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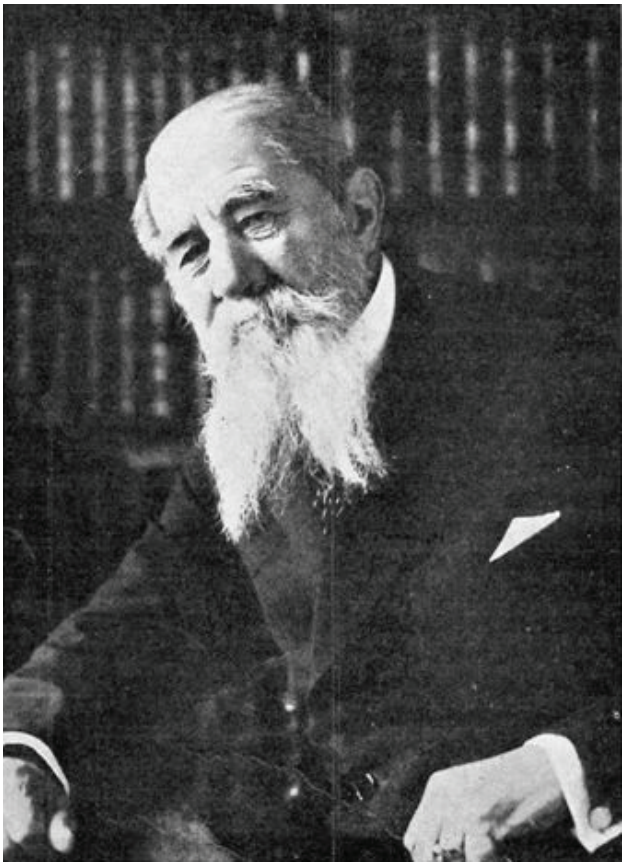

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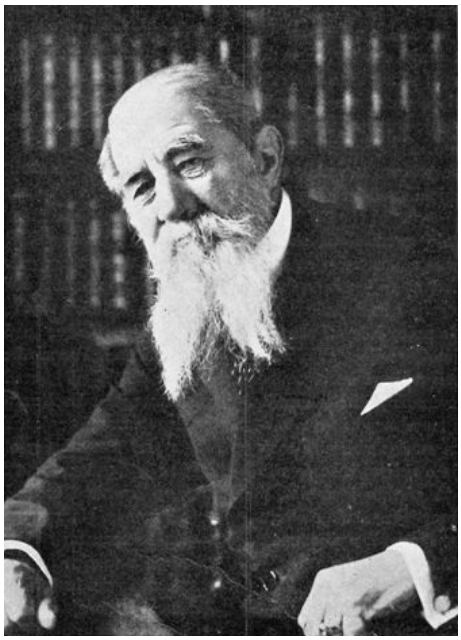

Milan Jovanović Batut (Sremska Mitrovica, October 10, 1847 – Belgrade, September 11, 1940), a great Serbian physician and humanist, was the founder of the Faculty of Medicine in Belgrade and its first Dean. In his rich professional career, he was also the president of the Serbian Medical Association, the Association for the Protection of Public Health, a long-term member, and later the president of the Main Sanitary Council. On his initiative, after the First World War, the Ministry of Public Health was established. Today, the Institute of Public Health of Serbia bears his name.
September this year marks the 80th anniversary of his death.

Milan Jovanović Batut (Sremska Mitrovica, 10. oktobar 1847 – Beograd, 11. septembar 1940), veliki srpski lekar i humanista bio je osnivač Medicinskog fakulteta u Beogradu i njegov prvi dekan. U svojoj bogatoj profesionalnoj karijeri bio je i predsednik Srpskog lekarskog društva, Društva za čuvanje javnog zdravlja, višegodišnji član, a kasnije i predsednik Glavnog sanitetskog saveta. Na njegovu inicijativu, posle Prvog svetskog rata osnavano je Ministarstvo javnog zdravlja. Danas, Institut za javno zdravlje Srbije nosi njegovo ime.
U septembru ove godine navršava se 80 godina od njegove smrti.

ORIGINAL ARTICLES


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# Survival outcomes in surgically treated patients with advanced laryngeal cancer in Serbia

## Preživljavanje hirurški lečenih bolesnika sa odmaklim karcinomom larinksa u Srbiji

**Jovica Milovanović*†, Ana Jotić*†, Ljiljana Tešić Vidović‡, Vojko Djukić*†, Aleksandar Trivić*†, Sanja Krejović Trivić*†, Zorana Radin§, Katarina Savić-Vujović‖, Andjela Milovanović¶, Bojan Banko**, Vera Artiko††**

**Clinical Center of Serbia, *Clinic for Otorhinolaryngology and Maxillofacial Surgery, ¶Clinic for Physical Medicine and Rehabilitation, **Center for Radiology and Magnetic Resonance Imaging, ††Institute for Nuclear Medicine, Belgrade, Serbia; University of Belgrade, Faculty of Medicine, †Department of Otorhinolaryngology and Maxillofacial Surgery, ‖Department of Pharmacology, Clinical Pharmacology and Toxicology, Belgrade, Serbia; ‡General Hospital Loznica, Loznica, Serbia; §General Hospital "Dr Djordje Jovanović", Zrenjanin, Serbia**

**Abstract**

**Background/Aim.** Laryngeal carcinomas make 1%–3% of all head and neck malignancies.Treatment outcome and survival rates depend greatly on established stage of the disease. The purpose of this study was to examine the survival of the patients with advanced laryngeal carcinoma depending on gender, age, common risk factors (tobacco and alcohol use), primary tumor localization, histopathological tumor grade, clinical TNM (tumor, node and metastasis) stage and surgical treatment of the disease. **Methods.** Retrospective study included 252 patients treated surgically for advanced squamocellular carcinoma of the larynx in a three-year period with five-year follow-up. Patients included in the study were treated primary with surgery, with postoperative radiotherapy and chemotherapy depending on the stage of the disease, intraoperative findings and tumor resection borders. Overall survival and disease-specific five-year survival of patients was calculated for demographical and clinical characteristics of the patients. **Results.** Overall 5-year survival of patients with operable advanced laryngeal cancer included in the study was 86.14% and disease-specific survival 86.51%. Lower overall and the disease-specific survival was associated with age, higher histological tumor grade and more extensive neck dissections. **Conclusion.** Primary total laryngectomy results in higher survival outcomes in cases of transglottic T3 and T4a laryngeal tumors. Patients should be informed of the likely increased mortality risks tied to the choice of surgical resection and treatment modality before their decision.

**Key words:**
**laryngeal neoplasms; postoperative period; survival; risk factors; neoplasm staging.**

**Apstrakt**

**Uvod/Cilj**. Karcinomi larinksa čine 1%–3% svih malignitета glave i vrata. Terapijski rezultati i preživljavanje umnogome zavise od stadijuma bolesti. Cilj rada bio je da se ispita preživljavanje bolesnika, hirurški lečenih od odmaklih karcinoma larinksa, u zavisnoti od starosti, pola, uobičajenih faktora rizika (pušenje, konzumiranje alkohola), primarne lokalizacije tumora, patohistološkog stadijuma bolesti, tumor, nodus, metastaza (TNM) stadijuma bolesti, i primenjene hirurške terapije bolesti. **Metode.** Retrospektivnom studijom bilo je obuhvaćeno 252 bolesnika, hirurški lečenih od odmaklih karcinoma glave i vrata, sa periodom praćenja od pet godina. Bolesnici uključeni u studiju primarno su lečeni hirurški, sa sprovođenjem postoperativne radio- i hemioterapije, u zavisnosti od lokalne i regionalne proširenosti bolesti, intraoperativnog nalaza i granica linija resekcija. Petogodišnje ukupno preživljavanje i preživljavanje bez znakova bolesti računato je u zavisnosti od demografskih i kliničkih karakeristika bolesnika. **Rezultati.** Ukupno petogodišnje preživljavanje bolesnika sa odmaklim karcinomima larinksa je iznosilo 86,14%, a petogodišnje preživljavanje bez znakova bolesti 86,51%. Smanjeno preživljavanje bilo je značajno povezano sa starošću bolesnika, višim histološkim gradusom tumora i opsežnijim disekcijama vrata. **Zaključak.** Primarna totalna laringektomija

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rezultira dužim preživljavanjem u slučajevima transglotisnih T3 i T4a tumora larinksa. Pre donešenja odluke, bolesnici bi trebali biti informisani o mogućim većim rizicima od smrtnog ishoda povezanim sa izborom hirurške metode i drugih načina lečenja.

**Ključne reči:**
**larinks, neoplazme; postoperativni period; preživljavanje; faktori rizika; neoplazme, određivanje stadijuma.**

## Introduction

Laryngeal carcinomas make 1%–3% of all head and neck malignancies [1]. Treatment outcome and survival rates depend greatly on established stage of the disease. In the recent decades treatment concept of advanced laryngeal cancer was shifted toward organ preservation therapy, suggesting radiotherapy with concurrent chemotherapy as a preferable method for laryngeal preservation [2]. Some studies suggested that in clinical setting, organ preservation protocols were not as efficient in providing enough survival rates as the surgical treatment combined with radiotherapy or chemoradiotherapy [3]. Some authors argue that traditional treatment of advanced laryngeal cancer, which most commonly includes total laryngectomy with postoperative radiotherapy, is still the best for ensuring most favorable oncological results [4, 5].

The purpose of this study was to examine the overall and disease- specific survival of patients surgically treated of operable advanced laryngeal carcinoma depending on gender, common risk factors (tobacco and alcohol use), histopathological tumor grade, clinical tumor (T), node (N), matastasis (M) – TNM stage, and treatment of the disease.

## Methods

A retrospective study included 252 patients treated at the Clinic for Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia in Belgrade. The data were obtained by processing medical charts of patients with squamocellular carcinoma of the larynx surgically treated in the period from January 1, 2010 to January 1, 2012. This study was approved by the Institutional Ethics Committee (440/IX-3/09), and all patients signed the informed consent form prior to their inclusion in the study. Patients were divided into age groups according to International Cancer Survival Standard (ICSS) using the Five Default Age Groups (15–44, 45–54, 55–64, 65–74, 75+) [6].

The diagnosis of laryngeal carcinoma was confirmed by otorhinolaryngological clinical examination and laryngomicroscopic examination of the larynx with the biopsy and histopathologic examination of the tissue. Additional diagnostics like ultrasonography of the neck and of the abdomen, chest x-ray (radiography) and computed tomography (CT) of the neck were performed to determine the TNM stage of the disease [7]. Study included patients diagnosed with advanced stages of operable laryngeal carcinoma (T2N1-N2, T3N0-N2 and T4aN0-N2), without previous treated malignancies and distant metastases. The modality of treatment for every patient was decided on the Oncological Board (consisting of a radiotherapist, head and neck surgeons, an oncologist and a histopathologist). Choice of primary and adjuvant treatment was decided based on the National Comprehensive Cancer Network (NCCN) and the American Society for Radiation Onclogy (ASTRO) guidelines [7, 8] which are recommended and used at the Clinic for Otorhinolaryngology and Maxillofacial Surgery and the Institute for Oncology and Radiology of Serbia in Belgrade. Surgical therapy involved resection of the tumor with some form of the neck dissection in case of cervical lymphadenopathy. Radiotherapy consisted of external radiotherapy with total dose of 60 to 70 Gy in 30–35 fractions for 6–7 weeks. Patients received concomitant chemotherapy consisted of at least three courses of cisplatin (CDDP) with 5-fluorouracil (5-FU) intravenously. Follow-up period was from 63 to 82 months. Demographic characteristics (age and gender) and risk factors (cigarette and alcohol consumption) were noted. Histopathological tumor grade, the beginning of treatment with or without tracheotomy, TNM classification, type of surgical treatment, type of neck dissection, and therapy modality were also examined. Five-year overall and disease-specific survival (DSS) of patients was determined depending on all previously mentioned factors.

For statistical analysis of data, the program SPSS v20 (Statistical Package for Social Sciences, SPSS Inc, Chicago, Illinois) was used. Descriptive statistics was used for demographic characteristics, risk factors and other parameters and presented as frequencies and proportions. Overall survival (OS) and DSS rates were calculated using the Kaplan–Meier method. A Cox proportional hazards regression model along with univariate and multivariate analyses were used for estimating the impact of prognostic factors on DSS rate. Risk estimates are presented as hazard ratios (HR) with 95% confidence intervals (CI). Statistical significance was considered at $p < 0.05$.

## Results

The study comprised 230 (91.3%) males and 22 (8.7%) females of an average age of 59.98 years [standard deviation (SD) ± 8.85 years]. The youngest patient was 38, and the oldest one 84 years old. Two hundred thirty three (92.5%) of the patients were smokers, and 19 (7.5%) were non-smokers. Alcohol consumption was noted in 87 (34.5%) of the patients, while 165 (65.5%) were non-drinkers. Diagnostics of the tumor started with tracheotomy in 51 (20.2%) of the patients. Majority of carcinomas were histologically moderately differentiated tumors (74.2%) and transglottic tumors (40.9%) Out of all surgical procedures used to treat advanced laryngeal carcinoma in our study, total laryngectomy was most frequently done procedure in 216 (85.7%) of the patients. Fifty nine (23.4%) of the patients underwent neck dissection. Most of the patients were treated with surgery fol-

lowed by postoperative radiotherapy, while there were only small number of patients treated only surgically (3.6%), or with surgery with concomitant chemo-radiotherapy (5.2%) (Table 1).

**Table 1**
**Demographic and clinical characteristics of the patients**

| Patients' characteristcs | Patients n (%) |
|---|---|
| Age (years) | |
| < 45 | 9 (3.6) |
| 45–54 | 64 (25.4) |
| 55–64 | 101 (40.1) |
| 65–74 | 65 (25.8) |
| > 75 | 13 (5.1) |
| Gender | |
| male | 230 (91.3) |
| female | 22 (8.7) |
| Smoking | |
| smokers | 233 (92.5) |
| non-smokers | 19 (7.5) |
| Alcohol | |
| consumers | 87 (34.5) |
| non-consumers | 165 (65.5) |
| Histological grade | |
| G1 | 44 (17.5) |
| G2 | 187 (74.2) |
| G3 | 21 (8.3) |
| Tumor localization | |
| supraglottis | 53(21) |
| glottis | 94(37.3) |
| subglottis | 2(8) |
| transglottic | 103(40.9) |
| Diagnostics started with tracheotomy | |
| yes | 51 (20.2) |
| no | 201 (79.8) |
| Tumor stage | |
| T2 | 9 (3.6) |
| T3 | 213 (84.5) |
| T4a | 30 (11.9) |
| Nodus stage | |
| N0 | 203 (80.6) |
| N1 | 43 (17.1) |
| N2 | 6 (2.4) |
| Type of surgery | |
| total laryngectomy | 216 (85.7) |
| subtotal laryngectomy | 16 (6.3) |
| haemilaryngectomy | 11 (4.4) |
| supraglottic laryngectomy | 9 (3.6) |
| Neck dissection | |
| none | 193 (76.6) |
| selective or modified radical dissection | 27 (10.7) |
| radical dissection | 13 (5.2) |
| expanded radical dissection | 19 (7.5) |
| Treatment | |
| OP | 9 (3.6) |
| OP+ RT | 230 (91.3) |
| OP+RT+CT | 13 (5.2) |

**OP – surgery; RT – radiotherapy; CT – chemotherapy.**

Overall 5-year survival of patients with operable advanced laryngeal cancer included in the study was 86.14% and the DSS 86.51%. OS did not differ much from the DSS in relation to different age groups, except in the group of patients older the 75 years, mostly because of other comorbidities (Tables 2 and 3). Females had lower 3-year and 5-year survival rates comparing to male patients (3-year OS and DSS were 81.8 and 87.4, respectively; 5-year OS was 72.7 for females and 80.9 for males, and DSS was 72.7 for females and 81.2 for males). Non-smokers had better DSS compared to smokers included in the study, but the difference was not statistically significant. Patients who did not consumed alcohol had higher OS and DSS comparing to those who consumed alcohol. Patients with poorly differentiated tumors had lower survival rates comparing to patients with good and moderately differentiated tumors. Tumor localization significantly influenced on OS (Log rank, $p = 0.017$) and DSS (Log rank, $p = 0.025$). Tumors with primary supraglottic localization had significantly lower survival rates comparing to other localization. Totally, 20.1% of patients started their diagnostic process with tracheotomy, which significantly influenced their DSS (Log rank, $p = 0.041$). Patients with T2 advanced laryngeal tumors had lower OS and DSS comparing to those with T3 and T4a tumors (3-year DSS was 77.8, 88.2 and 83.3, respectively; 5-year DSS was 66.7, 81.1 and 80, respectively), but the regional spreading of the disease present in these patients should be taken into account in explaining the results. OS (Log rank $p = 0.046$) and DSS (Log rank $p = 0.037$) were significantly lower in patients with the N2 nodal disease comparing to those with the N0 and N1 stages of the disease (3-year OS and DSS were 88.1, 86 and 66.7, respectively; 5-year OS and DSS were 82.7, 74.4 and 50, respectively). 5-year OS and DSS were lower in patients who underwent partial supraglottic laryngectomy comparing to other operative procedures conducted. 5-year OS (Log rank $p = 0.032$) and DSS (Log rank p=0.024) in patients who underwent radical and expanded radical neck dissection were significantly lower comparing to other patients, which was directly associated with the advancement of the nodal disease. Patients who were treated only surgically, without postoperative radiotherapy or chemotherapy had higher OS and DSS one year, three years and five years after the treatment.

Multivariate analysis revealed that age, histological grade of the tumor and undertaken selective or modified radical neck dissection were significant prognostic factors for DSS in patients with advanced laryngeal cancer (Table 4). Age of the patients (HR 1.042, $p = 0.013$), histological G2 (moderately differentiated) (HR 3.453, $p = 0.027$) and G3 (poorly differentiated HR 4.069, $p = 0.036$) tumor grade had a negative impact on DSS. Undertaken selective or modified radical neck dissection had a positive impact on DSS (HR 0.132, $p = 0.02$). DSS was significantly better for patients with supraglottic (HR 0.405, $p = 0.009$) and glottic localization of the tumor (HR 0.478, $p = 0.023$), but only by univariate analysis.

**Table 2**

**Overall survival for patients included in the study**

| Patients' characteristcs | 1-year survival (%) | 3-year survival (%) | 5-year survival (%) | Log rank |
|---|---|---|---|---|
| Age (years) | | | | |
| < 45 | 100 | 100 | 100 | |
| 45–54 | 100 | 88.5 | 85.2 | $p = 0.127$ |
| 55–64 | 99 | 88.3 | 81.6 | |
| 65–74 | 97 | 84.8 | 74.2 | |
| ≥ 75 | 92.3 | 69.2 | 61.5 | |
| Gender | | | | |
| male | 98.3 | 87.4 | 80.9 | $p = 0.453$ |
| female | 100 | 81.8 | 72.7 | |
| Smoking | | | | |
| smokers | 98.7 | 87 | 80 | $p = 0.996$ |
| non-smokers | 94.7 | 84.2 | 78.9 | |
| Alcohol | | | | |
| consumers | 96.5 | 83.7 | 75.6 | $p = 0.327$ |
| non-consumers | 99.6 | 88.5 | 82.4 | |
| Histological grade | | | | |
| G1 | 100 | 95.5 | 90.9 | $p = 0.083$ |
| G2 | 98.4 | 86.6 | 78.6 | |
| G3 | 95.2 | 71.4 | 71.4 | |
| Tumor localization | | | | |
| supraglottis | 98.1 | 79.2 | 66 | |
| glottis | 96.8 | 90.4 | 85.1 | $p = 0.017$* |
| subglottis | 100 | 100 | 100 | |
| transglottic | 100 | 87.4 | 82.5 | |
| Diagnostics started with tracheotomy | | | | |
| yes | 96.1 | 86.3 | 70.6 | $p = 0.05$ |
| no | 99 | 87.1 | 82.6 | |
| Tumor stage | | | | |
| T2 | 100 | 77.8 | 66.7 | $p = 0.265$ |
| T3 | 98.6 | 87.8 | 80.8 | |
| T4a | 100 | 83.3 | 80 | |
| Nodus stage | | | | |
| N0 | 98.5 | 87.7 | 82.8 | $p = 0.046$* |
| N1 | 97.7 | 86 | 74.4 | |
| N2 | 100 | 66.7 | 50 | |
| Type of surgery | | | | |
| total laryngectomy | 98.1 | 86.6 | 80.1 | |
| subtotal laryngectomy | 100 | 87.5 | 81.3 | $p = 0.724$ |
| haemilaryngectomy | 100 | 100 | 90.9 | |
| supraglottic laryngectomy | 100 | 77.8 | 66.7 | |
| Neck dissection | | | | |
| none | 98.4 | 87.6 | 83.4 | |
| selective or modified radical dissection | 96.3 | 85.2 | 74.1 | $p = 0.032$* |
| radical dissection | 100 | 93.3 | 61.5 | |
| expanded radical dissection | 100 | 78.9 | 68.4 | |
| Treatment | | | | |
| OP | 100 | 100 | 100 | $p = 0.105$ |
| OP+RT | 98.7 | 86.5 | 80 | |
| OP+RT+CT | 92.3 | 84.6 | 69.2 | |

**OP – surgery; RT – radiotherapy; CT – chemotherapy.**
***p* < 0.05.**

**Table 3**

**Disease-specific survival for patients included in the study**

| Patients' characteristcs | 1-year survival (%) | 3-year survival (%) | 5-year survival (%) | Log rank |
|---|---|---|---|---|
| Age (years) | | | | |
| < 45 | 100 | 100 | 100 | |
| 45–54 | 100 | 88.5 | 85.2 | $p = 0.241$ |
| 55–64 | 99 | 88.3 | 81.6 | |
| 65–74 | 97 | 84.8 | 74.2 | |
| ≥ 75 | 100 | 75 | 66.7 | |
| Gender | | | | |
| male | 98.7 | 87.8 | 81.2 | $p = 0.422$ |
| female | 100 | 81.8 | 72.7 | |
| Smoking | | | | |
| smokers | 98.7 | 87 | 80 | $p = 0.629$ |
| non-smokers | 100 | 94.4 | 83.3 | |
| Alcohol | | | | |
| consumers | 97.7 | 84.7 | 76.5 | $p = 0.427$ |
| non-consumers | 99.4 | 88.5 | 82.4 | |
| Histological grade | | | | |
| G1 | 100 | 95.5 | 90.9 | $p = 0.086$ |
| G2 | 98.9 | 87.1 | 79 | |
| G3 | 95.2 | 71.4 | 71.4 | |
| Tumor localization | | | | |
| supraglottis | 98.1 | 79.2 | 66 | |
| glottis | 97.9 | 91.4 | 86 | $p = 0.025$* |
| subglottis | 100 | 100 | 100 | |
| transglottic | 100 | 87.4 | 82.5 | |
| Diagnostics started with tracheotomy | | | | |
| yes | 96.1 | 86.3 | 70.6 | $p = 0.041$* |
| no | 99.5 | 87.5 | 83 | |
| T stage | | | | |
| T2 | 100 | 77.8 | 66.7 | $p = 0.246$ |
| T3 | 98.6 | 88.2 | 81.1 | |
| T4a | 100 | 83.3 | 80 | |
| N stage | | | | |
| N0 | 99 | 88.1 | 82.7 | $p = 0.037$* |
| N1 | 97.7 | 86 | 74.4 | |
| N2 | 100 | 66.7 | 50 | |
| Type of surgery | | | | |
| total laryngectomy | 98.6 | 87 | 80.5 | |
| subtotal laryngectomy | 100 | 87.5 | 81.3 | $p = 0.703$ |
| haemiaryngectomy | 100 | 100 | 90.9 | |
| supraglottic laryngectomy | 100 | 88.9 | 66.7 | |
| Neck dissection | | | | |
| none | 99 | 88 | 83.9 | |
| selective or modified radical dissection | 96.3 | 85.2 | 74.1 | $p = 0.024$* |
| radical dissection | | | | |
| expanded radical dissection | 100 | 92.3 | 61.5 | |
| | 100 | 78.9 | 68.4 | |
| Treatment | | | | |
| OP | 100 | 100 | 100 | $p = 0.099$ |
| OP+RT | 99.1 | 86.9 | 80.4 | |
| OP+RT+CT | 92.3 | 84.6 | 69.2 | |

**OP – surgery; RT – radiotherapy; CT – chemotherapy.**
***p* < 0.05.**

**Table 4**

**Cox proportional hazard for disease-specific survival in patients with advanced laryngeal cancer**

| Patients parameters | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| Age (per year) | 1.044 | 1.009–1.074 | 0.005* | 1.042 | 1.009–1.077 | 0.013* |
| Gender | | | | | | |
| female | 1 | 0.310-1.694 | 0.457 | 1 | 0.306-1.975 | 0.597 |
| male | 0.724 | | | 0,778 | | |
| Smoking | | | | | | |
| non-smokers | 1 | 0.360–2.274 | 0.996 | 1 | 0.354–3.776 | 0.810 |
| smokers | 0.998 | | | 1.156 | | |
| Alcohol consumption | | | | | | |
| non-consumers | 1 | 0.758–2.278 | 0.331 | 1 | 0.720–2.604 | 0.338 |
| consumers | 1.314 | | | 1.369 | | |
| Histological grade | | | | | | |
| G1 | 1 | 0.972–7.543 | 0.057 | 1 | 4.153–10.339 | 0.027* |
| G2 | 2.708 | 1.058–13.29 | 0.041* | 3.453 | 1.093–15.146 | 0.036* |
| G3 | 3.749 | | | 4.069 | | |
| Tumor localization | | | | | | |
| transglottic | 1 | | | 1 | | |
| supraglottis | 0.405 | 0.206–0.797 | 0.009* | 0.498 | 0.232–1.072 | 0.075 |
| glottis | 0.478 | 0.253–0.904 | 0.023* | 0.499 | 0.243–1.028 | 0.059 |
| subglottis | 0.000 | 0.00– - | 0.975 | 0.000 | 0.00– - | 0.984 |
| Diagnostics started with tracheotomy | | | | | | |
| yes | 1 | | | 1 | | |
| no | 0.563 | 0.313–1.012 | 0.055 | 1.736 | 0.886–3.402 | 0.108 |
| Tumor stage | | | | | | |
| T2 | 1 | 0.626–7.862 | 0.217 | 1 | 0.086–12.243 | 0.985 |
| T3 | 2.218 | 0.415–2.289 | 0.952 | 1.024 | 0.426–2.567 | 0.923 |
| T4a | 0.974 | | | 1.045 | | |
| Nodus stage | | | | | | |
| N0 | 1 | 0.097–1.022 | 0.054 | 1 | 0.245–1.245 | 0.496 |
| N1 | 0.315 | 0.154–1.898 | 0.317 | 2.115 | 0.199–1.425 | 0.989 |
| N2 | 0.541 | | | 0.989 | | |
| Type of surgery | | | | | | |
| total laryngectomy | 1 | | | 1 | | |
| subtotal laryngectomy | 0.448 | 0.1561–1.246 | 0.124 | 1.098 | 0.109–11.035 | 0.936 |
| haemilaryngectomy | 0.404 | 0.090–1.804 | 0.235 | 0.863 | 0.066–11.239 | 0.936 |
| supraglottic laryngectomy | 0.366 | 0.067–2.001 | 1.247 | 0.872 | 0.085–8.085 | 0.908 |
| Neck dissection | | | | | | |
| none | 1 | | | 1 | | |
| selective or modified radical dissection | 0.378 | 0.174–0.818 | 0.014* | 0.132 | 0.024–0.723 | 0.02* |
| radical dissection | 0.608 | 0.220–1.676 | 0.336 | 0.288 | 0.069–1.199 | 0.087 |
| expanded radical dissection | 0.867 | 0.283–2.650 | 0.802 | 0.496 | 0.145–1.700 | 0.264 |
| Treatment | | | | | | |
| OP | 1 | 0.000- - | 0.964 | 1 | 0.0- - | 0.969 |
| OP+RT | 0.000 | 0.202–1.273 | 0.148 | 0.00 | 0.195–1.559 | 0.262 |
| OP+RT+CT | 0.507 | | | 0.552 | | |

**HR – hazard ratio; CI – confidence interval; OP – surgery; RT – radiotherapy; CT – chemotherapy.**
***p* < 0.05.**

## Discussion

In our study OS and DSS were significantly lowered with age. Age of the patients proven to be an important risk factor which other studies confirmed as well [9, 10]. In female patients in our study, 3-year and 5-year survivals were lower comparing to those in male patients, which differs from other studies where females had significantly higher survival rates [11, 12]. Smoking and alcohol consumption were also associated with lower survival in our patients. In this study, gender, smoking and alcohol consumption were not significant prognostic factors for DSS. Less differentiated advanced carcinoma had higher risk for mortality. These data do not differentiate significantly from other research data available [9, 10]. This study involved patients with advanced laryngeal carcinoma which included the T2 stage with nodal disease, and

the T3 and T4 stages with and without nodal disease. Patients with higher T stage of the disease had, as expected, lower OS and DDS survival. Presence of the nodal disease also lowered survival in the patients included in our study.

The evolution of treatment for advanced laryngeal carcinoma in Serbia was interesting during the last half of the century. In the 1960s' radiotherapy was considered to be primary treatment for laryngeal cancer. In the 1970s' functional and radical laryngeal surgery started to emerge as the primary curative treatment, with postoperative radiotherapy and chemotherapy [13]. It was considered that extended surgery was needed for successful oncological treatment of advanced laryngeal cancer. With the advances of chemotherapeutic drugs and radiotherapy, new treatment protocol for organ preservation were presented in the last three decades [2, 14, 15]. Nonsurgical therapy was suggested to be as efficient as surgical therapy in treating advanced laryngeal cancer. Since then, numerous studies showed that in the clinical settings, surgical therapy still resulted in better OS and DSS in patients treated for advanced laryngeal cancer comparing to chemoradiotherapy and radiotherapy alone [3, 16, 17].

Patients with T4a tumors in our study had high 3-year and 5-year OS and DSS (83.3% and 80%, respectively) and were all treated with total laryngectomy with postoperative chemoradiotherapy. In the recent systematic review which included 24 studies, Francis et al. [18] reported that OS for the T4a laryngeal carcinoma, treated with total laryngectomy, varied from 30% to 100% at 2-years and from 10% to 80.9% at 5-years. In 2010, Olsen [19] stated that tumors that extend through the laryngeal cartilage should be treated with total laryngectomy, followed by postoperative RT or chemoradiotherapy depending on primary tumor pathologic findings and presence of neck metastases.

In Serbian leading medical centers, surgery is considered the preferable primary method of treatment for advanced laryngeal carcinomas. Adherence to new recommended protocols for treating advanced laryngeal carcinoma is significantly influenced by health care organization. Small number of radiology and oncology centers and prolonged waiting period for radiotherapy, is surely influencing the decision on treatment choice, leaving surgery as the best and reliable method. There are limited number of papers written on the matter, without significant stratification of data. In older studies done in Serbia, 3-year OS in patients with advanced laryngeal carcinoma treated with total laryngectomy from 1971 to 1981 was 68.49% [13]. In the period from 1990 to 1997, 5-year OS in patients with advanced laryngeal carcinomas who underwent total laryngectomy reported to be 63% [20]. Stankovic et al. [21] reported DSS of 61.3% in 387 patients with advanced laryngeal cancer who underwent total laryngectomy from 1995 to 2007. In this study, patients treated with total laryngectomy had 3-year OS of 86.6% and DSS of 87%, and 5-year OS of 80.1% and DSS of 80.5%.

Most of the patients included in the study had clinically negative enlarged lymph nodes of the neck (203 patients, 80.6%), and neck dissection was not done in 193 (76.6%) of the patients. Current recommendations support selective neck dissection in advanced laryngeal carcinoma without clinically positive neck [22, 23] which should be implemented in our clinical practice. In our study, 31 (12.7%) of the patients underwent radical of extended radical neck dissection. 5-year OS was significantly lower in these patients, which is surely connected with the advanced regional spread of the disease. Selective or modified radical neck dissection positively influenced DSS. There are few studies that suggest that with the careful selection of patients without massive lymphadenopathy, nodal fixation, gross extracapsular spread and history of previous neck surgery or radiotherapy, selective neck dissection could resolve the N1 to N3 nodal disease [24].

## Conclusion

This study indicates that lower OS and DSS were significantly associated with patients' age, higher histological grade of the disease and more extensive neck dissections. Primary total laryngectomy results in higher survival outcomes in cases of transglottic T3 and T4a laryngeal tumors. Patients should be informed of the likely increased mortality risks tied to the choice of surgical resection and treatment modality before their decision.

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# Evaluation of a three-month trial of thyroxine replacement in symptomatic subclinical hypothyroidism: An impact on clinical presentation, quality of life and adoption of long-term therapy

## Evaluacija tromesečne supstitucije levotiroksinom u simptomatskoj supkliničkoj hipotireozi: uticaj na kliničku sliku, kvalitet života i prihvatanje dugoročne terapije

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**Abstract**

**Background/Aim.** Although subclinical hypothyroidism (SCH) is frequently a biochemical diagnosis, some symptoms and signs of overt disease may be present, influencing our decision to start the treatment with levothyroxine (LT4). The aim of this study was to examine the effect a 3-month LT4 treatment on clinical presentation and quality of life in symptomatic SCH with thyroid-stimulating hormone (TSH) < 10 mIU/L. We also considered whether treatment discontinuation additionally improves reliability of these findings. **Methods.** Clinical parameters (disease-specific score) and quality of life (Short Form-36 questionnaire) were measured in 35 patients with persistent symptomatic SCH (TSH 7.0 ± 2.1 mIU/L) before the intervention (LT4 substitution), 3 months after the euthyroid state had been achieved and 3 months after cessation of LT4 substitution. **Results.** The median of the Zulewski index significantly decreased after the treatment with LT4: 5.0 (4.0–7.0) vs. 3.0 (2.0–5.0) ($p < 0.001$) representing a reduction of symptoms. The most common ailments before the treatment were dry skin (71.4%), hoarseness (65.7%) and rough skin (54.3 %). After the treatment, there was a significant reduction in the frequency of constipation ($p = 0.004$), dry skin ($p = 0.022$), hoarseness ($p = 0.002$), decreased sweating ($p = 0.006$), and delayed Achilles reflex ($p = 0.002$). Quality of life was not changed significantly after LT4 treatment. In the group of 18 patients who discontinued the treatment, many symptoms and signs reappeared with the TSH increasing (6.8 ± 1.1 mIU/L): periorbital edema, constipation, weight gain, decreased sweating, slow motion and delayed Achilles reflex. The median of the Żulewski index after discontinuation of LT4 was 6.0 (4.0–9.0) ($p = 0.010$). Also, there was a statistically significant reduction in the general health score, and vitality, role emotional and mental health scores. **Conclusion.** Clinical score, based on symptoms and signs, is a sensitive and reproducible test for objective estimation of LT4 treatment effects in symptomatic SCH patients with TSH <10 mIU/L and supports individually adjusted treatment. Symptomatic SCH is not necessarily associated with a quality of life impairment that may be significantly improved by LT4 treatment. Changes in general health, vitality, mental health and emotional role after LT4 cessation suggest that some aspects of life quality can be affected by subtle variations in thyroxine availability.

**Key words:**
**hypothyroidism; thyroxine; surveys and questionaires; withholding treatment; disease progression; quality of life.**

**Apstrakt**

**Uvod/Cilj.** Mada je supklinički hipotiroidizam (SCH) najčešće biohemijska dijagnoza, neki simptomi i znaci manifestne bolesti mogu biti prisutni i uticati na našu odluku o započinjanju lečenja levotiroksinom (LT4). Cilj ove studije bio je da se ispita efekat tromesečne supstitucije LT4-om na kliničku sliku i kvalitet života u simptomatskoj SCH sa

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vrednostima tiroidnog stimulišućeg hormona (TSH) < 10 mIU/L. Takođe, razmatrano je da li prekid lečenja dodatno doprinosi pouzdanosti dobijenih nalaza. **Metode.** Klinički parametri (bolest-specifičan upitnik) i kvalitet života (Short Form-36 upitnik – SF-36 ) procenjivani su kod 35 bolesnika sa perzistentnom simptomatskom SCH (TSH 7,0 ± 2,1 mIU/L) pre intervencije (LT4), tri meseca nakon postizanja zadovoljavajućeg kvaliteta supstitucije i tri meseca nakon prekida lečenja. **Rezultati.** Medijana Zulewski indeksa bila je značajno snižena nakon lečenja LT4-om: 5,0 (4,0–7,0) vs. 3,0 (2,0–5,0) ($p < 0,001$), što je bilo praćeno smanjenjem tegoba. Najučestalija tegoba pre tretmana bila je suva koža (71,4%), nagluvost (65,7%) i gruba i perutava koža (54,3%). Nakon lečenja, zabeležen je značajan pad u učestalosti opstipacije ($p = 0,004$), suve kože ($p = 0,022$), nagluvosti ($p = 0,002$), smanjenog znojenja ($p = 0,006$) i produženog Ahilovog refleksa ($p = 0,002$). Kvalitet života nije značajno promenjen ovim tretmanom. U grupi od 18 bolesnika koji su prekinuli lečenje, sa porastom TSH (6,8 ± 1,1 mIU/L) brojni simptomi i znaci su se ponovo javili: periorbitalni edem, opstipacija, porast telesne mase, smanjeno znojenje, usporenost i produžen Ahilov refleks. Medijana Žulewski indeksa nakon prekida uzimanja LT4 iznosila je 6,0 (4,0–9,0) ($p = 0,010$). Takođe, došlo je do značajnog pada u skorovima SF-36 upitnika koji se odnose na opšte zdravstveno stanje, vitalnost, emocionalnu komponentu i mentalno zdravlje. **Zaključak.** Klinički skor baziran na simptomima i znacima je senzitivan i reproducibilan test za objektivizaciju procene efekata supstitucije LT4-om kod bolesnika sa simptomatskom SCH (TSH < 10 mIU/L), što govori u prilog individualnom pristupu u lečenju. Simptomatska SCH nije neophodno udružena sa oštećenim kvalitetom života, ali on može biti značajno poboljšan lečenjem. Promene u opštem zdravstvenom statusu, vitalnosti, mentalnom stanju i emocionalnoj ulozi nakon prekida lečenja sugerišu da neki aspekti kvaliteta života mogu biti zahvaćeni suptilnim promenama u nivou dostupnog tiroksina.

**Ključne reči:**
**hipotireoidizam; tiroksin; ankete i upitnici; lečenje, prekid; bolest, progresija; kvalitet života.**

## Introduction

Subclinical hypothyroidism (SCH) is defined as normal serum free thyroxine (FT4) and elevated thyroid-stimulating hormone (TSH), suggesting mild thyroid hypofunction. Normal serum thyroid hormone levels may not adequately represent its effects on peripheral tissues in each individual with SCH [1]. In the majority of cases, SCH is a biochemical diagnosis, and mild symptoms and signs of hypothyroidism seem to be present in about 30% of patients [2]. Although the number and intensity of the symptoms may vary widely, when they are present in patients with TSH < 10 mIU/L, it is suggested that a 3-month trial of levothyroxine (LT4) therapy with the assessment of response can be carried out [3]. In the elderly, avoidance of the treatment is advised since such a mild increase of TSH is found to be associated with the greater longevity and unchanged quality of life [4–5]. Rationale for the trial approach in symptomatic patients younger than 65–70 years is based on many interventional studies which demonstrated an improvement of symptoms and potential benefit from LT4 treatment [6–10]. However, in a smaller number of randomized studies, no evidence for symptoms improvement was found [11, 12].

Generally, benefit of the treatment depends on the degree of thyroid failure in SCH and the risk for progression to overt hypothyroidism. Relevant factors for treatment decision even in the absence of symptoms are serum TSH values, thyroid antibody (tAb) levels, goitre size and comorbidities [3, 13]. Complaints of patients with mild hypothyroidism may be occasionally misleading, since they are similar to those of the general age-matched population. Prevalence of SCH in the general population is high, with a peak of 17% reported in women and older adults [14]. For this reason, it is useful to objectify effects of both SCH and its resolution in treated patients by the clinical scale for the hypothyroidism assessment. Health-related quality of life (HR-QOL) questionnaires may provide additional tool for the assessment of the need for therapy of SCH and the evaluation of its effects.

The aim of our study was to evaluate the reliability and relevance of present symptoms and signs of hypothyroidism in the group of SCH patients with TSH < 10 mIU/L, and their relationship to the quality of life on three different occasions: before treatment, after a 3-month trial with LT4 and after the treatment withdrawal. We also considered patients' opinion on the treatment benefit. We used general questionnaire of World Health Organization (WHO) for the subjective assessment of the health status, Short Form-36 (SF-36).

## Methods

This prospective, open-label study was a part of a larger study that evaluated different effects of thyroxine substitution in SCH [15]. It included 35 consecutive symptomatic patients with persistent (3–6 months) untreated SCH with TSH < 10 mIU/L, caused by chronic autoimmune thyroiditis (positive tAbs and/or typical ultrasound scan). All patients had at least two symptoms of hypothyroidism. The exclusion criteria were: previous history of thyroid disease and treatment, conditions and drugs that affect thyroid, past or current serious medical diseases that might affect study parameters, smoking and patient's refusal to participate in the study. All criteria were configured to avoid the confounding effects of other factors on clinical presentation of SCH, quality of life in SCH and biochemical parameters in SCH. The group was ranked by disease-specific score for hypothyroidism clinical grading validated by Zulewski and al. [6]. The score includes 12 symptoms and signs (periorbital edema, constipation, weight gain, cold skin, paresthesia, dry skin, rough/flaky skin, hoarseness, decreased sweating, slow motion, hearing loss, delayed Achilles reflex); the maximum score is 12 and the sum is interpreted as follows: less than 3 points – clinically eu-

thyroid patients; 3–5 points – intermediate, more than 5 points – symptoms and signs of overt hypothyroidism [6].

Quality of life was assessed using a self-administered SF-36 questionnaire validated in Serbian language [16–17]. SF-36 provides data on general health status and well-being, both physical and mental. The higher total SF-36 score represents a better quality of life.

The study design included two phases. After the initial investigation, in the first phase, LT4 treatment was started in doses sufficient to normalize TSH. Three months after TSH normalization, all measurements were repeated. In the second phase, patients were asked to stop the treatment with LT4 and three months later, thyroid function, degree of hypothyroidism and quality of life were reassessed. The Ethics Committee of the Faculty of Medicine of the Belgrade University approved the study protocol. All patients gave informed consent before participating in the study.

Serum FT4 (12–20 pmol/L), FT3 (3.95–6.8 pmol/L), and TSH (0.3–4.0 mIU/L) levels were measured by the commercially available automated chemiluminescence system and associated kits (ACS: 180, Chiron Diagnostics, East Walpole, MA, USA); tAbs (TPO Ab and Tg Ab) were measured by RIA method (Inep, Belgrade, Serbia); normal level for TPO Abs is < 35 U/mL.

Data were analyzed using methods of descriptive and analytical statistics. To assess the significance of differences we used the Mc Nemar test for categorical variable and the paired *t*-test or Wilcoxon test for numerical variables. The Spearman's correlation coefficient was used to analyze the relationship between study variables. Central tendency was presented by mean and median and variability by standard deviation and interquartile range. The $p < 0.05$ was considered statistically significant. All statistical analyses were performed in SPSS 20.0 (SPSS Inc., Chicago, Illinois).

## Results

Our study included 29 female and 6 male patients (82.9% vs. 17.1%, r4spectively), mean age 51.6 ± 15.4 years. The anthropometric data and thyroid status before and after therapy are shown in Table 1.

Doses sufficient to normalize TSH were ranged from 25 mcg to 75 mcg daily, with a mean dose of 50 mcg.

After correction of TSH, the median of the Zulewski index was reduced significantly ($p < 0.001$) from 5.0 (4.0–7.0) before the treatment to 3.0 (2.0–5.0) after the treatment, representing a reduction of symptoms and signs in the whole group of patients. A distribution of all three Zulewski index degrees is presented in Table 2. Before the treatment, most patients fell into intermediate and overt hypothyroidism groups. After the treatment, the highest frequencies were recorded in clinically euthyroid and intermediate categories.

LT4 treatment significantly reduced the percentage of patients with symptoms and signs of overt hypothyroidism and significantly increased the number of individuals classified as intermediate and euthyroid ones. The frequency of symptoms and signs of hypothyroidism before and after the substitution are shown in Table 3.

The most common ailments before the treatment were dry skin (71.4%) and hoarseness (65.7%). After the treatment, there was a significant reduction in the frequency of constipation ($p = 0.004$), dry skin ($p = 0.022$), hoarseness ($p = 0.002$), decreased sweating ($p = 0.006$), and delayed Achilles reflex ($p = 0.002$) (Figure 1). The frequency of weight gain was reduced, but that was close to statistical significance ($p = 0.057$).

**Table 1**

**The anthropometric data and thyroid status before and after therapy with levothyroxine**

| Parameters | Before (n = 35) mean ± SD | After (n = 35) mean ± SD | *p*-value (*t*-test) |
|---|---|---|---|
| Body mass (kg) | 72.6 ± 18.4 | 71.4 ± 16.6 | 0.030 |
| Body mass index (kg/m$^2$) | 25.5 ± 4.0 | 24.9 ± 4.0 | 0.320 |
| Waist circumference (cm) | 87.1 ± 16.0 | 85.7 ± 16.2 | 0.422 |
| Free thyroxine – FT4 (pmol/L) | 13.4 (12.5–14.7) | 16.6 (15.4–18.8) | < 0.001* |
| Thyroid-stimulating hormone – TSH (mIU/L) | 7.0 ± 2.1 | 3.0 ± 1.0 | < 0.001 |
| Free triiodothyronine – FT3 (pmol/L) | 1.7 ± 0.2 | 1.6 ± 0.2 | 0.019 |
| Thyroid peroxidase antibodies – TPO Abs (U/mL) | 208.4 (16.2–903.1) | 25.4 (0.1–87.5) | < 0.001* |

**SD – standard deviation; **p* – value from the Wilcoxon test.**

**Table 2**

**Zulewski index before and after therapy with levothyroxine**

| Zulewski index | Before (n = 35) n (%) | After (n = 35) n (%) |
|---|---|---|
| 1–2 (clinically euthyroid patients) | 4 (11.4) | 13 (37.1) |
| 3–5 (intermediate) | 16 (45.7) | 18 (51.4) |
| > 5 (symptoms and signs of overt hypothyroidism) | 15 (42.9) | 4 (11.4) |

**Table 3**

**The frequency of symptoms and signs asassessed by the Zulewski score before and after therapy with levothyroxine**

| Simptoms and signs | Before (n = 35) n (%) | After (n = 35) n (%) | *p*-value |
|---|---|---|---|
| Periorbital edema | 17 (48.6) | 13 (37.1) | 0.388 |
| Constipation | 15 (42.9) | 6 (17.1) | 0.004 |
| Weight gain | 17 (48.6) | 9 (25.7) | 0.057 |
| Cold skin | 16 (45.7) | 16 (45.7) | 0.999 |
| Paresthesia | 15 (42.9) | 13 (37.1) | 0.774 |
| Dry skin | 25 (71.4) | 16 (45.7) | 0.022 |
| Rough / flaky skin | 19 (54.3) | 18 (51.4) | 0.999 |
| Hoarseness | 23 (65.7) | 10 (28.6) | 0.002 |
| Decreased sweating | 11 (31.4) | 1 (2.9) | 0.006 |
| Slow motion | 10 (28.6) | 8 (22.9) | 0.625 |
| Hearing loss | 11 (31.4) | 6 (17.1) | 0.125 |
| Delayed Achilles reflex | 11 (31.4) | 1 (2.9) | 0.002 |

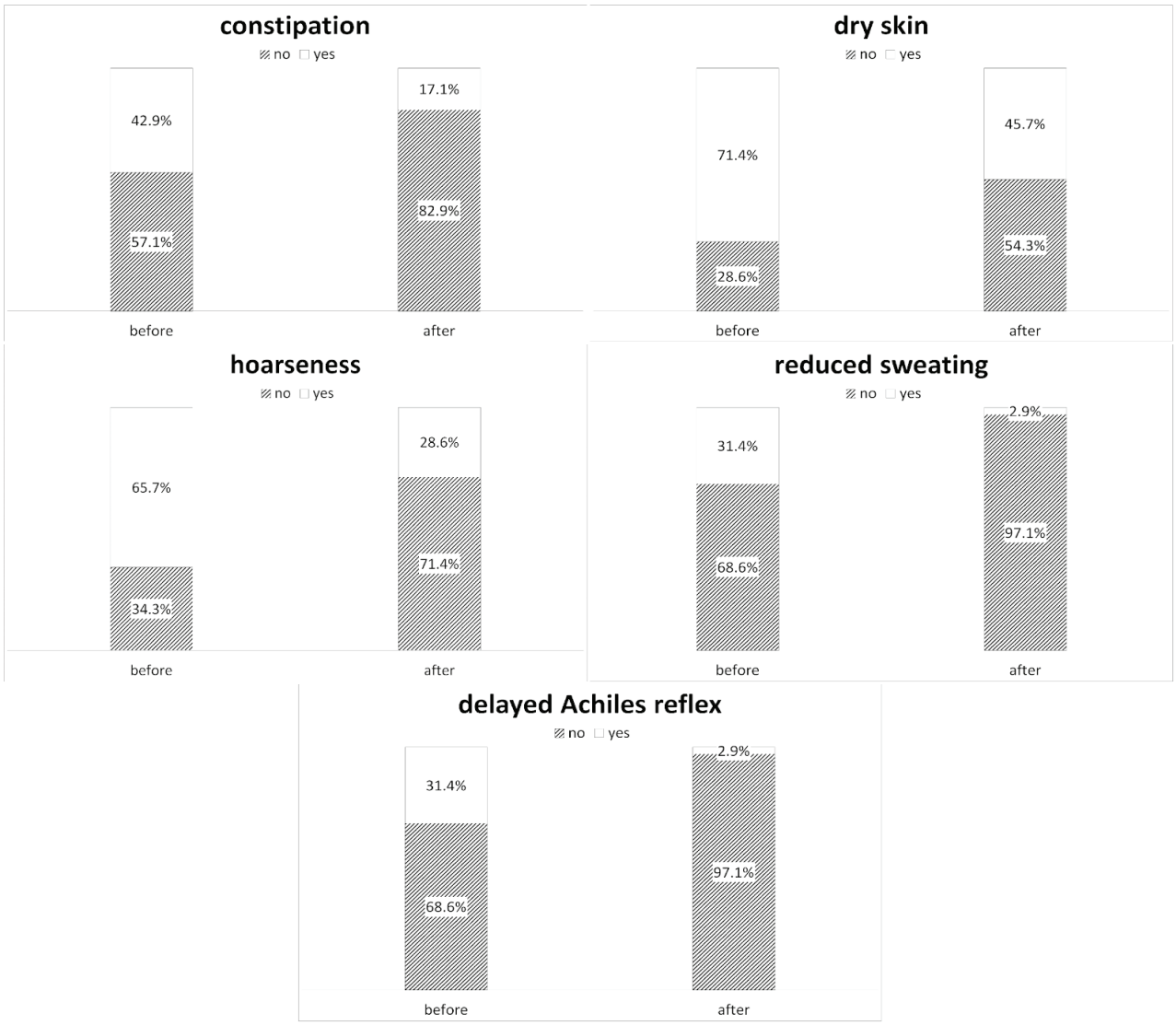


**Fig. 1 – The frequency of constipation, dry skin, hoarseness, decreased sweating and delayed Achilles reflex in patients with subclinical hypothyroidism before and after therapy with levothyroxine.**

There was no correlation among changes in the Zulewski index and changes of TSH, FT4, FT3, thyroid autoantibodies (TPO Abs) levels and average dose of levothyroxine (Table 4).

**Table 4**
**The correlation of changes in the Zulewski index with changes in TSH, FT4, FT3, TPO Abs and the average dose of levothyroxine**

| Thyroid hormones | Zulewski index | |
|---|---|---|
| | *r* | *p* |
| Thyroid-stimulating hormone (TSH) | -0.105 | 0.550 |
| Free thyroxine (FT4) | -0.057 | 0.752 |
| Free triiodothyronine (FT3) | 0.103 | 0.589 |
| Thyroid peroxidase antibodies (TPO Abs) | 0.330 | 0.061 |
| Average dose of levothyroxine | 0.165 | 0.343 |

***r* – Spearman's rank correlation coefficient.**

We also estimated the quality of life by SF-36 before and after LT4 substitution. The results are shown in Table 5.

Emotional roles improved the most after the therapy, the change being close to statistical significance ($p = 0.065$). The improvement of other scores was not significant.

In the phase 2, all 35 patients were asked to give their opinion about treatment benefit based on symptom correction and to stop the treatment in order to re-evaluate the effects of LT4 therapy. Among them, 17 patients were quite satisfied with the improvement of their symptoms and were not willing to stop the treatment. The remaining 18 patients accepted additional checking and continued the study. There were no statistically significant differences in any measured parameters between these two groups of patients. In the group of patients who discontinued treatment, many symptoms and signs reappeared (Table 6) with the increase of TSH (6.8 ± 1.1 mIU/L). Patients had no insight into their earlier test responses.

**Table 5**
**The Short Form-36 (SF-36) questionnaire scores obtained before and three months after euthyroidism had been achieved**

| SF-36 domain | Before (n = 35) mean ± SD | After (n = 35) mean ± SD | *p*-value (*t*-test) |
|---|---|---|---|
| Physical functioning | 62.4 ± 37.3 | 73.1 ± 27.6 | 0.364 |
| Role-physical | 64.3 ± 47.1 | 62.1 ± 48.6 | 0.842 |
| Body pain | 72.8 ± 23.9 | 74.5 ± 25.6 | 0.891 |
| General health | 53.9 ± 9.2 | 53.8 ± 8 | 0.837 |
| Vitality | 75.1 ± 24.7 | 83.3 ± 19.2 | 0.182 |
| Social functioning | 64.8 ± 26.8 | 67 ± 23.2 | 0.647 |
| Role-emotional | 47.6 ± 49.4 | 74.3 ± 45.1 | 0.065 |
| Physical health | 65.6 ± 21.0 | 69.3 ± 19.6 | 0.675 |
| Mental health | 63.7 ± 19.7 | 72.5 ± 17.9 | 0.106 |
| Total SF-36 score | 64.8 ± 21.4 | 71.6 ± 20.2 | 0.379 |

**SD – standard deviation.**

**Table 6**
**Incidence of symptoms according to the Disease-specific questionnaire with clinical assessment scale for hypothyroidism before and after stopping therapy with levothyroxine**

| Symptoms from the questionnaire | Before (n =18) n (%) | After (n =18) n (%) | *p*-value |
|---|---|---|---|
| Periorbital edema | 4 (23.5) | 12 (70.6) | 0.021 |
| Constipation | 4 (23.5) | 12 (70.6) | 0.039 |
| Weight gain | 1 (5.9) | 7 (41.2) | 0.008 |
| Cold skin | 6 (35.3) | 7 (41.2) | > 0.999 |
| Paresthesia | 7 (41.2) | 7 (41.2) | > 0.999 |
| Dry skin | 9 (52.9) | 18 (51.4) | 0.688 |
| Rough / flaky skin | 10 (35.3) | 7 (41.2) | 0.375 |
| Hoarseness | 6 (35.3) | 12 (70.6) | 0.109 |
| Decreased sweating | 0 (0) | 7 (41.2) | 0.016 |
| Slow motion | 4 (23.5) | 11 (64.7) | 0.039 |
| Hearing loss | 4 (23.5) | 3 (17.6) | > 0.999 |
| Delayed Achilles reflex | 0 (0) | 12 (70.6) | < 0.001 |

**Table 7**
**The Short Form-36 (SF-36) questionnaire scores obtained before and after stopping therapy with levothyroxine**

| SF-36 domain | Before (n = 18) (mean ± SD) | After ± SD (n = 18) (mean ± SD) | *p*-value (*t*-test) |
|---|---|---|---|
| Physical Function | 69.4 ± 30.9 | 67.1 ± 25.7 | 0.781 |
| Role-Physical | 52.9 ± 51.4 | 35.3 ± 49.3 | 0.257 |
| Body Pain | 73.9 ± 27.2 | 74.1 ± 21.6 | 0.972 |
| General Health | 52.9 ± 10.5 | 71.6 ± 25.4 | 0.007 |
| Vitality | 82.1 ± 21.0 | 66.8 ± 17.8 | 0.01 |
| Social Functioning | 69.4 ± 26.5 | 82.4 ± 13.9 | 0.11 |
| Role emotional | 68.6 ± 46.4 | 43.1 ± 45.3 | 0.033 |
| Mental Health | 83.5 ± 21.0 | 68.5 ± 23.7 | 0.023 |
| Physical health | 66.1 ± 22.8 | 62.9 ± 24.3 | 0.535 |
| Mental Health (dimension) | 71.3 ± 20.2 | 66.5 ± 22.7 | 0.379 |
| Total SF-36 Score | 69.2 ± 23.4 | 63.6 ± 24.8 | 0.325 |

**SD - standard deviation.**

**Table 8**
**The Zulewski index and total Short Form 36 (SF-36) questionnaire score compared between patients who continued levothyroxine treatment and those who quitted it**

| Score | Treatment continued (n =17) | Treatment quitted (n = 18) | *p*-value (*t*-test) |
|---|---|---|---|
| Zulewski index | 3.0 ( 2.0–5.0) | 4.0 (3.0–6.0) | 0.460 |
| Total SF-36 | 73.9 ± 17 | 69.2 ± 23.4 | 0.493 |

**Note: Results are present as median (range) or mean ± standard deviation.**

After the therapy discontinuation, there was a statistically significant increase in the frequency of following parameters: periorbital edema, constipation, weight gain, decreased sweating, slow motion and delayed Achilles reflex. The median of the Zulewski index on LT4 treatment was 3.0 (2.0–5.0) and three months after the therapy discontinuation it was 6.0 (4.0–9.0). There was again a statistically significant change of the score ($p = 0.010$).

Therapy withdrawal was associated with a non-significant change in the total SF-36 score, but there was a statistically significant reduction in the general health score. The significant decrease was also recorded in vitality, role emotional and mental health scores. There were no significant changes in other parameters of the SF-36 questionnaire (Table 7). Also, there were no significant differences in the Zulewski index and the total SF-36 score between the patients who continued LT4 treatment and those who quitted it (Table 8).

