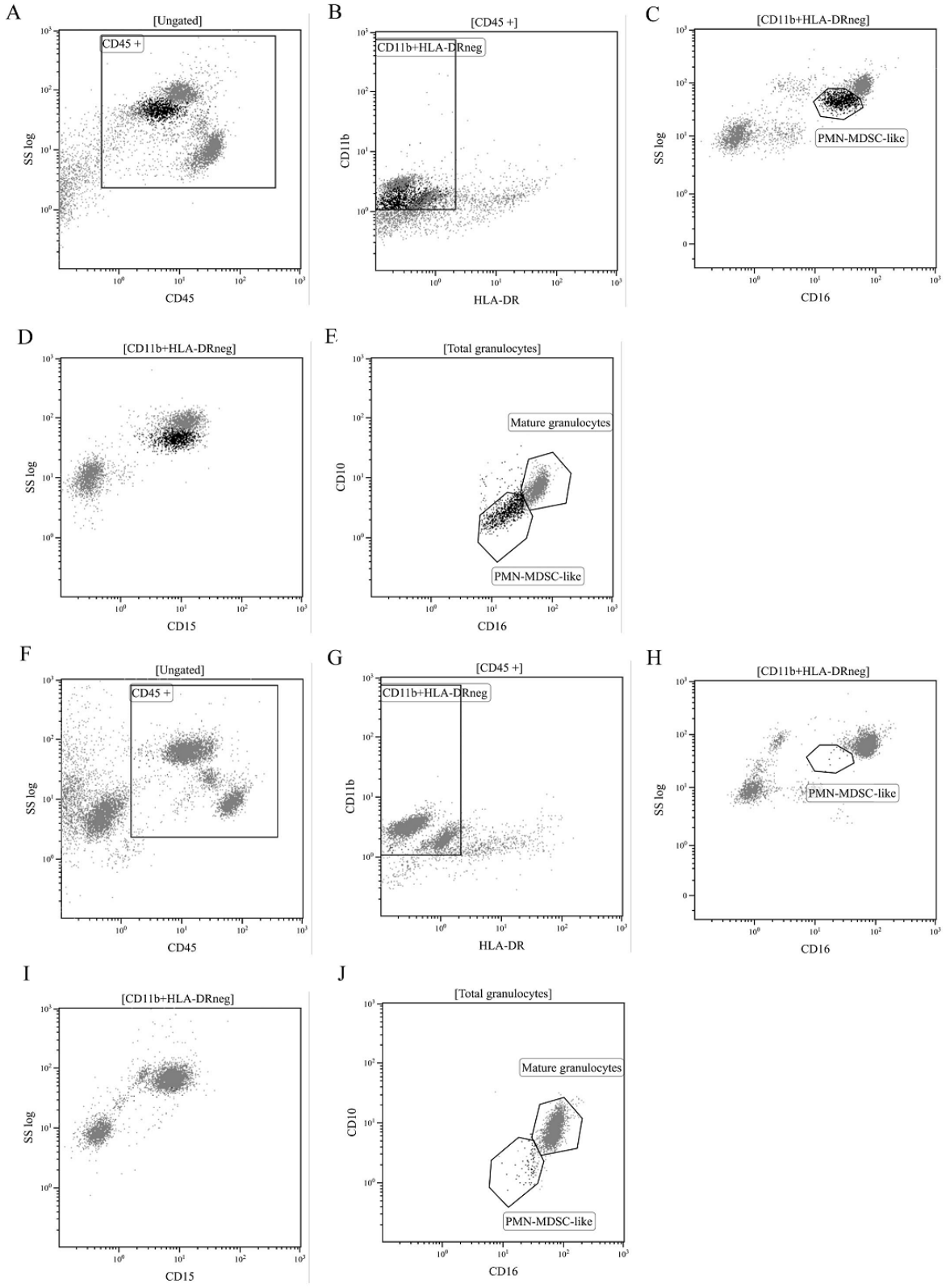


**Fig. 1 – Detection of PMN-MDSC-like cells: In the representative CRC patient PB sample, the CD45 positive events are selected for analysis (A) and plotted on to HLA-DR vs. CD11b dot plot, next, $CD11b^{+}$ and $HLA\text{-}DR^{-/low}$ events (B) are further evaluated for CD16 expression, showing clearly lower signal of the examinee, as well as lower side scatter value (C); Strong expression of polymorphonuclear CD15 marker (D) and negative expression of mature granulocytes marker – CD10 (E); The healthy donor PB sample subjected to the same gating strategy, showing the substantially lower quantity of PMN-MDSC-like cells (F-J). PMN-MDSC – polymorphonuclear myeloid-derived suppressor cells; CRC – colorectal carcinoma; PB – peripheral blood.**

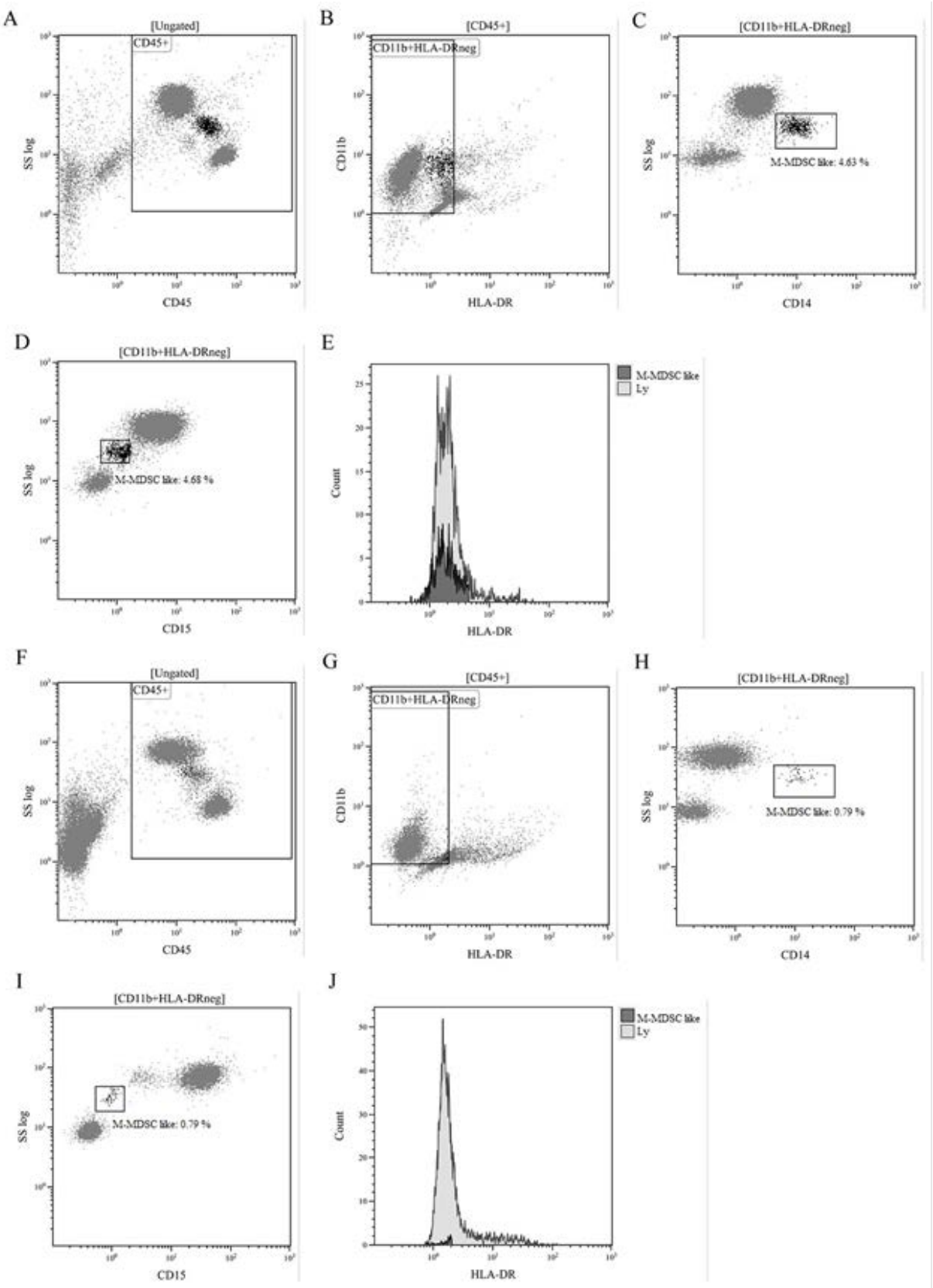


**Fig. 2 – Detection of M-MDSC-like cells: In the representative CRC patient PB sample, the CD45 positive events are selected for analysis (A) on CD11b and HLA-DR expression (B); Selected CD11b$^{+}$/HLA-DR$^{neg/low}$ events showing positive CD14 staining (C) and negative CD15 signal (D); Overlay histogram showing HLA-DR negative signal in CD14 positive cells compared with lymphocytes as an internal control (E); The healthy donor PB sample subjected to the same gating strategy (F-J).**
**M-MDSC – monocytic-MDSCs. For other abbreviations, see Figure 1.**

The study involved 83 patients with CRC and 12 healthy subjects who made up the control group; their descriptive statistics are presented in Table 1. It is known that CRC mainly affects a slightly older population, more often males, which is confirmed in this study as well. Namely, the average age of our patients was 63.6 years, while there was a statistically significant difference between the number of male (65.1%) and female (34.9%) patients with CRC (Chi-Square = 5.568; $p$ = 0.018). Furthermore, despite some observed differences between our patients and the control group, statistical testing showed that there was no significant difference between these groups regarding their average age ($t$ = 0.263, $p$ = 0.793), average BMI (Mann-Whitney, $U$ = 555.5, $p$ = 0.381), or proportion of males and females (Chi-Square = 2.438; $p$ = 0.118). The study lasted 19 months, and the patients were divided into groups depending on the stage of the disease for further testing. The lowest number of subjects belonged to stage I, according to the TNM/AJCC classification, and the highest to stage IV of the disease (Table 1).

First, we compared the percentages and absolute values of both MDSC-like cell subtypes in healthy and all diseased individuals, and complete statistical results are depicted in Figure 3. Patients with CRC had a highly significant increase in the percentage and absolute number of PMN-MDSC-like cells compared to healthy individuals (4.21 ± 4.30 vs. 1.46 ± 1.24, $p < 0.0001$ for percentage and 0.36 ± 0.59 vs. 0.09 ± 0.09, $p < 0.0001$, for absolute number, respectively). On the contrary, a statistically significant relationship was not observed in the case of M-MDSC-like cells (0.70 ± 0.33 vs. 0.39 ± 0.33, $p$ = 0.3027 for percentage and 0.05 ± 0.06 vs. 0.03 ± 0.21, $p$ = 0.4346 for absolute number of M-MDSC-like cells in patients with CRC and healthy persons) (Figure 3).

**Table 1**

**Demographic and clinical characteristics of study participants**

| Parameters | Groups | |
|---|---|---|
| | CRC (n = 83) | Control (n = 12) |
| Age (years), mean | 63.6 | 62.6 |
| Gender, n (%) | | |
| female | 29 (34.9) | 7 (58.3) |
| male | 54 (65.1) | 5 (41.7) |
| BMI, kg/m$^2$ | 26.0 | 24.9 |
| CRC stage (TNM classification), n | | |
| I | 5 | – |
| II | 31 | – |
| III | 18 | – |
| IV | 29 | – |

**TNM – Tumor Node Metastasis; CRC – colorectal carcinoma; BMI – body mass index; n – number of patients.**

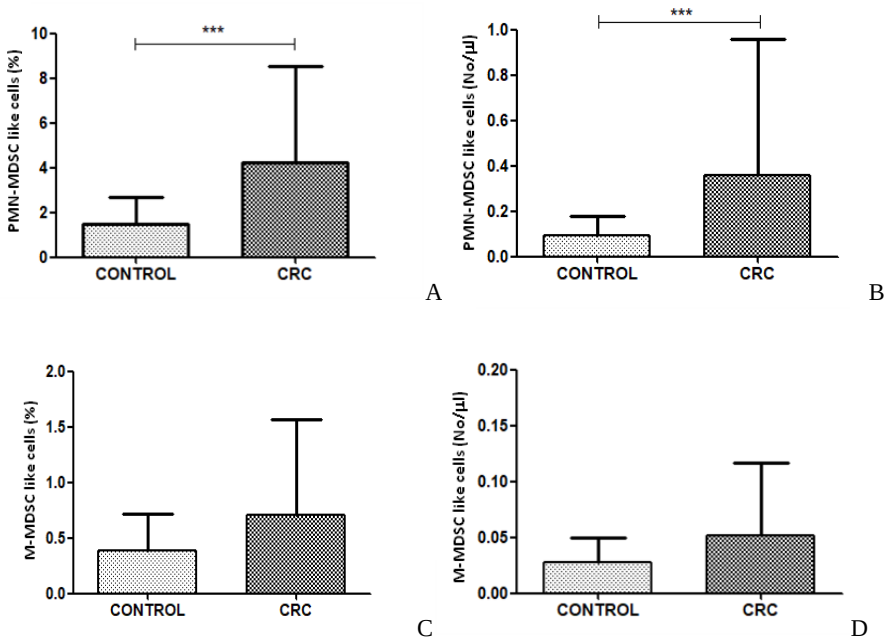


**Fig. 3 – Comparison of the numbers of PMN-MDSC- like and M-MDSC-like cells between healthy subjects (control group) and CRC patients. The average percentage (A) and the absolute number (B) of PMN-MDSC-like cells; The average percentage (C) and the absolute number (D) of M-MDSC-like cells. Mann-Whitney *U* test was used (***$p < 0.0001$). Some data points are outside the axis limits and excluded for better figure presentation (number of excluded data points ≤ 3). For abbreviations, see Figures 1 and 2.**

Then, we refined our analysis by comparing the percentages and absolute numbers of PMN-MDSC-like cells and M-MDSC-like cells in patients with different clinical stages according to the TNM/AJCC classification. All participants were divided into five groups, and the first group comprised healthy individuals. The other four groups consisted of patients in different stages of CRC according to the TNM/AJCC classification, starting from the lowest (I) to the highest (IV) stage of the disease. Descriptive statistics for these groups are presented in Table 2.

After a descriptive analysis was performed and the normality of the distribution was checked, more rigorous statistical testing was completed with the Mann-Whitney *U* test, and the complete results are shown in Figure 4. Comparing the data of healthy individuals and patients in different stages of the disease according to the TNM classification, a statistically significant difference of PMN-MDSC-like cells in relative and absolute numbers was noticed between the control group and patients in stage III and IV (1.46 ± 1.2 vs. 3.72 ± 1.64, $p = 0.0032$ and 1.46 ± 1.24 vs. 6.78 ± 6.15, $p < 0.0001$ for percentage and 0.09 ± 0.09 vs. 0.32 ± 0. 29, $p = 0.0003$ and 0.09 ± 0.09 vs. 0.64 ± 0.92, $p < 0.0001$ for absolute numbers). Likewise, there was a significant difference in relative and absolute numbers of PMN-MDSC-like cells between patients in stage I and stage IV of the disease (2.02 ± 1.85 vs. 6.78 ± 6.15, $p = 0.0161$ for percentage and 0.12 ±

**Table 2**

**Descriptive data by stages based on the latest AJCC classification**

| Subjects | | PMN-MDSC-like cells | | M-MDSC-like cells | |
|---|---|---|---|---|---|
| | | % | No/μL | % | No/μL |
| Control group (n = 12) | mean ± SD | 1.46 ± 1.24 | 0.09 ± 0.09 | 0.39 ± 0.33 | 0.03 ± 0.02 |
| | median | 1.01 | 0.06 | 0.29 | 0.03 |
| CRC stage | | | | | |
| I ( n = 5) | mean ± SD | 2.02 ± 1.85 | 0.12 ± 0.10 | 0.55 ± 0.54 | 0.03 ± 0.03 |
| | median | 1.09 | 0.06 | 0.48 | 0.02 |
| II (n = 31) | mean ± SD | 2.45 ± 1.66 | 0.16 ± 0.12 | 0.40 ± 0.53 | 0.03 ± 0.05 |
| | median | 2.07 | 0.13 | 0.15 | 0.01 |
| III (n = 18) | mean ± SD | 3.72 ± 1.64 | 0.32 ± 0.29 | 0.61 ± 0.62 | 0.04 ± 0.04 |
| | median | 3.70 | 0.24 | 0.30 | 0.04 |
| IV (n = 29) | mean ± SD | 6.78 ± 6.15 | 0.64 ± 0.92 | 1.12 ± 1.13 | 0.08 ± 0.08 |
| | median | 4.32 | 0.36 | 0.63 | 0.05 |

**AJCC – American Joint Committee of Cancer; n – number of subjects; SD – standard deviation; % – relative number of cells; No/μL – absolute number of cells.**
**For other abbreviations, see Figures 1 and 2.**

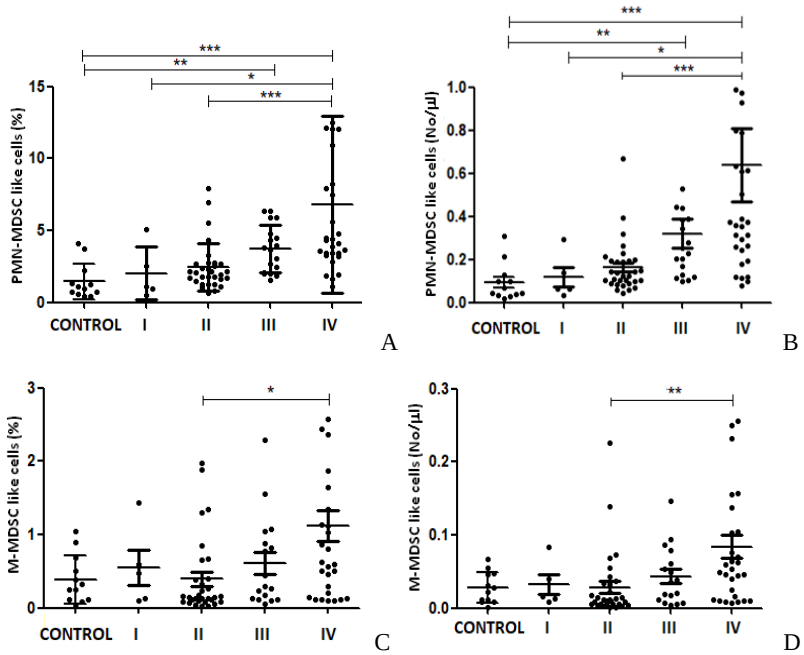


**Fig. 4 – Comparison of the numbers of PMN-MDSC-like and M-MDSC-like cells between healthy subjects (control group) and patients with CRC divided into four stages of the disease. The average percentage (A) and the average absolute number (B) of PMN-MDSC-like cells; The average percentage (C) and the average absolute number (D) of M-MDSC-like cells. Mann-Whitney *U* test (*$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$). Some data points are outside the axis limits and excluded for better figure presentation (number of excluded data points ≤ 3).**
**For abbreviations, see Figures 1 and 2.**

0.10 vs. 0.64 ± 0.92, $p = 0.0065$, for absolute numbers, respectively).

Statistical significance in M-MDSC-like cells was observed when we compared the percentages and absolute numbers of these cells between CRC patients within stage II and stage IV (0.40 ± 0.53 vs. 1.12 ± 1.13, $p = 0.0014$ for percentage and 0.03 ± 0.05 vs. 0.08 ± 0.08, $p = 0.0002$, for absolute numbers, respectively). The highest values of both MDSC-like cell subtypes were observed in patients with disseminated disease in phase IV of CRC (6.78 ± 6.15 and 0.64 ± 0.92 for the percentage and absolute number of PMN-MDSC-like cells and 1.12 ± 1.13 and 0.05 ± 0.08 for the percentage and absolute number of M-MDSC-like cells, respectively). As illustrated in Figure 4, the differences between other analyzed groups were not found to be statistically significant in our sample.

In the next step, we looked at the correlation between the percentages and absolute numbers of MDSC-like cells and the metastatic spread of the disease. For the purpose of this analysis, all patients were divided into four groups. The first group (group 0) consisted of the largest number of patients who did not have a metastatic spread of CRC. Then we made the division into three more groups depending on whether metastatic changes were observed in one organ (group 1), two organs (group 2), or three or more organs (group 3). Descriptive statistics for these groups are presented in Table 3.

Based on Spearman's correlation coefficient and statistical tests, it was observed that there was a positive correlation between the presence of MDSC-like cells and the number of organs affected by metastatic changes. This relationship is moderately strong for PNM-MDSC-like cells, while it is somewhat weaker for M-MDSC-like cells ($p < 0.0001$ for the relative and absolute number of PMN-MDSC-like cells and $p = 0.003$ and $p = 0.0004$ for the relative and absolute number of M-MDSC-like cells) (Table 4; Figure 5).

## Discussion

MDSCs make up about 0.5–1% of peripheral blood neutrophils in healthy individuals [8]. A higher frequency of these cells has been observed in people with malignancies – breast cancer [14], pancreas cancer [15], lung cancer [16], but also in many inflammatory diseases such as chronic hepatitis C [17], active ulcerative colitis [18], or sepsis [19].

Patients diagnosed with CRC require further treatment. It often includes surgery, but sometimes, besides surgical treatment, depending on the stage and localization of the disease, patients also require chemotherapy and radiotherapy. The effect of chemotherapy and radiotherapy, as well as immunosuppressive drugs, on MDSCs in cancer patients, is well known [20, 21]. Therefore, none of our subjects, regardless of the stage of the disease, underwent these types of treatments prior to MDSCs sampling. Peripheral

**Table 3**

**Descriptive data for patients with a different number of organs affected by metastases**

| Groups | | PMN-MDSC-like cells | | M-MDSC-like cells | |
|---|---|---|---|---|---|
| | | % | No/μL | % | No/μL |
| 0 (n = 54) | mean ± SD | 2.82 ± 1.75 | 0.21 ± 0.20 | 0.49 ± 0.56 | 0.03 ± 0.04 |
| | median | 2.18 | 0.14 | 0.20 | 0.02 |
| | min–max | 0.51–7.93 | 0.03–1.35 | 0.02–2.29 | 0.00–0.23 |
| 1 (n = 12) | mean ± SD | 3.99 ± 2.90 | 0.33 ± 0.23 | 1.12 ± 1.48 | 0.08 ± 0.11 |
| | median | 3.45 | 0.26 | 0.46 | 0.05 |
| | min–max | 1.09–12.13 | 0.12–0.93 | 0.10–4.35 | 0.01–0.31 |
| 2 (n = 13) | mean ± SD | 8.53 ± 7.33 | 0.67 ± 0.61 | 1.21 ± 1.01 | 0.09 ± 0.08 |
| | median | 7.48 | 0.61 | 0.87 | 0.06 |
| | min–max | 1.86–30.14 | 0.10–2.43 | 0.12–3.15 | 0.01–0.26 |
| ≥ 3 (n = 4) | mean ± SD | 9.99 ± 6.83 | 1.51 ± 2.17 | 0.81 ± 0.54 | 0.09 ± 0.06 |
| | median | 9.06 | 0.50 | 0.87 | 0.09 |
| | min–max | 3.42–18.42 | 0.27–4.76 | 0.14–1.34 | 0.01–0.16 |

**0 – patients with CRC and without metastases; 1 – patients with CRC and metastases in only one organ; 2 – patients with CRC and metastases in two organs; 3 – patients with CRC and metastases in three or more organs. % – relative number of cells; No/μL – absolute number of cells; min-max – value range minimum to maximum.**
**For abbreviations, see Figures 1 and 2.**

**Table 4**

**Spearman's correlation coefficient with statistical significance testing between PMN-MDSC-like cells and M-MDSC-like cells and the number of organs affected by metastases in patients with CRC**

| Parameter | Metastases and PMN-MDSC-like cells % | Metastases and PMN-MDSC-like cells No/μL | Metastases and M-MDSC-like cells % | Metastases and M-MDSC-like cells No/μL |
|---|---|---|---|---|
| Spearman's coefficient | 0.5126 | 0.5115 | 0.3234 | 0.3793 |
| *p*-value | <0.0001 | <0.0001 | 0.003 | 0.0004 |

**% – relative number of cells; No/μL – absolute number of cells.**
**For abbreviations, see Figures 1 and 2.**

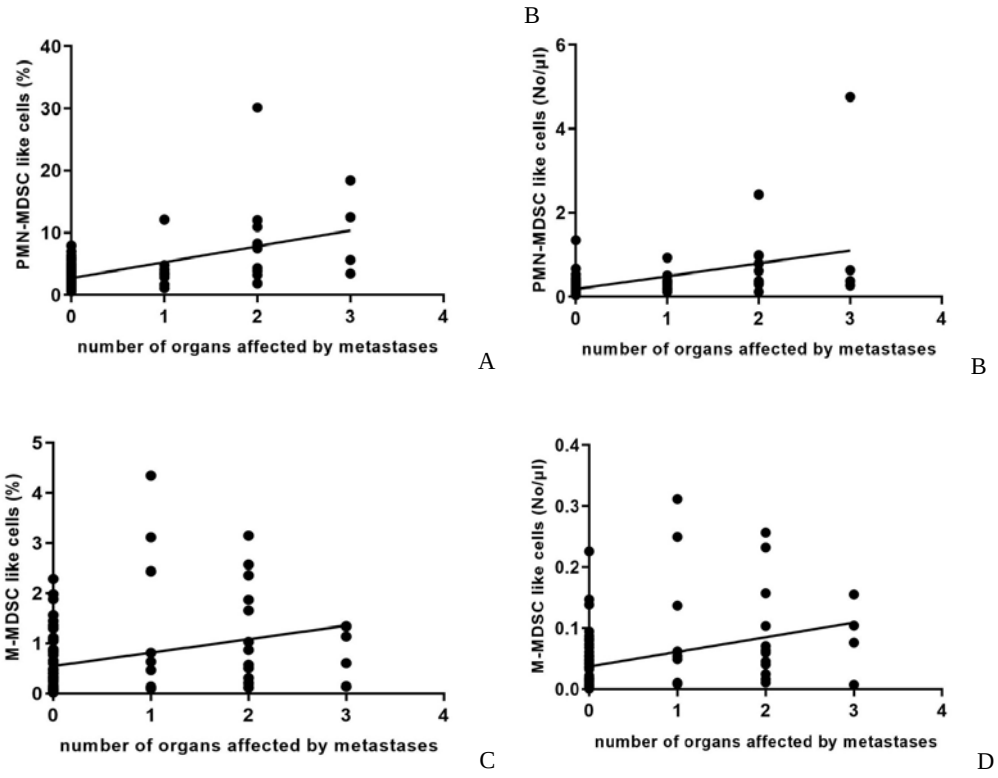


**Fig. 5 – Graphic presentation of the Spearman's correlation between MDSC-like cells and the number of organs affected by metastases in patients with CRC. The relative number (A) and the absolute number (B) of PMN-MDSC-like cells; The relative number (C) and the absolute number (D) of M-MDSC-like cells. For abbreviations, see Figures 1 and 2.**

blood samples were taken immediately after the diagnosis of CRC was made and before possible surgery treatment because such treatment has an impact on the frequency of MDSCs [22, 23].

First, we compared the percentages and absolute numbers of PMN-MDSC-like cells and M-MDSC-like cells in circulation in healthy individuals and patients with CRC. Our results showed a highly statistically significant increase in the percentages and absolute numbers of PMN-MDSC-like cells in CRC patients compared to the control group and are in agreement with the study by Zhang et al. [24], who also indicated an increased presence of MDSCs in patients with colon cancer compared to the healthy population. Such a conclusion was also reached in the study by Toor et al. [25]. However, when measuring the number of M-MDSC-like cells that were otherwise less present in peripheral blood compared to PMN-MDSC-like cells, no statistical significance was observed between the control group and total CRC patients. The obtained results are in agreement with the reports of other studies. Namely, data from studies by Hossaini et al. [26] indicated that, in CRC patients, the MDSCs subpopulation with the highest percentage was PMN-MDSCs. They also found that M-MDSCs were present in a smaller percentage compared to PMN-MDSCs and were not increased in CRC patients. It should be emphasized that some studies have indicated an increase in the number of both types of MDSCs in CRC, not only PMN-MDSCs [27].

We divided patients by cancer stage using TNM classification. It was assumed that the number of PMN-MDSCs and M-MDSCs would increase with disease progression. Significant differences were seen in the percentage and absolute number of circulating MDSC-like cells between healthy donors and patients with advanced stages – III and IV. Moreover, significant differences were seen between patients within stages I and IV, as well as between patients in stages II and IV. Looking at M-MDSC-like cells, patients in stage IV had a statistically significant increase in both absolute and relative cell numbers compared to those who were in stage II, according to the TNM classification. A positive correlation between the presence of MDSCs and the number of organs affected by metastatic changes clearly indicates a significant relationship between tumor burden and spread of the disease on the one hand and MDSCs accumulation on the other.

The highest values of PMN and M-MDSC-like cells were registered in stage IV of CRC in patients with advanced disease involving other organs. Observing the relationship between the stage of the disease and the presence of PMN-MDSCs, OuYang et al. [12] showed that increased presence in the peripheral blood in patients with CRC was associated with more severe clinical stages of the disease and lymph node metastasis. However, this observation is not unique to colon cancer and can be observed in other malignancies as well [13, 16]. The correlation between MDSCs

and disease progression is a consequence of the communication between these cells and cancer cells. Many mediators secreted by different tumors in a hypoxic environment lead to an increase in the number and activation of MDSCs. MDSCs also promote tumor survival and expansion through various immunosuppressive effects [28]. Malignant tumors often lead to increased myelopoiesis due to disorders in the regulation of the production of growth factors that affect hematopoiesis [29, 30]. The immunosuppressive effect of MDSCs is achieved in the tumor microenvironment and the peripheral blood, and it includes the mobilization and induction of other suppressive cells (i.e., macrophages) and altered metabolism of amino acids such as arginine and cysteine [7]. However, it should be emphasized that MDSCs also accelerate tumor progression. These effects of MDSCs take action through the process of angiogenesis and metastasis [31]. MDSCs activate growth factors such as vascular endothelial growth factor, basic fibroblast growth factor, Bombina variegata peptide 8, and platelet-derived growth factor, which are important for angiogenesis. MDSCs help cancer cells enter the circulation by releasing proteolytic enzymes – metalloproteinases, that transform vascular structures. An increase in the permeability of the vascular wall results in easier entry of cancer cells into the circulation and easier extravasation of the cells into the tissues. Even the "seeding" of malignant cells into other organs is not a random choice but is preceded by soil preparation, referred to as a premetastatic niche. MDSCs help its formation by influencing neovascularization by enabling oxygenation and nutrient supply to the future metastatic lesion [32].

It can be seen that the "cooperation" between the tumor and MDSCs is present at several levels, which may result in an increase in the number of these cells as the disease progresses. Our data confirm higher MDSC-like cells numbers in patients with advanced and metastatic disease in CRC. This fact may indicate that a possible blockade of the immunosuppressive effect of MDSCs could enhance the treatment of patients with CRC. In recent years, many different preclinical and clinical studies have tested various therapeutic approaches, such as the following: inhibition of MDSCs expansion and proliferation; differentiation of MDSCs into mature, less suppressive myeloid cells; inhibition of their immunosuppressive function and depletion of MDSCs in tumor microenvironment [33–38]. Targeting these cells as a treatment modality requires additional research.

## Conclusion

Based on our results, we can conclude that the role of MDSCs is important in the process of progression and very likely in the formation of CRC. We showed for the first time significant correlations between the absolute and relative number of both subtypes of MDSC-like cells with the number of organs affected by metastases in CRC by using an original clinical protocol for identifying MDSC-like cells in total blood leukocytes. The increase in the absolute and relative numbers of MDSC-like cells registered in the advanced disease in our CRC patients, especially in patients with multiple organs affected by metastases, confirms the presumed link between the accumulation of these cells and CRC progression and may be helpful in monitoring these patients.

