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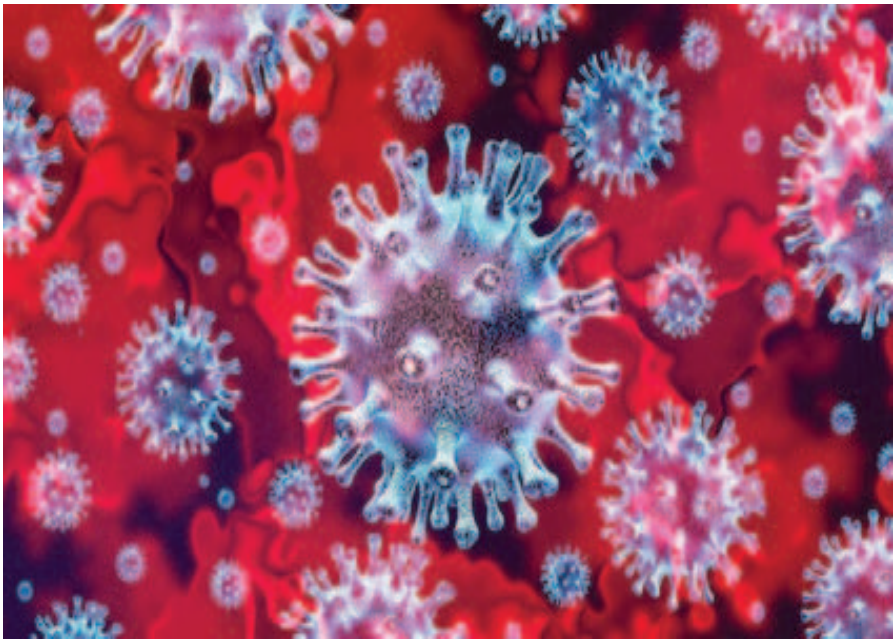

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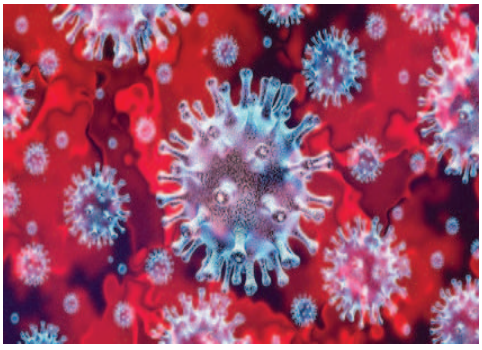

A novel coronavirus, SARS-CoV-2, emerging in the Chinese city of Wuhan, has sparked fears of a global pandemic. The present occurrence of infections of the lower respiratory tract including respiratory distress syndrome, is the third "spillover" of an animal coronavirus to humans, in just last two decades, leading to a large-scale epidemic (see Editorial, p. 139).

Novi koronavirus, SARS-CoV-2, koji se pojavio u kineskom gradu Wuhan, izazvao je strah od globalne pandemije. Sadašnja pojava infekcija donjih disajnih puteva, uključujući respiratorni distres sindrom, treće je „prelivanje" životinjskog koronavirusa na čoveka u samo poslednje dve decenije, koji dovode do epidemije velikih razmera (vidi Uvodnik, str. 139).

EDITORIAL

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Coronaviruses – new emerging pathogens

Koronavirusi – novoiskrsli, preteći patogeni

The appearance of new coronavirus (CoV) designated 2019-nCoV has opened the new chapter in the research of this group of viruses. 2019-nCoV was identified as the cause of many pneumonia cases in Wuhan, a city in the Hubei Province of China, at the end of 2019. This infection spread across China and other countries and becomes global epidemic. The 2019-nCoV has been officially named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses. It is considered that the virus has genetic similarity to the SARS virus from 2002-3 but there may be differences in disease spectrum and transmission.

Coronaviruses (CoV) are a group of RNA viruses that cause illnesses ranging from the common cold to more severe diseases such as the Middle East Respiratory Syndrome (MERS-CoV) and Severe Acute Respiratory Syndrome (SARS CoV). They are wide spread among animals (birds and mammals). In certain circumstances, as with the MERS and SARS, animal coronaviruses can evolve and infect people and then spread among them.

Human coronaviruses were first isolated in 1965 from a volunteer with the common cold.