## Discussion

The treatment of SCH, especially a mild form, is still a matter of debate. In spite of very extensive research over the last two decades, the relationship between subclinical hypothyroidism, lipid metabolism and cardiovascular diseases is still controversial. Many studies showed that mild thyroid hypofunction may represent a risk factor for metabolic and cardiovascular diseases. The largest epidemiologic Colorado study confirmed a positive relationship between serum TSH and dyslipidaemia [2]. A larger number of observational studies demonstrated that the lipid profile is unfavorably changed in SCH compared to the number of those which showed that it is unchanged [18]. Previous analyses of small interventional studies did not confirm a significant influence of LT4 on lipid profile [12, 18]. One of the largest studies which demonstrated a significant reduction of total cholesterol and low density lipoprotein cholesterol (LDL-C) on LT4 was a randomized, double-blind, cross-over study by Razvi et al. [10]. A recent meta-analysis of randomized controlled trials showed that LT4 treatment has clear benefits on total cholesterol and LDL-C in SCH patients, including those with mild SCH [19].

The relationship of SCH and cardiovascular events was also a focus of many studies. Selmer et al.[20] demonstrated, in a large population study, that a risk of major adverse cardiovascular events in SCH is elevated. Analyses of prospective cohort studies showed that SCH is associated with increased coronary heart disease mortality and an increased risk of stroke and heart failure [21]. A double-blind, placebo-controlled study by Monzani et al. [22] showed the existence of functional disorder of the left ventricle which was resolved by LT4 treatment . After the already mentioned study of Razvi et al.[10] who demonstrated the beneficial effect of LT4 on cardiovascular risk factors and endothelial function, recent meta-analysis [23] has supported the positive influence of the treatment on the progression of carotid intima-media thickness, possibly by reducing risk factors for its occurrence in SCH . The limitation of this analysis is a small number of randomized controlled trials with the overall small number of respondents included, indicating that there is still a need for larger interventional studies to confirm those findings.

Although, there are controversies about the efficacy of LT4 treatment in SCH, the drug is widely used for potential beneficial effect on symptoms, protection from asymptomatic complications or prevention of the insidious development of

overt hypothyroidism. The decision to treat SCH is mostly based on patients' TSH and tAb levels, their complaints, quality of life and present co-morbidities [3]. Unfortunately, complaints of patients with mild hypothyroidism are similar to those of the general age-matched population. Some studies estimated frequency and consistency of complaints in a large number of patients with SCH. Among the many frequent symptoms, not specific to mild hypothyroidism alone, dry skin and poor memory were the most common [2, 6, 24]. Patients with SCH may tolerate their symptoms in different ways, which also may enhance the importance of the quality of life assessment.

Clinical evaluation by the standardized score used in this study provides information about the severity of thyroid dysfunction and allows better evaluation of treatment effects. Patients with SCH may be ranged as overt hypothyroidism according to the Zulewski score (> 5) and the therapeutic target could be based not only on TSH correction but also on clinical improvement (euthyroid state defined by the Zulewski score 1–2). The majority of patients in our study were classified as clinically intermediate and hypothyroid before and showed significant improvement after the treatment, with a large number of respondents reaching the clinically euthyroid state. Some studies support our results emphasizing that SCH patients treated with LT4 improved their score when regained normal TSH, even if they did not feel many symptoms at the beginning [6–8].

To provide better objectivity in the assessment of LT4 effects in this study, patients were tested on three different occasions with the time distance. About 57% of symptomatic patients in our study were interested to suspend LT4 use and re-evaluate the influence of treatment on their ailments. This group of patients did not significantly differ from the other in any measured parameters. The retested group showed consistency in reporting symptoms of untreated SCH and the same degree of severity was measured by the Zulewski index on these two occasions. Despite a lesser confidence in the benefit of LT4 therapy in this group of patients, objective scoring demonstrated significant improvement of symptoms.

The individuals in our study mostly reported problems related to the dry, rough skin and hoarseness. In the Colorado study including 2,336 subjects with SCH, many symptoms were significantly more often reported than in euthyroid subjects, but dry skin and cognitive impairment predominated [2]. After the treatment, frequency of constipation, dry skin, hoarseness, decreased sweating and delayed Achilles reflex were significantly reduced in our study. These results are in accordance with report from two randomized controlled trials that SCH subjects treated with LT4 had significantly greater improvement in general hypothyroid symptom scores than did subjects treated with placebo [7, 8].

A few very large studies provide insight into the influence of SCH on quality of life. LifeLines Cohort study on 9,491 participants showed no significant difference in HR-QOL scores of 973 subjects with SCH compared to euthyroid controls, but also compared to the groups with overt hypothyroidism and hyperthyroidism [25]. In a cross-sectional study which included women aged 18–75 years, SCH was neither associated with lower well-being nor impaired health-related quality of life [26].

As for LT4 treatment, there are many controversies about the success of the intervention to improve the quality of life and symptoms in SCH. In the study of Jorde et al. [11], LT4 treatment was not found to improve hypothyroid symptoms and cognitive and emotional functions in subjects with SCH compared to placebo group. But, in that study, the mean TSH level in the SCH group was 5.5 mIU/L, slightly higher than normal. Also, even before LT4 treatment there were no differences between SCH patients and healthy controls in symptoms related to hypothyroidism and neuropsychological dysfunction. A double-blind, randomized, placebo-controlled trial involving 737 adults who were at least 65 years of age (TRUST study) suggested that the treatment of older patients with subclinical hypothyroidism does not change significantly quality of life or hypothyroid symptoms [27]. We recruited for our study only symptomatic patients with persistent SCH, which were otherwise healthy nonsmokers. Even though the old age was not an exclusion criterion, our study did not cover patients with SCH older than 65 years eventually, due to exclusion criteria related to their co-morbidities and the use of drugs which might influence thyroid function or biochemical parameters analyzed and presented elsewhere [15].

In our study, emotional roles were mostly improved after therapy, but the change was just close to statistical significance. Total score and many other subscales of the SF-36 questionnaire (physical and social functioning, vitality, physical and mental health) showed only a trend toward improvement. The study of Razvi et al. [10] also showed partial improvement following LT4 treatment. Some patient-reported outcomes as tiredness related symptoms were significantly improved, some others, as all subscales of the SF-36 questionnaire (apart from role emotional) just tended toward the improvement. Quality of life measured by general questionnaires in patients with unselected SCH may not be significantly disturbed or improved by LT4 treatment in the large studies. Our patients, who had at least two recognizable sustained symptoms of hypothyroidism, also did not show significant changes of their quality of life on LT4 treatment. Still, patients who stop the treatment could experience worsening of their life quality in some aspects, according to our finding.

The main limitation of our study is a small number of patients and this finding need to be confirmed in larger population of symptomatic SCH patients. Also, the lack of significant differences between groups related to the measured variables such as the Zulewski index and SF-36 questionnaire scores indicates the influence of unmeasured confounders, e.g. long-term drug compliance, and the intensity, type and duration of symptoms.

Some data suggest that the physical and mental component scores of the SF-36 questionnaire are influenced by smoking status, co-morbidity, and body mass index (BMI) or waist circumference [28]. Our study does not include smokers and patients with co-morbidities and since there were no significant changes in BMI and waist circumference (WC) during the study, the potential changes of the SF-36 questionnaire scores could be attributed to LT4 treatment *per se*.

**Conclusion**

Clinical score based on symptoms and signs is a sensitive and reproducible test for objective estimation of LT4 treatment effects in symptomatic SCH patients with TSH < 10 mIU/L. The improvement in clinical score may not be associated with the patient's clear sense of treatment benefit. Measured by general health-related SF-36 questionnaire, symptomatic subclinical hypothyroidism is not necessarily associated with a quality of life impairment that may be significantly improved by LT4 treatment. Still, after withdrawal from the therapy, some aspects of life quality, such as general health, vitality, mental health and emotional role, may be significantly affected according to our findings.

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# Electroclinical characteristics of MRI negative focal epilepsy: A video-EEG study

## Elektrokliničke karakteristike MRI negativnih fokalnih epilepsija: video-EEG monitoring studija

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**Abstract**

**Background/Aim.** Epileptogenic lesions carry intrinsic epileptogenicity or epileptogenic potential in their close vicinity. One third of patients with focal epilepsy have no epileptogenic lesions magnetic resonance imaging [MRI(-)]. The aim of this study was to determine the epileptogenic zone investigating electrical and clinical properties of MRI- patients. **Methods.** In 180 patients with focal epilepsy we analyzed 1,712 seizures for interictal and ictal electroencephalography (EEG) and seizure semiology. If multiple seizures occurred we took the best seen on video as an example, with secondary generalized tonic-clonic seizures (GTCS) if it occurred. Brain MRI was focused to investigate the zone of ictal EEG onset. Electroclinical properties of the MRI- patients were compared to lesion positive patients [MRI(+)]. **Results.** A single epileptogenic lesion was identified in 68.89% [hippocampal sclerosis (HS) in 58, focal cortical dysplasia (FCD) in 28 and other pathologies in 38 patients]. MRI(-) patients had significantly less interictal epileptiform abnormalities, and presented more often ($p < 0.001$) with secondary GTCS as the only seizure. Eye opening, hypermotor seizure, bilateral asymmetric clonic seizure, vocalization, and contralateral body turning occurred more frequently in the MRI- group compared to the MRI+ one. MRI- patients share some semiological features with FCD as opposed to HS patients. **Conclusion.** MRI- epilepsy patients frequently present with electroclinical features seen in frontal lobe epilepsy or in epilepsy associated with FCD.

**Key words:**
**brain; diagnosis; electroencephalography; epilepsies, partial; hippocampus; magnetic resonance imaging.**

**Apstrakt**

**Uvod/Cilj.** Epileptogene lezije nose unutrašnji epileptogeni potencijal u svojoj neposrednoj blizini. Jedna trećina bolesnika sa fokalnom epilepsijom nema epileptogenu leziju – negativan nalaz magnetno-rezonantnog snimanja (MRI-). Cilj rada bio je određivanje epileptologene zone ispitivanjem električnih i kliničkih svojstava bolesnika sa MRI-. **Metode.** Ispitivanjem je obuhvaćeno 180 bolesnika sa fokalnom epilepsijom kojima su analizirani interiktalni i iktalni EEG zapis i semiološke karakteristike kod ukupno 1 712 napada. U slučaju više napada analizirani su najbolje vidljivi napadi na video snimku, sa sekundarnim generalizovanim toničko-kloničkim napadom (GTKN), ukoliko se dogodio. MRI mozga imalo je za cilj istraživanja zone početka napada zabeleženog na EEG zapisu. Elektrokliničke osobine bolesnika sa MRI- upoređivane su sa MRI nalazom bolesnika sa epileptogenom lezijom (MRI+). **Rezultati.** Jedna epiloptegena lezija je identifikovana kod 68,89% bolesnika [hipokampusna skleroza (HS) kod 58, fokalna kortikalna displazija (FKD) kod 28 bolesnika, druga patologija kod 38 bolesnika]. Bolesnici sa MRI- imali su značajno manje interiktalnih EEG abnormalnosti, a klinički su bili prezentovani značajno češće ($p < 0.001$) sa sekundarno GTKN. Otvorene oči, hipermotorni napad, bilateralni asimetrični klonički napad, vokalizacija i kontralateralno okretanje tela bili su semiološki znaci viđeni češće kod bolesnika sa MRI- u poređenju sa onima sa MRI+. Bolesnici sa MRI- imali su zajedničke semiološke osobine sa bolesnicima sa FKD, a ne sa bolesnicima sa HS. **Zaključak.** Epilepsija bolesnika sa MRI- često se prezentuje elektrokliničkom osobinama kakve se viđaju kod epilepsija frontalnog režnja ili epilepsija sa FKD.

**Ključne reči:**
**mozak; dijagnoza; elektroencefalografija; epilepsija, parcijalna; hipokampus; magnetska rezonanca, snimanje.**

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## Introduction

Epileptogenic lesions carry intrinsic epileptogenicity or epileptogenic potential in their close vicinity. Pharmacoresistant focal epilepsy with well-defined cortical lesions, owing to improved acquisition and interpretation of brain magnetic resonance imaging (MRI) positive (MRI+), is a subject of surgical treatment frequently leading to favorable outcome. However, there exists a significant proportion of patients with MRI not showing a lesion potentially causative of chronic epilepsy. Patients studied presurgically with MRI negative (MRI-) focal epilepsy and epileptogenic zone potentially located anywhere in the cerebral cortex account for 18% to 43% [1–3]. In theory, in those patients an epileptogenic zone could be located anywhere in the cerebral cortex. Nevertheless, some authors found a high prevalence of frontal lobe epilepsy in a group of consecutively recruited MRI- refractory epilepsy patients [3].

The analysis semiology of symptoms during epileptic seizures helps to determine the epileptogenic zone. Some studies of seizure semiology helped to differentiate between seizures arising in the frontal region from the mesial temporal regions [4–5]. Although MRI- patients represent a significant subgroup of epileptic patients, its overall semiology is not extensively studied. In the setting of long-term video-EEG monitoring (vEEG), the aim of this study was to investigate a seizure semiology and EEG findings in MRI- and MRI(+) epileptic patients with the premise that they may differ.

## Methods

Patients were selected from the database of the Epilepsy Center EEG Monitoring Unit at the Neurology Clinical Center of Serbia in Belgrade, covering the period from August, 2009 till May, 2012. We used the Vyasis Nicolet 64-channel acquisition system with 10–20 electrode placement system with anterior temporal electrodes added. Antiepileptic drugs (AEDs) were discontinued in the absence of patient-specific contraindications in all patients, in a well-structured way: 50% of the prescribed daily dose was withdrawn upon admission; for patients on polytherapy the complete withdrawal of one drug was favored. We studied 180/310 (58.04%) of patients who underwent long-term vEEG monitoring in whom focal epileptic seizures were recorded. One hundred thirty patients were excluded due to focal epilepsy with isolated auras (65 patients), psychogenic nonepileptic seizures (30 patients), generalized epilepsy (28 patients), brain MRI not performed (3 patients), syncope (3 patients) and *epilepsia partialis continua* (1 patient).

In patients with multiple seizures recorded, one was taken as a "reference" [defined as the best seizure seen on video, with secondary generalized tonic-clonic (sGTC) phase if this occurred]. Semiological signs, recorded only in other (non-reference) seizures, were added according to time of the occurrence in the final sequence of semiological signs of the "reference" seizure. Clinical onset of the seizure was determined by the first visible change in behavior, or when the patient announced an aura or pressed the seizure alarm. Data concerning quality of auras were obtained during the aura, immediately at the end of the seizure or from the medical history. We defined the onset of generalization as the brief period between the focal seizure and the remaining sGTC phase characterized by head version or vocalization [6]. Determination was used as the cut-off point for analysis.

We analyzed seizure duration in all patients. Seizure duration was analyzed in the "reference" seizure if multiple seizures occurred. Seizure onset was defined as the first ictal EEG change or first subjective/objective clinical change, whichever happened first. Ictal EEG was defined as rhythmic electrical activity with evolution in frequency, amplitude and distribution. Ictal onset zone (ictal EEG), and location and number of the interictal epileptiform abnormality populations were included in the analysis.

The ictal characteristics listed by Kotogal et al. [5] were looked for in every seizure. Lateralization of the semiological signs was determined according to the side of the lesion or ictal EEG.

In all patients we performed brain MRI (Avanto Siemens or Achieva Phillips 1.5T) including MPRAGE or T1W isotropic volume examination, T1W sagittal slices (5 mm), FLAIR and T2W coronal slices (3 mm), FLAIR and T2W axial slices (5 mm), Gradient Echo Image axial slices (5 mm), and Inversion Recovery coronal slices (5 mm), with proper angulations. Two experienced neuroradiologists defined quality and location of the lesion by visual inspection.

Descriptive statistics including numbers and percentages of categorical variables or mean, median, and standard deviation were used to characterize the study sample. Differences between groups in number and frequency of semiological signs were analyzed using Student's *t*-test, one-way ANOVA followed by Tukey's *post hoc* test and Pearson's $\chi^2$ test or Fisher exact probability test when appropriate. Temporal relations between most frequent symptoms were analyzed using log-linear models. Hierarchical cluster analysis with Furthest neighbour and Euclidean distance metric were used to describe clustering of semiological signs which were included in analysis only if frequency was > 20%. Statistical analyses were performed using SPSS for Windows, version 22. The significance level was set at 0.05 in all analyses. The research was approved by the Ethical Committee of the Clinical Center of Serbia. Written informed consent was obtained from all patients.

## Results

A single epileptogenic lesion was detected in 124 (68.89%) MRI+ patients (34.9 ± 11.2 years, 68 females). Radiological findings were distributed as follows: hippocampal sclerosis – HS (58 patients), focal cortical dysplasia – FCD (28 patients), dual pathology (11 patients), remote brain infarcts (6 patients), dysembryoplastic neuroepithelial tumor (6 patients), cavernoma (4 patients), astrocytoma/oligodendroglioma (3 patients); posttraumatic gliosis (4 patients), ganglioglioma, dermoid cyst, nodular heterotopia and hamartoma (4 patients). FCD were located in different brain regions: mesial temporal lobe (28.6%), lateral temporal lobe

(17.9%), dorsolateral frontal lobe (21.4%), occipital lobe (14.3%), and equally in mesial frontal lobe, orbitofrontal region, dorsolateral parietal lobe, mesial parietal lobe and insula (3.6%). MRI- was found in 54 (30%) patients (32.8 ± 10.3 years, 19 females). In two MRI+ patients multiple demyelinating lesions were demonstrated.

Average duration of long-term vEEG monitoring was 3.98 days (range 2–4 days). A total of 1,712 seizures were recorded (median 5 seizures per patient, range 1–384 seizures). A single seizure was recorded in 11.1% of patients. A total of 184 sGTC seizures were recorded (median 2 seizures per patient, range 1-10 seizures) in 74 patients (41.1%). In 12.7% of patients only sGTC seizures were recorded (single sGTC seizure in 5%). Secondary GTC seizures as the only seizure type were more likely in MRI- patients compared to patients with HS or FCD ($p < 0.001$) (Figure 1).

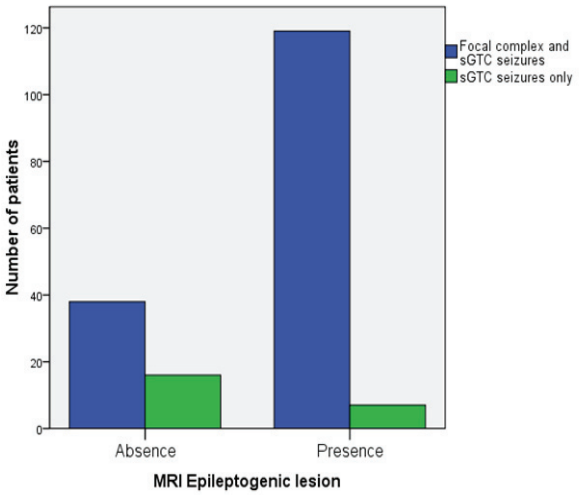


**Fig. 1 – Focal complex seizures with and without secondary generalization and secondary generalized tonic-clonic seizures only in analyzed patients.**
**sGTC – secondary generalized tonic-clonic; MRI – magnetic resonance imaging; MRI negative (MRI-) patients had sGTC seizures more frequent than patients with MRI detected epileptogenic lesions (MRI+) ($p < 0.001$).**

Median seizure duration was 90.5 s (range 18–2,603 s). We did not find a significant difference in median seizure duration between the MRI+ (95 s) and MRI- (85.5 s) groups. Average sGTC phase duration was 80 ± 35 s (range 38–301 s). There did not find a significant difference in average sGTC phase duration between the MRI+ (83.9 ± 40 s) and MRI- (76.3 ± 29 s) groups.

In both the MRI+ and MRI- patients ictal EEG showed lateralized and localized activity in 96.66%: 4 patients in the MRI+ and 2 patients in the MRI- group had a nonlateralized ictal EEG pattern. Ictal onset zone was localized as follows: temporal region (37.36%), fronto-temporal region (34.48%), frontal region (12.64%), temporo-posterior region (4.02%), temporo-occipital, fronto-central and parietal region (2.3%), temporo-central and vertex region (1.15%), and lateralized to one hemisphere (2.3%). Ictal EEG was lateralized to the side of the epileptogenic lesion with a high probability [odds ratio (OR) 8.84; confidence interval (CI) 95% 1.2–64.8]. Ictal EEG onset zone in the MRI- group was significantly more frequent in frontal, fronto-central and temporo-posterior regions. There was a difference in distribution of the ictal EEG onset patterns between MRI+ and MRI- patients ($p < 0.001$) (Table 1).

**Table 1**
**Distribution of the ictal EEG onset patterns between MRI+ and MRI- patients**

| Ictal EEG onset pattern | MR- patients n (%) | MRI+ patients n (%) |
|---|---|---|
| Frontal | 12 (23.1) | 9 (7.5) |
| Fronto-temporal | 17 (32.7) | 43 (35.8) |
| Temporal | 11 (21.2) | 53 (44.2) |
| Parietal | 1 (1.9) | 3 (2.5) |
| Temporo-posterior | 5 (9.6) | 2 (1.7) |
| Temporo-occipital | 0 | 4 (3.3) |
| Temporo-central | 0 | 2 (1.7) |
| Fronto-central | 4 (7.7) | 0 |
| Vertex | 0 | 2 (1.7) |
| Lateralized | 2 (3.8) | 2 (1.7) |
| Total number | 52 | 120 |

**EEG – electroencephalogram; MRI – magnetic resonance imaging, positive (MRI+) and negative (MRI-).**

Interictal epileptiform abnormalities were detected in 93.3% of patients. One, two or three interictal spike populations were recorded in 65%, 27.81% and 7.19% of patients, respectively. The lack of interictal epileptiform abnormalities in MRI- patients (8/54) was more likely compared to patients with HS (2/58) or FCD (0/28) ($p < 0.001$).

The sum of 1,431 signs from 180 seizures was detected (average 7.95 per patient/seizure). Number of detected signs was significantly lower in MRI-)(7.1 ± 3; range 2–14) compared to MRI+ patients (8.3 ± 2.9; range 2–17) ($p = 0.014$) and significantly higher in HS (9.3 ± 2.6; range 5–17) compared to FCD (7 ± 2.7; range 2–14) ($p = 0.001$) and MRI- patients ($p < 0.001$) with no significant difference between the FCD and MRI- groups ($p = 0.98$).

In the whole group, three most frequent signs were behavioral arrest (52.2%), oroalimentary automatisms (44.4%), and vocalization (40%). Total of 5 signs occurred more common in the MRI- group compared to the MRI+ group: eye opening (44.4% vs. 17.5%; $p < 0.001$), hypermotor seizure (9.3% vs. 1.6%; $p = 0.027$), vocalization (55.6% vs. 33.3%; $p = 0.008$), contralateral body turning (13% vs. 3.2%; $p = 0.018$), and bilateral asymmetric clonic seizure (29.6% vs. 14.3%; $p = 0.022$).

Less common symptoms in the MRI- group compared to the MRI+ group were: cephalic aura (3.7% vs. 15.1%; $p = 0.040$), epigastric aura (5.6% vs. 30.2%; $p < 0.001$), oroalimentary automatisms (24.1% vs. 53.2%; $p < 0.001$), contralateral hand dystonia (11.1% vs. 25.4%; $p = 0.045$), unilateral arm automatisms (13% vs. 29.4%; $p = 0.023$), contralateral (3.7% vs. 15.9%; $p = 0.024$), unilateral head turning (9.3% vs. 32.5%; $p = 0.001$), and unilateral body turning (1.9% vs. 14.3%).

Results indicated a significant difference for various two-sequential signs that appeared anywhere in the seizures in MRI+ and MRI- groups. Frequencies are reported in Table 2.

We found 67 different signs in the MRI- group (in total 385 in 54 analyzed seizures). Three most frequent signs in the MRI- group were vocalization (55.6%), behavioral arrest (50%), and eye opening (44.4%). In the HS group, 70 different signs were identified (in total 541 from 58 seizures) with the three most frequent signs being oroalimentary automatisms (65.5%), behavioral arrest (60.3%), and epigastric aura (46.6%). In the FCD group, 61 different signs were found (in total 196 from 28 seizures). The three most frequent signs in the FCD group were behavioral arrest (57.1%), oroalimentary automatisms (35.7%), and contralateral arm clonic seizure (32.1%). Signs presenting in significantly different frequency between MRI- patients, HS patients, and FCD patients are shown in Table 3.

As the first symptom, epigastric aura was more common in the HS group and eye opening in the MRI- and FCD groups ($p < 0.001$). First three most common sequential symptoms and signs were different in analyzed groups (Table 4).

Cluster analysis yielded patterns of symptom grouping that were different for the MRI- group, the HS group, and the FCD group (Figure 2). Two major clusters of signs appeared in the majority of seizures in the MRI- group (Figure 2A): contralateral tonic-clonic arm seizure or GTC seizure associated with eye opening, and oroalimentary automatisms with behavioral arrest. Similarly, in the FCD group two clusters of signs emerged (Figure 2B): isolated and less frequent GTC seizure cluster, and complex cluster consisting of eye opening and cephalic aura closely associated with smiling/laughing, unilateral hand automatisms and head turning, or more distant oroalimentary automatisms with behavioral arrest. Four well demarcated cluster signs appeared in the HS group (Figure 2C): epigastric aura with behavioral arrest and oroalimentary automatisms, as a frequent cluster, was associated with the cluster of similar frequency consisting of contralateral arm immobility or hand dystonia, unilateral head or body turning and arm automatisms; the cluster consisting of staring, vocalization and contralateral clonic arm seizure was associated with a less frequent cluster of GTC seizures.

**Table 2**

**Difference for various two-sequential signs that appeared anywhere in the seizure in the MRI+ and MRI- groups**

| Two-sequantial signs anywhere in the seizure | Whole group (n = 180) | MRI+ (n = 126) | MRI- (n = 54) | *p* |
|---|---|---|---|---|
| | n (%) | n (%) | n (%) | |
| Oroalimentary automatisms→ behavioral arrest | 30 (16.7) | 26 (20.6) | 4 (7.4) | 0.048 |
| Epigastric aura→oroalimentary automatisms | 21 (11.7) | 21 (16.7) | 0 (0.0) | 0.002 |
| Contralateral hand dystonia→ unilateral arm automatisms | 12 (6.7) | 12 (9.5) | 0 (0.0) | 0.019 |

**MRI – magnetic resonance imaging, positive (MRI+) and negative (MRI-).**

**Table 3**

**Difference in symptoms and sings of the disease: MRI-, HS and FCD**

| Symptom/sign | MRI- group[a] | HS group[b] | FCD group[c] | *p* | | |
|---|---|---|---|---|---|---|
| | n (%) | n (%) | n (%) | a:b | a:c | b:c |
| Eye opening | 24 (44.4) | 6 (10.3) | 7 (25.0) | **< 0.001** | 0.10 | 0.11 |
| Cephalic aura | 2 (3.7) | 7 (12.1) | 6 (21.4) | 0.16 | **0.017** | 0.34 |
| Epigastric aura | 3 (5.6) | 27 (46.6) | 4 (14.3) | **< 0.001** | 0.22 | **0.004** |
| Oroalimentary automatisms | 13 (24.1) | 38 (65.5) | 10 (35.7) | **< 0.001** | 0.31 | **0.011** |
| Behavioral unrest | 12 (22.2) | 22 (37.9) | 3 (10.7) | 0.10 | 0.24 | **0.011** |
| Staring | 3 (5.6) | 12 (20.7) | 3 (10.7) | **0.025** | 0.41 | 0.37 |
| CL immobility of the arm | 5 (9.3) | 14 (24.1) | 2 (7.1) | **0.045** | 1.00 | 0.08 |
| CL hand dystonia | 6 (11.1) | 19 (32.8) | 3 (10.7) | **0.007** | 1.00 | **0.035** |
| UL arm automatisms | 7 (13.0) | 19 (32.8) | 6 (21.4) | **0.015** | 0.35 | 0.32 |
| UL arm large automatisms | 1 (1.9) | 8 (13.8) | 1 (3.6) | **0.033** | 1.00 | 0.26 |
| Vocalization | 30 (55.6) | 21 (36.2) | 6 (21.4) | **0.057** | **0.005** | 0.22 |
| BL tonic seizure | 23 (42.6) | 17 (29.3) | 4 (14.3) | 0.17 | **0.013** | 0.18 |
| CL head turning | 2 (3.7) | 10 (17.2) | 5 (17.9) | **0.030** | **0.043** | 1.00 |
| UL head turning | 5 (9.3) | 21 (36.2) | 6 (21.4) | **0.001** | 0.17 | 0.22 |
| UL body turning | 1 (1.9) | 12 (20.7) | 2 (7.1) | **0.002** | 0.27 | 0.13 |
| Automatic walking | 2 (3.7) | 0 (0.0) | 3 (10.7) | 0.23 | 0.33 | **0.032** |

**MRI(-) – magnetic resonance imaging negative; HS – hippocampal sclerosis;**
**FCD – focal cortical dysplesia; CL – contralateral; UL – unilateral; BL – bilateral.**
**Statistically significant differences are bolded.**

**Table 4**

**First three most common sequential symptoms and signs in analyzed groups of patients**

| Variable | MRI- group | HS group | FCD group |
|---|---|---|---|
| Age[a] (years),mean ± SD | 32.9 ± 10.4 | 37.2 ± 11.3 | 33 ± 11.3 |
| Gender [b] (F/M), n | 19/35 | 32/26 | 15/13 |
| Epilepsy onset[b] (years), mean ± SD | 15 ± 9.3 | 12.7 ± 9.7 | 16 ± 11.2 |
| Epilepsy duration[c] (years), mean ± SD | 17.8 ± 10.8 | 24.4 ± 13 | 16.9 ± 9.5 |
| First three most frequent sequential symptoms/signs, (%) | | | |
| eyes opening → somatosensory aura (whole body) → behavioral arrest (3.7) | | epigastric aura → behavioral arrest → head turn (unilateral) (5.2) | eyes opening → cephalic aura → behavioral arrest (7.1) |
| eyes opening → vocalization → hypermotor seizure (3.7) | | eyes opening → epigastric aura → behavioral arrest (3.4) | |
| | | epigastric aura → psychic aura → behavioral arrest (3.4) | |

**MRI- – magnetic resonance imaging negative; HC – hippocampal sclerosis; FCD – focal cortical dysplasia.**
**[a]Patients with HS were significantly older than MRI- patients ($p$ = 0.037); [b]There was no difference across variables;**
**[c]Epilepsy duration in HS patients was significantly longer than in MRI- patients ($p$ = 0.004) and FCD patients ($p$ = 0.008).**

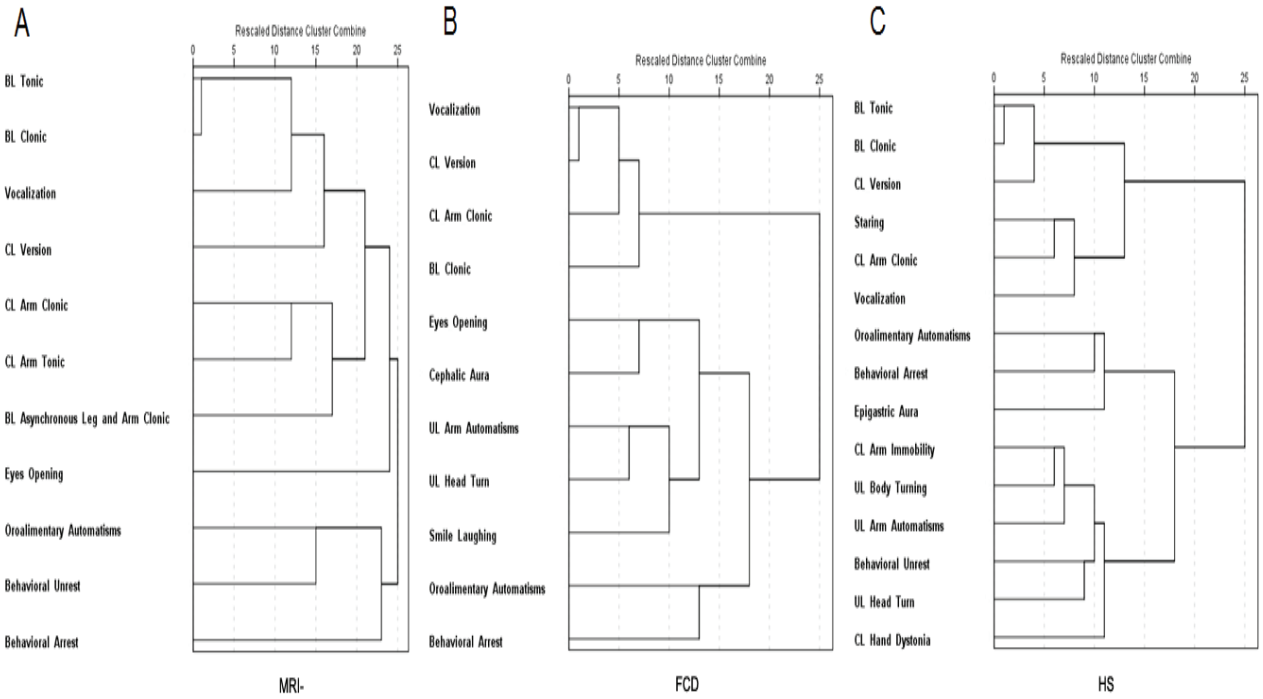


**Fig. 2 – Cluster analysis results in analyzed groups.**
**MRI- – magnetic resonance imaging negative; FCD – focal cortical dysplasia; HS – hippocampal sclerosis; BL – bilateral; CL – contralateral; UL – unilateral.**
**Number of seizures in which these symptoms and signs clustered with one another is indicated by vertical lines (also shown on X axis). Cluster analysis results in: (A) the MRI- group, (B) the FCD group, and (C) the HS group.**

## Discussion

For seizure expression the site of etiological lesion is the most critical even if its electroclinical presentation appears to be remote [7]. However, semiology and EEG findings remain major objective measures that help delineate the epileptogenic zone in pharmacoresistant focal epilepsy patients with normal MRI findings. In this study we contrasted overall electroclinical features of MRI- patients to the MRI+ group and its two distinctive representations – patients with HS and FCD. Although several studies analyzed different aspects of MRI- patients [1, 3, 8, 9], we focused on electroclinical characteristics of patients with focal epilepsy and negative MRI findings.

Our MRI- patients, to a certain extent, have similar features seen in frontal lobe epilepsy (FLE). We found a common presence of sGTC seizures as an exclusive seizure type in MRI- patients. A very high percentage of sGTC seizures

(50%–90%) was reported in studies of frontal lobe epilepsy [10–13]. Furthermore, single photon emission computed tomography (SPECT) images of GTC seizure during electroconvulsive therapy revealed the greatest signal increase in the frontal and parietal cortices [14]. Furthermore, MRI- patients exhibit a significant absence of the interictal epileptiform abnormalities. This could be due to the generation of spikes in deep extratemporal cerebral tissue (midline or supraorbital frontal cortices) as discussed in a previous report [15]. Finally, our MRI- patients, compared to MRI+ patients, had different ictal EEG onset localization, i.e. MRI- patients had more common ictal onset zone in the frontal or frontocentral region.

Some clinical features commonly occurred in MRI- patients. Eye opening as an early sign occurs in nocturnal seizures typically associated with frontal lobe epilepsy [16]. Similarly, hypermotor seizures, and ictal vocalization are a common consequence of the symptomatogenic zone activity in different frontal lobe regions [17–18]. Still, the significance of contralateral body turning and bilateral asymmetric clonic seizure in MRI- patients is not clear. Bilateral asymmetric clonic seizure corresponds to the pretonic phase of GTC seizure. GTC seizures whose clinical heterogeneity suggest that full expression is less common than fragmentary events [6]. Therefore, bilateral asymmetric clonic seizure may represent a distinctive phase of the GTC seizure in MRI- patients with uncertain localizing value.

In our cohort. the proportion of MRI- patients and distribution of detected cortical etiology in the MRI+ group (highest prevalence of HS) are comparable with earlier findings [2, 19.] Therefore, signs commonly present in the MRI+ group correspond to those habitually seen in mesial temporal lobe epilepsy. Similarly, our results of the two-sequential signs that appeared anywhere in the seizures, are those that appear in temporal lobe epilepsy patients [20, 21].

In order to better understand the clinical characteristics of our MRI- patients, we compared semiological differences with the HS and FCD subgroups, which are the most common pathologies seen in large cohorts [19]. Patients in the MRI- group significantly differed from HS patients. This finding is consistent with the hypothesis that MRI- patients most commonly do not have mesial temporal lobe epilepsy. Interestingly, FCD patients share similar differences from HS patients as MRI- patients. In contrast, the MRI- and FCD group distinction is not that noticeable.

Cluster analysis indicates two major clusters of signs in the MRI- and FCD groups, and three major clusters of signs in the HS group. One cluster is identical in all analyzed groups, with-signs depicting sGTC seizure. It is worth noting that the secondary GTC seizure subcluster is the most complex in the MRI- group. The HS group showed a distinct cluster of signs (epigastric aura, oroalimentary automatisms and behavioural arrest, or contralateral hand immobility/dystonia, unilateral arm automatisms, unilateral head and body turning), well described as typical in mesial temporal lobe epilepsy [4]. Although the MRI- and FCD groups shared some common signs in the remaining subcluster, these were not equally distributed. This finding can be attributed to the limitation of the cluster analysis dendogram presentation; rearranging the ordering of symptoms in a dendogram can to some degree be feasible without having an effect on the meaning of the diagram [5].

## Conclusion

Our results indicate that MRI- patients present some electroclinical features seen commonly in frontal lobe epilepsy. Further, some of the clinical characteristics of MRI- patients resemble those reported in epilepsy associated with FCD. It seems possible that higher-resolution MRI (i.e. higher field-strength magnet with thinner slices) may increase the chances to detect small frontal lobe lesions not seen in our scanning protocol. However, a well-defined clinical syndrome in the MRI- group remains to be identified. Our study improves knowledge about focal MRI- epilepsy. Further research comparing MRI- epilepsy and larger sample of FCD patients, should establish the presence of a clinically distinct entity in focal epilepsy patients with no epileptogenic lesion.

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# Assessment of age-related influences on the quality of life of breast cancer patients before and after surgical treatment

## Procena uticaja životnog doba na kvalitet života bolesnica sa rakom dojke pre i posle hirurškog lečenja

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**Abstract**

**Background/Aim.** Breast cancer comprises about 25% of all female cancers, and its incidence is increasing. New diagnostic procedures and therapeutic modalities have increased treatment success rates as well as patient survival. The goal of contemporary treatment is not only patient survival, but also a better quality of life (QoL). The objective of this study was to assess the effect of age at diagnosis on the QoL of patients with breast cancer before and after surgery. **Methods**. We analyzed QoL in 170 female patients (43 patients < 50 and 127 patients ≥ 50 years) diagnosed with breast cancer (I and II stage) a month before and after surgical treatment, using the European Organisation for Research and Treatment of Cancer (EORTC QLQ-C30) questionnaire and specific version for breast cancer patients (EORTC QLQ-BR23). **Results.** The QLQ-C30 questionnaire showed that surgical treatment significantly decreased all domains of the patients' QoL in both age groups. Age-related differences were present in sexual functioning and pleasure independently of surgical treatment, with higher scores in the group of younger women. The analysis of data obtained using the QLQ-BR23 questionnaire revealed a lower QoL after surgical treatment in almost all dimensions regardless of patients' age. **Conclusion.** The results of our study pointed out statistically significant differences in the QoL domains of sexual functioning, and sexual enjoyment between women in both age groups independently of surgical treatment. The QoL was better in the younger age group. Surgical breast cancer treatment negatively affected patients QoL independently of age.

**Key words:**
**breast neoplasms; quality of life; age groups; surgical procedures, operative; surveys and questionnaires.**

**Apstrakt**

**Uvod/Cilj.** Rak dojke čini oko 25% svih malignih tumora žena, sa incidencom u porastu. Novije dijagnostičke procedure i terapijski modaliteti lečenja rezultiraju uspešnijim lečenjem i većom stopom preživljavanja žena obolelih od raka dojke. Cilj savremenog lečenja podrazumeva, ne samo bolje preživljavanje, već i bolji kvalitet života. Cilj ove studije bio je da se proceni kvalitet života bolesnica obolelih od karcinoma dojke pre i posle hirurške intervencije u zavisnosti od njihovog životnog doba pri postavljanju dijagnoze. **Metode.** Analizirali smo kvalitet života 170 bolesnica sa karcinomom dojke (43 starosti do 50 godina i 127 starosti 50 i više godina) u I i II kliničkom stadijumu bolesti, mesec dana pre i nakon hirurškog lečenja, primenom uputnika Evropske organizacije za istraživanje i lečenje karcinoma (EORTC QLQ-C30) i specifične verzije upitnika za karcinom dojke (EORTC QLQ-BR23). **Rezultati.** Analizom upitnika QLQ-C30 pokazano je da je hirurško lečenje značajno negativno uticalo na kvalitet života bolesnica u obe starosne grupe. Nezavisno od hirurškog lečenja, između bolesnica mlađih i starijih postojale su razlike u seksualnom funkcionisanju i seksualnom uživanju, sa višim skorovima u grupi mlađih žena. Analiza uticaja hirurškog lečenja na kva-

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litet života, primenom upitnika QLQ-BR23, pokazala je statistički značajno pogoršanje kvaliteta života nakon hirurškog lečenja u skoro svim domenima kod bolesnica obe starosne grupe. **Zaključak.** Rezultati našeg istraživanja ukazali su na statistički značajne razlike u kvalitetu života između žena obe starosne grupe nezavisno od hirurškog lečenja i to u domenima seksualnog funkcionisanja i uživanja. Pritom je bolji kvalitet života bio u grupi žena mlađe životne dobi. Hirurško lečenje bolesnice sa karcinomom dojke negativno je uticalo na kvalitet života, nezavisno od starosnog doba.

**Ključne reči:**
**dojka, neoplazme; kvalitet života; životno doba; hirurgija, operativne procedure; ankete i upitnici.**

## Introduction

Breast cancer makes up approximately 25% of all cancers in the female population in Europe and 28% in the most developed European countries with mortality rate of 14%–15%. In Serbian females, 26% of all cancer diagnoses and 17.5% of all cancer deaths are due to breast cancer [1]. Globally, the incidence of breast cancer has been continuously rising by 3.1% *per* year over the last 30 years, while its mortality has varied [1]. The age-standardized incidence rate of breast cancer, adjusted to the world population, in central Serbia in 2015 was 61.0 *per* 100,000 population, while the age-standardized breast cancer mortality rate, adjusted to the world population, was 19.8 *per* 100,000 [2].

Most breast cancers (85%) are diagnosed early (stage I or II), a smaller number with locally progressive disease (stage III), and the fewest initial diagnoses are performed when the disease has already significantly progressed and is already the stage IV disease. Breast cancer therapy and treatment depend on the clinical stage of the cancer and on individual disease characteristics, comorbidities, as well as the patient's overall state [1].

Appropriate surgical intervention, followed by radiotherapy, chemotherapy, endocrinological and biological therapy dramatically increase the survival rates of breast cancer patients. The 5-year survival rate for patients diagnosed with the stage I or II disease is 92% [3]. New diagnostic procedures and therapies have lead to an increased number of women undergoing successful breast cancer therapy and improved overall survival rates [4].

Current concepts in breast cancer therapy take into account not only disease progression, but also the patient's quality of life (QoL). The concept of QoL has a broader connotation than the concept of health, and it also includes living and material stipulations. It is defined as the patient's total welfare. Its goal is for the patient to live their life with satisfaction and with the ability to attain personal goals [5]. QoL includes cognitive and emotional perceptions regarding oneself and their environment. There is no single definition of QoL. However, it is generally accepted that it implies the functional status of the individual, as well as a positive feeling of wellbeing [6].

For cancer patients, QoL is a complex and multidimensional concept which depends upon physical, psychological, social and sexual factors [6, 7]. Fundamental health dependent QoL dimensions include physical factors, disease and treatment dependent symptoms, psychological factors (emotional and cognitive states), as well as the patient's social milieu and interactions. Social support is generally defined as information, advice and/or assistance *via* contact with a social network which has a beneficial effect for the individual in question [6, 8, 9].

Available literature suggests points to significant psychosocial stress that patients experience following a breast cancer diagnosis [10]. If breast cancer is diagnosed in an early clinical stage, the initial treatment is mastectomy or breast-conserving therapy with or without axillary lymph node dissection. Subsequent treatment is dependent upon the patient's histopathological results. Irrespective of treatment modality (radiotherapy, chemotherapy, hormone or biological therapy), all patients endure a period of fear, anxiety and a degree of depression, all of which influence QoL.

The normal aging process is accompanied with QoL changes. Data suggests that younger breast cancer patients experience greater QoL changes than their older counterparts [11]. Age-related differences in the psychological reactions to breast cancer and its therapy can be observed between younger breast cancer patients (under 50 years of age) and their older counterparts (50 years of age or older). The objective of our study was to assess the breast cancer patients' QoL before and after surgical treatment.

## Methods

The study was carried out at the Oncology Clinic of the Clinical Hospital Center "Bežanijska Kosa" in Belgrade, Serbia. From 2017 to 2018, 170 newly diagnosed early stage breast cancer patients were included in the study. The study and informed consent form were approved by the Ethics Committee of the Clinical Hospital Center "Bežanijska Kosa" (approval number 3441/3 dated May 5, 2017). The study was carried out as *per* the Declaration of Helsinki. All participants signed the informed consent form. All participants were given a questionnaire with instructions as to how to complete the questionnaire. The questionnaires used in the study were in the Serbian language and were approved and certified by the European Organization for Research and Treatment of Cancer (EORTC). The purpose of the study was explained to each patient in an individual interview before the questionnaire was completed. The patients were informed as to the confidentiality of all data obtained as well as their right not to respond either partially or totally.

Inclusion criteria for the study were newly diagnosed breast cancer patients older than 18 years with the operable cancer stage I or II. Patients were divided into two groups, those under the age of 50 (n = 43) and those 50 years and older (n = 127). Exclusion criteria were: locally progressive or metastatic disease, chronic diseases including diabetes,

cardiomyopathy, or coronary disease, as well as pre-diagnosed psychological illness (depression or anxiety).

QoL questionnaires EORTC QLQ-C30 and EORTC QLQ-BR23 were administered after histopathological, clinical and radiological verification of the breast cancer stage I or II, before surgical intervention. The same questionnaires were then readministered one month after surgical treatment. In addition to the QoL questionnaires, the participants also filled out a socio-demographic background questionnaire.

The EORTC created a cancer specific questionnaire, QLQ-C30, which is composed of 30 questions designed to measure cancer patients' QoL. The QLQ-C30, version 3.0 is composed of nine multi-item scales and six single-item measures. These include a global health status/QoL scale, five functional scales (physical, role, emotional, cognitive, and social functioning), three symptom scales (fatigue, pain, and nausea and vomiting), and six single items (dyspnea, insomnia, appetite loss, constipation, diarrhea and financial difficulties) [12].

The EORTC has also created the QLQ-BR23, a 23 question breast cancer specific QoL questionnaire. The QLQ-BR23 measures QoL in various breast cancer stages as well as varying therapeutic modalities. It measures five domains: body image, sexuality, shoulder-related symptoms, breast cancer symptoms and therapeutic side effects.

The analysis of the answers to both questionnaires were performed as per the EORTC QLQ-C30 Scoring Manual [12–14]. All of the scales and single-item measures range from 0 to 100 scores. A high scale score represents a higher response level. Thus, a high score for the global health status represents a high QoL, a high score for a functional scale represents a high or healthy level of functioning, but a high score for a symptom scale or item represents a high level of symptomatology/problems.

*Statistical analysis*

Results are presented as count (%) or means ± standard deviation (SD) depending on data type. Groups are compared using parametric (Student's *t*-test) and nonparametric (Mann-Whitney *U* test) tests. Within group comparisons were performed using the Student's *t*-test and the Wilcoxon Signed Ranks test. All $p$ values < 0.05 were considered significant ($p < 0.05$). All data were analyzed using SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, IBM Corp.).

## Results

Total of 170 female patients with diagnosis of breast cancer participated in the study. Most of the patients (51.2%), were 50–69 years old, cohabitated with their partners (58.8%), had children (84.1%), and middle or higher education (90.0%), lived in an urban setting (73.5%), and were religious (85.3%). Less than half (39.4%) of the patients were actively employed. Stage IA breast cancer was the most frequent diagnosis (31.8%), breast conserving therapy was performed in 65.9% of patients, and axillary lymph node dissection was performed in 38.2% of them.

For the purpose of this study patients were divided into two groups: those under the age of 50 (n = 43) and those 50 years or older (n = 127). Sociodemographic and clinical characteristics of patients were shown in Table 1.

There were statistically significant differences between age groups in marital status ($p = 0.041$), number of children ($p = 0.043$), education level ($p = 0.028$), employment status ($p < 0.001$) and salary ($p = 0.003$), but not in clinical features (Table 1).

Both younger and older patients experienced a decline in their QoL after undergoing surgery. This effect was present in every QoL dimension. However, when comparing two age groups before and after surgery, the only significant difference was in pre-surgery cognitive functioning domain. The better cognitive functioning was in younger women. This difference was no longer observed after surgery (Table 2).

QoL assessment showed that surgical treatment was significantly associated with financial difficulties and following disease symptoms: fatigue, pain and insomnia, and a loss of appetite and diarrhea in older women. Financial difficulties and above mentioned symptoms were more prominent after surgical treatment. Analysis of the disease symptoms did not show age-related differences (Table 3).

Analysis of the association between surgical treatment of breast cancer and QoL by use of the QLQ BR-23 showed statistically significant pre- and post-surgery differences in all categories except emotional reactions to hair loss. After surgical treatment all categories of QoL were in decline (Table 4).

Statistically significant differences were found in the body image perception and future perspective in all patients with the above-mentioned dimensions being more expressive in the older age group. Additionally, significant differences were found between age groups with regard to sexual functioning and sexual enjoyment, which were better in younger women. It is notable that these differences were significant both before and after surgery. Furthermore, there was a difference between two age groups with respect to their future perspective (only after surgery), with higher score for older women and emotional reaction to hair loss (only before surgery) which was higher in the younger women.

## Discussion

The age at diagnosis, therapeutic modality, as well as therapeutic sequelae all contribute to breast cancer patient's QoL changes [11, 15]. In this study we analyzed QoL of 170 early stage breast cancer female patients surgically treated, before and after the treatment. The results of our analysis showed that surgical treatment lead to decline of QoL across all categories regardless of age. However, there was no significant age-related differences in QoL changes after surgery in terms of patients' global health status/QoL, physical functioning, role functioning, or emotional and social functioning. The only observed difference between younger and older patients was in their pre-surgical cognitive functioning. Due to differences between examined groups regarding their sociodemographic characteristics (marital status, education, employment status, salaries), there is possibility that these variables are confounding factors in relationship between patients'age and QoL.

**Table 1**

**Sociodemographic and clinical characteristics of the study population**

| Parameters | Age (years) | | | | *p*-value* |
|---|---|---|---|---|---|
| | < 50 (n = 43) | | ≥ 50 (n = 127) | | |
| | n | % | n | % | |
| Marital status | | | | | |
| married/living with partner | 31 | (72.1) | 69 | (54.3) | 0.041 |
| †living alone | 12 | (27.9) | 58 | (45.7) | |
| Number of children | | | | | |
| 0 | 12 | (27.9) | 15 | (11.8) | |
| 1–2 | 23 | (53.5) | 86 | (67.7) | 0.043 |
| 3+ | 8 | (18.6) | 26 | (20.5) | |
| Education | | | | | |
| none or elementary school | 2 | (4.7) | 15 | (11.8) | |
| middle school | 20 | (46.5) | 77 | (60.6) | 0.028 |
| college/university | 21 | (48.8) | 35 | (27.6) | |
| Place of residence | | | | | |
| urban | 28 | (65.1) | 97 | (76.4) | 0.148 |
| countryside | 15 | (34.9%) | 30 | (23.6) | |
| Employment status | | | | | |
| employed | 30 | (69.8) | 37 | (29.1) | |
| unemployed | 12 | (27.9) | 16 | (12.6) | < 0.001 |
| retired | 1 | (2.3) | 74 | (58.3) | |
| Salary/pension | | | | | |
| lower | 21 | (48.8) | 93 | (73.2) | 0.003 |
| higher | 22 | (51.2) | 34 | (26.8) | |
| Religion | | | | | |
| religious | 39 | (90.7) | 106 | (83.5) | 0.247 |
| atheist | 4 | (9.3) | 21 | (16.5) | |
| Disease stage | | | | | |
| IA | 14 | (32.6) | 0 | (31.5) | |
| IB | 5 | (11.6) | 11 | (8.7) | 0.867 |
| IIA | 13 | (30.2) | 36 | (28.3) | |
| IIB | 11 | (25.6) | 40 | (31.5) | |
| Surgical intervention | | | | | |
| mastectomy | 13 | (30.2) | 45 | (35.4) | 0.534 |
| breast-conserving | 30 | (69.8%) | 82 | (64.6) | |
| Axillary lymph node dissection | | | | | |
| yes | 18 | (41.9) | 47 | (37.0) | 0.571 |
| no | 25 | (58.1) | 80 | (63.0) | |
| Lymph node metastasis | | | | | |
| no | 31 | (2.1) | 87 | (68.5) | 0.659 |
| yes | 12 | (7.9) | 40 | (31.5) | |

***p*-value between age groups; †living alone: divorced, widowed and unmarried.**

**Table 2**
**Age-related differences in quality of life of breast cancer patients, analyzed using the QLQ-C30, before and after surgical intervention**

| Questionnaire domain | Age (years) | | *p-value |
|---|---|---|---|
| | < 50 (n = 43) | ≥ 50 (n = 127) | |
| Global health status | | | |
| pre-surgery | 73.45 ± 19.27 | 67.32 ± 21.12 | 0.137 |
| post-surgery | 61.63 ± 20.10 | 62.01 ± 19.55 | 0.949 |
| †*p*-value | **< 0.001** | **0.006** | |
| Physical functioning | | | |
| pre-surgery | 90.339 ± 11.39 | 83.67 ± 18.78 | 0.103 |
| post-surgery | 81.24 ± 16.73 | 76.54 ± 18.74 | 0.134 |
| †*p*-value | **< 0.001** | **< 0.001** | |
| Role functioning | | | |
| pre-surgery | 86.05 ± 19.56 | 82.55 ± 22.99 | 0.446 |
| post-surgery | 72.87 ± 20.26 | 70.47 ± 23.02 | 0.645 |
| †*p*-value | **< 0.001** | **< 0.001** | |
| Emotional functioning | | | |
| pre-surgery | 63.18 ± 20.99 | 70.01 ± 21.98 | 0.080 |
| post-surgery | 51.74 ± 24.64 | 56.10 ± 27.45 | 0.544 |
| †*p*-value | **0.013** | **< 0.001** | |
| Cognitive functioning | | | |
| pre-surgery | 86.82 ± 18.39 | 80.18 ± 19.89 | **0.034** |
| post-surgery | 66.28 ± 24.80 | 67.72 ± 25.95 | 0.737 |
| †*p*-value | **< 0.001** | **< 0.001** | |
| Social functioning | | | |
| pre-surgery | 79.07 ± 23.60 | 83.20 ± 23.60 | 0.248 |
| post-surgery | 61.24 ± 24.05 | 70.21 ± 22.97 | 0.063 |
| †*p*-value | **< 0.001** | **< 0.001** | |

**Results are given as mean ± standard deviation.**
**QLQ-C – Quality of Life Questionnaire-Cancer specific.**
***p*-value between age groups; †*p*-value before and after surgery in the same age group.**

**Table 3**
**Age-related differences in disease symptoms of breast cancer patients, analyzed using QLQ-C30, before and after surgical intervention**

| Symptom | Age | | *p-value |
|---|---|---|---|
| | < 50 (n = 43) | ≥ 50 (n = 127) | |
| Fatigue | | | |
| pre-surgery | 22.22 ± 17.82 | 24.32 ± 20.61 | 0.678 |
| post-surgery | 31.52 ± 17.81 | 33.59 ± 18.86 | 0.649 |
| †*p*-value | 0.002 | < 0.001 | |
| Nausea and vomiting | | | |
| pre-surgery | 3.88 ± 10.18 | 5.64 ± 14.12 | 0.707 |
| post-surgery | 4.26 ± 10.97 | 3.54 ± 11.04 | 0.548 |
| †*p*-value | 0.951 | 0.101 | |
| Pain | 12.02 ± 18.30 | 14.17 ± 20.37 | 0.619 |
| Pre-surgery | 30.62 ± 19.56 | 28.21 ± 20.63 | 0.436 |
| Post-surgery | | | |
| †*p*-value | < 0.001 | < 0.001 | |
| Dyspnea | | | |
| pre-surgery | 3.10 ± 9.80 | 4.20 ± 16.26 | 0.816 |
| post-surgery | 3.88 ± 16.60 | 5.25 ± 17.53 | 0.531 |
| †*p*-value | 1.000 | 0.303 | |
| Insomnia | | | |
| pre-surgery | 20.93 ± 24.15 | 26.25 ± 27.74 | 0.312 |
| post-surgery | 34.11 ± 29.54 | 33.60 ± 27.70 | 0.950 |
| †*p*-value | 0.012 | 0.001 | |

**Table 3** ***(continued)***

| Symptom | Age | | *$p$-value |
|---|---|---|---|
| | < 50 (n = 43) | 50 ≥ (n = 127) | |
| Appetite loss | | | |
| pre-surgery | 14.73 ± 23.35 | 13.65 ± 23.89 | 0.650 |
| ost-surgery | 17.05 ± 22.27 | 20.47 ± 27.87 | 0.733 |
| †$p$-value | 0.495 | 0.002 | |
| Constipation | | | |
| pre-surgery | 6.20 ± 19.59 | 11.81 ± 23.20 | 0.084 |
| post-surgery | 7.75 ± 20.36 | 10.50 ± 20.01 | 0.282 |
| $p$-value | 0.589 | 0.429 | |
| Diarrhea | | | |
| pre-surgery | 6.98 ± 17.15 | 5.77 ± 13.99 | 0.876 |
| post-surgery | 1.55 ± 7.10 | 2.10 ± 8.13 | 0.692 |
| †$p$-value | 0.053 | 0.006 | |
| Financial difficulties | | | |
| pre-surgery | 19.38 ± 29.31 | 15.75 ± 26.17 | 0.448 |
| post-surgery | 31.78 ± 33.30 | 21.00 ± 26.83 | 0.057 |
| †$p$-value | 0.007 | 0.024 | |

**Results are given as mean ± standard deviation.**
**QLQ-C – Quality of Life Questionnaire-Cancer specific.**
***$p$-value between age groups; †$p$-value before and after surgery in the same age group.**

**Table 4**
**Age-related differences in quality of life of breast cancer patients, analyzed using QLQ-BR23, before and after surgery intervention**

| Parameters | Age (years) | | *$p$-value |
|---|---|---|---|
| | < 50 (n = 43) | ≥ 50 (n = 127) | |
| Body image functioning | | | |
| pre-surgery | 83.92 ± 21.00 | 88.91 ± 18.46 | 0.227 |
| post-surgery | 55.43 ± 26.35 | 67.52 ± 27.09 | 0.011 |
| †$p$-value | < 0.001 | < 0.001 | |
| Sexual functioning | | | |
| pre-surgery | 39.15 ± 28.38 | 13.91 ± 18.87 | < 0.001 |
| post-surgery | 27.13 ± 24.94 | 8.53 ± 14.37 | < 0.001 |
| †$p$-value | 0.006 | < 0.001 | |
| Sexual enjoyment | | | |
| pre-surgery | 42.64 ± 38.71 | 16.54 ± 25.15 | < 0.001 |
| post-surgery | 24.03 ± 31.98 | 6.82 ± 16.97 | < 0.001 |
| †$p$-value | 0.002 | < 0.001 | |
| Future perspective | | | |
| pre-surgery | 44.19 ± 29.75 | 49.61 ± 32.22 | 0.320 |
| post-surgery | 24.81 ± 30.07 | 39.37 ± 33.97 | 0.013 |
| †$p$-value | 0.003 | 0.006 | |
| Breast symptoms | | | |
| pre-surgery | 11.24 ± 11.19 | 10.96 ± 14.19 | 0.437 |
| post-surgery | 23.84 ± 13.68 | 25.26 ± 16.33 | 0.452 |
| †$p$-value† | < 0.001 | < 0.001 | |
| Arm symptoms | | | |
| pre-surgery | 9.30 ± 14.93 | 9.71 ± 16.26 | 0.951 |
| post-surgery | 27.91 ± 14.42 | 22.75 ± 15.95 | 0.065 |
| †$p$-value | < 0.001 | < 0.001 | |
| Upset by hair loss | | | |
| pre-surgery | 15.50 ± 28.50 | 5.77 ± 17.36 | 0.011 |
| post-surgery | 10.85 ± 24.90 | 5.51 ± 15.58 | 0.208 |
| †$p$-value | 0.15 | 0.929 | |

**Results are given as mean ± standard deviation.**
**QLQ-BR – Quality of Life Questionnaire-Breast specific.**
***$p$-value between age groups; †$p$-value before and after surgery in the same age group.**

Gavric [6] analyzed the influence of breast cancer on patients' QoL in 161 women with newly diagnosed breast cancer and compared the results with an age matched segment of the general population (n = 949). Using the QLQ-C30 questionnaire at the time of diagnosis, as well as three and 12 months postoperatively, she showed that breast cancer patients had a significantly lower QoL in term of emotional, cognitive and social functioning ($p < 0.01$) than age-matched controls.