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# Incidence of nonsyndromic congenital heart defects in the Republic of Srpska in the period 2015–2016

## Učestalost nesindromskih urođenih srčanih mana u Republici Srpskoj u periodu od 2015. do 2016. godine

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**Abstract**

**Background/Aim.** Congenital heart defect (CHD) is the most common type of birth defect and one of the leading causes of infant mortality. It can be isolated or occur as a part of multiple different syndromes. The reported worldwide incidence of isolated CHD is between 70 and 120 *per* 10,000 live births. The aim of the study was to estimate the incidence of nonsyndromic CHD in the Republic of Srpska (RS), Bosnia and Herzegovina and compare it with other countries. **Methods.** The fetuses, live-born and stillborn infants with CHD during 2015 and 2016 in the RS, were analyzed using data from a cross-sectional study. **Results.** The total incidence of CHD was 163.95 *per* 10,000 total births, and the incidence of live-born with CHD was 136.64 *per* 10,000. The diagnosis was established prenatally in 8.09% of cases. The most common type of anomaly was ventricular septal defect (45.63%), followed by an atrial septal defect (31.40%), patent ductus arteriosus (7.44%), and pulmonary valve stenosis (5.18%). A significant difference in the incidence of CHD between regions and different maternal age groups was found. **Conclusion.** The incidence of CHD in the RS found in this study is higher than in other studies, with marked heterogeneity between different regions. This study provides baseline data for future monitoring of the risk factor changes and the implementation of primary preventive measures.

**Key words:**
**bosnia and herzegovina; fetus; heart defects, congenital; incidence; infant, newborn.**

**Apstrakt**

**Uvod/Cilj**. Urođena srčana mana (USM) je najčešći tip urođene mane i jedan od vodećih uzroka umiranja odojčadi. Može biti izolovana ili deo mnogobrojnih sindroma. Učestalost izolovane USM u svetu iznosi između 70 i 120 na 10 000 živorođene dece. Cilj rada bio je da se utvrdi učestalost nesindromskih USM u Republici Srpskoj (RS), Bosna i Hercegovina i da se uporedi sa drugim državama. **Metode.** Korišćenjem podataka iz studije preseka, analizirani su slučajevi fetusa, živorođene i mrtvorođene dece sa USM u 2015. i 2016. godini u RS. **Rezultati.** Ukupna učestalost USM iznosila je 163,95 na 10 000 porođaja, a učestalost novorođenčadi sa USM 136,64 na 10 000 živorođenih novorođenčadi. Dijagnoza je postavljana prenatalno u 8,09% slučajeva. Najčešći tip anomalije bio je ventrikularni septalni defekt (45,63%), zatim atrijalni septalni defekt (31,40%), otvoreni duktus arteriozus (7,44%) i stenoza plućne valvule (5,18%). Utvrđena je značajna razlika u učestalosti USM među regijama i između majki različitog životnog doba. **Zaključak**. Učestalost USM u RS utvrđena u ovoj studiji značajno je veća od učestalosti USM utvrđene u drugim studijama i značajno se razlikuje među regijama. Ova studija pruža osnovne podatke koji bi se mogli koristiti za praćenje USM i promenu faktora rizika, kao i sprovođenje primarnih preventivnih mera.

**Ključne reči:**
**bosna i hercegovina; fetus; srce, kongenitalne mane; incidenca; novorođenče.**

### Introduction

Congenital heart defect (CHD) is a structural abnormality of the heart and great vessels that is present at birth [1]. It is the most common type of major birth defect and the leading cause of birth defect-associated infant death and illness [2]. The underlying causes of CHD remain poorly understood and are thought to be genetic and environmental. In the majority of cases, CHD is isolated, and in about one-third of cases occurs as a part of a syndrome [3].

The incidence of CHD at birth (sometimes referred to as birth prevalence) varies between studies. According to the

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latest reports, the worldwide incidence of CHD is between 70 and 120 *per* 10,000 births [4–7]. Owing to the improvement in diagnostics, the reported incidence of mild CHD is slightly increasing over time due to a higher detection rate [8]. On the other hand, the incidence of the most severe CHD, like hypoplastic left heart syndrome, is decreasing consistently with better prenatal diagnostics and consequent termination of pregnancy [9]. Prenatal diagnosis of CHD generally improves treatment success by allowing appropriate preparation for birth and fetal cardiac intervention.

Nowadays, primary prevention measures include rubella vaccination, good glycemic control, avoiding known teratogenic drugs, folic acid supplementation [10], avoiding contact with influenza and febrile illnesses, as well as exposition to organic solvents [11]. There is growing evidence of a link between maternal obesity [12] and limited evidence of a link between maternal smoking and advanced maternal age [13].

The aim of this study was to determine the incidence of nonsyndromic CHD in the Republic of Srpska (RS), which is a part of Bosnia and Herzegovina, and to compare our findings with other reports.

## Methods

The study was cross-sectional and involved all the fetuses, live-born and stillborn children with CHD in the RS from 1 January 2015 to 31 December 2016.

The study was conducted according to the principles of the Declaration of Helsinki. There was no written informed consent obtained from the parents of the participants; however, they were verbally informed that their child's condition and other relevant information about the child and mother would be reported to the Clinic for Children's Diseases and that their personal information would not be available to the public. Bearing in mind that not all of the institutions involved in the study have an Ethics Committee, the study was approved by the Ministry of Health and Social Welfare of the RS from September 17, 2015.

Data were collected in the form of a questionnaire survey conducted by 52 physicians, 46 of whom were pediatricians and 6 were gynecologists working at university centers, general hospitals, or primary healthcare institutions from all six regions of the RS. Physicians reported the presence and the type of CHD, additional anomalies and chromosomal aberrations, time of diagnosis of the anomaly (prenatally or postnatally), child's birth date and gender, duration of pregnancy, and mother's age and place of living. These questionnaire surveys were sent by regular mail to the Clinic for Children's Diseases in the University Clinical Center of the RS, where they were collected and analyzed.

Diagnosis of CHD was established by a pediatric cardiologist using a color doppler echocardiogram. Infants and fetuses with chromosomal aberrations were excluded. In addition, a clinical geneticist manually reviewed the records of cases with multiple anomalies to exclude the ones in which CHD was suspected to be a part of a genetic syndrome. Since they were not considered structural abnormalities, normal physiological findings in premature or newborn infants younger than six weeks, such as patent ductus arteriosus, patent *foramen* ovale, or valve insufficiency unrelated to structural valve abnormality, were excluded from the analysis. Patent ductus arteriosus was only considered a CHD if it occurred in the term infants and was not maintained patent to ensure survival due to another cardiac condition.

Infants and fetuses with CHD were grouped in the following way: into 15 groups according to the type of defect (common arterial truncus, double outlet right ventricle, transposition of great vessels, single ventricle, ventricular septal defect, atrial septal defect, atrioventricular septal defect, tetralogy of Fallot, pulmonary valve stenosis, pulmonary valve atresia, hypoplastic left heart, coarctation of aorta, aortic atresia/interrupted aortic arch, total anomalous pulmonary venous return, and patent ductus arteriosus); into three groups according to the number of CHD (single, double, and triple); into six groups according to the region of the RS where the mother of the child was living (Banja Luka, Prijedor, Doboj, Bijeljina, Istočno Sarajevo, and Trebinje); into two groups according to the birth year (2015 and 2016); into three groups according to the gender of the child or fetus (male, female, and unknown); into two groups according to the maternal age (younger than 35 years and 35 years and older); into two groups according to the time of diagnosis (prenatally and postnatally).

The total incidence of CHD was defined as the total number of CHD among live-born and stillborn children plus the number of pregnancies terminated for severe fetal heart anomaly *per* 10,000 total births. Live birth incidence of CHD was defined as the number of live-born children with CHD *per* 10,000 live births.

The total number of births in the RS was as follows: 9,357 in 2015 and 9,452 in 2016 [14].

We compared the incidence of CHD between groups and data reported by other studies and discussed the possible causes for the differences found.

### *Statistical analysis*

Statistical analyses were performed using the statistical package SPSS. The obtained data were expressed as counts and percentages. Differences among groups were evaluated using the chi-square ($\chi^2$) test. A two-tailed $p \leq 0.05$ was considered to indicate statistical significance. The total numbers of live-born and stillborn infants have been taken from the official website of the RS Institute of Statistics [14].

## Results

During the period of study, the total number of births in the RS was 18,841 (9,374 in 2015 and 9,467 in 2016), of which the number of live births was 18,809.

The total number of infants and fetuses with CHD was 263. Single CHD was found in 224 (85.17%), double CHD in 32 (12.17%), and triple CHD in 7 (2.66%) infants and fetuses. Therefore, the total number of CHD was 309 (160 in 2015 and 149 in 2016) (Table 1).

Six pregnancies (three each year) with a prenatal diagnosis of CHD were electively terminated, and 257 infants with CHD (141 in 2015 and 116 in 2016) were live-born (Table 2). Therefore, the total incidence of CHD in these two years was 163.95 *per* 10,000 total births, and the total incidence of live-born children with CHD was 136.64 *per* 10,000 live births. In 2015, the incidence of CHD was 170.63 *per* 10,000 total births, and the incidence of live-born children with CHD was 150.69 *per* 10,000 live births. In 2016, the incidence of CHD was 157.34 *per* 10,000 total births, and the incidence of live-born children with CHD was 122.73 *per* 10,000 live births.

There have been significant differences in the total incidence of CHD between regions. The highest was in Prijedor (340.26 *per* 10,000 live births), and the lowest was in Istočno Sarajevo (80.23 *per* 10,000 live births) (Table 3).

In our study, we found 15 different types of CHD. The most common were ventricular septal defects (45.63%), atrial septal defects (31.40%), patent ductus arteriosus (7.44%), and pulmonary valve stenosis (5.18%) (Table 4).

**Table 1**

**Number of infants and fetuses with CHD and the total number of CHD**

| Period | Infants and fetuses with CHD (LB+TOPFA) | | | | Total CHD |
|---|---|---|---|---|---|
| | single CHD n (%) | double CHD n (%) | triple CHD n (%) | Total n | n |
| 2015 | 129 (89.58) | 14 (9.72) | 1 (0.69) | 144 | 160 |
| 2016 | 95 (79.83) | 18 (15.13) | 6 (5.04) | 119 | 149 |
| Total | 224 (85.17) | 32 (12.17) | 7 (2.66) | 263 | 309 |

**CHD – congenital heart defect; LB – live-born children; TOPFA – terminations of pregnancy for a fetal anomaly; n – number.**

**Table 2**

**Number of live-born infants with CHD and number of terminations of pregnancy for CHD**

| Period | LB n (%) | TOPFA n (%) | Total n |
|---|---|---|---|
| 2015 | 141 (97.92) | 3 (2.08) | 144 |
| 2016 | 116 (97.48) | 3 (2.52) | 119 |
| Total | 257 (97.72) | 6 (2.28) | 263 |

**For abbreviations, see Table 1.**

**Table 3**

**Number and incidence of CHD per region**

| Period | Banja Luka | Prijedor | Doboj | Bijeljina | Istočno Sarajevo | Trebinje | Total |
|---|---|---|---|---|---|---|---|
| 2015 | 61 (38.13) | 31 (19.38) | 30 (18.75) | 27 (16.88) | 7 (4.38) | 4 (2.5) | 160 |
| 2016 | 63 (42.28) | 30 (20.13) | 10 (6.71) | 34 (22.82) | 8 (5.37) | 4 (2.68) | 149 |
| Total | 124 (40.13) | 61 (19.74) | 40 (12.94) | 61 (19.74) | 15 (4.85) | 8 (2.59) | 309 |
| Total incidence* | 164.34 | 340.26 | 128.96 | 152.13 | 80.23 | 142.85 | 163.95 |
| *p*-value | 0.911 | < 0.05 | 0.051 | 0.569 | < 0.05 | 0.271 | |

**All values are expressed as numbers (percentages). CHD – congenital heart defect. * – per 10,000 total births.**

**Table 4**

**Number and incidence of different types of congenital heart defect (CHD)**

| Type of CHD | 2015 | | 2016 | | Total | |
|---|---|---|---|---|---|---|
| | n | Inc. | n | Inc. | n (%) | Inc. |
| Common arterial truncus | 0 | 0 | 1 | 1.06 | 1 (0.32) | 0.53 |
| Double outlet right ventricle | 1 | 1.07 | 1 | 1.06 | 2 (0.65) | 1.06 |
| Transposition of great vessels | 4 | 4.27 | 2 | 2.11 | 6 (1.94) | 3.18 |
| Single ventricle | 2 | 2.13 | 2 | 2.11 | 4 (1.29) | 2.12 |
| Ventricular septal defect | 74 | 78.92 | 67 | 70.75 | 141 (45.63) | 74.81 |
| Atrial septal defect | 48 | 51.19 | 49 | 51.74 | 97 (31.40) | 51.47 |
| Atrioventricular septal defect | 2 | 2.13 | 0 | 0 | 2 (0.65) | 1.06 |
| Tetralogy of Fallot | 4 | 4.27 | 1 | 1.06 | 5 (1.62) | 2.65 |
| Pulmonary valve stenosis | 7 | 7.47 | 9 | 9.50 | 16 (5.18) | 8.49 |
| Pulmonary valve atresia | 0 | 0 | 1 | 1.06 | 1 (0.32) | 0.53 |
| Hypoplastic left heart | 2 | 2.13 | 0 | 0 | 2 (0.65) | 1.06 |
| Coarctation of aorta | 6 | 6.40 | 1 | 1.06 | 7 (2.27) | 3.71 |
| Aortic atresia /interrupted aortic arch | 1 | 1.07 | 0 | 0 | 1 (0.32) | 0.53 |
| Total anomalous pulmonary venous return | 0 | 0 | 1 | 1.06 | 1 (0.32) | 0.53 |
| Patent ductus arteriosus | 9 | 9.60 | 14 | 14.78 | 23 (7.44) | 12.20 |
| Total | 160 | 170.63 | 149 | 157.34 | 309 (100) | 163.95 |

**n – number; Inc. – incidence per 10,000 total births.**

**Table 5**

**Time of establishing the diagnosis of congenital heart defect**

| Period | Prenatally | Postnatally |
|---|---|---|
| 2015 | 11 (6.87) | 149 (93.13) |
| 2016 | 14 (9.40) | 135 (90.60) |
| Total | 25 (8.09) | 284 (91.91) |
| *p*-value | 0.417 | |

**All values are expressed as numbers (percentages).**

**Table 6**

**Number of CHD in infants and fetuses depending on maternal age**

| Parameter | 2015 | | 2016 | | Total | |
|---|---|---|---|---|---|---|
| | < 35 y | ≥ 35 y | < 35 y | ≥ 35 y | < 35 y | ≥ 35 y |
| Total births + TOPFA, n | 7,981 | 1,396 | 8,041 | 1,429 | 16,022 | 2,825 |
| CHD, n (%) | 129 (1.62) | 31 (2.22) | 117 (1.46) | 32 (2.24) | 246 (1.54) | 63 (2.23) |
| *p*-value | 0.107747 | | < 0.00001* | | 0.007343* | |

**CHD – congenital heart defect; TOPFA – terminations of pregnancy for a fetal anomaly; y – years. * – statistically significant; n – number.**

The diagnosis was made prenatally in 8.09% of cases. The higher proportion of prenatally diagnosed CHD found in 2016 (6.87%) compared to 2015 (9.40%) was not significantly different (Table 5).

We found no significant difference in gender distribution between infants and fetuses with CHD. There were 123 males, 134 females, and 6 infants and fetuses with unknown gender.

The incidence of CHD was significantly higher in infants and fetuses of mothers who were 35 years old or above (2.23%) compared to the infants and fetuses of younger mothers (1.54%) in 2016 and in a two-year period (Table 6).

## Discussion

The incidence of CHD at birth is considered to vary significantly, depending on how a population is studied. According to Hoffman [4], the global incidence of CHD at birth is between 100 and 120 *per* 10,000 births. Other worldwide studies, such as those by Van der Linde et al. [5] and Liu et al. [9], reported CHD incidences of 91 and 94 *per* 10,000 births. The study from China reported an incidence of 110 *per* 10,000 live births [6]. In the latest European network of population-based registers for the epidemiological surveillance of congenital anomalies (EUROCAT) report, the average total incidence of nonsyndromic CHD in Europe is significantly lower compared to other reports, 70 *per* 10,000 live births [7]. In our study, the incidence of live-born children with CHD was 136.64 *per* 10,000, and the total incidence of CHD was 163.95 *per* 10,000 total births, which is higher than the incidence in other reports.

The difference in the incidence between our and other studies is mainly due to the higher reported incidence of mild types of CHD in our study. Compared to EUROCAT [7] and reports from Hoffman [4] and Van der Linde et al. [5], we found a much higher incidence of ventricular and atrial septal defects. The incidence of the ventricular septal defect in our study is 74.81 *per* 10,000, while in other studies, it is between 26.2 and 32.37 [4, 5]. In our study, the incidence of atrial septal defect is found to be 51.47 *per* 10,000, which is significantly higher than the incidence between 5.6 and 16.4 reported by others [4, 5]. On the other hand, the incidence of severe CHD (transposition of great vessels, tetralogy of Fallot, coarctation of the aorta, hypoplastic left heart, tricuspid atresia, etc.) in our study is similar to these reports. These findings lead us to the conclusion that the difference in total CHD incidence between our and other studies could be due to differences in research methods rather than representing the true difference in incidence. It is well known that the routine use of echocardiography increases the diagnosis of minor heart defects. Although we do not have data on the number of echocardiographic examinations performed during the study, it is assumed that, in some regions, this diagnostic method is widely used, often as screening in infants with or without mild symptoms of CHD, which significantly increased the detection rate of the ventricular septal defect and, therefore, the total incidence of CHD. On the other hand, the high incidence of atrial septal defect can be most likely explained by including a trivial type of this defect by some cardiologists.

We found significant differences in the incidence of CHD between different regions of the RS. That may be due to differences in the availability of echocardiography and reporting methods, but genetic causes, the impact of various environmental factors, and dissimilar implementation of preventive measures between regions cannot be ruled out. That could be a subject for future studies.

Prenatal detection of CHD results in less morbidity and mortality, but even in developed countries, the rate of prenatal detection for severe CHD ranges between 30% and 60% [15, 16]. Moreover, it is higher in countries with prenatal screening programs, like the Netherlands [17]. In our study, CHD was prenatally detected only in 8.09% of cases.

The incidence of CHD in our study was similar in males and females.

We found a higher incidence of CHD in infants and fetuses of mothers aged 35 years or above (2.23%) compared to younger mothers (1.54%). This result is consistent with the Reefhuis and Honein [13] report, in which advanced maternal age has been associated with an increased risk of non-chromosomal CHD. It is well known that advanced maternal age increases the incidence of chromosomal disorders and, therefore, the incidence of CHD related to these disorders. Since we studied only cases without chromosomal anomalies, a possible explanation for this finding could be other risk factors associated with advanced maternal age, such as poor glycemic control, higher body mass index, and the advanced age of their partners [18–21]. Furthermore, the higher incidence of premature birth in older women increases the incidence of ventricular septal defect at birth by detecting those defects that would close spontaneously until term [22].

*Limitations of the study*

The major limitation of our study was our inability to estimate the degree of overreporting and underreporting in data received from other hospitals; however, we believe that this limitation did not have a major effect on the results of the study. Second, the results of only two years cannot reflect the occurrence of CHD for every consecutive year. Finally, the data used for the study are relatively outdated. Nevertheless, although we have noticed that there were some changes in the meantime in the number of echocardiographic examinations, maternal age, and usage of folic acid during pregnancy, we believe that these changes could not have influenced the current incidence of CHD so much to make them significantly different from the results of this study.

## Conclusion

The incidence of nonsyndromic CHD in the RS is higher than reported in other studies. A high detection rate of mild defects, differences in ascertainment methods, and the impact of environmental and genetic factors might be the reasons for this difference. The low percentage of prenatally detected CHD cases underscores the need to improve the prenatal detection rate of this anomaly through public health programs in order to enhance perinatal outcomes in children with CHD.

This study provides baseline data that could be used in future monitoring of the incidence of CHD in the RS to determine the risk factor changes and implementation of primary preventive measures.

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# The factors associated with mild cognitive impairment in outpatient practice

## Faktori povezani sa blagim kognitivnim oštećenjem kod ambulantno lečenih bolesnika

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**Abstract**

**Background/Aim.** Previous studies showed that mild cognitive impairment (MCI) was more common in patients with comorbidities and those using medications that disrupt the homeostasis of vitamin B12. The aim of our study was to determine which of these factors are significantly associated with MCI, as well as which are the most significant risk factors for predicting its occurrence. **Methods.** The data have been prospectively collected for 200 adults (35–65 years old) in primary care settings enrolled in the clinical study with the case-control approach. **Results.** By applying the $\chi^2$ test for independence, we have determined that the MCIs and the use of proton-pump inhibitors (PPIs) ($p < 0.0005$), as well as metformin ($p < 0.0005$), are independent factors. In addition, a significantly higher percentage of subjects who had MCI also had a peptic ulcer and diabetes mellitus type 2 (T2DM). Direct logistic regression has been implemented in order to estimate the influence of many probability factors on whether the study patients would have the MCI. Two variables made statistically significant contributions to the model, and these are the serum concentrations of vitamin B12 [odds ratio (OR) = 0.953; 95% confidence interval (CI) 0.936–0.971; $p < 0.001$] and T2DM (OR = 6.681; 95% CI 1.305–34.198; $p = 0.023$). **Conclusion.** The absolute and relative risk associations of exposure to medicines and MCI is lower than those of comorbidities and MCI. Serum concentrations of vitamin B12, as well as the presence of T2DM, have the greatest statistically significant influence on predicting MCI.

**Key words:**
**cognitive dysfunction; comorbidity; diabetes mellitus, type 2; risk assessment; risk factors; vitamin b 12.**

**Apstrakt**

**Uvod/Cilj.** Prethodne studije pokazale su da je blago kognitivno oštećenje (BKO) češće kod bolesnika sa komorbiditetima, kao i kod bolesnika koji koriste lekove koji remete homeostazu vitamina B12. Cilj rada bio je da se utvrdi koji od tih faktora su značajno povezani sa BKO i koji su najznačajniji za predviđanje njegovog nastanka. **Metode.** Podaci o 200 odraslih osoba (starosti 35–65 godina) uključenih u kliničku studiju po tipu anamnestičke studije u ustanovi primarne zdravstvene zaštite su prikupljeni prospektivno. **Rezultati.** Primenom $\chi^2$ testa nezavisnosti, utvrđeno je da da su BKO i upotreba inhibitora protonske pumpe (IPP) ($p < 0{,}0005$), kao i metformina ($p < 0{,}0005$), nezavisni faktori. Takođe, značajno viši procenat ispitanika koji su imali BKO su imali peptički ulkus i tip 2 dijabetes melitus (T2DM). Da bi se procenio uticaj mnogih faktora verovatnoće na to da li će ispitani bolesnici imati BKO primenjena je direktna logistička regresija. Dve varijable dale su statistički značajan doprinos modelu, a to su koncentracije vitamina B12 u serumu [*odds ratio* (OR) = 0,953; 95% *confidence interval* (CI) 0,936–0,971; $p < 0{,}001$] i T2DM (OR = 6,681; 95% CI 1,305–34,198; $p = 0{,}023$). **Zaključak.** Apsolutna i relativna povezanost rizika izloženosti lekovima sa BKO niža je od povezanosti komorbiditeta sa BKO. Koncentracije vitamina B12 u serumu i prisustvo T2DM imaju najveći statistički značajan uticaj na predviđanje BKO.

**Ključne reči:**
**saznanje, disfunkcija; komorbiditet; dijabetes mellitus, insulin nezavisni; rizik, procena; faktori rizika; vitamin b12.**

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## Introduction

Mild cognitive impairment (MCI) is a clinical-cognitive syndrome that includes concern regarding a change in cognition, impairment in one or more cognitive domains, and preservation of independence in functional abilities without dementia [1]. MCI does not always precede dementia; it can revert to normal cognition or remain stable. It is characterized by cognitive dysfunction in different domains including the memory domain. It does not disable everyday independent functioning, but the deteriorations are inappropriate for a patient's age and education [2].

Some of the clinical syndromes associated with MCI can be cured, but, on the other hand, drugs used for treating these conditions can also have adverse effects opposing the improvement of the existing features of MCI [3, 4].

One of the possible causes of the appearance of MCI can be the deficiency of vitamin B12, as its insufficient body levels can adversely affect brain functions. If there is a deficiency in an organism of the nutrient, it is necessary to compensate for it by intaking appropriate dietary supplements. The causes of vitamin B12 deficiency are in a wide spectrum of pathological conditions such as reduced consumption because of stomach pathology, which includes pernicious anemia, atrophic gastritis caused by chronic inflammation of *Helicobacter (H.) pillory* infection, gastrectomy, Zollinger-Ellison syndrome, bowel diseases, such as Crohn's disease, celiac disease, tropical sprue, bowel resections, congenital selective malabsorption of vitamin B12 with proteinuria, or Imerslund-Grasbeck syndrome.

Deficiency of vitamin B12, besides hematological and gastrointestinal disorders, can lead to neuropsychiatric disorders and symptoms such as neuropathy, cerebellar ataxia, dementia, and disorders of mood occurring as a result of insufficient intake, inadequate absorption, and reduced function. Cyanocobalamin participates in the metabolism of amino acids, myelin regeneration, and growth and differentiation of bone marrow cells. Due to the deficiency of vitamin B12, degeneration of lateral and dorsal columns of the spinal cord with consequent neuropathy occurs. Cognitive dysfunction of these patients is often followed by irritability, depression, and in some cases, psychotic behavior [5].

Among the causes of vitamin B12 deficiency are some medicaments such as biguanides (metformin) and proton-pump inhibitors (PPI), which significantly disturb its metabolism. That can have secondary consequences to hematopoiesis, gastrointestinal system and nervous system, including cognitive consequences, which it has on healthy patients [6, 7]. The aim of our research was to investigate the factors which are associated with MCI in patients within primary healthcare settings, particularly including comorbidities and drugs. We proposed that insufficient vitamin B12 body levels play a prominent role in predicting MCI existence because of the influence of many factors commonly encountered in ambulatory patients which disturb the homeostasis of that essential nutrient.

## Methods

In this clinical research, we implemented the case-control approach on the level of primary health care. We conducted the research at the Health Center "Dr. Milenko Marin" in Loznica, Serbia, with the approval of the Ethics Committee, number 1117/1 / V-1, issued on 8 July 2021. In the case group, there were patients with MCI (MCI+), as confirmed using appropriate screening tests, while in the control group, there were patients without MCI (MCI-).

The subjects were selected from the population of adult patients of the Health Center Loznica who fulfilled the inclusion criteria and did not have exclusion criteria. The inclusion criteria were the following: male or female gender, 35–65 years of age, and those who gave consent for participating in the study and were previously fully informed.

The exclusion criteria were the following: patients younger than 35 or older than 65 years of age (since physiological aging is associated with the weakening of cognitive functions, we did not include people over 65 years of age in our research), patients with a confirmed diagnosis of MCI before this study, presence of other neuropsychiatric illness for which MCI was diagnosed, the respondents who did not give consent for participation or who already participate in another study, and the presence of any illness or condition which disrupts the participation in the study.

All the respondents who satisfy the criteria for participation in the study were given to fill in a screening test by educated doctors in order to reveal the presence of cognitive deficiency, and the scores were measured by Mini-Mental State Examination (MMSE), Montreal Cognitive Assessment (MoCA), and Lawton Instrumental Activities of Daily Living (IADL) scale. The participants who had values lower than 26 points for MoCA, 20 to 24 points for MMSE, which indicates MCI, and values higher than 8 points for women and 5 points for men for Lawton IADL scale, which marks that the patients were functional in doing everyday activities, but with a present cognitive impairment, were referred to a neurologist for the confirmation of the diagnosis of MCI.

MoCA is a test for the fast assessment of cognition. The test includes the domain of concentration and attention, executive functions, language, memory visuoconstructive skills, conceptualization, and calculation of orientation. In this work, the Serbian version of the test was used with a structure and description, scheme, and instructions for the application and scoring of the test. The Serbian version of MMSE consists of questions in which the following cognitive functions are examined: memory, attention and calculation, orientation in space and time, naming, recalling, and complex actions. It represents the most frequently and widely used screening test, which correlates well with other neuropsychological tests. Testing time is less than 10 min for a trained clinician (4–21 min). Lawton IADL is an appropriate instrument to assess independent living skills [8–10]. These skills are considered more complex than the basic activities of daily living as measured by the Katz Index of activities of

daily living. The instrument is most useful for identifying how a person is functioning at present and for identifying improvement or deterioration over time. There are eight domains of function measured with the Lawton IADL scale. The Lawton IADL is an easy-to-administer assessment instrument that provides self-reported information [8–10].

The study subjects, for which the diagnosis of MCI was confirmed by the consulted neurologist, were allocated to the case group while the others, without the presence of MCI, were allocated to the control group in the proportion 1 : 3. While allocating to the study groups, the participants were matched according to their gender and age group.

The primary dependent variable (outcome) was the presence of MCI, which was demonstrated as a binary variable (present/absent). The primary independent variable was the presence of deficiency of vitamin B12 expressed as its concentration in the serum of an examinee under the given limit value. In serum samples of study subjects, the concentration of vitamin B12 was measured using a routine method of clinical biochemistry at the authorized laboratory of the Health Center. The limit values and the concentration units of vitamin B12 were determined according to the local laboratory standards. They were determined by taking blood samples and analyzing the serum with chemiluminescent microparticle immunoassay (CMIA) using the analyzer Abbott Alinity; its reference values were from 138.00 to 652.00 pmol/L.

The prospectively collected data for the study subjects were analyzed by the methods of descriptive statistics, hypothesis testing, and logistic regression. The descriptive statistics include determinations of central tendency measures (e.g., the mean, median) and variability values [e.g., standard deviation (SD), confidence limits]. Continuous variables, such as age and vitamin B12 value, were presented using minimum and maximum values, mean, and SD. Categorical variables, such as MCI, the use of certain medications, and the presence of certain comorbidities, were presented using absolute and relative frequencies.

We analyzed the data in relation to whether the patients have MCI in the following way: when analyzing continuous variables, we used the *t*-test for independent samples, and when analyzing categorical variables, we used the $\chi^2$ test. We analyzed the data in relation to vitamin B12 values in patients in the following way: we used linear correlation and regression methods for analyzing continuous variables, and we used the *t*-test for independent samples when analyzing categorical variables.

Standard multiple regression was used to assess the ability to predict the value of vitamin B12 in patients. In order to evaluate the influence of several factors on the probability that the respondents will have MCI, a direct logistic regression was conducted. We used the receiver operating curve (ROC) to determine the size of the influence of the administered drugs and associated diseases on the occurrence of MCI. The results were considered statistically significant if the *p*-value was less than or equal to 0.05.

## Results

The study population consisted of 200 adult persons. The main characteristics of the patients are presented in Table 1.

The values of the level of vitamin B12 varied from 73.8–1,476.0 pmol/L for the total number of respondents, 173.8–326.6 pmol/L for the case group of the study subjects, and 198.0–1,476.0 pmol/L for the control group of the patients. The average value and SD of vitamins for all the respondents, for the patients with the diagnosed MCI, and patients with preserved cognition are shown in Table 1. Charslon's comorbidity index (CCI) gives us two results, a total score and an estimate of the probability that a person will live for the next ten years according to the groups presented in Table 1.

The sociodemographic characteristics of the study population and the data of the $\chi^2$ test for the descriptive characteristics of the population were presented in Table 2, where the value of $p > 0.05$ for the entire category was variable, so these variables did not prove to be significant for that research.

Table 3 shows the numerical and percentage frequencies of the subjects' associated diseases and the results of the $\chi^2$ test for comorbidities for MCI. The results were not statistically significant ($p > 0.005$) for sideropenic anemia and other types of anemia, hypertension, angina pectoris, acute myocardial infarction, heart failure, vascular diseases, diseases of the thyroid gland, diseases of cerebral blood vessels, asthma, and other chronic obstructive lung diseases, liver diseases, diseases of the musculoskeletal system and joint tissue, chronic kidney diseases, and benign prostatic hyperplasia, while for the peptic ulcer ($\chi^2 = 24.000$, df = 1, $p < 0.001$) and diabetes mellitus (DM) ($\chi^2 = 21.258$, df = 1, $p < 0.001$), $\chi^2$ test results were found to be significant.