The name “coronavirus” was introduced in 1968. Coronaviruses derive their name from the fact that under electron microscopic examination, each virion is surrounded by a “corona” or halo. This is due to the presence of viral spike peplomers emanating from each proteinaceous envelope. At present, there are six coronaviruses that have been associated with diseases in humans: 229E, OC43, NL63, HKU1, SARS-CoV, MERS-CoV. Coronaviruses are ubiquitous. Although CoV respiratory infections occur primarily in the winter, or early spring, they can occur at any time during the year.

Coronaviruses probably spread in the fashion similar to that of rhinoviruses, via direct contact with infected secretions of large aerosol particles. Reinfection is common. All age groups are affected. Common signs of the CoV infection are fever, cough, shortness of breath, and difficulty in breathing. More severe cases can cause pneumonia, severe acute respiratory syndrome, kidney failure, and even death. The World Health Organization announced that the name of the illness from novel coronavirus is COVID-19, short for coronavirus disease.

Studies in the 1970s and 1980s linked them to as much as one-third of upper respiratory tract infections during winter, 5% to 10% of overall colds in adults, and some proportion of lower respiratory diseases.

Identification of CoV in Serbia was done only during 1980s in Military Medical Academy in Belgrade.

There has long been speculation about association of human CoV with more serious disease (multiple sclerosis, hepatitis or enteric diseases), but none of these early associations has been substantiated.

However, until the pathogen identified as a cause of SARS was isolated, the previously known human CoVs (229E and OC43) were considered to play a marginal clinical role and cause only mild respiratory diseases. The other new CoVs have emerged as a major global health threats since 2002. Namely severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002 spread to 37 countries, and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 spread to 27 countries. SARS-CoV caused more than 8,000 infections and 800 deaths, and MERS-CoV infected 2,494 individuals and caused 858 deaths worldwide. Both are zoonotic viruses with similar epidemic characteristics. Symptomatic cases of both viral infections usually present with moderate-to-severe respiratory symptoms that often progress to severe pneumonia. With the occurrence of the SARS, MERS and the new 2019 n-CoV epidemic, CoV may now be considered “emerging pathogens”. Future directions for CoV research include further understanding of the mechanism of replication, elucidation of the molecular determinates of virulence and tropism and immune response, development of vaccine strategies and antiviral therapies.

New research in CoV will provide new insight into this important virus family and perhaps lead to better understanding of the potential of CoV for revival or emergence of other CoV in human population.

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ORIGINAL ARTICLES

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Clinicopathological retrospective analysis of thymoma in Serbia: A single center experience

Kliničkopatološka retrospektivna analiza timoma u Srbiji: iskustvo jednog centra

Natalija Samardžić*, Dragana Jovanović*†, Ljiljana Marković-Denić†‡, Sanja Šarac§‖, Vesna Škodrić-Trifunović*†, Jelena Stojšić*, Mihailo Stjepanović*, Spasoje Popević*†, Branislav Ilić*, Vesna Ćeriman*, Marina Roksandić Milenković*†, Milija Gajić*, Ivan Soldatović†¶

Clinical Center of Serbia, *Clinic for Pulmonology, Belgrade, Serbia; University of Belgrade, †Faculty of Medicine, Belgrade, Serbia; University of Belgrade, Faculty of Medicine, ‡Institute of Epidemiology, ¶Institute for Medical Statistics and Informatics, Belgrade, Serbia; University of Defence, §Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia; Military Medical Academy, ‖Clinic for Pulmonology, Belgrade, Serbia

Abstract

Background/Aim. Thymoma is the most common mediastinal tumor. The treatment procedures are based on the results from the research of retrospective studies because they are not frequent tumors. The aim of this work was to define common clinical features, therapeutic aspects, survival and recurrence free survival. **Methods**. This study was performed in the Clinic for Pulmonology, Clinical Centre of Serbia, Belgrade from January 1993 to December 2013. We analyzed 62 patients with histopathologically proven thymoma. The results were obtaind from medical history, physical exam, chest X-ray and/or computed tomography and operational findings or diagnostic procedure reports. Thymomas were clasiffied according to the World Health Organization classifying system, based on histopathological findings, and staged according to the Masaoka-Koga staging system. **Results.** There were more female (54.8%) patients. Patients were mostly in the seventh decade of life. One third (29%) of the patients were asymptomatic. Cough was the dominant symptom. Myasthenia gravis was the most common paraneoplastic syndrome (12.9%). Solitary tumor was the most common in our patients (61.3%), as well as the tumors larger than 5 cm (52.5%), and noninvasive thymomas (52.5%). The majority of patients (40%) were in the stage I of the disease. The operative approach was conducted in most of the patients (88.7%). A statistically significant difference in survival was in women, patients with solitary tumor, non-invasive thymomas, patients in the stage I of the disease, and those who were operated. The dimension of the tumor mass approached the conventional level of significance. **Conclusion.** In patients with thymomas, statistically significant survival rate predictors are gender, presence of solitary tumor mass, tumor invasiveness, clinical stage and surgical treatment of the disease.