Most QoL studies of breast cancer patients have shown that younger age was a risk factor for lower QoL as well as for significant stress after treatment [15–19]. On the other hand, it has been observed that there was an improvement in the perceived QoL in all domains regardless of therapeutic modality. Bantema-Joppe et al. [11] studied the influence of age on breast cancer patients' QoL after radiotherapy. The average follow-up time was 34 months (range 6–70 months), and results were compared to age-matched controls in the general population in Holland. They showed an improvement in QoL which was greatest after the initial stress of cancer diagnosis and the first treatment phase. A greater improvement was observed in younger patients, a fact that was attributed to a better overall physical state, as well as their superior capacity for recovery. Hau et al. [18] performed a 12-year follow-up study on a cohort of women with an average age of 49.8 years at the time of breast cancer diagnosis. The patients showed a dramatic improvement in all QoL dimensions over the follow-up period, to the point that 12 years after breast cancer treatment no QoL differences were observed in terms of overall QoL, physical or emotional functioning, or fatigue [18].

In this study, no age-related differences in the symptoms were noted after surgery. Furthermore, surgery was associated with physical and psychological changes in all patients. Goldstein et al. [20] studied breast-cancer treatment associated fatigue in 218 patients and concluded that fatigue was a common symptom and generally a call for help. On the basis of multivariate analysis, they suggested that patient age was no longer a fatigue-related risk factor six months after the completion of adjuvant treatment [20].

Our results from the QLQ-BR23 questionnaire showed that surgical treatment changed patients' QoL in all categories except emotional reactions to hair loss (after surgical treatment all categories declined), that can be explained by the stress of a diagnosis of breast cancer. The extent of the surgical intervention, whether complete mastectomy or breast-conserving therapy, has a negative effect on body image, sexual relations, and social activities, and it can be said that a breast cancer diagnosis represents one predictor of depression [21]. As society favors physical appearance and attractiveness, it is generally accepted that surgical intervention leads to an impaired QoL, including social interactions [22–24]. Psychological effects can have an influence on breast cancer patients' day-to-day lives and can affect their social and vocational interactions. Additionally, marital status, support of family and friends, education, vocation, as well as financial situation all influence a patient's QoL.

Avis et al.[25] pointed out that there is a lack of information regarding the emotional, social and psychological repercussions on younger patients who face a breast cancer diagnosis. They followed 202 women diagnosed with stages I to III breast cancer at age 50 or younger from 4–42 months after diagnosis and identified factors that impact their QoL. Factors which were found to negatively impact women's ability to deal with breast cancer diagnosis included the relationship with a partner, sexual functioning and body image issues. A poor relationship with a partner, especially poor communication, was negatively associated with nearly all QoL dimensions [25].

The importance of stress management strategies has been the focus of numerous breast cancer studies [10, 26]. Younger patients who have never been faced with a potentially life-threatening illness were found to require assistance to develop effective strategies to handle the psychological consequences of their diagnosis. Kerr et al. [27] noted that patients younger than 50 years of age required more social and psychological assistance than their older counterparts. Younger patients were less satisfied with the information given regarding breast cancer treatment, as well as its effects during and after completion. It was suggested that more information is required for younger age groups [27]. Other studies have confirmed that after a breast cancer diagnosis, younger women need more psychological mechanisms in order to adapt to their new situation, than their older counterparts [28, 29].

Several published studies focused specifically on younger women with breast cancer. Bloom et al.[30] and Allen et al. [31] performed intervention studies among younger women with breast cancer to address their specific psychological needs. The Cancer and Menopause Study (CAMS) [32] confirmed a substantial degree of psychological distress which persisted many years after the initial diagnosis of breast cancer in young women, especially those aged 25–34 at the time of diagnosis. It was also shown that better general health was positively correlated with more than a high level of education, better emotional and psychological functioning, less comorbid conditions and an unchanged menopausal status over the course of treatment. The authors determined that the emotional functioning was of essential importance in the youngest women who faced diagnosis of malignant disease, such as breast cancer. Older women at diagnosis (the oldest group in the study cohort) with more life experience may have developed better emotional resistance and reacted better to a diagnosis of malignant disease. However, older women may have weaker physical resiliency due to underlying comorbidities or greater physical limitations associated with age which limit their tolerance to various treatment modalities [32].

The results of this study showed a statistically significant difference in sexual functioning and enjoyment between younger and older patients. However, surgical treatment was not associated with age-related differences in these QoL aspects. Bantema-Joppe et al. [11] showed that radiotherapy can also be associated with changes in sexual functioning. They analyzed three age groups and found that immediately after radiotherapy the sexual functioning was best in the youngest group and the worst in the oldest group. Contrary to other

QoL dimensions, sexual functioning did not show improvement over time in any age group. The lack of change over time cannot be explained by the use of systemic treatment, resulting in vaginal dryness since the authors controlled for systemic treatment [11].

Ganz et al. [32] used standardized questionnaires to study QoL in 691 breast cancer patients over 65 years of age. They applied the questionnaire three months after surgery and twice more over the course of the next year. Just three months after surgery, their results showed a high degree of physical and emotional functioning. They also showed that chemotherapy by itself in the presence of comorbid conditions had a significant negative impact on the physical functioning of older patients. Due to a higher number of comorbidities, they found that three months after surgery older patients had worse physical functioning regardless of the extent of the surgery or radiotherapy [33]. Their results are in congruence with other studies which examined the physical functioning of older breast cancer patients [34–36]. The results of our study showed that surgical treatment for early-stage breast cancer influenced the changes in physical, emotional, cognitive and social functioning independently of the patients' age.

On the other hand, younger patients who have undergone breast-conserving therapy with radiotherapy or mastectomy experienced a more significant drop in physical functioning than patients who have only undergone breast-conserving therapy. Additionally, breast cancer may be the only illness in younger patients, resulting in a relatively fast recovery of physical functioning in the months following treatment [37]. Ganz et al. [32] noted that in older breast cancer patients, when all factors were considered in the determination of QoL, the extent of surgical intervention had less influence on QoL changes than other factors, such as emotional and social QoL dimensions. However, age is a more significant factor than the extent of surgery on changes in physical, emotional and social dimensions when considering a patient's QoL [33]. In our study, there were no statistically significant differences in physical functioning between older and younger patients before or after surgery. Although, it should be noted that our cohort included only breast cancer patients without preexisting comorbidities.

Our research represents one of the few studies of QoL in women with breast cancer in the Republic of Serbia, which provides insight into the physical and psychological characteristics of this group of patients. However, this is one center study, which has some limitations: a small number of patients, and a relatively short period of readmitting of questionnaires. Another limitation is that we did not include other important variables such as social support, current mood, and fear of recurrence.

## Conclusion

The results of this study point out significant differences between patient age groups in QoL dimensions specific to cancer and breast cancer patients before and after surgery that enhance our knowledge of factors which influence patients' QoL and help us to consider potential possibilities for its improvement.

Surgical breast cancer treatment negatively affected patients' QoL, especially in the physical and psychological domains. These domains are sensitive and require a multidisciplinary treatment approach. There is a need for further research, primarily in the psychological approach of women with breast cancer.

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# Postoperative urinary tract infection after ureteroscopic lithotripsy in patients with asymptomatic bacteriuria

## Postoperativna urinarna infekcija nakon ureteroskopske litotripsije kod bolesnika sa asimptomatskom bakteriurijom

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**Abstract**

**Background/Aim.** Postoperative urinary tract infection (UTI) is one of the most common infective complications of ureteroscopic lithotripsy. Preoperative asymptomatic bacteriuria is not a contraindication for performing ureteroscopic lithotripsy but it can be a significant risk factor for occurrence of severe forms of postoperative urinary infection. **Methods.** From January 2010 until December 2014 at the Urology Clinic of the Military Medical Academy in Belgrade, 389 patients undergoing ureteroscopic lithotripsy were analyzed, and their postoperative infective complications were monitored. From the group, the incidence of posteoperative urinary infection was analysed in 52 patients with preoperative asymptomatic bacteriuria. **Results.** Infective complications occured in 18.7% of patients, and postoperative UTI in 10% of patients. Out of 52 patients with preoperative asymptomatic bacteriuria, 36.5% had postoperative urinary tract infection ($\chi^2 = 46.773$; $p < 0.001$). In these patients, we registered higher frequency of severe forms of postoperative UTI, systemic inflammatory response syndrome (SIRS) and sepsis. **Conclusion**. Preoperative asymptomatic bacteriuria represents a significant risk factor for developing postoperative UTI following ureteroscopic lithotripsy and is associated with increased risk for occurence of severe forms of SIRS and sepsis. It is desirable that every patient with indicated ureteroscopic lithotripsy has sterile urine culture, and if this is impossible to achieve, a special caution and an adequate antibiotic therapy and prophylaxis are needed before and during the surgical procedure.

**Key words:**
**bacteriuria; lithotripsy; ureteroscopy; postoperative complications; urinary tract infections.**

**Apstrakt**

**Uvod/Cilj**. Postoperativna urinarna infekcija jedna je od najčešćih infektivnih komplikacija ureteroskopske litotripsije. Asimptomatska preoperativna bakteriurija nije kontraindikacija za izvođenje ove metode, ali može biti značajan faktor rizika za nastanak teških oblika postopertivne urinarne infekcije. M**etode.** Od januara 2010. do decembra 2014. godine, u Klinici za urologiju Vojnomedicinske akademije u Beogradu, analizirano je 389 bolesnika kod kojih je urađena ureteroskopska litotripsija i praćene su postoperativne infektivne komplikacije. Kod 52 bolesnika iz ove grupe sa preoperativnom asimptomatskom bakteriurijom, analizirana je učestalost postoperativne urinarne infekcije. **Rezultati.** Infektivne komplikacije javile su se kod 18,7% bolesnika, a postoperativnu urinarnu infekciju imalo je 10% bolesnika. Od 52 bolesnika sa asimptomatskom preoperativnom bakteriurijom, postoperativnu urinarnu infekciju imalo je njih 36,5% ($\chi^2 = 46.773$; $p < 0.001$). Kod ovih bolesnika zabeležena je veća učestalost teških oblika postoperativne urinarne infekcije, sindroma sistemskog inflamatornog odgovora (SSIO) i sepse. **Zaključak.** Asimptomatska preoperativna bakterurija predstavlja značajan faktor rizika za pojavu postoperativne urinarne infekcije nakon ureteroskopske litotripsije i udružena je sa povećanim rizikom za nastanak teških oblika SSIO i sepse. Poželjno je da svi bolesnici kod kojih je indikovana ureteroskopska litotripsija imaju sterilnu urinokulturu, a ako je to nemoguće postići, potreban je poseban oprez i adekvatna antibiotska terapija i profilaksa pre i tokom operativne procedure.

**Ključne reči:**
**bakteriurija; litotripsija; ureteroskopija; postoperativne komplikacije; urinarni trakt, infekcije.**

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## Introduction

Ureteroscopic lithotripsy is one the most common surgical methods for treating kidney and ureter stone [1]. The multicentric study of the Endourological Society (*Clinical Research Office of the Endourological Society – CROES*), which was done at 114 hospitals in 32 countries, with 11,885 patients, has found that the incidence of this procedure success, when the patients lose the stone (stone free rate – SFR) is 85.6%, and the incidence of postoperative complications is 3.5% [2].

According to recent studies, the frequency of infective complications following ureteroscopic lithotripsy is between 1.7% and 18.8% [3–5].

Patients with stone in the kidney or ureter often have asymptomatic bacteriuria with values from 10,000 to 100,000 colonies *per* millilitre, but without local and general signs of urinary tract infection (UTI). This condition is explained by existence of bacterial biofilm at the surface of the stone or at the prevoiously placed ureteral JJ stent or at the nephrostomy catheter [6, 7]. Untreated preoperative UTI presents absolute contraindication for performing ureteroscopic lithotripsy [1], while it can be done in patients with asymptomatic bacteriuria with the application of a short term preoperative antibiotic therapy recommended in order to mitigate risks for developing possible infective complications [8].

The aim of this study was to analyze significance of preoperative asymptomatic bacteriuria as a risk factor for development of infective complications following ureteroscopic lithotripsy.

## Methods

The research was done at the Urology Clinic of the Military Medical Academy in Belgrade, Serbia through retrospective analysis of medical records of 389 patients, male and female, who had pneumatic and laser ureteroscopic lithotripsy in the ureter or kidney, by a semirigid and/or a flexible ureteroscope, in the period from January 2010 until December 2014. During preparations for ureteroscopic lithotripsy the detailed anamnesis was taken from all the patients, and physical examination and laboratory tests (erythrocyte sedimentation rate, complete blood analyses, biochemical analyses, miscroscope exam of the urine sediment and urine culture) were done.

In patients who developed infective complications, their gradation was done according to the Modified Clavien Classification System – MCCS. According to the MCCS all infective complications following ureteroscopic lithotripsy in this study were divided into 4 degrees: gradus I – temporary febrile condition not requiring additional treatment besides applying antipyretics; gradus II – postoperative urinary tract infection, non-obstructive pyelonephritis, systemic inflammatory response syndrome (SIRS) or sepsis requiring applying additional antibiotics, and sepsis requiring also inotropic drugs; gradus III – obstructive sepsis requiring applying additional endoscopic procedures and multi- pharmacological treatment, and gradus IV – severe sepsis (IVa) and septic shock (IVб) requiring staying and treating patients in an intensive care unit [9].

The criteria of the International Conference for Sepsis and Organs Collapsing and Guidelines for Using Inovative Therapies in Sepsis of the American College of Chest Physicians and the Society of Critical Care, established in 1992, where sepsis is defined as presence and verification of infection source and SIRS, were used in this study. The existence of two or more criteria is characteristic for SIRS: body temperature > 38 ºC or < 36ºC; heart rhythm > 90 beats/min; respiration rate number > 12/min or partial pressure of $CO_2$ < 32 mmHg; leukocytosis > 12,000/mm$^3$ or < 4,000/mm$^3$. Organ dysfunction is characteristic for severe sepsis and acute circulatory collapse with persistent arterial hypotension is characteristic for septic shock [10, 11].

The examinees were classified into two groups: the first group – patients with preoperative asymptomatic bacteriuria subjected to the pneumatic or laser ureteroscopic lithotripsy, who did not have postoperative urinary infection, and the second group – patients with preoperative asymptomatic bacteriuria subjected to the pneumatic or laser ureteroscopic lithotripsy who had postoperative urinary infection.

All data in the study were processed in SPSS 20.0 (IBM corporation) software package. The chosen level of significance, i.e. possibility of the first type mistake was 0.05.

## Results

The study comprised 389 patients, 200 (51.4%) male and 189 (48.6%) female, with unilateral ureteral or kidney calculosis (one or more stones in clearly defined levels of the upper urinary tract in which the stone was located: kidney, upper ureter, middle ureter and lower ureter), subjected to ureteroscopic lithotripsy by a semirigid and/or a flexible instrument. Patients without preoperative urinary tract infection were analysed.

Average age of the patients in this study was 55. The youngest patient was 13 and the oldest one 92. Average body mass index (BMI) of the patients was 26 kg/m$^2$ (minimum 14 and maximum 37 kg/ m$^2$). Average size of stones was 13 mm, and in 94 (24.2%) patients lithotripsy was performed in the kidney and in 295 (75.8%) patients in the ureter. Laser lithotripsy was performed in 237 (60.9%) patients, and breaking by a pneumatic probe in 152 (39.0%) patients. Average duration of surgery was 40 minutes, the shortest one lasted 5 minutes, the longest one 185 minutes. A semirigid ureteroscope was used in 357 (91.7%) patients, a flexible one in 28 (7.2%), and in 4 (1.1%) patients the both types of ureteroscope were used.

Infective complications in this study developed in 73 (18.7%) patients, and postoperative urinary tract infection in 39 (10%) patients. Temporary febrile condition not requiring additional treatment besides applying antipyretics occured in 34 (8.7%) patients.

Postoperative urinary tract infection, according to definitions of the Section of Infection in Urology of the European Association of Urology [11] and the International Conference for Sepsis and Organ Collapsing and the Guidelines for

Using Inovative Therapies in Sepsis of the American Chest Physicians and the Society of Critical Care [10], had 39 (10%) patients. In these patients the treatment implied using antipyretics, additional antibiotic therapy, additional infusion of inotropic and supportive therapy, and in 2 (0.4%) patients additional procedures were performed – placing JJ stent and percutaneous nephrostomy catheter. In Table 1 the incidence of infective complications and postoperative urinary infections and the treatment method are shown.

Out of total number of patients, 52 (13.4%) were with preoperative asymptomatic bacteriuria, while 337 (86.6%) patients had sterile urine culture. In the group of patients without postoperative urinary infection, in 33 (63.5%), asymptomatic bacteriuria was verified before the surgery, and in the group of patients with postoperative urinary infection, 19 (36.5%) had asymptomatic bacteriuria before the surgery. These data were analysed by $\chi^2$ test which confirmed that there was a statistically highly significant difference between the groups ($\chi^2 = 46.773$; $p < 0.001$).

In 19 patients with postoperative urinary infection, the most common bacterium present in preoperative urine cultures was *Escherichia coli* (52.6%), the second most common one was *Pseudomonas aeruginosa* (21.0%), then mixed bacterial flora with *Proteus mirabilis, Pseudomonas aeruginosa and Enterococcus faecalis* (10.5%), *Pseudomonas aeruginosa and Enterococcus faecalis* (5.3%), *Proteus mirabilis* (5.3%) *and Enterococcus faecalis* (5.3%). In postoperative urine cultures of these patients the following bacteria were isolated: *Escherichia coli* (43.7%), *Klebsiella species* (17.8%), *Pseudomonas aeruginosa* (15.0%), *Enterococcus faecalis* (13.2%) and *Proteus mirabilis* (10.3%).

Out of 7 patients with sepsis in this study, 5 (1.4%) patients were treated at the Urology Department. Out of these 5 patients, 3 (0.9%) patients received antibiotic therapy, infusion solutions and drugs for regulating circulatory collapse and cardiorespiratory dysfunction, but it was not necessary to keep them in the Intensive Care Unit. In all three patients the *Staphylococcus coag.* (-) bacterium was isolated in the hemoculture. In the rest 2 (0.5%) patients treated at the Urology Department, postoperative obstructive pyelonephritis and sepsis were verified and it was necessary to place a JJ stent in one case and a percutaneous nephrostomic catheter in another case besides applying antibiotic therapy.

In 2 (0.4%) patients (out of 7 patients with sepsis), the treatment required monitoring and their staying in the Intensive Care Unit because of circulatory collapse and cardiorespiratory dysfunction, under diagnosis of severe sepsis and septic shock, with intubation and putting on respiratory device and applying several antibiotics, inotropic drugs and infusion of colloid solutions and nutritive solutions simultaneously. In both patients *Escherchia coli* was isolated from the hemoculture, and the same bacterium was found in the preoperative asymptomatic bacteriuria results.

## Discussion

The impact of preoperative asymptomatic bacteriuria on development of urinary tract infection in patients following ureteroscopic lithotripsy was analyzed in this study. Infective complications following ureteroscopic lithotripsy are the most common complications occuring after this procedure [2], which is why it is important to identify patients with a risk for their developing following the procedure. Reasons for secondary postoperative urinary tract infection may also be disseminaton of bacteria from the lower to the upper urinary tract in the course of the procedure, also performing the procedure on an infectious stone and using irigation solution under high pressure which generates bacteriemia [9]. Preoperative antibiotic therapy mitigates the risk of postoperative urinary infection [8].

**Table 1**

**Incidence of infective complications and postoperative urinary infections and the treatment method**

| Complication | Patients n (%) | Treatment |
|---|---|---|
| Gradus I | | |
| Temporary febrile condition | 34 (8.7) | Antipyretics |
| Gradus II | | |
| SIRS | 32 (8.3) | Antibiotic therapy |
| Sepsis | 3(0.9) | Antibiotic therapy<br>Parenteral solution infusion<br>Inotropic drugs |
| Gradus III | | |
| Obstructive sepsis pyelonephritis | 2 (0.4) | Endoscopic intervention<br>Placement of JJ stent or percutaneous nefrostomic catheter |
| Gradus IVa | | |
| Severe sepsis | 1 (0.2) | Intensive Care Unit management |
| Gradus IVb | | |
| Septic shock | 1 (0.2) | Intensive Care Unit management |

**SIRS – systemic inflammatory response syndrome.**

Infective complications following ureteroscopic lithotripsy comprises: temporary febrile condition not requiring applying additional antibiotic therapy, postoperative urinary tract infection, SIRS and sepsis [8, 12, 13]. Temporary febrile condition requires applying only antipyretics and usually passes spontaneously in 24–48 hours, but postoperative urinary tract infections which comprise also severe forms of postoperative urinary infection – SIRS and sepsis, are complications extending time of a patient's hospitalizing, requiring expensive antibiotics therapy, requiring sometimes even additional procedure, significantly rising the price of treatment, and in the case of organ dysfunction are life threatening complications [5, 14]. Even with abiding basic principles in the preoperative preparation of a patient and applying antibiotic prophylaxis in line with recommendations of the Guidelines on Urological Infections – EAU Guidelines, patients frequently get unpredictable severe forms of postoperative urinary tract infections.

The incidence of postoperative urinary infection following ureteroscopic lithotripsy in the study was 10%, and the frequency of all infective complications, taking into account also temporary febrile condition was 18.7%. This incidence in our study is in line with the Japanese authors [12] that analyzed factors associated with occurence of postoperative febrile urinary tract infection following ureteroscopic stone breaking in 153 patients. In their study, the incidenece of all infective complications was 18.3%, and the incidence of postoperative infective complications requiring additional antibiotics and supportive therapy or endoscopic procedures was 7.8% [12]. However, other authors reported lower incidence of infective complications following ureteroscopic procedures. Sohn et al. [13] analyzed 531 patients that were subjected to ureteroscopic procedures from 2002 to 2010, including also ureteroscopic lithotripsy. In that study, the incidence of infective complications was 3.8%. Higher frequency of infective complications in our study can be explained by differences between the groups of patients analyzed and also by different definitions of infective complications and postoperative urinary infections among studies. In our study we analyzed only patients subjected to ureteroscopic lithotripsy, and the Sohn et al. [13] study comprised also 154 (29.0%) patients that were subjected to diagnostic ureteroscopy. In the study of Bloom et al. [14], the infective complications comprised only complications requiring additional antibiotics or other treatment (Gradus ≥ II complications according to the MCCS). In our study, infective complications Gradus ≥ II were defined as postoperative urinary infections and their frequency in the study was 10%. Also, the difference in frequency of infective complications between these two studies may be due to using different types of ureteroscopes [15]. The size of a stone could also have impact on developing postoperative infection, because the average stone size in our study in patients with postoperative urinary infections was 15 mm, which was more than in the studies dealing with complications following ureteroscopy and endourological procedures [5, 16].

An absolute contraindication for performing ureteroscopic lithotripsy was untreated urinary tract infection. Preoperative asymptomatic bacteriuria with positive findings of bacteria in the urine culture, from 10.000 to 100.000 colonies *per* mL, but with no local, general and clinic signs of urinary infection, was not an excluding factor because in a number of patients, sterile urine culture could not be achieved, which is explained by increasing bacterial colonies at the stone surface or at the ureteral JJ stent and on the nephrostomic catheter which the patients had to wear before the surgery [6, 7]. Totally, 52 (13.4%) patients in our study had preoperative asymptomatic bacteriuria and in these patients an antibiotic therapy was applied 1–12 days preoperatively according to the antibiogram results. Most frequently, a third generation cephalosporin, ceftriaxon was used – in 17 (32.7%) patients. In these patients, the most frequently isolated bacterium in preoperative urine cultures was *Escherichia coli* (40.4%). Among 52 patients with preoperative asymptomatic bacteriuria, a postoperative urinary tract infection had 19 (36.5%) of them. In these patients, the most commonly preoperatively used antibiotics were aminoglycosides (amikacin, gentamycin), ie. they were applied in 6 (31.6%) patients, and the most frequently isolated bacterium in preoperative urine cultures was also *Escherichia coli* (52.6%). In our study we found statistically significant difference between the group without and the group with postoperative urinary infection following ureteroscopic lithotipsy in comparison to patients that had asymptomatic bacteriuria and patients with sterile urine culture ($p < 0.001$). Moses et al. [8] in their study examined 16.5% patients that had preoperatively positive urine cultures and received antibiotic therapy 3–7 days preoperatively. Preoperative bacteriuria was examined in other studies as a risk factor following ureteroscopic lithotripsy. Uchida et al. [17] proved, by using multivariate analysis, that positive preoperative findings of urine cultures in patients that were subjected to ureteroscopic laser lithotripsy, were associated with higher risk of postoperative SIRS. In that study, 12.4% patients, subjected to ureteroscopic laser lithotripsy, had positive preoperative findings of urine cultures, but with no signs of urinary tract infection. In those patients, an adequate antibiotic therapy was applied in duration prescribed by an urologist, but the study did not bring forward the therapy duration. It was proved in the study, through a multivariate logistic regression analyses, that positive preoperative urine culture, was a significant risk factor for occurrence of SIRS following laser ureteroscopic lithotripsy ($p = 0.005$). The patients with preoperative asymptomatic bacteriuria, subjected to ureteroscopic lithotripsy, were also analyzed by Sohn et al. [13]. They examined 20.9% patients with preoperative bacteriuria, and 10.8% of these patients had infective complications ($p = 0.000$). Matsumoto et al. [18], in their study also have concluded that preoperative bacteriuria is a statistically significant risk factor for occurrence of infective complications following urologic procedures in the upper urinary tract. Blackmur et al. [15], in their analysis of risk factors for developing sepsis following ureteroscopic lithotripsy, which was done in 462 patients, found that 34 (7.4%) patients had sepsis and that positive preoperative findings of urine cultures were associated with occurrence of postoperative urosepsis, although an ade-

quate antibiotic preoperative therapy was applied ($p < 0.001$). All these studies showed results in line with our analysis. The study proving the opposite was not found in the existing literature.

Out of 19 patients that preoperatively had asymptomatic bacteriuria and developed infective complications following ureteroscopic lithotripsy, 7 (1.8%) patients were treated for severe postoperative urinary infections with signs of sepsis. All these patients preoperatively received an adequate antibiotic prophylaxis. Sepsis following ureteroscopic lithotripsy is one of the most severe complications. In other studies that analyzed risk factor for developing postoperative urinary infection, the frequency of sepsis was also between 1%–3%. Mitsuzuka et al. [12] reported 1.3% patients with sepsis developed after ureteroscopic lithotripsy. In the existing literature only few studies analyzed the frequency of sepsis following ureteroscopic lithotripsy. Geavlete et al. [19] brought forward the data of 1.13% out of 2,735 patients with sepsis occurred following ureteroscopic lithotripsy made by a semirigid ureteroscope. Eswara et al. [20] analyzed 328 patients that were subjected to endourological procedures, out of which 11 (3.0%) had sepsis. However, Blackmur et al. [15] in their analysis of risk factors for developing sepsis after ureteroscopic lithotripsy, in which they examined 462 patients, published data on 34 (7.4%) patients with sepsis. This somewhat larger number of patients with sepsis developed following ureteroscopic lithotripsy in that study was explained by the fact that the study comprised patients with both sides ureteroscopic lithotripsy also and a great number of patients with associated cardiovascular diseases and diabetes, high the American Society of Anesthesiology (ASA) score and larger stones.

Out of 19 patients in our study that had asymptomatic bacteriuria preoperatively and developed postoperative infective complications, the rest 12 (3.1%) patients had postoperative urinary infection and SIRS. Their treatment required applying additional antibiotic therapy according to findings of urine cultures, but not infusion of solutions and supportive therapy, nor additional endoscopic procedures.

From the above mentioned results it can be concluded that patients with asymptomatic bacteriuria preoperatively had also higher frequency of severe forms of urinary infections postoperatively.

Use of standardized system for infective complications classification (MCCS) enabled easier and more precised comparison with the referent studies. This study provided initial results of infective complications following ureteroscopic lithotripsy. A prospective multicenter study should be conducted that would analyze, in addition to preoperative bacteriuria, other risk factors for development of infective complications following ureteroscopic lithotripsy, in order to find ways to prevent these complications.

## Conclusion

In our study, out of all patients with preoperative asymptomatic bacteriuria, 36.5% of the patients had postoperative urinary infection which presented a statistically significant number. It was found a statistically significant difference between the group without and the group with postoperative urinary infection developed following ureteroscopic lithotripsy compared to patients who had asymptomatic bacteruria and patients who had sterile findings of urine culture. This result proves that asymptomatic preoperative bacteriuria is a significant risk factor for developing postoperative urinary infection following ureteroscopic lithotripsy and is associated with higher risk for developing severe forms of SIRS and sepsis. This great risk must be taken into account in an observant preoperative preparation of patients for ureteroscopic lithotripsy. It is desirable that all patients indicated with ureteroscopic lithotripsy have sterile urine culture, but if this is impossible to achieve, a special caution and an adequate antibiotic therapy and prophylaxis are necessary in these patients before and in the course of the operative procedure. Immediate postoperative monitoring is also very important in order to timely prevent severe infective complications.

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# Do nature of bacteremia and origin of secondary sepsis in critically ill patients determine subset of myeloid-derived suppressor cells expansion?

## Da li vrsta bakterija i poreklo sekundarne sepse kod kritično obolelih određuju tip supresorskih ćelija mijeloidnog porekla?

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**Abstract**

**Background/Aim.** Gram-positive and Gram-negative bacteria may induce different inflammatory patterns. The aim of this study was to examine the association of the myeloid-derived suppressor cells (MDSCs) with the type of infecting microorganisms (Gram positive, Gram negative, polymicrobial) and underlying cause of secondary sepsis (peritonitis, pancreatitis, trauma). **Methods.** Totally, 40 critically ill patients with secondary sepsis were enrolled in the prospective study. Two patients without documented positive blood culture were excluded. We detected and enumerated both main subsets of MDSCs: granulocytic (G)-MDSCs and monocytic (M)-MDSCs on the Days 1 and 5. Blood was simultaneously drawn for a blood culture. The patients with different underlying causes of sepsis (peritonitis, pancreatitis, trauma) were perceived as separated groups and the frequencies and absolute numbers of their G-MDSCs and M-MDSCs were compared. **Results.** Both main MDSC subpopulations were accumulated significantly in Gram-positive sepsis. Univariate logistic regression analyses of investigated variables regarding Gram-positive sepsis on the Day 5 revealed that G-MDSCs absolute number along with both M-MDSCs frequency and absolute number had statistically significant power for predicting Gram-positive sepsis. Stepwise multivariate logistic regression analyses of the variables on the Day 5 determined that M-MDSCs absolute number was independent predictor of Gram-positive sepsis [odds ratio (OR) 1.012; $p < 0.05$]. Clinical accuracy of neutrophil (Ne)/G-MDSCs (Ne/G-MDSCs) and monocyte (Mo)/M-MDSCs (Mo/M-MDSCs) ratios in predicting nature of bacteremia and outcome were investigated. Discriminative power of both Ne/G-MDSCs and Mo/M-MDSCs ratios in predicting Gram-positive blood culture was statistically significant both on the Day 1 and Day 5 [areas under curve (AUCs): 0.684 and 0.692, and 0.707 and 0.793, respectively). Ne/G-MDSCs both on the Day 1 and Day 5 were statistically significant predictors of lethal outcome (AUCs: 0.694 and 0.678, respectively). There were no statistically significant differences in G-MDSCs and M-MDSCs among different three groups of patients regarding peritonitis, pancreatitis and trauma as causes of sepsis neither on the Day 1 nor on the Day 5. **Conclusion.** Gram-positive infectious agents were powerful inducers of MDSCs generation in sepsis. Also, underlying causes of secondary sepsis might not seem to influence the MDSCs accumulation.

**Key words:**
**gram-negative bacteria; gram-positive bacteria; critical illness; myeloid-derived suppressor cells; sepsis.**

**Apstrakt**

**Uvod/Cilj.** Gram-pozitivne i Gram-negativne bakterije mogu indukovati različit imunoinflamatorni odgovor. Cilj istraživanja bio je da se utvrdi da li kod kritično obolelih bolesnika sa sekundarnom sepsom postoji povezanost učestalosti i/ili apsolutnih brojeva supresorskih ćelija mijeloidnog porekla (MDSC) sa vrstom bakterijskog prouzrokovača i poreklom sekundarne sepse. **Metode.** Prospektivnom studijom bilo je obuhvaćeno ukupno 40 kritično obolelih bole-

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snika sa sekundarnom sepsom. Dva bolesnika bez dokazanog prisustva bakterija u sistemskoj cirkulaciji bila su isključena iz daljih analiza. Detektovane su i kvantifikovane obe glavne podvrste MDSC: granulocitne (G)-MDSC i monocitne (M)-MDSC) 1. i 5. dana. Istovremeno je uzimana i krv za određivanje hemokultura. **Rezultati.** Utvrdili smo da su obe glavne podvrste koje odgovaraju MDSCs bile značajno akumulirane u Gram-pozitivnoj sepsi. Univarijantna logistička regresiona analiza ispitivanih varijabli pokazala je da su 5. dana apsolutni broj G-MDSC, kao i učestalost i apsolutni broj M-MDSC bili značajni prediktori Gram-pozitivne sepse. Multivarijantna logistička regresiona analiza pokazala je da je 5. dana apsolutni broj M-MDSC bio nezavisni prediktor Gram-pozitivne sepse [*odds ratio* (OR) 1,012; $p < 0,05$]. Odnosi neutrofili (N)/G-MDSC i monociti (M)/M-MDSC bili su značajni prediktori Gram-pozitivne sepse u oba termina [*area under curve* (AUC) 0,684 i 0,692, odnosno 0,707 i 0,793]. Takođe, N/G-MDSC odnos je u oba termina bio značajan prediktor smrtnog ishoda (AUC 0,694, odnosno 0,678). Posmatrajući bolesnike sa različitim poreklom sekundarne sepse (peritonitis, pankreatitis, trauma) kao zasebne grupe, i poređenjem učestalosti i apsolutnog broja G-MDSC i M-MDSC, nisu utvrđene statistički značajne razlike ni prvog ni petog dana. **Zaključak.** Gram-pozitivne bakterije su snažni induktori akumulacije MDSC u sepsi. Takođe, izgleda da poreklo sepse ne utiče na akumulaciju MDSC.

**Ključne reči:**
**gram-negativne bakerije; gram-pozitivne bakterije; kritična stanja; kostna srž, ćelije, supresorske; sepsa.**

## Introduction

Sepsis is a principal cause of death in critical care units worldwide and consumes considerable healthcare resources. There is evidence suggesting that there are different mechanisms of clinical manifestations of Gram-positive and Gram-negative sepsis to the extent that they may represent different disease entities [1]. Some microbial challenges may elicit levels of mediators that damage both the infecting microorganism and the host. Lipoteichoic acid (LTA) of Gram-positive bacteria as well as lipopolysaccharide (LPS) of Gram-negative bacteria elicit different response from the host. Furthermore, Gram-positive and Gram-negative bacteria may induce different inflammatory patterns. But, it is not physiologically or clinically apparent because of the fact that signs of systemic inflammatory response syndrome and routine laboratory markers of infection are nonspecific [1–3].

Myeloid-derived suppressor cells (MDSCs), with its two main subsets being monocytic (M-MDSCs) and granulocytic (G-MDSCs) are important regulators of intricate and complex immuno-inflammatory response to various insults such as bacteria [4].

The aim of this study were to examine the association of the MDSCs with the type of infecting microorganism (Gram positive, Gram negative, polymicrobial) and underlying cause of secondary sepsis (peritonitis, pancreatitis, trauma).

## Methods

Totally, 40 critically ill patients with secondary sepsis due to peritonitis, pancreatitis and severe trauma, admitted to a surgical intensive care unit (SICU), were enrolled in prospective study conducted in a tertiary university hospital (Military Medical Academy, Belgrade, Serbia). Approval in concordance with Declaration of Helsinki was obtained from local Ethics Committee and informed consent from a patient or first-degree relative. Detailed description of the study population is reported elsewhere [5]. Blood samples for MDSCs analysis were collected on admission (the Day 1) and on the Day 5. These two specific time points were chosen because dynamic changes in MDSCs function during sepsis were expected. Blood was simultaneously drawn for a blood culture. The Sequential Organ Failure Assessment (SOFA) score, the Simplified Acute Physiology Score (SAPS) II and the Acute Physiology and Chronic Health Evaluation (APACHE) II score were calculated and recorded within the first 24 hours after admission to the SICU (the Day 1). SOFA score was recorded daily during SICU stay to assess severity of organ dysfunction in secondary sepsis [6–8]. The use of antibiotics, circulatory volume replacement and vasoactive support were performed according to guidelines [9]. Various modes of mechanical ventilation and surgical procedures were performed if and when necessary in all patients.

Detailed description of demographic and clinical data of examined patients was presented in our previous study [5].

Fresh peripheral blood samples were analyzed, frequency and absolute number of MDSCs were determined. Both main subsets of MDSCs were detected, G-MDSCs and M-MDSCs. MDSCs analysis is described elsewhere [5].

Complete statistical analysis of data was done with the statistical software package, SPSS Statistics 18. Most of the variables were presented as frequency of certain categories, while statistical significance of differences was tested with the $\chi^2$ test. In case of continuous data, variables were presented as mean value ± standard deviation (SD), median, minimal and maximal values. Kolmogorov-Smirnov test was used for evaluation of distribution of continual data. Statistical significance between groups was tested by Wilcoxon or Mann-Whitney test. Spearman's Rank Correlation analyses were used to establish the relation between parameters. Receiver operating characteristic (ROC) curves were constructed and analyzed to determine the sensitivity and specificity of variables for prediction of bacteremia nature and outcome. Calculations of odds ratios (OR) and their 95% confidence intervals (CI) were done to determine the strength of the association between variables and nature of bacteremia. For that purpose, the most promising independent variables, as single or combined, were incorporated into binary logistic regression analyses.

All the analyses were estimated at $p < 0.05$ level of statistical significance.

## Results

Demografic and clinical data of 40 patients are shown in Table 1. Two patients with sterile blood cultures were excluded from further analysis.

Baseline characteristics of the patient population according to nature of bacteremia on the Day 1 and Day 5 are shown in Table 2.

*Both main MDSC subpopulations accumulate significantly in Gram-positive sepsis*

We compared frequencies and absolute numbers of G-MDSCs and M-MDSCs in sepsis patients according to the nature of bacteremia (Gram-positive, Gram-negative and Polymicrobial groups) (Figure 1).

**Table 1**

**Demographic and clinical data of critically ill patients with secondary sepsis**

| Parameter | Values |
|---|---|
| Age (years), mean (range) | 59.3 (27–86) |
| Sex, n (%) | |
| male | 28 (70) |
| female | 12 (30) |
| Scores | |
| Simplified Acute Physiology Score II (SAPS II), mean ± SD | 57.05 ± 9.37 |
| Acute Physiology and Chronic Health Evaluation II (APACHE II), mean ± SD | 21.65 ± 3.360 |
| Sequential (Sepsis) Organ Failure Assessment (SOFA), mean ± SD | 6.850 ± 2.832 |
| Reason for ICU admission due to severe sepsis, n (%) | |
| pancreatitis | 16 (40) |
| peritonitis | 14 (35) |
| trauma | 10 (25) |
| Blood cultures, n (%) | |
| Gram-positive | 20 (50) |
| Gram-negative | 8 (20) |
| polymicrobial | 10 (25) |
| sterile | 2 (5) |
| Overall hospital mortality, n (%) | 20 (50) |

**ICU – Intensive Care Unit; SD – standard deviation.**

**Table 2**

**Presence of MDSCs subpopulations in patients with secondary sepsis according to nature of bacteremia on the Day 1 and Day 5**

| Parameters | Gram-positive bacteremia (n = 20) mean ± SD; M (min-max) | Gram-negative bacteremia (n = 8) mean ± SD; M (min-max) | Polymicrobial bacteremia (n = 10) mean ± SD; M (min-max) |
|---|---|---|---|
| G-MDSCs | | | |
| frequencies (%) | | | |
| Day 1 | 2.00 ± 2.72; 0.88 (0.02–9.35) | 0.56 ± 0.77; 0.20 (0.02–1.99) | 0.58 ± 0.50; 0.37 (0.19–1.58) |
| Day 5 | 1.69 ± 1.12; 1.39 (0.17–3.86) | 2.55 ± 3.61; 0.81 (0.25–9.00) | 0.49 ± 0.35; 0.45 (0.03–1.13) |
| absolute number | | | |
| Day 1 | 237.42 ± 306.16; 153.44 (5.20–991.10) | 57.92 ± 68.11; 31.12 (2.35–178.50) | 72.09 ± 80.53; 48,12 (5.92–229.10) |
| Day 5 | 273.91 ± 236.53; 194.58 (12.56–864.24) | 205.34 ± 282.57; 71.67 (19.40–708.30) | 75.12 ± 97.00; 34.85 (2.05–267.81) |
| M-MDSCs | | | |
| frequencies (%) | | | |
| Day 1 | 0.66 ± 0.83; 0.30 (0.04–2.56) | 0.19 ± 0.15; 0.19 (0.02–0.39) | 0.58 ± 0.82; 0.21 (0.04–2.18) |
| Day 5 | 0.94 ± 0.69; 0.84 (0.13–2.49) | 0.63 ± 0.93; 0.19 (0.01–2.17) | 0.39 ± 0.41; 0.13 (0.01–0.99) |
| absolute number | | | |
| Day 1 | 106.57 ± 153.82; 55.12 (4.81–533.92) | 29.02 ± 28.35; 21.21 (1.67–74.61) | 66.97 ± 98.00; 13.96 (3.52–255.06) |
| Day 5 | 200.51 ± 216.24; 109.76 (3.51–689.73) | 49.46 ± 73.44; 14.45 (0.89–170.78) | 58.47 ± 76.61; 15.39 (0.68–215.67) |

**MDSCs – meloid derived suppressor cels; G – granulocytic; M – monocytic;**
**SD – standard deviation; M – Median; min – minimum; max – maximum.**

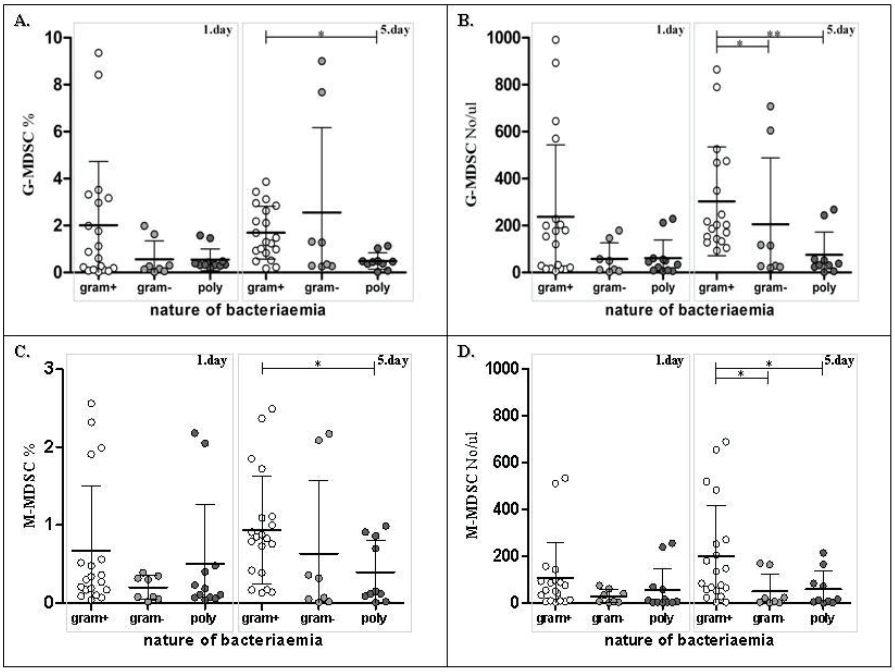


**Fig. 1 – Comparison of MDSCs frequencies between groups of patients with different nature of bacteraemia (relative and absolute numbers are given as mean ± standard deviation; Mann Whitney test, *$p < 0.05$, **$p < 0.01$).**
**A. Relative number of G-MDSC (%); B. Absolute number of G-MDSC (N/μL); C. Relative number of M-MDSC (%); D. Absolute number of M-MDSC (N/μL).**
**MDSC – myeloid-derived suppressor cells; G – granulocytic; M – monocytic; poly – polymicrobial.**

Initially, on the Day 1, patients with Gram-positive sepsis had more G-MDSCs and M-MDSCs (both relative and absolute number) comparing to other two groups, but without significant difference. Accumulation of G-MDSCs and M-MDSCs in patients with Gram-positive sepsis has become more intensive on the Day 5. This group had significantly more both G-MDSCs and M-MDSCs comparing to the Polymicrobial sepsis group ($p < 0.05$) (Figures 1 A,B,C,D). Also, patients with Gram-positive sepsis had significantly more G-MDSCs and M-MDSCs (absolute number) than patients with Gram-negative sepsis ($p < 0.05$) (Figures 1B and 1D).

Univariate logistic regression analyses were performed in order to determine whether associations of each individual variable with Gram-positive sepsis exist. Standardized regression coefficient (β) and OR with 95% CI were calculated for each variable. Forward stepwise multivariate logistic regression model was performed in order to determine the independent predictors of Gram-positive sepsis, without the effect of possible confounders. In Table 3 univariate ORs of variables for predicting Gram-positive sepsis in the patient population on the Day 1 and Day 5 are shown.

**Table 3**
**Univariate odds ratio (ORs) of variables for predicting Gram-positive sepsis in the patient population on the Day 1 and Day 5**

| Variables | Standard β value | OR | 95% CI | | *p* |
|---|---|---|---|---|---|
| | | | lower bound | upper bound | |
| G-MDSCs | | | | | |
| frequencies | | | | | |
| Day 1 | 0.709 | 2.033 | 0.974 | 4.242 | 0.023 |
| Day 5 | 0.084 | 1.087 | 0.767 | 1.542 | 0.638 |
| absolute number | | | | | |
| Day 1 | 0.007 | 1.007 | 0.999 | 1.014 | 0.039 |
| Day 5 | 0.003 | 1.003 | 1.000 | 1.007 | 0.043 |
| M-MDSCs | | | | | |
| frequencies | | | | | |
| Day 1 | 0.580 | 1.786 | 0.689 | 4.624 | 0.232 |
| Day 5 | 1.012 | 2.752 | 0.915 | 8.275 | 0.038 |
| absolute number | | | | | |
| Day 1 | 0.005 | 1.006 | 0.998 | 1.013 | 0.166 |
| Day 5 | 0.009 | 1.009 | 1.001 | 1.017 | 0.030 |

**β – standardized regression coefficient; MDSCs – myeloid-derived suppressor cells; G-granulocytic; M – monocitic; OR – odds ratio; CI – confidence interval.**

Univariate logistic regression analyses of investigated variables regarding Gram-positive sepsis on the Day 1 revealed that both G-MDSCs frequencies and absolute number had statistically significant power for predicting Gram-positive sepsis. Univariate logistic regression analyses of investigated variables regarding Gram-positive sepsis on the Day 5 revealed that G-MDSCs absolute number along with both M-MDSCs frequencies and absolute number had statistically significant power for predicting Gram-positive sepsis. Stepwise multivariate logistic regression analyses of the variables on the Day 5 determined that M-MDSCs absolute number was independent predictor of Gram-positive sepsis which is shown in Table 4.

The Spearman's rho test of correlation between frequencies and absolute numbers of G-MDSCs and M-MDSCs on one hand, and Gram-positive sepsis on the other hand, was performed to assess strength of association. On the Day, absolute numbers of G-MDSCs and M-MDSCs correlated significantly with Gram-positive sepsis. That positive correlation is shown in Table 5 and Figure 2.

On the Day 5, there were significantly positive correlations between all investigated variables and Gram-positive sepsis (Table 6).

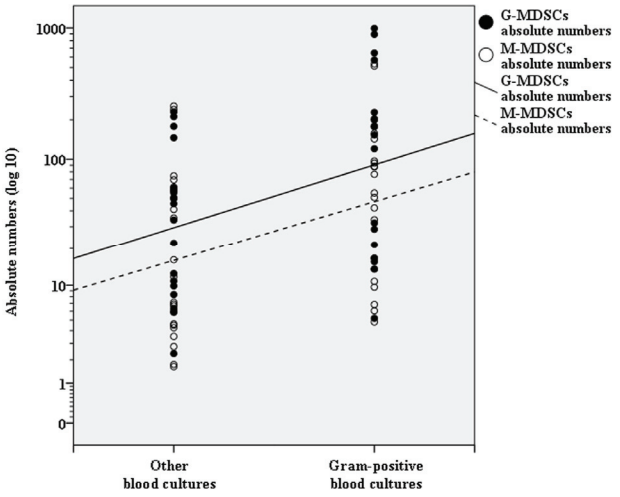


**Fig. 2 – Scattergram on $log_{10}$ scales of G-MDSCs and M-MDSCs absolute numbers vs. blood cultures in patients with secondary sepsis on the Day 1.**

**MDSCs – myeloid-derived suppressor cells; G – granulocytic; M – monocitic.**

**Table 4**

**Independent predictor of Gram-positive sepsis in the patient population by multivariate logistic regression analysis on the Day 5**

| Variables | Standard β value | OR | 95% CI | | *p* |
|---|---|---|---|---|---|
| | | | lower bound | upper bound | |
| M-MDSCs absolute number | 0.012 | 1.012 | 0.999 | 1.026 | 0.035 |

**β – standardized regression coefficient; MDSCs – myeloid-derived suppressor cells; M – monocitic; OR – odds ratio; CI – confidence interval.**

**Table 5**

**Spearman's rho correlations between variables and Gram-positive sepsis in the patient population on the Day 1**

| Variables | G-MDSCs frequencies | G-MDSCs absolute number | M-MDSCs frequencies | M-MDSCs absolute number |
|---|---|---|---|---|
| Gram positive blood culture | 0.185; $p = 0.261$ | 0.328; $p = 0.040$ | 0.258; $p = 0.113$ | 0.378; $p = 0.018$ |
| G-MDSCs frequencies | | 0.854; $p = 0.000$ | -0.152; $p = 0.356$ | -0.221; $p = 0.177$ |
| G-MDSCs absolute number | | | 0.043; $p = 0.797$ | 0.154; $p = 0.350$ |
| M-MDSCs frequencies | | | | 0.866; $p = 0.000$ |

**MDSC – myeloid-derived suppressor cells; G-granulocytic; M – monocitic.**

**Table 6**

**Spearman's rho correlations between variables and Gram-positive sepsis in the patient population on the Day 5**

| Variables | G-MDSCs frequencies | G-MDSCs absolute number | M-MDSCs frequencies | M-MDSCs absolute number |
|---|---|---|---|---|
| Gram positive blood culture | 0.401; $p = 0.013$ | 0.428; $p = 0.007$ | 0.440; $p = 0.006$ | 0.466; $p = 0.003$ |
| G-MDSCs frequencies | | 0.818; $p = 0.000$ | 0.484; $p = 0.002$ | 0.389; $p = 0.016$ |
| G-MDSCs absolute number | | | 0.663; $p = 0.000$ | 0.749; $p = 0.000$ |
| M-MDSCs frequencies | | | | 0.899; $p = 0.000$ |

**MDSCs – myeloid-derived suppressor cells; G – granulocytic; M – monocitic.**

Positive correlations between G-MDSCs and M-MDSCs frequencies and Gram-positive sepsis are shown in Figure 3.

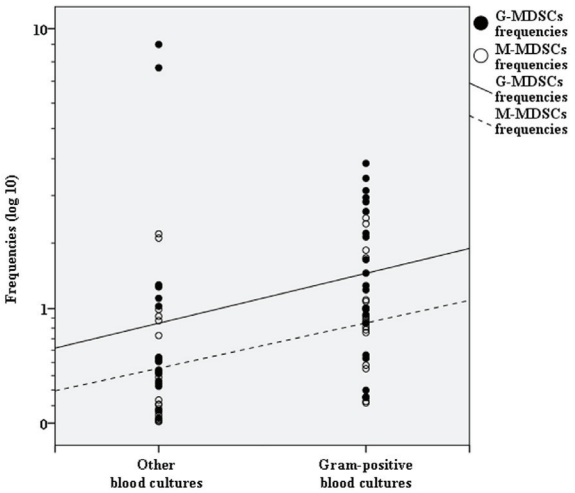


**Fig. 3 – Scattergram on $log_{10}$ scales of G-MDSCs and M-MDSCs frequencies vs. blood cultures in patients with secondary sepsis on the Day 5.**

**MDSC – myeloid-derived suppressor cells; G – granulocytic; M – monocitic.**

*Neutrophil (Ne) to G-MDSCs ratio and monocyte (Mo) to M-MDSCs ratio*

Baseline characteristics of patient population regarding Ne/G-MDSCs and Mo/M-MDSCs according to nature of bacteremia on the Day 1 and Day 5 are shown in Table 7.

Ne/G-MDSCs and Mo/M-MDSCs ratios were lowest in critically ill patients with Gram-positive bacteremia both on the Day 1 and Day 5. On the first day, that difference did not reach statistical significance, but on the Day 5 both ratios were statistically significantly lower in patients with Gram-positive bacteremia compared to patients with Gram-negative or polymicrobial blood culture (Ne/G-MDSCs: $\chi^2$ = 6.806, $p < 0.05$; Mo/M-MDSCs: $\chi^2 = 9.070$, $p < 0.01$).

*Post hoc* Mann-Whitney test revealed that on the Day 5 Mo/M-MDSCs ratio was significantly lower in patients with Gram-positive compared to Gram-negative blood culture (Z = -2.389; $p < 0.05$) Also, patients with Gram-positive blood culture had significantly lower both Ne/G-MDSCs and Mo/M-MDSCs ratios compared to patients with polymicrobial blood culture (Ne/G-MDSCs: Z = -2.781, $p < 0.01$; Mo/M-MDSCs: Z = -2.493, $p < 0.01$).

Also, levels of Ne/G-MDSCs were significantly lower in nonsurvivors, both on the Day 1 (Z = -1.921; $p < 0.05$) and the Day 5 (Z = -1.815; $p < 0.05$).