Figures 1 and 2 present the distribution of disease frequency of peptic ulcer and DM, respectively, with the appearance of MCI. By applying the $\chi^2$ test, we have determined that a significantly higher percentage of respondents with MCI also had peptic ulcer and DM.

**Table 1**

**Basic characteristics of study subjects**

| Variable | All subjects | Case group | Control group |
|---|---|---|---|
| Age (years) | 54.18 ± 5.90 (35–65) | 55.34 ± 3.85 (45–60) | 53.79 ± 6.41 (35–65) |
| Vitamin B12 (pmol/L) | 372.2 ± 153.1 (73.8–1476.0) | 229.9 ± 55.5 (73.8–326.6) | 419.6 ± 145.8 (198.0–1476.0) |
| CCI-score | 2.03 ± 1.26 (0–5) | 2.66 ± 1.27 (0–5) | 1.82 ± 1.18 (0–5) |
| CCI-10-year survival rate | 83.95 ± 17.19 (21–98) | 75.24 ± 23.65 (21–98) | 86.85 ± 13.30 (21–98) |

**CCI – Charslon Comorbidity Index. All values are expressed as mean ± standard deviation (minimum-maximum).**

**Table 2**

**Sociodemographic characteristics of the subjects**

| Variable | All subjects | Case group | Control group | $\chi^2$ test | *p*-values |
|---|---|---|---|---|---|
| Gender | | | | | |
| male | 98 (49.0) | 24 (48.0) | 74 (49.3) | 0.027 | 0.870 |
| female | 102 (51.0) | 26 (52.0) | 76 (50.7) | | |
| Marital status | | | | | |
| single | 19 (9.5) | 3 (6) | 16 (10.7) | | |
| married | 147 (73.5) | 39 (78) | 108 (72) | 1.083 | 0.582 |
| widower | 34 (17) | 8 (16) | 26 (17.3) | | |
| Education | | | | | |
| elementary school | 20 (10) | 4 (8) | 16 (10.7) | | |
| high school | 129 (64.5) | 35 (70) | 94 (62.7) | 0.921 | 0.820 |
| college | 38 (19) | 8 (16) | 30 (20) | | |
| master's degree | 13 (6.5) | 3 (6) | 10 (6.7) | | |
| Monthly income | | | | | |
| minimal | 31 (15.5) | 5 (10) | 26 (17.3) | | |
| middle | 96 (48) | 31 (62) | 65 (43.3) | 5.343 | 0.069 |
| high | 73 (36.5) | 14 (28) | 59 (39.3) | | |
| Settlement | | | | | |
| city | 96 (48) | 21 (42) | 75 (50) | 0.962 | 0.327 |
| countryside | 104 (52) | 29 (58) | 75 (50) | | |
| Occupation | | | | | |
| employed | 140 (70) | 33 (66) | 107 (71.3) | | |
| unemployed | 35 (17.5) | 10 (20) | 25 (16.7) | 0.510 | 0.775 |
| retired | 25 (12.5) | 7 (14) | 18 (12) | | |
| Tobacco | 101 (50.5) | 26 (52) | 75 (50) | 0.060 | 0.806 |
| Alcohol | 81 (40.5) | 21 (42) | 60 (40) | 0.062 | 0.803 |
| Immunized | 132 (66) | 33 (66) | 99 (66) | < 0.001 | 1.000 |
| COVID-19 positive | 60 (30) | 15 (30) | 45 (30) | < 0.001 | 1.000 |

***p* – probability. All values are expressed as numbers (percentages).**

**Table 3**

**Associated diseases of the subjects**

| Variable | | All subjects | Case group | Control group | $\chi^2$ test | *p*-values |
|---|---|---|---|---|---|---|
| D50 | yes | 12 (6) | 3 (6) | 9 (6) | 0.000 | 1.000 |
| D51-64 | yes | 3 (1.5) | 2 (4) | 1 (0.7) | 2.820 | 0.093 |
| I10 | yes | 128 (64) | 34 (68) | 94 (62.7) | 0.463 | 0.496 |
| N18 | yes | 5 (2.5) | 3 (6) | 2 (1.3) | 3.350 | 0.067 |
| I20 | yes | 30 (15) | 7 (14) | 23 (15.3) | 0.052 | 0.819 |
| J45 | yes | 4 (2) | 1 (2) | 3 (2) | 0.000 | 1.000 |
| E00-07 | yes | 9 (4.5) | 4 (8) | 5 (3.3) | 1.900 | 0.168 |
| N40 | yes | 22 (11) | 6 (12) | 16 (10.7) | 0.068 | 0.794 |
| I21 | yes | 11 (5.5) | 2 (4) | 9 (6) | 0.289 | 0.591 |
| I50 | yes | 8 (4) | 3 (6) | 5 (1.3) | 0.694 | 0.405 |
| I70-89 | yes | 86 (43) | 25 (50) | 61 (40.7) | 1.333 | 0.248 |
| I60-69 | yes | 1 (0.5) | 1 (2) | 0 (0) | 3.015 | 0.082 |
| J44 | yes | 4 (2) | 1 (2) | 3 (2) | 0.000 | 1.000 |
| M00-99 | yes | 3 (1.5) | 1 (2) | 2 (1.3) | 0.113 | 0.737 |
| K27 | yes | 40 (20) | 22 (44) | 18 (12) | 24.000 | < 0.001 |
| K70-77 | yes | 1 (0.5) | 1 (2) | 0 (0) | 3.015 | 0.082 |
| E10-14 | yes | 42 (21) | 22 (44) | 20 (13.3) | 21.258 | < 0.001 |

**D50 – Iron deficiency anemia; D51-64 – Non-Iron deficiency anemia; I10 – Essential (primary) arterial hypertension; N18 – Chronic kidney disease; I20 – Angina pectoris. J45 – Asthma; E00-07 – Diseases of the thyroid gland; N40 – Prostate enlargement; I21 – Acute heart attack; I50 – Heart insufficiency; I70-89 – Diseases of arteries, veins, small blood vessels, lymphatic vessels, and lymph nodes; I60-69 – Diseases of blood vessels of the brain; J44 – Chronic obstructive pulmonary disease; M00-99 – Diseases of the musculoskeletal system and connective tissue; K27 – Peptic ulcer; K70-77 – Liver disease; E10-14 – Diabetes mellitus. *p* – probability. All values are expressed as numbers (percentages).**

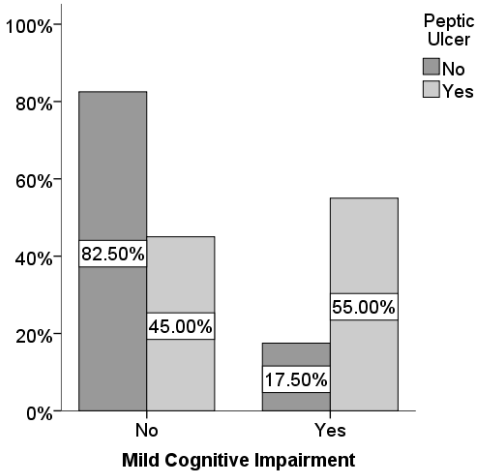


**Fig. 1 – Influence of peptic ulcer on the occurrence of mild cognitive impairment.**

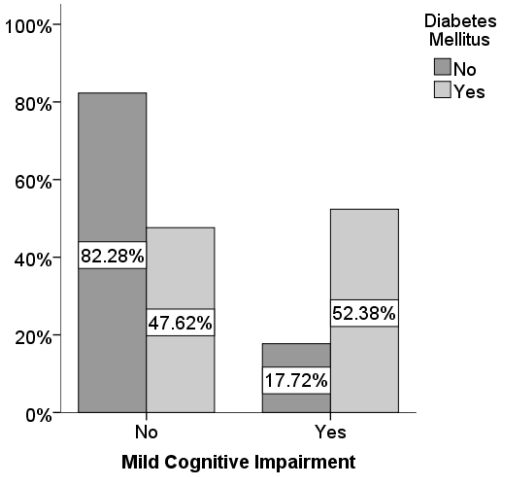


**Fig. 2 – Influence of diabetes mellitus on the occurrence of mild cognitive impairment.**

In Table 4, we have presented absolute values and percentage frequencies of drug use among the study subjects, and by using the $\chi^2$ test, the distribution between drug use and the occurrence of MCI was examined. For mesalazine, the combination of progestin and estrogen, estrogen, tetracycline, penicillin, erythromycin, phenobarbital, methotrexate, diuretics, iron, vitamin B1, vitamin B12, folic acid, levothyroxine sodium, bronchodilators, angiotensin-converting enzyme (ACE) inhibitors, antiarrhythmic agents, beta-adrenergic receptor blockers, trimetazidine, calcium channel blockers, isosorbide mononitrate, allopurinol, acetylsalicylic acid, clopidogrel, hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins), glyceryl trinitrate, the results were not statistically significant ($p > 0.005$), while for PPIs ($\chi^2 = 22.256$, df = 1, $p < 0.001$) and metformin ($\chi^2 = 21.258$, df = 1, $p < 0.001$), high statistical significance was found.

**Table 4**

**Drugs used by subjects**

| Drug | | All subjects | Case group | Control group | $\chi^2$ test | *p*-values |
|---|---|---|---|---|---|---|
| Proton pump inhibitors | yes | 53 (26.5) | 26 (52) | 27 (18) | 22.256 | < 0.001 |
| Mesalazine | yes | 3 (1.5) | 0 (0) | 3 (2) | 1.015 | 0.314 |
| Metformin | yes | 42 (21) | 22 (44) | 20 (13.3) | 21.258 | < 0.001 |
| Progestogens and estrogens | yes | 6 (3) | 2 (4) | 4 (2.7) | 0.229 | 0.632 |
| Estrogens | yes | 14 (7) | 4 (8 ) | 10 (6.7) | 0.102 | 0.749 |
| Tetracycline | yes | 13 (6.5) | 3 (6) | 10 (6.7) | 0.027 | 0.868 |
| Penicillins | yes | 40 (20) | 11 (22) | 29 (19.3) | 0.167 | 0.683 |
| Erythromycin | yes | 6 (3) | 2 (4) | 4 (2.7) | 0.229 | 0.632 |
| Phenobarbital | yes | 1 (0.5) | 1 (2) | 0 (0) | 3.015 | 0.082 |
| Methotrexate | yes | 4 (2) | 1 (2) | 3 (2) | <0.001 | 1.000 |
| Diuretics | yes | 79 (39.5) | 20 (40) | 59 (39.3) | 0.007 | 0.933 |
| Iron | yes | 13 (6.5) | 4 (8) | 9 (6) | 0.247 | 0.619 |
| Vitamin B1 | yes | 15 (7.5) | 4 (8) | 11 (7.3) | 0.024 | 0.877 |
| Vitamin B12 | yes | 15 (7.5) | 4 (8) | 11 (7.3) | 0.024 | 0.877 |
| Folic acid | yes | 8 (4) | 2 (4) | 6 (4) | <0,001 | 1.000 |
| Levothyroxine sodium | yes | 8 (4) | 4 (8) | 4 (2.7) | 2.778 | 0.096 |
| Bronchodilators | yes | 4 (2) | 1 (2) | 3 (2) | <0.001 | 1.000 |
| ACE inhibitors | yes | 95 (47.5) | 25 (50) | 70 (46.7) | 0.167 | 0.683 |
| Antiarrhythmics | yes | 15 (7.5) | 4 (8) | 11 (7.3) | 0.024 | 0.877 |
| Beta blockers | yes | 73 (36.5) | 19 (38) | 54 (36) | 0.065 | 0.799 |
| Trimetazidine | yes | 14 (7) | 2 (4) | 12 (8) | 0.922 | 0.337 |
| Anti-cholinesterase | yes | 1 (0.5) | 0 (0) | 1 (0.7) | 0.335 | 0.563 |
| Tamsulosin | yes | 31 (15.5) | 8 (16) | 23 (15.3) | 0.013 | 0.910 |
| Calcium channel blockers | yes | 64 (32) | 16 (32) | 48 (32) | <0,001 | 1.000 |
| Isosorbide mononitrate | yes | 19 (9.5) | 5 (10) | 14 (9.3) | 0.019 | 0.889 |
| Allopurinol | yes | 13 (6.5) | 3 (6) | 10 (6.7) | 0.027 | 0.868 |
| Acetasalicylic acid | yes | 86 (43) | 21 (42) | 65 (43.3) | 0.027 | 0.869 |
| Clopidogrel | yes | 9 (4.5) | 2 (4) | 7 (4.7) | 0.039 | 0.844 |
| HGM inhibitors | yes | 89 (44.5) | 23 (46) | 66 (44) | 0.061 | 0.805 |
| Glyceryl trinitrate | yes | 7 (3.5) | 2 (4) | 5 (3.3) | 0.049 | 0.824 |

**ACE – angiotensin-converting enzyme; HGM – hydroxymethylglutaryl.**

**All values are expressed as numbers (percentages).**

Applying the $\chi^2$ test of independence, we determined that the values of MCI and the use of PPI ($p < 0.0005$) were dependent variables. An increase in the use of PPIs was associated with an increase in the incidence of MCI (Figure 3). We also found that MCI and the use of metformin ($p < 0.0005$) were related, too. The increase in the use of metformin was associated with an increase in the incidence of MCI (Figure 4).

By analyzing the comorbidities and use of medications of the respondents, we have determined that the MCI was influenced by two comorbidities and the application of two medications. We shall consider which of these four factors had a greater influence on the presence of the MCI (Table 5).

Using the ROC curve, it has been determined which risk factors (DM, peptic ulcer, and use of the medicaments of metformin and the PPI) had the greatest influence on the MCI, as shown in Figure 5.

Since the test area under the curve was occupied by the PPI, we concluded that the PPI had the greatest influence on the presence of the MCI; however, it should be noticed that all the areas had approximately similar values.

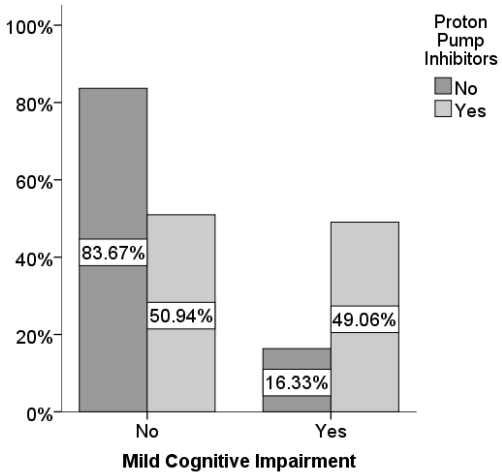


**Fig. 3 – Influence of proton pump inhibitors on the occurrence of mild cognitive impairment.**

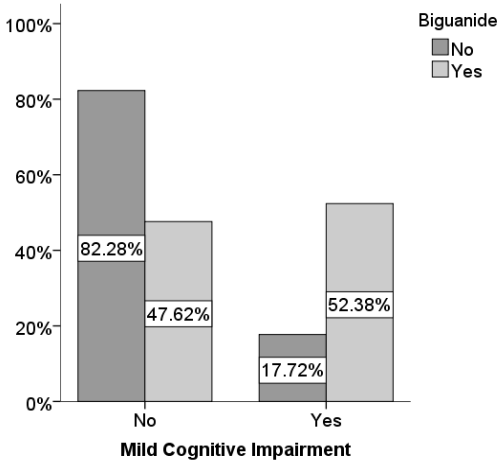


**Fig. 4 – Influence of biguanide (metformin) on the occurrence of mild cognitive impairment.**

**Table 5**

**Influence of drugs and diseases on the occurrence of mild cognitive impairment**

| Variable | AUC | SE | *p* | 95% confidence interval | |
|---|---|---|---|---|---|
| | | | | lower bound | upper bound |
| Proton pump inhibitors | 0.670 | 0.047 | < 0.001 | 0.578 | 0.762 |
| Metformin | 0.653 | 0.048 | 0.001 | 0.559 | 0.748 |
| Peptic ulcer | 0.660 | 0.048 | 0.001 | 0.5660 | 0.754 |
| Diabetes mellitus | 0.653 | 0.048 | 0.001 | 0.559 | 0.748 |

**AUC – area under the curve; SE – standard error; *p* – probability.**

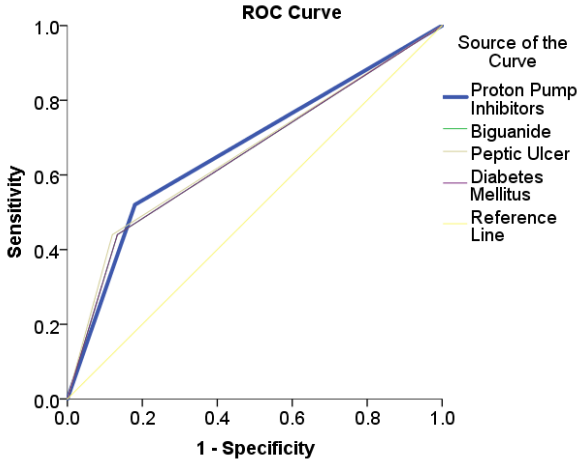


**Fig. 5 – Influence of drugs and diseases on the occurrence of mild cognitive impairment. ROC – receiver operating characteristic curve.**

**Table 6**

**Prediction of mild cognitive impairment**

| Variable | B | SE | Wald | df | *p* | OR | 95% confidence interval of OR | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | lower limit | upper limit |
| Vitamin B12 | -0.048 | 0.009 | 26.565 | 1 | 0.000 | 0.953 | 0.936 | 0.971 |
| Peptic ulcer | 0.950 | 1.096 | 0.751 | 1 | 0.386 | 2.585 | 0.302 | 22.130 |
| Diabetes mellitus type 2 | 1.899 | 0.833 | 5.197 | 1 | 0.023 | 6.681 | 1.305 | 34.198 |
| CCI | -0.147 | 0.314 | 0.218 | 1 | 0.640 | 0.863 | 0.466 | 1.599 |
| PPI | -0.072 | 0.987 | 0.005 | 1 | 0.942 | 0.930 | 0.134 | 6.438 |
| Constant | 12.592 | 2.684 | 22.003 | 1 | 0.000 | 294,278.263 | | |

**CCI – Charlson Comorbidity Index; PPI – proton pump inhibitors; df – degree of freedom; *p* –probability; OR – odds ratio; SE – standard error; B – regression slope (unstandardised coefficient).**

The absolute and relative risk of being exposed to medications was lower than the risk of comorbidities and conditions for the presence of the MCI at the same time in patients in primary health protection.

The following variables had a statistically important influence on the prediction of the MCI: the serum level of vitamin B12 ($p < 0.001$; Wald = 26.565) and type 2 DM (T2DM) ($p = 0.023$; Wald = 5.197). Considering that the highest value of the Wald coefficient had the serum level of vitamin B12, we came to the conclusion that this factor had the highest and statistically important influence on the prediction of the MCI. Besides this result, in this model, T2DM also had an important influence on the prediction of the MCI (Table 6).

The whole model (with all the predictors) was statistically important, c2 (8, H = 200) = 12.352, $p < 0.0005$, which shows that the model could help successfully in distinguishing the patients who have or do not have the MCI: the model, as a whole, explains between 52.7% (R squared Cox-Snell) and 78.1% (R squared Nagelkerke) variance of the MCI. Two variables have given statistically important contributions to the model, and these were the serum values of vitamin B12 and T2DM.

The serum value of vitamin B12 is the strongest factor in predicting the MCI, of which the quotient probability is 0.953. That means that the study patients who had a value of vitamin B12 higher by one unit had 4.7% less frequent MCI compared to those who had a value of vitamin B12 lower by one unit.

## Discussion

Our study presents evidence that low serum concentrations of vitamin B12 and the presence of T2DM are associated independently with the clinical features of MCI in patients of primary healthcare settings. Our statistical models, which also included CCI, the use of PPI, and peptic ulcer disease (which did not show significant association with the MCI), were fairly reliable, explaining between 52.7% and 78.1% variability of the MCI appearances. Other researchers, by using a standard approach for estimating global cognition in examining Parkinson's disease (PD), investigated previously the relationships between cognitive status, comorbidities, metabolic variables, and lifestyle factors of 533 participants with PD from the data of the COPPADIS study. They found that the cognitive outcome was negatively connected to the levels of interleukin (IL)-2, IL-6, iron, and homocysteine ($p < 0.05$) and positively connected to the levels of vitamin B12. The results of this study are similar to our research, as they establish a connection between the increase in the presence of MCI and vitamin B12 deficiency. They also do not indicate the greater importance of the impact of iron deficiency on cognitive abilities [11].

The estimation of the potential value of some microRNAs as diagnostic biomarkers for MCI among patients with T2DM and the identification of other risk factors for MCI was the aim of the research of another study. In this trial, researchers included 163 adult persons with the disease, and they found significantly excessive expression of microR-132 among the study subjects with T2DM with MCI compared to those with normal cognition. In this research, the association between T2DM and MCI was proven, which also proved to be a significant result in our final research model [12]. Other research groups tried to estimate the associations between CCI, polypharmacy, inappropriate use of medications, and cognitive impairments of 105 patients from institutions for long-term care. An important difference was reported between genders, CCI, and cognitive impairment, while every increase of the CCI by one point added the risk of cognitive impairment 3.1 times (95% CI 1.8–5.4), hypertension increases the risk 12 times (95% CI 2.5–67.8). The study conducted by these researchers examines the impact of CCI on the occurrence of MCI, which was also part of our study, but this variable did not stand out as significant compared to the other variables we looked at in our study [13]. The methodological differences, including the sample sizes, could contribute to the differences between the results of the two studies.

The deficiency of cobalamin (vitamin B12) could be connected to the *H. pylori* infection. It had been reported that the serum levels of hemoglobin and cobalamin were significantly increased after the treatment of *H. pylori* infections, regardless of the status of its eradication. This research points to the direct and/or indirect relationships between *H. pylori* gastrointestinal infection, vitamin B12 homeostasis, and PPI use, which was also the assumption in our research that turned out to be correct [14].

It is also known that homocysteine is a risk factor for brain atrophy, cognitive impairment, and dementia and that vitamin B12 and folate are necessary for the methylation of

homocysteine. The previous study, enrolling elderly adult Chinese people, demonstrated that the low level of folates and vitamin B12 in the blood increased serum levels of homocysteine, which were, in turn, significantly related to MCI and Alzheimer's disease. This is another study that proves the connection between MCI and vitamin B12 deficiency, which was the basic hypothesis of our study [15].

In our study, besides the PPI, the use of metformin was significantly associated with the presence of MCI in the univariate analysis, but this connection disappeared when other factors had been analyzed simultaneously within the final statistical model. In the previously published observational study of 200 patients with T2DM treated with metformin, the deficiency of vitamin B12 and diabetic neuropathy were very frequent, and they were related to the increased anti-gastric parietal cell antibodies, tumor necrosis factor (TNF)-α, and dyslipidemia [16]. While using the screening for the B12 deficiency by two markers, methylmalonic acid and homocysteine, one research group noticed a prevalence of 23.3% of the deficiency of vitamin B12 within 490 hospitalized patients. In this study, it was shown that the prolonged use of metformin and PPI was significantly related to vitamin B12 deficiency [17]. The use of these drugs also has a more significant impact in our study on vitamin B12 deficiency compared to other drugs.

The importance of metabolic functions on vitamin B12 deficiency and, consequently, its association with MCI was the subject of our research. Our results are in concordance with many previously published pieces of evidence, either directly or indirectly. The highest risk of weak cognitive function was noticed in the comorbidity group (study subjects with depressive symptoms and metabolic syndrome) in our study, as well as in the previously published study [18].

T2DM was the key factor independently associated with the MCI in our study. In the meta-analysis, this disease was also identified as the key risk factor for dementia and MCI, so the growing prevalence of glycemic disorders had the potential to additionally increase the prevalence of cognitive impairment in the general population [19]. Other researchers reported similar results, and some of them pointed to the mitochondrial abnormalities in patients with DM as the pathogenic pathways for neuronal damage and subsequent cognitive impairment [20, 21]. Our research showed that vitamin B12 deficiency leads to MCI, which suggests that vitamin B12 supplementation can prevent this damage. Some scientists have already dealt with this topic, investigating the proposed neuroprotective effects of vitamin B12 for cognition. There are reports that vitamin B12 affects the function of memory, especially in patients with MCI, so they also point out that supplements of vitamin B12 can be efficient strategies for the prevention and/or treatment of Alzheimer's dementia [22].

Again, the researchers focused on the relationships between the deficiency of vitamin B12 and the use of metformin, including the long-term prescribing settings. Some of them reported that anemia was more frequent in patients using metformin but without significant effects on the status of vitamin B12. On the other hand, the prevalence of neuropathy was higher in the patients using metformin, which, at the same time, had low levels of vitamin B12. This research recommends measures to be applied when using metformin, which, in our research, presented as a significant factor in the occurrence of MCI [23].

Furthermore, the results of another study showed that the levels of vitamin B12 in the serum had a strong negative correlation with the duration of using metformin. At the same time, the status of vitamin B12 did not influence the presence and severity of polyneuropathy, which is one of the important complications of long-standing, uncontrolled DM [24].

Overall, it seems that the use of PPI and metformin, as well as peptic ulcer disease, could be indirectly associated with MCI. The presence of damaged stomach mucosa caused by *H. pylori* infection or other aggressive agents and conditions necessitates the prescription of gastroprotective medications, such as PPIs. On the other hand, gastric mucosal injury disturbs vitamin B12 homeostatic pathways, which, on its own, has a plethora of negative effects on nervous system functions, including cognitive impairment. Similar circumstances probably exist for the use of metformin which represents the first choice of the majority of patients with T2DM. In addition, there is a possibility that metformin could interfere directly with vitamin B12 intestinal absorption, although the exact mechanism is yet to be clarified [25]. Some inconsistency of results between different studies about the relative importance of various risk factors for MCI and/or vitamin B12 deficiency is probably influenced by the heterogeneity of enrolled patient populations. A wide spectrum of drug prescription patterns, disease clinical features, and healthcare settings exist, thus making direct comparisons rather difficult. Nevertheless, we consider that our study contributed significantly to the novel scientific knowledge in the field, quantifying exactly the associations with MCI for at least two factors commonly encountered in primary health care patients, serum concentration of vitamin B12 and T2DM.

## Conclusion

The results of this study revealed that there is an association between the low serum level of vitamin B12 and the presence of MCIs in patients in primary health care. In addition, we have found that a significantly higher percentage of the study patients who had MCI also used PPI and metformin and had peptic ulcer disease, but that associations could have indirect pathways. On the other hand, the increasing number of patients with T2DM also manifested the symptoms of MCI. Overall, the absolute and relative risk of being exposed to the medications and having MCI was lower than the corresponding risks of comorbidities and conditions for the presence of MCI at the same time. The low levels of vitamin B12 and T2DM had the greatest, most unique, and statistically significant influence on predicting the outcome of MCI. These results could contribute to the realization of additional research and medical protective measures for preventing the occurrence of MCI.

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# Comparison of the efficiency of cleaning and disinfection protocols for hand endodontic instruments

## Upoređivanje efikasnosti protokola čišćenja i dezinfekcije ručnih endodontskih instrumenata

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**Abstract**

**Background/Aim**. There is no standard protocol for cleaning and disinfection of used endodontic instruments before their sterilization and reuse. The aim of this study was to determine the efficiency of the different methods of removing biological debris from different types of used hand stainless steel endodontic instruments. **Methods**. A total of 120 hand stainless steel endodontic instruments: Kerr™ reamers, Kerr™ files, and Hedström™ files, each forty ISO 25, used for root canal treatment on extracted teeth, were analyzed. The used instruments were divided into four groups based on different decontamination protocols. The evaluation of the efficiency of the cleaning methods was based on the evaluation of the amount of stained organic residues on the instruments (Van Gieson staining). Samples were analyzed by stereomicroscopy (x40). Statistical analysis was performed using the Mann-Whitney *U* test for the Kerr™ reamers and Hedström™ files, while the One-Way ANOVA/Bonferroni test was used for the Kerr™ files, at a significance level of 5% ($\alpha = 0.05$). **Results**. Residual biological debris was observed on 93.3% of all the samples taken. The thermal disinfectant cleaning method showed the lowest contamination values for all types of instruments. The method of mechanical cleaning showed that the mean value of maximum biologic contamination (MBC) was 58.5% for the Kerr™ reamers and 56.2% for Kerr™ files, while for Hedström™ files, the highest MBC (50.2%) was shown by the ultrasonic method of cleaning. **Conclusion**. The use of a thermal disinfectant was the most efficient cleaning method for all three types of hand endodontic instruments.

**Key words:**
**decontamination; dental instruments; endodontics; infection control; root canal preparation.**

**Apstrakt**

**Uvod/Cilj.** Ne postoji standard za čišćenje i dezinfekciju upotrebljenih endodontskih instrumenata pre njihove sterilizacije i ponovnog korišćenja. Cilj rada bio je da se ustanovi efikasnost različitih metoda uklanjanja ostataka biološkog materijala sa radnih površina upotrebljenih ručnih endodontskih instrumenata od nerđajućeg čelika. **Metode**. Analizirano je ukupno 120 ručnih endodontskih instrumenata od nerđajućeg čelika: po četrdeset *Kerr*™ proširivača, *Kerr*™ turpija i *Hedström*™ turpija ISO 25, upotrebljenih za obradu kanala korena na ekstrahovanim zubima. Na osnovu protokola koji je korišćen za dekontaminaciju instrumenata, upotrebljeni instrumenti su podeljeni u četiri grupe. Procena efikasnosti korišćenih metoda čišćenja zasnovana je na proceni količine prebojenih organskih ostataka na instrumentima (bojenje po Van Gieson-u). Uzorci su analizirani stereomikroskopijom (x40). Statistička analiza dobijenih rezultata izvršena je Mann-Whitney *U* testom za *Kerr*™ proširivače i *Hedström*™ turpije dok je za *Kerr*™ turpije korišćen *One-Way ANOVA*/Bonferoni test, na nivou pouzdanosti od 5% ($\alpha = 0,05$). **Rezultati.** Prisustvo rezidualnih ostataka biološkog materijala uočeno je na 93,3% svih analiziranih instrumenata. Metoda čišćenja toplotnim dezinfikatorom pokazala je najniže vrednosti kontaminacije za sve tipove instrumenata. Metoda mehaničkog čišćenja pokazala je da je srednja vrednost parametra maksimum biološke kontaminacije (MBK) bila 58,5% za *Kerr*™ proširivače i 56,2% za *Kerr*™ turpije, dok je za *Hedström*™ turpije najviša vrednost MBK (50,2%) bila posle ultrazvučne metode čišćenja instrumenata. **Zaključak**. Primena toplotnog dezinfikatora je najefikasnija metoda čišćenja za sva tri tipa ručnih endodontskih instrumenata.

**Ključne reči:**
**dekontaminacija; stomatološki instrumenti; endodoncija; infekcija, kontrola; zub, korenski kanal, priprema.**

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## Introduction

The success of endodontic therapy depends not only on the correct diagnosis, adequate mechanical and medical treatment of the endodontic space, and hermetic obturation but also on the correct implementation and maintenance of the aseptic work protocol [1].

Stainless steel hand endodontic instruments [Kerr™ reamers (KR), Kerr™ files (KF), and Hedström™ files (HF)] are generally accepted as reusable instruments [1–3], although there are literature references that support their single-use [4–6]. During instrumentation, organic and inorganic debris (residues of vital and necrotic tissue, dentin chips, bacteria, blood, and its decomposition products) may remain on the threads of endodontic instruments, which may have antigenic, infectious, and nonspecific irritating potential [7–9].

Prevention of the possibility of irritation of periapical tissues, cross-infections between patients, and avoidance of additional introduction of foreign microorganisms into the endodontic space is achieved by sterilization of used, contaminated endodontic instruments before their reuse [10]. About 700 species of microorganisms persist in the oral cavity, and the reuse of endodontic instruments potentially carries the risk of transmitting bacterial and viral diseases (*Fusobacterium nucleatum*, *Porphiromonas Gingivalis*, and *Streptococcus mutans* are the most common bacteria isolated from infected root canals) [10–12].