Key words:
thymoma; diagnosis; neoplasm staging; treatment outcome; survival; serbia.

Apstrakt

Uvod/Cilj. Timomi su retke bolesti, ali najčešći tumori medijastinuma. Terapijski vodiči su zasnovani na rezultatima analiza retrospektivnih studija. Cilj ove studije je bio da se definišu osnovne kliničke karakteristike obolelih od timoma, terapijski aspekti, preživljavanje i pojava relapsa bolesti. **Metode.** Ovom retrospektivnom studijom analizirana su 62 bolesnika sa patohistološki verifikovanim timomom u Klinici za plućne bolesti Kliničkog centra Srbije u Beogradu, u periodu od januara 1993. do decembra 2013. Popunjavani su upitnici koji su obuhvatali podatke iz istorije bolesti, fizički pregled bolesnika, nalaze radiografije i/ili kompjuterizovane tomografije grudnog koša, operativne liste i/ili patohistološke nalaze biopsija. Analizirani su klinička slika, terapijski aspekti, preživljavanje i pojava relapsa bolesti. Timomi su klasifikovani prema patohistološkoj klasifikaciji Svetske zdravstvene organizacije i prema Masaoka-Koga sistemu. **Rezultati.** Više je bilo obolelih žena (54,8%). Najveći broj bolesnika je bio u sedmoj deceniji života

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(32,3%). Skoro trećina bolesnika je bila asimptomatska (29%), a ostali su imali bar jedan simptom, najčešće kašalj. Najčešći paraneoplastički sindrom bila je miastenija gravis (12,9%). Najčešće je konstatovano prisustvo solitarne promene u grudnom košu (61,3%), tumora većeg od 5 cm (52,5%) i neinvazivnih timoma (52,5%). Većina bolesnika je bila u I stadijumu bolesti (40%), a najčešći terapijski pristup operacija (88,7%). Statistički značajna razlika u preživljavanju konstatovana je kod žena, bolesnika koji su imali solitarni tumor, neinvazivni timom, I stadijum bolesti i bolesnika kojima je učinjena operacija. Dimenzija tumora je bila blizu granice konvencionalnog nivoa značajnosti. **Zaključak**. Statistički značajni prediktori preživljavanja kod obolelih od timoma su pol, diseminovanost bolesti, invazivnost tumora, klinički stadijum bolesti i operativno lečenje.

Ključne reči:
timom; dijagnoza; neoplazme, određivanje stadijuma, lečenje, ishod; preživljavanje; srbija.

Introduction

Thymomas are not so common mediastinal tumors. They are developing from epithelial thymic cells and present 0.2–1.5% of all malignancies [1]. On one hand they are very rare, but on the other hand they present 20% of all mediastinal masses and up to 50% of all anterior mediastinal masses [2]. Average incidence of thymomas is 0.15 per 100,000 persons/year. The disease is usually developed locally but metastases are most often found on the pleura, pericardium, or diaphragm [3, 4]. The World Health Organization (WHO) classifies thymmomas on the basis of histopathological (HP) findings which differ thymomas from thymic carcinomas. This classification was made in 1999 and updated in 2003. Thymomas are subdivided into five main types (called A, AB, B1, B2, B3) and a few less frequent other types based upon the morphology of the epithelial tumor cells and on the proportion of the non-tumoral lymphocytic component, which may indicate an aggressiveness of the tumor. The greatest probability of a good outcome has the type A, and decreases going to the type B3 [5, 6]. Staging of thymomas is based on the Masaoka-Koga system (1994) which was adapted in 2011 by the International Thymic Malignancy Interest Group (ITMIG) [7]. Clinical presentation of thymomas can be asymptomatic, with the symptoms of local spread of the tumor and within paraneoplastic syndromes. Autoimmunity is a very frequent clinical feature [8], especially myasthenia gravis (MG) which is present in 30% of patients, but also other autoimmune diseases can be seen [9–12]. Treatment options for this patients are: operational approach, radiotherapy (RT), chemotherapy and the best supportive care (BSC), and/or combinations of these regimens [13] depending on the disease location, thymoma stage, general condition of a patient, as well as a presence of comorbidities.