**Table 7**

**Baseline characteristics of the patient population according to nature of bacteremia on the Day 1 and Day 5**

| Parameter | Gram-positive bacteremia (n = 20) mean ± SD, M (min-max) | Gram-negative bacteremia (n = 8) mean ± SD, M (min-max) | Polymicrobial bacteremia (n = 10) mean ± SD, M (min-max) |
|---|---|---|---|
| Neutrophils × $10^6$/L | | | |
| Day 1 | 11,743.33 ± 8,567.86 | 11,701.25 ± 8,265.44 | 9,909.09 ± 4,823.16 |
| | 9,410 (1,390–28,600) | 8,700 (1,960–22,800) | 10,300 (1500–18300) |
| Day 5 | 12,893.33 ± 8,141.56 | 6,810.00 ± 3,868.92 | 9,585.00 ± 5,160.65 |
| | 14,500 (1,610–26,000) | 6,265 (2,110–14,800) | 7,960 (4,500–21,800) |
| Neutrophil to G-MDSC ratio | | | |
| Day 1 | 481.05 ± 1,039.91 | 863.99 ± 1,502.29 | 465.90 ± 818.14 |
| | 61.32 (5.55–4655.64) | 375.70 (33.24–4,553.19) | 229.67 (39.72–2,909.38) |
| Day 5 | 128.10 ± 250.63 | 143.12 ± 139.56 | 589.28 ± 890.21 |
| | 37.57 (9.60–1070.24) | 91.52 (9.65–336.13) | 217.10 (27.71–2625.67) |
| Monocytes × $10^9$/L | | | |
| Day 1 | 718.09 ± 706.23 | 566.50 ± 341.08 | 533.63 ± 346.21 |
| | 600 (43–2,610) | 510 (100–1090) | 537 (50–1,120) |
| Day 5 | 801.00 ± 742.35 | 430.25 ± 189.21 | 699.80 ± 449.03 |
| | 697 (43–3,460) | 411 (170–670) | 525 (310–1,810) |
| Monocyte to M-MDSC ratio | | | |
| Day 1 | 16.02 ± 23.86 | 93.98 ± 128.28 | 47.59 ± 62.44 |
| | 8.38 (0,60–114.09) | 12.72 (5.96–347.72) | 16.09 (2.24–209.17) |
| Day 5 | 9.25 ± 10.64 | 134.07 ± 237.85 | 133.29 ± 222.34 |
| | 6.54 (0.65 – 36.47) | 23.80 (2.32–671.14 ) | 37.95 (4.03–702.78) |

**MDSCs – myeloid-derived suppressor cells; G-granulocytic; M – monocitic.**
**SD – standard deviation; M – median; min – minimum; max – maximum.**

*Clinical accuracy of Ne/G-MDSCs and Mo/M-MDSCs ratios in predicting nature of bacteremia and outcome*

Clinical accuracy of Ne/G-MDSCs and Mo/M-MDSCs ratios in predicting nature of bacteremia and outcome was investigated. Discriminative power of both Ne/G-MDSCs and Mo/M-MDSCs ratios in predicting Gram-positive blood culture was statistically significant both on the Day 1 and Day 5. Results are shown in Table 8 and Figures 4 and 5.

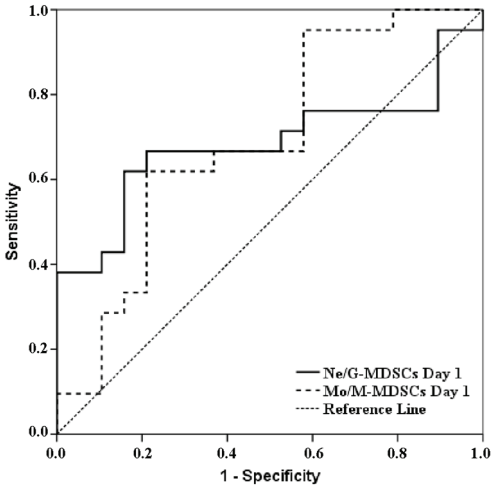


**Fig. 4 – Receiver operating characteristic (ROC) curve for Ne/G-MDSCs and Mo/M-MDSCs on the Day 1 (Gram-positive blood culture).**

**MDSCs –myeloid-derived suppressor cells; G – granulocytic; M – monocytic; Ne – neutrophil; MO – monocyte;**

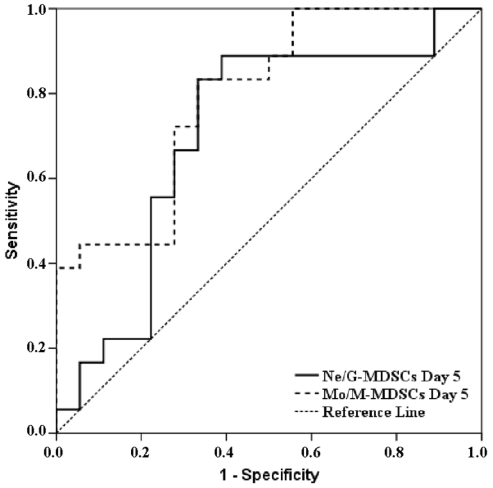


**Fig. 5 – Receiver operating characteristic (ROC) curve for Ne/G-MDSCs and Mo/M-MDSCs on the Day 5 (Gram-positive blood culture).**

**MDSCs –myeloid-derived suppressor cells; G – granulocytic; M – monocytic; Ne – neutrophil; MO – monocyte.**

Ne/G-MDSCs and Mo/M-MDSCs ratios lower than cut-off values were moderate predictors of Gram-positive blood culture both on the Day 1 and Day 5 in critically ill patients with secondary sepsis.

Clinical accuracy of Ne/G-MDSCs and Mo/M-MDSCs ratios in predicting polymicrobial blood culture was statistically significant on the Day 5 (Table 9 and Figure 6).

**Table 8**

**Clinical accuracy of Ne/G-MDSCs and Mo/M-MDSCs ratios in predicting Gram-positive blood culture in patients with secondary sepsis on the Day 1 and Day 5**

| Parameter | AUC ROC | *p* | 95% CI | | Cut-off value | Sensitivity (%) | Specificity (%) | Youden index |
|---|---|---|---|---|---|---|---|---|
| | | | lower bound | upper bound | | | | |
| Day1 | | | | | | | | |
| Ne/G-MDSCs | 0.684 | < 0.05 | 0.510 | 0.858 | 121.86 | 61.9 | 84.2 | 0.46 |
| Mo/M-MDSCs | 0.692 | < 0.05 | 0.524 | 0.860 | 9.98 | 61.9 | 78.9 | 0.41 |
| Day 5 | | | | | | | | |
| Ne/G-MDSCs | 0.707 | < 0.05 | 0.527 | 0.887 | 185.53 | 88.9 | 61.1 | 0.50 |
| Mo/M-MDSCs | 0.793 | < 0.01 | 0.648 | 0.939 | 13.69 | 83.3 | 66.7 | 0.50 |

**MDSCs – myeloid-derived suppressor cells; G – granulocytic; M – monocitic; Ne – neutrophil; Mo – monocyte; AUC – area under curve; ROC – reciever operating characteristic; CI – confidence interval.**

**Table 9**

**Clinical accuracy of Ne/G-MDSCs and Mo/M-MDSCs ratios in predicting polymicrobial blood culture in patients with secondary sepsis on the Day 5**

| Parameter | AUC ROC | *p* | 95% CI | | Cut-off value | Sensitivity (%) | Specificity (%) | Youden index |
|---|---|---|---|---|---|---|---|---|
| | | | lower bound | upper bound | | | | |
| Ne/G-MDSCs Day 5 | 0.773 | < 0.01 | 0.606 | 0.940 | 185.53 | 80.0 | 81.0 | 0.61 |
| Mo/M-MDSCs Day 5 | 0.719 | < 0.05 | 0.533 | 0.906 | 45.64 | 50.0 | 92.3 | 0.42 |

**MDSCs – myeloid-derived suppressor cells; G – granulocytic; M – monocitic; Ne – neutrophil; Mo – monocyte; AUC – area under curve; ROC – reciever operating characteristic; CI – confidence interval.**

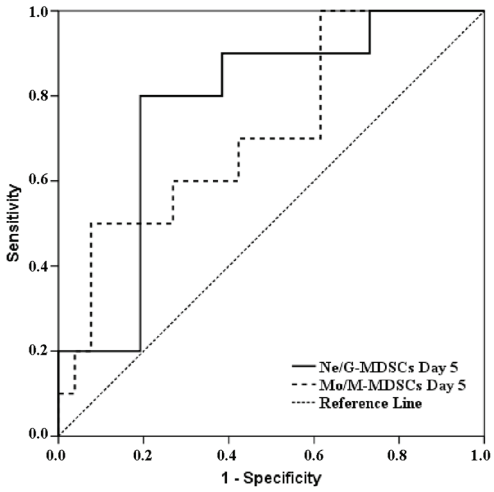


**Fig. 6 – Receiver operating characteristic curve for Ne/G-MDSCs and Mo/M-MDSCs on the Day 5 (polymicrobial blood culture).**

**MDSCs –myeloid-derived suppressor cells; G – granulocytic; M – monocytic; Ne – neutrophil; MO – monocyte.**

Ne/G-MDSCs and Mo/M-MDSCs ratios higher than respective cut-off values were predictors of polymicrobial blood culture on the Day 5 in critically ill patients with secondary sepsis. Ne/G-MDSCs ratio has very good discriminative power while Mo/M-MDSCs ratio has moderate one.

Clinical accuracy of both ratios in predicting lethal outcome was investigated. Ne/G-MDSCs ratio lower than cut-off value both on the Day 1 and Day 5 was moderate predictor of lethal outcome in this patient population. Discriminative power of Mo/M-MDSCs regarding outcome was not significant. Results are shown in Table 10 and Figure 7.

*Underlying causes of secondary sepsis might not seem to influence the MDSCs accumulation*

The underlying causes of secondary sepsis in examined patients were pancreatitis, peritonitis and trauma. The patients with different underlying causes of sepsis were perceived as separated groups and frequencies and absolute numbers of their G-MDSCs and M-MDSCs were compared. There were no statistically significant differences among these three subgroups neither on the Day 1 nor on the Day 5. So, MDSCs expansion was related to secondary infection regardless of nature of primary insult (pancreatitis, peritonitis, trauma).

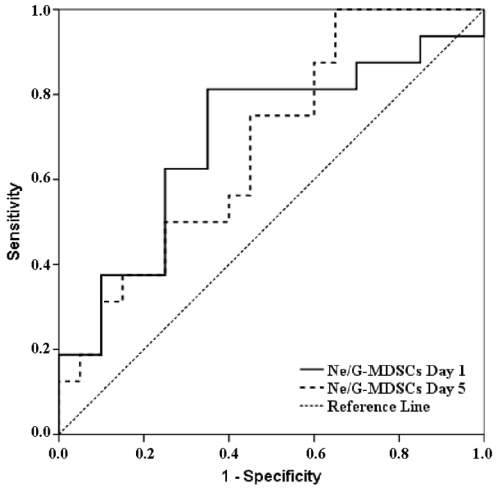


**Fig. 7 – Receiver operating characteristic curve for Ne/G-MDSCs on the Day 1 and Day 5 and lethal outcome in patients with secondary sepsis**

**MDSCs –myeloid-derived suppressor cells; G – granulocytic; M – monocytic; Ne – neutrophil; MO – monocyte.**

## Discussion

Immune dysfunction is common in critically ill patients and it may modulate immune response and affect patient morbidity and mortality, particularly in severe trauma and/or sepsis. Immune cells' and mediators' role in immune response in critical illness is not yet fully elucidated [10, 11].

Expansion and activation of MDSCs, as part of immune response, are under the influence of several different factors, including infectious agents [12–14]. It seems that there is a difference between Gram-positive and Gram-negative sepsis regarding cytokine profile, for instance [1, 15]. It has also been shown that different types of microbes can induce specific subsets of MDSCs, with different impact on disease outcome [14].

A study by Janols et al. [16] showed predominant accumulation of $CD14^{low}$ polymorphonuclear MDSCs in patients with Gram-positive sepsis and septic shock. They also showed that the $CD14^{low}$ polymorphonuclear MDSCs accumulate in both, Gram-negative and Gram-positive sepsis, but are significantly more potent suppressors of T-cell proliferation when isolated from Gram-positive sepsis patients [16].

**Table 10**

**Clinical accuracy of Ne/G-MDSCs ratio in predicting lethal outcome in patients with secondary sepsis on the Day 1 and Day 5**

| Parameter | AUC ROC | *p* | 95% CI | | Cut-off value | Sensitivity (%) | Specificity (%) | Youden index |
|---|---|---|---|---|---|---|---|---|
| | | | lower bound | upper bound | | | | |
| Ne/G-MDSCs Day 1 | 0.694 | < 0.05 | 0.513 | 0.875 | 241.53 | 81.3 | 65.0 | 0.46 |
| Ne/G-MDSCs Day 5 | 0.678 | < 0.05 | 0.504 | 0.853 | 262.90 | 100.0 | 35.0 | 0.35 |

**MDSCs – myeloid-derived suppressor cells; G – granulocytic; M – monocitic; Ne – neutrophil; Mo – monocyte; AUC – area under curve; ROC – reciever operating characteristic; CI – confidence interval.**

The findings of Janols et al. [16] suggest that different types of bacteria can influence myeloid response of the septic host, and accordingly, generation of specific MDSCs subset with possible distinct functions. In our study, we found significantly higher frequencies of both detected MDSCs subpopulations, G-MDSCs and M-MDSCs, in patients with Gram-positive sepsis when compared with Polymicrobial sepsis patients on the Day 5. Also, patients with Gram-positive sepsis had significantly more both G-MDSCs and M-MDSCs (absolute number) than patients with Gram-negative sepsis ($p < 0.05$). Stepwise multivariate logistic regression analyses of variables on the Day 5 determined that M-MDSCs absolute number was independent predictor of Gram-positive sepsis. Positive correlations between G-MDSCs and M-MDSCs frequencies and Gram-positive sepsis are confirmed by the Spearman's rho test. Possible explanation of these differences may lie in the basic understanding of MDSCs expansion seen in malignant diseases and protracted infections [12, 17]. Prompt reaction of the bone marrow in response to Gram-negative, and possible to polymicrobial causative infectious agents, may leave no time for different proinflammatory factors to act on myeloid precursors in different stages of maturation and to activate/convert them into immunosuppressive cells. On the contrary, more indolent, in terms of an acute inflammatory response, Gram-positive infectious agents could lead to prolonged bone marrow exposure, creating the environment conducive for MDSCs accumulation [18]. In addition, our finding that there were no significant differences in MDSCs accumulation between patients with different underlying causes of secondary sepsis (pancreatitis, peritonitis or trauma injury as primary insults) also speaks in favor of the causative infectious agent being more important for MDSCs generation than the type of primary insult leading to secondary sepsis.

Uhel et al. [19] performed peripheral blood transcriptomic analysis on 29 patients with sepsis and 15 healthy donors and in a second cohort of 94 patients with sepsis, 11 severity-matched ICU patients and 67 healthy donors, they performed functional analysis in order to clarify phenotype, suppressive activity, origin and clinical impact of MDSCs in patients with sepsis. Their results showed that MDSCs were major players in sepsis-induced immunosuppression. In sepsis patients they demonstrated up-regulation of gene profile associated with MDSCs regrutation and immunosuppression (MMP8, MMP9, ARG1, S100A8, S100A9, S100A12, PD-L1, IL-4R, and IL-10), but down-regulation of gene profile associated with inflammatory response (CD4, CD20, CD8, CD3, IL-8 and IL-6). They concluded that $CD14^{+}HLA\text{-}DR^{low/-}$ M-MDSCs and $CD15^{+}$ G-MDSCs strongly contributed to T-cell dysfunction in patients with sepsis. Contrary to our results, they found no association with Gram-staining of the causative organism. Interestingly, they also demonstrated that expression of two, among key MDSCs parameters, ARG1 and S100A9, significantly directly correlated to granulocyte count and inversely correlated to number of lymphocytes. Furthermore, Uhel et al. [19] showed that beside MDSCs, $CD14^{+}$ monocytes and $CD15^{+}$ low density granulocytes from sepsis patients were suppressive *in vitro*, similarly to MDSCs. They also showed that population of low density granulocytes is very heterogenous, being composed of immature and mature granulocytes both expressing degranulation markers.

In other words, beside MDSCs, mature monocytes and granulocytes of investigated patients demonstrated function and phenotype alterations. These findings are hard to explain from the aspect where MDSCs increment is a consequence of emergency myelopoesis followed by export of immature myeloid cells from bone marrow into blood stream. But, several recent articles pointed out that MDSCs increase could be achieved by reprogramming of existing monocytes, arguing that monocyte to M-MDSCs relation is very dynamic and plastic [4, 20]. Of course, both mechanisms could be operative at the same time, they are not mutually exclusive. According to this, we have analyzed ratio of monocytes to M-MDSCs and neutrophils to G-MDSCs, in every individual patient and in both time points. All sepsis patients from our study demonstrated decrement of Ne/G-MDSCs ratio and increment of Mo/M-MDSCs ratio from the 1th to 5th day. But, stratification of patients according to the type of microbial culture demonstrated significant differences. Patients with Gram-positive sepsis demonstrated significant decrement of Ne/G-MDSCs ratio and less prominent decrement of Mo/M-MDSCs ratio from the 1th to 5th day. Although Gram-negative sepsis patients also demonstrated significant Ne/G-MDSCs ratio decrement, the number of their monocytes increased comparing to detected number of M-MDSCs. Contrary to both previous groups, sepsis patients with polymicrobial cultures on the Day 5 demonstrated increase of both Ne/G-MDSCs and Mo/M-MDSCs ratios. All these indicate that type of microbial infection in sepsis is significantly associated with particular profile of MDSCs, dynamic of their change and their relation to mature – like counterpart cells. Finally, in our investigation, decrease of Ne/G-MDSCs ratio was associated with worse outcome, being significantly lower in nonsurvivors comparing to survivors.

Bergenfelz et al. [21] demonstrated that systemic M-MDSCs are generated from monocytes and that their number correlates with disease progression in breast cancer patients. Additionaly, they observed significant increase of monocytes with altered phenotype both in breast cancer group as well as in control sepsis group. These monocytes exhibited $CD14^{+}HLA^{-}DR^{low/-}$ phenotype, which is specific for M-MDSCs, and were already documented in few earlier studies in sepsis patients with compensatory antiinflammatory response syndrome [20, 22–25]. Gene profiling further delineated that these populations of monocytes/M-MDSC were similarly immunosuppressive in both breast cancer and sepsis patients, but not other infective diseases and healthy controls [21]. Monocytes from early breast cancer group produced comparable levels of IL-1β, IL-6, IL-8 and TNF as monocytes from metastatic group, indicating change of monocyte function early in the disease. Furthermore, sepsis patients had significantly more total $CD14^{+}$ cells, $CD14^{+}CD16^{-}$ cells, $CD14^{++}CD16^{+}$ intermediate monocytes and $CD14^{++}CD16^{++}$ nonclassical monocytes comparing to both early and metastatic breast cancer patients and healthy controls, with in-

creased $CD16^{+}/CD16^{-}$ monocyte ratio. Authors concluded that these Mo/M-MDSCs were induced early during the tumor growth and progression and that monocytes are affected by the tumor much before their extravasation into the tumor tissue. Based on observation of similar phenotypic and molecular findings in breast cancer and sepsis patients, we could assume that sepsis progression could reprogramme monocytes and granulocytes in the same way.

Reprogramming process is not a rare event and could have physiological implications. Zhao et al.[26] demonstrated that human trophoblast cells efficiently change differentiation programme in monocytes, inducing their maturation toward dendritic cells. Those trophoblast cells induced monocyte derived dendritic cells display altered, hypostimulatory capacity to T lymphocytes and induce generation of inhibitory regulatory T lymphocytes. Sepsis itself induces numerous changes in monocyte functions. Shalova et al.[27] demonstrated that sepsis patients' monocytes exert significant upregulation of genes associated with inflammation (IL-1b, IL-6, CCL3, CCL5), but also with tissue remodeling genes (VEGF, MMPs). Authors found that hypoxia inducible factor-1 (HIF-1a) was specifically upregulated in sepsis patients' monocytes but not in the control ones. HIF-1a negatively regulated Toll-like receptors (TLR) monocyte activation, resulting in diminished proinflammatory response to endotoxin challenge, so called endotoxin tolerance. Although that study did not investigate MDSCs, authors concluded that HIF-1a is important regulator of monocyte reprogramming toward immunosuppressive functions in sepsis patients.

The main limitation of our study is sample size. Significant number of critically ill patients with secondary sepsis due to diffuse peritonitis had to be excluded because of malignant disease.

## Conclusion

Gram-positive infectious agents were powerful inducers of MDSCs generation in sepsis. Also, underlying causes of secondary sepsis might not seem to influence the MDSCs accumulation. Larger trial is essential for possible confirmation of our findings.

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# Risk factors profile for liver damage in cardiac inpatients

## Profil faktora rizika od oštećenja jetre kod hospitalizovanih kardioloških bolesnika

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**Abstract**

**Background/Aim.** Liver damage, with potentially serious consequences, is not uncommon in hospitalized cardiac patients. The aim of our study was to determine the risk factor profile for liver damage in patients hospitalized from a deterioration of their acute or chronic cardiac illness. **Methods.** The study had observational case-control design with retrospective data collections from medical files of adult patients hospitalized in a tertiary health care center. The cases (n = 140) were subjects with novel liver injury (which emerged during hospital stay) and three control subjects were matched (age, date) for each case subject (n = 420). The primary outcome was hepatotoxicity (present or absent) and independent variables were proposed risk factors. Statistical analysis included descriptive methods, hypothesis testing and univariate and multivariate binary logistic regression, with $p \leq 0.05$. **Results.** In the whole study population, there were 432 (77.1%) females and the mean age of patients was 64.1 years [standard deviation (SD) = 10.7, range 24–85 years]. The most common illnesses were coronary heart disease (n = 385), hypertension (n = 334) and arrhythmia (n = 115). Mean value of Charlson Comorbidity Index (CCI) score was 3.8 (SD=1.7; range 1-10) corresponding to estimated CCI 10-years survival rate of 54.4% (SD = 33.5%). In the group of cases, 114 (81.4%) of the patients had hepatocellular, 9 (6.4%) cholestatic and 17 (12.2%) mixed type of hepatic injury. Factors independently associated with hepatotoxic event were previous occasional alcohol intake odds ratio (OR) 96.47; 95% confidence interval (CI) 28.95–321.43; $p < 0.001$), amiodarone (OR 3.70; 95% CI 1.82–7.53; $p < 0.001$), enoxaparin (OR 3.29; 95% CI 1.79–6.05; $p < 0.001$), obesity (OR 2.78; 95% CI 1.15–6.71; $p < 0.023$), atorvastatin (OR 2.67; 95% CI 1.33–5.38; $p < 0.006$) and CCI total score (OR 1.89; 95% CI 1.53–2.34; $p < 0.001$). **Conclusion.** Major factors associated with acute liver damage in patients hospitalized in cardiology ward of a tertiary health care institution were patient's constitutional and habitual characteristics (occasional alcohol intake, obesity, CCI total score) and drugs with known hepatotoxic properties (amiodarone, enoxaparin, atorvastatin).

**Key words:**
**alcohol drinking; amiodarone; cardiovascular diseases; chemical and drug induced liver injury; drug toxicity; inpatients; obesity; risk factors.**

**Apstrakt**

**Uvod/Cilj.** Oštećenje jetre, sa potencijalno ozbiljnim posledicama, nije retka pojava kod hospitalizovanih kardioloških bolesnika. Cilj studije bio je ispitivanje profila faktora rizika od oštećenja jetre kod bolesnika hospitalizovanih zbog pogoršanja akutne ili hronične kardiološke bolesti. **Metode.** Studija je bila opservacionog dizajna, tipa slučaj-kontrola, uz retrospektivno prikupljanje podataka uvidom u istorije bolesti odraslih bolesnika lečenih u tercijarnoj zdravstvenoj ustanovi. Slučajevi (n = 140) su bili bolesnici sa novonastalim oštećenjem jetre (koja se razvila tokom hospitalizacije), a po tri kontrolna bolesnika (n = 420), komparabilna po godinama i datumu hospitalizacije, pridruženi su svakom slučaju. Primarni ishod je bila hepatotoksičnost (simptomatska ili asimptomatska), a nezavisne varijable su bile predložene kao faktori rizika. Statistička analiza je uključivala deskriptivne metode, ispitivanje hipoteze i univarijantnu i multivarijantnu binarnu logističku regresiju, sa $p \leq 0.05$. **Rezultati.** Od ukupne studijske populacije, 432 (77,1%) osobe su bile ženskog pola, a srednja vrednost godina bolesnika iznosila je 64,1 godina [standardna devijacija (SD) = 10,7; opseg 24–85]. Najčešće bolesti su bile koronarna bolest (n = 385), hipertenzija (n = 334) i aritmija (n = 115). Srednja vrednost Charlson Comorbidity Index-a (CCI) bila je 3.8 (SD = 1,7; opseg 1–10), što je bilo u skladu sa procenjenim CCI 10-ogodišnjim preživljavanjem od 54,4% (SD = 33,5%). U grupi slučajeva,

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114 (81.4%) bolesnika imalo je hepatocelularni tip, 9 (6,4%) holestatski tip, a 17 (12,2%) mešoviti tip oštećenja jetre. Nezavisni prediktori hepatotoksičnog događaja su bili: prethodna povremena konzumacija alkohola [odds ratio (OR) 96,47; 95% interval poverenja (IP) 28,95–321,43; $p < 0,001$], upotreba amiodarona (OR 3,70; 95% IP 1,82–7,53; $p < 0,001$), enoksaparina (OR 3,29; 95% IP 1,79–6,05; $p < 0,001$) i atorvastatina (OR 2,67; 95% IP 1,33–5,38; 0,006), gojaznost (OR 2,78; 95% IP 1,15–6,71; 0,023) i ukupni CCI skor (OR 1,89; 95% IP 1,53–2,34; $p < 0,001$). **Zaključak.** Glavni faktori povezani sa akutnim oštećenjem jetre kod bolesnika hospitalizovanih na kardiološkom odeljenju u institucijama tercijarne zdravstvene nege su konstitucionalne karakteristike i navike bolesnika (povremeni unos alkohola, gojaznost, CCI skor) i lekovi za koje se zna da imaju hepatotoksični potencijal (amjodaron, enoksaparin, atorvastatin).

**Ključne reči:**
**alkohol, pijenje; amjodaron; kardiovaskularne bolesti; jetra, oštećenje, hemijsko i lekovima izazvano; lekovi, toksičnost; hospitalizacija; gojaznost; faktori rizika.**

## Introduction

Cardiac patients represent a population that is very prone to developing manifestations of the liver damage because they have many characteristics which are, in essence, risk factors for hepatic injury. The liver receives up to a quarter of cardiac output and any cardiovascular disease which causes significant reduction of arterial perfusion and increased cardiac preload could lead to concomitant hypoxia of the hepatic tissue and features of congestive hepatopathy. Such risk factors could be, for example, any cause of right ventricular heart failure, including constrictive pericarditis, tricuspid regurgitation, mitral stenosis, cardiomyopathy, and cor pulmonale [1]. In addition, physicians usually prescribe numerous drugs to patients suffering from cardiovascular disease, particularly within hospital settings and some of such pharmaceuticals have more or less the ability to induce liver injury. Recognizing particular risk factors for drug-induced hepatotoxicity in cardiac inpatients is an important clinical task. Such host factors can be divided into two groups: genetic (eg. polymorphism or variant involving drug-metabolizing enzymes and transport proteins) and non-genetic (eg. age, gender, concomitant somatic disease, pregnancy, alcohol, smoking, obesity) [2].

In general, drug-induced liver damage is nowadays recognized as one of the greatest problems in pharmacovigilance. Its incidence in developed countries on annual basis is significant, it is the major reason for drug withdrawal from the market as well as for stopping drug therapy due to safety issues and it causes important economic losses [3]. Cardiological drugs such as amiodarone, hydralazine, methyldopa, statins (atorvastatin, simvastatin), quinidine and ticlopidin as well as some other medicines frequently prescribed in hospitalized cardiac inpatients with associated comorbidity such as antibiotics (amoxicillin plus clavulanate, nitrofurantoin, sulfamethoxazole plus trimethoprim, sulfonamides), antigout agents (allopurinol) and nonsteroidal antiinflammatory drugs (diclofenac, ibuprofen, nimesulide) have been classified in the group of the pharmaceuticals with the most frequent reports of liver damage [4]. Other drugs, commonly prescribed to these patients are sometimes associated with hepatic damage. For example, among all reports of adverse events associated with the use of enoxaparin in a pharmacovigilance database, about 4% cases involve hepatic events [5].

Although liver toxicity of amiodarone (which is among the main cardiological drugs with known hepatotoxic potential) has been well described so far, additional research is needed for some features. Firstly, the mutual relationships of predisposing factors, which play synergistic role in the development of the amiodarone-induced hepatotoxicity, are still not completely understood. Drug-related (cumulative dose, pharmaceutical formulation, administration route), patient-dependent (age, gender, nutritional status, comorbidity, genetic polymorphism of metabolizing enzymes and target receptors) and treatment-associated issues (other hepatotoxic medications, adverse drug interactions) are examples of such factors [2, 6, 7].

Secondly, rare studies included exclusively cardiac inpatients and data in that subpopulation were rather limited [8]. Previous researchers examined hepatotoxicity caused by amiodarone in variety of ambulatory and/or inpatient groups including subjects with comorbid gastrointestinal, liver and other internal diseases [9, 10]. The patients primarily hospitalized from cardiovascular diseases have rather unique risk factor patterns for liver injury. Coronary heart disease, heart failure, coagulation disorders (eg. unstable prothrombin time), inflammatory illnesses (eg. bacterial endocarditis, myopericarditis), endocrine disturbances (eg. thyrotoxic cardiac disease) and circulatory instability due to extreme bradycardia or tachyarrhythmias are some of circumstances highly predisposing the inpatients to organ-specific or systemic ischemia. Frequent use of diagnostic and therapeutic vascular procedures (cardiac surgery, percutaneous coronary interventions) and medical devices (eg. intraaortic balloon pump, pacemakers, cardiac ablation and electrostimulation equipment) as well as high prescription rate of drugs with possible hepatic adverse reactions (eg. antilipemic drugs, anticoagulants, analgesics) add further risks for clinically-important liver damage.

Taking the above-mentioned facts into account, the aim of this study was to determine the risk factor profile for liver injury in patients hospitalized due to a deterioration of their acute or chronic cardiac illness.

## Methods

This research was based upon a retrospective data collection and observational case-control design, similar to other studies in the field [10, 11]. The study was conducted in the

Clinic of Cardiology, the Clinical Center "Kragujevac" in Kragujevac, Serbia. It complied with the ethical principles of the scientific research and it was approved by the Institutional Ethics Committee. The medical records of all patients treated at the institution throughout the period of four years (2011–2014) was screened. The study cases were the subjects with novel liver injury, which emerged during the hospital stay ("the index day") and three control subjects were randomly chosen for each case subject among all patients from the ward that were matched with this case. The control subjects had no recorded signs of liver injury at admission nor until the index day. They were matched with case patients for gender and age (5-year intervals), and taking into account the inclusion and exclusion criteria. The selection of patients was performed successively in the described manner, until the estimated number of study subjects was fulfilled.

The case patient was included if he or she was male or female, 18 to 75 years old and had a hepatotoxic event during hospitalization which was identified as any of the following: a liver enzyme level increases more than three times above the upper limit of the reference values, a total bilirubin level two times higher than the upper limit of the reference values and clinically manifested symptoms of the acute liver damage (pain under the right rib, nausea, feeling sick, vomiting, jaundice, hemorrhagic syndrome, abdominal pain, hepatomegaly). The exclusion criteria for both the case and the control subjects were the following: age younger than 18 or older than 75 years, confirmed diagnosis of either acute or chronic liver disease such as liver cirrhosis, Wilson's disease, porphyry, alpha-1 antitrypsin deficiency, hepatitis virus infection, primary biliary cirrhosis, primary sclerosing cholangitis, substance abuse, biliary calculosis, cholecystitis, pancreatitis, abdominal trauma, the increased values of aspartate aminotransferase (AST) at baseline with an AST/alanine aminotransferase (ALT) ratio > 2 upon admission, increased values of ALT, gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin above the upper normal limits at admission and decreased platelet counts below the lower normal limits at admission. AST/ALT ratio > 2 was the exclusion criterion because it was considered highly suggestive for alcohol abuse and consequent patient's liver injury [12].

The probability of supposed drug-induced/associated liver damage was assessed using the Council for International Organizations of Medical Sciences/Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) scale, a purposefully designed questionnaire aimed at evaluation of hepatotoxic effect of medications, herbal products and other xenobiotics. This questionnaire had already been used in numerous clinical studies as a valid method [13]. Drugs were classified according to the Anatomical Therapeutic Chemical (ATC) classification system used by the World Health Organization (http://www.whocc.no/atc_ddd_index) and the medication exposure was expressed as the number of defining daily doses (DDD) per 100 patient's days (PD) of hospitalization. We used Charlson Comorbidity Index (CCI) in order to assess the influence of patients´ multiple comorbidities [14]. The composite score of CCI was calculated with assessing age and existence of diabetes mellitus, liver disease, malignancy, acquired immunodeficiency syndrome (AIDS), moderate to severe chronic kidney disease, chronic heart failure, myocardial infarction, chronic obstructive pulmonary disease, peripheral vascular disease, cerebrovascular accident or transitory ischemic attack, dementia, hemiplegia, connective tissue disease and peptic ulcer disease of study patients.

The primary dependent variable was the hepatotoxicity, expressed as a binary variable (present or absent). The primary independent variable was treatment with amiodarone, identified as the prescription of at least one of its oral or parenteral dose. There were numerous other secondary independent or confounding variables (eg. route of amiodarone administration, number of DDD of amiodarone per 100 PD, type of cardiovascular disease, important comorbid illness, prescription of drugs with known hepatotoxic properties, particularly hypolipidemics, nonsteroidal antiinflammatory drugs, antiepileptics and antibiotics, patients' sociodemographic characteristics, smoking, caffeine intake as well as use of dietary supplements, herbal remedies and other over-the-counter-preparations) [15, 16].

We performed study sample calculation using the appropriate computer program, setting up the alpha error at 0.05 and the study power at 0.8 for dichotomous variable ($\chi^2$-test). The expected difference in the frequency of hepatotoxic-drug prescription rate (amiodarone) relating to the presence of primary variable (liver injury) was presumed based on a preliminary analysis of a small patient sample at the same institution [42.5% vs. 32.5%, odds ratio (OR) 1.49]. The calculation with above-mentioned input parameters gave the total sample size of 280 case patients and 840 control subjects. However, we prespecified the interim analysis after the inclusion of the half of study subjects with the study-ending rule if the analysis confirmed the statistically significant difference in the amiodarone-exposure rate (eg. significant OR) between the study groups. Therefore, the final study sample included 560 cardiology inpatients.

Statistical analysis of collected data included the descriptive methods (measures of central tendency and variability, frequencies), the methods for hypothesis testing (Student's *t*-test or Man-Whitney *U* test, $\chi^2$-test, or Fisher's exact test) and the calculation of crude and adjusted ORs [with 95% confidence intervals (CI)] using univariable and multivariable binary logistic regression. The probability level of significance for observed differences between study groups for all statistical analyses was established at 5% (0.05) or less.

## Results

The study included 2,500 hospital files of the patients treated during the study period of four years in the Clinic of Cardiology, Clinical Center "Kragujevac", Kragujevac, Serbia. After the first assessment for eligibility, we excluded a total of 1,500 patient records due to the presence of the exclusion criteria. The final sample included 140 patients' files in the case group and 420 patients' records in the control group, after exclusion of non-matched control patients and subsequent identification of noneligible criteria.

Most of the patients were females (432 of 560, 77.1%) and the mean age of patients in the whole study sample was 64.1 ± 10.7 years (from 24 to 85 years). Obesity was not significantly represented in the study population because only 46 (8.2%) of the study patients of whole study population were obese. Morphological hepatic lesion, ultrasonographic verified as fatty liver or hepatomegaly were observed in 116 (20.7%) of the patients from the whole study population, while clinically symptomatic hepatotoxicity had 28 (20.0%) of the case group patients.

Patients were hospitalized due to cardiovascular illnesses, among which the most common was coronary heart disease, followed by hypertension, arrhythmia and heart failure. Myocardial infarction with ST-segment elevation (STEMI) was the most frequent diagnosis of all coronary heart diseases, followed by myocardial infarction without ST-segment elevation (non-STEMI), non-stable angina, stable angina and ischemic cardiomyopathy. Among the patients who had arrhythmia, atrial fibrillation was the most frequent reason for commencement of drug treatment, followed by tachycardia and extrasystoles. Of all patients who had heart failure, about the three quarter of the patients had ejection fraction (EF) > 45%, while the others had heart failure with ejection fraction ≤ 45%. The most common non-cardiovascular comorbid disorder was diabetes mellitus. Only a minority of patients consumed alcohol periodically, and majority were non-smokers. The mean value of CCI score in the whole study population was 3.8 [standard deviation (SD) 1.7, from 1 to 10) with the estimated CCI 10-years survival rate of 54.4% (SD = 33.5%).

The patients' characteristics and laboratory values in all the study subjects and in the patients within study subgroups are presented in Tables 1 and 2. The median of days of hospitalization was 4 days (range 1–45 days) and the mean value 6.0 (SD = 5.6) days for all patients in the study group. In the case group, the median of hospitalization days was 7 (range 2–30 days) and the mean 7.7 (SD = 5.0) days while for the control group these values were 3 days (range 1–45 days) and 5.4 (SD = 5.7) days, respšectively. Overall mortality rate was 4 (0.7%) in the group of all study patients, considering that no fatal outcome was observed in the control group.

**Table 1**

**Demographic and clinical characteristics of study patients**

| Variable | Case group (n = 140) | Control group (n = 420) | $p^*$ |
|---|---|---|---|
| Gender (male) | 32 (22.9) | 96 (22.9) | 1.000‖ |
| Age (years) | 64.2 ± 11.3 | 64.0 ± 10.5 | 0.914§ |
| Obesity | 25 (17.9) | 21 (5.0) | < 0.001‖ |
| Fatty liver | 32 (22.9) | 18 (4.3) | < 0.001‖ |
| Hepatomegaly | 36 (25.7) | 20 (4.8) | < 0.001‖ |
| Heart failure | 32 (22.9) | 62 (14.8) | 0.026‖ |
| EF < 45% | 12 (8.6) | 12 (2.9) | 0.010‖ |
| Hypertension | 79 (56.4) | 255 (60.7) | 0.371‖ |
| Coronary heart disease | 108 (77.1) | 277 (66.0) | 0.013‖ |
| STEMI | 68 (48.6) | 107 (25.5) | < 0.001‖ |
| non-STEMI | 21(15.0) | 52 (12.4) | 0.425‖ |
| unstable angina | 13 (9.3) | 54 (12.9) | 0.260‖ |
| stable angina | 4 (2.9) | 36 (8.6) | 0.023‖ |
| Arrhythmia | 47 (33.6) | 68 (16.2) | < 0.001‖ |
| atrial fibrillation | 39 (27.9) | 54 (12.9) | < 0.001‖ |
| tachycardia | 4 (2.9) | 7 (1.7) | 0.480¶ |
| extrasystole | 4 (2.9) | 7 (1.7) | 0.480¶ |
| Diabetes mellitus | | | |
| type 1 | 11 (7.9) | 68 (16.2) | 0.014‖ |
| type 2 | 25 (17.9) | 46 (11.00) | 0.033‖ |
| Alcohol intake† | 73 (52.1) | 4 (1.0) | < 0.001‖ |
| Smoking habit | 35 (25.0) | 47 (11.2) | 0.001‖ |
| CCI score (points) | 4.9 ± 1.5 (5; 1–10) | 3.4 ± 1.6 (3; 1–7) | < 0.001** |
| CCI estimated survival (percent) | 32.0 ± 30.2 (21; 0–96) | 61.0 ± 31.1 (77; 0–96) | < 0.001** |
| Time to the index day‡ | 4 ± 3 (3; 1–14) | 4 ± 5 (2; 1–44) | < 0.001** |
| Hospital stay (days) | 8 ± 5 (7; 2–30) | 5 ± 6 (3; 1–45) | 0.001** |

**Results are present as mean ± standard deviation (median; range) for continuous variables, and number (percent) of patients (frequencies), as appropriate; * – probability for difference between the values of the case and the control group; † – occasionally, not satisfying exclusion criteria (regular alcohol use was exclusion criterion, see methods); CCI – Charlson Comorbidity Index; ‡ – index day-day on which novel liver injury emerged during the hospital stay); EF – ejection fraction; STEMI – myocardial infarction with ST segment elevation; nonSTEMI – myocardial infarction without STsegment elevation; ‖ – $\chi^2$ test, § – *t*-test; ¶ – Fisher's exact test; ** – Mann–Whitney U test.**

**Table 2**
**Laboratory parameters in patients of study groups (Case – hepatotoxicity, Control – without hepatotoxicity)**

| Variables | All patients (n = 560) | Case group (n = 140) | Control group (n = 420) | $p^*$ |
|---|---|---|---|---|
| Alanine transaminase (U/L) | 95 ± 226 (24; 6–2,760) (n = 560) | 305 ± 382 (221; 150–2760) (n = 140) | 25 ± 15 (20; 6–125) (n = 420) | n.a.† |
| Aspartate transaminase (U/L) | 98 ± 463 (23; 9–8,811) (n = 560) | 303 ± 895 (129; 16–811) (n = 140) | 29 ± 34 (20; 9–372) (n = 420) | na† |
| Gamma-glutamyltransferase (U/L) | 38 ± 64 (22; 5-858) (n = 312) | 45 ± 41 (29; 7–224) (n = 131) | 33 ± 76 (20; 5–858) (n = 181) | < 0.001‡ |
| Total bilirubin (μmol/L) | 15 ± 10 (12.3; 3–83) (n = 560) | 24 ± 15 (20, 5.7–83) (n = 140) | 12 ± 5 (11; 3–35.5) (n = 420) | n.a.† |
| Alkaline phosphatase (U/L) | 71 ± 85 (56.5; 3.24–1,185) (n = 306) | 91 ± 126 (62; 31–1,185) (n = 128) | 57 ± 21 (55; 3–227) (n = 178) | < 0.001‡ |
| Lactate dehydrogenase (U/L) | 452 ± 233 (419; 13–1,723) (n = 294) | 464 ± 280 (449; 55–1,723) (n = 123) | 442 ± 192 (407; 13–1,228) (n = 171) | 0.728‡ |
| Creatine phosphokinase (U/L) | 505 ± 1027 (118; 8.2–8,030) (n = 386) | 885 ± 1384 (289.5; 25–8,030) (n = 136) | 299 ± 686 (103; 8–5,680) (n = 250) | < 0.001‡ |
| Creatine phosphokinase-MB (U/L) | 84 ± 310 (16.3; 3.4–3,869) (n = 381) | 161 ± 497 (31; 4–3869) (n = 135) | 42 ± 94 (14.35; 3–908) (n = 246) | < 0.001‡ |
| Amylase (U/L) | 70 ± 56 (60; 4–603) (n = 218) | 63 ± 44 (56; 4–404) (n = 104) | 75 ± 65 (64; 16–603) (n = 114) | 0.036‡ |
| Troponin (ng/mL) | 16.1 ± 60.9 (0.95; 0.002–797) (n = 234) | 24.2 ± 84.7 (2.49; 0–797) (n = 102) | 9.8 ± 31.1 (0.3; 0–242) (n = 132) | < 0.001‡ |
| Proteins (g/L) | 66 ± 8 (n = 361) | 67 ± 7 (129) | 65 ± 8 (n = 232) | 0.006‖ |
| Albumins (g/L) | 40 ± 6 (n = 376) | 39 ± 6 (n = 132) | 40 ± 6 (n = 244) | 0.526‖ |
| Fibrinogen (g/L) | 4.2 ± 7.3 (3.76; 0.54–127) (n = 294) | 3.8 ± 1.4 (3.66; 0.5–11) (n = 116) | 4.6 ± 9.3 (3.85; 1.48–127) (n = 178) | 0.352‡ |
| International normalized ratio (INR) | 1.3 ± 0.7 (1.083; 0.9–6) (n = 326) | 1.4 ± 0.7 (1.1, 0.9–4.9) (117) | 1.3 ± 0.7 (1.08; 0.9–6) (n = 209) | 0.344‡ |
| C-reactive protein (mg/L) | 22 ± 36 (7; 0.2–256) (n = 367) | 31 ± 40 (12.15; 1.3–196) (n = 130) | 17 ± 32 (5.5; 0.2–256) (n = 237) | < 0.001‡ |
| Glucose (mmol/L) | 6.4 ± 2.5 (n = 465) | 6.7 ± 2.6 (n = 138) | 6.2 ± 2.4 (n = 327) | 0.067‖ |
| Cholesterol (mmol/L) | 4.9 ± 1.2 (n = 425) | 5.0 ± 1.3 (n = 133) | 4.8 ± 1.1 (n = 292) | 0.037‖ |
| Triglycerides (mmol/L) | 1.8 ± 1.0 (1.4; 0.5–7.4) (n = 419) | 1.8 ± 1.0 (1.52; 0.66–6) (n = 131) | 1.7 ± 1.0 (1.3; 0.5–7.4) (n = 288) | 0.092‡ |
| Urea (mmol/L) | 7.7 ± 8.3 (6.1; 1.9–145) (n = 415) | 7.5 ± 3.8 (7; 2.4–24.5) (139) | 7.8 ± 9.6 (6; 1.9–145) (n = 276) | 0.073‡ |
| Creatinine (μmol/L) | 100 ± 45 (n = 417) | 100 ± 36 (n = 139) | 99 ± 49 (n = 278) | 0.880‖ |
| Leukocytes ($x10^9$/L) | 8.6 ± 2.5 (n = 409) | 9.4 ± 2.4 (n = 136) | 8.3 ± 2.5 (n = 273) | < 0.001‖ |
| Platelets ($x10^9$/L) | 219 ± 64 (n = 408) | 213 ± 61 (n = 135) | 222 ± 64 (n = 273) | 0.122‖ |

**Results are present as mean ± standard deviation and (median; range) with (number of patients); * – probability for difference between the values of the case and the control group; † n.a. – not applicable (testing was not done as the values above upper normal limits during the whole study period were an exclusion criterion for the control group); ‡ – Mann–Whitney U test; ‖ – *t*-test.**

The most frequently prescribed drug in the study population was acetylsalicylic acid, followed by the inhibitors of angiotensin-converting enzyme (ACE), selective beta blocking agents, clopidogrel, atorvastatin, proton pump inhibitors, organic nitrates, enoxaparin sodium, trimetazidine, high-ceiling diuretics, amiodarone, benzodiazepines, dihydropyridines, $H_2$ receptor antagonists, metformin, xanthines, spironolactone and sulphonylureas. Other drugs were prescribed in less than 5% of the study patients and due to low frequency of the use, they were excluded from further analysis.

The primary analysis of factors associated with liver injury was performed with hypothesis testing of differences of study variables between the case and control groups of study patients (Tables 1–3). In the case group, 114 (81.4%) of the patients had hepatocellular type of the liver injury, 9 (6.4%) cholestatic and 17 (12.2%) mixed-type of the hepatic injury. Numerous demographic and clinical characteristics, laboratory parameters and drugs were differently distributed between two study groups with statistical significance. Previous occasional alcohol intake and current obesity, type of arrhythmia and diabetes mellitus type 2, cholesterol levels and leukocytosis as well as amiodarone and enoxaparin had higher magnitude of association with the liver injury within their risk-factor groups (Table 1).

In the model of multivariable binary logistic regression among nine putative risk factors for hepatotoxicity, which

we selected based on statistical significance, existing knowledge and clinical reasoning, six (alcohol intake, amiodarone, enoxaparin, obesity, atorvastatin, CCI score) had independent association with the liver injury (Table 4).

**Table 3**

**The most used drugs in the Case group (patients with hepatotoxicity) and the Control group (patients without hepatotoxicity)**

| Drugs | Case group (n=140) | Control group (n=420) | *p* ($\chi^2$-test) |
|---|---|---|---|
| $H_2$ receptor antagonists | 32 (22.9) | 40 (9.5) | < 0.001 |
| Proton pump inhibitors | 96 (68.6) | 174 (41.4) | < 0.001 |
| Metformin | 8 (5.7) | 62 (14.8) | 0.005 |
| Sulphonylureas | 7 (5.0) | 33 (7.9) | 0.256 |
| Enoxaparin sodium | 89 (63.6) | 104 (24.8) | < 0.001 |
| Clopidogrel | 105 (75.0) | 225 (53.6) | < 0.001 |
| Acetylsalicylic acid | 124 (88.6) | 326 (77.6) | 0.005 |
| Amiodarone | 70 (50) | 66 (15.7) | < 0.001 |
| Organic nitrates | 75 (53.6) | 192 (45.7) | 0.107 |
| Trimetazidine | 54 (38.6) | 120 (28.6) | 0.027 |
| High-ceiling diuretics | 62 (44.3) | 108 (25.7) | < 0.001 |
| Spironolactone | 16 (11.4) | 44 (10.5) | 0.752 |
| Beta blocking agents, selective | 93 (66.4) | 256 (61.0) | 0.247 |
| Dihydropyridine derivatives | 16 (11.4) | 75 (17.9) | 0.074 |
| ACE inhibitors | 97 (69.3) | 253 (60.2) | 0.055 |
| Atorvastatin | 105 (75.0) | 222 (52.9) | < 0.001 |
| Benzodiazepine derivatives | 31 (22.1) | 87 (20.7) | 0.720 |
| Xanthines | 25 (17.9) | 42 (10.0) | 0.013 |

**Results are present as the number (percentage) of patients.**
**ACE – angiotensin converting enzyme.**

**Table 4**

**Factors significantly associated with liver injury according to the univariable and multivariable binary logistic regression analysis**

| Variable | Logistic regression | |
|---|---|---|
| | univariable | multivariable |
| Obesity | 4.13 (2.23–7.65; < 0.001) | 2.78 (1.15–6.71; 0.023) |
| Coronary heart disease | 1.74 (1.12–2.71; 0.014) | n.a. |
| Arrhythmia | 2.62 (1.69–4.05; < 0.001) | n.a. |
| Heart failure (EF< 45%) | 1.71 (1.06–2.76; 0.028) | n.a. |
| Diabetes mellitus | | |
| type 1 | 0.44 (0.23–0.86; 0.016) | n.a. |
| type 2 | 1.77 (1.04–3.00; 0.035) | n.a. |
| CCI total score | 1.80 (1.58–2.07; < 0.001) | 1.89 (1.53–2.34; < 0.001) |
| Occasional alcohol intake | 113.31 (40.09–320.27; < 0.001) | 96.47 (28.95–321.43; < 0.001) |
| Smoking | 2.64 (1.62–4.31; < 0.001) | 1.92 (0.84–4.38; 0.121) |
| $H_2$ receptor antagonists | 2.82 (1.69–4.70; < 0.001) | n.a. |
| Proton pump inhibitors | 3.08 (2.06–4.63; < 0.001) | n.a. |
| Metformin | 0.35 (0.16–0.75; 0.007) | 0.14 (0.04–0.51; 0.003) |
| Enoxaparin | 5.30 (3.52–7.98; < 0.001) | 3.29 (1.79–6.05; < 0.001) |
| Clopidogrel | 2.60 (1.70–3.99; < 0.001) | n.a. |
| Acetylsalicylic acid | 2.24 (1.26–3.95; 0.006) | n.a. |
| Amiodarone | 5.36 (3.51–8.19; < 0.001) | 3.70 (1.82–7.53; < 0.001) |
| Trimetazidine | 1.57 (1.05–2.34; 0.027) | n.a. |
| High-ceiling diuretics | 2.30 (1.54–3.42; < 0.001) | 1.04 (0.53–2.03; 0.916) |
| Atorvastatin | 2.68 (1.74–4.10; < 0.001) | 2.67 (1.33–5.38; 0.006) |
| Xanthines | 1.96 (1.14–3.35; 0.014) | n.a. |
| Number of hepatotoxic drugs | 1.77 (1.41–2.21; < 0.001) | n.a. |

**Results are present as odd ratios (95% confidence interval; probabilty); n.a. – not applicable (the variable was not included in the multivariable model).**
**EF ejection fraction; CCI – Charlson Comorbidity Index.**

The whole model (with all putative predictors) was statistically significant ($p < 0.001$) with Cox & Snell R Square $p = 0.457$ and Hosmer-Lemeshow test $p = 0.279$. There was no significant multicollinearity between the predictors. The model was also stable after the introduction of interaction of amiodarone and CCI score which was insignificant ($p = 0.251$). Alkaline phosphatase, creatine phosphokinase, creatine phosphokinase-MB, total serum proteins, C-reactive protein, cholesterol, white blood cell count were also statistically associated with the liver injury within univariate binary logistic regression analysis, but the magnitudes of their Ors were very tiny and their lower confidence intervals touched the one and they were excluded from the model.

The analysis placed the drugs with possible hepatotoxic effects on the top among other risks for the liver injury in hospitalized cardiac patents. Causal assessment of drug-associated liver injury in the case group using CIOMS/RUCAM scoring scale additionally confirmed these findings. The average value of the total score in patients of the case group was 7.8 (SD = 1.3, from 5.0 to 11.0). Out of 140 cases, in 39 (27.8%) of the patients, the medicine causality was assessed as highly probable (CIOMS/RUCAM score ≥ 9), in 100 (71.4%) patients as probable (score 6-8) and in 1 (0.7%) patient as possible (score 3–5). Amiodarone had the highest prescription rate and the median of defined daily dose of amiodarone was 112.5 (range 3.6–800) per 100 patients' hospital days. In the case group amiodarone utilization was 193.8 (range 8.3–412.5) DDD per 100 patients' hospital days and 75.0 (range 3.6–800.0) DDD per 100 patients' hospital days in the control group ($p < 0.001$). Amiodarone was administered parenterally, orally and via both routes in 44 (31.4%), 5 (3.6%) and 21 (15.0%) of the case study subjects and in 11 (2.6%), 45 (10.7%) and 10 (2.4%) of the control study subjects, respectively ($p < 0.001$).

## Discussion

The result of our research showed that occasional alcohol intake, obesity, combined significant comorbidities and prescription of amiodarone, enoxaparin and atorvastatin were independently associated with the liver injury in hospitalized patients with cardiac diseases. In addition, we established the rank order for hepatotoxicity of commonly prescribed drugs in patients of cardiology wards with amiodarone representing the greatest risk. We also noted significant strength of the association of drug prescription and liver damage, which has been little studied so far for the investigated study population.

Prehospital alcohol intake was the most significant independent risk factor for hepatotoxicity in subjects of our study despite the fact that manifested alcoholism was an exclusion criterion. Therefore, the study patients were those who either consumed alcohol infrequently or in small quantities, but who, at the time of being included in the study, had neither symptomatic nor asymptomatic liver damage. Unique characteristics of hospitalized cardiac patients (eg. hypotension, hepatic ischemia, liver congestion, hepatotoxic cardiovascular drugs) probably potentiate well-known hepatotoxic action of alcohol even if it has been consumed in minute quantities before hospital admission [17–19].

In our study, many patients had both obesity and ultrasound findings of fatty liver, which suggested the presence of nonalcoholic fatty liver degeneration. We did not obtain pathological findings of patients' liver tissues and hospital patient's record usually does not include data necessary for evaluation of visceral (central) type of obesity (eg. waist circumference) which is primarily associated with liver disease [20]. However, numerous previous published studies provided the strong association of obesity-triggered nonalcoholic fatty liver degeneration and coronary heart disease, the later being very prevalent in our study subjects [21–24].

Patients with a large number of comorbidities in our study, as assessed with CCI score, had significantly higher probability of the liver injury, independently of other factors. Several cardiovascular conditions associated with the case study subjects in univariable analysis; however, we decided to include variables of two main cardiac disorders (coronary heart disease, heart failure) as well as other condition related to atherosclerosis and metabolic disbalance (peripheral vascular disease, cardiovascular accident, transient ischemic attack, hemiplegia, diabetes) within the composite, comorbidity assessment tool in order to decrease confounding by indication (indication bias) and increase the model performance for detecting drug-induced liver injury. For example, congestive heart failure is a common cause of acute liver injury in hospitalized patients [25]. Previous studies confirmed that higher CCI scores did put the patient in increased mortality risk, but the association with acute hepatotoxic damage was little investigated, at least in patients treated in cardiology wards [26]. Therefore, our findings could be considered a novelty in the field, which deserves further validation research.

Three drugs in patients of our study, amiodarone, enoxaparin and atorvastatin, were strongly associated with newly appeared, acute hepatic damage. Drug pharmacological profiles and accumulated, overall knowledge about the role of pharmaceuticals, in general, for various types of hepatic injuries support such results [27]. Many patient-specific factors in subjects with advanced cardiovascular disease and/or cardiac emergencies admitted to hospital mutually interplay, predisposing to drug induced hepatotoxicity. For example, unstable coronary heart disease causes worsening of existing arrhythmia or emergence of novel rhythm disorders which need escalation of drug treatment. Indeed, prescription of amiodarone led other, numerous drugs with hepatotoxic actions.

Our study was neither designed nor adequately powered to discriminate hepatotoxic action of two amiodarone formulations, but some issues in our results and literature data (eg. short duration of hospital stay until the appearance of liver injury, higher defined daily doses, significant differences in route of use between study group, known facts about possible hepatotoxicity of pharmaceutical excipients in parenteral formulation) indirectly suggest that parenteral administration was a primary risk factor [28, 29].

Enoxaparin and atorvastatin had also positive and significant association with hepatotoxicity in our study in compa-

rison with the use in the control subjects. Enoxaparin could increase liver transaminase levels and, in some cases, may cause toxic hepatitis due to temporary necrosis of hepatocytes, usually around one week after the treatment initiation and in a dose-dependent manner [5, 30–32]. Atorvastatin had well-known hepatotoxic potential, which could manifest with a wide range of clinical features, from asymptomatic increase of liver enzymes to drug-induced hepatitis in different periods from the time of treatment initiation [4]. Prescription of lower doses (not exceeding 40 mg daily), delayed action and contribution of numerous other strong risks could diminish the magnitude of association of atorvastatin use with liver injury in our study subjects in comparisons with other two drugs.

The limitations of our study are mainly inherited from its observational design, which well comprehends feature of case-control research. Many important data, necessary for better characterization of the type and time course of liver injury were missing in medical records (eg. liver tissue pathology and higher-performance biomarkers). Although our study includes information from several hundreds of patients it seems that the final sample size was sufficiently powered to detect only the major determinates of acute hepatic damage. Even within our analysis we noted numerous significant associations of putative risks, but the majority of them were not included in the final regression model due to presumed statistical constraints and/or clinical reasoning (eg. confounding, collinearity). For example, it seems that the associations of factors such as smoking and high-ceiling diuretics use (almost exclusively furosemide) had been confounded with obesity and decompensated heart failure requiring escalation of drug treatment, respectively, rather than caused by their direct hepatotoxic actions [33].