To be reused, endodontic instruments must undergo a process of cleaning and disinfection because the presence of organic material and debris on the surface can compromise their sterilization [10]. A strict treatment protocol for the instruments used should be followed to eliminate or reduce the risk of cross-contamination between patients as efficiently as possible [5]. Only clean endodontic instruments can be effectively sterilized, so cleaning is a particularly important step in the cycle of preparing instruments for reuse [6]. Spaulding's classification according to the potential risk for infection transmission describes three categories of instruments (critical, semi-critical, and non-critical), each of which has specific requirements for reprocessing [12]. Infection control and quality management in the dental office classify endodontic instruments as critical and are subject to stricter requirements for processing and reuse [7, 8].

Evaluation of the success of different methods of cleaning and disinfection of endodontic instruments before their sterilization has been the subject of numerous scientific studies [1–3, 13–24] in the constant need to improve aseptic work and provide the most efficient cleaning and disinfection techniques. Studies by Aslam et al. [25] and Mustafa [26] emphasize the importance of involving dental assistants and dentists in training programs for the preparation of endodontic instruments for sterilization and the application of a successful cleaning and disinfection protocol.

The aim of the study was to check the efficiency of different methods of removing biological debris from work surfaces of used hand stainless steel endodontic instruments.

## Methods

This study was conducted with the consent of the Ethics Committee of the Faculty of Dentistry, University of Belgrade (No. 36/2; 2020). A total of 120 new instruments were used in the study: forty KR, forty KF, and forty HF (all of Shenzhen Denco Medical Co., Ltd. Guangdong, China), size #25. Directly from the original packaging, the instruments were subjected to ultrasonic (US) decontamination to be cleaned of inorganic and organic debris that occurs during the production process.

The instruments were used for the manual treatment of root canals on 120 intact single-rooted teeth extracted for orthodontic reasons or advanced periodontitis. After the formation of the access cavity, a certain working length was determined (0.5 mm shorter than the length at which the tip of the instrument appears at the apex). All teeth were processed with manual instruments, KR #10–20 (Dentsply, Sirona, USA), and between each instrument, irrigated with 2 mL of 2% NaOCl (Chloraxid 2%, Cerkamed, Polska). Instruments #10 and #15 were used in a clockwise motion, while instrument #20 was used in a combination of clockwise motion and balanced force motion.

Each #25 instrument was used to process one canal until the working length was reached. KR were used in a combination of clockwise and balanced force motion. KF and HF were activated in the canals by the motion of filing and scraping (up and down). During the instrumentation, NaOCl was used as an irrigant using a plastic syringe and an endodontic irrigation needle with a closed tip and side openings (side-vented needle, SmearClear, SybronEndo) in the amount of 2 mL before applying the instrument to the canal.

After instrumentation, the instruments were stored in closed endodontic boxes. The instruments were randomly divided into four groups of thirty instruments (ten KR, ten KF, and ten KH) and were subjected to different cleaning and disinfection methods/protocols: Method 1 – the instruments were immersed in a 3% solution of Gigasept Instru AF disinfectant (Schulke & Mair GmbH, Nordstedt, Germany) for 15 min and then mechanically cleaned with a brush under running water for two min for each instrument; Method 2 – the instruments were immersed in a 3% solution of Gigasept Instru AF disinfectant for 15 min and then cleaned in a US bath with the same disinfectant for 10 min; Method 3 – the instruments were immersed in a 3% solution of Gigasept Instru AF disinfectant for 15 min, then mechanically cleaned with a brush under running water and finally cleaned in a US bath with 3% solution of the same disinfectant for 10 min; Method 4 – the instruments were immersed in a 3% solution of Gigasept Instru AF disinfectant for 15 min and then subjected to a thermal disinfection treatment with water disinfectant "Miele PG 8582 CD" (Miele & Cie. KG, Gutersloh, Germany) (compliant to EN ISO 15883).

Used instruments were disposed of in a Biohazard Sharps container in disinfectant. Cleaning agent (Neodisher® Mediclean Forte 0.5%) and rinse aid (Neodisher® Mediklar Special 0.03%) were used with a program washer/disinfector

cleaning cycle for 10 min at 93°C completed by hot air drying for 15 min at 110°C.

After the cleaning and disinfection protocol, all instruments were subjected to the Van Gieson solution staining method (1% aqueous solution of acid magenta and a saturated aqueous solution of picric acid, which stains collagen red orange) for three min. After rinsing in distilled water for one min, the instruments were dried on an endodontic stand (at room temperature).

The instruments were placed in a square holder, which enabled the rotation of the instruments by 90°. The working surfaces of the examined instruments were recorded with a stereomicroscope with an integrated digital camera (Boeco BSZ-405, Germany) at x40 magnification. Digital images were saved as JPG format files and then processed and analyzed in Scopeimage 9.0 software (Telescope, Austria).

The evaluation of the efficiency of the applied cleaning and disinfection methods was performed by analyzing saved digital images using the method of Linsuwanont et al. [13] (based on the number of residual debris).

The found debris was recognized as a biological risk factor and was evaluated by grades based on the amount of repainted material: Grade 0 – clean surface without any debris; Grade 1 – the presence of an organic film (a thin non-textured layer that covers part of the surface of the instrument and turns red); Grade 2 – slight discoloration in the form of individual, rare particles of debris, scattered on the surface of the working part of the instrument); Grade 3 – medium discoloration, organic particles covering the surface of the instrument in the form of a continuous cover; Grade 4 – pronounced discoloration with fields on the instruments where the grooves of the work surfaces are completely filled with debris.

The instruments were observed at three levels: apical, middle, and coronary. At each level, the samples were tested on four sides, gradually rotating by 90° so that each sample had twelve measurements and thus covered the entire working surface of the instrument. The results of all positions were summed, so the minimum grade value was 0 (without organic material), and the maximum was 48 (all surfaces were heavily contaminated with debris). For each instrument, the mean value was calculated and converted into the percentage mean value of the maximum biological contamination (% MBC).

The assessment of the detected contamination was performed by two independent researchers, and the harmonization of the results was performed by Cohen Kappa analysis. Statistical analysis of the results obtained by the Mann-Whitney *U* test and the One-Way ANOVA/Bonferroni test was performed.

## Results

After analyzing the obtained images, a different degree of % MBC was observed on 112 (93.3%) instruments. A completely clean surface was observed on 8 (6.7%) instruments cleaned with a thermal disinfectant.

The fourth method (method with thermal disinfectant) showed the lowest values of contamination for all types of instruments. After the application of the thermal disinfectant, a completely clean instrument surface was obtained on eight instruments (3 KR, 2 KF, and 3 HF). The highest contamination of KR instruments (Table 1, Figure 1) was shown for the first method (mechanical cleaning) with 58.5 ± 8.9% MBC value. Comparing all four methods of cleaning for KR, a statistically significant difference at the level of $p < 0.005$ was observed.

**Table 1**

**Contamination of different types of instruments after different methods of cleaning and disinfection (% MBC)**

| Type of instrument | Method 1 | Method 2 | Method 3 | Method 4 |
|---|---|---|---|---|
| Kerr reamers | 58.5 ± 8.9 | 49.4 ± 5.9 | 42.1 ± 7.8 | 12.9 ± 9.3 |
| Kerr files | 56.2 ± 12.3 | 51.9 ± 8.9 | 39.8 ± 8.1 | 14.6 ± 7.9 |
| Hedström file | 37.7 ± 5.8 | 50.2 ± 7.8 | 38.9 ± 5.8 | 11.2 ± 7.9 |

**MBC – maximum biological contamination.**
**Description of the methods is given in the paragraph Methods.**
**Results are expressed as mean ± standard deviation.**

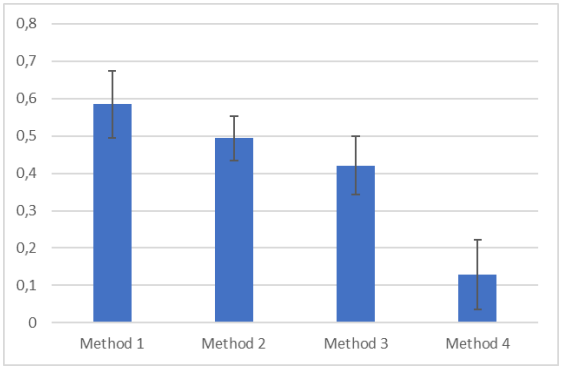


**Fig. 1 – Prevalence of Kerr reamers contamination after four different cleaning and disinfection methods (expressed in % MBC).**
**MBC – maximum biological contamination. Description of the methods is given in the paragraph Methods.**

Analyzing the success of different methods of cleaning and disinfection of KF instruments (Table 1; Figure 2), the highest contamination was shown for the first method (mechanical cleaning), with 56.2 ± 12.3% MBC value. Comparing all four methods of KF cleaning, a statistically significant difference was observed at the level of $p < 0.005$, except between the first and the second method.

For HF-type instruments, the highest contamination was shown for the second, US method, with a 50.2 ± 7.8% MBC value (Table 1; Figure 3). Comparing all four methods of cleaning for HF, a statistically significant difference was observed at the level of $p < 0.005$, except between the first and third methods.

Analyzing the success of different methods of cleaning and disinfection on the apical third of the instruments (Table 2), a statistically significant minimum amount of contamination was observed for all types of instruments after the application of thermal disinfectant compared to all other methods ($p < 0.005$) (KR 15.6 ± 13.9%, KF 18.1 ± 10.4%, and HF 16.2 ± 11.8%). The highest contamination was observed on the apical third of the KR after the first, mechanical method (61.9 ± 19.6%) and on the apical surface of the KF (61.2 ± 12.4%) and HF (55.0 ± 9.7%) after the US method (Figures 4 and 5).

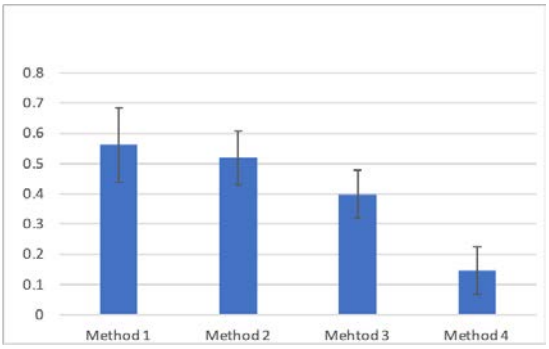


**Fig. 2 – Prevalence of Kerr files contamination after four different cleaning and disinfection methods (expressed in % MBC).**
**MBC – maximum biological contamination. Description of the methods is given in the paragraph Methods.**

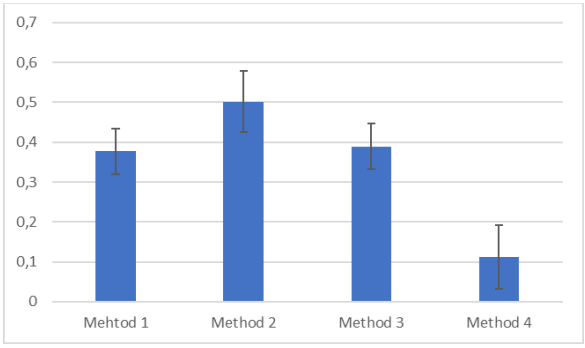


**Fig. 3 – Prevalence of Hedström files contamination after four different cleaning and disinfection methods (expressed in % MBC).**
**MBC – maximum biological contamination. Description of the methods is given in the paragraph Methods.**

**Table 2**
**Contamination of different types of instruments after different methods of cleaning and disinfection on the apical, middle, and coronal third of the instruments (% MBC)**

| Type of instrument | Instrument third | Method 1 | Method 2 | Method 3 | Method 4 |
|---|---|---|---|---|---|
| Kerr reamers | A | 61.9 ± 19.6 | 60.0 ± 9.4 | 46.2 ± 12.6 | 15.6 ± 13.9 |
| | M | 58.8 ± 6.7 | 43.8 ± 7.8 | 37.5 ± 9.8 | 13.1 ± 9.5 |
| | C | 55.0 ± 8.2 | 44.4 ± 10.8 | 42.5 ± 10.5 | 10.0 ± 9.9 |
| Kerr files | A | 55.6 ± 14.3 | 61.2 ± 12.4 | 40.0 ± 12.9 | 18.1 ± 10.4 |
| | M | 51.9 ± 12.5 | 48.8 ± 12.4 | 38.8 ± 10.5 | 13.1 ± 9.9 |
| | C | 61.2 ± 14.9 | 45.6 ± 11.4 | 40.6 ± 7.9 | 12.5 ± 12.2 |
| Hedström file | A | 45.6 ± 13.2 | 55.0 ± 9.7 | 40.0 ± 9.4 | 16.2 ± 11.8 |
| | M | 31.9 ± 4.6 | 51.2 ± 11.3 | 36.9 ± 6.9 | 9.4 ± 8.9 |
| | C | 35.6 ± 6.6 | 44.4 ± 9.1 | 40.0 ± 5.3 | 8.1 ± 8.9 |

**A – apical third, M – middle third, C – crown third; MBC – maximum biological contamination.**
**Results are shown as mean ± standard deviation.**
**Description of the methods is given in the paragraph Methods.**

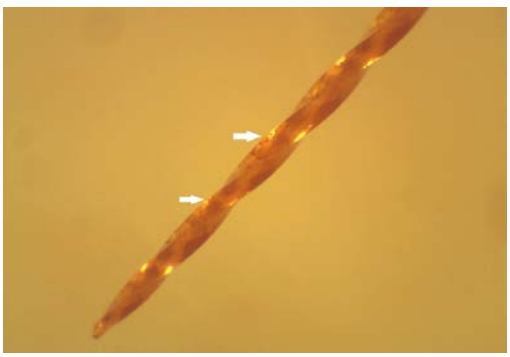

**Fig. 4 – Apical third Kerr reamer instrument after combining mechanical and ultrasonic cleaning methods (red spots, white arrows).**

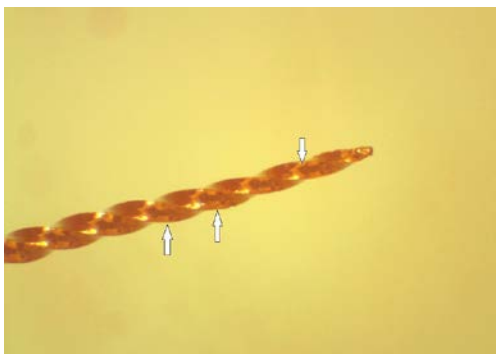

**Fig. 5 – Apical third of Kerr file instrument after mechanical cleaning method (red spots, white arrows).**

A statistically significant minimum amount of contamination was observed on the middle and coronal thirds for all types of instruments after the application of thermal disinfectant compared to all other methods ($p < 0.005$) (Table 2; Figure 6).

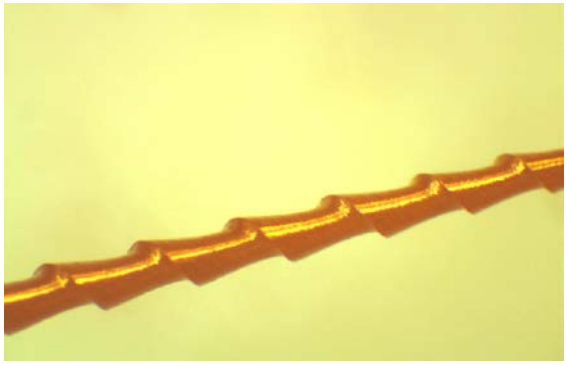

**Fig. 6 – Middle third completely clean in Hedström-type instruments after the application of the thermal disinfectant.**

## Discussion

Respecting and providing aseptic conditions during endodontic treatment is important, not only in preventing the risk of infection but also in ensuring the success of this therapy. The application of an adequate disinfection and sterilization control protocol prevents and reduces the possibility of the spread of infections, and the greatest risk is represented by blood-borne infections (human immunodeficiency virus/acquired immunodeficiency syndrome, hepatitis C virus, hepatitis B virus) [8]. Endodontic instruments come into close contact with biological fluids and are applied in a working field that is often bacterially contaminated, so it is important to study and apply all the factors that ensure aseptic work [7–9].

The complex geometry and gracefulness of endodontic instruments, as well as the demanding technical production conditions, affect the appearance of inorganic and organic material on new, unused instruments [14, 16, 27]. The presence of metal chips of nickel-chromium and organic material (various lubricants and human material) was noticed on new instruments; possible bacterial contamination was also noticed [14, 16, 27]. Therefore, new instruments must also be subjected to cleaning of inorganic and organic debris that has occurred during the production and packaging or storage process, which in this study was done using a US bath with a mild disinfectant. This procedure was applied following the literature recommendations that suggest decontamination with a US bath or thermal disinfectant [18–20]. The recommendation is that instruments should not be placed in special containers during the application of the US method to improve the removal of detritus, which was observed in this study.

In cases where the recommendations for single use of endodontic instruments are not followed, and the endodontic instrument is used again on another patient, it is necessary to apply a strict cleaning and sterilization protocol to eliminate or reduce the risk of cross-contamination between patients [12]. The presence of debris on the instruments can compromise their sterilization due to the impossibility of steam penetration, but also due to the nature of biologically active material which, with minimal moisture content, in its vegetative forms (spores) becomes more resistant to heat [21].

The high percentage (93.3%) of contamination on the examined instruments in this study confirms the difficulties in adequate cleaning of endodontic hand instruments and preparation for their sterilization and reuse in clinical conditions [3–6, 13–16]. This finding is consistent with the studies of Popović et al. [14] (84%), Linsuwanont et al. [13] (90%), Khullar et al. [15] (94%), Buchanan et al. [16] (94%), and Smith et al. [3] (98%), who also noticed a high prevalence of contamination after various methods of cleaning endodontic instruments. The results of a study by Aasim et al. [17] showed the impossibility of complete decontamination of endodontic instruments (especially the removal of calcium hydroxide medications).

This study aimed to assess which of the applied method is the most effective for a given type of instrument. Examining the contamination of three types of hand endodontic instruments (KR, KF, and HF) after applying four different methods of cleaning and disinfection, the most successful method is convincingly the application of thermal disinfectant for all three types of instruments. Only this method obtained a completely clean surface of the tested instruments.

This result agrees with the results of Vassey et al. [18], who confirmed the effectiveness of thermal disinfectants for cleaning endodontic instruments. The aforementioned authors showed that disinfection devices are significantly more efficient in cleaning endodontic instruments than a combination of manual and US cleaning. The use of thermal disinfectants in a routine procedure can primarily improve the efficiency of cleaning and disinfection and increase productivity, but also reduce the exposure of dental staff to contaminated sharp instruments [19, 20]. According to Assaf et al. [20], thermal disinfectants can remove detritus more effectively than other methods (but cannot remove it completely). Furthermore, Assaf et al. [20] and Souza et al. [21] pointed out that the effectiveness of the thermal disinfectant decreases with the decrease in the diameter of the endodontic file. The results of this study also highlighted a lower decontamination efficiency depending on the diameter of the instrument (the apical segment is more difficult to clean and disinfect).

Analyzing the success of the cleaning protocols of KR and KF, the methods of mechanical removal of impurities with a steel brush under running water and US decontamination were singled out as insufficiently successful if performed individually. However, combined, these two methods give statistically better success in removing debris from the surface of these instruments. The explanation for this probably lies in the design of these instruments, where the cutting edge of the reamers is placed more longitudinally in relation to the axis of the instrument (an angle of about 20°), while the cutting edge of the KF is under 40°, which certainly makes it difficult to access the fibers of the cleaning brush. Therefore, the combination of the application of a US bath with a disinfectant after mechanical cleaning with a steel

brush under running water gives the best results for cleaning KR and KF if we are unable to apply a thermal disinfectant. Most chemical methods and the use of strong disinfectants during the decontamination process potentially damage the metal surfaces of endodontic instruments (corrosion, potentiation of existing defects), so the use of mild disinfectants in the US tub is recommended [3, 22]. Disinfectants have a double effect, breaking down biological contamination and removing detritus from the blades of endodontic instruments.

The US bath uses vibrational energy that transmits sound waves in the liquid to remove biological material from the surface of the instrument. US cleaning is an efficient method that saves time and saves dental staff, although it is not able to remove all contamination [28]. Van Eldik et al. [23] have also confirmed the harmfulness of using instrument containers during US decontamination, which by their design, can dampen sound waves and reduce the effect of cleaning and disinfection.

The results of the study by Souza et al. [21] also indicate that the best results in the decontamination of KR (ISO 25) are achieved by the combined use of the mechanical method of brushing with a US bath.

Although the use of plastic, nylon, and metal brushes for cleaning endodontic instruments is a common and most used method, many researchers have confirmed its ineffectiveness [3, 13, 21]. Linsuwanont et al. [13] observed that brushing instruments while in the stand restricts access to all surfaces, and often the bristles of the brush are larger than the width of the grooves of the instruments (apical thirds and instruments of small dimensions). In addition, the brushing is performed perpendicular to the longitudinal axis of the instruments while they are held between the fingers of the dental assistant, and the fibers of the metal brush do not move along the blade of the instrument but over them. In addition, the results of research by Souza et al. [21] and Smith et al. [3] point out that the application of this mechanical, manual brush cleaning poses a risk to operators due to possible injury and infection, but also the formation of aerosols during cleaning and decontamination procedures. However, according to the results of these studies, the presence of organic and inorganic detritus does not interfere with the sterilization process by creating a protective layer for bacteria because the heat of the autoclave is able to destroy all microorganisms (except in the case of prions) [3, 7, 8].

Analyzing the cleaning and disinfection protocols of HF, the method of US decontamination is the least successful. Compared to this method, the method of mechanical cleaning-brushing is statistically significantly more successful, as well as the combined method (brushing and US method). The reasons for the difference in the efficiency of different methods of decontamination of HF in relation to KR and KF should be sought in the different designs of these instruments. KR and KF are created by twisting a triangular, i.e., quadrangular wire profile which twists counter-clockwise, while HF are created by milling round wire profiles, which results in spirally twisted blades, so they are one of the most efficient hand tools due to their specific design (blade edges are almost at right angles, an angle bigger than 65°). The results of this study show that for HF, in the absence of a thermal disinfectant, the dominant method is the mechanical removal of impurities because brush fibers can penetrate better between cutting edges. This finding confirms that the design of the instruments can influence the success of the cleaning method and the selection of the best protocol for their decontamination. The disadvantages of this mechanical method are, in addition to the long-time protocol, the disruption of the surface structure of the instruments as well as the risk of additional contamination and injury to the dental staff performing it [12].

The result highlighted in this study is the influence of instrument design on the efficiency of the cleaning and disinfection method. While analyzing the influence of the design of the working part of endodontic instruments, it was noticed that the instruments of HF type had the lowest degree of contamination. This finding is consistent with the results of a study by Van Eldik et al. [23], who showed a cleaner surface of HF compared to rotating instruments after the application of a thermal disinfectant (88.6%). According to the same study, the size and conicity of endodontic instruments have no effect on their cleaning efficiency, unlike the design of cutting surfaces. Due to the specific design of HF (the cutting edges are at right angles), this study showed the necessity of mechanical cleaning of instruments of this type because the application of a US tub only gave the worst result.

Another result highlighted in this study is the different degrees of % MBC observed on different parts of the work surface of hand endodontic instruments (apical, middle, and coronal third). The analysis of the results showed the highest degree of contamination on the apical third of KR and HF after using all four methods and of the KF after using the US and thermal disinfectant methods. Mechanical cleaning (method 1) was the least efficient to remove impurities from the coronal third of KF (maximum % MBC). The combined mechanical US technique (method 3) performed best in terms of cleaning all types of instruments in their middle third. This method showed similar results on the apical and coronal thirds of KF and HF. Although no parameters have been found in the available literature with which the results of this study can be compared, this result can be explained by different designs of examined instruments (different cross-sections, different cutting edges, blade depths, different cutting angles).

Plasma cleaning is the most modern method of cleaning and disinfection described by Whittaker et al. [24], involving the use of ionized gases. The advantages of this technique are its nonaggressiveness on the working surface of the instruments and the fact that its application does not release toxic substances (the remaining gases are usually $CO_2$, $H_2O$, and $N_2$).

The reuse of endodontic instruments has the basic goal of reducing material costs [4–6, 10, 16]. However, if the cost of disposable instruments is compared to the additional costs arising from reuse, such as the cost of cleaning instruments, sterile storage, and keeping proper records, the projected savings are reduced to a minimum [6, 12, 28, 29]. The impossibility of completely removing the biologically active material in this study supports the shortcomings of multiple uses of instruments. Ana-

lyzing mandatory quality management and the possibility of prolonged infections, this important procedure in dental practice is increasingly a topic of clinical discussion.

## Conclusion

The most efficient method of cleaning and disinfection was thermal disinfection for all three types of hand endodontic instruments – KR, KF, and HF, and the efficiency of this technique depends on the diameter of the endodontic instrument. Removing biological material was more difficult to perform in the apical portion of manual instruments, regardless of the instrument type.

In the absence of thermal disinfection, the combination of US bath and mechanical cleaning gives the best results for cleaning all types of hand instruments.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgment

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# Practical skills of persons with vision impairment

## Praktične veštine osoba sa oštećenjem vida

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**Abstract**

**Background/Aim.** The acquisition of practical skills (PS), as well as adaptive behavior (AB) in general, is affected by an array of personal and environmental factors. The aim of this study was to determine the level of acquisition of practical adaptive skills (PAS) among adults with vision impairment (VI), in comparison to the norms among the general population and with regard to the vision status (low vision and blindness), age of vision loss onset, gender, age, living arrangements, education, and employment status. **Methods.** Seventy-nine (62.2%) participants who were blind and forty-eight (37.8%) participants with low vision and typical intellectual abilities voluntarily took part in the study. The respondents were aged from 19 to 60 years, with a mean age of 36.1 ± 11.8 years. PAS were assessed using the PS domain which is part of the Adaptive Behavior Assessment System II – ABAS II. **Results.** The scores achieved in the skill areas of the PS domain range from extremely low to average. Extremely low scores were detected in the Work skill area, while for the skill areas of Community use, Home living, and Health and safety, the scores were below average, and average scores were noted in the Self-care skill area. The degree of PS acquisition among participants with VI depended primarily on the vision status, but a significant connection with living arrangements and employment status was also established. **Conclusion.** Persons with VI showed significant limitations in the area of PS, which indicates the need for support programs designed to foster the development of self-reliance.

**Key words:**
**adaptation, psychological; aptitude; surveys and questionnaires; vision, low; vision disorders.**

**Apstrakt**

**Uvod/Cilj.** Niz ličnih i činilaca sredine utiče na usvajanje praktičnih veština (PV), kao i na adaptivno ponašanje (AP) u celini. Cilj rada bio je da se utvrdi nivo usvajanja praktičnih adaptivnih veština (PAV) kod odraslih osoba sa oštećenjem vida (OV), u poređenju sa normama u opštoj populaciji, kao i u odnosu na vizuelni status (slabovidost i slepilo), vreme gubitka vida, pol, životno doba, porodični status, nivo obrazovanja i radni status ispitanika. **Metode.** U istraživanju je dobrovoljno učestovalo 79 (62,2%) slepih i 48 (37,8%) slabovidih ispitanika tipičnih intelektualnih sposobnosti. Ispitanici su bili starosti od 19 do 60 godina, srednja vrednost 36,1 ± 11,8 godina. Za procenu nivoa usvajanja PAV korišćen je domen PV koji pripada sistemu *Adaptive Behavior Assessment System* II (ABAS II). **Rezultati.** Vrednosti skorova ostvarenih na subtestovima/oblastima koji pripadaju domenu PV kretali su se u rangu od ekstremno niskih do prosečnih. Ekstremno niske vrednosti su zabeležene na subtestu Posao. Na subtestovima Život u zajednici, Život u kući i Zdravlje i bezbednost, skorovi su bili u rangu vrednosti ispod proseka, a na subtestu Briga o sebi, postignuti rezultati bili su u rangu prosečnih. Stepen usvajanja PV kod osoba sa OV prvenstveno je zavisio od kategorije OV, a utvrđena je i značajna povezanost sa porodičnim statusom i radnim iskustvom. **Zaključak.** Osobe sa OV ispoljavaju značajna ograničenja u oblasti PV, što ukazuje na potrebu podrške programa koji podstiču razvoj njihovog nezavisnog funkcionisanja.

**Ključne reči:**
**adaptacija, psihološka; sposobnost; ankete i upitnici; vid, oslabljen; vid, poremećaji.**

### Introduction

People with vision impairment (VI) face many difficulties in different areas of life, such as daily living skills, orientation, mobility, leisure activities, social interaction, and career choice [1,2].

According to the results of some studies, VI greatly affects the development of motor skills, which in turn directly affects the acquisition of practical skills (PS) [3–6], relevant for self-care and instrumental daily living skills [7]. Among conceptual and social skills, PS represent an integral component

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of adaptive behavior (AB), which is essential for the person's independence and safety [8–10].

The acquisition and maintenance of PS is particularly difficult for people with VI, as the learning process requires observation, demonstration, and practice in everyday situations. Children and adults who are blind have a more narrow repertoire of acquired PS compared to those with low visio [4, 11, 12].

Besides confirmed differences related to the level of VI, daily functioning, vitality, and outdoor participation among young adults (18 to 25 years old) with VI significantly are lower than the norms of the age-related general population [13].

Adults and elderly people with VI face difficulties with certain practical life skills two to three times more frequently than individuals from the general population. Binns et al. [14] point out that persons with VI have a more limited set of PS, that they use the PS less often, feel insecure, and exhibit greater dependence on assistance from others. That primarily pertains to those PS which require good eyesight, such as personal hygiene, meal preparation, and movement outside the home (e.g., visits to a physician or shopping) [15, 16]. Elderly people with vision loss (both partial and total) primarily face difficulties when it comes to independent movement, the use of public transport, completing tasks in their wider surroundings, and regular visits to the doctor [17–20]; however, difficulties concerning household chores are not insignificant either.

PS, like AB in general, are affected by an array of personal and environmental factors [21]. That is why the creation of more efficient programs aimed at improving the self-reliance of people with VI requires a good understanding of the degree to which they succeed in acquiring a wide spectrum of practical adaptive skills (PAS) and the factors that affect this process [22].

The aim of this study was to determine the level of acquisition of PAS among adults with VI in comparison to the norms among the general population and with regard to the vision status (low vision and blindness), age of vision loss onset, gender, age, living arrangements, education, and employment status.

## Methods

### *Study sample*

The participants are members of the Organization of Citizens with Visual Impairment of Belgrade (OCVIB), which is the largest in Serbia by number of members. The research sample consisted of 127 volunteers with VI of both genders, aged 19–60 years, mean age of 36.1 ± 11.8 years. The group included 79 (62.2%) participants who were blind [visual acuity lower than 0.05 (20/400)] and 48 (37.8%) participants with low vision [visual acuity ranging from 0.05 (20/400) to 0.3 (20/60)] according to World Health Organization, 2016 [23]. Seventy-nine participants (62.2%) had VI since birth. All participants lived in an urban environment and were involved in some of the rehabilitation programs within the OCVIB (computer work, massage training, psychosocial counseling, orientation and mobility training, and daily life skills training). According to data obtained by the psychosocial services of the OCVIB, all participants exhibited typical intellectual abilities with no additional impairment.

The research sample was almost completely balanced regarding gender (51.2% of female, 48.8% of male participants). Concerning living arrangements, 60 (47.2%) persons lived with their parents, 41 (32.3%) lived with their spouses, and 26 (20.5%) lived alone. The majority had graduated from high school (n = 86; 67.7%) and a third of them had higher education (n = 41; 32.3%). Considering that only 38 (29%) respondents were employed at the time of the testing, we considered their work experience as well. More than half of the group confirmed that they had prior work experience (n = 80; 63%).

### *Instrument and procedure*

To assess PS as a component of AB, we used the PS domain from the Adaptive Behavior Assessment System II – ABAS II [24]. This domain consists of five skill areas (Community use, Self-care, Health and safety, Home living, and Work), encompassing a total of 116 items. Community use assesses the use of community resources and skills required for shopping and moving around. Self-care subtest involves the skills needed for eating, getting dressed, hygiene, and face and body care. Activities such as cleaning, doing repairs, housekeeping, and preparing food all relate to the Home living subtest. The skills necessary for maintaining health and responding to injury are part of the Health and safety subtest and include following safety rules and general caution. The Work subtest is applied only when a participant is employed part-time or full-time. It refers to the skills necessary for successful functioning and keeping a job (completing work tasks, working with a supervisor, and keeping up with the work schedule).