Here we retrospectively analyzed the clinical presentation and overall survival in patients with histologically confirmed thymoma diagnosed during an 20-year period in our institution.

Methods

This retrospective study was performed in the Clinic for Pulmonology, Clinical Centre of Serbia, Belgrade from January 1993 to December 2013. We analyzed 62 patients who were hospitalized and treated in our hospital. All the cases with thymoma were confirmed by the histopathological (HP) analysis of the tissue obtained during surgery or by fine needle aspiration biopsy (FNAB). Thymomas were classified according to the WHO histopathological classification and staged on the basis of the Masaoka-Koga staging system. The results were obtained from medical history, physical exam, chest X-ray and/or computed tomography, and operational findinga or reports of a diagnostic procedure with HP proof of thymoma. We analyzed potential influence of gender, age and smoking on thymoma and whether they affect the survival rate in thymoma patients. We also analyzed the incidence of paraneoplastic disorders, paying particular attention to myasthenia gravis, comorbidities in thymoma patients and the presence of certain symptoms and their duration before establishing the diagnosis. The overall survival rate was analyzed in relation to the tumor size, tumor spread and invasiveness of the disease, its clinical stage and HP findings as well as to the therapy approaches, i.e. whether tumor was treated by surgical methods, especially if the radical operation or incomplete resection (palliative surgical approach) was done, or it was treated by other methods of treatment when surgical approaches could not be carried out. We also studied thymoma recurrence in patients who had complete surgical resection in relation to the possible influence of smoking, clinical stage of the disease and HP findings.

Results were presented as count (percent), mean ± standard deviation (SD) or median, depending on data type. Log rank test was performed to assess significant predictors of survival rate. Survival rate was presented using medians or means with confidence intervals (CI) if appropriate. Due to a small sample size, Cox regression was performed using the Forward method. Also, due to large difference between arithmetic means and medians, some results were presented as medians without confidence interval and medians were taken for calculating survival rate. All data were analyzed using SPSS 20.0 (IBM corp.) statistical software. All p values less than 0.05 were considered significant.

The Institutional Ethics Committee approved the study.

Results

The study included 34 (54.8%) women and 28 (45.2%) men, with the proportion of 1.2 : 1. The average age was 55.7 ± 13.0 years (ranging from 21 to 75 years). The highest percentage of patents was in their seventies, 20 (32.3%), and the next group were the patients in their sixties, 17 (27.4%). The basic characteristics of the patients are shown in Table

1. The clinical presentation of thymomas varied. Nearly one third (29%) of the patients had no symptoms and rest of the patients had minimum symptoms (Figure 1): the dominant one was cough in 24 (38.7 %) of the patients followed by general weakness and chest pain, in 22 (35.5%) of the patients each. Polyneuropathia as paraneoplastic syndrome was present in 1 (1.6%) patient, while myasthenia gravis was present in 8 (12.9%) of the patients, 5 (62.5%) of whom were women. The duration of symptoms prior to diagnosis of thymoma varied from 1 month to more than one year.

Table 1
Basic characteristics of 62 patients with thymoma

Characteristics	Values
Gender (male/female), n (%)	28 (45.2) / 34 (54.8)
Age (years), mean ± SD	55.7 ± 13.0
Smoking habits, n (%)	36 (58.1)
Symptoms, n (%)	44 (71)
Symptom duration (n = 44), n (%)	
1 month	12 (26.7)
6 months	21 (46.7)
1+ year	11 (25)
Paraneoplastic disorders, n (%)	9 (14.5)
Comorbidities, n (%)	30 (48.4)

n (%) – number (percentage) of patients; SD – standard deviation.