Our finding that metformin takes independent and protective associations suggests that it was justifiable to exclude the majority of factors with doubtful direct influence on hepatic tissue from the final regression model. There are exceptional case reports of metformin-induced hepatotoxicity in humans, but the estimated incidence is extremely low, particularly considering the widespread use of this drug [34, 35]. In fact, true mechanism of hepatic damage due to metformin is unknown and evidence from both the animal models and the clinical settings clearly demonstrated its hepatoprotective effects, too [36–40]. It seems that the inclusion of a drug with the effects of direction opposite to other factors provide the approach more realistic to clinical practice. Taking into account the abovementioned facts as well as the values of parameter estimation of the model, we consider our results accurate and clinically significant.

## Conclusion

Major factors associated with acute liver injury in patients hospitalized in cardiology ward of a tertiary health care institution are patient's constitutional and habitual characteristics (occasional alcohol intake, obesity, CCI total score) and drugs (amiodarone, enoxaparin, atorvastatin) with known hepatotoxic potential. Type and severity of primary cardiovascular disease or comorbid condition can increase the risk for liver injury primarily in synergy with other risks, jointly acting with of each other and/or other major hazards. Future studies focusing on individual factors are justified in order to better characterize their effects in different subpopulations of patients with particular cardiac illnesses.

## Conflict of interest

There is no conflict of interest for any author.

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# Lung ultrasound for volume status assessment in chronic hemodialysis patients

## Ultrasonografija pluća u proceni hipervolemije kod bolesnika na hemodijalizi

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**Abstract**

**Background/Aim.** Assessing volume status in chronic hemodialysis (HD) patients is difficult despite several techniques have been developed. The aim of this study was to demonstrate the adequacy and efficacy of lung ultrasound (LUS) and B line score (BLS) in the assessment of volume status in patients on HD in comparison to other techniques: ultrasonographic determination of inferior vena cava diameter (IVCD), echocardiography (ECHO) and B-type natriuretic peptide (BNP) determination. **Methods.** LUS, ECHO, ultrasonography of inferior vena cava in inspiration (IVCDi) and expiration (IVCDe), and BNP sampling were performed before and after HD in 83 patients. **Results.** A significant reduction of BLS, IVCDi, IVCDe, BNP and several ECHO parameters such as left atrium diameter (LA), left ventricular internal dimension in diastole and systole (LVIDd and LVIDs, respectively), and left atrial volume in systole (LAVs), was registered ($p < 0.001$). There was a significant correlation between BLS and BNP before ($p = 0.01$) and after HD ($p = 0.05$), and a weaker but significant correlation between BLS and IVCDi and IVCDe before HD ($p = 0.05$). **Conclusion.** All techniques assessed hypervolemia before and after HD successfully. BNP correlated with LUS before and after HD, and IVCDi and IVCDe correlated with LUS only before HD. LUS is cheap and simple to perform, can be performed bedside and can be reliably used for assessing volume status in HD patients.

**Key words:**
**renal hemodialysis; lung; ultrasonography; natriuretic peptide, brain; vena cava, inferior; echocardiography; water-electrolyte imbalance.**

**Apstrakt**

**Uvod/Cilj.** Procena stanja volemije bolesnika na hroničnom programu hemodijalize (HD) je teška uprkos postojećim razvijenim tehnikama. Cilj ove studije je bio da pokaže adekvatnost i efikastnost ultrazvuka pluća (UZP) i B linijskog skora (BLS) u proceni stanja volemije kod bolesnika na HD u poređenju sa drugim tehnikama: određivanjem dijametra donje šuplje vene (DŠVD) ultrazvukom, ehokardiografijom (ECHO) i određivanjem nivoa B tipa natriuretskog peptida (BNP) u krvi. **Metode.** UZP, ehokardiografija sa ultrasonografijom donje šuplje vene u inspirijumu (DŠVDi) i ekspirijumu (DŠVDe) i uzorkovanje krvi za određivanje BNP su učinjeni pre i posle HD kod 83 bolesnika. **Rezultati.** Registrovano je značajno smanjenje BLS, DŠVDi i DŠVDe, nivoa BNP i nekoliko ehokardiografskih (EHO) parametara (dimenzije leve pretkomore, komore u sistoli i dijastoli i zapremina leve pretkomore) ($p < 0.001$). Postojala je značajna korelacija između BLS i BNP pre i posle HD ($p = 0.01$), između BLS i EHO parametara ($p = 0.01$), i slabija, ali ipak značajna korelacija između BLS i DŠVDi i DŠVDe pre HD ($p = 0.01$). **Zaključak.** Svim tehnikama je uspešno procenjena hipervolemija pre i posle HD. Vrednosti BNP su korelisale sa nalizom UZP pre i posle HD, a vrednosti DŠVDi i DŠVDe su korelisale sa UZP, uglavnom, pre HD. UZP je jeftina i jednostavna tehnika, može se izvoditi pored postelje i pouzdano koristiti za procenu stanja volemije kod bolesnika na HD.

**Ključne reči:**
**hemodijaliza; pluća; ultrasonografija; natriuretski peptid, moždani; v. cava inferior; ehokardiografija; voda-elektroliti, disbalans.**

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## Introduction

Hemodialysis (HD) has two important goals in restoring homeostasis in patients with end stage renal disease (ESRD). One is the removal of metabolic waste products from the circulation. The other is removal of excess fluid, which is removed by ultrafiltration (the amount of fluid removed based on the patient's "dry weight"). Dry weight represents a "clinically determined lowest body weight that the patient can tolerate without symptoms of hypovolemia during HD, in absence of clear signs of hypervolemia" [1]. Adequate fluid balance in HD patients is very difficult to achieve without an accurate dry weight. Under- and overestimating dry weight leads to hypo- and hypervolemia, respectively. Both hypo- and hypervolemia cause numerous serious complications and increase morbidity and mortality in HD patients [2]. Hypovolemia manifests as intradialytic hypotension, while hypervolemia presents most commonly as high interdialytic gain, arterial hypertension (HTA), peripheral edema and bibasilar crackles on lung auscultation. Complications include left ventricular myocardial hypertrophy (LVMH), heart failure (HF), pulmonary hypertension and pulmonary edema [3].

Treatment of HTA in patients on HD begins with dry weight reduction. This measure is not without risk, as complications of overshooting the target dry weight include intradialytic hypotension, vascular access thrombosis and accelerated decline of residual renal function. All of these are related to low quality of life and worse prognosis [4].

Studies have shown that clinical assessment of fluid status has low specificity and sensitivity in detecting both under- and overhydration [5]. Patients on HD have 10 to 20 times greater cardiovascular mortality in comparison to the general population [4]. Both hypovolemic hypotensive episodes and chronic hypervolemia greatly contribute to it. A new, more specific and sensitive method for volume status assessment is needed in HD setting.

An ideal volume status assessment method would have to be reliable, simple, fast, non-invasive and inexpensive [6]. No currently available method has all of these characteristics, but all perform better than clinical assessment alone [5]. Some of these methods are bioimpedance, continuous volume monitoring, ultrasonographic measurement of inferior vena cava diameter (IVCD), echocardiography (ECHO), B type natriuretic peptide (BNP) and its N terminal prohormone (NT- proBNP) blood levels [7]. IVCD correlates with volume status in patients on HD. Wider IVCD are registered before HD. Respiratory variations in IVCD, or their absence are also noted before HD, while after HD a decrease in these diameters is noted, as well as an increase of the index of collapsibility [5]. The use of ECHO is not as clearly defined as ultrasonographic measurement of IVCD, but some ECHO parameters show a significant reduction after HD (diameter and volume of the left atrium, end-diastolic and end-systolic left ventricular volume) [8]. BNP, a polypeptide secreted by cardiomyocytes in response to their stretching, can be used as a marker for hypervolemia in patients on HD. Elevated BNP levels are registered in 100% of patients on chronic HD, and these levels decline after dialysis [9]. This reduction can be explained by increased dialytic clearance of BNP or by better volume control leading to a reduction in left ventricle wall stretching and reduced BNP secretion [10].

Some of the flaws of these methods are high price of equipment, high price of supplies and laboratory reagents, longer duration of the examination and the need for specially trained medical staff to perform these ultrasonographic examinations [7]. A new method for assessing volume status in this patient group is needed.

Lung ultrasound (LUS) has emerged as a new method for volume status assessment in patients on HD. LUS works by detecting extravascular lung water (EVLW). EVLW represents the amount of water present in lungs outside of the pulmonary vasculature. It shows the amount of lung congestion, and is elevated in states of hypervolemia, low oncotic pressure and/or increased lung capillary permeability [10]. LUS detects EVLW by registering it as sonographic artifacts called B lines [5]. A B line represents an acoustic shadow that forms when an ultrasonic wave meets edematous interlobulus septa in the lungs [9]. The exam is performed by placing the probe on 28 predetermined points on the chest and then adding the number of registered B lines, thus getting the B line score (BLS) [7].

LUS is a method that has numerous benefits: it is fast, simple, low-cost and readily accessible to secondary health centers, requiring only an ultrasound machine with a convex, linear or sector probe [5].

This article did not focus on the cause of hypervolemia and did not take the patients' cardiologic status, New York Heart Association (NYHA) class, comorbidities or pharmacotherapy into account. Our focus was volume status determined by BLS as a new method and its correlation to volume status assessed by other methods. This approach is justified by the fact that lung congestion quantified by LUS represents a significant predictor of patient survival, irrespective of heart function [2].

Our goal was to demonstrate the adequacy and efficacy of LUS in the assessment of volume status in patients on HD in comparison to other methods: ultrasonography of IVC, ECHO and BNP levels.

## Methods

The study was performed from April 2016 to June 2017 on 84 patients in the Dialysis Unit of the Department of Internal Medicine, General Hospital in Kikinda. Inclusion criteria were: age equal to or greater than 18 years and time on chronic HD program longer than 3 months. Exclusion criteria were: patient declining or unable to sign the informed consent, chronic pleuritis, pulmonary fibrosis and pneumectomy. One patient met the exclusion criteria due to dementia, being unable to sign the informed consent, the other 83 were enrolled in the study.

Every patient was clinically evaluated before, during and after dialysis. Lung and heart auscultation, arterial blood pressure, body weight before (pre HD) and after (post HD) dialysis were recorded.

Measurements were made on the first weekly dialysis. LUS, ECHO, ultrasonography of IVC and collection of blood samples for BNP measurements were made immediately prior to dialysis. All patients then underwent HD according to their usual dialysis protocols. After dialysis all measurements were repeated.

LUS was performed before HD using a Samsung Medison MySono U6 portable ultrasound system, with a 3.5 MHz convex probe. Measurement was done in a semi recumbent position. This was chosen as this is how most of our patients lie down during HD, so that the method could later be used in the bedside setting. The probe was placed longitudinally in the 2nd intercostal space on the left parasternal line, and the "bat sign" was seen on screen (Figure 1). The "bat sign" represents a normal LUS finding where the upper and the lower ribs represent the "wings" and the pleural line represents the "back" of the bat. Placing the probe was continued along the 2nd intercostal space in the medioclavicular, anterior axillar and midaxillary line and then in the same fashion along the 3rd and 4th intercostal spaces on the left and the 2nd, 3rd, 4th and 5th intercostal spaces on the right.

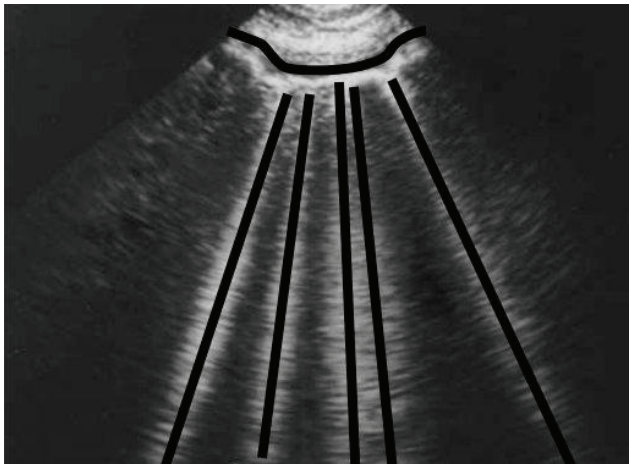

**Fig. 1 – Lung ultrasound with visible B lines. Bat sign marked with horizontal curved black line; B lines highlighted with straight vertical lines (Modified image, original image by Lichtenstein et al. [12], Creative Commons License 2.5).**

B lines were seen as hyperechogenic dynamic vertical wedge-shaped lines that start at the pleural line and spread to the bottom of the screen without loss of intensity (Figure 1). B lines were counted and noted in a form (Table 1) for each of the 28 positions on the anterolateral chest. The sum of these numbers represents the B line score (BLS). The BLS of less than 8 is considered normal. Lung congestion is estimated as light if the BLS is between 8 and 13, medium if it is between 14 and 30 and severe if the BLS equals more than 30 [7].

Echocardiography and IVC ultrasound were performed using Esaote MyLab™Six cardiovascular ultrasound system. ECHO diameters were measured in parasternal view using M mode, volumes were measured in four chamber view, using the area length formula. Systolic dysfunction was defined as ejection fraction of les then 55%. Diastolic dysfunction was determined and graded according to recommendations of the American Society of Echocardiography from April 2016 [13]. IVC diameters were measured in subxiphoid view, 2,5 cm from the right chamber, in non-forced expirium and inspirum. Values were indexed by body surface area, calculated using Du Bois formula.

Blood samples for determination of pre HD BNP levels were collected just prior to initiation of HD. Blood samples for post HD BNP levels were collected immediately before the second dialysis of the week (next HD session), in order to avoid the rise of BNP immediately after HD because of hemoconcentration. BNP blood levels were measured by an immunochemical method on a Siemens ADVIA Centaur® CP Immunoassay System.

We expressed continuous variables as mean ± standard deviation, except when otherwise noted. Categorical variables are presented as numbers and percentages. The distribution of obtained data was tested for normality using the Shapiro-Wilk test. Based on the distribution, differences between techniques pre and post HD were determined using either the paired-sample *t*-test or Wilcoxon test. Correlations between continuous variables were assessed either with the Pearson's coefficient of correlation for normally distributed data or with Spearman's coefficient of correlation for non-normally distributed data. Statistical significance was set at $p < 0.05$. Statistical analysis of the data was performed by use of the commercial statistical software IBM SPSS 23.

**Table 1**

**B line score form**

| Right hemithorax | | | | | Left hemithorax | | | |
|---|---|---|---|---|---|---|---|---|
| Midaxilar | Anterior axilar | Midclavicular | Parasternal | Intercostal space | Parasternal | Midclavicular | Anterior axilar | Midaxilar |
| | | | | II | | | | |
| | | | | III | | | | |
| | | | | IV | | | | |
| | | | | V | / | / | / | / |

## Results

Totally, 83 patients were enrolled in this study. Their anthropometric and clinical characteristics are shown in Table 2.

**Table 2**
**Anthropometric and clinical characteristics of patients**

| Patients' characteristics | Values |
|---|---|
| Number of patients | 83 |
| Age (years), mean ± SD | 61.02 ± 11.74 |
| Male/female, n/n | 58/25 |
| Hi-flux/low-flux filters, n/n | 65/18 |
| Kt/V, mean ± SD | 1.14 ± 0.22 |
| MAPpre (mmHg), mean ± SD | 94.10 ± 13.10 |
| MAP post (mmHg), mean ± SD | 90.86 ± 14.00 |
| Ultrafiltration (L), mean ± SD | 3.05 ± 1.00 |
| Weight pre HD (kg), mean ± SD | 76.68 ± 16.64 |
| Weight post HD (kg), mean ± SD | 73.91 ± 16.36 |
| Hgb (g/L), mean ± SD | 105.78 ± 17.78 |
| Alb (g/L), mean ± SD | 38.03 ± 4.00 |
| Normal systolic function n (%) | 41 (49%) |
| Systolic dysfunction, n (%) | 42 (51) |
| Normal diastolic function, n (%) | 27 (33%) |
| Diastolic dysfunction, n (%) | 56 (67%) |

**MAP – mean arterial pressure; HD – hemodialysis, Hgb – hemoglobin, Alb –albumin; SD – standard deviation.**

Effects of dialysis on the BLS, IVC diameters, collapsibility index, ECHO parameters and BNP levels are shown in Table 3.

**Table 3**
**Differences in volume status parameters before hemodialysis (pre HD) and after hemodialysis (post HD)**

| Method | Reference interval | Pre HD | Post HD | *p* |
|---|---|---|---|---|
| BLS* | < 8 | 18.85 | 7.3 | < 0.0001 |
| BNP (pg/mL)* | < 400 | 914.07 | 486.57 | < 0.0001 |
| MAP (mmHg)† | 70–100 | 94.1 | 90.86 | 0.0094 |
| LA (cm)* | 2.7–3.8 (f)<br>3.0–4.0 (m) | 3.78 | 3.53 | < 0.0001 |
| LVIDd (cm)* | 3.9–5.3 (f)<br>4.2–5.9 (m) | 5.21 | 4.96 | < 0.0001 |
| LVIDs (cm)† | 2.0–2.6 | 3.69 | 3.43 | < 0.0001 |
| LAVs (mL)* | 22–52 (f)<br>18–58 (m) | 60.54 | 52.36 | < 0.0001 |
| IVCDe (mm/m$^2$)† | 8–11.5 | 10.45 | 7.85 | < 0.0001 |
| IVCDi (mm/m$^2$)† | - | 7.19 | 4.41 | < 0.0001 |
| CCI (%)* | > 50 | 0.32 | 0.45 | < 0.0001 |

***Paired sample *t*-est; †Wilcoxon signed-rank test.**
**BLS – B line score; BNP – B type natriuretic peptide; MAP – mean arterial pressure; LA – left atrium; LVIDd – left ventricular internal dimension (diastole); LVIDs – left ventricular internal dimension (systole); LAVs – left atrial volume (systole); IVCDe – inferior vena cava diameter (expirium); IVCDi – inferior vena cava diameter (inspirium); CCI – inferior vena cava collapsibility index; m – males; f – females.**

*Lung ultrasound*

We were able to perform LUS in all patients (100% feasibility). Pre HD BLS ranged from 1 to 159 (mean 19). According to LUS 22/83 (27%) of the patients had normal volume status, 30/83 (36%) had mild hypervolemia, 19/83 (23%) had moderate hypervolemia and 12/83 (14%) had severe hypervolemia. Post HD BLS values ranged from 0 to 109 (mean 7). According to LUS, 65/83 (78%) of the patients had normal volume status post HD, 8/83 (10%) had mild, 8/83 (10%) had moderate and 2/83 (2%) still had severe hypervolemia. A significant reduction of the BLS of 64% was achieved post HD ($p < 0.001$).

The distribution of B lines can be seen in Figure 2.

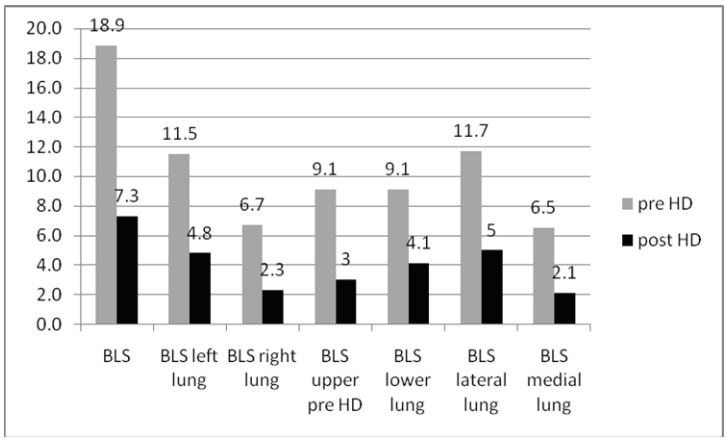


**Fig. 2 – Number of B lines detected over each hemithorax, left and right, upper and lower and lateral and medial hemithorax, before and after dialysis.**
**BLS – B lines score; preHD – pre hemodialysis; postHD – posthemodialysis.**

There was a significant difference in the BLS pre HD between the right and left lung (11.46 ± 14.33 vs. 6.75 ± 10.53, respectively; $p < 0.001$) and medial and lateral parts of the chest (6.48 ± 12.08 vs. 11.72 ± 12.88, respectively; $p < 0.001$), while there was no significant difference between upper and lower parts of the chest (9.08 ± 13.66 vs. 9.14 ± 10.54, respectively; $p = 0.9$). Post HD BLS differed significantly between the left and right lung (4.77 ± 6.90 vs. 2.31 ± 6.90, respectively; $p < 0.001$), upper and lower parts of the chest (2.99 ± 7.78 vs. 4.10 ± 6.17, respectively; $p = 0.01$) and medial and lateral parts of chest (2.11 ± 7.13 vs. 4.98 ± 6.95, respectively; $p < 0.001$). There was a significant correlation between the BLS preHD and BNP pre HD ($\rho = 0.49$, $p < 0.001$), as well as between the BLS pre HD and BNP post HD ($\rho = 0.45$, $p < 0.001$). A weaker significant positive correlation was noted between IVCDe pre HD and the BLS both pre HD and post HD, IVCDi pre and post HD and the BLS pre HD, as shown in Table 4.

**Table 4**
**Spearman's correlation between lung ultrasound and other volume status assessment techniques**

| Parameter | BLS pre HD | BLS post HD |
|---|---|---|
| BLS pre HD | - | $\rho = 0.81^{\dagger}$ |
| BLS post HD | $\rho = 0.81^{\dagger}$ | - |
| BNP pre HD | $\rho = 0.49^{\dagger}$ | $\rho = 0.45^{\dagger}$ |
| BNP post HD | $\rho = 0.44^{\dagger}$ | $\rho = 0.42^{\dagger}$ |
| IVCDe pre HD | $\rho = 0.29^{\dagger}$ | $\rho = 0.22$* |
| IVCDe post HD | $\rho = 0.27^{ns}$ | $\rho = 0.29^{ns}$ |
| IVCDi pre HD | $\rho = 0.26^{*}$ | $\rho = 0.21^{ns}$ |
| IVCDi post HD | $\rho = 0.23^{*}$ | $\rho = 0.23^{*}$ |
| CCI pre HD | $\rho = -0.20^{ns}$ | $\rho = -0.23^{*}$ |
| CCI post HD | $\rho = -0.16^{ns}$ | $\rho = -0.13^{ns}$ |

**BLS – B line score, BNP – B type natriuretic peptide; IVCDe – inferior vena cava diameter (expirium); IVCDi – inferior vena cava diameter (inspirium); CCI – inferior vena cava collapsibility index; HD – hemodialysis. Statistically significant difference: $^{*}p < 0.05$, $^{\dagger}p < 0.01$, ns – no significance.**

*Echocardiography and inferior vena cava ultrasonography*

Left ventricular ejection fraction (LVEF) ranged from 29% to 70% (mean 54 ± 8%). Systolic dysfunction was present in 42/83 (51%) of the patients, while 41/83 (49%) of the patients had normal systolic function. Normal diastolic function was present in 27/83 (33%), while diastolic dysfunction was present in 56/83 (67%) of the patients. Grade I diastolic dysfunction was present in 36/83 (43%) of the patients, grade II diastolic dysfunction in 9/83 (11%) and grade III in 11/83 (13%) of the patients.

IVC dimensions in expirium pre HD ranged from 5.74 mm to 16.14 mm (mean 10.45 ± 2.28 mm) and from 3.57 mm to 13.69 mm (mean 7.85 ± 2.18 mm) post HD. Inferior vena cava dimension in inspirium pre HD ranged from 0.96 mm to 15.12 mm (mean 7.19 ± 2.70 mm) and from 0.65 mm to 15.12 mm (mean 4.41 ± 2.19 mm) post HD. The inferior vena cava collapsibility index (CCI) was calculated according to the formula:

$$CCI = \frac{IVCDe - IVCDi}{IVCDe} \times 100\%$$

and ranged from 6% to 91% (mean 32±15%) pre HD and from 11% to 92% (mean 45 ± 17%) post HD. A significant reduction of both IVCDe (25%) and IVCDi (38%) was registered post HD ($p < 0.0001$), as well as significant increase in the CCI of 58% ($p < 0.0001$).

According to IVCDe dimensions (cutoff value of 11.5 mm/m$^2$) 24/83 (29%) of the patients were hypervolemic pre HD, while 59/83 (71%) had normal volume status. After dialysis only 5/83 (6%) were hypervolemic, while 78/83 (94%) had normal hydration status.

A significant positive correlation detected between IVCe pre HD and IVCe post HD (r = 0.77, $p < 0.001$), IVCi pre HD and IVCi post HD (r = 0.77, $p < 0.001$), IVCe pre HD and IVCi pre HD (r = 0.62, $p < 0.001$), IVCi pre HD and IVCe pre HD (r = 0.81, $p < 0.001$), IVCi pre HD and IVCe post HD ($p = 0.67$, $p < 0.001$), IVCe post and IVCi post HD (r = 0.77, $p < 0.001$) was noted.

A significant positive correlation was found between the BLS pre HD and post HD and several ECHO parameters (Table 5).

**Table 5**
**Spearman's correlation between lung ultrasound and echocardiographic parameters**

| Parameter | BLS pre HD | BLS post HD |
|---|---|---|
| LA pre HD | $\rho = 0.31^{\dagger}$ | $\rho = 0.31^{\dagger}$ |
| LA post HD | $\rho = 0.30^{\dagger}$ | $\rho = 0.28^{*}$ |
| LVIDdpre HD | $\rho = 0.28^{\dagger}$ | $\rho = 0.23^{*}$ |
| LVIDd post HD | $\rho = 0.27^{*}$ | $\rho = 0.17^{ns}$ |
| LVIDs pre HD | $\rho = 0.31^{\dagger}$ | $\rho = 0.23^{*}$ |
| LVIDs post HD | $\rho = 0.32^{\dagger}$ | $\rho = 0.21^{ns}$ |
| LAVS pre HD | $\rho = 0.32^{\dagger}$ | $\rho = 0.28^{*}$ |
| LAVS post HD | $\rho = 0.33^{\dagger}$ | $\rho = 0.29^{\dagger}$ |

**BLS – B line score; LA – left atrium diameter; LVIDd – left ventricular internal dimension in diastole; LVIDs – left ventricular internal dimension in systole; LAVS – left atrial volume in systole.**
**Statistically significant difference: $*p < 0.05$, $\dagger p < 0.01$, ns = no significance.**

*BNP*

Pre HD BNP ranged from 59.3 pg/mL to 5,000 pg/mL [median 484,5, interquartile range (IQR) 954,7]. Post HD BNP ranged from 16.4 to 4,253.2 (median 197.9, IQR 473.3). We considered BNP levels above 400 pg/mL elevated. There were normal BNP levels before HD in 36/83 (43%) of the patients and 47/83 (57%) of the patients had elevated BNP levels. Post HD, 54/83 (65%) of the patients had normal BNP levels and 29/83 (35%) of the patients had elevated BNP levels.

## Discussion

The aim of our study was to validate LUS for assessment of volume status in patients on chronic hemodialysis by performing it pre and post HD and by comparing it to other

methods of volume status assessment - ECHO, BNP, IVCD in inspirium and expirium including the CCI [5]. Their flaw is the requirement of specially trained staff (cardiologist), and an adequate echocardiographic window, which not all patients have. This goes even more for other ECHO parameters, such as diameters of LA, LV, volume of LA, LV, which require the use of echocardiographic cabinet, a cardiologist, an adequate chest anatomy and more time for the exam.

Our sample population showed a male predominance. Half of our patients had systolic and two thirds had diastolic dysfunction. Other authors had similar samples [2, 11, 14–20], while others had samples with low number of cardiac patients [7, 16]. Since 80% of all hemodialysis patients have some cardiac disease [ischemic heart disease (39%), congestive heart failure (40%), arrhythmia (31%) and other heart conditions (63%)] this sample is acceptable [17].

What we found is that all techniques for volume assessment showed its significant reduction post HD. This shows that the techniques in question can detect hypervolemia and can detect changes in volume status. This conclusion is supported by a number of studies [6, 7, 11, 14–16, 18–22]. What was also established was that a significant correlation exists between these methods, albeit of varying degree. This is also supported by studies by Vitturi et al. [22], Alexiadis et al. [23], Basso et al. [7] and Donadio et al. [6]. What was interesting was that no method in our study showed a correlation with reduction of weight during HD. Similar results were reported by several studies [2, 14]. Others found this not to be the case [16, 22]. This could be explained by the notion that IVC diameters are representative of intravascular filling grade, and LUS via BLS quantifies the level of extravascular imbibition of the lung interstitium. IVC diameters are not sensitive to detect volume change in a short amount of time, as in HD. An equilibrium between compartments takes time to be established after dialysis [22]. On the other hand, LUS measures changes in real time, therefore being more practical, as it can be performed immediately after dialysis, or even during dialysis [22].

LUS also takes less time to perform and is not as dependent on patients' anatomy. The staff can be educated in this method in a relatively short time [5]. It can be performed at patient bedside. The flaw of this method is that it detects extravascular lung water index (EVLW) in the same manner as fibrosis of the lung interstitium so it cannot be used for volume assessment in patients with interstitial lung disease [5].

The BLS was the greatest in the more lateral parts of the lung. Left lung had a greater number of B lines detected. This information can be used to streamline the LUS process and make it even simpler by focusing on the regions where B lines are more numerous.

We found that the BLS correlates with ECHO parameters (LA, LAVS, LVIDd and LVIDs,). This is supported by a study of Mallamaci et al. [11] , who found that the BLS correlates with LVMI, LVES, E/e', LAV, EDLVL. On the other hand, other authors like Siriopol et al. [2] and Weitzel et al. [14] found no association between change of the BLS and ECHO measurements, despite similar study population.

High concentrations of BNP were also reported pre and post HD. While other authors did not report post HD increase of BNP we detected it at the start of our study and adjusted the time of blood sampling for determination of post HD BNP. BNP has an advantage in the setting of HD because of its lower molecular weight, meaning it is readily dialyzed, as opposed to NT- proBNP which is not cleared by low flux filters because of higher molecular weight [24]. We know that the number of B lines correlates with natriuretic peptide levels [5].We also know that despite differences in BNP clearance across filter membranes, several studies support direct association between natriuretic peptides and overhydration in dialysis patients [25]. In our study a positive correlation was shown between the BLS and BNP levels, both pre and post HD. Donadio et al. [6] supported this by reporting similar results in their study, where they established a positive correlation only between pre HD BLS and pre HD BNP. Paudel et al. [20] showed similar results on a positive correlation between the BLS and NT-pro BNP in patients on peritoneal dialysis. On the other hand, Basso et al. [7] found no correlation between the BLS and BNP levels.

Some authors argue that increased levels of circulating BNP and NT- proBNP in patients on HD and their reduction after dialysis indicate that both BNP and NT-proBNP act as composite markers of hypervolemia, systolic dysfunction and left ventricular hypertrophy [9]. It is proven that increased levels of BNP and NT-proBNP are reliable predictors of all-cause and cardiovascular mortality [9].

We found a weaker but significant correlation between pre HD and post HD BLS and pre HD IVCDe and between pre HD and post HD BLS and pre HD IVCDi. No correlation was found between the BLS and CCI. These results are supported by Basso et al. [7] who report positive correlation between IVCD in inspiration (pre and post HD) and expiration (only pre HD) and the CCI (only pre HD). Vitturi et al. [22] report a positive relationship between BLS changes and changes in IVCDe and IVCDi, but not the CCI. However, Trezzi et al. [16] did not find these relationships.

Adjusting of dry weight based on IVC measurements resulted in a significant reduction of intradialytic events such as cramps, nausea, hypotension, an improvement in quality of life, and delayed left ventricle hypertrophy and chamber dilatation in hypervolemic patients on HD when compared with clinical assessment of dry weight [26].

LUS has a few limitations. It cannot differentiate edematous (wet) B lines of pulmonary congestion from fibrotic (dry) B lines, which are related to interstitial pulmonary fibrosis. Integration with other methods can help here, as well as a repeat LUS after an intervention which changes the volume state of the patients, because wet B lines change with volume expansion or depletion, while dry B lines are fixed. It is also not possible to distinguish EVLW accumulation due to heart failure and that of acute respiratory distress syndrome, even though the latter has a more irregular fragmented pattern with alternating spaces of highly concentrated B lines and those that are spared. The third limitation is that LUS cannot detect underhydration [5].

Ther are two flaws of this study. First, it was a single center cross-sectional study on a somewhat heterogeneous sample in terms of cardiac function. Another flaw is that

LUS, like other ultrasonographic methods, is observer dependent.

## Conclusion

LUS can be used to assess volume status in patients on chronic hemodialysis. Our data show that LUS correlates well with BNP levels and slightly less well with IVCD. Overhydration is associated with both greater IVCD and BNP levels, and thus the BLS can also be considered a marker of volume status. LUS is cheap, simple to perform, can be performed bedside and thus can be a reliable indicator for a patient's volume status and can easily be made part of routine patient care protocol.

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# Analysis of personality disorder profiles obtained by five-factor personality model

## Analiza profila poremećaja ličnosti primenom petofaktorskog modela ličnosti

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**Abstract**

**Background/Aim.** In spite of the growing body of evidence in the field of personality disorders, these disorders still retain the lowest diagnostic reliability of any major category of mental disorders. The aim of this study was to investigate the differences of personality profiles in patients diagnosed with personality disorder in comparison with the group of healthy control subjects, as well as to establish to what extent the five-factor personality model domains determine the specific clusters of personality disorders. **Methods.** The study group comprised 97 patients diagnosed as personality disorders (according to the *Diagnostic and Statistical Manual of Mental Disorders* – DSM-IV criteria), aged between 18 and 65 years [mean = 35.78 years, standard deviation (SD) = 13.72 years], 67% were female. Control group included 58 healthy subjects (student population) aged between 20 to 35 years (mean = 22.48 years, SD = 2.56 years), 56% were female. The assessment was carried out by the new version of the NEO Personality Inventory-Revised (NEO-PI-R), form S, and the Structured Clinical Interview (SCID II) for DSM-IV disorders. **Results.** The three clusters were found by the use of regression analysis: cluster A – eccentrics (low scores in agreeableness), cluster B – dramatics (high score in extroversion, low score in agreeableness, and cluster C – anxious (low score in extroversion). The findings showed that the high level of neuroticism was a non-specific predictor of all three clusters, while dimension openness to experience had no predictive power for any of the three clusters. **Conclusion.** Our findings support the meta-analysis which suggests consistently high level of neuroticism and low level of agreeableness in most personality disorders. The study showed that it is possible to conceptualize personality disorders by using five-factor personality model of normal personality. Integrating the psychiatric classification with the dimensional model of general personality structure could enable the uncovering of essential parameters for setting the diagnosis.

**Key words:**
**personality disorders; personality assessment; neuroticism; surveys and questionnaires.**

**Apstrakt**

**Uvod/Cilj.** Uprkos rastućem broju istraživanja u oblasti poremećaja ličnosti, ove poremećaje karakteriše najniža dijagnostička pouzdanost u odnosu na sva druga psihijatrijska oboljenja. Cilj ovog rada bio je da se ispitaju razlike profila ličnosti kod ispitanika sa dijagnozom poremećaja ličnosti u odnosu na kontrolnu grupu zdravih ispitanika i da se utvrdi koliko domeni petofaktorskog modela ličnosti doprinose određivanju specifičnih klastera poremećaja ličnosti. **Metode.** Studijsku grupu činilo je 97 ispitanika sa dijagnozom poremećaja ličnosti [prema kriterijumima Dijagnostičkog i statističkog priručnika za mentalne poremećaje (DSM-IV)], starosti od 18 do 65 godina [srednja vrednost = 35,78 godina, standardna devijacija (SD) = 13,72 godina], od kojih je 67% bilo ženskog pola. Kontrolnu grupu činilo je 58 zdravih ispitanika (studentska populacija), starosti od 20 do 35 godina (srednja vrednost = 22,48 godina, SD = 2,56 godina) od kojih je 56% bilo ženskog pola. Primenjeni su Revidirani novi upitnik ličnosti (NEO-PI-R), forma S, i Instrument za procenu poremećaja ličnosti – Strukturisani klinički intervju (SCID II) za DSM-IV poremećaje. **Rezultati.** Na osnovu regresione analize dobijeno je rešenje za tri klastera: klaster A – ekscentrici (niski skorovi na saradljivosti), klaster B – dramatici (visoki skor na ekstroverziji i nizak skor na saradljivosti) i klaster C – strašljivci (nizak skor na ekstroverziji). Rezultati su pokazali da je visok nivo neuroticizma nespecifični prediktor sva tri klastera, a da dimenzija otvorenost nema prediktorsku snagu ni za jedan klaster poremećaja ličnosti. **Zaključak.** Dobijeni rezultati su u skladu sa nalazima meta-analiza koji ukazuju na konzistentno visok nivo neuroticizma i niske saradljivosti kod većine poremećaja ličnosti. Naša studija je pokazala da je na ispitivanoj populaciji moguće konceptualizovati poremećaj lično-

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sti primenom petofaktorskog modela normalne ličnosti. Integracija psihijatrijske klasifikacije i dimenzionalnih modela ličnosti omogućila bi iznalaženje bitnih parametara za postavljanje dijagnoze.

**Ključne reči:**
**ličnost, poremećaji; ličnost, procena; neuroticizam; ankete i upitnici.**

## Introduction

Current nosological systems (Diagnostic and Statistical Manual of Mental Disorders – DSM, International Classification of Disease – ICD) assume that there are qualitative differences between healthy personality and personality disorder, as well as between specific types of personality disorders [1]. The existing ICD-10 categorical perspective, based on the arbitrary nature of the given criteria and their threshold limit values, leads to a significant diagnostic overlapping, insufficient homogeneity and insufficient stability of diagnostic categories of personality disorders [2].

The need to reconceptualize personality disorders as dimensional taxonomies came as a result of numerous empirical studies conducted both on clinical and general population [3]. According to the available literature, the basic domains of five-factor model have consistently proven to represent the common dimensions of healthy personality structure and personality disorders [4]. In addition, the healthy personality domains could account for a significant part of variance of personality disorder syndrome [5].

Widiger and Costa [6] suggested a model which implies that the personality disorder categories (Diagnostic and Statistical Manual of Mental Disorders – Text revision – DSM-IV-TR) are maladaptive and/or are extreme versions of the domains and facets of the five-factor personality model. Some other authors directed their research more specifically towards particular categories of personality disorders. Hence, Samuel et al. [7] find that borderline personality symptoms lie alongside the same latent dimension as the neuroticism dimension of the five-factor model [7]. In addition to neuroticism, the existence of significant comorbidity of personality disorders as well as other five-factor model dimensions were found [7].

The aims of this study were: a) to investigate the differences in personality profiles by applying the Revised Neo Personality Inventary (NEO-PI-R) [8] in subjects diagnosed with personality disorder in comparison with the control group of healthy subjects, and b) to establish to what extent each NEO five-factor model domain contributes in determining the specific personality disorder clusters – eccentric (A), dramatic (B), and anxious (C).

## Methods

### *Sample*

The study included 155 subjects divided into two groups. The study group comprised 97 patients of the Institute for Mental Health in Belgrade diagnosed as personality disorders (according to DSM-IV criteria), aged between 18 and 65 years [mean = 35.78 years, standrad deviation (SD) = 13.72 years], 67% were female. Control group included 58 healthy subjects (student population) from the Psychology Department of the Faculty of Media and Communications, Singidunum University, Belgrade, aged between 20 to 35 years (mean = 22.48 years, SD = 2.56 years), 56% were female.

### *Assessment*

The Revised NEO Personality Inventory, form S [9]

The NEO-PI-R is a questionnaire with 240 statements and a broad range of answers: the level of agreement or disagreement with item content is shown on Likert 5-point scale ranging from 0 (strongly disagree with the statement) to 5 (strongly agree with the statement). The Questionnaire is based on the five-factor model of personality interpreting the five basic dimensions (domains): neuroticism, extroversion, openness to experience, agreeableness and conscientiousness. Each measurement scale includes six subscales which measure so-called facets or aspects, with eight items per subscale (five domains and 30 specific traits – one domain comprises six specific personality traits).

Structured Clinical Interview (SCID II) [10]

The 'Structured Clinical Interview for DSM-IV Axis II Personality Disorders was used to assess personality disorders. The interview includes 125 'yes' or 'no' questions. Afterwards, the positive answers are tested by using a semi-structured interview. Positive answers which indicate pathological, permanent, and all-embracing quality of conduct covered by the question are accepted as a sign of the presence of the symptoms. The instrument shows the total number of symptoms (0-9) in a subject on every of the 10 categories of personality disorders, as well as severity of personality disorder through the total number of personality disorder diagnoses (subject scores threshold limit value for the diagnosis on one, or more than one of 10 categories of personality disorders).

### *Data analysis*

Cronbach's α was used to estimate the reliability, while the results were analyzed by the use of descriptive statistics and the one-way analysis of variance (ANOVA). In order to check the predictive role of personality domains in subjects with personality disorders, regressive analysis was carried out with domains such as neuroticism, extroversion, openness to experience, agreeableness and conscientiousness as predictive variables, and personality disorder clusters A, B, and C as criterion variables.

## Results

According to Cronbach's α coefficient, high reliability/internal consistency was found for domains N (α = 0.928) and C (α = 0.920), while the reliability of remaining NEO domains, E (α = 0.877), O (α = 0.872) and A (α = 0.871) was good.

In our study group, the average number of personality disorder diagnoses was 2.84 (from 1 to 7), whereas one third of the subjects were diagnosed with a personality disorder (n = 29; 29.9%).

Descriptive statistic factors and F-multipliers of the NEO-PI-R are shown in Table 1.

The ANOVA statistical test detected statistically significant differences in all domains of the NEO-PI-R questionnaire. Moreover, the subjects in our study had higher scores for N dimension (F = 83,421, $p < 0.001$), and lower scores for remaining NEO domains in comparison with those in the control group of healthy subjects.

Regressive analysis results showed that the coefficient of determination obtained was statistically significant for all three criterion variables: cluster A – eccentrics ($R^2 = 0.268$, F = 10,888, $p < 0.01$), cluster B – dramatics ($R^2 = 0.427$, F = 22,227, $p < 0.01$) and cluster C – anxious ($R^2 = 0.313$, F = 13.570, $p < 0.01$).

After establishing statistical significance of all three models, specific predictive structures were set up for all three criterion variables, as well. For cluster A – eccentrics, statistically significant predictors were N (β = 0,006, $t$ = 2,832, $p < 0.005$) and A domains (β = -0.010, $t$ = -4.091, $p < 0.01$). The most important predictors for cluster B – dramatics were N (β = 0.013, $t$ = 4.932, $p < 0.001$), E (β = 0.008, $t$ = 2.298, $p < 0.001$) and A (β = -0.017, $t$ = -5.607, $p < 0.001$), while predictive variables N (β = 0.013, $t$ = 4.974, $p < 0.001$) and E (β = -0.008, $t$ = -2.398, $p < 0.005$) accounted for 31.3% of variance of cluster C – anxious.

## Discussion

Our findings showed that people with and those without personality disorder diagnosis differ regarding intensity of all NEO domains, which is in accordance with previous findings – higher scores for N and lower scores for E, A, O and C have been confirmed in subjects diagnosed with personality disorders [11].

Based on the personality profile we obtained by applying NEO-PI-R, the subjects diagnosed with personality disorders were upset, low-spirited, perceiving life as difficult (low cores for N dimension combined with low scores for E dimension), suspicious of other people's intensions, cynical, egocentric, vindictive, antagonistic, competitive, preferring familiar environment, less prepared for any change (inflexibility), leading to frequent experience of negative affectivity in stressful situations (combination of low O and A dimensions). If lower scores for O dimension are interpreted as rigidity (having in mind that cognitive and affective inflexibility lead to numerous disorders), then the results of our study showing that the subjects with personality disorders who had lower scores for this dimension in comparison with healthy subjects could be regarded as convincing [8].

A combination of high N and low C scores in subjects diagnosed with personality disorders suggests more impulsive reactions, riskier behavior, and greater inclination towards substance abuse comparing to the control group of healthy subjects. Additionally, a combination of high N and low A scores suggests a specific style of anger control in subjects diagnosed with personality disorders in comparison with the group of healthy subjects: it is easier for them to get angry; they are more direct in expressing their rage; they are inconsiderate of how their rage affects others; they are more prone to physical violence and verbal abuse [8].

NEO domains were accountable for a third of variance referring to each personality disorder cluster (from 26.8 to 42.7%), which is compatible with resent findings of Nestadt et al [12]. Our findings support other authors' claim that possible solution of this problem could be integration of dimensional models of personality disorders and those of healthy personalities [13, 14].

High neuroticism (emotional instability) is a common feature of all personality disorder clusters. Differential diagnostic relevance is attributed to extraversion which makes diagnostic difference between dramatic cluster (positive pole) and anxious cluster (negative pole).

**Table 1**

**Descriptive statistic factors and F-multipliers of NEO-PI-R domains**

| NEO domains | Sample | Mean | SD | Min | Max | F | df | *p* |
|---|---|---|---|---|---|---|---|---|
| Neuroticism (N) | N1 | 164.53 | 25.527 | 86 | 223 | 83.421 | 153 | 0.000 |
| | N2 | 126.22 | 24.816 | 75 | 190 | | | |
| Extroversion (E) | N1 | 142.23 | 22.453 | 77 | 193 | 27.881 | 153 | 0.000 |
| | N2 | 160.03 | 16.094 | 110 | 195 | | | |
| Openness to experience (O) | N1 | 160.51 | 21.807 | 103 | 208 | 6.375 | 153 | 0.013 |
| | N2 | 169.52 | 20.985 | 117 | 212 | | | |
| Agreeableness (A) | N1 | 161.07 | 21.556 | 101 | 206 | 11.372 | 153 | 0.001 |
| | N2 | 172.52 | 18.430 | 132 | 214 | | | |
| Conscientiousness (C) | N1 | 157.54 | 23.824 | 103 | 214 | 27.106 | 153 | 0.000 |
| | N2 | 176.76 | 19.291 | 129 | 213 | | | |

**NEO-PI-R – Revised Neo Personality Inventary; N1 = 97 (study group diagnosed with personality disorders); N2 = 58 (control group without personality disorder diagnosis); SD – standard deviation; Min – minimum; Max – maximum.**

Low agreeableness is typical of eccentric and dramatic clusters, which is not the case with anxious cluster. Our findings support the meta-analysis which suggests consistently high level of neuroticism and low level of agreeableness in most personality disorders [15].

This study has several limitations that have to be considered in the interpretation of the results. The study sample was relatively small. We did not perform objective assessment of comorbid mood and anxious disorders, therefore influence of state on a personality trait was conducted only by clinical assessment, which was made in the phase of clinical remission.

## Conclusion

The results of this study confirmed that it was possible to conceptualize a personality disorder by the use of five-factor model of normal personality in the studied population. High neuroticism has diagnostic value for personality disorders, and other domains have differential diagnostic relevance. Integrating the psychiatric classification with the dimensional model of general personality structure could enable the uncovering of essential parameters for setting the diagnosis.

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# Validity of cytology in the diagnosis of small cell lung carcinoma

## Vrednost citologije u dijagnostici mikrocelularnog karcinoma pluća

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**Abstract**

**Background/Aim.** Small cell lung carcinoma (SCLC) is the most aggressive form of lung cancer. Patients with SCLC generally appear in a locally advanced or disseminated stage, when small biopsies and/or cytological materials are the only possibility for diagnosis. The aim of this study was to evaluate the validity of cytology in the initial diagnosis of SCLC, comparing cytological with histological findings of small biopsies. **Methods.** The retrospective study included 200 patients with cytological diagnosis of SCLC, established in the period from 2016 to 2018 based on examination of the exfoliative material (sputum), as well as abrasive and aspiration materials obtained during bronchoscopy. In the same act, bronchoscopic materials were taken for cytological and histological diagnosis. Cytological materials were stained by May Grünwald Giemsa and histological ones using hematoxylin-eosin and immunohistochemical stains. **Results.** The most frequently sampled materials were: transbronchial needle aspiration (TBNA) in 72.2% of the patients and bronchial brushing in 18.54% of the patients, in the following order: bronchial aspirate in 4.88%, tru-cut needle biopsy in 5.37%, and sputum in 2.44% of the patients. In 91.5% (183/200) of the patients cytological diagnosis of SCLC was histopathologically confirmed. Among 17 patients whose cytological diagnosis of SCLC was not confirmed histopathologically, another type of tumor was histopathologically proved for 12 (6%) of them: in 6 cases non SCLC not otherwise specified, and in each *per* one squamocellular carcinoma, adenocarcinoma, large cell carcinoma, mixed tumor (NSCLC with a neuroendocrine component), lymphoma and sarcoma. Finally, in five patients histological material was false-negative. **Conclusion.** Cytological diagnosis of SCLC is a reliable method which yields satisfactory accuracy. The best way is to be interpreted in conjunction with histology of small biopsies. When only cytological materials are available, in doubtful cases, other small round cell tumors, and poorly differentiated NSCLC, must be considered in the differential diagnosis.

**Key words:**
**bronchoscopy; carcinoma, non-small-cell lung; cytological techniques; diagnosis; diagnosis, differential; histological techniques; small cell lung carcinoma.**

**Apstrakt**

**Uvod/Cilj.** Mikrocelularni karcinom pluća (MCKP) je najagresivnija forma karcinoma pluća. Bolesnici sa MCKP se uglavnom javljaju u lokalno uznapredovalom ili diseminovanom stadijumu, kada su male biopsije i/ili citološki materijali jedina mogućnost za dijagnostiku. Cilj rada je bio procena validnosti citologije u inicijalnoj dijagnostici MCKP, upoređivanjem citoloških sa histološkim nalazima malih biopsija. **Metode.** Retrospektivnom studijom obuhvaćeno je 200 bolesnika, kojima je u periodu od 2016. do 2018. godine postavljena citološka dijagnoza MCKP, na temelju pregleda eksfolijativnog materijala (sputum), kao i abrazivnog i aspiracionog materijala dobijenog prilikom bronhoskopije. Bronhoskopski materijal je u istom aktu uziman za citološku i za histološku dijagnostiku. Citološki materijal bojen je May-Grünwald Giemsa, metodom, a histološki hematoksilin-eozinom i imunohistohemijskim bojenjima. **Rezultati**. Najčešće uzorkovani materijali bili su transbronhijalna iglena aspiracija (TBNA) kod 72,2% bolesnika i bris bronha kod 18,54% bolesnika, a zatim: aspirat bronha kod 4,88%, *true cut* iglena biopsija kod 5,37% i sputum kod 2,44% bolesnika. Kod 183/200 (91,5%) bolesnika citološka dijagnoza MCKP potvrđena je patohistološki. Od 17 bolesnika kojima citološka dijagnoza MCKP nije potvrđena patohistološki, kod 12 (6%) je patohistološkim pregledom dokazan drugi tip tumora: kod 6 nemikrocelularni karcinom pluća (NMCKP) bez druge specifikacije, kod po jednog bolesnika skvamocelularni karcinom, adenokarcinom, karcinom velikih ćelija, mešoviti tumor (NMCKP sa neuroendokrinom komponentom), limfom i sarkom, a kod 5 bolesnika se radilo o lažno negativnom histološkom materijalu. **Zaključak.** Citološka

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dijagnostika MCKP je pouzdana metoda zadovoljavajuće tačnosti. Najbolje je da se interpretira sa histologijom malih biopsija. U spornim slučajevima, kada je na raspolaganju samo citološki materijal, diferencijalno dijagnostički se moraju uzeti u obzir drugi tumori malih okruglih ćelija, ali i slabo diferentovani NMCKP.

**Ključne reči:**
**bronhoskopija; pluća, nesitnoćelijski karcinom; citološke tehnike; dijagnoza; dijagnoza, diferencijalna; histološke tehnike; pluća, sitnoćelijski karcinom.**

## Introduction

Lung cancer, as the most common type of cancers in the world and the leading cause of mortality among all types of carcinomas, is a global health problem [1]. It is the second most common cancer in both men (after prostate cancer) and women (after breast cancer) [2]. A high percentage of deaths from lung cancer is mainly the consequence of the fact that the disease is most frequently diagnosed in the advanced stage.

Serbia belongs to the group of the Central and Eastern European countries with high rates of morbidity and mortality, and also with the trend of increasing incidence of lung cancer [3, 4].

Besides advanced age, which is the most important risk factor for most cancers, there are a lot of other risk factors for lung cancer. Nowadays, it is known that lung carcinoma is a multifactorial disease originated from associate effects of more risk factors in combination with the individual characteristics of the human organism [5, 6]. The main risk factor (in 85% of patients) is tobacco smoking (active and passive) [6, 7].

Lung cancer is a clinical, biological and molecular heterogeneous disease [8]. In the diagnosis of lung cancer, it is essential to separate small cell lung carcinoma (SCLC) from non SCLC (NSCLC) because biological differences between these two types of lung carcinoma cause different clinical course and require different therapeutic modalities.

NSCLC accounts for 80–85% of lung cancers, among which the most common are adenocarcinoma (40%), squamous cell carcinoma (25–30%) and large cell carcinoma (5–10%). SCLC comprises for 15–20% of lung cancers [8]. The development of new treatments based on molecular tumor characteristics (molecular targeted therapy and antiangiogenic agents) led to the necessity of precise diagnosis of the histopathological type in the NSCLC group, and thus, for this group of lung cancers, it opened possibility of personalized therapy, depending on histological diagnosis and molecular tumor status [9, 10]. Unlike the NSCLC group, treatment of SCLC patients has not changed significantly for more than 30 years [11].

Most patients with SCLC have clinically disseminated or extensive disease at the time of diagnosis, when chemotherapy without radiation is recommended method of therapy [12]. In recent years many efforts have been made to discover specific therapeutic goals for SCLC. Immunotherapy tries to find its place in the treatment of SCLC. Increased PD-L1 expression was found in SCLC, underlying potential efficacy of anti PD-1/PD-L1 agents [12].

SCLC is the most aggressive type of lung cancers with a five-year and a 10-year survival rate of about 10% and 5%, respectively [13]. Due to clinical behaviour, systemic nature and good response to chemotherapy and radiotherapy, it is important to distinguish SCLC from other types of lung carcinomas [6, 14].

It is believed that SCLC cells are most likely derived from stem cells of the bronchial epithelium, which undergoes partial differentiation to neuroendocrine cells in the process of neoplastic transformation [14].

At about 5% to 30% of SCLC, a non small cell component can be found, and those are combined SCLC. Most often it is a component of squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [15, 16].

The diversity and complexity of the lung tumor histogenesis led to the need for their classification as precisely as possible. Over time, with new knowledge, the classification of lung tumors has also changed. The latest classification of lung tumors according to the World Health Organization (WHO) is based not only on the great progress in genetics, immunohistochemistry and lung cancer therapy, but also on the fact that about two thirds (70%) of lung cancers are established on samples of small biopsies and cytological samples, due to the disseminated or extensive disease at presentation [17].

Patients with SCLC are mainly presented in a locally advanced or disseminated stage, when small biopsies and/or cytology materials are the only possibility for diagnosis. Concordance of lung cancer diagnosis based on cytological materials compared to resectional or autopsy material ranges from 94%–100%, and concordance of bronchoscopic cytological material and small biopsies up to 97.4% [13, 18].

From small biopsies, it is possible to obtain multiple cuts which allow additional cytochemical and immunocytochemical staining in unclear cases, when the diagnosis can not be established based on the review of hematoxylin-eosin (HE) stained sections. This type of aid is largely not possible in cytodiagnostics. Cytological preparations are commonly stained only by one method, Papanicolaou or Romanowsky, so diagnosis is established exclusively on the basis of cell morphology. The question arises now is how much cytological diagnosis is reliable, that is, how much we can rely on well-known and defined cytological criteria in the diagnosis of SCLC.

The aim of this study was to evaluate the validity of cytology in the initial SCLC diagnosis by comparing cytological with histological findings of small biopsies.

## Methods

### *Study design*

In this retrospective study, the cytological diagnosis of SCLC established during a two-year period (January 2016 to

December 2017) was correlated with a histopathological diagnosis.

The cytological diagnosis was based on the examination of the exfoliative material (sputum) as well as the abrasive and aspiration materials obtained during bronchoscopy. The materials were taken in the same act for the cytological [bronchial aspirate and bronchial brushing, transbronchial needle aspiration (TBNA) of mediastinal or hilar lymph nodes, imprint of bioptic material], as well as for the histopathological diagnosis (TBNA, transbronchial and endobronchial biopsy). For both types of diagnostics, the material was also obtained by percutaneous needle biopsy.

The cytological and histological diagnoses were established separately and independently in the Department of Cytology and the Pathology Department of the Institute of Pathology and Forensics Medicine of the Military Medical Academy (MMA) in Belgrade, Serbia.

The bronchoscopy was performed in the Department of Interventive Pulmology at the Clinic for Pulmonary Disease of the MMA, Belgrade, Serbia. The bronchoscopic material was taken after a short analgosedation during video bronchoscopy (Olympus BF260, aspirate and bronchial brushing), while TBNA and transbronchial biopsy (histological needle, 19 G, crocodile forceps-type Machida) for both types of diagnostics, was performed during rigid bronchoscopy (Karl StorzGmbH&Co.KG, Tuttlingen, Germany). Percutaneous needle biopsy was done with tru-cut needle under the control of computed tomography.

*Material processing*

The cytological material was air-dried and stained with May-Grünwald-Giemsa (MGG). For histological analysis, the material was processed in the usual manner (fixation in 4% formaldehyde, routine process of incorporation into paraffin and cutting to cuts of thickness of 4 μm). The histopathological diagnosis of SCLC was first performed on materials stained with HE, and then, in order to confirm the diagnosis, immunohistochemical staining was carried out with chromogranin, sinaptofizin, thyroid transcription factor-1 (TTF-1), cytokeratin 8 (CK8), and neuron-specific enolase (NSE).