The respondents graded a statement by selecting one of the four answers provided: 0 – not applicable, 1 – never, 2 – sometimes, and 3 – always. The answers were added up after each skill area, providing a raw score which was subsequently converted to a standard score based on the chronological age of the participant. The standard score for each separate domain was obtained by adding up all the raw scores from the separate skill areas.

The values of the composite scores in each domain, based on the achievement of the American population, are sorted into one of the following categories: "very superior" (130 or more), "superior" (120–129), "above average" (110–119), "average" (90–109), "below average" (80–89), "borderline" (71–79), and "extremely low" (70 or less). The standard scores in skill areas belonging to the PS domain are distributed across the following categories: "superior" (15 or more), "above average" (13–14), "average" (8–12), "below average" (6–7), "borderline" (4–5), and "extremely low" (3 or less).

The study was conducted over six months, directly, through an interview with each participant, in the daytime

period, at the moment which was the most suitable for them. Professionals of the Organization assisted in making the initial contacts with the participants. We contacted 170 adults with VI by phone, and 131 of them accepted participation in the research, and for four participants, the data was not completed. After acquiring the data relevant to the visual status, age of vision loss onset, gender, age, education, living arrangements, and work experience, the participants were familiarized with the structure of the scale and the answers provided. The statements were read aloud to the participants, and their responses were recorded.

The study was approved by the Ethics Committee of the University of Belgrade – Faculty of Special Education and Rehabilitation (No. 17/30)

*Data analysis*

The Statistical Package for Social Science (SPSS, version 19) was used for data analysis. The achievements of participants with VI in the PS domain are presented in the form of basic descriptive measurements: arithmetic mean, standard deviation, and minimum and maximum values. The relationship between the variables was determined using the correlation coefficient. The significance of the differences in the achievements in the applied skill areas in accordance with the defined independent variables was tested through the application of one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA).

**Results**

Observing the range of values of the standard scores achieved by participants with VI in different skill areas belonging to the PS domain, it is evident that the minimal values range from 0 to 2, which is extremely low in comparison to the norm-referenced score provided by the ABAS II, while the maximum values fall within the "above average" (13–14) to "average" (8–12) level. In comparison to the norm-referenced skill area scores, the mean values fall within the category of "extremely low" in the skill area of Work and "below average" in the skill areas of Community use and Home living. The scores achieved in the skill areas of Health and Safety and Self-care fall within the "average" category (more details in Table 1).

In comparison to the norm-referenced score, the minimum values of the composite scores achieved by participants with VI in the PS domain are at the "below average" level, while maximum values are "average". The mean value of the score in the PS domain falls within the "borderline" category (4–5) (more details in Table 1).

Table 2 shows the intercorrelation between the skill areas belonging to the PS domain (more details in Table 2).

*The relationship between the Practical skills domain scores and independent variables*

Pearson coefficient showed no significant correlation between the age of a respondent and any of the assessed PS areas.

**Table 1**

**Achieved scores for the skill areas of the Practical skills domain presented with descriptive parameters**

| Practical skills domain | Values | |
|---|---|---|
| | min-max | mean ± SD |
| Skill areas | | |
| Community use | 1 –13 | 6.9 ± 2.8 |
| Home living | 1 –13 | 7.3 ± 2.9 |
| Health and safety | 1 –13 | 7.6 ± 2.8 |
| Self-care | 2 –12 | 9.6 ± 2.9 |
| Work | 0 –13 | 2.9 ± 3.9 |
| Domain composite score | | |
| Practical skills | 49 –100 | 79.2 ± 10.6 |

**min – minimum; max – maximum; SD – standard deviation.**

**Table 2**

**Correlation between different skill areas of the Practical skills domain**

| Skill areas | | Community use | Home living | Health and safety | Self-care | Work |
|---|---|---|---|---|---|---|
| Community use | *r* | / | 0.475 | 0.577 | 0.359 | 0.042 |
| | *p*-value | | **0.000** | **0.000** | **0.000** | 0.638 |
| Home living | *r* | 0.475 | / | 0.319 | 0.427 | 0.159 |
| | *p*-value | **0.000** | | **0.000** | **0.000** | 0.075 |
| Health and safety | *r* | 0.577 | 0.319 | / | 0.314 | 0.019 |
| | *p*-value | **0.000** | **0.000** | | **0.000** | 0.834 |
| Self-care | *r* | 0.359 | 0.427 | 0.314 | / | 0.114 |
| | *p*-value | **0.000** | **0.000** | **0.000** | | 0.201 |
| Work | *r* | 0.042 | 0.159 | 0.019 | 0.114 | / |
| | *p*-value | 0.638 | 0.075 | 0.834 | 0.201 | |

***r* – correlation coefficient; *p* – statistical significance.**
**Bolded values are statistically significant.**

The results of variance analysis indicate that there were no statistically significant differences between male and female participants with VI ($p = 0.769$). The relation analysis between the participants' gender and the achievements in individual skill areas revealed a significant score difference in favor of the female participants when it comes to the Home living skill area ($p = 0.002$), while the differences in other skills areas were not statistically significant (from $p = 0.142$ to $p = 0.998$).

Analysis of the relationship between visual status and the scores in the area of PAS is shown in Table 3.

The visual status has the most profound effect on the scores achieved in the Community use skill area ($\eta^{2part} = 0.412$).

No significant relation was found between the age of vision loss onset and the composite score in the PS domain, nor in the individual skill areas.

The level of education of the respondents turned out to be a statistically significant factor only in the Work skill area – F(1) = 31.39, $p \leq 0.000$. The participants who graduated from high school estimate their Work skills significantly less favorably (mean value = 1.84) in comparison to those with higher education (mean value = 5.22). The scores of the respondents who graduated from high school and those with higher education did not differ significantly in other skill areas or the PS domain.

A similar result was observed through analysis of the relationship between participants' work experience and the PS. It was determined that work experience significantly affects only the estimation of work skills included in the Work skill area [F(1) = 33.82, $p \leq 0.000$]. Those with work experience estimated their work skills significantly better (mean value = 4.10) in comparison to those with no work experience (mean value = 0.94). In other skill areas and the PS domain, no significant differences were found with regard to whether the participants had prior work experience.

The analysis of variance also revealed a statistically significant relationship between living arrangements and PS (more details in Table 4).

**Table 3**

**The relationship between the visual status and the scores in the Practical skills domain**

| Practical skills domain | Visual status | Values | | F(1) | *p*-value | $\eta^{2part}$ |
|---|---|---|---|---|---|---|
| | | min-max | mean ± SD | | | |
| Skill areas | | | | | | |
| Community use | blindness | 1–12 | 5.5 ± 2.4 | 87.660 | **0.000** | 0.412 |
| | low vision | 5–13 | 9.2 ± 1.6 | | | |
| Home living | blindness | 1–12 | 6.4 ± 2.8 | 22.407 | **0.000** | 0.152 |
| | low vision | 3–13 | 8.8 ± 2.4 | | | |
| Health and safety | blindness | 1–13 | 6.7 ± 2.5 | 28.165 | **0.000** | 0.184 |
| | low vision | 1–13 | 9.2 ± 2.5 | | | |
| Self-care | blindness | 2–12 | 71.4 ± 4.3 | 5.876 | **0.017** | 0.045 |
| | low vision | 6–12 | 73.4 ± 2.3 | | | |
| Work | blindness | 7–39 | 2.8 ± 3.9 | 0.116 | 0.734 | 0.001 |
| | low vision | 21–45 | 3.1 ± 3.9 | | | |
| Domain composite score | | | | | | |
| Practical skills | blindness | 49–95 | 24.1 ± 7.7 | 58.433 | **0.000** | 0.319 |
| | low vision | 70–100 | 33.9 ± 5.7 | | | |

**$\eta^{2part}$ – partial eta-squared; F – coefficient of ANOVA. min – minimum; max – maximum; SD – standard deviation. Bolded values are statistically significant.**

**Table 4**

**Relationship between living arrangements and the scores in the Practical skills domain**

| Practical skills domain | Visual status | Values | | F(1) | *p*-value | $\eta^{2part}$ |
|---|---|---|---|---|---|---|
| | | min-max | mean ± SD | | | |
| Skill areas | | | | | | |
| Community use | family of origin | 1 –13 | 6.7 ± 3.2 | 3.479 | **0.034** | 0.054 |
| | alone | 4 –12 | 7.5 ± 2.1 | | | |
| | married | 3 –11 | 6.9 ± 2.6 | | | |
| Home living | family of origin | 2 –12 | 6.5 ± 2.7 | 6.982 | **0.001** | 0.102 |
| | alone | 5 –12 | 8.7 ± 1.9 | | | |
| | married | 1–13 | 7.7 ± 3.2 | | | |
| Health and safety | family of origin | 1–13 | 7.2 ± 2.4 | 6.263 | **0.003** | 0.092 |
| | alone | 1 –13 | 9.0 ± 3.3 | | | |
| | married | 1 –13 | 7.5 ± 2.6 | | | |
| Self-care | family of origin | 2–12 | 8.6 ± 3.0 | 7.143 | **0.001** | 0.104 |
| | alone | 5–12 | 10.7 ± 2.2 | | | |
| | married | 4–12 | 10.2 ± 2.7 | | | |
| Work | family of origin | 0–11 | 2.4 ± 3.6 | 0.611 | 0.545 | 0.010 |
| | alone | 0–13 | 3.4 ± 3.9 | | | |
| | married | 0–12 | 3.5 ± 4.5 | | | |
| Domain composite score | | | | | | |
| Practical skills | family of origin | 7–45 | 24.7 ± 8.6 | 13.763 | **0.000** | 0.183 |
| | alone | 20–44 | 32.6 ± 5.9 | | | |
| | married | 13–42 | 29.4 ± 7.9 | | | |

**min – minimum; max – maximum; SD – standard deviation. Bolded values are statistically significant.**

*Post hoc* analysis revealed a statistically significant difference between Domain composite scores of PS found between the participants who live with their family of origin and those who live alone (mean difference = 7.23, $p = 0.004$). The same analysis of the relationship between living arrangements and individual skill areas of the PS domain identified significant differences in the Self-care skill area between those who live with their families of origin and both those who live alone ($p = 0.007$) and those who are married ($p = 0.017$). In the Home living skill area, there was also a statistically significant difference ($p = 0.004$) between those who live in their families of origin and those who live alone. Furthermore, it was determined that the self-estimation of successfulness in carrying out household chores differed significantly between participants who lived alone and those who lived in their family of origin ($p = 0.001$). The difference between those who live alone and those who live in their family of origin with regard to the degree of acquisition of skills necessary for maintaining personal safety and health is also significant ($p = 0.013$).

## Discussion

The mean value of the score (borderline) in the domain of PS indicates the significant limitations in this area of AB according to self-evaluation of the participants with VI, which is in line with the findings of other studies of both children and adolescents [3, 5, 25], young adults [13], as well as elderly persons with VI [16, 17]. It is possible that, along with the primary impairment, environmental factors also have an impact on the limited acquisition of PS since it is known that young and adult people with VI are not independent in their decision-making or in performing daily tasks such as doing chores and caring for their own health [6, 7, 11–13, 19, 26–28].

Observing the scores of the participants in the skill areas in the domain of PS, it is clear that the lowest scores are seen in the skill area of Work, which may be connected to the fact that most of the participants are unemployed. The high percentage of unemployment among people with VI may be a result of the poor job market, lack of motivation with regard to searching for work, and also the inability to get about independently as a significant reason for leaving an existing job [29]. Among the employed participants, we identified difficulties in accessing the workplace and work materials. We should also take into consideration the fact that the limited scope of work for people with VI dictates, to a degree, their choice of profession, which may impact their motivation [30]. Furthermore, previous research found that negative attitudes toward disability and unfavorable economic situations were additional factors for the low employment rate among people with VI in our society [31].

The mean score achieved by the participants' self-evaluation in the skill area of Community use is also lower than that of the norm for the typical population, and here the greatest difficulties were caused by activities that require good motor skills and mobility. The Orientation and Mobility training is not available for all people with blindness in Serbia, especially those who lost vision in adulthood; therefore, it could be one of the reasons for the lower score in this skills area. The results of other studies indicate that only 30–45% of adults with VI are active in their communities [20, 32]. Authors point out that most elderly persons with VI spend their time at home without going out or performing other daily activities in their community [33], which is in accordance with our observations from the conversations we had with our participants. The studies that have focused on daily functioning in adults and elderly people who lost their sight in adulthood indicate that they have difficulties performing activities such as self-care, going to the bank or a social gathering, shopping, and using public transport independently [17, 18]. The participants with VI had the highest average score in the domain of PS in the skill area of Self-care, which corroborates the results of previous studies [18, 19, 26]. Qualitative analysis has shown that the participants have trouble performing tasks dependent on good vision and established aesthetic criteria. Although most claim to successfully care for their clothes, some believe that their attire is not following social norms; thus, they rely on help from family members or friends to choose their clothing. The results of previous studies show that VI negatively affects daily functioning at home [12, 16, 34]. The average scores of our participants in the skill area of Home living and Health and safety are in the category of "below average" compared to the norm for the typical population. The parental overprotection of children with VI in their early years can lead to difficulties in performing household tasks [35]. As they grow up, the demands upon them increase. Regardless of their visual status, the adults are expected to find a way and create strategies for performing household tasks independently due to new living arrangements [33]. In our cultural and social environment, families tend to overprotect even adult people with VI, especially those who have lost vision later in life. The family members' expectations regarding the functioning and independence of people with VI are usually quite low.

Most of our participants are able to care for their own safety and maintain a high level of caution, which is to be expected given the higher risk of injury due to the difficulty or impossibility of visually monitoring the environment and events occurring within it. In the skill area of Health and safety, many difficulties concerning the care for their health were observed, as was a general divergence between the two groups of participants in this area. The interviews showed that one group exhibits great uneasiness and dependence on others, while the second group, in contrast, tends to neglect their health. Medical treatment outcomes may sometimes result in a loss of faith in public health institutions or the development of resistance towards medical services due to the nature of treatment and frequent hospitalization [36]. Taking into consideration the dynamics of the acquisition, maintenance and weakening of PS through life, and the results of other studies [17, 37], the absence of a relationship between age and PS is somewhat surprising. As the age range (from 19 to 60 years) was encompassed in this research, we could have expected that the young adult persons acquired PS with more success than older participants since motor skills and physical activity decreased by the age [3, 5, 17, 28, 34, 37, 38]. This can likely be explained by the stronger influence of other factors such as visual status, living arrangements, etc. Comparison of the results of female and male participants shows a significant difference only in the domain of

household tasks, which may be attributed to cultural gender roles in the country [39, 40]. Female participants did not exhibit greater care for their health, hygiene, or appearance, even though some authors consider them more responsible and more successful in these areas of PS [41]. Following the results of previous studie [17, 18, 42], it has been established that persons with low vision acquire PS with more success than persons who are blind. The statistically significant differences in favor of the participants with low vision were established in all skill areas in the domain of PS, except in the skill area of Work. The results of other studies also confirm that for most persons who are blind, daily living activities, as well as leisure, are, in fact, a greater difficulty than for people with low vision [2, 7, 15, 16].

Although participants with low vision express more doubts about their success with completing household chores, the results in the skill area of Home living tell us that persons who are blind perform significantly fewer tasks in the home and they require help from family members or friends, as was established in the previous study [35]. It was noted that good orientation in a familiar space and adaptation might help the person with blindness to be more independent in performing household tasks and feel safer [37]. The significantly lower results of the respondents who are blind in the skill area of Health and safety may be attributed to difficulties in caring for their health (e.g., going to the doctor, getting information on their health condition, and taking prescribed medication) [43] and the decreased safety in open spaces due to a lack of visual information, which can cause them to overlook dangerous situations or obstacles and fail to protect themselves adequately [35, 38]. The results in the skill area of Self-care indicate that persons who are blind have significantly more difficulties in maintaining personal hygiene and choosing appropriate clothing than persons with low vision, which confirms the findings of earlier studies [7, 19] but is not in accordance with Langelaan et al. [18] who claim that this visual status does not significantly affect the area of self-care. The age of vision loss onset might also influence the acquisition and practice of acquired PS. Partial or complete loss of vision in adulthood often leads to functional difficulties because the person is unable to perform practical tasks for which they relied on their vision before the impairment [19, 38]. One of the greatest difficulties for persons who lose sight in adulthood is the mobility decrease, which influences their engagement in their environment. Nevertheless, our research detected no significant differences between participants with congenital and acquired VI concerning the acquisition of PS. Engaging in the practical aspects of life might decrease after vision loss, while participants with congenital VI have never acquired PS at an optimal level. A significant relationship between PS and the level of education, as well as work experience, was not found, which brings into question the assumption that for persons with VI, independence in daily tasks is a precondition to developing a career. Analyzing the influence of living arrangements, it was found that respondents with a VI who live alone have acquired better PS than the other two groups of participants (who live in a family of origin/parents and married participants), with the difference being statistically significant when compared to the group of participants who live with their parents. It is possible that, under the opinions of some authors, participants who experienced independence during childhood had the opportunity to acquire PS and a sufficient level of self-confidence to begin independent life [44, 45]. In accordance with our results, Desrosiers et al. [33] did not find any differences in the level of PS attainment between persons with VI who are married and those who are not.

Although family life requires a higher degree of responsibility and engagement in the practical aspect of life, the participants who are married have significantly lower results in the skill area of Self-care compared to those who live alone, which may be attributed to the possibility that this group of participants is protected by their spouses. Other authors point out that spouses and other family members often take on health care, household chores, and grocery shopping instead of persons with VI [27].

The limitations of this study include the fact that many participants (n = 47; 37%) did not have any work experience, which may have influenced the achievements in the Work subtest. Furthermore, some quality of life parameters, which might have been significant for the participants' achievements in the PS domain, were not included.

## Conclusion

Based on the analysis of the results, we can conclude that adults with VI exhibit significant limitations in the domain of PAS. The scores achieved in the skill areas of the PS domain range from "extremely low" to "average". Extremely low scores were detected in the Work skill area, while for the skill areas of Community use, Home living, and Health and safety, the scores were "below average", and for the Self-care skill area, the scores were "average".

The study showed that the degree of PS acquisition among persons with VI depends primarily on the visual status, but a significant relationship between living arrangements and work experience was also established.

In summary, these results allow us to make recommendations for support services. It is necessary to establish specialized programs of instruction in everyday activities for children and adults who are blind, and for persons with low vision to make adaptations in the home, the environment, and the workplace. The training for the use of aids should become part of clinical practice in acquired VI, and enable more effective and motivated performance of PS. It is of particular importance for the parents of children with VI to be made aware of the importance of providing their children with opportunities to acquire PS since they are among the preconditions of future independence.

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## Conflict of interest

The authors declare no conflict of interest.

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# Versatile clinical application of radial artery perforator flap for hand and wrist reconstruction

## Višestruke mogućnosti kliničke primene perforator režnjeva radijalne arterije za rekonstrukciju defekata ručnog zgloba i šake

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**Abstract**

**Background/Aim.** Radial artery perforator flap (RAPF) is a type of fasciocutaneous or adipofascial reverse pedicle flap, which proved to be a versatile flap for the reconstruction of small and moderate size soft tissue defects of the forearm, wrist, and hand. RAPF provides suitable skin coverage with elastic subcutaneous tissue thus enabling the protection of exposed or damaged functional structures and their simultaneous repair. The aim of this study was to summarize and analyze the results of the treatment of patients with upper extremity soft tissue defects caused by trauma, infection, burn, or tumor removal, which were reconstructed with RAPF. **Methods.** This retrospective study included 20 consecutive patients with skin and soft tissue defects of the hand and wrist, treated at the University Clinical Center of Vojvodina from 2012 to 2022. The design of the flaps and length of the pedicles were determined by the recipient site. Tourniquet-induced exsanguination was used during surgery for better visualization. The flaps were elevated and placed at the site of the defect. Data on patients and flaps were summarized upon their collection. **Results.** Distally based fasciocutaneous RAPF was used in all cases. The average age of the patients was 48 years, predominantly (64%) males. Defects were most often localized on the dorsal part of the hand (60%) and wrist (20%). The most common indications for surgery were trauma (45%) and tumor resection (25%). A satisfactory coverage of the defect was achieved in all 20 patients with no flap loss. Venous congestion was noted in 4 (20%) patients, which resulted in partial necrosis of the flap in 3 (15%) patients. Wound healing was achieved upon conservative treatment by secondary intention in two patients and with a secondary suture in the last one. A surgical site infection occurred in 2 (10%) patients who withdrew after proper local and systemic therapy. **Conclusion.** RAPF proved to be a workhorse flap for soft tissue reconstruction of the upper limb. This surgical solution led to an excellent functional and aesthetic outcome in the majority of patients. Complex surgical procedures could be done simultaneously, together with the reconstruction of tendons, joints, or fracture stabilization. The reliability and safety of these flaps are confirmed through our clinical data.

**Key words:**
**hand; radial artery; surgical flaps; surgical procedures, reconstructive; wrist joint.**

**Apstrakt**

**Uvod/Cilj.** Perforator režanj radijalne arterije (PRRA) je vrsta fasciokutanog ili adipofascijalnog reverznog peteljkastog režnja, koji je potvrdio svoju višestruku primenu u rekonstrukciji malih i srednjih defekata mekih tkiva podlaktice, ručnog zgloba i šake. PRRA obezbeđuje odgovarajući kožni pokrivač sa elastičnim potkožnim tkivom, i time omogućava zaštitu eksponiranim ili povređenim funkcionalnim strukturama i njihovu istovremenu reparaciju. Cilj rada bio je da se sumiraju i analiziraju rezultati lečenja bolesnika sa defektima mekih tkiva gornjeg ekstremiteta nastalih usled traume, infekcije, opekotine ili uklanjanja tumora, koji su rekonstruisani primenom PRRA. **Metode.** Retrospektivnom studijom obuhvaćeno je 20 bolesnika sa defektima mekih tkiva šake i ručnog zgloba, koji su lečeni u Univerzitetskom Kliničkom Centru Vojvodine od 2012. do 2022. godine. Dizajn režnjeva i dužina peteljki određivani su u odnosu na recipijentnu regiju. Sve operacije izvedene su pod Esmarch-ovom bandažom u bledoj stazi, radi bolje vizualizacije. Režnjevi su podizani i pozicionirani na mesto defekta. Podaci o režnjevima i bolesnicima su neposredno beleženi i sumirani. **Rezultati.** Distalno bazirani fasciokutani

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PRRA korišćen je kod svih slučajeva. Bolesnici su bili stari prosečno 48 godina, pretežno (64%) muškog pola. Defekti su bili najčešće lokalizovani na nadlaničnoj strani šake (60%) i ručnog zgloba (20%). Najčešće indikacije za operaciju bile su povreda (45%) i resekcija tumora (25%). Zadovoljavajuće pokrivanje defekta postignuto je kod svih 20 bolesnika, bez izgubljenog režnja. Venska kongestija je zabeležena kod 4 (20%) bolesnika, što je izazvalo delimičnu nekrozu režnja kod 3 (15%) bolesnika. Odloženo zarastanje je postignuto kod dva bolesnika, dok je kod poslednjeg postavljen sekundarni šav. Infekcija na mestu intervencije se razvila kod 2 (10%) bolesnika. Znaci infekcije su se povukli nakon sprovedene odgovarajuće lokalne i sistemske terapije. **Zaključak.** PRRA su se dokazali kao vrlo korisni režnjevi za rekonstrukciju defekata mekog tkiva na gornjim ekstremitetima. Ovo hirurško rešenje dovodi do odličnog funkcionalnog i estetskog ishoda u većini slučajeva. Složene hirurške procedure se mogu uraditi istovremeno, zajedno sa rekonstrukcijom tetiva, zglobova ili stabilizacijom preloma. Naša klinička studija potvrđuje pouzdanost i sigurnost korišćenja tih režnjeva.

**Ključne reči:**
**šaka; a. radialis; režnjevi, hirurški; hirurgija, rekonstruktivna, procedure; ručje, zglob.**

## Introduction

Reconstructive surgery of the hand is still a challenge in everyday clinical practice. Closure of soft tissue and skin defects, where the functional structures of the hand are often exposed or damaged, must be performed in a timely manner and in the best possible way for the patient. At the same time, it is necessary to enable the simultaneous repair of tendons, joints, bones, nerves, or blood vessels [1].

The radial forearm flap, also called the "Chinese flap", was first described in the literature as a free flap by Yang et al. in 1981 [2]. In the following year, a distally based pedicle flap, whose vascularization is based on retrograde blood flow from the radial artery and accompanying veins (venae comitantes), was presented [3]. Regardless of its wide use at the beginning, the main disadvantage of this reverse flap is the necessary sacrifice of the main artery. This problem was solved by introducing the radial artery perforator flap (RAPF) into clinical practice [4]. Based on the increasing knowledge of the topographic anatomy of the arterial perforators of the forearm and hand over the years, several surgical approaches have been put forward, enabling the versatile use of perforator flaps in reconstructive surgery [5].

Pedicled perforator flap combines the advantages of local (complement skin), regional flaps (arc of rotation up to 180°), and distant flaps (reliable vascularisation) without the need for microsurgical vascular anastomosis. Like-with-like reconstruction is the basic principle in plastic surgery. RAPF matches the surrounding skin with color, texture, and contour, especially for the dorsal aspect of the wrist and hand [6]. It provides suitable skin coverage with elastic subcutaneous tissue to protect exposed hardware and allow all degrees of motion. In some patients, a drawback may be the rich subcutaneous fatty tissue of the forearm, which will require trimming of the flap in the second act [7].

The aim of this study was to present the results of the treatment of our patients with soft tissue defects of the hand and wrist caused by trauma, tumor resection, chronic infection, or deep burn, which were reconstructed with RAPF.

## Methods

Data for this retrospective study was collected from the Clinic for Plastic and Reconstructive Surgery, University Clinical Center of Vojvodina, from January 2012 to April 2022. The study was approved by the Ethics Board of the aforementioned institution (No. 00-133). It included 20 consecutive patients for whom we used RAPF. All the obtained data were statistically processed upon their collection.

### *Topographic anatomy*

On its way through the forearm, the radial artery gives numerous septocutaneous and musculocutaneous arterial perforators (range 9 to 16). Two clusters of clinically useful cutaneous perforators arise at the proximal and distal third of the forearm. In the distal part of the forearm, at least six septocutaneous perforators can be found [8]. For planning and elevation of the RAPF, the most important septocutaneous perforators of the radial artery arise within 2 cm proximal to the radial styloid, where the pivot point of the flap is. Circulation is achieved due to a retrograde axial flow from at least two clinically significant (> 0.5 mm) septocutaneous perforators, which anastomoses with the longitudinal chain-link vascular plexus in the overlying deep fascia and subcutaneous tissue. Linking vessels are also connected with an intrinsic superficial vascular network that surrounds the lateral cutaneous nerve and cephalic vein, which altogether provide sufficient blood supply to the RAPF [9].

### *Surgical technique*

Although a preoperative Allen test is unnecessary since flap circulation is independent of ulnar-radial palmar vascular communication, patency of the radial artery at the site of septocutaneous perforator branching is crucial for flap survival. Doppler ultrasound examination was performed in all patients to identify and map the arterial perforators, which can be aggravated by the dominant sound of the superficially placed radial artery at the level of the distal forearm [10].

The patient is placed in a supine position on the operating table with a hand laid on a well-padded arm board. Exsanguination is achieved with a brachial tourniquet. After adequate debridement of the wound or tumor resection site, the size and position of the defect are measured. The design of the flap and the length of the pedicle are determined. Extra pedicle length should be considered in order to rotate the flap without tension or kinking. A lazy "S" shaped skin incision

along the axis of the radial artery is made over the volar forearm with an elevation of thin skin flap equally on both sides to expose the adipofascial pedicle. Lifting the flap starts at its proximal end. Dissection continues towards the distal forearm and pivot point in the subfascial plane. The longitudinal adipofascial pedicle should be 3–4 cm wide and include the cephalic vein and the lateral cutaneous nerve [11]. It needs to be separated from the intertendinous septum, located between the flexor carpi radialis and brachioradialis tendons. Several septocutaneous perforators originating from the radial artery located in the dissection pathway need to be carefully ligated. A small skin bridge is usually kept as a flap extension to minimize pressure on the pedicle following flap transfer. Care should be taken to avoid injury to the sensitive branch of the radial nerve. After elevation to the pivot point, the flap is rotated for 180° to reach the distal defect through the subcutaneous tunnel. In the case of a larger flap or a rich layer of subcutaneous adipose tissue, the intervening skin bridge between the defect and the pivot point should be opened, and, after passing, the flap subsequently closed [12]. Direct closure of the donor site can be achieved if the width of the defect does not exceed 4–5 cm; otherwise, a split skin graft is required. The hand and wrist are immobilized for several days in a neutral position following surgery to promote healing.

**Results**

Twenty patients underwent reconstruction of the hand and wrist with radial artery perforator fasciocutaneous island flaps. The mean follow-up period was 18 months (range 12–24 months). Coverage of the defect was successful in all cases, with a few minor complications. The average age of the patients was 48 years, with most (65%) being male. The largest size of the fasciocutaneous island flap was 11 × 6.5 cm, and the smallest was 5 × 3.5 cm. The average time duration for the flap elevation and placement was 1 h and 15 min if there was no need for other surgical procedures, including tendon and joint reconstruction or fracture stabilization. The patients usually stayed at the hospital for six days after surgery.

The clinical summary of the patient and flap data is shown in Table 1. The dorsal side of the hand was the most common localization of the defect (60%). In nine (45%) patients, the flap was used entirely for soft tissue defect coverage; however, in all other cases, a complex surgical procedure was required simultaneously with extensor tendons, joint reconstruction, or fracture stabilization. The most common indications for surgery were reconstruction after trauma (45%) and tumor resection (25%).

**Table 1**

**Clinical summary of patient and flaps' data**

| Features | Values |
|---|---|
| Number of flaps | 20 |
| Age, year, mean (minimum-maximum) | 48 (26–77) |
| Gender ratio | 2.9 |
| male/female | 13 (65)/7 (35) |
| Localization | |
| dorsal side of the hand | 12 (60) |
| wrist | 4 (20) |
| thumb | 3 (15) |
| palm | 1 ( 5) |
| Etiology | |
| trauma | 9 (45) |
| tumor | 5 (25) |
| chronic infection | 3 (15) |
| burn | 3 (15) |
| Type of reconstruction | |
| only soft tissue coverage | 9 (45) |
| tendon | 7 (35) |
| bone | 3 (15) |
| joint | 1 (5) |
| Time of reconstruction | |
| delayed | 12 (60) |
| immediate | 9 (45) |
| Size of the flap in $cm^2$, mean (minimum-maximum) | 24 (17.5–71.5) |
| Tunneling of the pedicle | 17 (75) |
| Skin extension harvesting | 11 (55) |
| Donor site | |
| split-thickness skin grafts | 13 (65) |
| primary closure | 7 (35) |
| Complications | |
| distal venous congestion | 4 (20) |
| partial necrosis | 3 (15) |
| infection | 2 (10) |
| Follow-up in months, mean (minimum-maximum) | 18 (12–24) |
| Long/Wide ratio, mean (minimum-maximum) | 3.7 (3–5) |

**All values are given as numbers (percentages) except age, size of the flap, follow-up, and long/wide ratio.**

Tunneling of the pedicle under a skin bridge was performed in the majority (75%) of patients. Small skin extension of the flap in order to relax the tension on the pedicle was used in 55% of patients.

The immediate pedicled flap was performed in 9 (45%) patients, for acute traumatic defects with exposed bones and tendons or for planned operations after malignant tumor resection. A delayed procedure was performed on most patients, mostly as they arrived at our institution from other departments or regional hospitals as unsolved cases.

Distal venous congestion was the most common (20%) complication in the early postoperative period. Spontaneous recovery of the flap occurred in one patient. Three cases resulted in marginal necrosis of the most distal part of the flap. Wound healing was achieved upon conservative treatment by secondary intention in two patients and with a secondary suture in the last one. This kind of complication did not develop in patients in whom the cephalic vein was ligated at the base of the flap pedicle during the operation.