*Cytological criteria for diagnosing SCLC/suspected SCLC*

The cytological diagnosis of SCLC was established if individual cells and/or group of cells were found with subsequent morphological characteristics: nuclear size about 1.5–3 nuclei of small lymphocytes with fine structure of uniformly distributed chromatin without visible nucleolus, scant cytoplasm with high nucleo-cytoplasmatic ratio, well developed nuclear molding. The main criteria were the absence of the nucleolus and the presence of nucleus molding (Figure 1). The suspicion of SCLC was set if the diagnostic material was scant: if it contained a very small number of cells that had these morphological characteristics with or without the presence of the crash phenomenon, and if in addition to the microcellular component that prevailed, there was also a suspicion of a nonmicrocellular component.

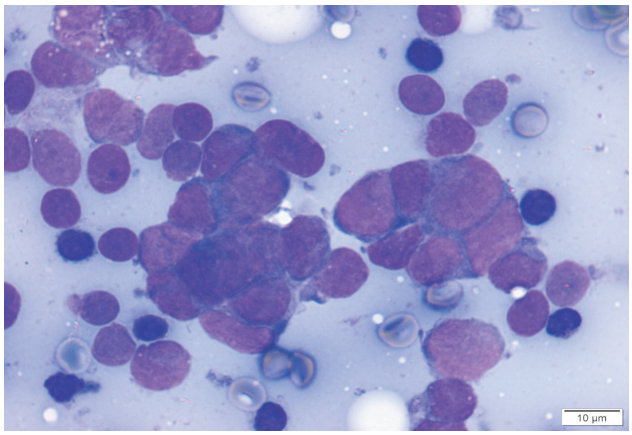


**Fig. 1 – Transbronchial needle aspiration finding points out cytology of small cell lung carcinoma: cluster of cells with finely granular and uniformly distributed chromatin, absence of nucleoli, nuclear molding and scant cytoplasm (May-Grünwald-Giemsa, ×1000).**

*Statistical analysis*

The data were statistically processed using descriptive statistics for the age of patients [mean value ± standard deviation (SD)], and the Student's *t*-test and Mann-Whitney test, for the evaluation of statistical significance of certain parameters (at the level of $p < 0.05$). Analyses were performed with the computer program IBM SPSS 20 and Microsoft Office Excel 200.

The unit of analysis was a patient. For statistical analysis, a finding suspected of SCLC was considered positive.

## Results

Over a two-year period, out of a total of 3,773 patients, 5,277 samples of materials for cytological diagnosis of lung lesions and/or hilar and mediastinal lymphadenopathies were taken. There were 3,600 (68.22%) benign and 1,237 (23.44%) malignant samples; 164 (3.1%) of the samples were suspicious to malignancy. Atypical cells were found in 59 (1.12%) of the samples, whereas 217 (4.12%) of the samples were not representative for the analysis.

Out of a total of 1,237 malignant cytological samples taken from 926 patients, in 222 samples taken from 200 (21.59%) of the patients, diagnosis of SCLC/suspected for SCLC was established. They were the subject of this retrospective study. There were 140 (68.3%) men and 65 (31.7%) women with a mean age (± SD) of 63.41 ± 11.3 (34–84) years. There was neither statistically significant difference between the number of men and women ($p = 0.317$), nor between the age of male and female patients ($p = 0.352$).

Depending on the localization of lesions in the lungs, hilum of the lungs or the mediastinum, as well as the clinical condition of the patient, one or more types of material were sampled. In 18 (8.78%) of the patients, the diagnosis of SCLC was made in several different types of materials, and

in 187 (91.22%) only in one type of material. The most frequently sampled materials were TBNA in 148 (72.2%) of the patients, followed by bronchial brushing in 38 (18.54%) of the patients, and then bronchial aspirate in 10 (4.88%), tru-cut needle biopsy in 11 (5.37%), and sputum in 5 (2.44%) of the patients (Figure 2).

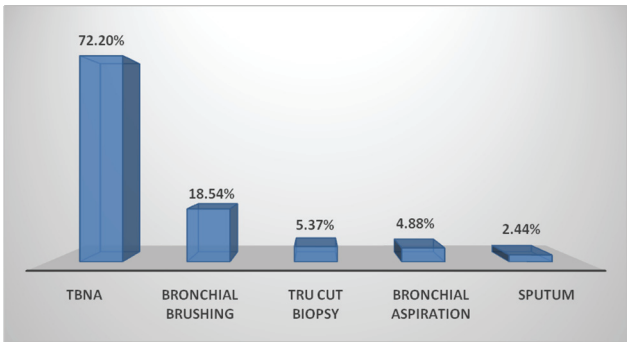


**Fig. 2 – Types of the most frequently sampled cytological material for diagnosis of small cell lung carcinoma.**

Among total of 200 patients, 184 (92.0%) had a cytological diagnosis of SCLC and 16 (8%) were susceptible to SCLC (cytologically positive). In 183 (91.5%) of the patients, SCLC diagnosis was confirmed histopathologically. There was no statistically significant difference in the number of patients with established diagnosis of SCLC between cytology and histopathology ($p = 0.068$).

Cytological diagnosis of SCLC was not confirmed histopathologically in 17 (8.5%) of the patients. In 12 of them, the other type of tumor was diagnosed: in 6 patients NSCLC not otherwise specified (NOS), and in another 6 patients squamous cell carcinoma, adenocarcinoma, large cell carcinoma (LCC), mixed tumor (NSCLC with neuroendocrine component), lymphoma and sarcoma, each *per* one. In histopathological material of 5 patients, no malignant but benign changes (inflammation, fibrosis) were revealed. Those were the cases of falsely negative histopathological findings.

Review of five misdiagnosed SCLC from 2017 was made by two cytologists. In 3 cases both cytologists confirmed the initial cytologic diagnosis of SCLC or suspected SCLC (Figure 3, a-c), and in 2 cases the initial diagnosis was not confirmed and NSCLC was diagnosed (Figure 3d, and Figure 4, a, b).

In Figure 3, a-c, cells had round nucleus without visible nucleolus, scant cytoplasm with high nucleo-cytoplasmatic ratio and prominent nuclear molding, but histopathological diagnosis was lymphoma, sarcoma and NSCLC-NOS.

Figure 3d shows the group of cells with increased cytoplasm which lacks definite borders, absence of clear nucleus molding, cell overlap and three-dimensionality; histopathological diagnosis was NSCLC, most probably adenocarcinoma.

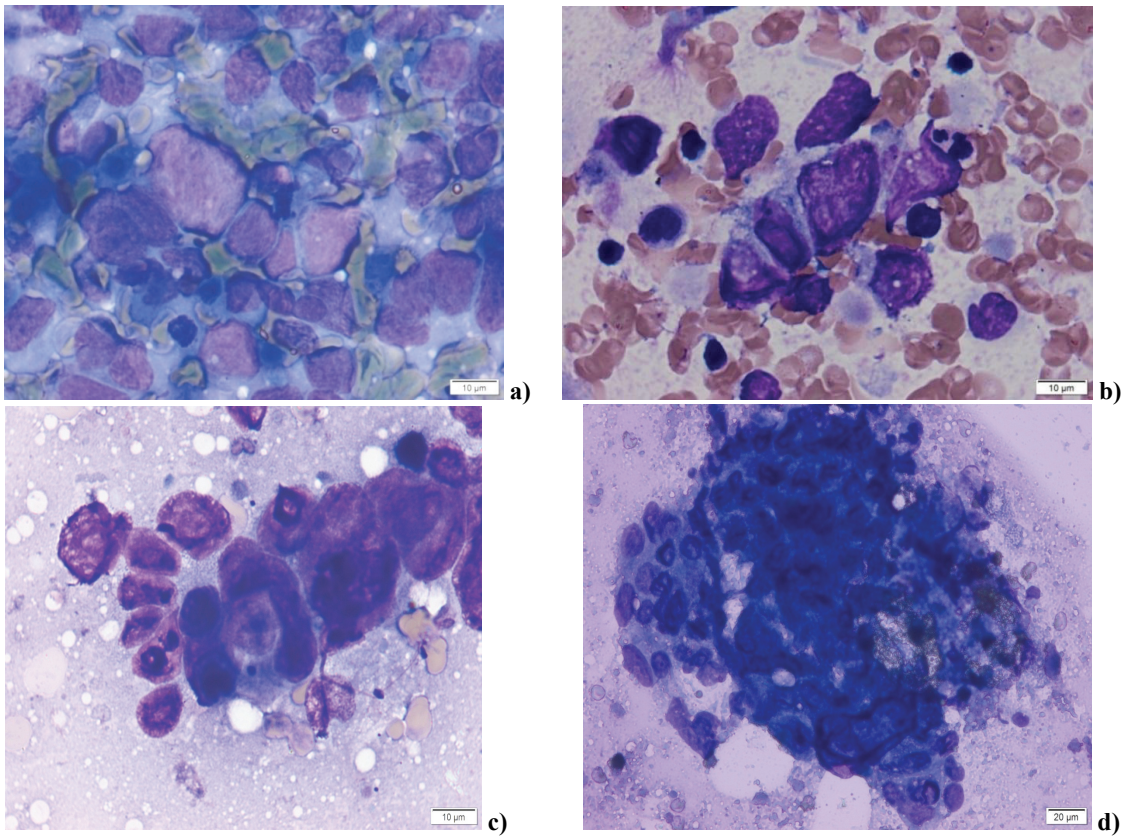


**Fig. 3 – Cases with misdiagnosed small cell carcinoma: a) parafollicular T cell lymphoma [transbronchial needle apiration (TBNA), May-Grünwald-Giemsa (MGG), ×1000]; b) nondifferentiated sarcoma (TBNA, MGG, ×1000); c) non small cell lung carcinoma (not otherwise specified – high grade) (tru-cut, MGG ×1000); d) non small cell lung carcinoma, most probably adenocarcinoma (TBNA, MGG, ×400).**

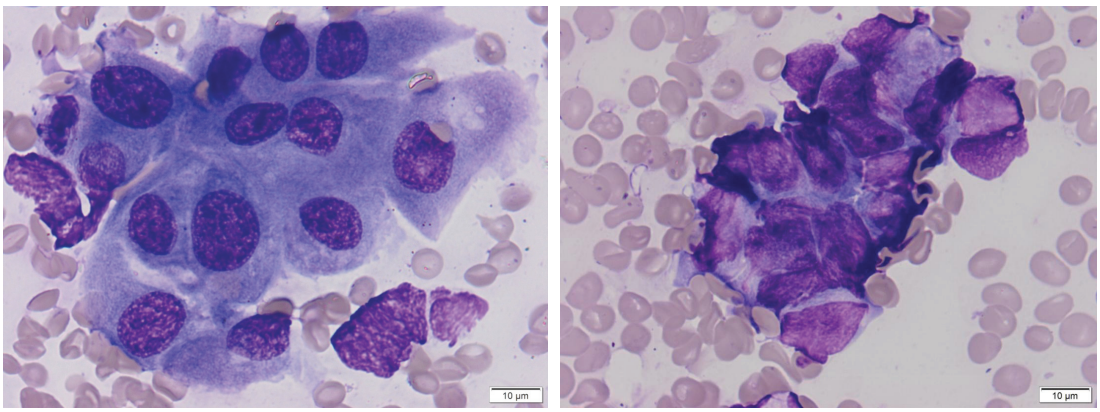

**Fig. 4 – Case with misdiagnosed small cell carcinoma: two groups of cells in the same sample of bronchial brushing; nonkeratinizing squamous cell carcinoma intraepithelial basaloid type, with microinvasion (May-Grünwald-Giemsa, ×1000).**

Figure 4 are cytological samples of one patient. The groups of cells in Figure 4 belongs to the same sample (bronchial brushing). On the other hand, while the group of cells in Figure 4 (left) is poorly differentiated and morphologically meet the criteria for SCLC, another group of cells on the same sample (Figure 4, right) is characteristic for squamocellular differentiation (large tumor cells with central, irregular hyperchromatic nuclei and abubdant cytoplasm, gaps between cells and distinct cell borders). On a bioptic sample taken in the same act, histopathological diagnosis of nonkeratinized squamous cell carcinoma, basaloid type – intraepithelial with microinvasion, was established.

Figure 5 presents a smear of transcarineal puncture performed in the same patient after a month. In the background of necrosis and cellular debris there are tumor cells with a clear morphology of keratinized squamous cell carcinoma (mostly isolated bizarre shapes cells with hyperchromatic or pyknotic nuclei and keratinized cytoplasm). In the material taken in the same act for histopathological analysis, there was no tumor tissue.

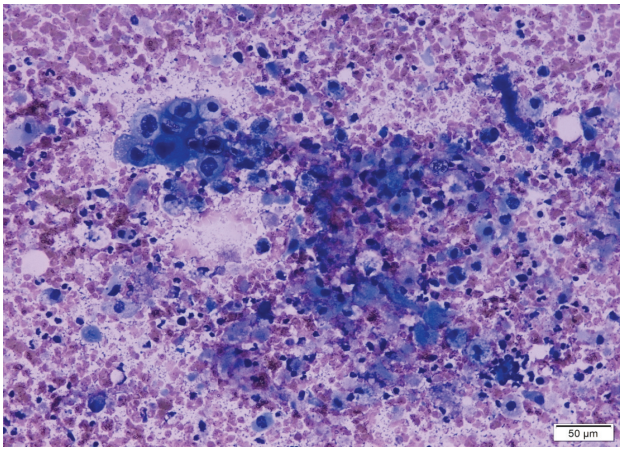

**Fig. 5 – Squamous cell lung carcinoma with keratinization (transcarinal puncture, May-Grünwald-Giemsa, ×200).**

**Discussion**

In patients with lung carcinoma, the only significant tumor parameters affecting the therapeutic procedure are the type of malignancy and stage of illness [19].

Since two thirds of patients with lung cancer are present in advanced stages when the cancer is unresectable, the decision on therapy is made on the basis of small biopsies and/or cytological samples obtained with less invasive methods, which are the primary method of diagnosis for the majority of lung cancer patients [10, 20]. Due to different therapeutic approaches and different prognoses, the first step in the diagnosis of lung carcinoma is the separation of SCLC from NSCLC.

Previous research has shown that the accuracy in differentiation between SCLC and NSCLC in cytologic diagnosis ranges from 94–100%, with a mean error rate of 9% (range 0% to 33%) for SCLC, and 2% (1–7%) for NSCLC, in comparison with resectional or autopsy samples [21].

The accuracy of SCLC diagnosis on cytologic samples is similar to that achieved with small biopsies, that is, sufficiently high to start with treatment [19]. The most recent study by Li et al. [23], based on a comparative analysis of the diagnostic value of cytology and histology taken during the same bronchoscopic procedure, concluded that the value of cytology (bronchial brushing and TBNA) was superior to histology (small biopsy stainded with HE and immunohistochemically) [4].

Of the former 20–25%, today the percentage of patients with SCLC has dropped to around 14–15%, probably due to a reduced number of smokers [6, 23]. However, in the examined two-year period, the percentage of patients with SCLC diagnosed in our hospital is still high (22.03%). There was neither statistically significant difference by sex nor by age between male and female patients. SCLC is an older age disease, but among our patients there were ones younger than 40, the youngest was only 34 years old. The mean age of our patients was 63.41 and it is not different from the mean age of patients with SCLC in similar studies [24, 25].

The most common sampled cytological material in our patients was TBNA lymph node number 7, which is understandable, since SCLC was mainly positioned centrally and submucosally, and in almost all patients the disease was extended to surrounding lymph nodes at the time of diagnosis. Tru-cut needle biopsy was done only in those cases where diagnostic material could not be obtained by any other methods.

In our study, the concordance between cytology and histology (bioptic samples) was 91.5%, slightly higher than in similar studies like tose of Sakr et al. [26] (83%) and Miličić et al. [24] (76%), but slightly less than Delgado et al. [19] (96%).

The disagreement between cytological and histological diagnosis was found in 17 (8.5%) of our patients. Of this number, 12 (6%) was a histopathologically proven NSCLC or another type of tumor, confirmed by immunohistochemistry.

Unlike our results, and those of Miličić et al. [24] who found disagreement between cytology and histology in 12/50 (23%) of the patients in similar investigation of the value of cytology in SCLC diagnosis, and Sakr et al. [26], who found incorrect cytological diagnoses of SCLC in 1/11 (9%) of the cases, Delgado et al. [19] in their study, comparing the accuracy of fine-needle aspiration cytology in the diagnosis of SCLC with the diagnosis of other lung malignancies, did not have any interpretative error**.** However, in their study, 221 patients had 242 fine-needle aspiration cytology, and all 18 (7%) of the smears interpreted as SCLC were correctly diagnosed, which is a far smaller number of patients with SCLC than in our study.

Rewiew of five misdiagnosed SCLC from 2017, found two cases with a clear interpretive error, that is, the wrong classification of the tumor type. In one case (Figure 3d), it is obvious that morphology and cell architecture did not satisfy cytological criteria for SCLC, in other words, it indicated NSCLC. But in another case, in the same sample (bronchial brushing), besides the groups of poorly differentiated cells (Figure 4, left) there are also other groups of cells with the clear squamocellular differentiation (Figure 4, right). On a bioptic sample, the histopathological diagnosis of nonkeratinized squamous cell carcinoma, basaloid type – intraepithelial with microinvasion, was established. After a month, the cytological finding of a transcarineal puncture performed in the same patient, revealed a clear morphology of keratinized squamous cell carcinoma, but the histopathological material was negative for malignancy. This case represents an interpretative error of a cytologist who overlooked a clear nonmicrocellular component in the bronchial brushning, as well as a limitation of small biopsies that represent only a small part of the tumor tissue. It was clear from the cytological sample obtained by transcarineal puncture, that it was a keratinized, most likely invasive squamous cell carcinoma, which could not be confirmed histopathologically, as histological sample was false negative.

However, in Figure 3, a-c, the morphology of cells and the manner of clustering were such, that SCLC could not be excluded only on the basis of morphological criteria, which was also a cytological diagnosis, but pathology revealed lymphoma, sarcoma and NSCLC-NOS.

In cytological samples, malignant lymphomas are presented mainly as uniform individual cells, usually with present lymphoglandular bodies. Lymphatic cells, depending on the type of lymphoma can have clearly visible nucleolus, and phenomena of nucleus molding, typical for the SCLC is lacking. However, in cytological samples, occasionally, tissue fragments or cellular grouping with nucleus molding phenomena can also be obtained, which can objectively lead to the misinterpretation in terms of SCLC, as it happened in our case (Figure 3a).

The main diagnostic problem in our study was to distinguish SCLC form NSCLC-NOS, and in a study of Miličić et al. [24], from squamous cell carcinoma and adenocarcinoma. These authors also had an incorrect diagnosis of SCLC in sarcoma. Domagała-Kulawik et al.[9] had similar difficulties in differentiating SCLC from undifferentiated, anaplastic NSCLC, and Delgado et al. [19] from poorly differentiated squamous cell carcinoma and large cell carcinoma.

In the material obtained by fine needle aspiration, Renshaw et al. [27] studied cytological characteristics of those cases of SCLC which are most often incorrectly classified as NSCLC. They concluded that this was mostly often the case with those SCLC that had some NSCLC characteristics, such as increased amounts of cytoplasm, or the presence of paranuclear blue bodies and/or some architectural features such as pseudoglandular or squamous cell grouping.

Sturgis et al. [28], studying the cytomorphologic features useful for separating SCLC from NSCLC in the bronchial brushing and aspirate, found that the three most sensitive and specific cytomorphologic features traditionally used to separate SCLC from NSCLC are nucleus molding, finely granulated chromatin, and scant delicate cytoplasm. However, they also found that some features which are classically associated with certain types of neoplasms, e.g. 3-dimensional groups with nuclear overlapping in lung adenocarcinoma, were also noted in SCLC (they noted 3-dimensional tumor fragments in 73% and nuclear overlap in 53% of SCLC cases). These studies have shown that SCLC may have some cytological features of NSCLC, as well as some other neoplasms, e.g. lymphoma or sarcoma, may have occasionally some of morphological characteristics of SCLC, such as nucleus molding.

The above-mentioned examples show the complexity of morphological, cytological and also histopathological diagnostics on small biopsies. This complexity comes from the possibility that some of morphological characteristics of NSCLC could be found in SCLC, as well as from the histological heterogeneity of lung carcinoma. In addition to SCLC and neuroendocrine LCC, the latest WHO classification of lung tumors, in the group of neuroendocrine tumors recognizes combined SCLC and combined large cell neuroendocrine carcinoma [17].

It was estimated that 70% of resected SCLC were pure and 30% combined. In a series of 100 surgical biopsies or SCLC resections, Nisholson et al. [16] found combined SCLC in 28% cases (16% combined with LCC, 9% with adenocarcinoma and 3% with squamous cell carcinoma). While combined small cell/LCC require at least 10% of the tumor show LCC, no percentage requirement is needed if there is a clear adeno- or squamocellular component [16, 29, 30].

In the most combined tumors, a small cell component is predominant. Since the presence of a small cell component will define patient therapy, the most important decision for a pathologist is to determine whether a small cell component is present.

In the light of these facts, except for the possibility of overlapping morphological characteristics of SCLC and NSCLC, small diagnostic samples do not need to be representative of the entire tumor that may be morphologically heterogenous, consisting of well- and poorly differentiated parts (like in Figures 4). If, in these small diagnostic materials, different parts of the tumor are obtained, this may be the reason of an inadequate diagnosis or disagreement in cytological and/or histological diagnosis of small biopsies, with a definite histological diagnosis on the resection material [10]. The most accurate diagnosis can only be set on the resected material. However, this type of material is available only in patients with early stage disease at the time of diagnosis, who are candidates for surguical resection.

In five of our patiens with benign histopathological findings (inflammation, fibrosis), abundant well preserved cytological material with a clear morphological characteristic of SCLC as well as a clinical finding and a further course of the disease, pointed out a false negative histopathological result; there was no tumor tissue in the material for histopathological analysis, respectively.

The discrepancy between cytological and histological diagnosis can also be the result of sampling (sample quality, size, representativity) or misinterpretation. In our research, we found sampling errors in bioptic material of five patients (nonrepresentative falsely negative bioptic material), and interpretative errors on cytological samples in 12 (6%) of patients. Due to the design of the study, in which the patients with the cytological diagnosis of SCLC were the starting point, we were not able to assess sampling errors on cytological specimens, as well as to evaluate if there were cases with cytological diagnosis of NSLC in histopathologically proved SCLC.

We could say that the part of committed cytological interpretive errors were objective, because they fell into overlapping zone of morphological, cytological characteristics of SCLC and NSCLC, or other types of small cell tumors, which was difficult to resolve without the aid of immunohistochemistry and/or detailed clinical data.

However, it is well known that problems in the SCLC diagnosis, that is, in the separation of SCLC from NSCLC, exist in the histological HE material in about 5–7% of the cases, even among experienced pathologists involved in the diagnosis of lung cancer [29]. Factors that contribute to variability in separating SCLC from NSCLC among pathologists can be of technical nature, such as: extensive crush phenomenon in small biopsies, ischemic changes, poor fixation, too thin or stained preparations, but also a reflection of the variability in the size of SCLC cells that are approaching the size of LCC cells, or the basaloid variant of LCC and squamous cell carcinoma [30].

Besides combined tumors (SCLC with a non small cell component) that may be the reason for misinterpretation (subjective or objective, if only one component of the tumor is in the sample), differential diagnosis of SCLC encompasses NSCLC, lymphoma, melanoma, chronic inflammation, other neuroendocrine lung tumors, metastatic breast and prostate carcinomas and metastatic neuroendocrine carcinomas from other localizations [30]. In addition, SCLC should also be separated from small round cell neoplasms, such as neuroblastoma, embryonic rhabdomyosarcoma, desmoplastic small round cell tumor and primitive peripheral neuroectodermal tumor [31].

## Conclusion

The cytological diagnosis of SCLC is a reliable method with satisfactory degree of accuracy. The best way is to be interpreted in conjunction with histology of small biopsies, so that invasive procedures are not indispensable in the diagnosis of lung cancer. When only cytological material is available, in doubtful cases, other type of small round cell tumors, but also poorly differentiated NSCLC must be considered for differential diagnosis. If in these cases it is not possible to do immunohistochemical and molecular studies, then the finding should be interpreted in conjunction with anamnestic, clinical and radiological parameters.

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# Radiation exposure during neurointerventional procedures in modern angiographic systems: A single center experience

## Izloženost radijaciji tokom neurointerventnih procedura u modernim angiografskim sistemima: iskustvo jednog centra

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**Abstract**

**Background/Aim.** Interventional neuroradiology procedures expose patients to ionizing radiation. The aim of this study was to assess doses received by patients during interventional neuroradiology procedures and to establish dose range with an estimate of risk from adverse consequences of irradiation. **Methods.** Our study describes series of patients submitted to diagnostic and/or therapeutic procedures at the Department of Interventional Neuroradiology, Clinical Center Kragujevac, Serbia, from December 1, 2014 to December 1, 2016. The following variables were considered for this study: kerma-area product, air kerma and fluoroscopy exposure time; peak skin dose and effective dose calculated from the kerma-area product. **Results.** Median kerma-area product was 87.802 Gy·cm², 78.567 Gy·cm², 117.626 Gy·cm²; effective dose was 12.731 mSv, 11.392 mSv, 17.056 mSv; peak skin dose was 0.456 Gy, 0.409 Gy, 0.612 Gy, and estimated brain dose was 254.62 mGy, 227.84 mGy, 341.12 mGy, for diagnostic, therapeutic and combined procedures, respectively. **Conclusion.** Interventional neuroradiology procedures show significant variability in radiation dose, due to patient constitution, radiologist expertise and equipment factors. Knowing the doses can have a great benefit for patients and medical and paramedical stff in terms of prevention of possible deterministic and stochastic effects of the radiation.

**Key words:**
**dose-response relationship, radiation; neuroradiography; radiation dosage; radiation protection.**

**Apstrakt**

**Uvod/Cilj.** U toku interventnih neuroradioloških procedura bolesnici su izloženi jonizujućem zračenju. Cilj ove studije bio je da utvrdi doze jonizujućeg zračenja koje bolesnik primi tokom interventnih neuroradioloških procedura i proceni rizik od negativnih efekata i posledica jonizujućeg zračenja. **Metode.** Studijom su obuhvaćeni svi bolesnici kojima su urađene dijagnostičke i/ili terapijske procedure na Odseku interventne neuroradiologije u Kliničkom centru Kragujevac, Srbija, u periodu 1.12.2014–1.12.2016. godine. Beležene su sledeće vrednosti: *kerma-area product*; *air kerma*; vreme izloženosti jonizujućem zračenju; maksimamalna kožna doza i efektivna doza. **Rezultati.** Srednja vrednost *kerma-area product*-a iznosila je 87,802 Gy·cm², 78,567 Gy·cm², 117,626 Gy·cm²; efektivne doze 12,731 mSv, 11,392 mSv, 17,056 mSv; maksimalne kožne doze 0,456 Gy, 0,409 Gy, 0,612 Gy i procenjene doze za mozak 254,62 mGy, 227,84 mGy, 341,12 mGy, za dijagnostičke, terapijske i kombinovane procedure, redom. **Zaključak.** Interventne neuroradiološke procedure pokazuju izrazitu varijabilnost u emitovanoj i primljenoj dozi zračenja, u zavisnosti od konstitucije bolesnika, opreme, kao i iskustva radiologa. Poznavanje veličine ovih doza u različitim uslovima, može biti od velike koristi za bolesnike, kao i za medicinsko i paramedicinskog osoblje u smislu smanjenja mogućih determinističkih i stohastičkih efekata zračenja.

**Ključne reči:**
**zračenje, odnos doza-reakcija; neuroradiografija; zračenje, doziranje; zračenje, zaštita.**

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## Introduction

Interventional neuroradiology (INR) procedures are guided by imaging techniques and both are performed as diagnostic and/or therapeutic [1]. Their use show constant increase, because of the great benefit they have for patients [2]. However, INR procedures expose patients to ionizing radiation [3]. The radiation risk is presented as deterministic effect, which happens after exceeding a radiation dose threshold, and stochastic effect, which does not have a threshold [4, 5]. Despite technological improvements, there are other risk factors such as procedure complications, longer time of fluoroscopy and high dose rates, which contribute to increase of the skin injuries and to occurrence of stochastic effects such as carcinoma [3–6]. Units that are provided by the INR angiographic system are kerma-area product (KAP) historically known as dose-area product, air kerma (AK) and fluoroscopy time (T) [7]. Since none of them is directly related to the patient organ doses, it is necessary to estimate the peak-skin dose (PSD) and effective dose (ED), which are associated with deterministic and stochastic effects, respectively [8–10].

The International Commission on Radiologic Protection (ICRP) proposed that the threshold for dose absorbed by patients' brain should be 0.5 Gy [11, 12]. It was also suggested that high doses could be avoided by real-time observation of doses by INR specialists, following proper consultation to their patients and optimization of risk factors [13]. Still, there are practical limitations to the direct measurement, such as inconvenient dosimeters [14]. Because of that, indirect assessment of radiation doses is currently used in a form of a KAP meter [15]. KAP does not supply us with direct radiation risk effect, but can be used to create dose reference level, together with AK and T [14, 16]. Dose reference levels are usually set at 75% and are defined as a degree of radiation exposure which should not be surpassed during procedures [14]. KAP can also be used to estimate the effect of ionizing radiation on patients, by calculating ED and PSD [17]. Previously published study has shown that for cerebral embolization, the average brain dose was 500 mGy and third quartile was 856 mGy, while for cerebral angiography, the average brain dose was 100 mGy [11]. That study did not show the exact formula or conversion factor from KAP to ED, but cite the website with formula that was used for calculation [18].

There is little information available regarding patient exposure to the radiation during INR procedures. Most of studies that were already published were conducted on patients subjected to cardiac and other vascular procedures, or showed variations in number of patients and dose calculations [8–10, 14, 17, 19, 20]. To our knowledge, there is limited data on radiation indicators during INR procedures and especially on brain doses. Accordingly, the aim of this study was to assess doses received by patients during INR procedures and to establish INR dose range with an estimate of risk from adverse consequences of irradiation.

## Methods

Our study describes series of patients submitted to diagnostic and/or therapeutic procedures at the Department of Interventional Neuroradiology, Clinical Center Kragujevac, Serbia, from December 1, 2014 to December 1, 2016. The study was approved by our Institutional Ethics Committee. Data used in the study were collected from the angiographic database.

We included all patients who underwent diagnostic procedures (cerebral angiographies) and therapeutic INR procedures: aneurism embolizations and embolizations of arteriovenous malformations (AVM). Follow-up diagnostic procedures, after therapeutic ones, were excluded. All procedures were performed by a team of two experienced interventional neuroradiologists. Both of radiologists had performed over 1,000 aneurysm and AVM embolizations and had over 10 years of experience.

The angiographic system used was a biplane angiographic unit (Allura Xper FD20, Philips, Philips Medical Systems, Veenpluis, The Netherlands) with a flat panel detector: frontal and lateral planes (48 cm) with variable fields of view of 42-37-31-26-22-19-15 cm. The system is provided with the high-power X-ray tube and Spectra Beam filtration (Copper filters: 0.2, 0.5, and 1.0 mm CU) which reduces patient X-ray (radiographic) dose and provides great image quality. The angiography unit has three pulsed fluoroscopy modes, of 10, 30, and 60 P/s, of which 30 P/s is used most frequently. The system includes real-time relevant dose information.

Data were collected separately for frontal and lateral views but were added together and compared for analysis.

The following variables were taken into account for this study: KAP, AK and fluoroscopy exposure time. Also, PSD and ED were calculated, since they are not routinely measured. PSD is a good indicator of the potential for deterministic injury. The radiation dose parameter associated with the risk of stochastic effects is ED. KAP was used to estimate both ED and PSD in previous studies, although conversion factors normally entail a degree of uncertainty or error [11, 14, 19, 21–23]. PSD was calculated from a published dose conversion formula for interventional procedures as follows: PSD (mGy) = 249 + 5.2 x KAP ($Gy \cdot cm^2$) [21, 24]. We estimated ED from KAP using a dose conversion factor (DCC), where DCC= ED (mSv)/KAP ($Gy \cdot cm^2$) [21]. We calculated brain dose using ED and tissue weighing factor provided by ICRP-103 [25]. In this calculation, the distribution of probability was considered to be normal, but due to the somewhat skewed distribution of our data, a coverage factor of 3 was used.

The study data were analyzed using the SPSS version 21 statistical software (SPSS Inc, Chicago, IL) [26]. Descriptive statistics was performed. The significance of difference between values of examined variables by groups (diagnostic, therapeutic and combined procedures) was tested with the Kruskal-Wallis nonparametric analysis of variances, since data was not normally distributed. We performed *post hoc* test using the Mann Whitney test with the Bonferroni correction of critical value for significance of every test.

## Results

From the angiographic database, totally 300 diagnostic and therapeutic INR procedures were identified. There were 224 cerebral angiographies, 55 therapeutic procedures (52 aneurism embolizations and 3 AVM embolizations) and 21 combined procedures. In total, there were 245 patients. Out of them, 55 patients [males (m)= 17; females (f) = 38, mean age = 49.35 ± 13.73 years] were exposed to radiation twice, 21 patients (m = 6; f = 15, mean age = 52.05 ± 13.23 years) were exposed to both diagnostic and therapeutic doses, while 169 patients (m = 77; f = 92, mean age = 51.62 ± 13.98 years) were exposed only to diagnostic radiation doses.

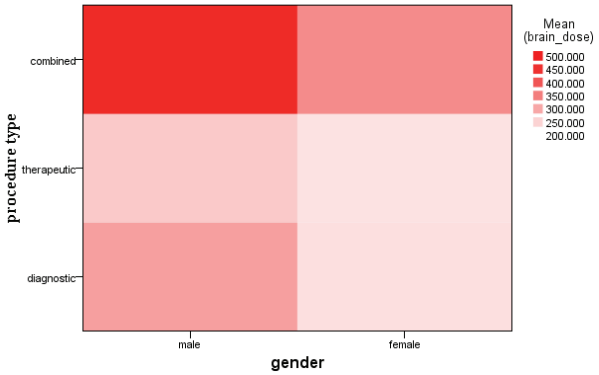


**Fig. 1 – Heat map for mean (estimated) brain dose depending on patient' gender and procedure type.**

We calculated total mean ± standard deviation and third quartiles for all dependent variables: KAP (93.95 ± 50.48 Gy·cm$^2$; 116.23 Gy·cm$^2$), AK (595.23 ± 382.07 Gy; 680.94 Gy); T (7.43 ± 7.37 min; 9.26 min); ED (13.62 ± 7.32 mSv; 16.85 mSv) and PSD (0.49 ± 0.26 Gy; 0.60 Gy). Estimated brain doses for diagnostic, therapeutic, combined and all procedures in total were: 254.62 ± 181.72 mGy, 227.84 ± 167.35 mGy, 341.12 ± 185.41 mGy and 272.4 ± 183.82 mGy, respectively. Figure 1 presents mean brain doses depending on the patients' gender and procedure type, using colors instead of numbers. Estimated brain doses for all three procedure types did not show normal distribution ($p = 0.001$), and frequency histogram is presented in Figure 2. Main statistical parameters for all three procedures types, as well as the Kruskal-Wallis test results are presented in Table 1.

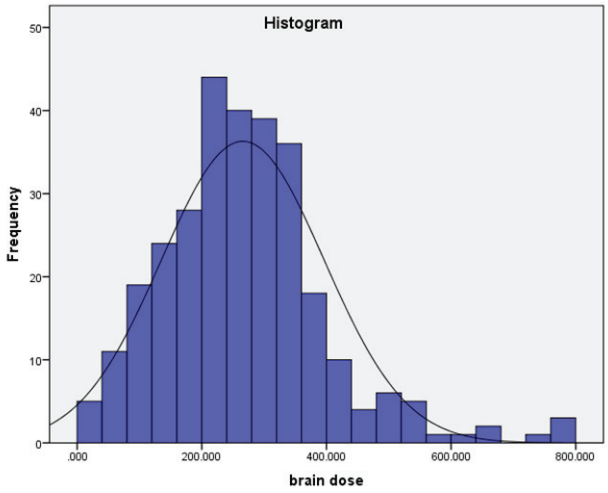


**Fig. 2 – Frequency histogram for estimated brain dose.**

**Table 1**

**Mean, minimum and maximum values for KAP, AK, T, ED and PSD for all three procedures types**

| Parameters | Diagnostic procedure (n = 224) | Therapeutic procedures (n = 55) | Both diagnostic and therapeutic procedures (n = 21) | Kruskal-Wallis test |
|---|---|---|---|---|
| KAP (Gy·cm$^2$) | | | | |
| median | 87.802 | 78.567 | 117.626 | Median = 86.514 |
| minimum | 2.710 | 11.685 | 29.673 | $\chi^2 = 6.075$ |
| maximum | 342.301 | 263.005 | 322.655 | $p = 0.048$ |
| AK (Gy) | | | | |
| median | 495.019 | 642.631 | 860.280 | Median = 524.685 |
| minimum | 8.586 | 35.170 | 135.188 | $\chi^2 = 15.63$ |
| maximum | 1905.900 | 2486.620 | 2984.920 | $p = 0.000$ |
| T (min) | | | | |
| median | 3.690 | 11.670 | 12.880 | Median = 4.8 |
| minimum | 0.53 | 2.380 | 2.820 | $\chi^2 = 81.488$ |
| maximum | 25.070 | 42.870 | 43.230 | $p = 0.000$ |
| ED (mSv) | | | | |
| median | 12.731 | 11.392 | 17.056 | Median = 12.544 |
| minimum | 0.393 | 1.694 | 4.303 | $\chi^2 = 6.075$ |
| maximum | 49.634 | 38.136 | 46.785 | $p = 0.048$ |
| PSD (Gy) | | | | |
| median | 0.456 | 0.409 | 0.612 | Median = 0.450 |
| minimum | 0.014 | 0.061 | 0.154 | $\chi^2 = 6.075$ |
| maximum | 1.780 | 1.368 | 1.678 | $p = 0.048$ |

**KAP – kerma-area product; AK – air kerma; T – fluoroscopy time; ED – effective dose; PSD – peak skin dose.**

The Kruskal-Wallis nonparametric analysis of variance showed that there was a significant difference between groups in term of dependent variables (KAP, AK, T, ED and PSD) and procedure type. *Post hoc* analysis determined by the Bonferroni correction of critical values was significant for each test: KAP and procedure type ($p = 0.016$), AK and procedure type ($p = 0.000$), T and procedure type ($p = 0.000$), ED and procedure type ($p = 0.016$), PSD and procedure type ($p = 0.016$).

## Discussion

Our study presented radiation exposure of patients during INR procedures by analyzing measured values by angiographic units (Kap, AK and T). We used KAP to assess and estimate ED and PSD, since previous studies have shown that it is the most effective way for determination of stochastic and deterministic effects of radiation during INR procedures.

The ICRP 103 states that ED should not be used for individual dose estimates nor for retrospective studies of individual radiation risk [25]. There are numerous formulas and DCCs for conversion of KAP to ED [11, 14, 24]. Choosing the right one is not easy, especially for neuroradiology procedures, because of limited number of published data and different angiographic units used. Also, comparison of ED is possible only with optimum DCC. Our study estimated that total mean absorbed dose by the brain was 272.4 mGy while brain dose during therapeutic procedures was 227.84 mGy. Previous study [11] presented that in 34% of patients, this dose was higher than 500 mGy, which is a threshold set by the ICRP [25]. The authors of that study used dose conversion factor different from the one we used and no clear information was given about the conversion formula, although the same angiographic unit was used as in our study. The study [21] that used the same DCC as us, due to accords with tube geometry and the beam quality, showed that their mean ED was 12.4 mSv which is much more than previously mentioned study, and in accordance with our mean therapeutic ED (11.392 mSv). In that study total mean EDs during interventional vascular procedures were: 6.2 mSv, 12.7 mSv, 27 mSv and 11.7 mSv [21].

Our estimated total mean PSD was 0.489 Gy, while maximum and minimum values were: 1.78 Gy and 0.014 Gy. PSD allows us to determine the possibility for a patient to receive a radiation skin injury [10, 23]. Suggested threshold is 2 Gy [11, 21, 23]. Our study showed results below the threshold. Only 3 patients had PSD higher than 1.5 Gy and none of the skin injuries, like erythema were reported. Other studies showed that their estimated total mean PSDs were 0.44 Gy and 1.01 Gy [21, 23]. Still, estimating PSD from KAP is problematic because during interventional procedures X-ray tube is moved around the patient, thus irradiating different areas of the skin. Also, there are different conversion formulas used for conversion of KAP to PSD. Even though, our estimation of PSD showed that suggested threshold was not reached, which complied with absence of skin injuries in our patients.

Our study showed that median KAP and T during intracranial aneurism and AVM embolizations were 78.567 Gy·cm$^2$ and 11.670 min, respectively. A study that had included patients with aortic aneurism showed that their KAP and T were 106.765 Gy·cm$^2$ and 17.32 min, respectively [27]. Average KAP in one of INR studies was 230 Gy·cm$^2$ [11], while our total average KAP was 93.95 Gy·cm$^2$.

Differences between our results and those in previously published studies may exist due to different methods in calculation of ED. This is the main limitation of our study. Nevertheless, we consider that ED and brain dose can give us some sense of direction, which might be better than having none.

## Conclusion

INR procedures show significant variability in radiation doses due to a patient constitution, radiologist expertise and equipment factors. Knowing a radiation dose during INR procedures can have a great benefit for patients and also for medical and paramedical staff. There are cases where medical indication can justify the dose, but in other cases it is important to do anything we know to reduce the risk of deterministic and stochastic effects of ionizing radiation.

In our study statistically significant difference was noted between procedures (diagnostic, therapeutic, and combined), although threshold values were never reached. A mean total absorbed dose by the brain was far less than the threshold value, which was also never reached in our study, although previous studies suggested that excessive amount of radiation (> 500 mGy) occurs in about a third of patients. PSD over 1.5 Gy, which was close to the threshold value, was present in a few cases, however, not causing any skin injuries.

Our study suggests that INR procedures are safe in terms of radiation exposure even when a patient undergoes combined interventions.

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# Gene expression of chemokines CX3CL1 and CXCL16 and their receptors, CX3CR1 and CXCR6, in peripheral blood mononuclear cells of patients with relapsing-remitting multiple sclerosis – A pilot study

## Ekspresija gena za hemokine CX3CL1 i CXCL16 i njihove receptore, CX3CR1 i CXCR6, u mononuklearnim leukocitima periferne krvi bolesnika sa relapsno-remitentnom multiplom sklerozom – pilot studija

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**Abstract**

**Background/Aim.** *In vitro* and *in vivo* studies show that CX3CL1 and CXCL16 chemokines and their specific receptors, CX3CR1 and CXCR6, respectively, mediate mechanism of neuroinflammation during the pathogenesis of multiple sclerosis (MS). The aim of this study was to investigate relative messenger ribonucleic acid (mRNA) levels of CX3CL1, CXCL16, CX3CR1 and CXCR6 in peripheral blood mononuclear cells, as potential molecular markers of relapsing-remitting (RR) MS. **Methods.** The study included 43 unrelated RR MS patients, 20 of them with clinically active disease (relapse) and 23 with clinically stable disease (remission), and 28 unrelated healthy subjects as controls. Real-time polymerase chain reactions (PCR) were performed using TaqMan® gene expression assays. Relative expression (mRNA) level of each target gene in each sample of peripheral blood mononuclear cells was calculated as the mean normalized expression. **Results.** The levels of CX3CR1 mRNA were significantly higher in clinically active RR MS patients compared to controls [fold change = 1.38, $p$ (Mann-Whitney $U$ test) = 0.009], and significantly lower in clinically stable *vs* active RR MS patients [fold change = -1.43, $p$ ($t$-test) = 0.03]. Stable RR MS patients had significantly higher CXCL16 mRNA levels than controls [fold change = 1.33, $p$ (Mann-Whitney $U$ test) = 0.006]. A trend of increased CXCR6 gene expression was found in active RR MS patients compared to controls [fold change = 1.23, $p$ (Mann-Whitney $U$ test) = 0.08]. In either active or stable RR MS patients there were no significant correlations of the clinical parameters with expression levels of the target genes. **Conclusion.** The current results show that increased CX3CR1 mRNA levels in peripheral blood mononuclear cells could represent a proinflammatory molecular marker of clinically active RR MS.

**Key words:**
**blood; chemokines; disease progression; gene expression; leukocytes; multiple sclerosis; rna, messenger.**

**Apstrakt**

**Uvod/Cilj.** Studije *in vitro* i *in vivo* pokazuju da hemokini CX3CL1 i CXCL16 i njihovi specifični receptori, CX3CR1 i CXCR6, posreduju u mehanizmu neuroinflamacije tokom patogeneze multiple skleroze (MS). Cilj studije bio je ispitivanje relativnih nivoa informacione ribonukleinske kiseline (iRNK) za CX3CL1, CXCL16, CX3CR1 i CXCR6 u mononuklearnim leukocitima periferne krvi, kao potencijalnim molekularnim markerima relapsno-remitentne (RR) MS. **Metode.** Studijom su bila obuhvćena 43 bolesnika sa RR MS, koji nisu bili u srodstvu, od kojih je 20 bilo u klinički aktivnoj fazi bolesti (relaps), a 23 u klinički stabilnoj fazi bolesti (remisija), dok su 28 zdravih ispitanika, koji nisu bili u srodstvu, bili kontrola. Za izvođenje lančanih reakcija polimeraze u realnom vremenu korišćeni su genski ekspresioni eseji TaqMan®. Relativni nivo ekspresije svakog ciljnog gena (iRNK) u svakom uzorku mononuklearnih leukocita

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periferne krvi bio je računat kao srednja normalizovana ekspresija. **Rezultati.** Nivoi CX3CR1 iRNK bili su značajno viši kod bolesnika u fazi relapsa u poređenju sa kontrolama ["*fold change*" = 1,38, *p* (Mann-Whitney *U* test) = 0,009] i značajno niži kod bolesnika u fazi remisije u poređenju sa bolesnicima u relapsu ["*fold change*" = -1,43, *p* (*t*-test) = 0,03]. Bolesnici u remisiji su imali značajno više nivoe CXCL16 iRNK nego kontrole ["*fold change*" = 1,33, *p* (Mann-Whitney *U* test) = 0,006]. Trend povećanja nivoa ekspresije CXCR6 gena je bio nađen kod bolesnika u relapsu u poređenju sa kontrolama ["fold change" = 1,23, *p* (Mann-Whitney *U* test) = 0,08]. Ni kod jednog bolesnika, ni u fazi relapsa ni u fazi remisije, nije bilo značajnih korelacija između vrednosti kliničkih parametara i nivooa ekspresije ciljnih gena. **Zaključak.** Rezultati pokazuju da povećanje nivoa CX3CR1 iRNK u mononuklearnim leukocitima periferne krvi može predstavljati proinflamatorni molekularni marker relapsa, tj. klinički aktivne faze relapsno-remitentne MS.

**Ključne reči:**
**krv; hemokini; bolest, progresija; geni, ekspresija; leukociti; multipla skleroza; rnk, informativna.**

## Introduction

Chemokines are a family of cytokines, representing small soluble proteins that have an essential role in the stimulation of cell migration and intercellular communication [1]. Changes in expression of certain chemokines and chemokine receptors in the central nervous system (CNS) can be associated with the pathogenesis of chronic neuroinflammatory and autoimmune diseases, such as multiple sclerosis (MS) [2].

Both CX3CL1 (fractalkine) and CXCL16 chemokines are expressed in vascular endothelial cells [3], while CXCL16 is also produced by monocytes/macrophages [4], B cells [5] and T cells [6]. Specific receptors for these two chemokines, CX3CR1 and CXCR6, respectively, are expressed on the surface of leukocytes, T cells [7, 8], monocytes/macrophages [9, 10] and non-killer (NK) cells [7, 8]. CX3CL1 and CXCL16 represent structurally and functionally unique chemokines. Each can exist as a soluble or a membrane-bound molecule and so can act as either a soluble chemoattractant or a membrane adhesion molecule, regulating both leukocyte migration and leukocyte adhesion to the vascular wall, which are key events in the inflammatory process [5, 11]. Conversion of the transmembrane into the soluble form of these two chemokines is achieved through regulated proteolysis of their transmembrane forms, by ADAM10 and ADAM17 extracellular metalloproteinases [12].

CX3CL1 and CX3CR1 are constitutively expressed in the CNS − CX3CL1 predominantly in neurons and CX3CR1 in microglia, so they are important for the formation of intercellular connections between neurons and microglial cells [1]. Inducible CX3CL1 expression was detected in astrocytes of the CNS inflammatory lesions in experimental autoimmune encephalomyelitis (EAE) [13], while elevated levels of soluble CX3CL1 were measured in the cerebrospinal fluid and serum of MS patients [14, 15]. CX3CL1 significantly increased the gene expression of proinflammatory cytokines in CD4+ T cells derived from patients with relapsing-remitting (RR) MS [14]. In the inflammatory brain lesions of rats and mice with EAE, there was accumulation of microglial cells and peripheral leukocytes expressing CX3CR1 messenger ribonucleic acid (mRNA), and CX3CR1 was responsible for the selective recruitment of NK cells into the CNS of these animals [13, 16]. A significantly higher percentage of CD4+ CX3CR1+ T cells was detected in blood of RR MS patients compared to healthy controls [14], and CX3CR1 mediated the recruitment of cytotoxic T cells into the brain tissue of patients with MS [17]. In normal CNS tissue, CXCL16 expression is low and mostly restricted to endothelial cells [18]. However, production of CXCL16 in the CNS has been increased during both preclinical and acute EAE [19]. It was found that CXCL16 could act as a proinflammatory chemokine in the pathogenesis of MS because, in animals with EAE, application of CXCL16 monoclonal antibodies resulted in: reduced disease incidence, decreased infiltration of mononuclear leukocytes into the CNS, decreased level of serum interferon gamma (IFNγ) and decreased production of myelin-specific T cells [20]. The levels of soluble CXCL16 in the human serum and cerebrospinal fluid were significantly increased in MS and other neuroinflammatory autoimmune diseases [21]. Expression of CXCR6 was typically detected in myelin-reactive IFNγ-producing CD4+ Th1 cells of EAE mice [20]. Also, CXCR6 was upregulated in neutrophils, which accumulated in the brain prior to and during the acute EAE attacks [19].

*In vitro* and *in vivo* studies indicate that CX3CL1 and CXCL16 chemokines and their specific receptors, CX3CR1 and CXCR6, respectively, are involved in the mechanism of neuroinflammation during the pathogenesis of MS. We had previously shown association of the single nucleotide variants in CXCL16 and CX3CR1 genes with susceptibility and progression of MS [22, 23]. The aim of the current study was to investigate changes in relative gene expression of CX3CL1 and CXCL16 chemokines and their receptors at mRNA levels in peripheral blood mononuclear cells (PBMC), as potential molecular markers of RR MS.

## Methods

### *Subjects*

The study included 43 unrelated patients with MS, from the Clinic for Neurology of the Military Medical Academy (MMA), Belgrade, Serbia. All patients were diagnosed with clinically definite MS [24] and the clinical course of the disease was defined [25]. For estimation of the disease severity, the Expanded Disability Status Scale [26] and the Multiple Sclerosis Severity Score [27] parameters were calculated, according to clinical data obtained at the time when blood specimens for genetic analysis were collected. Of 43 patients, 20 had clinically active RR MS (relapse) and 23 had clinically stable

RR MS (remission). Patients with stable RR MS were treated with 0.25 mg of interferon beta-1b (Betaferon®, Bayer Pharma AG), every other day, over a period of at least 12 months. The control group consisted of 28 unrelated healthy volunteers of the Military MMA personnel. Both controls and patients were of Serbian ethnic origin. The Ethics Committee of the MMA approved the study, and each participant gave his/her written informed consent to participate in the study.

*Real-time reverse transcription-quantitative polymerase chain reaction (PCR) and calculation of relative gene expression (mRNA) levels of CX3CL1, CXCL16, CX3CR1 and CXCR6*

Fresh blood samples (3 mL) were used for separation of PBMC, with lymphocyte separation medium (PAA, GE Healthcare), and extraction of PBMC total RNA, with TRI Reagent (Ambion, Life Technologies). The quality and quantity of total RNA were assessed using RNA 6,000 Nano Kit, on 2,100 Bioanalyzer (Agilent, US).

Each PBMC sample total RNA (500 ng) was treated with DNAseI (Fermentas, Thermo Fisher Scientific) and the reverse transcription was done using First strand cDNA synthesis kit, with oligo-dT18 and random hexamer primers (Fermentas, Thermo Fisher Scientific), in a reaction volume of 20 μL. Real-time polymerase chain reactions (PCR) were performed on Applied Biosystems 7500 Real-Time PCR system, by use of the following TaqMan® gene expression assays: Hs00171086_m1 (for CX3CL1), Hs01055223_g1 (for CXCL16), Hs01922583_s1 (for CX3CR1), Hs01890898_s1 (for CXCR6), Hs99999905_m1 (for GAPDH), Hs99999904_m1 (for PPIA) and Hs99999901_s1 (for 18S rRNA). Each real-time PCR reaction contained 1 μL of the reverse transcription product, in a total reaction volume of 13 μL. All samples were run in duplicates.

NormFinder algorithm [28] was used for identification of the optimal endogenous control among the candidate genes (GAPDH, peptiddylprolylcis-trans-isomerase A PPIA and 18S rRNA), according to their expression stability in a given sample group and a given study design. Based on the input data, representing Ct values were transformed to linear scale expression quantities by delta-Ct method. NormFinder calculated the stability value for each candidate gene, which was a direct measure of the estimated gene expression variation.

The relative expression level of each target gene in each PBMC sample was calculated as the mean normalized expression (MNE), according to the following formula [29]: $MNE = (E_{reference})^{Ct\ reference,\ mean} / (E_{target})^{Ct\ target,\ mean}$, where E represented PCR amplification efficiency for the reference (endogenous control) gene ($E_{reference}$) and the target gene ($E_{target}$), and Ct represented an average cycle threshold value from the two replicates, for the reference gene (Ct reference, mean) and for the target gene (Ct target, mean). TaqMan® gene expression assays provide the amplification efficiency of 100%, meaning that $E_{reference} = E_{target} = 2$, so the above formula for calculating the relative expression level of each target gene in each sample has become: $MNE = 2^{(Ct\ reference,\ mean\ -\ Ct\ target,\ mean)}$, $MNE = 2^{-(Ct\ target,\ mean\ -\ Ct\ reference,\ mean)} = 2^{-dCt}$. For verifying the relative gene expression results, relative expression software tool REST 2009 was used [30].

*Statistical analysis*

The statistical analysis was performed using Statistica 8.0 software package (StatSoft, Inc. 1984–2007). Comparisons of continuous variables between the tested sample groups were done by *t*-test and Analysis of Variance or by Mann-Whitney *U* test and Kruskal-Wallis test, depending on whether variable values had a normal or a non-normal distribution. Correlations between continuous variables were tested by product-moment and partial correlations. In all statistical tests, $p < 0.05$ values were considered statistically significant. Graphs were designed using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA).

## Results

*Controls and RR MS patients*

Characteristics of controls and RR MS patients who participated in the study are shown in Table 1. There was no significant difference in age between controls, patients with clinically active and patients with clinically stable RR MS [Table 1; *p* (Kruskal-Wallis test) = 0.72]. In each of the three groups, female-to-male ratio was > 1 (Table 1).

**Table 1**

**Characteristics of the study participants**

| Parameter | Controls (n = 28) | Active RR MS patients (n = 20) | Stable RR MS patients (n = 23) |
|---|---|---|---|
| Age (years) | 33.0 (25.0−64.0) # | 32.5 ± 8.2 | 35.2 ± 7.5 |
| Gender (female/male) | 21/7 | 16/4 | 12/11 |
| Disease onset age (years) | − | 29.0 ± 7.9 | 27.5 ± 8.6 |
| Duration of disease (years) | − | 2.0 (1.0−15.0) # | 6.0 (1.0−31.0) # |
| Expanded disability status scale | − | 1.5 (1.0−3.5) # | 2.0 (1.0−4.5) # |
| MS severity score | − | 3.5 ± 1.9 | 4.0 ± 1.6 |

**Values of continual parameters with a normal distribution are presented as mean ± standard deviation; #values of continual parameters with a non-normal distribution are presented as median (minimum–maximum).**
**MS – multiple sclerosis; RR – relapsing-remitting.**

*Relative expression (mRNA) levels of CX3CL1, CX3CR1, CXCL16 and CXCR6 genes in PBMC of controls and patients with RR MS*

In all tested PBMC samples, we detected the amplification of each target gene's mRNA (complementary DNA), except of CX3CL1 mRNA. By comparing the values of statistical parameter that represents the expression stability for each of the three tested endogenous control genes and by comparing the amplification profiles of endogenous control genes with amplification profiles of target genes, PPIA was found to be the optimal endogenous control for normalizing the results of expression of target genes in the analyzed PBMC samples. The statistical analysis of relative expression levels of the target genes in PBMC of controls and MS patients is presented in Figure 1 and Table 2. The levels of CX3CR1 mRNA were significantly higher in clinically active RR MS patients compared to controls [fold change = 1.38, $p$ (Mann-Whitney $U$ test) = 0.009], and significantly lower in clinically stable *vs* active RR MS patients [fold change = -1.43, $p$ ($t$-test) = 0.03] (Figure 1 A, Table 2). Stable RR MS patients had significantly higher CXCL16 mRNA levels than controls [fold change = 1.33, $p$ (Mann-Whitney $U$ test) = 0.006] (Figure 1 B, Table 2). A trend of increased CXCR6 gene expression was found in active RR MS patients compared to controls [fold change = 1.23, $p$ (Mann-Whitney $U$ test) = 0.08] (Figure 1 C, Table 2).

**Table 2**
**Statistical analysis of CX3CR1, CXCL16 and CXCR6 relative gene expression (mRNA) levels in peripheral blood mononuclear cells**

| Parameter | Fold change | $p$ |
|---|---|---|
| CX3CR1 relative gene expression | | |
| active RR MS patients *vs* controls | 1.38 | 0.009 ** |
| stable *vs* active RR MS patients | -1.43 | 0.03 * |
| stable RR MS patients *vs* controls | -1.03 | 0.66 |
| CXCL16 relative gene expression | | |
| active RR MS patients *vs* controls | 1.20 | 0.39 |
| stable *vs* active RR MS patients | 1.11 | 0.20 |
| stable RR MS patients *vs* controls | 1.33 | 0.006 ** |
| CXCR6 relative gene expression | | |
| active RR MS patients *vs* controls | 1.23 | 0.08 |
| stable *vs* active RR MS patients | -1.22 | 0.26 |
| stable RR MS patients *vs* controls | 1.01 | 0.97 |

**RR MS – relapsing-remitting multiple sclerosis.**
**Fold change – mean relative gene expression level (mean $2^{-dCt}$) of the target sample group to mean relative gene expression level of the reference sample group ratio (* – statistically significant difference when $p < 0.05$; ** – statistically significant difference when $p < 0.01$).**

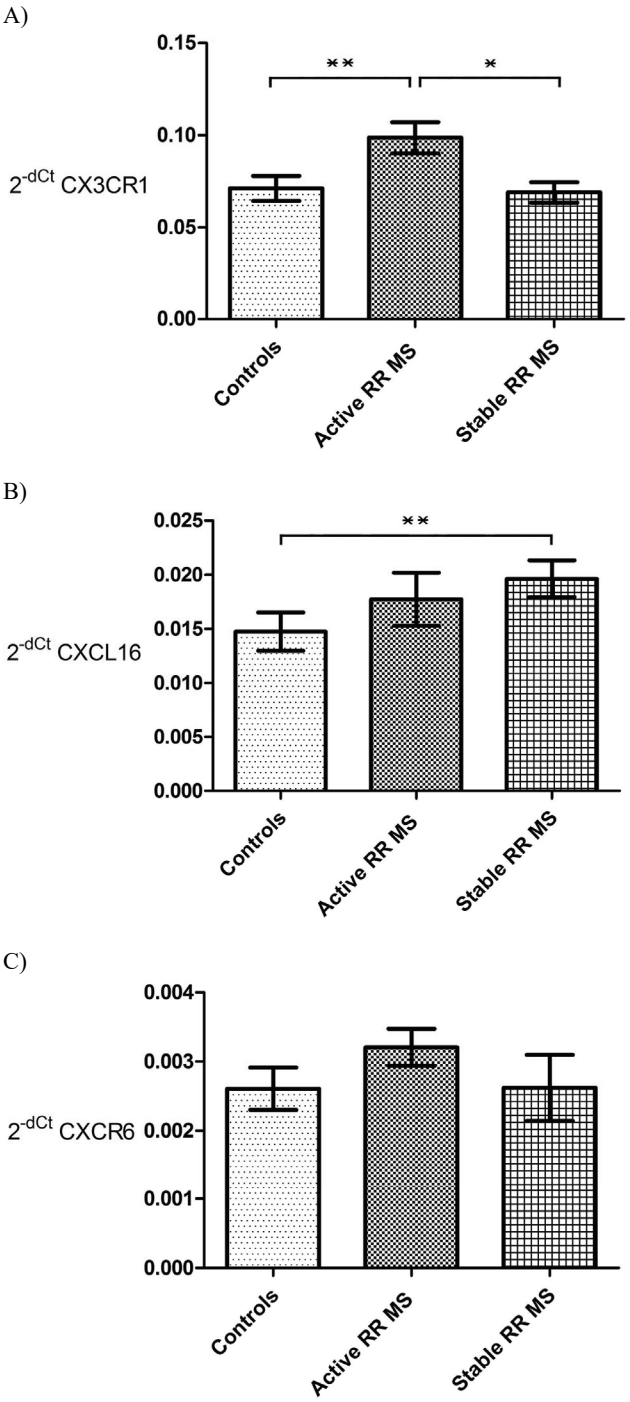


**Fig. 1 – Relative expression (mRNA) levels of the target genes in peripheral blood mononuclear cells.**

**A) Relative expression of CX3CR1 gene; B) Relative expression of CXCL16 gene; C) Relative expression of CXCR6 gene. The analyzed groups are controls (n = 28), active relapsing-remitting multiple sclerosis (RR MS) patients (n = 20) and stable RR MS patients (n = 23). For each target gene, the expression levels are normalized to endogenous control gene peptidylprolyl cis-trans-isomerase A (PPIA) and shown for each analyzed sample group (graph bars) as mean relative gene expression level (mean $2^{-dCt}$) with its standard error (* – statistically significant difference when $p < 0.05$; ** – statistically significant difference when $p < 0.01$).**

*Correlations between levels of the target mRNAs in controls and patients with RR MS*

No significant correlations were established between CXCL16 and CXCR6 mRNA levels or between CX3CR1 and CXCR6 mRNA levels, within each analyzed sample group – controls, active RR MS patients or stable RR MS patients (product-moment and partial correlations, $p > 0.05$).

*Correlations of clinical parameters with levels of the target mRNAs in patients with RR MS*

In either active or stable RR MS patients there were no significant correlations between values of clinical parameters (Expanded Disability Status Scale, Multiple Sclerosis Severity Score) and mRNA levels of CX3CR1, CXCL16 or CXCR6 in PBMC (product-moment and partial correlations, $p > 0.05$).

**Discussion**

Important purpose of research regarding the molecular basis of MS is to identify gene expression changes in the immune cells of peripheral blood, which may reflect the pathological changes in the target CNS tissue. The aim of the present study was to analyze the relative gene expression of CX3CL1 and CXCL16 chemokines and their specific receptors, CX3CR1 and CXCR6, respectively, at the mRNA levels in PBMC samples of patients with RR MS and healthy control subjects. We found that changes in PBMC CX3CR1 mRNA levels may represent a molecular marker of RR MS.

A proposed proinflammatory role of CX3CL1 in the pathogenesis of MS is supported by the fact that CX3CL1 significantly increased the expression of IFNγ gene in CD4+ T cells and secretion of IFNγ from these cells, derived from RR MS patients but not healthy individuals [14]. In rheumatoid arthritis, representing a T cell-mediated inflammatory and autoimmune disease, such as MS, CX3CL1 was expressed in T cells of patients. Yet, there was a low overall proportion of CX3CL1-expressing peripheral T cells in both rheumatoid arthritis patients and controls [31]. Likewise, in the current study, expression of CX3CL1 mRNA was not detectable in PBMC of either MS patients or controls. On the other hand, inducible expression of CX3CL1 in endothelial cells, as well as CX3CL1 proteolytic cleavage from the surface of these cells, was demonstrated *in vitro*, in response to inflammatory mediators [32]. This finding [32], along with ours, proposes that the inducible endothelial expression of CX3CL1 followed by its inducible proteolytic cleavage from the endothelial surface represents the main cause of a significant increase in circulating soluble CX3CL1 levels, having been shown in patients with RR MS [14, 15].

We detected significantly higher levels of PBMC CX3CR1 mRNA in patients with clinically active RR MS compared to both controls and patients with clinically stable RR MS. The study of relative gene expression, analyzed by real-time PCR, in PBMC samples of 28 healthy controls and 25 patients with RR MS showed that the levels of CX3CR1 mRNA, as well as percentage of CX3CR1+ cells, were significantly lower in patients compared to controls [33]. This decreased CX3CR1 gene expression in patients may be explained by the fact that most of them were clinically stable [33]. Likewise, we found a significant decrease in CX3CR1 mRNA levels in stable RR MS patients compared to the active ones. Furthermore, in microarray analysis by Infante-Duarte et al. [33], the only patient who suffered a relapse 14 days after venipuncture had an increased CX3CR1 gene expression in comparison to healthy individuals, which is consistent with our finding. Previous research indicated the proinflammatory properties of peripheral CX3CR1+ CD4+ T cells and CX3CR1+ NK cells, by which these cells should contribute to the process of neuroinflammation in RR MS, especially during relapse [14, 17, 33]. Accordingly, our finding suggests that the increase in PBMC CX3CR1 mRNA levels represents a potential proinflammatory molecular marker of active RR MS.

Both CXCL16 chemokine and its receptor, CXCR6, are widely expressed in PBMC [4–6, 8, 20]. CXCL16 from monocytes infiltrated in the CNS of EAE mice was suggested to induce chemotaxis and accumulation of activated myelin-specific CXCR6+ CD4+ Th1 cells in the CNS tissue, indicating a proinflammatory action of CXCL16 in the pathogenesis of EAE [20]. Production of CXCL16 in the CNS was increased during the acute EAE [19], and severity of EAE positively correlated with CNS mRNA and protein levels of CXCL16 and CXCR6 [20]. The analysis of brain tissue from MS patients demonstrated an increase in CXCL16 expression by foamy macrophages in the rims of chronic active brain lesions [34]. Considering the foregoing results obtained *in vivo* and *in vitro*, we expected to find out elevated CXCL16 and CXCR6 gene expression levels in PBMC of the patients. In clinically active RR MS patients compared to controls, we detected no significant differences in either CXCL16 or CXCR6 mRNA levels, although there was a trend of increased CXCR6 gene expression. CXCR6 was an indicator of IFNγ production, since the intracellular synthesis of IFNγ significantly positively correlated with expression of CXCR6 on the surface of MBP-reactive CD4+ Th1 cells [35]. The trend that we found, along with this finding of Calabresi et al. [35], suggests further investigation in order to evaluate the hypothesised role of CXCR6 as a proinflammatory marker of RR MS, typically of the active phase of the disease. In the present analysis of PBMC CXCL16 gene expression, we detected significantly higher mRNA levels only in patients with stable RR MS in comparison to controls. These patients were receiving interferon-beta treatment, which is known to suppress the secretion of proinflammatory cytokines [36]. An *in vitro* study showed no correlation between CXCL16 expression and interferon-beta treatment [37]. Considering the hypothesised proinflammatory action of CXCL16 in the pathogenesis of MS [20], the largest increase in CXCL16 gene expression is expected to be found in clinically active RR MS patients. As the intensity of inflammation and autoimmune response decreases during the stable phase of the disease, CXCL16 expression should also decrease. Yet, we did not find its decrease in

stable RR MS. Therefore, the increased PBMC CXCL16 mRNA levels in patients with clinically stable disease in the current study could be due to some pleiotropic effect(s) of CXCL16 on pathogenesis of RR MS, which is(are) not directly related to inflammation.

We aware that our study has limitations. One is the restricted inclusion of patients with clinically stable disease who are treatment-naïve. Thus, to create a homogenous group of clinically stable patients, we deliberately selected only those receiving interferon-beta treatments. We did not show which subpopulation(s) of PBMC underlay the detected significant changes in gene expression, and this represents another limitation of the study. Still, the expression changes were detected in total PBMC, which represent a valid source of potential MS biomarkers quantified at the level of mRNA and/or protein [38–40].

## Conclusion

The current study demonstrates that the increased CX3CR1 mRNA expression in PBMC could represent a pro-inflammatory molecular marker of clinically active RR MS. The results should be verified in future studies with a larger number of samples. In addition, more functional research is needed to fully clarify the roles of CX3CL1, CXCL16, CX3CR1 and CXCR6 in the pathogenesis of MS. Despite limitations, the present study adds value to investigation of CX3CL1, CXCL16, CX3CR1 and CXCR6 gene expression in the immune cells of RR MS patients, with respect to the disease activity.

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META-ANALYSIS


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# Efficacy and safety of triazoles *versus* echinocandins in the treatment of invasive aspergillosis: A meta-analysis

## Poredjenje efikasnosti i bezbednosti triazola sa ehinokandinima u lečenju invazivne aspergiloze: meta-analiza

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**Abstract**

**Backgroun/Aim.** Although majority of guidelines recommend triazoles (voriconazole, posaconazole, itraconazole and isavuconazole) as first-line therapeutic option for treatment of invasive aspergillosis, echinocandins (caspofungin, micafungin and anidulafungin) are also used for this purpose. However, head-to-head comparison of triazoles and echinocandins for invasive aspergillosis was rarely target of clinical trials. The aim of this meta-analysis was to compare efficacy and safety of triazoles and echinocandins when used for treatment of patients with invasive aspergillosis. **Methods.** This meta-analysis was based on systematic search of literature and selection of high-quality evidence according to pre-set inclusion and exclusion criteria. The literature search was made for comparison of treatment with any of triazoles (isavuconazole, itraconazole, posaconazole or voriconazole) versus any of echinocandins (caspofungin, anidulafungin or micafungin). The effects of triazoles (itraconazole, posaconazole or voriconazole) and echinocandins (caspofungin, anidulafungin or micafungin) were summarized using RevMan 5.3.5 software, and heterogeneity assessed by the Cochrane Q test and $I^2$ values. Several types of bias were assessed, and publication bias was shown by the funnel plot and Egger's regression. **Results.** Two clinical trials and three cohort studies were included in this meta-analysis. Mortality in patients with invasive aspergillosis who were treated with triazoles was significantly lower than in patients treated with echinocandins [odds ratio 0.29 (0.13, 0.67)], and rate of favorable response (overall treatment success) 12 weeks after the therapy onset was higher in patients treated with triazoles [3.05 (1.52, 6.13)]. On the other hand, incidence of adverse events was higher with triazoles than with echinocandins in patients treated for invasive aspergillosis [3.75 (0.89, 15.76)], although this difference was not statistically significant. **Conclusion.**Triazoles (voriconazole in the first place) could be considered as more effective and somewhat less safe therapeutic option than echinocandins for invasive aspergillosis: However, due to poor quality of studies included in this meta-analysis, definite conclusion should await results of additional, well designed clinical trials.

**Key words:**
**aspergillosis; triazoles; echinocandins; meta-analysis.**

**Apstrakt**

**Uvod/Cilj.** Iako većina vodiča preporučuje triazole (vorikonazol, itrakonazol, posakonazol i isavukonazol) kao primarnu terapijsku opciju za lečenje invazivne aspergiloze, ehinokandini (kaspofungin, mikafungin i anidulafungin) takođe se koriste u ovu svrhu. Uprkos ovoj činjenici, poređenje triazola i ehinokandina za lečenje invazivne aspergiloze retko je ispitivano u kliničkim studijama. Cilj ove meta-analize bio je da uporedi efikasnost i bezbednost triazola sa ehinokandinima kod bolesnika sa invazivnom aspergilozom. **Metode.** Ova meta-analiza je bazirana na sistematskoj pretrazi literature i biranju najkvalitetnijih studija prema uključujućim i isključujućim kriterijumima. Literatura je pretraživana za poređenje lečenja bilo kojim od triazola (isavukonazol, itrakonazol, posakonazol ili vorikonazol) naprema lečenju ehinokandinima (kaspofungin, anidulafungin ili mikafungin). Efekti triazola (itrakonazola, posakonazola i vorikonazola) i ehinokandina (kaspofungina, anidulafungina i mikafungin) sumirani su u RevMan 5.3.5 programu, a heterogenost je određena Cochrane Q testom i $I^2$ vrednostima. Nekoliko tipova sistematskih grešaka zbog pristrasnoti (*bias)* je ispitano, a sistematska greška u pogledu pristrasnosti u publikovanju je prikazana pomoću *funel plot*-a i Eger-ove regresije. **Rezultati.** Dve kliničke studije i tri kohortne studije bile su uključene u meta-analizu. Smrtnost kod bolesnika sa

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invazivnom aspergilozom, koji su tretirani triazolima, bila je značajno manja u poređenju sa onom kod bolsnika lečeniuh ehinokandinima [*odds ratio* 0.29 (0.13, 0.67)], i stopa povoljnog odgovora (uspeh lečenja) nakon 12 nedelja terapije bila je veća kod triazola [3.05 (1.52, 6.13)]. Sa druge strane incidencija neželjenih efekata bila je veća, ali ne statistički značajno, kod triazola nego kod ehinokandina u lečenju invazivne aspergiloze [3.75 (0.89, 15.76)]. **Zaključak.** Triazoli (pre svega vorikonazol) se mogu smatrati efikasnijom i, ponekad, manje bezbednom terapijskom opcijom nego ehinokandini za lečenje invazivne aspergiloze. Ipak, zbog slabog kvaliteta studija u ovoj meta-analizi, definitivni zaključak treba da sačeka dodatne, bolje dizajnirane studije**.**

**Ključne reči:**
**aspergiloza; triazoli; ehinokandini; meta-analiza .**

## Introduction

Invasive aspergillosis (IA) is the most frequent invasive mold infection caused by fungi belonging to the genus *Aspergillus*. It is a potentially life-threatening infection (usually taking place in the respiratory tract) with high mortality rate (80–90%) in high risk patients such as patients with hematological malignancies and patients undergoing hematopoietic stem cell transplant (HSCT) [1]. Without adequate therapy, invasive pulmonary aspergillosis is further complicated as a result of hematogenous dissemination or direct extension leading to infection of other tissues, the central nervous system (CNS) or cardiovascular system [2]. IA is the most common type of infection among stem cell transplant recipients, and the second most common type of fungal infection in organ transplant recipients. One-year survival in patients with IA was 59% in organ transplant recipients [3] and 25% among recipients of stem cells [4]. A major barrier to successful treatment of IA is delayed diagnosis. Due to the lack of reliable and feasible diagnostic techniques, over one third of *Aspergillus* infections still remain undiagnosed [5]. Members of the European Organization for Research in Treatment of Cancer/Invasive Fungal Infection Cooperative Group and National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) formed a Consensus Committee in order to develop standard definitions for invasive fungal infections for clinical research [6]. According to them, three levels of certainty of IA were defined: proven, probable, and possible. From the year 2008, the same study group recommended detection of serum biomarker galactomannan as one of the criteria of probable IA, which is very much helpful when diagnosing this infection in neutropenic patients before characteristic chest radiographic (x-ray) signs of aspergillosis become visible.

There are only four major classes of antifungal agents (polyenes, flucytosine, azoles and echinocandins) which could be used for systemic treatment of invasive mycoses. Primarily, amphotericin B and flucitosine were exploited, but due to their high toxicity, triazoles as efficient and safer drugs were later on usually recommended as the first-line choice in medical literature; however this recommendation was not based on comparative studies between triazoles and echinocandins [7, 8].

Triazoles are isomeric chemical compounds containing a five-membered ring with two carbon atoms and three nitrogen atoms. These drugs (posaconazole, isavuconazole, itraconazole and voriconazole) primarily inhibit synthesis of ergosterol by inhibition of lanosterol 14α-demethylase enzymes in the fungal membrane, but not in host cells. Currently, they are successfully used in clinical management of invasive mycoses, including prophylaxis, pre-emptive, empiric and targeted therapy. On the other hand, echinocandins, which were developed in the early 2000s, are also frequently used in the treatment of invasive mycoses (including aspergillosis) due to their low host toxicity and good efficacy, especially as salvage therapy for IA [9]. Three echinocandins, caspofungin, micafungin and anidulafungin, were the first antifungals that were created to selectively target the fungal cell wall. Echinocandins cause disruption in the b-(1,3)-D-glucan synthesis and increase permeability of cell wall that leads to a disbalance of the intracellular osmotic pressure of the fungal cell and the fungal cell lysis [10].

Although clinical trials comparing triazoles and echinocandins for curing IA were published, neither meta-analysis nor systematic review were performed on this topic up to date. Summarizing the available evidence about efficacy and safety of triazoles vs. echinocandins in this indication will be helpful for planning future clinical trials or observational studies with these drugs for IA.

The aim of this meta-analysis was to compare efficacy and safety of triazoles and echinocandins when used for treatment of patients with IA.

## Methods

Our study was registered at PROSPERO register of systematic reviews and meta-analyses under the number CRD42017081282 prior to commencement of the research.

The following criteria to include studies for this review were used: 1. types of studies – both randomized, controlled clinical trials and observational studies which compare any of triazoles with any of echinocandins in patients with IA; 2. types of participants – patients of both sex and any age with proven or probable IA (proven IA is characterised by documented histopathological and microbiological evidence of *Aspergillus spp*. infection, either at autopsy or in biopsied tissue or culture samples from a normally sterile site; probable IA is characterised by the presence of radiological [nodules, cavities, halos or air crescent signs on chest radiography or computed tomography (CT)] and microbiological (direct microscopy, culture) features in an immune-suppressed patient [absolute neutrophil count (ANC) < 500 cells/$mm^3$, prolonged steroid therapy, use of a T-cell suppressor or allogeneic stem transplantation]; 3. types of interventions – intravenous treatment with any of triazoles (isavuconazole, itraconazole, posaconazole or voriconazole) *versus* any of

echinocandins (caspofungin, anidulafungin or micafungin) for at least 7 days.

Search methods for identification of studies primarily included electronic databases, and collection of journal articles and books of University Library, University of Kragujevac, Kragujevac, Serbia. The literature search was made for comparison of treatment with any of triazoles (isavuconazole, itraconazole, posaconazole or voriconazole) *versus* any of echinocandins (caspofungin, anidulafungin or micafungin). Electronic searches of the literature were conducted in MEDLINE (PubMed, coverage from 1966 to present), Scopus/Elsevier (coverage from 1966 to present), EBSCO (Discovery Service, coverage from 1944 to present), SCINDEKS (Serbian Citation Index, coverage from 2001 to 2018), The Cochrane Central Register of Controlled Trials – CENTRAL (Wiley Online Library, coverage from 1966 to present) and a registry and results database of clinical studies of human participants ClinicalTrials. gov up to November 30, 2017. Additional searches were conducted up to March the 18th, 2018. Electronic databases were searched independently for relevant studies by two authors: SU and RŽZ. The searching strategies were presented in detail for each of the investigators in the Supplementary file. The most comprehensive strategy was used by the SU for the MEDLINE database, as following: (("voriconazole"[MeSH Terms] OR "voriconazole"[All Fields]) OR ("itraconazole" [MeSH Terms] OR "itraconazole"[All Fields]) OR ("posaconazole"[Supplementary Concept] OR "posaconazole"[All Fields]) OR ("isavuconazole"[Supplementary Concept] OR "isavuconazole"[All Fields])) AND (("aspergillosis"[MeSH Terms] OR "aspergillosis"[All Fields]) OR (invasive[All Fields] AND ("aspergillosis"[MeSH Terms] OR "aspergillosis"[All Fields]))) AND (("caspofungin"[Supplementary Concept] OR "caspofungin"[All Fields]) OR ("anidulafungin"[Supplementary Concept] OR "anidulafungin"[All Fields]) OR ("micafungin"[Supplementary Concept] OR "micafungin"[All Fields])). There were no restrictions on publication date, format or language in the search strategy. The references of the retrieved articles were searched for further similar studies ("snowball search"). The collection of journal articles and books of University Library, University of Kragujevac was hand searched for relevant studies by one author (RZZ).

*Data collection and analysis*

The data collection sheet was created and the articles included in the review were assessed for: 1. study identifier (ID); 2. report ID; 3. review author initials; 4. citation and contact details; 5. eligibility for review; 6. study design; 7. total study duration; 8. risk of bias (randomization if any, sequence generation, allocation sequence concealment, blinding, other concerns about bias); 9. total number of patients; 10. age of patients; 11. sex of patients; 12. settings; 13. country; 14. number of different intervention groups (triazole or echinocandin); 15. route of administration; 16. dose regimen; 17. duration of administration; 18. incidence of adverse events; 19. treatment discontinuation due to side effects; 20. mortality for each treatment group; 21. complete response at end of treatment for each treatment group; 22. partial response at end of treatment for each treatment group; 23. favorable response (overall treatment success) at 12 weeks after the start of treatment; 24. failure to respond at end of treatment; 25. failure at end of treatment; and 26. stable disease at end of treatment. Values provided as percentages were converted into actual patient numbers (n) for analysis, as well as standard errors into standard deviations using number of patients, when reported as such.

*Selection of studies*

Based on the searching strategy, all titles and abstracts retrieved were independently scanned by four authors (SU, RŽZ, MR and SJ). Eligibility of the retrieved articles was assessed at first from the title and the abstract, and if it was not possible, the full text of the articles was retrieved and searched. An article was included for review if all authors (SU, RŽZ, MR and SJ) agreed that eligibility criteria had been met. In case that the reviewers had different opinions about eligibility of a study for inclusion, the matter was resolved by the corresponding author (RŽZ).

*Data extraction and management*

The data were extracted from eligible studies using the data collection sheet described previously (under the "data collection and analysis" subheading). The data collection sheet was made in electronic form, using an Excel 2007 worksheet. The data were extracted by three investigators independently (SU, RŽZ and MR) and then collating of the four tables was done by another investigator (SJ), who produced the final extraction table. Meta-analysis was made for the following head-to-head comparisons found in the literature: itraconazole, posaconazole or voriconazole *versus* caspofungin, anidulafungin or micafungin.

*Assessment of risk of bias in included studies*

Risk of bias was assessed by two investigators independently (RŽZ and MR), and collating the assessments was done by the another investigator (SJ). The following sources of bias were assessed: 1. randomization if any; 2. sequence generation; 3. allocation sequence concealment; 4. blinding; 5. performance bias; 6. detection bias; 7. attrition bias; and 8. reporting bias. Although some of the studies had high risk of bias, none was excluded from further analysis due to small number of eligible studies (only five).

*Measures of treatment effect*

All of the outcomes used in the studies were dichotomous: mortality for each treatment group; complete response at the end of treatment for each treatment group; partial response at the end of treatment for each treatment group; favorable response (overall treatment success) at 12 weeks after the start of treatment; failure to respond at the end of

treatment; failure at the end of treatment; stable disease and adverse events frequency. For these outcomes the treatment effect was measured by risk ratio (RR).

*Unit of analysis issues*

Unit of analysis in the clinical trials or cohort studies that were included in this meta-analysis were individual patients. Individual participants were either randomized or simply allocated to one of two parallel intervention groups, and a single measurement for each outcome from each participant was collected and analyzed.

*Dealing with missing data*

Missing data were requested directly from the original investigators, however they did not respond to our requests except with courtesy. The missing data were then searched for among the results presented on ClinicalTrials.gov, when available. Finally, the potential impact of missing data on the findings of the meta-analysis will be addressed in the Discussion section.

*Assessment of heterogeneity*

Between-study heterogeneity was assessed with the Cochrane Q test using a $\chi^2$ function ($p$ values < 0.10 were considered significant). $I^2$ values were calculated to quantify inconsistency across studies. $I^2$ values of 30% or less may represent low heterogeneity, values from 30 to 50% may represent moderate heterogeneity, values from 50% to 90% substantial heterogeneity and values of 90% or more may represent considerably heterogeneity. An $I^2$ value > 30% was considered significant in this meta-analysis.

*Assessment of reporting biases*

The possibility of within-study selective outcome reporting was examined for each study included in this meta-analysis. First, by constructing matrix of the outcomes for all studies, we identified studies and specific outcomes that were not reported. Then we searched for published protocols of such studies at ClinicalTrials.gov and other forms of publications of the same studies, in order to find the missing outcomes. Finally, the authors were contacted with a request to provide the missing data, but they did not send us the data.The possibility of between-study publication bias was examined by construction of funnel plots for continuous outcomes and by the Egger's regression for discrete outcomes [11]. The Klein's number was also calculated for all outcomes [12].

*Data synthesis*

The random effects model (which includes both within-study and between-study variations in calculation of the weighted average) was used to combine the results from the studies. The Mantel-Haenszel method (fixed effect model) was also used to estimate how our conclusions could be influenced by assumptions about the model and by the study heterogeneity. Since significant heterogeneity of the studies was not found, subgroup analysis was not performed. All calculations were done by Review Manager (RevMan) software version 5.3.5 [13].

*Sensitivity analysis*

Sensitivity analysis was performed by excluding individual trials one at a time and recalculating the pooled odds ratio (OR) and mean difference estimates for the remaining studies. In this way we got insight how each of the included studies influenced our conclusions.

**Results**

Results of the literature search are shown in Figure 1. Only five studies fulfilled all inclusion and missed all exclusion criteria which were set prior the study commencement (two of the trials were published in the same publication, Raad et al. [14], and one trial was published in two publications [15, 16]). Characteristics of the included studies with risk of bias are shown in detail in Table 1 [14–18].

Summaries of differences in effects of triazoles vs. echinocandins for the main outcomes (using random effects model) were as following: triazoles were associated with lower mortality (OR 0.29), higher complete and partial response rate at end of treatment (ORs 2.38 and 2.83, respectively), more favorable response (overall treatment success) at 12 weeks after the start of treatment (OR 3.05), less failure to respond at the end of treatment (OR 0.38) and more stable disease at end of treatment (OR 1.16), but treatment discontinuation due to side effects and incidence of adverse events were higher with triazoles than with echinocandins (ORs 3.89 and 3.75, respectively). Details of summaries of differences in effects are shown in Table 2, expressed as RR. Sensitivity analysis did not show significant changes with exclusion of single trials.

Summaries of differences in effects of triazoles and echinocandins for the most important outcomes (mortality, complete response at the end of treatment and incidence of adverse effects) with heterogeneity estimates are shown by the Forest plots (Figures 2, 3 and 4).

The reporting bias was assessed by the Klein's number, Egger's regression and a funnel plot, using "trim and fill" method for mortality as the outcome. The central symmetry axis of a funnel plot for mortality rate did not change place significantly after "trim and fill" exercise. In Figure 5 funnel plots are shown before and after "trim and fill" exercise for mortality outcome. The Klein's number for mortality rate was 9.63, however the Egger's regression showed significant correction of the summary effect estimate: from OR = 0.29 to OR = 0.001 (Figure 6).

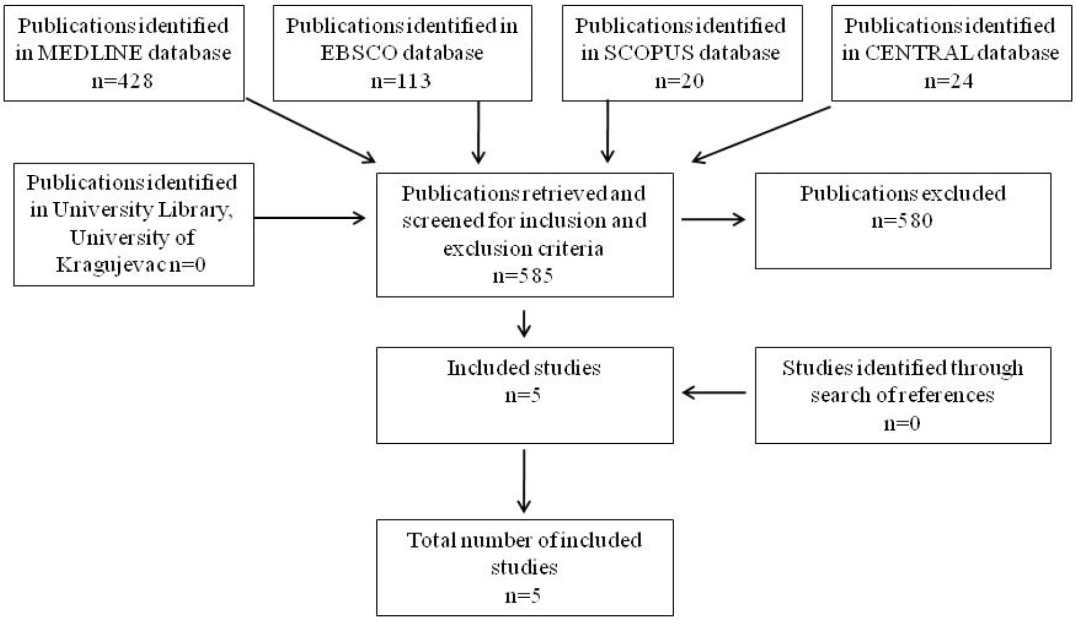


**Fig. 1 – Selection of studies included in the meta-analysis.**

**Table 1**

**Characteristics of the studies included in the meta-analysis**

| Study | Cornely et al.[17] | Walsh et al.[16] | Raad et al.[14] | Rabagliati et al[18] | van Burik et al. [15] |
|---|---|---|---|---|---|
| Methods | Phase II, multicentre, prospective, controlled, open-label, randomized and parallel arm clinical study | A retrospective chart review (retrospective cohort study) | A retrospective chart review (retrospective cohort study) | A retrospective chart review (retrospective cohort study) | Prospective, open-label, multicenter studywith external control group* |
| Participants | Patients 25–76 years old with invasive aspergillosis who received treatment intravenously 300 mg once-daily (QD) intravenous micafungin monotherapy, voriconazole (6 mg/kg twice daily loading dose, followed by 4 mg/kg twice daily); or caspofungin (70 mg loading dose followed by 50 mg (QD)" | Patients in caspofungin group 22–77 years old, voriconazole group 7–81 years old and combination group 22–74 years old, with invasive aspergillosis who received intravenously 4 mg/kg voriconazole every 12 h after 6 mg/kg twice daily on the first day; a loading dose of 70 mg and 50 mg thereafter for caspofungin; or both | Patients in caspofungin group 21–77 years old, voriconazole group 24–75 years old and combination group 7–80 years old, with invasive aspergillosis who received intravenously 4 mg/kg voriconazole every 12 h after 6 mg/kg twice daily on the first day; a loading dose of 70 mg and 50 mg thereafter for caspofungin; or both | Patients in voriconazole group 47.4 ± 17.1 years old, in caspofungin group 48.1 ± 18.6 years old with invasive aspergillosis who received therapy intravenously. | Patients with invasive aspergillosis who received posaconazole orally and comparators intravenously |
| Interventions | Two groups, micafungin (n=12) vs. caspofungin (n=4) or voriconazole (n=1) | Primary treatment: Caspofungin (n=15), voriconazole (n=38) and combination (n=33) | Salavage therapy: Caspofungin (n=17), voriconazole (n=24) and combination (n=35) | Voriconzole (n=46) patients, caspofungin (n=51) patients | Posaconazole n = 107), control group (n = 86) [amphotericin B (any formulation), itraconazole, and/or investigational agents when the study was conducted (eg, voriconazole and echinocandins)] |

**Table 1** ***(continued)***

| Study | Cornely et al.[17] | Walsh et al.[16] | Raad et al.[14] | Rabagliati et al[18] | van Burik et al. [15] |
|---|---|---|---|---|---|
| Outcomes | -Mortality for each treatment group;<br>-Complete response at end of treatment for each treatment group<br>-Favorable response (overall treatment success) at 12 weeks after the start of treatment | - Treatment discontinuation due to side effects<br>-Mortality for each treatment group<br>- Complete response at end of treatment for each treatment group | - Treatment discontinuation due to side effects<br>- Mortality for each treatment group<br>- Complete response at end of treatment for each treatment group | - Mortality for each treatment group<br>-Complete response at end of treatment for each treatment group<br>-Favorable response (overall treatment success) at 12 weeks after the start of treatment<br>-Failure at end of treatment<br>Stable disease at end of treatment | -Complete response at end of treatment for each treatment group<br>-Partial response at end of treatment for each treatment group<br>-Favorable response (overall treatment success) at 12 weeks after the start of treatment<br>-Failure to respond at end of treatment<br>-Stable disease at end of treatment |
| Risk of random sequence generation bias | Low: Randomized study | High: Observational design | High: Observational design | High: Observational design | High: Observational design |
| Risk of allocation concealment bias | Low: Randomized study | High: Observational design | High: Observational design | High: Observational design | High: Observational design |
| Risk of blinding of patients and personnel bias | High: There was no blinding | High: There was no blinding | High: There was no blinding | High: There was no blinding | High: There was no blinding |
| Risk of blinding of outcome assessment bias | High:There was no blinding of outcome assessment | High: There was no blinding of outcome assessment | High: There was no blinding of outcome assessment | High: There was no blinding of outcome assessment | Low: Measurement of all study outcomes were made by the Independent Data Review Board |
| Risk of incomplete outcome data bias | Low: There was no attrition bias. | Low: There was no attrition bias | Low: There was no attrition bias | Low: There was some attrition bias | High: High attrition bias, since in the micafungin group the attrition rate was 75% and in the active control group 80% |
| Risk of selective reporting bias | High: The authors did not pre-specify primary and secondary outcomes in the Methods section, which were later on reported in the Results | High reporting bias, as not all outcomes specified in the Methods were reported in the Results | High reporting bias, as not all outcomes specified in the Methods were reported in the Results | High: as not all outcomes specified in the Methods were reported in the Results | High: The authors did not pre-specify primary and secondary outcomes in the Methods section, which were later on reported in the Results |
| Risk of other bias | High: Efficacy outcomes were not reported for entire intention-to-treat population | High: Efficacy outcomes were not reported for entire intention-to-treat population | Low: Efficacy outcomes were reported for entire intention-to-treat population | Low: Efficacy outcomes were reported for entire intention-to-treat population | Low: Efficacy outcomes were reported for entire intention-to-treat population |

***external control group – since a control treatment could not have been compared with posaconazole in the same study, the patients from participating study sites who were treated by the control drugs, but not enrolled in the study, were used as controls if fulfilling pre-specified criteria. The control patients were matched with patients receiving posaconazole for important prognostic factors to allow for fair comparison between the treatments.**

**Table 2**

**Summary of findings of studies included in the meta-analysis**

| Triazoles (itraconazole, posaconazole and voriconazole) compared with echnocandins (caspofungin, anidulafungin or micafungin) for treatment of invasive aspergillosis | | | | | | |
|---|---|---|---|---|---|---|
| Patient or population: both sex and any age with proven or probable invasive aspergillosis<br>Settings: hospitalized patients.<br>Intervention: Triazoles (itraconazole, posaconazole and voriconazole)<br>Comparison: Echnocandins (caspofungin, anidulafungin or micafungin) | | | | | | |
| | Illustrative comparative risks* (95% CI) | | Relative effect (95% CI) | Number of participants (studies) | Quality of the evidence (GRADE) | Comments |
| | Assumed risk | Corresponding risk | | | | |
| | Echinocandins | Triazoles | | | | |
| Mortality (death rate) | 33.3%<br>60%<br>53%<br>32% | –<br>11%<br>33%<br>20% | <br>RR 0.18 (11-60%)<br>RR 0.62 (33-53%)<br>RR 0.63 (20-32%) | 17 (Cornely et al. [17])<br>53 (Raad et al. [14], primary therapy)<br>41 (Raad et al. [14], salvage therapy)<br>97 (Rabagliati et al. [18]) | ⊕⊖⊖⊖very low<br>⊕⊕⊕⊖moderate<br>⊕⊕⊕⊖moderate<br>⊕⊕⊖⊖low | In the study of Cornely et al. the authors did not state the outcome of treatment with voriconazole. |
| Incidence of adverse events | 25%<br>0.6%<br>5%<br>-<br>- | 20%<br>18%<br>16%<br>HSCT - 17%<br>non-HSCT - 25% | RR 0.8 (20- 25%)<br>RR 30 (0.6-18%)<br>RR 3.2 (5-16%)<br>-<br>- | 17 (Cornely et al. [17])<br>53 (Raad et al. [14], primary therapy)<br>41 (Raad et al. [14], salvage therapy)<br>193 (Burik et al. [15], Walsh et al. [16]) | ⊕⊖⊖⊖very low<br>⊕⊕⊕⊖moderate<br>⊕⊕⊕⊖moderate<br>⊕⊕⊖⊖low | |

**Table 2** ***(continued)***

| | Illustrative comparative risks* (95% CI) | | Relative effect (95% CI) | Number of participants (studies) | Quality of the evidence (GRADE) | Comments |
|---|---|---|---|---|---|---|
| | Assumed risk | Corresponding risk | | | | |
| | Echinocandins | Triazoles | | | | |
| Complete response at end of treatment | 25%<br>26%<br>29%<br>17.8%<br>9% | 60%<br>47%<br>45%<br>65%<br>6% | RR 2.4 (25-60%)<br>RR 1.8 (26-47%)<br>RR 1.6 (29-45%)<br>RR 3.7 (17.8-65%)<br>RR 0.7 (6% to 9%) | 17 (Cornely et al. [17])<br>53 (Raad et al. [14], primary therapy)<br>41 (Raad et al. [14], salvage therapy)<br>97 (Rabagliati et al. [18])<br>193 (Burik et al. [15], Walsh et al. [16]) | ⊕⊖⊖⊖very low<br>⊕⊕⊕⊖moderate<br>⊕⊕⊕⊖moderate<br>⊕⊕⊖⊖low<br>⊕⊕⊖⊖low | |
| Partial response at end of treatment | 16.2% | 35% | RR 2.2 (16.2-35%) | 193 (van Burik et al. [15], Walsh et al.[16]) | ⊕⊕⊖⊖low | |
| Favorable response (overall treatment success) at 12 weeks after the start of treatment | 50%<br>60.7%<br>26% | 20%<br>80%<br>42% | RR 0.4 (20-50%)<br>RR 1.3 (60.7-80%)<br>RR 1.6 (26-42%) | 17 (Cornely et al. [17])<br>97 (Rabagliati et al. [18])<br>193 (Burik [15], Walsh [16]) | ⊕⊖⊖⊖very low<br>⊕⊕⊖⊖low<br>⊕⊕⊖⊖low | |
| Failure to respond at end of treatment | 60% | 36% | RR 0.6 (36- 60%) | 193 (Burik [15], Walsh [16]) | ⊕⊕⊖⊖low | |

**Table 2** ***(continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| Failure at end of treatment | - | 15% | - | 97 (Rabagliati et al. [18]) | ⊕⊕⊖⊖low | |
| Stable disease at end of treatment | -<br>8.13% | 5%<br>10% | -<br>RR 1.2 (8.13-10%) | 97 (Rabagliati et al. [18])<br>193 (Burik [15], Walsh [16]) | ⊕⊕⊖⊖low<br>⊕⊕⊖⊖low | |

*The basis for the **assumed risk** (e.g. the median control group risk across studies) is provided in footnotes. The **corresponding risk** (and its 95% confidence interval) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).
**CI:** Confidence interval; **RR:** risk ratio

GRADE Working Group grades of evidence:
**High quality:** Further research is very unlikely to change our confidence in the estimate of effect.
**Moderate quality:** Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.
**Low quality:** Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.
**Very low quality:** We are very uncertain about the estimate.

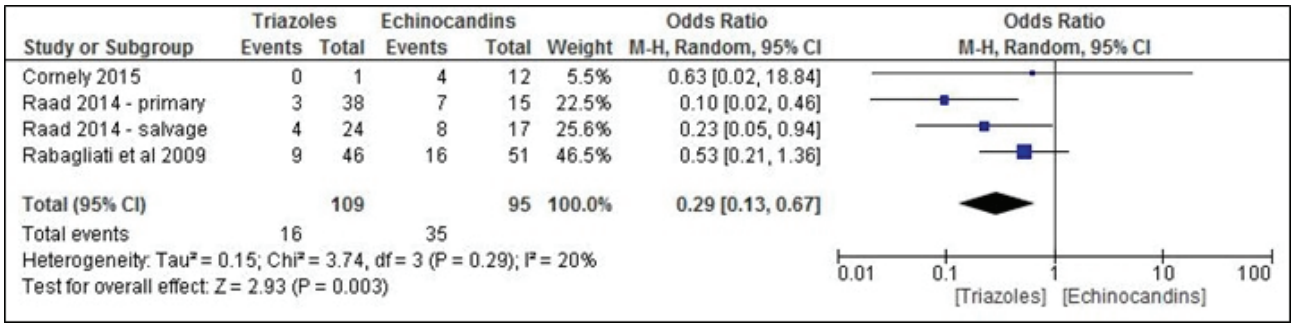


**Fig. 2 – Summary of differences in mortality rate of patients with invasive aspergillosis treated by triazoles or echinocandins.**
**M-H – Mantel-Haenszel method; CI – confidence interval.**

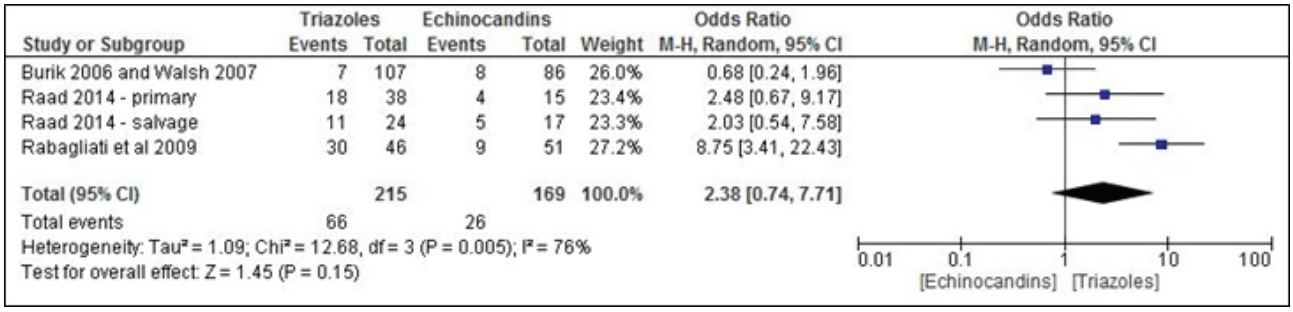


**Fig. 3 – Summary of differences in complete response rate at the end of treatment of patients with invasive aspergillosis treated by triazoles or echinocandins.**
**M-H – Mantel-Haenszel method; CI – confidence interval.**

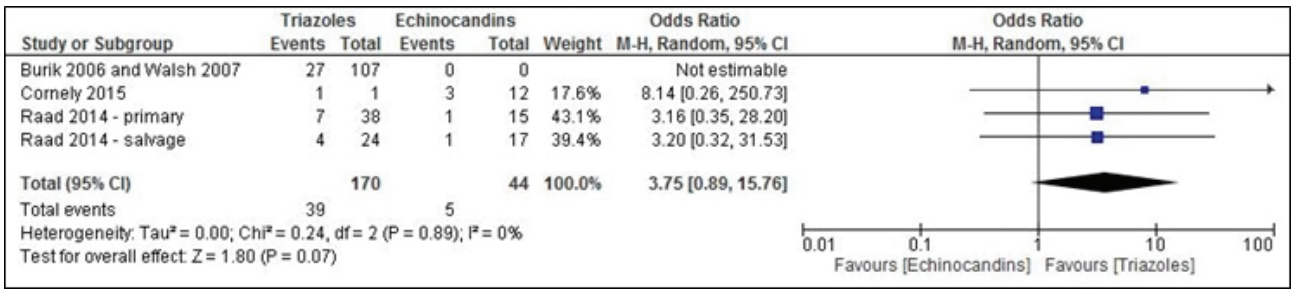


**Fig. 4 – Summary of differences in adverse effects rate observed in patients with invasive aspergillosis treated by triazoles or echinocandins.**
**M-H – Mantel-Haenszel method; CI – confidence interval.**

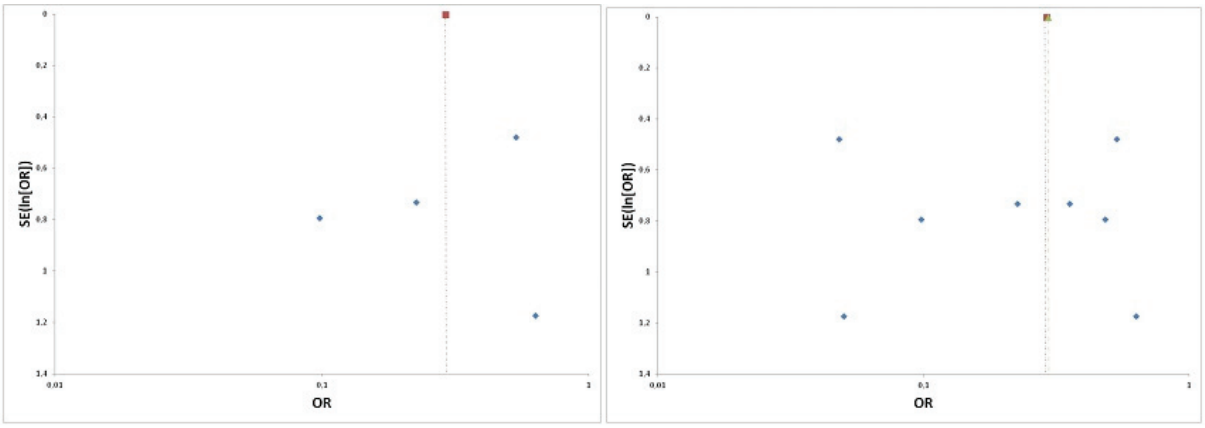


**Fig. 5 – Funnel plots before and after "trim and fill" exercise for mortality rate.**
**OR – odds ratio; SE – standard error.**

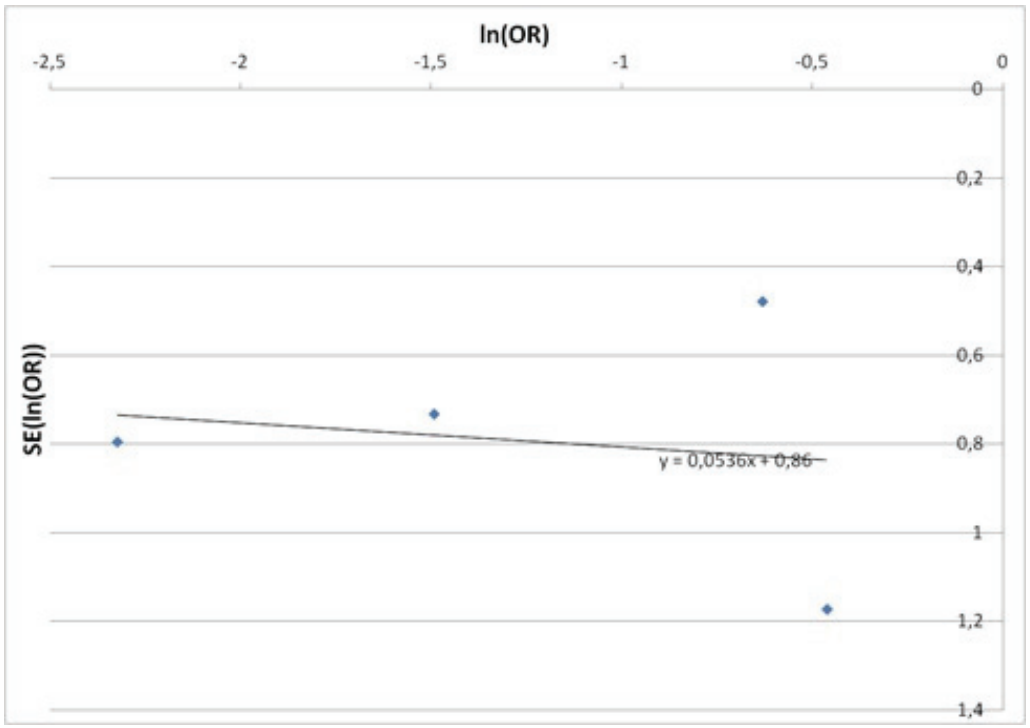


**Fig. 6 – Egger's regression for mortality in the included studies.**
**OR – odds ratio; SE – standard error.**

## Discussion

Our study showed that mortality in patients with IA who were treated with triazoles was significantly lower than in patients treated with echinocandins. However, among the other efficacy outcomes, only rate of favorable response (overall treatment success) 12 weeks after the therapy onset was significantly different between the patients treated with triazoles and echinocandins, triazoles being favored. Other efficacy outcomes invariably were more beneficial in triazole groups, but significance could not be reached because not all included studies recorded every outcome, and certain of them (e.g. failure at the end of treatment or stable disease at the end of treatment) were mentioned in only one or two studies. On the other hand, incidence of adverse events was higher in groups of patients receiving triazoles.

Systematic reviews and meta-analyses of clinical studies including patients with IA are rare, and mostly focused on comparison of combination therapy (triazoles or amphotericin B plus an echinocandin) with non-echinocandin-based monotherapy (i.e.triazoles) after first-line antifungals were ineffective (salvage therapy) [19, 20]. Although authors of these studies at first concluded that combination therapy had increased efficacy, later on they questioned their own conclusions and limited it to situations where antifungal drug resistance is suspected or adequate blood levels could not be achieved [19]. Good efficacy of triazoles (mostly voriconazole) was observed in these studies, as well as relatively high rate of their adverse reactions, but triazoles and echinocandins were not compared head-to-head as monotherapy. Our meta-analysis confirmed good efficacy of triazoles against IA and relatively high adverse events rate in both first-line and salvage settings, when used as monotherapy and compared with echinocandins. Voriconazole and posaconazole penetrate to tissues to high extent (especially to lungs, voriconazole 6.26 μg/g and posaconazole 87.7 μg/mL), while among echinocandins, only anidulafungin has comparable penetration (17.9 μg/g of the lung tissue); however, in studies included in our meta-analysis, only caspofungin and micafungin were used, which could additionally explain superior efficacy of triazoles [21]. Resistance of *Aspergillus* spp. is less frequent to triazoles (from no resistance of isolated *Aspergillus* spp. to posaconazole and voriconazole, to 17% resistance of isolated *Aspergillus fumigatus* to voriconazole) [22, 23] than to echinocandins (22% resistance of *Aspergillus fumigates* to caspofungin) [23], making the first more reliable therapeutic option, especially for second-line treatment of IA.

Increased incidence of adverse events in patients with IA treated by triazoles in comparison to those treated by echnocandins that was found in our study is related mostly to increased incidence of hepatic adverse effects [24]. Although both triazoles and echinocandins may cause either hepatocellular orcholestatic liver injury, frequency is higher with voriconazole, itraconazole, posaconazole or isavuconazole than with caspofungin, anidulafungin or micafungin (up to 24% vs. up to 9%, respectively). However, majority of patients experience only laboratory abnormalities, i.e. elevation of serum levels of aspartate aminotransferase, alanine aminotransferase and bilirubin, and serious liver injuries are rare with both drug groups [24]. Our study confirmed these findings, as none of the patients exposed to either triazoles or echinocandins in included studies had fulminant hepatitis or acute liver failure, yet adverse events rate was significantly higher in groups exposed to triazoles. Additionally, all triazoles interact with cytochrome P450, especially with CYP3A4 and CYP3A5, while voriconazole interacts also with CYP2C19 [25], and their potential to inhibit elimination of other drugs metabolized through the same enzymes is much higher than that of echinocandins [26]. Echinocandins are not metabolized through cytochromes (except micafungin in minor extent) and therefore do not influence elimination of other drugs that are oxidized by these enzymes in liver [21].

Our results should be taken conditionally, since some of the important clinical outcomes were reported in only one of the included studies (e.g. failure at the end of treatment or stable disease at the end of treatment), and overall number of the included studies was low, even after widening of inclusion criteria to encompass cohort studies, which are less reliable than clinical trials due to inherent limitations of observational design. Since clinical trials with triazoles in patients with IA are likely to be initiated in close future, new meta-analysis should be made to challenge our results.

## Conclusion

On the basis of published clinical trials and cohort studies triazoles (voriconazole in the first place) could be considered as more effective and somewhat less safe therapeutic option than echinocandins for invasive aspergillosis for the time being. Future studies which would include new clinical trials are necessary to confirm this conclusion.

## Acknowledgement

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GENERAL REVIEW

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# Large-format histology in diagnosing breast carcinoma

## Histološka tehnika velikog formata u dijagnostici karcinoma dojke

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**Key words:**
**breast neoplasms; histological techniques; cytodiagnosis.**

**Ključne reči:**
**dojka, neoplazme; histološke tehnike; citodijagnostika.**

### Introduction

Breast cancer comprises a remarkably diverse group of diseases in terms of morphology, molecular phenotype, clinical and radiological manifestations, and response to therapy. Management of breast cancer patients is based on prognostic and predictive parameters, which are essential for therapy planning. Key prognostic parameters are tumour size, histological grade, histological type, lymph node status, lymphovascular invasion, and presence or absence of distant metastases [1, 2]. In addition, a few next-generation prognostic parameters have been introduced into routine practice, of which the status of oestrogen and progesterone receptors (ER and PR, respectively), human epidermal growth factor receptor 2 (HER2/neu), and proliferation of cancer cells are the most prominent [3, 4]. Whereas steroid receptors, HER2 status, and proliferative activity are the major parameters for oncological therapy planning, breast cancer subgross morphological parameters such as tumour size, disease extent, and lesion distribution are essential for planning tailor-made surgery and radiation therapy [5, 9].

While breast cancer subgross morphological parameters can be determine with both pathological and radiological methods separately, the most effective is combination of these methods in the form of radiological-pathological correlation [9, 10]. The small block histology, which is the standard pathohistological method, is based on taking 1–2 cm sized representative tissue samples from breast specimens, which are selected under the control of only a pathologist's naked eye and, sometimes, using radiological marks. These samples represent selected pieces of the specimen, and interrelations of different tumour components, which are not present in the same block, are destroyed. Even though the examination of small standard histological samples enables precise determination of the type, grade, and hormone receptor status of the tumour, as well as detection of other molecular markers, this analysis may lack the adequate correlation with the radiological image [9–12].

Opposed to standard small sections, large-format histology is based on embedding and processing continuous tissue slices representing the entire cross-section of a quadrantectomy specimen that includes not only the tumour but also surrounding tissues together in one plane [9]. At the same time, this technique retains the advantages of standard sections and fulfils the requirements of multidisciplinary team needs for therapy planning. Large-section method is considered the most adequate modern diagnostical procedure in breast pathology [9, 13–18]. Some of the opponents of large-format histology state the costs of such technique as the principal argument against it. Recent cost-benefit analyses have shown that the large-format technique is less expensive in daily routine use than the conventional small block technique demonstrating equal tissue surface. The time needed for final pathology report is two weeks at average, which satisfy needs of medical oncologists as it does not prolong the time till adjuvant therapy or change therapeutic options for patients [17, 19].

### Methodological and technical aspects

The large-format histology technique has a long history, but only with the introduction of mammography screening, its importance has been realized, since this method allows precise access to all mammographically detected lesions [16, 18, 20–22]. Large-section method is a routine technique in a several

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pathology centres worldwide. This technique has been incorporated in the everyday practice at our institution, and the procedure has been successfully used to study neoplasms affecting various organs since 2011. Technical aspects of obtaining large-sections have been previously described in detail [23–25].

*Gross examination of the specimen*

In order to obtain an appropriate large-format histology section it is necessary to carefully plan the cut-up of the specimen. The size of the specimen (quadrant resection or mastectomy) and the type of the lesions (microcalcifications, solitary or multiple tumours) determine the way the unfixed specimen is processed.

A pathologist needs to have access to the specimen radiogram, which shows the location of abnormalities in the specimen. The specimen should be delivered to the pathology laboratory properly oriented in a way that enables correct determination of each surgical margin. The specimen radiogram helps a pathologist to map out a way of cutting-up that will include the entire lesion and surrounding tissue in a single cross-section. The specimen is macroscopically described and measured with special indication of present suturing and other markers, if any [22].