A surgical site infection occurred in two patients in whom delayed reconstruction had been performed. Signs of infection withdrew spontaneously after adequate local treatment and antibiotic therapy according to the wound swab antibiogram.

Primary closure of the defect was possible in 7 (35%) patients, while a split-thickness skin graft was used in 13 (65%) patients. There was no significant donor site morbidity.

All patients had stable scars and soft tissue coverage with a satisfactory color, texture, and contour of the flap in the follow-up period. Hypoesthesia and tingling were noticed in several patients at the area of skin, which was innervated by sacrificed lateral cutaneous nerve but without further consequences. Patients who underwent physical therapy showed significant improvement in the function of the involved fingers, with a good or satisfactory outcome. Two patients required flap trimming in a second surgical procedure due to a bulky appearance and aesthetic refinement.

*Case report 1*

A 55-year-old male patient was referred to our Clinic with a pigmented skin lesion on the volar aspect of the wrist. The tumor was first noticed by the patient two years prior. Vertical tumor growth started with intermittent secretion four months before he was admitted to the Clinic. Wide surgical excision (margins of 2 cm) was made all the way to the transverse carpal ligament. Reconstruction of the defect was achieved with a RAPF (size 4.5 × 4.5 cm). The donor site defect was closed with direct sutures. The histopathology report confirmed the clinically established diagnosis: melanoma, nodular type, Clark V, Breslow 4 mm, with ulceration. The function of the wrist was fully preserved with a satisfactory aesthetic outcome. In the two-year follow-up period, the patient was without local or systemic recurrence of the disease (Figure 1).

*Case report 2*

A 50-year-old male patient was admitted to our Clinic with soft tissue necrosis of the dorsal side of the right thumb. Three weeks earlier, the patient sustained a chainsaw injury with a dislocation fracture of the base of the proximal phalanx and section of the extensor tendons of the thumb. The patient was initially treated at another healthcare facility where fracture stabilization with a Kirschner wire was done. Radical surgical debridement was performed. Reconstruction of the tendon defect was achieved with an interposition tendon graft of palmaris longus. Soft tissue defect was covered after planning and elevation of the RAPF (size 6.0 × 3.5 cm). Direct closure of the donor site was done. The wound healing was completed with mild venous congestion of the flap in the first week after surgery. Six months later, a good functional result was achieved with a satisfactory range of motion in the right thumb (Figure 2).

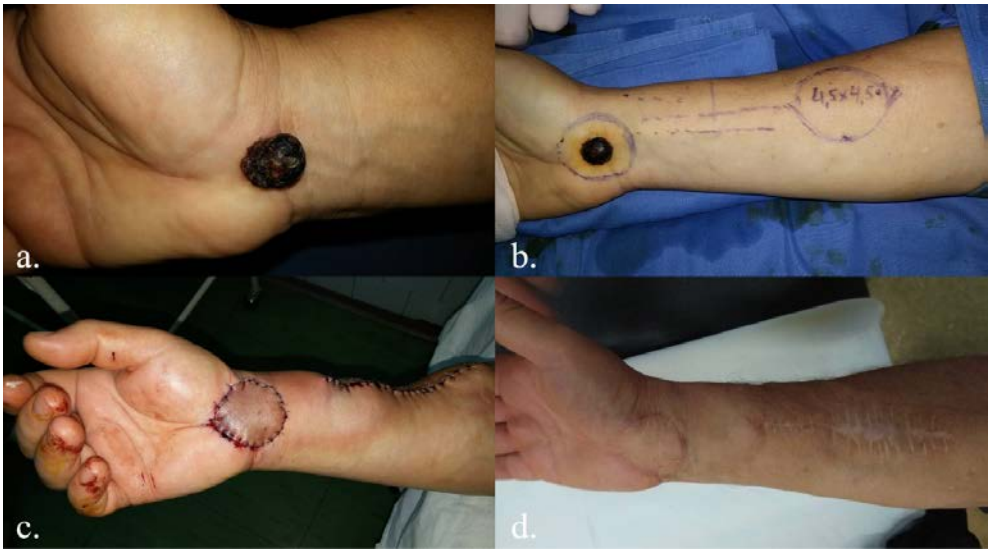


**Fig. 1 – a) Nodular type of wrist melanoma; Clark V, Breslow 4 mm, with ulceration; b) Planning the elevation of radial artery perforator flap (size 4.5 × 4.5 cm); c) Early postoperative result (two days post-op); d) Late postoperative result (one year post-op).**

*Case report 3*

A 74-year-old male patient was admitted to our Clinic with a soft tissue defect on the dorsal side of the right hand. Two months before, he was treated at the hematology department for Hodgkin's disease as an outpatient. A local chemotherapy agent induced perivenous extravasation injury, which led to necrosis. After radical surgical debridement, reconstruction of the defect was achieved with RAPF (size 8.5 × 5.5 cm). The donor side defect was covered with a split-thickness skin graft. In the early postoperative period, epidermolysis occurred in the most distal part of the flap without further complications. During the rehabilitation period, a full range of motion of all fingers was achieved (Figure 3).

*Case report 4*

A 57-year-old female patient was referred to our Clinic from another hospital after a compression injury of the right hand. The patient had a skin defect on the dorsal side of the hand and volar aspect of the wrist with the inability to actively extend the second, third, and fourth finger due to injury of the extensor tendons. After surgical treatment, delayed primary repair of extensor tendons was possible for the third and fourth fingers. An interposition tendon graft of *indicis communis* was used to reconstruct the extensor *indicis proprii* tendon. Soft tissue defect on the dorsal side of the hand was covered with RAPF (size 5.5 × 3.0 cm), while the skin defect on the volar side of the wrist was resolved with a split-

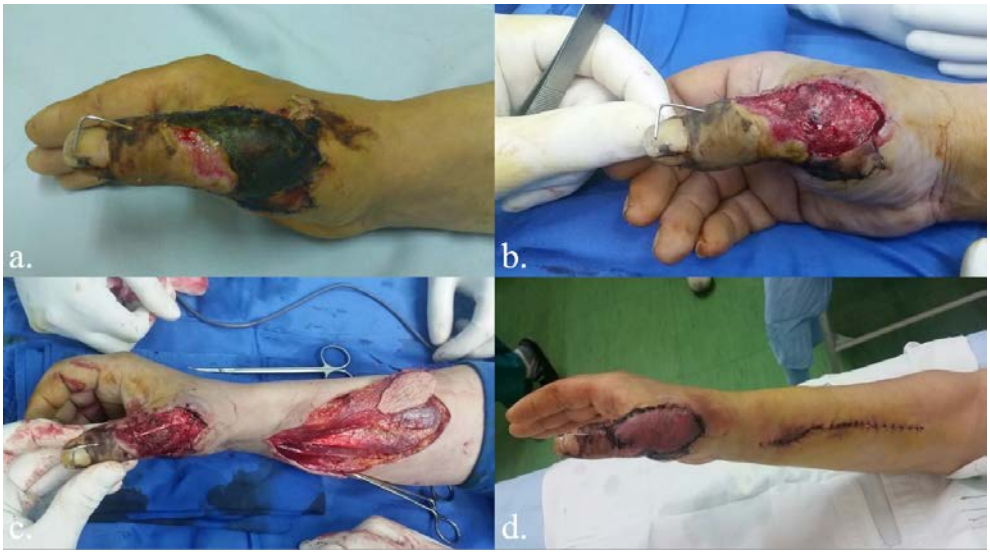


**Fig. 2 – a) Soft tissue necrosis of the dorsal side of the right thumb with dislocation fracture of the base of the proximal phalanx and extensor tendons injury; b) Wound after radical surgical debridement; c) Tendon reconstruction with interposition graft of palmaris longus and elevation of radial artery perforator flap (size 6.0 × 3.5 cm); d) Fully preserved vitality of the flap with mild venous congestion (two weeks post-op).**

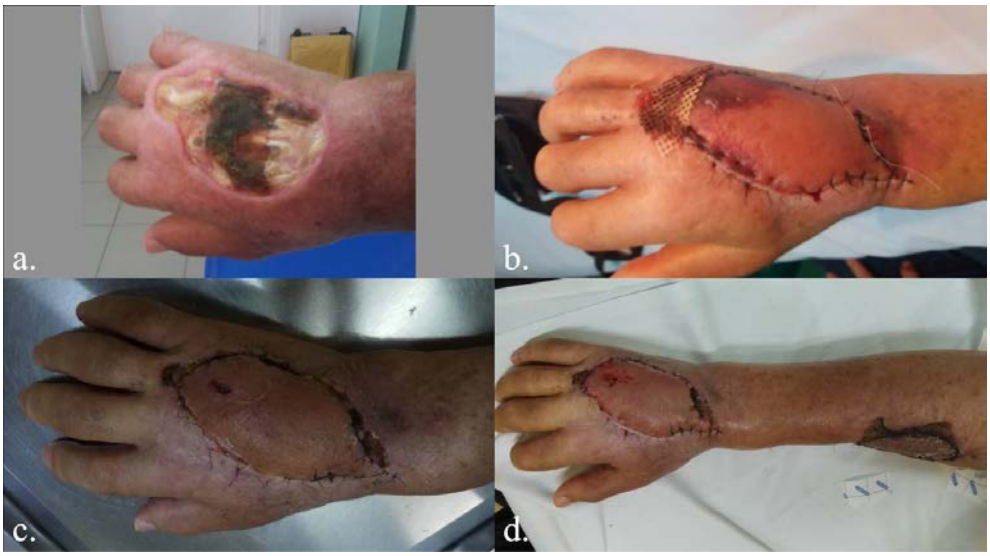


**Fig. 3 – a) Local chemotherapy drug-induced perivenous extravasation injury which led to necrosis of the dorsal side of the hand; b) Reconstruction of the defect with radial artery perforator flap (size 8.5 × 5.5 cm); c) Appearance of the flap two weeks post-op; d) Donor side defect covered with a split-thickness skin graft.**

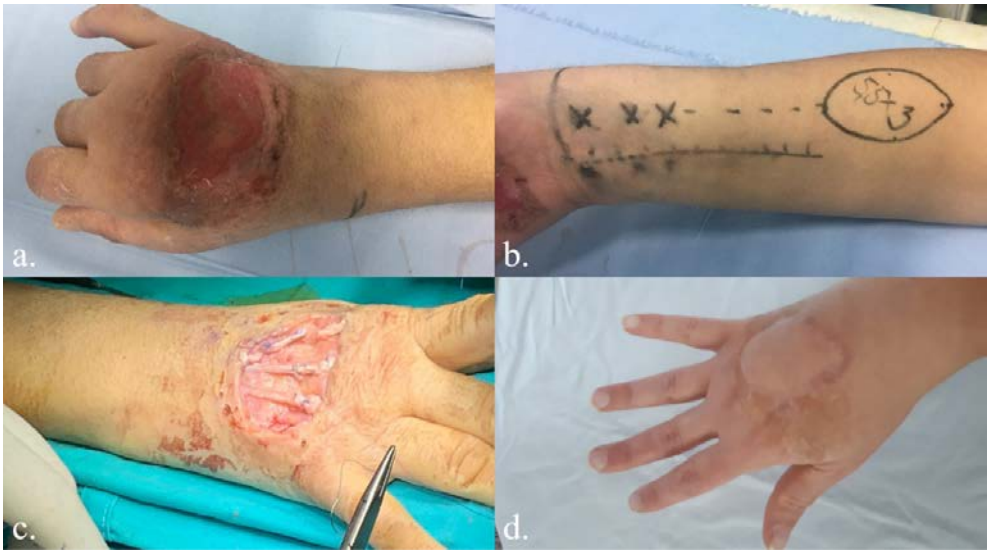


**Fig. 4 – a) Soft tissue defect on the dorsal side of the hand after compression injury with a lesion of extensor tendons for the second to fourth finger; b) Planning the elevation of radial artery perforator flap (size 5.5 × 3.0 cm) with mapping of arterial perforators with Doppler ultrasound; c) Delayed primary repair of extensor tendons for third and fourth fingers and with interposition graft for *indicis proprii* tendon; d) Good functional result with full extension of involved fingers (one year post-op).**

thickness skin graft. Direct closure of the flap donor site was done. The good functional result with full extension of involved fingers and the satisfactory cosmetic outcome was accomplished four months later (Figure 4).

## Discussion

RAPF allows the reconstruction of small- and medium-sized defects on the dorsal part of the hand up to the level of the metacarpophalangeal joints of the second to fifth finger and the proximal phalanx of the thumb. On the palmar side, it may reach a proximal or even distal transverse crease. According to most authors [8, 11], the maximum flap size that can be reliably planned is 8–12 cm wide and 15–20 cm long. The largest size of fasciocutaneous island in our patients was 11 × 6.5 cm. Localization of the top edge should be limited at the lower 2/3 of the forearm because of the perforator vascular cutaneous territory. Further increase in flap length would not be safe and reliable [13]. Relative contraindications for RAPF could be in patients at risk for microvascular arterial disease or venous insufficiency or thrombosis in the affected limb, smokers, diabetics, skin or systemic disorders, and others.

In 75% of cases in our series, a subcutaneous tunnel was used for positioning the flap at the recipient site. Dissection of the tunnel should be sufficiently wide to prevent compression or kinking of the vascular pedicle. This method of trans-passing the flap was not used only in cases of a thick layer of subcutaneous fatty tissue or unsuitable condition of the skin bridge. Otherwise, a longitudinal skin incision was performed through the shortest pathway of the flap with linear wound closure afterward. Small skin extension of the flap toward the pedicle was done in 11 (55%) patients. That is recommended to reduce tunnel tightness and avoid traction injury. Numerous authors believe these procedures can reduce the risk of possible complications [12, 14, 15].

Venous congestion is the most common complication of this surgical procedure [16]. When it is transient, it will not leave permanent consequences. Epidermolysis, marginal or complete flap necrosis may be a complication of a severe circulatory disorder. Intense venous blood inflow from a one-way magistral valvular system of the distal part of the hand may exceed the capacity of the smaller concomitant bypass veins that surround the cephalic vein. It remains controversial whether the cephalic vein should be ligated at the base of the flap pedicle. According to our clinical experience, it is necessary to consider this procedure if the cephalic vein is tense and voluminous with the presence of obvious venous stasis after tourniquet release during surgery. This procedure was not performed in any of the four patients from our series in whom partial flap necrosis was manifested. Some authors purpose “flow-through” venous flow by supercharging the free end of the vein on the distal part of the flap to a recipient’s vein [17]. Novel research suggests a hybrid perfusion mode by anastomosing the distal vein of the flap with the recipient artery [18]. An option for flap salvage can still be to apply medicinal leeches (*Hirudo medicinalis*). Despite the proposed solutions, venous congestion remains a challenge in distally based perforator flaps on both the upper and lower extremities. It has been shown that there is no positive role of the cephalic vein in the venous drainage of this flap [13]. On the other hand, the vascular plexus accompanying the cephalic vein contributes to the flap perfusion and should not be sacrificed [9, 19].

The lateral cutaneous nerve should be an integral part of the pedicle. Longitudinal intrinsic and extrinsic perineural vascularization of this nerve enhances flap nutrition [9]. The sacrifice of this nerve does not lead to a significant sensory

deficit and has never been emphasized by our patients. If there is a need for a greater degree of protective sensation return, such as in palmar reconstructions, the sensate flap can be achieved by the same nerve [20]. Neurorrhaphy of transected end of the antebrachial nerve should be done with a suitable recipient sensory nerve. Neurotization of the flap is not necessary in the majority of cases since the protective sensibility is established to a lesser or greater extent in all patients over time.

In over half of our cases, a more complex reconstruction was done, which included tendons [7 (35%)], bones [3 (15%)], and joints [1 (5%)]. Smooth tendon gliding can be obtained under this kind of flap. The palmaris longus tendon graft is most often used in secondary tendon repair. This tendon can be included and elevated together with the flap. In addition, the possibilities of using the tendon flexor carpi radialis and brachioradialis as part of the composite flap are described in literature [21, 22]. During the operation, it is necessary to ensure the appropriate tension and quality of the tendon sutures, which must be strong enough to withstand early protected motion. Excessive shortening of the tendon can result in loss or reduction of flexion mobility. On the other hand, insufficient tension on the suture will not allow complete extension of the fingers [23]. Adequate and timely physical therapy will certainly contribute to the best possible postoperative result.

Trauma [9 (45%)] is the leading etiological factor in our series. In addition, versatile clinical usage of RAPF is possible. Five (25%) of our patients underwent tumor surgical resection – three with squamous cell carcinoma, and two with melanoma, which are, together with basal cell carcinoma, the most common primary hand malignancies. In six (30%) patients, necrosis of the skin and soft tissue of the hand occurred after subdermal burn [3 (15%)] and chronic infection [3 (15%)]. Delayed reconstruction was performed in all of these patients. Hand infection is often encountered in clinical practice. Severe infections are more likely to develop in risk groups of patients such as drug addicts, patients with diabetes or immunodeficiency, oncologic patients, etc. [24]. As a complication of such conditions, deep necrosis can develop, with possible consequent tendon rupture. Successive necrectomy, adequate dressings, topical and systemic therapy according to the findings of wound swab antibiogram, as well as the use of a vacuum-assisted closure system, led to the optimization of the wound state [25]. After proper wound conditioning, complete reconstruction is most often performed in a single-stage procedure, using a RAPF, which is in line with the reference studies [1, 26]. In this group of patients, surgical site infection occurred in only 2 (10%) patients after delayed reconstruction. Signs of infection withdrew spontaneously due to appropriate topical wound care.

Applications of these flaps are varied. Adipofascial variation of RAPF also has widespread clinical use [11]. The advantage of this flap is that donor site morbidity is minimal because of the primary wound closure, although a split skin graft is needed to cover the adipofascial flap at the recipient site. Complications such as partial necrosis are slightly more common compared to standard RAPF. Wilson et al. [27] have used this flap to create a fascial tube to treat recurrent de Quervain's tendonitis. A similar surgical technique has been described for flexor tendon sheath reconstruction at the level of the wrist and distal forearm [8]. Recently, recurrent carpal tunnel syndrome has been treated by wrapping or padding the median nerve [28].

Based on the topographic anatomy of the perforators along the axis of the radial artery, several more surgical approaches have been put forward, such as the proximal RAPF, the posterolateral mid-forearm perforator flap, and the snuffbox radial perforator flap. The proximal RAPF, which enables the reconstruction of the elbow and antecubital fossa defects, stands out for its clinical importance [29].

Propeller perforator flap as a free-style flap is based on vascularization by only one septocutaneous or myocutaneous arterial perforator, which can be chosen along the course of the radial artery [30]. The limits of such flaps cover only small defects in the surrounding area.

Clinical use of the RAPF as a free flap has been described in only a few cases [8]. Contemporary knowledge about the vascular contribution of major perforators along the radial artery axis could enable the design of chimeric or segmental radial artery flaps with multiple skin islands that can be based on different clusters of perforators [31].

The most important flaps that can be a replacement for RAPF are certainly the ulnar artery perforator flap and retrograde posterior interosseous artery flap. Nutrition of the ulnar artery perforator flap is based on distal septocutaneous perforators of the ulnar artery [32]. It is suitable for covering defects on the ulnar side of the wrist and hand. The main disadvantage of this flap is the shorter pedicle length. The posterior interosseous flap is also very useful for hand reconstruction [33]. Technically demanding dissection and perfusion, which is dependent on adequate distal communication between the anterior and posterior interosseous arteries, are the reason why it is less often used in daily clinical practice.

In our series, RAPF was used for small and moderate-sized defects. For the minor defects on the specific localization of the hand, suitable alternative perforator flaps may be used, such as the reverse thenar perforator flap, the ulnar palmar perforator flap, and the dorsal metacarpal artery perforator flap [34, 35]. For each of them, the proper indication can be found in hand surgery. In the case of large defect reconstruction or if a regional flap could not be planned because of the poor condition of the wound environment and adjacent tissue, we mostly opted for distant pedicle flaps such as the groin and thoracoabdominal flap. These flaps are not technically demanding and can give satisfactory results. The main disadvantages are mandatory arm immobilization for three weeks and that the procedure has to be carried out in two stages [22]. With the development of microsurgery, the application of free flaps in hand reconstruction, particularly for large and composite defects, is gradually increasing [36]. Anterolateral thigh flap, tensor fascia lata flap, lateral arm flap, paraumbilical perforator flap, and *latissimus dorsi* flap are mostly used [37]. These demanding and time-consuming surgical procedures require an experienced microsurgical team with appropriate conditions and equipment.

## Conclusion

Algorithms and timeliness for coverage of the hand following various disease states will depend on the characteristics and localization of the soft tissue defect and the general condition of the patient. The versatile and frequent clinical application of RAPF proved that it became a workhorse flap for soft tissue reconstruction of the upper limb. This surgical solution yielded an excellent functional and aesthetic outcome in the majority of patients with limited donor site morbidity. Complex surgical procedures could be done simultaneously, together with the reconstruction of tendons, joints, or fracture stabilization. Straightforward elevation and perfusion based on cutaneous perforators alone, without the need to sacrifice the radial artery, are the main advantages of this flap. The reliability and safety of their versatile clinical application are confirmed through our clinical data.

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CASE REPORT


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# Conservative short-term treatment of non-cirrhotic and non-malignant portal vein thrombosis

## Kratkotrajno konzervativno lečenje bolesnika sa trombozom vene porte bez maligniteta i ciroze

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**Abstract**

**Introduction.** Portal vein (PV) thrombosis (PVT) is a partial or complete obstruction of blood flow as a result of a thrombus mass in the lumen of PV. In the acute phase, the symptoms such as right upper quadrant pain, nausea, and fever are unspecific. A diversity of subacute and chronic symptoms is associated with complications related to PVT and portal hypertension. **Case report.** A 50-year-old female was admitted to the hospital due to acute abdominal cramping pain. The pain lasted for 15 to 20 min and was followed by defecation of normal stool and hematochezia on three occasions. The abdominal pain recurred after several hours, again followed by hematochezia and tenesmus every 10 min. After admission, a colonoscopy was performed, and it revealed vulnerable, erythematous mucosa of the colon with contact bleeding from the lienal flexure to the rectosigmoid junction. During the colonoscopy, a biopsy was performed. A computed tomography (CT) scan revealed partial PVT of intrahepatic branches of PV, and thrombosis of the inferior mesenteric vein. After conservative treatment with low molecular weight heparin (LMWH) and other supportive measures, the digestive bleeding ceased, and defecation became normal. During the one-month follow-up, the patient had no complications, and the control CT scan revealed normal PV flow without thrombosis. **Conclusion.** Although rare, a non-malignant and non-cirrhotic PVT should not be neglected in the differential diagnosis because timely and vigilant therapy with LMWH can lead to complete resolution without serious complications.

**Key words:**
**diagnosis, differential; diagnostic techniques and procedures; heparin, low-molecular-weight; liver; treatment outcome; venous thrombosis.**

**Apstrakt**

**Uvod.** Tromboza vene porte (TVP) je delimična ili potpuna opstrukcija protoka krvi, nastala kao rezultat prisustva trombne mase u lumenu portalne vene (PV). U akutnoj fazi, simptomi kao što su bol u desnom gornjem kvadrantu, mučnina i groznica, nespecifični su. Raznolikost subakutnih i hroničnih simptoma povezana je sa komplikacijama vezanim za TVP i portalnu hipertenziju. **Prikaz bolesnika.** Žena starosti 50 godina, primljena je u bolnicu zbog akutnih bolova u stomaku u vidu grčeva. Bol je trajao od 15 do 20 min, nakon čega je usledila defekacija normalne stolice i hematohezija u tri navrata. Bol u stomaku se ponovio kroz nekoliko sati, nakon čega su ponovo usledili hematohezija i tenezmi, svakih 10 min. Nakon prijema, urađena je kolonoskopija pomoću koje je otkrivena vulnerabilna, eritematozna sluznica debelog creva, sa kontaktnim krvarenjem u dužini od lijenalne fleksure do rektosigmoidnog prelaza. Tokom kolonoskopije urađena je i biopsija. Primenom kompjuterizovane tomografije (KT) otkrivena je parcijalna TVP, tromboza intrahepatičnih grana PV i tromboza donje mezenterične vene. Nakon konzervativnog lečenja niskomolekularnim heparinom (NMH) i drugom suportivnom terapijom, krvarenje u digestivnom traktu je prestalo, a defekacija je postala normalna. Tokom mesec dana praćenja, bolesnica nije imala komplikacija, a kontrolni pregled KT pokazao je normalan protok kroz PV, bez tromboze. **Zaključak.** Iako je retka, TVP kod bolesnika bez maligniteta i ciroze ne treba da se zanemari u diferencijalnoj dijagnozi, jer pravovremena i adekvatna terapija NMH može dovesti do potpunog izlečenja, bez teških komplikacija.

**Ključne reči:**
**dijagnoza, diferencijalna; dijagnostičke tehnike i procedure; heparin, niskomolekulski; jetra; lečenje, ishod; tromboza, venska.**

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## Introduction

Portal vein (PV) thrombosis (PVT) is a partial or complete obstruction of blood flow as a result of a thrombus mass in the lumen of the portal vein (PV) [1]. In the morphological definition of PVT, it is more concise to define it as thrombosis of all or parts of the portal venous system, which includes the lienal vein, superior mesenteric vein, and inferior mesenteric vein (IMV), or extrahepatic and intrahepatic parts of PV and its branches.

The frequency of thrombosis of separate segments varies, but the most common one is the thrombosis of the PV itself (40%), followed by multiple vein thrombosis (38.5%), mesenteric vein thrombosis (9%), splenic vein thrombosis (7.5%), and hepatic vein thrombosis (5%) [2, 3].

Clinical presentation of PVT is diverse, depending on whether it is acute or chronic, complete or partial, and which part of the portal venous system is afflicted. Clinical features may range from asymptomatic to major complications with high morbidity and mortality rates. Complications of PVT are the results of consecutive portal hypertension [2, 4, 5] or are related to intestinal infarction [2, 6].

The symptoms of an acute PVT include persistent severe abdominal or lumbar pain associated with systemic inflammatory response syndrome, fever, general malaise, organ failure, metabolic acidosis, abundant ascites, rectal bleeding, abdominal contracture, nausea, postprandial fullness, intestinal infarction [7, 8]. The most common laboratory findings in PVT diagnosis are the slightly lowered prothrombin time and coagulation factors, while the D-dimer is elevated [9, 10]. PVT has a global incidence of 0.05–0.5% [11], and the most common causes are malignancies, progressive chronic liver diseases, processes localized to the epigastrium and hepatobiliary system, and acquired as well as inherited thrombophilia [12]. According to the etiological characteristics of PVT causes, there are three main categories: malignant PVT, cirrhotic PVT, and non-malignant and non-cirrhotic PVT [12]. PVT in non-cirrhotic and non-malignant individuals is rare. It can occur in the setting of abdominal inflammatory processes such as pancreatitis, infections, inflammatory bowel diseases, and after abdominal surgeries. In the absence of mentioned states, PVT may also occur; however, underlying causes may be found, such as clotting factor deficiencies, malignancy, or AIDS. In the absence of known factors possible for PVT occurrence, the prognosis and treatment are mainly empirically orientated. Literature is very sparse regarding this group of PVT, especially in those with "idiopathic" PVT, and there are no randomized controlled trials.

## Case report

A 50-year-old female was admitted to the hospital with acute abdominal cramping pain that spread diffusely after an initial physical examination. The pain itself lasted for 15 to 20 min and was followed by defecation of a harder consistency stool with hematochezia afterward on three occasions. The abdominal pain returned in several hours, again followed by hematochezia and tenesmus every 10 min.

The patient reported similar abdominal pain followed by defecation on several occasions 20 years ago when a colonoscopy was performed without any pathological findings. She was not on hormone replacement therapy. She stated that she had never had abdominal surgery.

On physical examination, the abdomen was painless and soft, with normal peristalsis on auscultation. Laboratory findings on admission showed elevated D-dimer of 23 μg/mL [reference range (RR): 0.22–0.46 μg/mL], leucocyte count of $12.2 \times 10^9$/L (RR: $4.5–11.0 \times 10^9$/L) and sedimentation rate of 18/45 mm/hr (RR: 20/25 mm/hr). Other biochemical parameters in serum, including protein C (125%), antithrombin III (74%), and coagulation factors, were in RR. The patient was tested for influenza virus, human immunodeficiency virus, hepatitis B and C virus, herpes simplex virus, and cytomegalovirus, and the results of acute viral infection were negative. Immunological analysis: antimitochondrial antibodies, anti-nuclear antibodies, anti-smooth muscle antibodies, anti-liver kidney microsome type 1 antibodies, perinuclear anti-neutrophil cytoplasmic antibodies, as well as anti-tissue transglutaminase antibodies, were also negative. The patient was not tested for JAK2 mutation because it had no clinical and laboratory indicators for myeloproliferative disease. After admission, a colonoscopy was performed, which determined vulnerable, erythematous mucosa of the colon, and contact bleeding, in part between the lienal flexure of the colon to the rectosigmoid junction. During the colonoscopy, a biopsy was performed, and pathohistological findings demonstrated ischemic colitis (Figure 1) and suspected bacterial infection. A computed tomography (CT) scan revealed partial main PVT, thrombosis of intrahepatic branches of PV, and thrombosis of the IMV (Figure 2). During the hospitalization, X-ray diagnostics were performed on the patient [Chest X-rays, CT aortography and

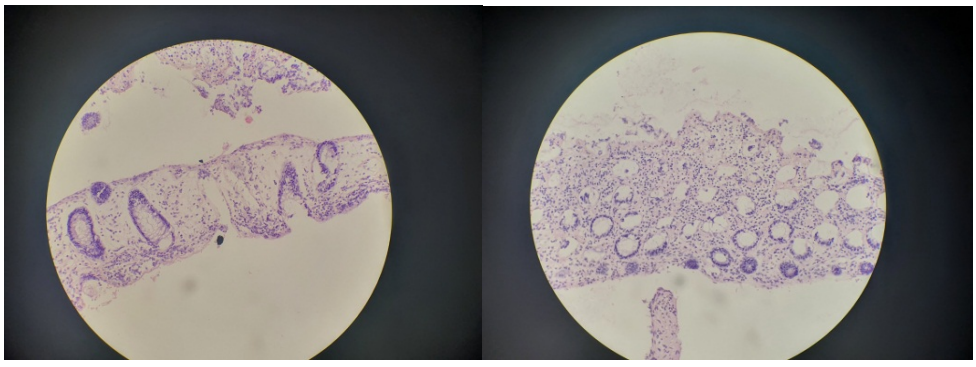

**Fig. 1 – Pathohistological finding: ischemic colitis (hematoxylin and eosin staining, ×20).**

splenoportography (SP), and ultrasound (US) of the upper abdomen], where there were no findings that would indicate a malignant disease. Thrombophilia was suspected only when it was discovered that the patient did not have any malignancies, that no infection was present, she was younger, and that there were also no findings that would indicate autoimmune or inflammatory diseases of the digestive tract. After that, heterozygous plasminogen activator inhibitor-1 (PAI-1) (carriers) was detected. Lupus anticoagulant and anticardiolipin antibodies were negative.

The patient was treated with low molecular weight heparin (LMWH) (enoxaparin sodium, Clexane®, 150 IU/kg) during hospitalization (seven days), anti-Xa (0.4 mL solution for injection, subcutaneous injection), antibiotics (ciprofloxacin, Ciprocinal® tablet 500 mg, twice a day and metronidazole, Orvagil® tablet 400 mg, three times a day), and proton pump inhibitors (pantoprazole, Nolpaza® tablet 20 mg, twice daily, half an hour before meals). After the applied measures, the digestive bleeding ceased, and defecation became normal.

A week after treatment initiation, the patient did not report any discomfort and was discharged from the hospital with a recommended therapy of novel oral anticoagulants (apixaban, Eliquis® tablet 5 mg, twice daily for six months), ciprofloxacin, and metronidazole.

One month after hospital discharge, the control CT scan showed a total resolution of the PVT (Figure 3). During the follow-up of three months, the patient was without any pathognomonic signs and symptoms.