In cases where a surgical specimen have only radiologically detected microcalcifications or a non-palpable tumour, the cut-up depends entirely on mammographic findings. It is recommended that specimens should be cut horizontally, in the plane of the specimen radiogram, parallel to the skin and pectoral muscle. This type of cutting is recommended even in cases of solitary tumour masses that are clearly-defined. Multiple tumours are more difficult to obtain in a single large-format section, and in those cases the way of cutting must be selected on the basis of palpation findings of a surgical specimen and findings of the mammogram [22].

The thickness of obtained slices should amount to approximately 3–4 mm. All slices are examined macroscopically and visible tumours are measured in millimetres and described. At the same time, the relation between the tumour and margins is defined [22–25].

Radiologist's markings on images help a pathologist in selecting representative tissue slices for further processing. After the formation of large tissue sections, the position of the selected section is marked on the radiogram, and at the same time, margins of the specimen are inked.

The recommended number of selected tissue slices is 2–4 *per* case. In addition to the formation of large specimens, a small tissue sample is usually taken for immunohistochemical and molecular analyses, but the most representative large sections must remain intact. If a tumour is smaller than 1 cm in the largest diameter, small slices should not be taken.

Slicing of mastectomy specimens for large-section histology is different for two reasons: dimensions of the slices are usually larger than dimensions of available large-format glass slides, and – more importantly – the dorsal resection margin, corresponding most often to the pectoral fascia (not the circumferential as in quadrant-resection) is the only important one. Therefore, the large-format section must show the dorsal surgical margin, so the specimen is sliced perpendicular to the skin. In this case, a pathologist must bear in mind that the radiogram of the entire specimen and the second specimen radiogram are taken in two different planes [22].

*Tissue processing*

The selected tissue slices are processed in the laboratory, using specific protocols for large-format histology. Process itself requires appropriate equipment and techniques adapted for handling large slides. Fixation is performed in formaldehyde solution for 24 hours as standard. The processing (dehydration) of large tissue sections is performed conventionally in any commercially available automated tissue processor for 22 hours. After the processing is completed, paraffin blocks are made using metal brackets placed on a glass plate. Cutting is performed on a special macrotome. The sections, 3-4 μm thick, are placed on commercially available large microscope slides, dimensions 12x9 cm. Staining is performed in modified slide racks, in the same automated stainer as for the small-block sections. Before archiving, the large sections must be properly dried in a well ventilated room, for 2–3 months [23–25].

**Morphological prognostic parameters – interpretation of findings in large section histology**

Many of the prognostic parameters used in assessing breast carcinomas are based on precise histopathological examination of specimens. It has also been shown that large-format sections are effective in the everyday breast routine practice for detailed evaluation of the size, extent, and distribution of the tumour, as well as the resection margins [9, 16, 26–29]. Foster et al. [26] demonstrated that large-sections gave more information than conventionally small blocks in 172 out of 656 cases, as they documented with additional findings of clinical significance, as minute multiple foci of carcinoma, involved margins, or change in size and extent of the disease [18, 26].

Correct tumour size measurement is very important and can be precisely determined on large format histology [16]. Foschini et al. [18] compared the tumour size of 102 consecutive quadrantectomies analyzed with both large-sections and standard small blocks. In 8.8% (9/102) of the cases, large-sections helped to definitely determine the size of the tumour better than conventional blocks, especially in invasive lobular carcinoma, where macroscopic borders of the tumour were ill defined and difficult to be measured at macroscopic level only [18]. Jackson et al. [16] demonstrated that the tumour size measured on large-format histology was larger at average than that measured with small blocks which was corresponding to the large-section size in only 63% of the cases in this series. In addition, ductal carcinoma *in situ* (DCIS) was found more frequently in association with invasive carcinomas in cases documented with large-sections than in cases assessed using conventional histological method.

If the surgical specimen is dissected into smaller pieces while grossing, the interrelation of multiple lesions is compromised, and the assessment of the extent of the tumour

disease becomes impossible. The extent of the disease is defined as the area including all invasive and *in situ* tumour foci, and is a robust prognostic parameter on its own. Tumours with limited extent have an extent less than 4 cm in largest dimension. Patients with this type of tumours are candidates for breast conserving surgery, as opposed to extensive breast carcinomas, where distance between tumour foci is greater, and often require mastectomy [30, 31]. Another important prognostic parameter is the distribution of breast carcinoma foci, which can be unifocal, multifocal and diffuse (Figures 1–3). *In situ* breast carcinomas, much like invasive carcinomas, show unifocal, multifocal and diffuse distribution [22, 32, 33].

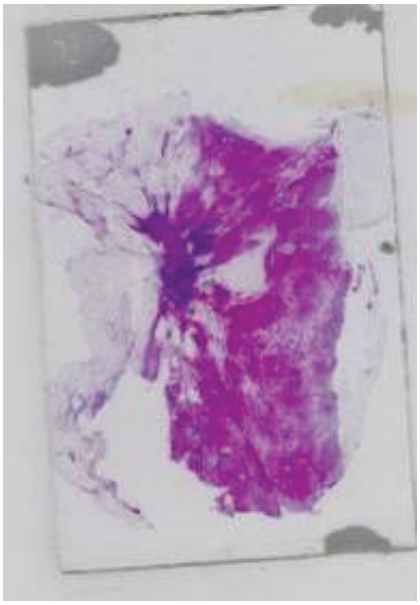

**Fig. 1 – Large-format histology section of an unifocal breast carcinoma.**

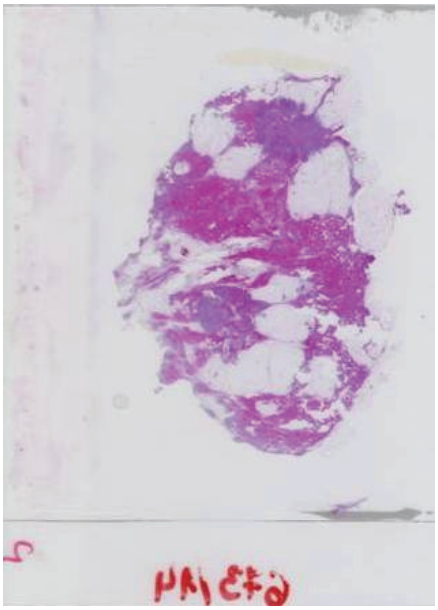

**Fig. 2 – Large-format histology section of a multifocal breast carcinoma.**

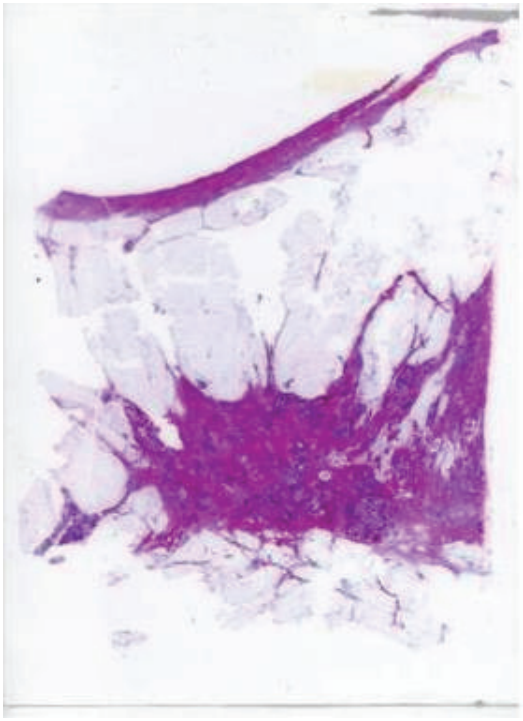

**Fig. 3 – Large-format histology section of a diffuse breast carcinoma.**

In everyday practice, pathologists should begin the gross examination of a specimen by summarizing radiological findings including the radiological disease extent.

For precise determination of the disease extent, the slice with the greatest disease area should be chosen and processed. Analysis of large-section slides begins on the peripheral part and is then directed towards the central area of the sections [22]. The most peripheral malignant lesions in the sample are marked. In order to properly determine the extent of the disease, a pathologist must summarize all findings related to the specimens and reconstruct the lesions as a whole. After this step the distribution of malignant lesions is assessed in the described area (*in situ* component and invasive tumour foci are identified).

The proportions of multifocal breast cancer cases vary among studies, depending on definition and methodology of assessment. According to large-format histopathology studies, multifocality of the invasive component occurs in ~35% of breast tumours [9, 30, 34]. Modern breast imaging techniques also support these results [35]. Several studies performed on large-sections have shown the prognostic value of the extent and distribution of lesions in breast carcinomas, as the metastatic capacity is higher in patients with multifocal and diffuse tumours when compared to those with unifocal carcinomas [36, 37]. Pekar et al. [34] using large-sections, have demonstrated that multifocality and diffuse distribution of invasive tumours were associated with significantly poorer survival in breast cancer patients compared to those with unifocal tumour disease.

The goal of histopathological examination of specimens resected after neoadjuvant chemotherapy is the detection of either the residual viable tumour or documenting the presence of the tumour bed. Large-format technique is especially

useful in the evaluation of post-neoadjuvant therapy surgical specimens with complete or near-complete response to therapy since these types of lesions are usually not visible on gross examination**.** Thus, residual cancer foci of small size may remain undetected on conventional blocks. The use of large-sections improves the accuracy of the assessment and increases the chance to detect even the smallest residual tumour foci [18, 38].

Precise evaluation of resection margins has become a very important issue especially in quadrantectomy cases. Standard blocking is based on gross inspection of the lesion and on palpation of the tissue. Small tumour foci may remain undetected during this examination. Large-format histology samples are ideal for determining the circumferential resection margins. Superficial and deep resection margins (close to the skin and close to the pectoral muscle, respectively) are not displayed directly due to the recommended way of slicing the specimen obtained with conservative surgery. The absence of any radiological or gross abnormalities in the first and the last horizontal slice is useful evidence of sufficient radicality in these directions. If one or both slices include suspicious macroscopic or radiological abnormalities, it is necessary to take small samples from that zone, based on which the status of margins over and under the tumor will be determined. Clarke et al. [25] reported that the large-sections method is more sensitive than conventional method for identifying positive margins or multifocal tumour disease in breast quadrantectomy specimens. The use of large-sections also helps distinguishing between the real inked margin and migration of ink through tissue clefts and fissures in the specimen surface [30].

Breast carcinoma is not necessarily composed of a identical monoclonal cells, but may represent a population of diverse tumour cell clones. This may result in different morphology in various parts of a tumour focus (intratumoural heterogeneity), or varying morphology of different tumour foci within the same breast (intertumoural heterogeneity). Heterogeneity is of the greatest importance when interpreting biomarkers, especially HER2 status, because many invasive breast cancers contain at the same time cells with and without HER2 amplification. Large-format histology allows a simple insight to inter- and intratumoural heterogeneity.

All cases of breast cancer documented with large-format histology should be regularly analysed at multidisciplinary meetings of pathologists, radiologists and surgeons, using an overhead projector [39].

### Oncology Institute of Vojvodina experience with large-format histology

Large-format histology has been introduced in routine use atthe Oncology Institute of Vojvodina in 2011, and since then it has become an integral part of our diagnostics protocols. Our six-year results with diagnostics of breast carcinoma are shown in Table 1.

In some of the cases, the use of large-format histology has led to detection of pathological changes that were undetected prior to surgical intervention and pathological work-up (Figure 4). Also, since the growth pattern of invasive carcinoma is sometimes infiltrative without clearly visible borders, macroscopic tumour size needed a substantial correction after analysis of large-format slides, which was in accordance to the observations of Foster et al. [26]. In our experience, tumour multifocality is easy to detect on large sections; its incidence in our series is comparable with that of other reported series. The status of the surgical margins is also accurately defined in large sections [9, 30, 40].

**Table 1**
**Six year experience with large-format histology at the Institute for Oncology of Vojvodina**

| Demographics | Values |
|---|---|
| Total number of patients (n) | 289 |
| Average age of patients (years) | 55.04 |
| Neoplastic lesions (n) | 215 |
| Non-neoplastic lesions (n) | 74 |
| Large-format slides (n) | 520 |
| Large-format slides *per* patient (n) | 1.78 |
| Tumour distribution (n) | |
| unifocal | 92 |
| multifocal | 73 |
| diffuse | 50 |
| Histological type (n) | |
| ductal carcinoma in situ | 41 |
| invasive carcinoma NST | 122 |
| only invasive carcinoma | 36 |
| invasive and in situ carcinoma | 86 |
| lobular | 28 |
| only invasive carcinoma | 14 |
| invasive and *in situ* carcinoma | 14 |
| mixed | 8 |
| papillary | 7 |
| mucinous | 4 |
| tubular | 2 |
| metaplastic | 2 |
| medullary | 1 |
| Complete response to NAT(n) | 4 |
| Histological grade (n) | |
| 1 | 46 |
| 2 | 114 |
| e 3 | 55 |
| T stage (TNM) (n) | |
| T0 | 3 |
| Tis | 39 |
| T1 | 82 |
| T1mi | 1 |
| T1a | 20 |
| T1b | 22 |
| T1c | 39 |
| T2 | 60 |
| T3 | 22 |
| T4 | 8 |

***NST – no special type; †NAT – neoadjuvant therapy; TNM – tumor, node, metastasis.**

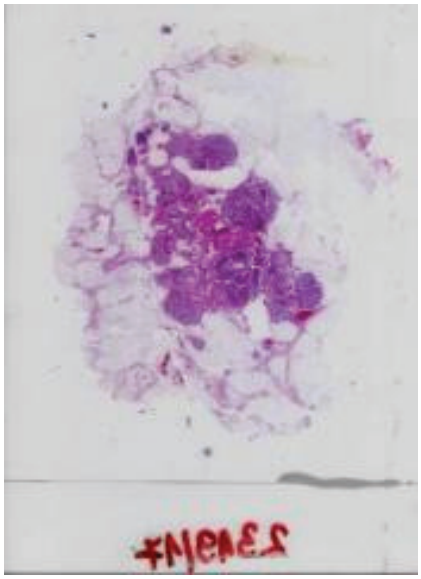

**Fig. 4 – Large-format histology section of an accidental finding of submilimeter invasive breast carcinoma surrounded with diffuse *in situ* intracystic papillary carcinoma.**

Large-format histology is a histo-technical method that allows pathologists to correctly identify the important morphological prognostic factors such as tumour size, the extent of the disease, the distribution of lesions, inter- and intratumoural heterogeneity, and the status of the circumferential surgical margin, while all of these parameters can be directly compared with radiograms. This technique is especially useful in the diagnosis of i*n situ* and early invasive carcinomas, as well as post-neoadjuvant therapy surgical specimens with complete or near-complete response to therapy, since these types of lesions are usually not visible while sampling the tissues. The too often expressed criticism regarding the presumed high costs and prolonged laboratory turn-around time due to this technique is not justified. Our experience and the growing body of scientific evidence show that it is the only cost-effective histo-technical method that meets the needs of modern multidisciplinary diagnostic approach in breast pathology. We propose including large-format histopathology into work-up of all breast surgical specimens as the standard method.

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CASE REPORTS


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# Aortoduodenal fistula after abdominal aortic aneurysm resection: Two case reports

## Aortoduodenalna fistula posle resekcije aneurizme abdominalne aorte

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**Abstract**

**Introduction.** Aortoenteric fistula (AEF) is rare and extremely difficult complication of aortic surgery. We presented two cases of secondary aortoduodenal fistula (SADF) as complication after aortic surgery. **Case reports.** In the first patient SADF happened 11 years after open abdominal aneurysmal resection with gastrointestinal tract (GIT) bleeding. After negative esophagogastroduodenoscopy (EGDS) we performed multislice computed tomography (MSCT) which revealed contrast leakage in duodenum from 10 cm wide visceral aortic aneurysm. The patient was treated with graft excision, aneurysmal reduction, sewing of proximal and distal aortal stumps, bowel repair followed by axillobifemoral bypass (AxFF). The patient dismissed on 30th postoperative day. The second case of ADF happened five months after endovascular reconstruction of abdominal aorta with GIT bleeding and fewer. During following 8 days, he had three negative EGDS. On MSCT we found signs of endoleak, free air in aneurysmal sac, and signs of blood in the intestine. On urgent operation we extracted stent graft, sewed proximal and distal aortal stumps, performed bowel repair and AxFF. The patient died a day after operation with signs of sepsis and multiple organ failure syndrome. **Conclusion.** Conventional treatment of ADF means extraanatomic AxFF with complete excision of infected graft or stent graft, with closure of aorta's proximal and distal stumps and duodenal repair. Because of high mortality, prompt diagnostic evaluation and quick decision of an adequate operative treatment is necessary. Although European Society of Vascular Surgery recommendations, as a guide, are very helpful, there is no unique attitude about management of AEF, so each patient should be specifically treated.

**Key words:**
**aortic aneurysm, abdominal; endovascular procedures; gastrointestinal hemorrhage; intestinal fistula; stents; treatment outcome; vascular surgical procedure.**

**Apstrakt**

**Uvod.** Aortoenterična fistula (AEF) je retka i izuzetno teška komplikacija operacije aorte. Prikazali smo dva bolesnika sa sekundarnom aortoduodenalnom fistulom (SADF) kao komplikacijom operacije aorte. **Prikazi bolesnika.** U prvom slučaju SADF je nastala 11 godina nakon resekcije aneurizme abdominalne aorte, sa znacima krvarenja iz gastrointestinalnog trakta (GIT). Posle negativnog ezofagogastroduodenoskopskog (EGDS) nalaza učinjena je multislajsna kompjuterizovana tomografija (MSCT) angiografija koja je otkrila curenje kontrasta u duodenum iz 10 cm široke aneurizme visceralne aorte. Bolesnik je hitno operisan, kada je izvađen graft, redukovana aneurizma sa prešivanjem proksimalnih i distalnih delova aorte, urađena je rekonstrukcija creva i aksilobifemoralni bajpas (AxFF). Bolesnik je otpušten 30. dana posle operacije. U drugom slučaju, SADF je nastala pet meseci nakon endovaskularne rekonstrukcije abdominalne aorte (EVAR) sa znacima krvarenja iz GIT i sepse. U sledećih osam dana učinjene su tri EGDS i viđen je normalan nalaz. Na MSCT aortografiji nađeni su znaci *endoleak*-a, partikule slobodnog gasa u aneurizmatskoj vreći i retroaortalno, kao i znaci prisustva krvi u crevima. Bolesnik je hitno operisan i odstranjen je stent graft, ušiveni proksimalni i distalni delovi aorte, učinjena reparacija crevnog zida i AxFF. Bolesnik je egzitirao prvog postoperativnog dana zbog sepse i sindroma multiorganske disfunkcije. **Zaključak.** Preporučeni tretman SADF podrazumeva ekstraanatomski AxFF sa kompletnom ekscizijom inficiranog grafta ili stent grafta, prešivanje aortnih bataljaka i reparaciju duodenuma. Zbog visoke smrtnosti, kod tih bolesnika neophodna je hitna dijagnostička procena

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i odluka o adekvatnom operativnom tretmanu. Iako su preporuke *European Society of Vascular Surgery*, kao vodiča, veoma korisne, ne postoji jedinstven stav o tretmanu AEF, tako da svaki bolesnik zahteva specifičan tretman.

**Ključne reči:**
**aorta, abdominalna, aneurizma; endovaskularne procedure: krvarenje, gastrointestinalno; fistula, crevna; stentovi; lečenje, ishod; hirurgija, vaskularna, procedure.**

## Introduction

An aortoenteric fistula (AEF) is a rare but dangerous cause of gastrointestinal bleeding. Abnormal communication between the aorta and the intestine represents AEF. Aortoduodenal fistula (ADF) is a case of aortic connection with duodenum as most frequently involved gut. If this communication was acquired spontaneously, mostly as an untreated abdominal aortic aneurysm (AAA), we talk about primary ADF (PADF). Secondary ADF (SADF) is a rare, late complication of aortic reconstructive surgery [1].

There are two types of AEF. Type 1, is a "true" fistula, with communication between lumen of the aorta and lumen of the intestine, and type 2, or a paraprosthetic-enteric fistula, better called erosion. In type 1, direct communication of the aorta and the intestine produces massive bleeding and untreated patient dies very soon. In type 2, there is no communication between the bowel and the blood stream, but only the intestine erosion with open lumen near intact blood vessel or graft. Bleeding occurs from the edges of the eroded bowel. Local infection and sepsis are main clinical signs associated with this type of fistula/erosion [2].

An AEF after aortic reconstructive surgery is suspicious when a patient presents with abdominal pain, gastrointestinal hemorrhage and sepsis [2]. Early detection of AEF is necessary and the first physician who evaluate gastrointestinal bleeding must consider AEF [1, 3]. Clinical presentation of bleeding varies as hematemesis, melena, hematochezia, anemia [4].

Unfortunately, exploratory laparotomy often is the only method to diagnose AEF.

We reported two cases of AEF treated in our hospital. The first case of SADF, was found 11 years after open surgical resection and reconstruction of AAA with tubular graft. The second case of SADF, was found five months after endovascular reconstruction of abdominal aorta (EVAR) procedure.

## Case reports

*Case 1*

A patient, male, 70 years old, was admitted in the Emergency Room in the morning with anamnesis of blood vomiting few hours earlier. Before one week, he had same episode with spontaneous remission, and day before admission he had dark stool. In anamnesis, the patient had resection of abdominal aortic aneurism 11 years before, with Dacron N°XVIII tubular graft reconstruction in our hospital. On physical exam the patient was normotensive, with sinus rhythm, heart rate 87 beats/min. In laboratory test the patient had erythrocyte (Er) count $3.41x10^{12}$ (normal values $4.34–5.72x10^{12}$/L), hemoglobin (Hgb) 107 g/L (normal values 130–160 g/L), hematocrit (Hct) 0.33 L/L (normal values 0.35–0.53 L/L). On urgent esophagogastroduodenoscopy (EGDS) there were empty stomach with no signs of active bleeding. The same finding was in the duodenum, in parts D1 and D2, with no active bleeding from distal part of the duodenum. A gastroenterologist prescribed him H2 blockers with diet and the patient dismissed home. In the evening, he came back complaining of pain in upper parts of the abdomen, nausea, hematemesis and melena 3 times in that afternoon. On exam he had pain in the abdomen on palpation, with clear peristaltic. On rectal exam, a clot of dark blood was found. In laboratory tests there were a small drop of Er ($2.92 x 10^{12}$/L, Hgb (94 g/L) and Hct (0.29 L/L). The patient had episode of hematemesis (about 300 $cm^3$) in the infirmary. On urgent multislice computed tomography (MSCT) angiography with contrast, an enlarged visceral part of the aorta, near the proximal anastomosis of earlier reconstruction or suprarenal aneurysm, 9.5 cm wide and 8 cm long, was found (Figure 1).

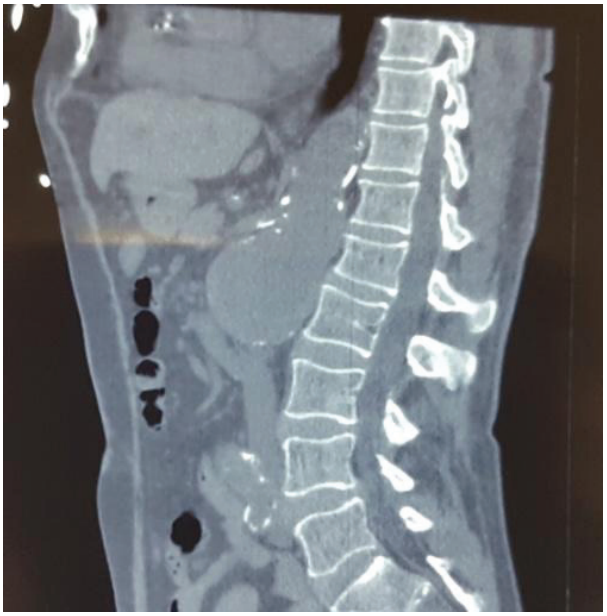

**Fig. 1 – Multislice computed tomography angiography of suprarenal aneurysm, 9.5 cm x 8 cm, and previous reconstruction of abdominal aneurysm with Dacron N°XVIII tubular graft.**

The aneurysm with parietal thrombus and 2.5 cm of circulating lumen because of a compression on D4 with clear signs of communication of AAA and the duodenum were seen as leakage of contrast in the duodenum and jejunum. There was no retroperitoneal or intraperitoneal leakage (Figure 2).

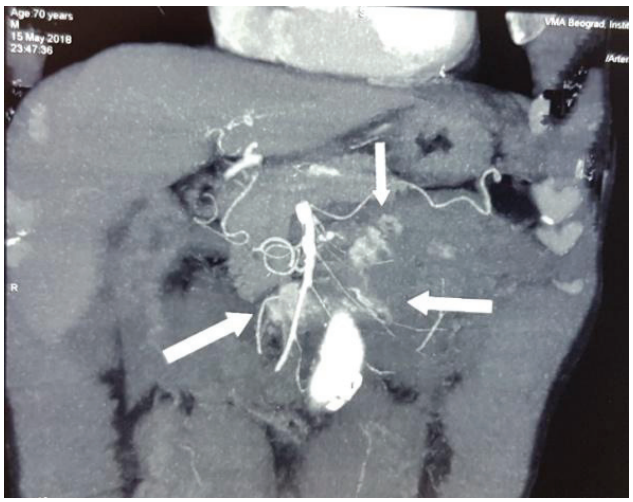


**Fig. 2 – Multislice computed tomography angiography with signs of contrast leakage from wide suprarenal aneurysm to the duodenum and jejunum.**

In the moment after scanning, the patient became unstable, hypotensive (70/45 mmHg), Htc 0.23 L/L, Hgb 87 g/L, with tachycardia (110 beats/min) and cold extremities. In the Intensive Care Unit, he had episode of hematemesis again and we sent him to the operative theater.

On prompt medial laparotomy we found 10 cm wide suprarenal aortic aneurysm with end above the celiac trunk. D3 part of the duodenum crossed over the aneurysm where was connected with small communication hole. After dissection of this communication joint, we temporary sewed hole on the aneurysm and sewed hall on the duodenum in two layers. Than we performed Mattox maneuver (left visceral rotation) from the sigmoid and left colon, the left kidney, the spleen with the pancreas to the diaphragm. After administration of 8,000 units of heparin we put a clamp on normal size aorta beneath the diaphragm and the second clamp beneath Dacron graft. Then we resected previous graft and reduced aneurysmal sac of the visceral aorta. Renal arteries, the superior mesenteric artery and the celiac trunk were perfused with 500 mL cold 0.9% NaCl. In the meantime, reduced aneurismal sac was sewn as aneurismorraphy to spare visceral arteries. Distal part of the aorta above aortal bifurcation was sewn. Then we performed right axillo-bifemoral bypass (AxFF) with Intervascular® ringed graft 8 x 8 mm, 100 x 55 cm (La Ciotat, France) impregnated with silver, in the standard manner through several skin incisions. The abdomen was closed and we drained left retroperitoneal space and Douglas recessus. Operation lasted six hours, supraceliac clamping lasted 22 min and intraoperative lost of blood was 3,000 mL. The patient was extubated on the zero postoperative day. After operation, nasogastric tube stayed in place for 5 days, and drains took out on 10th postoperative day. On 7th postoperative day the ptients discharged his guts with clots and dark old blood. One of skin incisions as a place for axillar part of the graft was infected with *Staphylococcus aureus* with leakage. The patient was treated everyday with low-molecular-weight heparin, nadroparin (Fraxiparine®) 0.6 units, dressing and antibiotics for two months. Triple antibiotic therapy (vancomycin, ceftriaxone, metronidazole) was used for 15 days, followed by ceftriaxone and metronidazole in next 15 days. Ciprofloxacine (*per os*) was used in the next month. Three weeks after operation, a control MSCT angiography was done. Patent bypass, both aortal stumps with patent all visceral arteries, and no liquid collections, gas or hematomas were found (Figure 3).

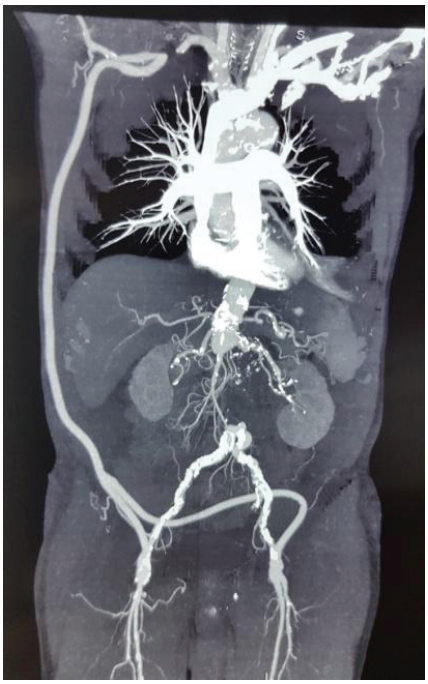

**Fig. 3 – Multislice computed tomography angiography with patent axillo-bifemoral bypass and all visceral arteries, and with no liquid collections, gas or hematomas.**

After closing of all skin wounds and removing of wound stiches, the patient was dismissed on 35th day after operation in a good condition. Few days after operation the patient had mild headache and dizziness in up-right position, but after several days these symptoms gone. Three months after the first control exam, the patient was with good patency of bypass and normal gastrointestinal function. All skin incisions were without signs of infection (Figure 4).

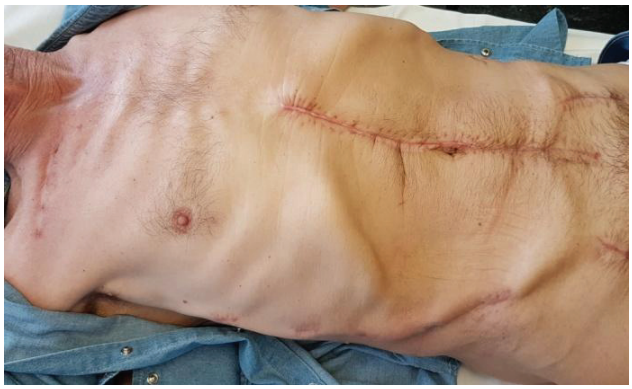

**Fig. 4 – All skin wounds were closed with no signs of infection three months after operation.**

*Case 2*

A male patient, 77 years old, was admitted in the Emergency Center with hematemesis, arrhythmia and signs of chronic obstructive pulmonary disease. Endovascular reconstruction of the abdominal aorta was done five months before, and one year ago he had episode of lumbal spondylodiscitis. A day before admission, the patient was admitted in a regional hospital where he had episode of massive blood vomiting. At the admission, the patient was immobile, with mild dyspnea, arrhythmia, hypotension (105/70 mmHg), signs of cardiomyopathy and atrial septal defect with initial kidney failure. On urgent gastroscopy, blood in the stomach was found, as well as an ulcer on the big curvature which was sclerosated with 1 : 100,000 solution of adrenaline. The finding on the duodenum was competent. Laboratory analyses were as follows: Er $2.25x10^{12}$/L, Hgb 63 g/L, Hct 0.20 L/L, and transfusion of blood was applied. On the control EGDS, the day after, bleeding in the stomach and duodenum was not found. Daily monitoring of laboratory tests in a period of eight days were as follows: Er $2.55x10^{12}$/L to $3.15x10^{12}$/L, Hct 0.23 L/L to 0.28 L/L and Hgb 73 g/L to 89 g/L.Three days after admission, the patient was febrile (37.5°C to 38.5°C), and sample for hemoculture was took. On 8th day after admission, on the control EGDS, the finding was normal without signs of bleeding and with competent duodenal wall. Ninth day after admission, hemorrhagic shock was developed. On urgent MSCT aortography signs of endoleak with contrast in aneurysmal sac were found (Figure 5).

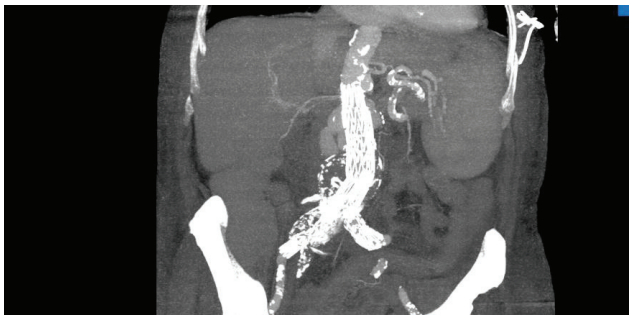

**Fig. 5 – Endovascular reconstruction of the abdominal aorta with signs of endoleak with contrast in the aneurismal sac.**

Also, findings included particles of free air in paraaortic and retroaortic regions, and radiographic signs of blood in the ileum and colon. Direct communication of AEF was not found. Laboratory tests showed Er $2.24x10^{12}$/L, Hgb 63 g/L and Hct 0.20 L/L. The patient was unstabile and underwent urgent operation. Intraoperatively, we found the duodenum in contact with infrarenal aneurysmal sac. We established bleeding control with supraceliac clamping. The bowel was dissconected from the aorta and the duodenal wall was repaired in two layers. After opening of aneurysmal sac, we found bleeding beneath the proximal part of stent graft and aortal wall (place of endoleak). After that, we extracted endovascular graft (Zenith Flex®, Cook Medical) and relocated clamp to infrarenal position. Duration of suprarenal clamping was 28 min. Bleeding from posterior side of the aorta, and lysis of L2 and L3 spine body was controlled with vax. Then, we sewed proximal and distal aortic stumps. Proximal aortic stump was disrupted twice intraoperatively with massive bleeding. Also, we found multiple spleen bleeding lesions and we done splenectomy. After that, the patient became hemodinamically stable. A part of endovascular graft and samples from the retroperitoneum and spine body L2/L3 were sent to microbiological analysis. After closing the operative wound, we assessed it was neccessary to make AxFF with Hemagard® silver AxFF graft 8/8, 100 x 50 cm. During the operation, the patient loss 7,000 mL of blood. Intraoperatively, we used cell saver and 4,100 mL washed erithrocytes were returned. Also, the patient got 150 mL of cryoprecipitate, and 1,390 mL of fresh frozen plasma. In the postoperative period, the patient was unstabile with muliple organ failure syndrom, pulmonal failure, cardiac failure, high temperature and signs of sepsis. The patient died in the Intensive Care Unit, the day after operation. Postmortem, *Enterococcus foecalis* was found in samples of aneurismal sac, and *Clostridium perfrigens* in hemoculture taken preoperatively.

**Discussion**

The first description of PAEF was given in 1817 by Sir Astely Cooper, and that of SAEF by Brock after a homograft aortic repair in 1953. The first successful repair of AEF was performed in 1957 [5]. AEFs are rare (2%) but devastating complications of prosthetic arterial reconstruction with high operative mortality rate (30% to 75%) and morbidity (amputation) rate (30% to 50%) [2, 6–9]. About 70% of patients previously have aortooclusive disease with some bypass, and 30% of patients are because of aneurismal disease [5]. The average interval between first operation and operation due to aortoenteric fistula is 3–5 years with a range of 2 weeks to 14 years [5–7]. In our cases these intervals were 11 years in the first and 3 years in the second case.

Diagnosis of AEF is preoperatively correct in about 30% of patients. In some cases, clinical signs are not so clear, so patients have normal arterial pressure, pulse, normal signs on pulmonary and heart systems, but with light drop in hemoglobin and hematocrit values. Katsinelos et al. [10] in 2005 pointed out that all their patients with AEF died despite presenting with classic "herald bleeding" as a result of delayed operative intervention. Japanese authors [7, 11] also presented the results of *postmortem* autopsy and identification of the cause of sudden death with massive bleeding. Bleeding in AEF has incidence from 1% to 2% of all gastrointestinal tract bleeding and 60% in cases of AEF [1]. Time interval between the initial "herald bleeding" and the massive gastrointestinal hemorrhage ranged from one hour to two days. [1] Our first patient on the initial and repeated exams had stabile condition with normal heart rate and arterial pressure, and normal laboratory values. He also had "herald bleeding" few days before. Only after profuse bleeding in the infirmary, a physician decided to perform complete diagnostic procedures. In the second case, situation was additionally complicated because the patient on initial "herald bleeding" were

treated in a regional hospital as gastric ulcer bleeding for one day. In our hospital he had three EGDS with negative finding and for next eight days treated with blood transfusions and proton pump blockers. On 8th day, the patient had profuse bleeding and became completely unstable. Urgent MSCT diagnostics revealed problem and we did prompt operation. However, we lost precious time before established right diagnosis. In false negative cases stool can be tested for occult blood and results can be helpful [12].

The main diagnostic procedure in gastrointestinal bleeding is EGDS. But, frequently, in cases of ADF, EGDS findings are negative and this method does not allow the bleding site to be found [10, 12]. Physicians can find the duodenum full of coagulum and fresh blood, but cannot identify an origin of the intestinal bleeding, and communication between the aorta and the intestine cannot be detected [7]. Nevertheless, most of AEFs are duodenal (81%) in the third and fourth portions, and complete examination of the duodenum is essential [6, 12]. In both our cases, EGDS findings were negative. In the first case there were no signs of bleeding and in the second case we performed EGDS three times. Only in the first EGDS finding we found blood in the stomach, and in other two EGDS, findings were negative. Gastrointestinal tract bleeding, negative endoscopic finding and previous aortic procedure are suspicion on SAEF and have a great value in establishing the right diagnosis.

Preoperative symptoms are mostly fever (in 70% of patients), gastrointestinal tract bleeding (in about 48% of patients) and only 22% of patients have both [5–7]. These febrile symptoms last in months and are related to longstanding graft infection. Patients with false aneurysms or graft failure have symptoms in the first week [5]. Blood cultures are also positive for different bacteria and with antibiotic therapy it can be false improvement. Urine cultures, chest X-ray and abdominal computerized tomography can also be normal [12]. Cultures of the aortic graft revealed enteric organisms in each instance [4]. In 30% of patients there is no signs of infection [7]. Our first patient had no fever, but second one was febrile. Also, the second patient had positive hemoculture finding on *Clostridium perfrigens* and positive microbiological samples of aneurysmal sac on *Enterococcus foecalis*.

True mechanism of developing AEF is unknown, but it develops on two ways. First, a chronic inflammation from mechanical pressure due to pulsating movements of graft on the bowel, in cases with no abscess or evidence of infection around fistula, may be the cause of fistula [13]. Second reason is adhesion of infected graft to the bowel. Contribution to AEF is infection of the suture line of the anastomosis and bad cover with retroperitoneal tissue around graft [2, 14]. Other reasons for ADF can be reoperation because of graft failure, inadequate reperitonealization of graft, use of aortic homograft, false aneurysm, renal infarction, etc [4]. Chronic mechanical irritation between the bowel and aneurysmal wall make watertight adhesion between these two tissues resulting in AEF. High pressure in the aorta creats an one-way fistula into the bowel lumen with massive arterial bleeding [1]. Adhesion of the duodenum is often larger than hall on the gut and aorta, and often is a near suture line with cut of suture string [7]. If this process destruct the bowel wall only, aortoenteric erosion becomes rather than fistula and lots of air bubbles can be find on MSCT finding around the aorta and this is a specific sign for diagnosis of the aortic graft infection [1, 10].

MSCT angiography with contrast is also very important diagnostic procedure and can be performed in an emergent situation and has been recommended as an initial diagnostic test for AEF. But, MSCT of the upper abdomen with bolus contrast often can show no signs of AEF. MSCT is not only diagnostic procedure for AEF but also reveled anatomical structures for later aortic surgery. Finally, visualization of the fistula is a very rare and means extravasations of contrast from the aorta to the intestinal lumen [15]. A periaortic air bubbles are a specific MSCT finding for the diagnosis of the aortic graft infection or AEF and were found in 80% of patients. It may be difficult to differentiate these two conditions because of the overlapping computed tomography features [1, 15]. Similar finding can be found in pseudoaneurysm, loss of retroperitoneal fat, and disruption of the aortic wall [15].

In our first case, the duodenum was adjacent to the proximal suture line, just above huge visceral aneurysm and we could see contrast leakage through communication from the aorta to the duodenum. The second case was very rare and interesting because of previous EVAR procedure. The duodenum was adjacent to belly of aneurysmal sac with a hole of 1 $cm^2$. Theoretically, space between graft and aneurysmal wall had no blood stream, but the patient had abundant bleeding. On MDCT, we found endoleak type 1, and revealed communication between the duodenum and blood stream from the aorta. Also, in the second case we found air bubbles on MSCT as indirect sign of AEF. Very similar case had Kao et al. [16].

There are strong recommendations what a surgeon must to do in situation with aortoenteric fistula with graft infection [4, 6]. With discovery of AEF, the old graft should be removed completely, the hall on intestine closed, and an extraanatomic bypass constructed, with survival rate about 36%–78% [3, 4, 9]. Wide resection of the proximal anastomotic site and adjacent infrarenal aorta must be done. Temporary, subdiaphragmatic (supraceliac or suprarenal) aortic clamping may be necessary to make the aortic stump closure in two layers. If there is inadequate infrarenal aortic length for closure, relocation of the renal pedicle to a higher aortic level is possible (anastomosis on hepatic or lienal artery). Thus, closure up to the level of the superior mesenteric artery orifice, far above the original infected anastomosis, is possible [6].

Control of bleeding is the most important primary step to rescue patients with this complication. Intra-aortic balloon occlusion (IABO) can be used as temporary control for intraabdominal bleeding. It is easy to implement with immediate control of bleeding from the aorta to the duodenum [13]. But, if balloon was placed above visceral arteries, an organ ischemia in short time and reperfusion will produce necrotic changes. Aortic occlusion over 45 min might cause irreversible dysfunction of organs and compartment syndrome [13].

In our first case, aortic clamp was placed above aneurysm of the visceral artery, just beneath the diaphragm. Vis-

ceral arteries were perfused with cold (+4 C°) Ringer's lactate and ischemic time was prolonged. Perfusion lasted about 20 min and clamp stayed 22 min. During that time, aneurysmal sac was reduced and sewed next to the orifice of visceral arteries to make minimal aortic stump. Distal part of the aorta was sewed one cm above bifurcation. In our second case, aortic clamp was put above the celiac trunk. Aortic clamping lasted about 28 min and relocated beneath renal arteries after EVAR prosthesis had been extracted.

Reconstruction after an aortic resection can be followed by AxFF [3, 17] *in situ* aortic reconstruction using a prosthetic graft (antibiotic-impregnated prosthetic graft) [9, 17], autogenous femoral vein graft, saphenous spiral graft [18, 19] or cryopreserved aortic allograft [1, 20].The European Society for Vascular Surgery (ESVS) has recommendations about AEF, where unstable patients should treat with stent graft, stable patients shoul receive staged procedure with extraanatomic bypass first and, finally, stable patients with aortoenteric erosion should receive *in situ* revascularization using autogenous femoral vein graft or aortoiliac allograft [14].

If abscesses are reason for ADF, mortality is very high, about 60%–75% [4, 5, 21]. After complete removal of infected graft and extraanatomic reconstruction, there is poor prognosis for survival and cure of infection resulted from aortic stump disruption, persistent infection, and especially with retained graft material [6, 20]. Some authors recommended a staged procedure with an initial AxFF followed by ligation of the aorta and iliac arteries [8, 21]. This can be done if it is no profuse bleeding and we have enough time for that comfort procedures order.

Other authors suggest more conservative procedure where uninvolved parts of the graft remain at the site and only with infection involved parts are resected, especially parts with proximal anastomosis. Reconstruction is performed either with short graft with old distal part or with aortobifemoral reconstruction. Short aortic grafts can completely be replaced with a brand new [3]. Someone debrides scar tissue near the fistula or graft, then sutures the defects of the aorta and the hole of the duodenum as the less effective way [8, 13]. AxFF is reserved for patients with periaortic purulence and an *in situ* aortic reconstruction is used in cases with minor purulence [1]. Harvesting of saphenous vein should be performed prior to clamping of the aorta, except in cases of profuse bleeding where clamping has priority [19]. There is no consensus on which approach is best.

By the *in situ* treatment, extraanatomic reconstruction and the aortic stump blowout are avoided. The *in situ* prosthetic replacement is appropriate in properly chosen patients, but autogenous reconstruction may be superior in reducing the risk of reinfection [9]. The *in situ* replacement with a "new" prosthetic (rifampin-bonded) graft was used more frequently than an autogenous vein [9, 17]. The aortic prosthesis was reexplanted in 8% of aortofemoral graft infections treated with the *in situ* replacement [9, 21]. For low-grade graft infections caused by s*taphylococci*, the *in situ* prosthetic or vein replacement is the preferred approach in about 52%–80% of cases [1, 9].

An addition to reconstruction with good results is interposition of the greater omentum as a protective barrier between the repaired small bowel and new aortic graft, especially in cases with partial short graft reconstruction [1, 5, 13].

Recent developments in interventional radiology and surgery as EVAR procedure, allow a two-stage operation. First, temporary control of bleeding with EVAR procedure and second, definitive delaying open surgery are possible [13]. Frequently, a lot of hospitals were not able to perform EVAR, and unstable patient can not be sent to another facility [3]. Authors from the region suggest to treat infected abscess and leakage in the thoracic aorta with endovascular graft (TEVAR) [3].

The right axillar artery was used as donor artery in our first case, and the one left in the second case. Right side is most common choice for donor artery in about 70% of cases for AxFF [22]. Some authors prefer left side using externally supported expanded polytetrafluoroethylene graft [12]. Headache and dizziness after operation can be explained by transitory "steal syndrome" where blood stream is redirected to AxFF and less amount of blood supplies brain. After several days redistribution of blood flow happens and a patient loses these symptoms.

Minimal surgical "refreshing" edges of the duodenal defect with doudenography is recommended option [8]. The gut reconstruction represents an important and integral part of the surgical treatment of patients with ADF. Depending on the location (D3 and/or D4 part of the duodenum) and size of the duodenal wall defect, surgical approach consists of the simple suturing of the duodenal wall and/or resection of the affected part and the duodenojejunal anastomosis. In both our cases, considering the size of the defect, around 1 cm on the duodenojejunal reflection, the duodenum was sewed in two layers.

The leading cause of perioperative and late deaths among AEF patients is aortic stump disruption [6, 20]. Kleinman et al. [4] described that all cases whom surgeons left infected graft in place died. More than half of postoperative deaths occurred in patients with SAEF [9]. Frequently, patients undergoing open repair of AAA have no adequate follow-up and late graft-related complications occurred in about 15.4% of such patients, as an aortoduodenal fistula (1.9%) resulting in death [23].

In cases where patients with aortic graft infections with periaortic purulence are complicated by sepsis or graft-enteric erosion/fistula, previous AxFF graft followed by total graft excision is option [1, 3, 9, 18, 20, 21]. Graft-enteric erosion is 4 time frequent than fistula [1, 9].

Those patients then should receive antibiotics orally for 3–6 months after leaving the hospital until C-reactive protein (CRO) values normalize and blood cultures remain negative [13, 19, 24]. Our first patient was treated with antibiotics in that manner.

Davidović et al. [22] reported a comparative study with two groups of patients, the AxFF group and the group with aortobifemoral bypass and there were no difference in early mortality and long term patency between those two groups. So, AxFF is standard reconstruction, especially in cases of

retroperitoneal infections such as ADF. Same authors suggest "Omega" or bypass in horseshoe shape, with anastomosis above the celiac trunk and arms of bypass in lateral retroperitoneal position [21].

Role of anesthesia is keeping of hemodynamic stability and targeted compensation for alogenic blood products. After anesthesia induction (etomidate, sufentanil, rocuronium) and antibiotic prophylaxis, a patient is heparinized. The optimum hemostasis balance is provided by correcting basic hemostatic parameters (temperature, acid base status, normocalcemia). The Hgb level is maintained above 80 g/L, and permissive hypotension due to massive bleeding (mean arterial pressure 65–70 mmHg) is maintained by continuous infusion of norepinephrine [25]. Blood derivative transfusion is targeted and based on "point of care" viscoelastic tests (ROTEM®). ROTEM® tests are performed in whole blood which mimic *in vivo* hemosthasis. These tests have faster turnaround time (results are available within 15 min) in comparison to conventional coagulation tests and provide assessment of whole kinetics of hemosthasis. Thus, goal-directed therapy based on viscoelastic tests is a way to avoid possible complications related to massive blood transfusion (Transfusion Related Acute Lung Injury – TRALI, Transfusion Associated Circulatory Overload – TACO and infection) [26].

## Conclusion

Although early diagnosis of AEF as well as timely intervention increase chance of survival, this condition usually has bad prognosis. Prompt operation of AEF is necessary to prevent fatal exsanguination. Vascular surgeons need to be familiar with multiple techniques for treating prosthetic graft infection and AEF. Conventional treatment of ADF means extraanatomic AxFF with complete excision of infected graft with closure of aorta's proximal and distal stumps and the duodenal repair. There are cases where ADF can develop after EVAR. Successful treatment means open surgery procedure. Although ESVS recommendations as a guide are very helpful, there is no unique attitude about management of AEF, so each patient should be treated specifically.

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CASE REPORT
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# To do or don't, to take or don't take: STN-DBS therapy in young PD patient

## Učiniti ili ne, primeniti ili ne primeniti: STN-DBS terapija kod mlađe osobe sa Parkinsonovom bolešću

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**Abstract**

**Introduction.** Parkinson's disease patients with impulse control disorders and dopamine dysregulation syndrome is increasingly recognized. There are reports that such disorders can sometimes be improved by using deep brain stimulation, but sometimes they can get worse. **Case report.** Our patient was a 30-year-old man with Parkinson's disease since the age of 23. The patient had motor fluctuations on the right with marked bradykinesia, bradymimia and rigidities in the off-periods. The patient's paraphilia and sexual indiscretions against women were apparent in the on-periods. The patient's eating habits were also changed. The patient underwent subthalamic nucleus-deep brain stimulation (STN-DBS). Significant improvements were seen in the motor and behavior signs of the patient after this procedure had been performed. **Conclusion.** STN-DBS may be a reasonable option in patients with Parkinson's disease when unwanted dopaminergic side effects occur, and motor disorders and impulse control disorders cannot be improved with drugs.

**Key words:**
**parkinson disease; young adult; subthlamic nucleus; deep brain stimulation; treatment outcome.**

**Apstrakt**

**Uvod.** Oboleli od Parkinsonove bolesti sa poremećajima kontrole impulsa i sindromom dopaminske disregulacije sve se više prepoznaju. Objavljeno je da se primenom duboke stimulacije mozga ovo stanje može ponekad rešiti, ali i pogoršati. **Prikaz bolesnika.** Naš bolesnik je bio 30-godišnji muškarac sa Parkisnonovom bolesti koja je dijagnostikovana u njegovoj 23. godini života. Bolesnik je imao motoričke fluktuacije s desne strane sa izraženom bradikinezijom, bradimijom i rigiditetom u „off" periodima. Njegova parafilija i seksualna indiskrecija prema ženama je bila izražena u „on" periodima. Prehrambene navike bolesnika bile su takođe izmenjene. Bolesnik je bio podvrgnit dubokoj moždanoj stimulaciji suptalamičkog jedra (engl. *subthalamic nucleus-deep brain stimulation* – STN-DBS). Nakon sprovedene procedure, značajna poboljšanja bila su uočena u motorici i ponašanju bolesnika. **Zaključak.** STN-DBS može biti razumna opcija kada se kod obolelih od Parkinsonove bolesti pojave neželjeni dopaminergički efekti, a motorički poremećaji i poremećaji kontrole impulse ne mogu poboljšati primenom lekova.

**Ključne reči:**
**parkinsonova bolest; mlade osobe; suptalamičko jedro; duboka stimulacija mozga; lečenje, ishod.**

### Introduction

Parkinson's disease (PD) patients with impulse control disorders (ICD) and dopamine dysregulation syndrome (DDS) is increasingly recognized. DDS occurs due to anti-parkinson therapy, and may include other psychomotor pathologies known as ICD: punding, pathological gambling, hypersexuality, binge eating and compulsive shopping. Such disorders may have dramatic effects on patient's family, and his/her personal and professional life. Drug replacement therapy (DRT) is believed to play an important role in the onset of these behavioral disorders [1, 2].

Binge eating phenomenon among PD patients has been defined by a few authors. A reliable prevalence of this disorder has not been reported. Binge eating implies continuous compulsive food intake. It is an increased diet in the form of uncontrollable consumption of more than normal food and a large amount to alleviate hunger. Zahodne et al. [3] reported that 1% of PD patients have binge eating disorder (BED).

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ICD and DDS relationship with subthalamic nucleus (STN) in PD is not exactly known. It has been reported that such disorders can sometimes be improved by using STN deep brain stimulation (STN-DBS), but sometimes they can get worse after this procedure. We report young PD patient who had improved DDS and ICD after STN-DBS application.

## Case report

Our patient was a 30-year-old man with PD since the age of 23. The patient recently complained of decreased motion, early termination and late onset of drug effect, difficulty in turning at night, extreme mobility and involuntary movements, especially after taking medication. The patient had motor fluctuations on the right with marked bradykinesia, bradymimia and rigidities in the off-periods. The patient's paraphilia and sexual indiscretions against women were apparent in on-periods. The patient's eating habits were also changed. His movements became faster and he took the food in excess. At that time, even when nothing was manifest, he was clumsy. He put everything he could find in his mouth; even he experienced the danger of drowning

Whenever he needed levodopa, eating disorder could be seen. Sometimes the patient did not wait for the time to expire; sometimes he was taking the extra levodopa. He said he got that pleasure. Even their relatives had to hide the medication.

The patient received orally 418.75 mg/day of levodopa/benserazide, 250 mg/day of levodopa/carbidopa/entecapone, 1 mg/day of rasagiline, 200 mg/day of amantadine, and intermittently 5 mg s.c. of apomorphine. The patient was Unified Parkinson's Disease Rating Scale (UPDRS) part I −1, UPDRS part II − Off 20, UPDRS part III − Off 24 and Hoehn&Yahr stage −Off 4. The dopamine agonists were removed from the treatment. Clozapine and quetiapine treatment, given by the psychiatrist, was discontinued due to worsening motor symptoms.

Despite this treatment, the patient could not sustain his life independently. He met the STN-DBS inclusion criteria as an idiopathic PD. Psychiatric statement was considered a contraindication for this procedure and before it the patient was re-evaluated. He was no psychiatric disease before the illness. There was no history of a similar disease in his family. We were concluded that behavioral problems may be related to dopaminergic therapy. The patient was diagnosed with ICD due to dopamine dysregulation syndrome. We decided that DBS administration and reduction of dopaminergic therapy would contribute to the improvement of motor disorders and DDS.

The patient underwent STN-DBS. Significant improvement was seen in the motor signs of the patient after the procedure performed. The patient's off–periods were reduced, and the patient was able to survive without assistance. In the second year after STN-DBS, levodopa was completely withdrawn from the therapy. After that, the patient used rasagiline 1 mg/day, and amantadine 200 mg/day *per os*. Significant improvement in DDS, ICD and BED symptoms was observed during the follow-up period of 3 years.

## Discussion

We reported here a young PD patient who developed ICD and DDS, such as sexual and eating disorders, after dopaminergic therapy. Significant resolution in psychiatric findings and motor symptoms improvement of our patient were seen after STN-DBS administration and dopaminergic treatment reduction. Our opinion is that STN-DBS administration in the PD patient with DDS and ICD, associated with dopaminergic therapy, may be important in disease management.

ICD, which occurs in a minority of patients with advanced PD, is rare, despite a rather regrettable psychiatric complication. It is estimated that ICD prevalence may be greater in PD patients than in the general population or healthy controls, and patients may have more than one ICD. ICD prevalence in PD was estimated to be 1.7–6% for pathological gambling, 2–10% for hypersexuality, 0.4–5.7% for compulsive shopping, 4.3% for binge eating and 3.9% for two or more ICD. ICD is a condition that leads to morbidity as a result of long-term dopaminergic therapy in PD patients [1].

Reward-seeking behaviors, including consumption of delicious food, are supported by the activation of the mesocorticolimbic dopamine neurocircuitry. The disorder of the mesocorticolimbic system forms the basis of binge eating – excessive consumption of delicious food behavior [4]. Dopamine overdose may increase nutrient motivation in PD patients treated with dopamine agonists leading to disturbed nutritional behavior such as an increased food intake, i.e. excessive eating. Dopamine replacement therapy and, in particular, D2/D3 selective dopamine agonists may cause behavioral changes, ICD and BED [5].

The effect of STN-DBS therapy in significant impulsive behaviors patients is largely unknown. ICD symptoms can be improved with the reduction of post-STN-DBS dopaminergic therapy, as well as the anew these symptoms may have emerged or in some cases may worsen pre-existing ICDs [6, 7].

## Conclusion

This report revealed the problem of how to treat ICD with PD. ICD and related behavior disorders such as BED and DDS may result in serious trouble to PD patients and their caregivers. STN-DBS may be a reasonable option when unwanted dopaminergic side effects occur and motor disorders cannot be improved with drugs including neuroleptics. Our case suggests that a background of drug-induced psychiatric disorders with motor function worsening does not constitute an obstacle to the STN-DBS application.

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C O R R I G E N D U M



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In the original article by **Raša Mladenović, Leonardo Pereira, Filip Djordjević, Zoran Vlahović, Kristina Mladenović, Andrijana Cvetković, Brankica Martinović, Jovan Mladenović, Julie Popovski**. The use of mobile-aided learning in education of local anesthesia for the inferior alveolar nerve block/ Primena učenja putem mobilnih uređaja u edukaciji mandibularne anestezije. **Vojnosanit Pregl 2020; 77(8):839-43.** (https://doi.org/10.2298/VSP180622154M),

the first name of the second author was misspelled as **Leonardo** instead of **Leandro.**

The correct name of the second author should be **Leandro Pereira**, so the list of the authors should have read:
**Raša Mladenović, Leandro Pereira, Filip Djordjević, Zoran Vlahović, Kristina Mladenović, Andrijana Cvetković, Brankica Martinović, Jovan Mladenović, Julie Popovski.**

We apologize for any inconvenience that this may have caused.

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Skraćenice i akronimi u rukopisu treba da budu korišćeni na sledeći način: definisati skraćenice i akronime pri njihovom prvom pojavljivanju u tekstu i koristiti ih konzistentno kroz čitav tekst, tabele i slike; koristiti ih samo za termine koji se pominju više od tri puta u tekstu; da bi se olakšalo čitaocu, skraćenice i aktinome treba štedljivo koristiti.

Abecedni popis svih skraćenica i akronima sa objašnjenjima treba dostaviti pri predaji rukopisa.

**Detaljno uputstvo može se dobiti u redakciji ili na sajtu: www.vma.mod.gov.rs/vsp**