**Discussion**

Consistent with every thrombosis, the basic pathophysiological mechanism that causes PVT is Virchow's triad (venous stasis, hypercoagulability, and endothelial injury) [2, 13]. The pathological entities that contribute to the development of this triad can be classified as local, in 70% of the PVT cases, and systemic, which cause PVT in 30% of the cases [9, 14–17]. The most common local risk factors of PVT are neoplasms, focal inflammatory lesions, neonatal omphalitis, umbilical vein catheterization, diverticulitis, appendicitis, pancreatitis, duodenal ulcer, cholecystitis, tuberculous lymphadenitis, Crohn's disease, ulcerative colitis, cytomegalovirus hepatitis, portal venous system injuries, splenectomy, colectomy, gastrectomy, liver transplantation, abdominal trauma, and cholecystectomy [9, 14]. On the other hand, the most common systemic risk factors of PVT are hereditary thrombophilia, factor V Leiden mutation, factor II mutation, protein C deficiency, protein S deficiency, antithrombin deficiency, acquired thrombophilia, myeloproliferative disorder, antiphospholipid syndrome, paroxysmal nocturnal hemoglobinuria, oral contraceptives, pregnancy, puerperium, and hyperhomocysteinemia [9, 14]. According to the guidelines of the American Association for the Study of Liver Diseases (AASLD) from 2009, the diagnostic approach to suspected PVT considers the methods of choice, such as B-mode US with a Doppler examination and CT scan with SP (Table 1) [18].

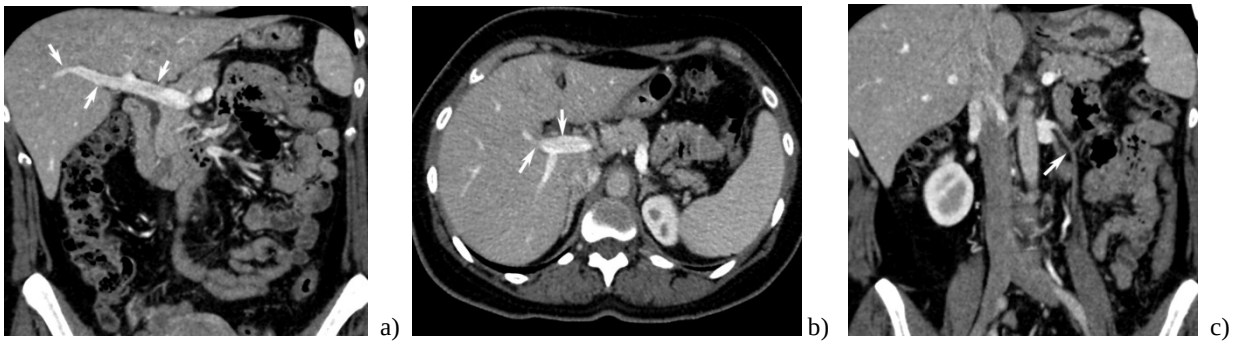

**Fig. 2 – Multislice computed tomography angiography of the abdomen on admission to the hospital: a) PVT-coronal plane (white arrows); b) PVT-axial plane (white arrows); c) IMVT-coronal plane (white arrows). PVT – portal vein thrombosis; IMVT – inferior mesenteric vein thrombosis.**

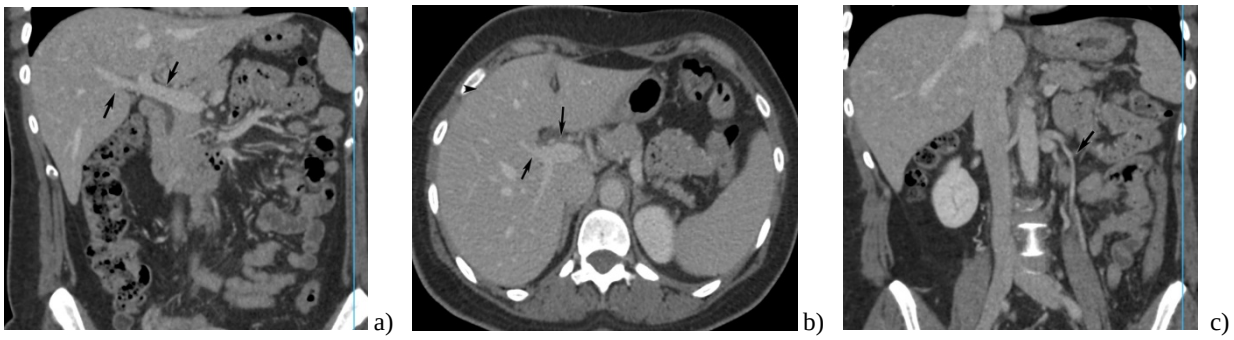

**Fig. 3 – Multislice computed tomography angiography of the abdomen, control scan, month after discharge from the hospital: a) Resolved PVT-coronal plane (black arrows); b) Resolved PVT-axial plane (black arrows); c) Resolved IMVT-coronal plane (black arrows).**
**For abbreviations, see Figure 2.**

**Table 1**

**Recommendations for the diagnosis of acute portal vein thrombosis (PVT)** [18]

| |
|---|
| Consider the diagnosis of acute PVT in any patient with abdominal pain longer than 24 hours, whether or not there is also fever or ileus. |
| If acute PVT is suspected, a CT scan, before and after injection of vascular contrast agent, should be obtained for early confirmation of diagnosis. If a CT scan is not rapidly available, obtain Doppler-sonography. |
| In patients with acute PVT and high fever and chills, septic pylephlebitis should be considered, whether or not an abdominal source of infection has been identified, and blood cultures should be routinely obtained. |
| In acute PVT, the possibility of intestinal infarction should be considered from presentation until the resolution of pain. The presence of ascites, thinning of the intestinal wall, lack of mucosal enhancement of the thickened intestinal wall, or the development of multiorgan failure indicates that intestinal infarction is likely and surgical exploration should be considered. |

**CT– computed tomography.**

The US is a harmless, fast, and easily available technique with sensitivity and specificity ranging from 80 to 100% depending on the objective circumstance and the experience of the radiologist [15, 19]. An eventual finding that supports the diagnosis of PVT is the presence of a homogeneous (in acute thrombosis) or a heterogeneous (in chronic thrombosis) formation in the anechogenic blood vessel lumen without a Doppler signal in the thrombosed vessel. The US is not suitable for peripheral vein examination, mostly pertaining to mesenteric vessels [17].

The multislice computed tomography (MSCT) SP, besides being more sensitive compared to the US, also offers information on possible bowel ischemia or perforation [10]. A finding that indicates PVT is the presence of hyperdense thrombus masses in the vein lumen, which, after intravenous application of the contrast agent, gives a filling effect disrupting the normal opacification in the vessel lumen. Bland thrombus will not enhance in radiodensity, while tumor thrombus will have a certain post-contrast enhancement. The thrombus masses will have different densities [expressed in Hounsfield Units (HU)] depending on the thrombus maturation, meaning that MSCT can help evaluate the thrombus age. Newly formed thrombus masses have a density of around 55 HU, while older ones have around 30 HU [10]. Generally, the sensitivity and specificity of detecting a PVT vary for the US from 66–100%, but in the combination of US with color Doppler, sensitivity is 100%, and specificity is 93% [20]. The sensitivity and specificity of CT scan with SP in diagnosing PVT amounts to at least 90% [21].

On the other hand, the treatment of PVT ranges from observation without active therapy, thrombectomy, and other interventional procedures such as transjugular intrahepatic portosystemic shunt (TIPS). The aim of the treatment consists of the resolution of symptoms, prevention and treatment of mesenteric ischemia, and prevention of thrombus extensions. Treatments and outcomes of an acute PVT depend on the involvement of the remaining splanchnic circulation, as well as associated factors such as liver cirrhosis or malignancy.

The American College of Chest Physicians (ACCP) and AASLD have separated clinical guideline recommendations for the treatment of non-cirrhotic and non-malignant acute PVT.

The first aforementioned society recommends anticoagulation for symptomatic PVT with a grade 1B level of evidence; however, they do not suggest anticoagulation for asymptomatic, incidentally diagnosed PVT, which has a grade 2C level of evidence. The second society suggests anticoagulation for all acute PVT regardless of symptomatology.

Plessier et al. [22] showed that the use of anticoagulants for treating non-cirrhotic and non-malignant PVT led to complete recanalization in 38.3% and partially recanalization in 14% of patients. Most patients were treated with LMWN or unfractionated heparin. Thrombosis of a proximal portal venous system portion showed a better response to thrombolysis, and nine patients had digestive bleeding. The length of therapy is not clearly defined; however, recanalization is recommended after 4–6 months of therapy initiation. Long-term therapy is recommended in case of a confirmed prothrombotic disorder (thrombophilia), recurrent thrombotic episodes, or positive family history of venous thrombosis [23].

The PVT thrombolysis is controversial without relevant data or guidelines. Various studies have shown different treatment modalities for thrombolysis with inconclusive, inconsistent, and controversial data. Those include the usage of tissue plasminogen activator, streptokinase, or urokinase, indirectly by catheterization of the superior mesenteric artery or directly, percutaneously, by trans-hepatic approach into the PV or a TIPS; all of this is available as a therapeutic course [24–27]. Since the incidence of bleeding as a complication of thrombolytic therapy remains high (up to 60%), this approach may be offered only in selected cases. Therefore, the principal therapy for non-malignant and non-cirrhotic PVT should be conservative [27]. Surgical thrombectomy is not recommended nowadays due to PVT recurrence, surgical morbidity, and high mortality rate [28].

A recently published study by Klinger et al. [29] showed that in 17 patients who underwent thrombolytic therapy with a transjugular approach, recanalization was achieved in 94%, without recurrence of PVT in 88% of patients during the two-year follow-up. This kind of treatment may be offered in cases when anticoagulation fails or in the setting of the occurrence of PVT complications.

Further studies are necessary to confirm the effectiveness of combined conservative and minimally invasive

treatment of this serious clinical condition. Furthermore, follow-up studies may evaluate the adequate length of conservative therapy in preventing PVT recurrence.

PVT, once considered a contraindication for TIPS, has indeed become an "indication" in cirrhotic and non-cirrhotic cases [18, 30, 31]. The potential concerns in performing TIPS in a patient with acute PVT would be the following: increased technical difficulty in performing the procedure as their blood cannot be freely aspirated from the portal vein after the puncture, a gradient across the stent cannot always be established, and the risk for pulmonary embolism when portal venous thrombolysis is done through TIPS tract. As experience has grown and technology has evolved, the US guidance of transvenous access to the portal vein from the hepatic vein contributes to the overall higher success rate of performing the TIPS procedure and reducing the procedure-related complications.

## Conclusion

Non-malignant and non-cirrhotic PVT is a rare and potentially serious clinical condition. The differential diagnosis for PVT should not be neglected because timely and vigilant therapy with LMWH can lead to complete resolution without serious complications.

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CASE REPORT


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# Open pleural decortication in a 12-day-old neonate with empyema thoracis

## Otvorena dekortikacija pleure kod novorođenčeta uzrasta 12 dana sa empijemom

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**Abstract**

**Introduction.** Empyema thoracis, defined as the accumulation of pus in the pleural space, is rare in the neonatal population. Limited data are reported in the medical literature, and still, no treatment guidelines are available for this age. **Case report.** We present a term 12-day-old neonate (born healthy) who developed sepsis caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and pneumonia associated with advanced-stage empyema. The child was admitted to our hospital with a few-hours history of difficulty breathing and lethargy. On admission, the child was cyanotic with desaturation and in severe respiratory distress; therefore, the child was intubated, and mechanical ventilation was started. Imaging tests were performed in an emergency, hence chest computed tomography (CT) scan was done without contrast. Suspected congenital pulmonary airway malformation with trapped air collections, significant mediastinal shift on CT scan, and deterioration of the patient's condition indicated urgent surgery. Intraoperatively, the diagnosis of stage II empyema was established, and decortication of thickened parietal and visceral pleura was performed. Afterward, the baby showed quick and progressive clinical improvement. **Conclusion.** The diagnosis and management of empyema in neonates may be challenging, especially in the case of unremarkable history, fulminant progression of the disease, and incomplete imaging tests.

**Key words:**
**diagnosis; infant, newborn; empyema, pleural; sepsis; thoracic surgical procedures; tomography, x-ray computed.**

**Apstrakt**

**Uvod.** Empijem pleure, definisan kao prisustvo gnoja u pleuralnom prostoru, retko se javlja u neonatalnom uzrastu. Ne postoji mnogo podataka u medicinskoj literaturi, kao ni smernica za lečenje u toj uzrasnoj grupi. **Prikaz bolesnika.** Prikazujemo zdravo terminsko novorođenče uzrasta 12 dana, koje je razvilo sepsu izazvanu meticilin rezistentnom bakterijom *Staphylococcus aureus* (MRSA) i pneumoniju, udruženu sa kasnim stadijumom empijema pleure. Dete je primljeno u bolnicu zbog otežanog disanja i letargije, koji su se javili nekoliko sati pre prijema u bolnicu. Na prijemu u bolnicu novorođenče je bilo cijanotično, u teškom respiratornom distresu, zbog čega je odmah intubirano i započeta je mehanička ventilacija. Hitno su sprovedena radiološka ispitivanja i urađena je kompjuterizovana tomografija (KT) grudnog koša bez kontrasta. Zbog sumnje na kongenitalnu malformaciju disajnih puteva sa „zarobljenom" kolekcijom vazduha, značajnog pomeranja medijastinuma viđenog na snimku KT, kao i zbog pogoršanja stanja deteta, sprovedena je hitna hiruška intervencija. Intraoperativno je dijagnostikovan empijem pleure drugog stadijuma i učinjena je dekortikacija parijetalne i visceralne pleure. Novorođenče se brzo oporavilo nakon intervencije. **Zaključak.** Dijagnoza i lečenje empijema kod novorođenčeta mogu predstavljati izazov, posebno u slučaju nejasne anamneze, fulminantne progresije bolesti i nekompletnog radiološkog ispitivanja.

**Ključne reči:**
**dijagnoza; novorođenče; empijem, pleuralni; sepsa; hirurgija, torakalna, procedure; tomografija, kompjuterizovana, rendgenska.**

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## Introduction

Empyema thoracis is defined as a pyogenic infection of the pleural cavity with an accumulation of pus in the pleural space [1]. It is frequently seen in children; however, it is very uncommon in the neonatal population. Only a few reports of neonatal empyema thoracis are described in the medical literature [2–5]. Due to the paucity of cases and their differences, the predisposing factors and etiopathogenesis in the neonate are still uncertain. Moreover, there are still no treatment guidelines for managing empyema in neonates. Various modalities of treatment, from antibiotics, chest tube drainage, intrapleural fibrinolytic agent instillation, video-assisted thoracoscopic surgery (VATS) to surgical decortication, have been suggested for treating different stages of empyema in children [6]; however, there are no data available for neonatal age. Although pleural empyema is rare in neonates, it is a life-threatening emergency with rapid progression and high mortality.

We present a term 12-day-old neonate (born healthy) who developed sepsis caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and pneumonia associated with stage II pleural empyema.

## Case report

A previously healthy term 12-day-old male neonate was admitted to our hospital due to difficulty breathing and lethargy. The mother noticed these symptoms the same morning the child was admitted to the hospital. The infant was born by spontaneous delivery at term gestation with an unremarkable antenatal history.

On physical examination on admission, the baby was cyanotic with desaturation and in severe respiratory distress. Blood gas analysis revealed hypoxia, hypercapnia, and acidosis, so the baby was immediately intubated, and mechanical ventilation was started. However, despite mechanical ventilation and administration of 100% oxygen, the baby had low oxygen saturation (Sat $O_2$ = 77%).

A supine babygram following intubation showed hyperlucent right hemithorax with triangular soft tissue opacity in the lateral aspect, increased intercostal spaces, depression of the diaphragm, and mediastinal shift to the left (Figure 1). On the chest ultrasound examination, an absence of lung sliding and comet tail artifacts was evident in most of the right hemithorax, with some pleural effusion and debris in its posterior aspect, without the possibility of differentiating pneumatoceles and pneumothorax. An emergency computed tomography (CT) scan was then performed but without contrast medium administration due to the patient's bad clinical condition in order to further characterize the lung pathology. On noncontrast emergency chest CT, a large septated "coffee bean-shaped" air collection in the anterior aspect of the hemithorax was visualized, extending from the lung apex to the diaphragm, with interposed compressed lung parenchyma (possibly part of the middle lobe) between these air collections and numerous cystic, air-filled spaces in the rest of the compressed lung parenchyma. The ipsilateral increased intercostal spaces, depression of the diaphragm, and contralateral mediastinal shift were confirmed. Differential diagnoses included right-sided congenital pulmonary airway malformation (congenital cystic adenomatoid malformation) vs. pneumothorax (Figure 2).

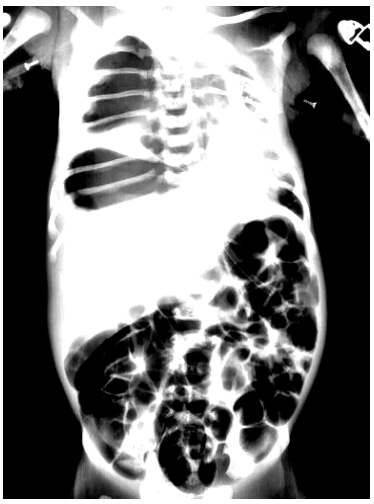

**Fig. 1 – Babygram on admission showing hyperlucent right hemithorax with triangular soft tissue opacity in the lateral aspect, ipsilateral increased intercostal spaces, depression of the diaphragm, and mediastinal shift to the left.**

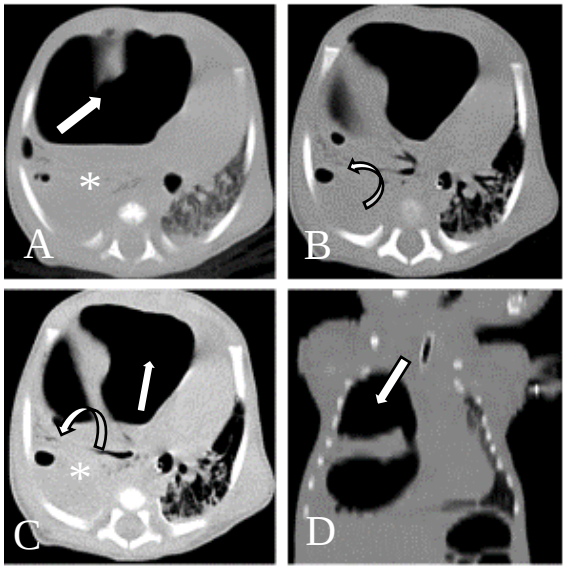


**Fig. 2 – Noncontrast chest computed tomography scans in axial (A, B and C) and coronal (D) views demonstrate a large septated "coffee bean-shaped" air collection in the anterior aspect of the hemithorax (white arrow on A, C and D), extending from the lung apex to the diaphragm, with interposed compressed lung parenchyma (possibly part of the middle lobe – asterisk on A and C) between these air collections and numerous cystic, air-filled spaces in the rest of the compressed lung parenchyma (curved arrow on B and C). The ipsilateral increased intercostal spaces, depression of the diaphragm, and contralateral mediastinal shift were evident.**

The initial laboratory analyses revealed leukocytosis [30.3 × $10^9$/L (reference range – RR 5.0–20.0 × $10^9$/L)] with 78% neutrophils (RR 25–55%), hyponatremia [121 mmol/L (RR 131.0–141.0 mmol/L)], and hyperglycemia [32 mmol/L (RR 3.0–6.49 mmol/L)]. C-reactive protein (CRP) was significantly elevated – 196 mg/L (normal values < 3 mg/L), whereas other serum chemistries were normal. The patient was managed with intravenous (iv) fluids, antibiotics (amikacin plus ceftriaxone), various ventilator modes and mechanical ventilation settings, and other supportive therapies.

In the following few hours, the patient's general condition got worse. The child had severe hypoxemia – $PaO_2$ = 42 mmHg (RR 83–108 mmHg), Sat $O_2$ = 72% (RR 95–98%), hypercapnia [$PaCO_2$ = 80 mmHg (RR 35–40 mmHg)], and combined respiratory and metabolic acidosis despite optimization of the ventilator settings and $FiO_2$ of 100%. Considering the deteriorating patient's condition and the high suspicion of progressively enlarging congenital lung malformation, the baby underwent urgent surgery on the same day. Thick fibrous parietal and visceral pleura were found. Frank pus and debris in the pleural cavity were found and all removed. Decortication of thickened parietal and visceral pleura was performed. The right lung expanded completely. Collections of pus in the lung were not found. Three air leaks detected were closed, and chest tube drains were placed. On postoperative babygram, the chest tube was *in situ*, and a small residual apical pneumothorax was evident. The right lung was completely expanded with diffuse, patchy, partially fused reticulonodular airspace opacities, mostly present in basal and central lung projections (Figure 3).

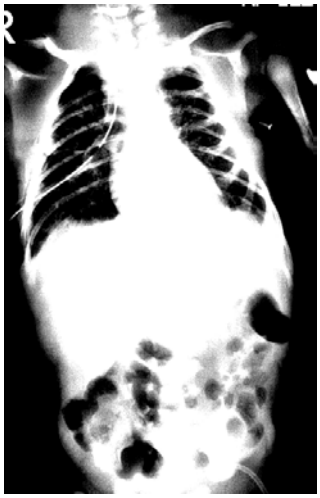

**Fig. 3 – Postoperative babygram showing chest tube *in situ* and small residual apical pneumothorax, the right lung completely expanded with diffuse, patchy partially fused reticulonodular airspace opacities, mostly present in basal and central lung projections.**

Pus was sent for analysis. Biochemical examination revealed pH = 7.0 (RR 7.60–7.64), glucose 1.9 mmol/L (RR 3.0–6.49 mmol/L), and LDH = 4,550 U/L (normal values < 113 U/L). Pus culture showed growth of MRSA. The blood cultures from admission grew MRSA with the same antibiotic sensitivity. At this point, vancomycin was added to the therapy, and iv immunoglobulin as well**.** The infant's nasal swab was negative for MRSA colonization. Cerebral spinal fluid analysis showed normal glucose and protein level with no pleocytosis, and there was no growth on bacterial culture. The echocardiogram was normal. Investigation for primary immunodeficiency for the infant was also normal.

In the days following surgery, the baby showed quick and progressive clinical improvement, inflammation markers and leukocyte count became normal, and the repeated blood cultures were negative. The intercostal drain was removed on the seventh postoperative day, and the baby was weaned from mechanical ventilation on the eighth postoperative day. Antibiotics were stopped after a total duration of 18 days, and the baby was discharged home.

Chest X-rays performed at hospital discharge and in a four-week follow-up revealed persistent patchy opacity in the central portion of the right lung (Figure 4). The patient, seen at the outpatient clinic at monthly intervals, was asymptomatic and steadily gaining weight.

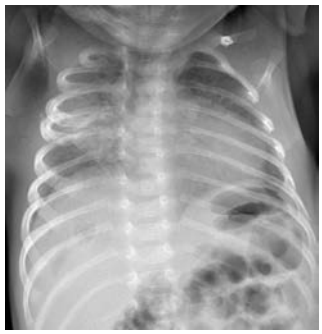

**Fig. 4 – Chest X-ray upon discharge showing persistent patchy opacity in the central portion of the right lung.**

On the follow-up chest CT scan performed six months after surgery, some pleural thickening and pleural adhesions as well as parenchymal fibrotic bands in the right middle and lower lobe were evident (Figure 5).

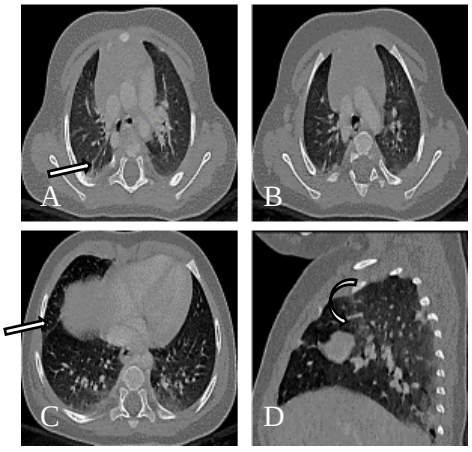


**Fig. 5 – Follow-up chest computed tomography scans in axial (A, B and C) and sagittal (D) view six months after surgery showed only pleural thickening and pleural adhesions (straight arrow) as well as parenchymal fibrotic bands in the right middle and lower lobe (curved arrow).**

## Discussion

In this report, we describe a 12-day-old neonate with MRSA sepsis and pneumonia associated with stage II empyema who was successfully treated with open decortication.

Our patient did not have potential risk factors for MRSA infection as in other reported cases [2, 3]. The patient was not previously hospitalized but was admitted from home. Antenatal history was unremarkable. The parents' nasal swabs were negative for MRSA colonization. The child had no siblings. Primary immunodeficiency was ruled out. The patient suddenly developed signs of severe respiratory distress without prodromal symptoms, so the baby was intubated on admission. This rapid progression of the disease and bad clinical condition on admission are unique from the other reviewed cases [2–5]. In a few case reports of empyema, neonates had stable vital signs, so all the imaging studies could be completely done [3–5].

In terms of diagnosis, chest radiography, ultrasound, and CT scan are utilized for evaluating possible empyema. In the presented case, we performed a CT scan in an emergency without contrast due to the patient's bad clinical condition. The iv contrast allows visualization of pleural inflammation, which is not normally possible with a noncontrast enhanced scan; therefore, split-pleural signs suggestive of pleural empyema could not be shown, which made diagnostics difficult. The literature emphasizes that iv contrast enhancement can be particularly helpful in young children who have poor natural tissue contrast, frequently enabling the differentiation of pneumonia from atelectasis, effusion, empyema, and adenopathy [7].

The management of empyema depends not only on clinical presentation but also on the stage of the disease. Empyema progresses through three stages: exudative, fibropurulent, and organizing stage. According to the American Thoracic Society (ATS), it was the fibropurulent stage (stage II) in our case in which frank pus was present and pleural surfaces became thickened due to fibrin deposition with the formation of loculations [8].

Determination of the empyema stage is important in choosing an appropriate therapeutic option. Symptoms duration has been suggested as one of the criteria for estimating the stage of empyema in children [9], which was challenging in our case due to the rapid progression of the disease. Furthermore, although ultrasound and CT have established roles in the investigation of pleural effusion, previous studies have shown some limitations regarding the determination of the stage of empyema. Kearney et al. [10], studying 50 adult patients with parapneumonic effusion, concluded that neither ultrasound nor CT reliably identifies the stage of pleural infection nor its likelihood of requiring surgical intervention. Additional evidence that CT is unable to distinguish stages of pleural infection in the pediatric population comes from studies by Donnelly and Klosterman [11] and Jaffe et al. [12]. In the presented case, due to a few-hours duration of symptoms and performed CT scan without contrast, it was very difficult to estimate the stage of the disease.

The management of empyema is further influenced by existing practice, surgical experience, availability of appropriate expertise, and local resources. For the advanced stage of empyema, as in our case, therapeutic options included chest tube drainage with or without fibrinolysis, VATS, or open decortication. Considering that our patient was in bad clinical condition and that we could not rule out underlying congenital lung malformation, VATS or open decortication were available treatment options. Even though VATS has been established as one of the standard modalities for the treatment of pleural empyema in older children, it is not routinely used in neonates due to the non-availability of small-sized instruments and other technical limitations of this age [13, 14]. Recently, Sanghvi et al. [5] reported the use of VATS in a 20-day-old newborn with staphylococcal pneumonia and empyema. In our case, due to the non-availability of the equipment for VATS in neonatal age, open decortication was done. Data regarding open decortication in neonates are limited as well since most of the reports were focused on older children beyond neonatal age [9, 15–17]. Open decortication frequently has some perioperative complications, such as persistent air leak from the lung, excessive bleeding, and bronchopleural fistula formation. However, we did not have any of the above complications in the presented case.

According to the literature, if the initial treatment is successful, children usually show complete clinical recovery, and chest radiographs return to normal in 3–6 months [18, 19], which is in accordance with our case.

## Conclusion

In conclusion, this case highlights the challenges of diagnostics and management of empyema in neonates, especially in the case of unremarkable history, fulminant and rapid progression of the disease, and incomplete imaging tests.

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# Remembrance of six prominent Serbian toxicologists who died during 2020 and 2021

## Sećanje na šest istaknutih srpskih toksikologa koji su preminuli tokom 2020. i 2021. godine

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**Key words:**
**death; history of medicine; serbia; toxicology; health personnel.**

**Ključne reči:**
**smrt; istorija medicine; srbija; toksikologija; zdravstveno osoblje.**

### Introduction

Mass deaths of the population, doctors, and other medical personnel usually occur in wars, epidemics of infectious diseases, and natural or technological disasters.

In World War I, in the great epidemic of "three typhus" (spotted, recurrent, and abdominal), from the end of 1914 to the middle of 1915, 120 doctors died in Serbia: 87 members of the Serbian military ambulance, 21 doctors from international humanitarian missions who worked as volunteers in the Serbian military ambulance, and 12 doctors of the Austro-Hungarian and German armies who were prisoners of war [1]. During World War II, from 1941 to 1945, 213 doctors died in the medical corps of the People's Liberation Army of Yugoslavia [2]. During the smallpox epidemic in 1972, 184 people fell ill on the territory of Serbia, while 40 of them died [3]. There were no doctors among the dead, but two nurses died – Dušica Spasić, who worked at the First Surgical Clinic in Belgrade, and Milka Đurisić, who worked at the hospital in Čačak, and they participated in the treatment and care of the sick.

In the last two years, more than 456 million people have fallen ill from the still current pandemic caused by the SARS-CoV-2, and more than six million have died. The first cases of the disease in Serbia were registered on March 6, 2020, and a total of 1,939,150 patients and 15,570 deaths have been recorded up to this day [4]. Statistical data have not yet been processed to provide an insight into the illness and death of doctors and other medical staff, who, by the nature of their work, are most exposed to the risks of illness and death from this contagious disease.

During the last 40 years in Serbia, despite the turbulent social events that took place in the last decade of the twentieth century – civil wars fought in one part of the former Yugoslavia and the aggression of the North Atlantic Treaty Organization (NATO) against Serbia and Montenegro – four times in a row, two or three prominent toxicologists passed away in two consecutive years, an example of which is given in the following text.

In two consecutive years, 1989 and 1990, two of the toxicologists who passed away were: Vojislav Ćosić (born 1920, in Vladimirci, Serbia – died 1990, in Belgrade, Serbia), a doctor, internist, cardiologist, and toxicologist, colonel, professor at the Military Medical Academy (MMA), head of the Department of Toxicology of the Clinic for Internal Medicine of the MMA from 1963 to 1983, and head of the Clinic for Emergency Internal Medicine from 1983 to 1986 [5]; Miloš Stanković (born 1926, in Belgrade – died 1989, in Belgrade), a pharmacist, specialist in toxicological chemistry, scientific advisor, expert of the International Labor Organization, and head of the Toxicology Laboratory at the Institute of Occupational Medicine "Dr. Dragomir Karajović" in Belgrade [6].

In the next two consecutive years, 2000 and 2001, two of the toxicologists that passed away were: Branko Banić (born 1931, in Belgrade – died 2000, in Novi Sad, Serbia), a doctor, pharmacologist, and toxicologist, professor at the Faculty of Medicine (FM) in Novi Sad, head of the Department of Pharmacology and Toxicology at the same Faculty [7]; Radivoje Kušić (born 1925, in Kraljevo, Serbia – died 2001, in Belgrade), a doctor, internist, clinical toxicologist, colonel, professor at the MMA, and head of the Clinic for Toxicology and Pharmacology at the MMA from 1981–1989 [8].

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Further down the timeline, in the year 2008, two toxicologists died: Vladimir Vojvodić (born 1930, in Cetinje, Montenegro – died 2008, in Belgrade), a doctor, toxicologist, and pharmacologist, lieutenant general of the Yugoslav People's Army, professor at the MMA, member of the Academy of Sciences and Arts of Montenegro, head of the MMA from 1980–1988, and head of the Sanitary Administration of the General Staff of the Yugoslav Army from 1988–1992 [9]; Branislav Toković (born 1930, in Dramče, near Delčevo, North Macedonia – died 2008, in Belgrade), a pharmacist and law graduate, toxicological chemistry specialist, colonel, professor at the MMA, and head of the Toxicological Chemistry Department at the MMA from 1968–1995 [10].

Finally, in the two consecutive years of 2010 and 2011, three toxicologists died: Danilo Soldatović (born 1927, in Strasbourg, France – died 2010, in Belgrade), a pharmacist, professor of the toxicological chemistry at the Faculty of Pharmacy (FPH) in Belgrade, director of the Institute of Toxicological Chemistry, and head of the Department of the Toxicology at the FPH in Belgrade – today, both institutions bear his name [11]; Dušan J. Jovanović (born 1953, in Belgrade – died 2010, in Belgrade), a doctor, toxicologist and pharmacologist, colonel, professor at the MMA, and head of the National Center for Poison Control from 1998–1999 and 2006–2009 [12]; Nedeljko Rosić (born 1932, in Belgrade – died 2011, in Belgrade), a doctor, toxicologist and pharmacologist, colonel, professor at the MMA, and head of the Sector for Scientific Research and Education of the MMA from 1986–1996 [13].

However, in less than two years, from January 2020 to October 2021, six top toxicologists died in Serbia: academician Dr. Milan Jokanović, professors Dr. Vesna Matović, Dr. Aleksandar Vidaković, and Dr. Dubravko Bokonjić, scientific advisor Dr. Neško Nešković, and colonel primarius Dr. Svetislav Ranđelović. Their professional and scientific work covered almost all areas of this multidisciplinary activity: analytical, professional, emergency, clinical, military, and experimental toxicology and ecotoxicology.

The aim of this paper was to present their life and work biographies.

**Professor Aleksandar Vidaković, MD, Ph.D.**

Professor Aleksandar Vidaković was a doctor, a specialist in internal and occupational medicine, a clinical toxicologist, a doctor of science, a full professor at the FM in Belgrade, and a full member of the Academy of Medical Sciences of the Serbian Medical Society. He was born on April 16, 1936, in Belgrade.

He finished elementary school and high school in Vrnjačka Banja and the FM in Belgrade in 1960. He spent his entire work time in various positions at the Institute of Occupational Medicine and Radiological Protection "Dr. Dragomir Karajović" in Belgrade: he was head of the Department of Professional Toxicology, head of the Clinical Laboratory, head of the Clinic for Professional Toxicology, assistant director of the Institute for Scientific Research, director of the Institute (1993–2001), and advisor to the director of the Institute (2001–2003). In his professional, scientific, and pedagogical work, he mostly dealt with professional toxicology [14]. He passed the specialist exam in Occupational Medicine in 1967 and the specialist exam in Internal Medicine in 1972. He defended his habilitation work "Contribution to the knowledge of blood coagulation disorders under the influence of carbon disulfide" in 1970 at the FM in Novi Sad and his doctoral dissertation "Contribution to the knowledge of the pathogenesis of anemia in chronic lead poisoning" in 1975 at the FM in Belgrade. Dr. Vidaković was elected assistant professor for Occupational Medicine at the FM in Belgrade in 1979 and full professor in 1991. He was the head of the Department of Undergraduate Teaching and the Department of Postgraduate Teaching for the subject of Occupational Medicine at the same Faculty. He organized a fifteen-day course in Emergency Toxicology, was the organizer and manager of the specialization in Clinical Toxicology at the FM in Belgrade, a mentor in the preparation of 11 doctoral dissertations, 10 master theses, and several subspecialist papers, as well as a member of the commissions for evaluation and defence of subspecialist and master papers and doctoral theses.

He published 330 papers in the country and abroad and authored four monographs: "Criteria for Assessing Working Ability in Occupational Diseases" (1987), "Occupational Toxicology" (2000), "Forensic Expertise of Non-Pecuniary Damage in the Case of Occupational Diseases and Injuries" (2005), and "Forensic Expertise in Occupational Medicine" (2011). He was one of the authors of the textbooks "Occupational Medicine" (1978, 1981, and 1984) and "Fundamentals of Occupational Medicine" (1998), as well as the "Diagnostic and Therapeutic Medical Manual" (1980, 1992). Professor Vidaković was the editor-in-chief of the postgraduate textbooks "Occupational Medicine I" (1996) and "Occupational Medicine II" (1997), which are major works in this field, and one of the editors of the manual "Assessment of Working Ability" (2003) and the script "Urgent Toxicology" (1991). He was also a contributor to the "Medical Lexicon" (1999).

As an independent researcher, he led projects on the toxic effects of vinyl chloride, copper, aluminum, mercury, polychlorinated biphenyls, and pesticides, funded by the Republic Science Association. He was co-organizer of the scientific conference "Toxicology of Metals" (1999) and the organizer of 15 seminars on "Forensic expertise of non-pecuniary damage in occupational medicine". He was very active in professional medical associations: President of the Section of Occupational Medicine of the Serbian Medical

Society from 1981 to 1983; one of the founders of the Section of Toxicology of the Serbian Medical Society; President of the Association of Toxicologists of Yugoslavia; member and president of the Association of Occupational Medicine of Yugoslavia; member of the Organizing Committee of the Sixth (Tara, 1994), Seventh (Igalo, 1998), and Eighth (Tara, 2002) Congress of Toxicologists of Yugoslavia. He was elected an associate member of the Academy of Medical Sciences of the Serbian Medical Society in 1994, and a full member in 1996.

He passed away on January 17, 2020, in Belgrade due to the worsening of previously established chronic diseases.

**Professor Vesna Matović, pharmacist, Ph.D.**

Professor Vesna Matović was a pharmacist, a specialist in toxicological chemistry, a doctor of science, and a full professor at the FPH in Belgrade. She was born on May 10, 1953, in Belgrade.

She finished elementary school, high school, and FPH (1977) in Belgrade. From 1978, she worked at the Department of Pharmaceutical Chemistry, where she was elected as an assistant trainee in 1980. She was elected assistant professor of Toxicological Chemistry and Clinical Toxicological Analysis in 1992, associate professor in 1997, and full professor in 2004 [15]. She defended her master's thesis titled "Changes in copper content in the organs of experimental animals in the conditions of saturnism and manganism" in 1981 and her doctoral dissertation "Antagonism of lead and magnesium in the conditions of saturnism" at the FPH in Belgrade in 1991. She passed the specialist exam in Toxicological Chemistry in 1994. She participated in the organization and implementation of teaching and numerous other activities at the FPH in Belgrade: she was head of academic doctoral studies, module Toxicology; she participated in the implementation and organization of theoretical classes in integrated academic studies in the study programs of Pharmacy and Pharmacy-Medical Biochemistry in six subjects – Toxicology, Clinical Toxicological Analysis, Introduction to Medical Biochemistry, Acute Drug Poisoning with Analytics, Human Health Risk Assessment, and Ecotoxicology; she organized and conducted classes for the following specializations: Toxicological Chemistry, Toxicological Risk Assessment of Environmental Pollutants, and Sanitary Chemistry. She was a mentor in the preparation of six doctoral dissertations and one master thesis and several times a member of examination commissions for the defence of doctoral dissertations.

Professor Matović was the author of four textbooks for pharmacy students: "Toxicology of Metals" (2010), "Drug Poisoning – Selected Chapters" (2013), "Practicum in Toxicological Chemistry" (2017, four editions), and "Practicum in Clinical Toxicological Analysis" (2017, four editions). She reviewed four textbooks for pharmacy students and numerous papers in journals from Science Citation Index list. She was the author of about 200 papers published in leading international and national journals or as Analytics chapters in books and thematic monographs. She gave several plenary lectures by invitation at international and national congresses of toxicologists. Professor Matović was the director of the Institute of Toxicology "Academician Danilo Soldatović" and the head of the Department of Toxicology at the FPH in Belgrade from 2001 until her death. She was a member of the Presidency of the Association of Toxicologists of Yugoslavia from 1994 and Deputy President of the Association from 2002 to 2010. When the Association of Toxicologists of Serbia was formed as a replacement for the Association of Toxicologists of Yugoslavia in 2010, she was elected the first president and performed that duty until she died in 2020. In the Section for Toxicological Chemistry at the Pharmaceutical Society of Serbia (later renamed the Pharmaceutical Association of Serbia), she was secretary from 1986 to 1994 and president from 1994 until her death. She was the vice president of the Pharmaceutical Association of Serbia from 2010 to 2015, and after that, the president until her death. During her term as president, the Association of Toxicologists of Serbia was admitted to the European Association of Toxicologists (EUROTOX) and the International Union of Toxicologists (IUTOX) in 2010. From 2014, she was a member of the Executive Board of the European Association of Toxicologists. She was the president of the Organizing Committee of the 10th (Palić, 2010), 11th (Sremski Karlovci, 2014), and 12th (Belgrade, 2018) Congress of Toxicologists of Serbia, and the 10th Congress of Toxicologists in Developing Countries (Belgrade, 2018). She spoke English, French, Italian, and Russian; she retired in 2018.

She passed away on January 29, 2020, in Belgrade after the worsening of previously established chronic diseases.

**Neško Nešković, agronomy engineer, Ph.D.**

Dr. Neško Nešković was an agronomy engineer, a doctor of science, an ecotoxicologist, and a scientific advisor. He

was born on October 24, 1943, in the village of Donja Ljubovidja, municipality of Ljubovija, Serbia.

He finished primary school in Donja Ljubovija and Ljubovija and secondary agricultural school in 1961 in Šabac. As a high school student, he participated in the work action in 1960 on the construction of the road Preljina–Čačak–Titovo–Užice. He graduated from the Faculty of Agriculture (Plant Protection Group) in Belgrade in 1966. He defended his master's thesis "Ecology of rats (Rattus sp) on agricultural-industrial facilities" at the Faculty of Natural Sciences in Belgrade (Department of Biological Sciences, Subdivision of Animal Ecology) in 1970 and his doctoral dissertation titled "Study of metabolism and toxicity of carbaryls and propoxur in experimental animals" at the Faculty of Agriculture in Belgrade in 1976 [16].

At the Institute for the Application of Nuclear Energy (INEP) in Agriculture, Veterinary Medicine, and Forestry in Belgrade, he worked as an expert associate (1967–1970), assistant (1971–1977), research associate (1978–1983), senior research associate (1984–1988), and scientific advisor (1989–1990). In the period 1991–2003, Dr. Nešković worked at the Institute for Plant Protection and the Environment in Belgrade, Topčider, and from 2003 until his retirement in 2008, as a scientific advisor and head of the Laboratory of Toxicology at the Pesticides Center of the Institute for Agricultural Research "Serbia", i.e., at the Institute for Pesticides and Environmental Protection, Zemun, Belgrade. During his work at INEP (1967–1990), he was the head of the Laboratory of Toxicology (1971–1978), the head of the Laboratory of Comparative Toxicology and Ecotoxicology (1979–1981), the acting director of the Institute for Pesticides and Protection Environment (which was part of INEP), director of INEP (1982–1987) and head of the Laboratory of Toxicology (1988–1990). During that period, he performed other duties, including the function of the President of the Scientific Council of the Institute. He was a member of numerous expert commissions at the Institute, but also in Belgrade, across the Republic of Serbia, and even in ex-Yugoslavia. During his work at the Institute for Plant Protection and Environment in Belgrade (1991–2003), he was a president of the Institute Council, a president of the Board (1993–1998), a member and a president of the Scientific Council, a deputy director (1998–2000), and an acting director of the Institute (2000–2002).

In the period 2002–2007, Dr. Nešković was a member, and from 2005–2007 a president of the Scientific Council of the Institute for Agricultural Research "Serbia" and the first President of the Scientific Council of the newly established Institute for Pesticides and Environmental Protection (2007–2009), in whose formation/independence he played a very important role.

In 1978, as a visiting researcher at the National Institute of Health and Environmental Protection in North Carolina, USA, and during July and August 1988, he completed a 30-day study tour of the State University of North Carolina.

He was the leader of the international scientific project "The Study of Bioavailability and Possible Toxicological Effects of Bound Pesticide Residues to Non-Target Organisms" (1986–1991), funded by the Vienna International Atomic Energy Agency, as well as a research project (in two cycles) "Pesticides and the Environment" (1991–1995 and 1996–2000), funded by the Ministry of Science and Technology of the Republic of Serbia. He was one of the founders of pesticide toxicology in the Socialist Federal Republic of Yugoslavia (SFRY) and the Republic of Serbia and the initiator of research in the field of pesticide ecotoxicology. He was the first in Serbia to deal with the problem of "bound" pesticide residues in food and the issues of their bioavailability and possible toxic effects on users.

He participated in teaching at the Faculty of Agriculture in Belgrade, as part of full-time and postgraduate studies, in the subjects of General Phytopharmacy, Special Phytopharmacy, Agricultural Toxicology, and Ecotoxicology. He was a lecturer on two international courses (1984 and 1986) in Pesticide Toxicology, organized by specialized agencies of the United Nations (World Health Organization, Food and Agriculture Organization, and the International Labor Organization) for experts from developing countries. He was a consultant and manager in the preparation of several graduate and master theses and doctoral dissertations at the Faculty of Agriculture and Faculty of Natural Sciences and Mathematics in Belgrade in the field of plant protection, phytopharmacy, and biology, and a member of commissions for evaluation and defence of several master theses and doctoral dissertations.

He published in domestic and international journals and presented over 140 papers at scientific and professional conferences. With his papers, he participated in 25 scientific conferences abroad, where he presented 30 papers, and in more than 30 scientific-professional conferences in SFRY and Serbia, where he presented 50 papers. He was one of the editors of the monograph "Plant Protection – Today and Tomorrow".

Dr. Nešković was one of the founders and a member of the editorial board of the scientific journal "Pesticides" (1986), and from 1987 to 2007, he was the editor-in-chief. The journal was later renamed "Pesticides and Phytomedicine". He was a member of the editorial board of the "Journal of Environmental Science and Health – Part B: Pesticides, Food Contaminants, and Agricultural Wastes" (1991–1994) and the domestic scientific journal "Archives of Toxicology, Kinetics, and Xenobiotic Metabolism" (1993–2002), and a member of the publishing council of the journal "Herbalist" (*Biljni lekar*). He was a reviewer of papers in renowned international scientific journals in the field of toxicology and ecotoxicology: "Environmental Toxicology and Pharmacology" and "Food and Cosmetic Toxicology". He participated in the organization and realization of numerous professional meetings: he was the president of the Organizing Committee of the Third Yugoslav Congress on Plant Protection (1995) and the president of the Organizing Committee of the Second Yugoslav Conference on Plant Protection (1994).

He was a member of the Section of Toxicology of the Serbian Medical Society, the Association of Toxicologists of Yugoslavia, the Association of Toxicologists of Serbia, the

Society for Plant Protection of Serbia, the Society of Biologists of Serbia, the Society of Ecologists of Serbia, and foreign scientific societies: Society of Environmental Toxicology and Chemistry (SETAC), EUROTOX, and International Society of Ecotoxicology and Environmental Safety (SECOTOX). In 1999, he was elected a member of the Research Board of Advisors of the American Biographical Institute, Raleigh, North Carolina.

He was an expert of the Federal Ministry for Development, Science, and Environmental Protection in the field of toxicology and ecotoxicology; member of the Committee for Environmental Protection of the Ministry of Science and Technology of the Republic of Serbia (1991–1995); member of the Federal Commission for the Registration of Pesticides (1991–2003), and the Federal Commission for Poisons (1992–2003). From 2003 to 2010, he was a member of the Poisons Commission of the Ministry of Science and Environmental Protection. From 2012 until his death, he was a member of the Committee on the Environment Board of the Serbian Academy of Sciences and Arts.

He was the winner of numerous social and guild awards and was awarded the Order of the Silver Wreath (1990). He was the first-class captain in reserve of the Serbian Army. He spoke English and Russian.

He passed away on February 19, 2021, in Belgrade as a result of the aggravation of previously established chronic diseases.

**Professor Dubravko Bokonjić, MD, Ph.D.**

Professor Dubravko Bokonjić was a doctor, a pharmacologist and toxicologist, a doctor of science, and a full professor at the MMA in Belgrade. He was born on December 6, 1950, in Zagreb, Croatia.

He started elementary school in Belgrade and finished it in Sarajevo, as well as high school. He graduated from the FM in Belgrade in 1976. He defended the master's thesis titled "Influence of central cholinergics and oximes on changes in conditioned behavior caused by non-lethal soman concentrations" in 1993 and his doctoral dissertation "Anticonvulsant and protective effects of diazepam and midazolam in animals treated with highly toxic organophosphorus compounds" at the MMA in Belgrade in 1995 [17].

After completing the obligatory medical internship, he was employed in 1977 at the Medical Department of the Sector for Nuclear-Chemical Protection of the Military Technical Institute in Belgrade, where he worked until 1998. During that time, he was engaged in research work in the Laboratory of Behavioural Pharmacology and Toxicology, where he was formed as a top researcher under the mentorship and with the support of prominent military pharmacologists and toxicologists, and led by colonels, professors Nedeljko Rosić, Bogdan Bošković, and Borivoje Stamenković. With the relocation of the Medical Department of the Military Technical Institute to the Poison Control Center at the MMA (1998), he served as head of the Department of Radiobiology (1999–2005), then head of the Department of Experimental Toxicology and Pharmacology (2005–2007), and head of the Institute of Toxicology and Pharmacology of the Poison Control Center (from 2007 until retirement in 2015).

During his professional career, he was engaged in research in the field of military toxicology (especially highly toxic organophosphorus compounds and psychochemical war poisons) and behavioral pharmacology. He was elected assistant professor at the MMA in 1995, associate professor in 2002, and full professor in 2007. He participated in various forms of education of personnel from various fields: medicine, pharmacy, dentistry, and veterinary medicine in basic academic studies and postgraduate training in the form of specialization and subspecialization, teaching Pharmacology and Toxicology, Scientific Research Methodology, and Medical Statistics. He gave lectures and exercises to students of the Faculty of Biology in Belgrade, FM in Foča (Republic of Srpska, Bosnia and Herzegovina), and from the establishment of doctoral studies at the FM in Banja Luka, he was a permanent teacher in Statistics in Biomedicine. He was one of the most beloved teachers; he always received the highest grades from students and teachers. From 2009 to 2014, he was the head of the Department of Pharmacological Sciences at the FM of the University of Defence in Belgrade. He was a mentor and collaborator in the preparation of several master theses and doctoral dissertations.

As an author or co-author, he published more than 300 papers, of which 54 were in international journals. His works have been cited 900 times by other authors. He was co-author with Professor Viktorija Dragojević-Simić and Professor Silva Dobrić on the textbook "Pharmaceutical Manual with Recipes" (2012 and 2015) and with Professor Jasmin Komić and Assistant Professor Nemanja Rančić on "Selected Methods of Statistical Analysis for Biomedical Research" (2018). He was the professional editor of the capital textbook "Dermatology 1–2" by Professor Dr. Đorđije Karadaglić (2016). Professor Bokonjić was an associate on two research projects of the Ministry of Science and Technology of the Republic of Serbia. He was a member of the Serbian Medical Society, the Serbian Pharmacological Society, the Association of Toxicologists of Yugoslavia, the Association of Toxicologists of Serbia, the European Association of Toxicologists, the European Association for Clinical Pharmacology and Therapy, and the International Union of Pharmacological Societies.

He was awarded the White Angel Medal in 2002 for exceptional personal achievement in the field of humanities. He was praised and awarded several times by the director of the Military Technical Institute and MMA.

He died on May 27, 2021, in Belgrade after the aggravation of a previously established chronic disease.

**Primarius Svetislav Ranđelović, MD, Ph.D.**

Primarius Svetislav Ranđelović was a doctor, a specialist in internal medicine, a clinical toxicologist and nephrologist, a doctor of science, a primarius, and a colonel of the Yugoslav People's Army. He was born on February 14, 1934, in Aleksinac, Serbia.

He spent his entire childhood in his birthplace, where he finished elementary school (1949) and high school (1953) as the best student of the generation. He graduated from the FM in Belgrade in 1959. After graduating from the Medical Officers School and completing his military service, he was promoted to the rank of lieutenant of the Yugoslav People's Army in 1960. He served as a general practitioner in Skopje for a short time, and, after that, from 1962 to 1967, in the Garrison ambulance in Kumanovo. He completed his specialization in internal medicine at the MMA in Belgrade in 1971, and from then, until 1981, he worked at the Department of Nephrology of the Clinic for Internal Diseases of the MMA, where he dealt with complete clinical nephrology and was the head of the Department of Hemodialysis. He was one of the first doctors in Serbia to perform percutaneous kidney biopsies in order to diagnose inflammatory and degenerative diseases. In late 1978 and early 1979, he spent three months in professional training at Hammersmith Hospital in London. From 1981 to 1999, he worked at the Clinic for Toxicology and Clinical Pharmacology of the MMA, initially as Deputy Chief, and from 1989 until his retirement in 1999 as head of Clinic [18].

He defended his doctoral dissertation, "Nephrotoxic manifestations of acute organophosphorus insecticide poisoning", in 1996 at the MMA.

As an author or co-author, he published 101 papers in domestic and foreign journals and collections of papers. He was the author of one and co-author of three chapters in the monograph of Associate Professor Luka Đorić (editor), Emergencies in Internal Medicine (1986). He was a member of the editorial board of the domestic scientific journal "Archives of Toxicology, Kinetics, and Xenobiotic Metabolism" (1993–2002). He was the head of the scientific research project of the Ministry of Defence, "Diagnosis and treatment of acute poisoning with organophosphorus compounds in humans". He was an active member of the Serbian Medical Society. He participated in founding the Section of Toxicology of the Serbian Medical Society and was its first president during two terms (from its founding in 1992 until 1995). He was a member of the Organizing Committee of the Sixth and a member of the Scientific Committee of the Seventh Congress of Toxicologists of Yugoslavia, which were held on Tara in 1994 and 1998, respectively. He enjoyed an exceptional reputation and authority in his profession. He was an expert in the field of clinical and military toxicology and nephrology. He loved and appreciated the military vocation very much; he was an honorable member of the officer corps. He proudly wore officer ranks. He was a top professional and patriot. Continuing the diligent work of his predecessors and encouraging the ability and creativity of his associates, he significantly contributed to the Clinic for Toxicology MMA, as the only clinical institution dealing with acute poisoning of adults in SFRY and the Republic of Serbia, highly positioned in the military and the civilian health system.

He was the holder of several decorations, including the Order of Military Merit with Silver Swords, the Order of the People's Army with a Gold Star, and the Order of Merit for the People with Silver Rays.

He retired in 1999 as a colonel. He passed away on September 6, 2021, in Belgrade, from the consequences of COVID-19 virus infection.

**Academician Milan Jokanović, pharmacist, Ph.D.**

Professor Milan Jokanović was a pharmacist, a toxicologist and pharmacologist, a university professor, a doctor of science, and a member of the Academy of Sciences and Arts of Republika Srpska, Bosnia and Herzegovina. He was born on December 6, 1955, in Dubrovnik, Croatia.

He finished elementary school and high school in Trebinje and FPH in Belgrade in 1979. He defended his master's thesis, "Influence of aliesterase inhibition and instruction on acute organophosphate toxicity in rats", in 1986 at the MMA in Belgrade and defended his doctoral dissertation, "Influence of phosphoramidite chemical structure on reaction with acetylcholinesterase and neurotoxic esterase *in vitro* and *in vivo*", at the FPH in Belgrade in 1991. He worked as an assistant at the Department of Analytical Chemistry of the FPH in Belgrade (1981–1982) and the Medical Department of the Military Technical Institute in Belgrade (1982–1994). He spent two years (1995–1996) as a visiting scientist

at the Institute of Occupational Medicine, University of Padua, Italy. He was the director of the Center for Biomedical Research at the Galenika Institute a.d. in Belgrade (1997–2006), where he managed the activities of preclinical and clinical development and drug registration [19]. He was elected a research associate at the Military Technical Institute in 1993 and an assistant professor at the MMA in Belgrade in 1994. At the FPH in Belgrade, he was elected a scientific advisor in 1998 and an associate professor of toxicology in 2000. He was elected a full professor at the FM in Niš (where he was the head of the Department of Pharmacy and head of accredited doctoral studies) and the FM in Banja Luka (where he was vice dean for Pharmacy from 2006 to 2010).

Academician Jokanović was elected full professor of Pharmacology and Pharmacokinetics at the FPH in Novi Sad in 2014. From 2005 to 2016, he also taught at the FM in Belgrade in postgraduate, specialist, and doctoral studies and academic specialization in the subject of Pharmaceutical Medicine. In 1989, he spent six months training at the Medical Research Council Toxicology Unit in Carshalton, UK. He spent six months training at ICN Pharmaceuticals Inc., Costa Mesa, California, USA (2000–2001). The main areas of scientific research and professional work of Academician Jokanović were: mechanisms of toxicity of anticholinesterase compounds (organophosphorus and carbamate insecticides and nerve agents) and their neurotoxic effects, research and development of new antidotes, pharmacokinetics and applications of existing and development of new tests in preclinical trials. He initiated two new areas of research in Serbia: research in the field of nanotoxicology and testing the biological compatibility of modern materials intended for use in medicine on cell cultures and experimental animals.

He published 66 papers in international scientific journals from the SCI list, 34 papers in international peer-reviewed journals, and 20 papers in scientific and professional journals in the Serbian language. He presented 150 papers at professional and scientific gatherings in the country and abroad. His works have been cited more than 2,200 times, of which about 200 times in books and monographs by foreign publishers. He gave 35 lectures in Serbia, Republika Srpska, and abroad.

In 2002, he published the textbook “Toxicology”, the first and, at that time, the only textbook of the entire toxicology of a domestic author in the Serbian language, which is used as a textbook at five faculties in Serbia. The expanded, supplemented edition of the same textbook from 2010, in 24 chapters and 400 pages, deals with general, clinical, and analytical toxicology. He published the book “Clinical Toxicology” in 2018. He published 18 chapters in toxicology in the books of the world’s leading publishers (Elsevier, John Wiley & Sons Ltd.) and eight chapters in books in the Serbian language. He was the editor-in-chief of the monograph “The Impact of Pesticides” (Academic Publishers, New York, 2012), which describes the physical and chemical properties of pesticides, their toxic effects on humans and the environment, and analytical methods for proving them. Academician Jokanović was elected a member of the Academy of Sciences and Arts of the Republika Srpska in Banja Luka in 2008. From 2003, he was a scientific expert in the biological sciences of the World Health Organization and the Food and Agriculture Organization of the United Nations. He was a mentor or a member of the commission in the preparation and defence of 17 doctoral dissertations and 10 master theses. He participated in the realization of 16 projects financed by the Ministry of Science of the Republic of Serbia and eight international projects. He participated in the preparation of a large number of internal scientific research and development projects at the Military Technical Institute and Galenika a.d. in Belgrade, whose results have not been published because they represent a business secret. He was a member of nine international and domestic scientific and professional associations and a member of the presidency of the Association of Toxicologists of Serbia and the Serbian Pharmacological Society. He was a member of the editorial board of “Toxicology” from 2003, “Scripta Medica” and “World Journal of Pharmacology” and a reviewer of 45 journals indexed in the SCI list and PubMed, including “Toxicology”, “Toxicology Letters”, “Biochemical Pharmacology”, “Basic & Clinical Pharmacology & Toxicology”, “Chemico-Biological Interactions”, “Food and Chemical Toxicology”, “Environmental Toxicology and Pharmacology”, “Expert Opinion in Pharmacotherapy”, “European Journal of Neurology”, “Journal of Applied Biomedicine”, “Medicinal Chemistry”, “Neurotoxicity Research”, “Letters in Drug Design & Discovery”, “Vojnosanitetski pregled”, “Drug and Chemical Toxicology”, “Archives of Toxicology and Occupational Medicine”, and “Neurochemistry International”, for which he did more than 300 reviews.

He passed away on September 17, 2021, in Belgrade, due to the COVID-19 virus infection.

## Conclusion

For the first time in the history of toxicology in Serbia, six prominent toxicologists, who worked in various fields and multidisciplinary science and profession, died, all in a very short period of less than two years (from the beginning of 2020 to October 2021). All of them were doctors of science with the highest academic and professional titles, researchers, educators, and successful managers of the institutions in which they worked and professional associations in which they were active. The causes of death were different: in four of the deceased, the cause of death was the worsening of preexisting chronic diseases, and in two, the cause of death was infection with the SARS-CoV-2.

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Papers should be written on IBM-compatible PC, using 12 pt font, and double spacing, with at least 4 cm left margin. **Bold** and *italic* letters should be avoided as reserved for subtitles. Original articles, reviews, meta-analyses and articles from medical history should not exceed 16 pages; current topics 10; case reports 6; short communications 5; letters to the editor and comments 3, and reports on scientific meetings and book reviews 2.

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**MS Word for Windows** (97, 2000, XP, 2003) is recommended for word processing; other programs are to be used only exceptionally. Illustrations should be made using standard **Windows** programs, **Microsoft Office** (**Excel**, **Word Graph**)**.** The use of colors and shading in graphs should be avoided.

Papers should be prepared in accordance with the **Vancouver Convention**.

Papers are reviewed anonymously by at least two editors and/or invited reviewers. Remarks and suggestions are sent to the author for final composition. Galley proofs are sent to the corresponding author for final agreement.

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Parts of the manuscript are: **Title page; Abstract with Key words; Text; Acknowledgements** (to the authors' desire), **References**, **Enclosures**.

**1.** ***Title page***

a) The title should be concise but informative, while subheadings should be avoided;

b) Full names of the authors signed as follows: ***, †, ‡, §, ‖, ¶, **, ††, ...** .

c) Exact names and places of department(s) and institution(s) of affiliation where the studies were performed, city and the state for any authors, clearly marked by standard footnote signs;

d) Conclusion could be a separate chapter or the last paragraph of the discussion;

e) Data on the corresponding author.

**2.** ***Abstract and key words***

The second page should carry a structured abstract (250-300 words for original articles and meta-analyses) with the title of the article. In short, clear sentences the authors should write the **Background**/**Aim**, major procedures – **Methods** (choice of subjects or laboratory animals; methods for observation and analysis), the obtained findings – **Results** (concrete data and their statistical significance), and the **Conclusion**. It should emphasize new and important aspects of the study or observations. A structured abstract for case reports (up to 250 words) should contain subtitles **Introduction, Case report, Conclusion**). Below the abstract **Key words** should provide 3–10 key words or short phrases that indicate the topic of the article.

**3.** ***Text***

The text of the articles includes: **Introduction**, **Methods**, **Results**, and **Discussion**. Long articles may need subheadings within some sections to clarify their content.

**Introduction**. After the introductory notes, the aim of the article should be stated in brief (the reasons for the study or observation), only significant data from the literature, but not extensive, detailed consideratuion of the subject, nor data or conclusions from the work being reported.

**Methods**. The selection of study or experimental subjects (patients or experimental animals, including controls) should be clearly described. The methods, apparatus (manufacturer's name and address in parentheses), and procedures should be identified in sufficient detail to allow other workers to reproduce the results. Also, give references to established methods, including statistical methods. Identify precisely all drugs and chemicals used, with generic name(s), dose(s), and route(s) of administration. State the approval of the Ethnics Committee for the tests in humans and animals.

**Results** should be presented in logical sequence in the text, tables and illustrations. Emphasize or summarize only important observations.

**Discussion** is to emphasize the new and significant aspects of the study and the conclusions that result from them. Relate the observations to other relevant studies. Link the conclusions with the goals of the study, but avoid unqualified statements and conclusions not completely supported by your data.

**References**

References should be superscripted and numerated consecutively in the order of their first mentioning within the text. All the authors should be listed, but if there are more than 6 authors, give the first 6 followed by *et al*. Do not use abstracts, secondary publications, oral communications, unpublished papers, official and classified documents. References to papers accepted but not yet published should be cited as "in press". Information from manuscripts not yet accepted should be cited as "unpublished data". Data from the Internet are cited with the date of citation.

*Examples of references:*

*Jurhar-Pavlova M, Petlichkovski A, TrajkovD, Efinska-Mladenovska O, Arsov T, Strezova A*, et al. Influence of the elevated ambient temperature on immunoglobulin G and immunoglobulin G subclasses in sera of Wistar rats. Vojnosanit Pregl 2003; 60(6): 657–612.

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*Primeri referenci:*

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**Tabele**

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

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

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