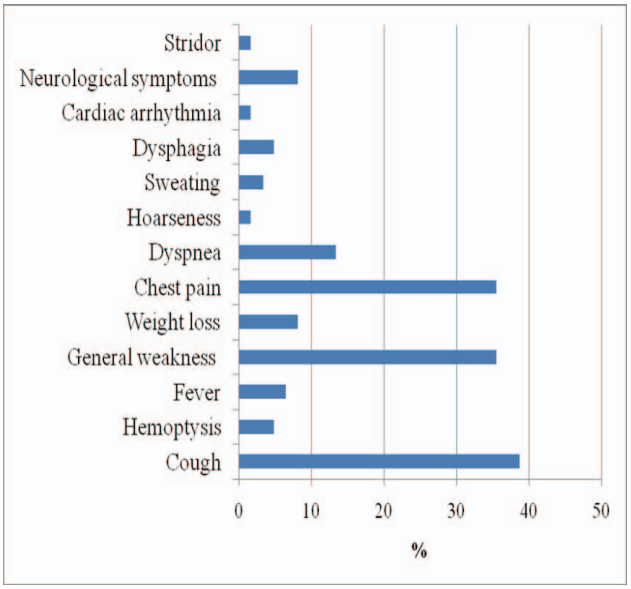

Fig. 1 – Symptoms distribution in 62 patients with thymoma.

The majority of the patients (nearly half, or 46.7%) had symptoms with a duration of 6 months, while a duration of one month and a duration of more than one year were approximately the same: 12 (26.7%) and 11 (25%) patients, respectively. Comorbidities were recorded in nearly half of the patients, 30 (48.4%), mainly in women, 21 (70%). The most common comorbidity was arterial hypertension in 13 (43.3%) of the patients, 4 (13.3%) of the patients had hyperplasia of the thyroid gland. Two patients (6.6%) experienced following diseases: bronchiectasis, angina pectoris, and cardiac arrhythmia. Other diseases (such as degenerative spinal diseases and alcoholism) were in individual cases. Secondary malignancies were present in 4 (13.3%) of the patients, and all were previously cured by a radical operational approach. A solitary finding was more frequent radiographic presentation in our patients (61.3%), while atypical lesions (21%) and metastatic process (17.7%) were less present (Table 2).

Table 2
Clinical characteristics of 62 patients with thymoma

Clinical characteristics	n (%)*
Radiographic finding	
solitary	38 (61.3)
atypical	13 (21)
metastatic	11 (17.7)
Tumor size (cm)	
> 5	36 (58.1)
< 5 m	26 (41.9)
Tumor invasiveness	
non-invasive	32 (52.5)
invasive in fatty tissue	11 (18)
metastatic	18 (29.5)
Masaoka-Koga stage	
I	29 (46.5)
II (IIA and IIB)	17 (27.4)
III	5 (8.1)
IV (IVA and IVB)	11 (17.7)
Diagnosis approach	
invasive	8 (12.9)
OP	54 (87.1)
WHO histology type	
A	16 (25.8)
AB	16 (25.8)
B1	18 (29)
B2	4 (6.5)
B3	8 (12.9)
Operation (n=55)	
radical/complete resection	50 (90.9)
palliative/incomplete resection	5 (10.1)
Therapy	
operation	55(90.9)
other treatments	16 (25.8)
Death (n = 61)	25 (40.9)

***Results are presented as number (n) and percentage (%) of patients. WHO – World Health Organization.**

Massive tumors, larger than 5 cm, verified by radiographic findings or /and during surgery, were the most commonly present in 58.1% of the patients. Noninvasive thymomas were the most common (in 52.5% of the patients). A majority of the patients were in the stage I of the disease (46.5%), while 27.4% were in the stage II, 8.1% in the stage III and 17.7% in the stage IV. The analysis of HP findings showed that most frequently present was the type B1 (in 29% of the patients), followed by the type A and type AB, and they were equally presented, each in 25.8% of the patients, followed by the type B3 and type B2, in 12.9 and 6.5% of the

patients, respectively. For the largest number of patients, the diagnosis, that is the HP verification of the process, was established during the operation itself (87.1%), and for other patients (12.9%) diagnosis was established using invasive diagnostic procedures, fine needle aspiration biopsy (FNAB) in all patients except the one who underwent mediastinotomy with tumor biopsy. The treatment in most cases (88.7%) was carried out by surgical approach, while radical operation (RO) was carried out in much greater number of patients compared to a incomplete resection (palliative surgery), in 90.9 and 10.1% f the patients, respectively. Therapeutic treatment modalities that were applied to others, non-operated patients, included the use of chemotherapy, radiotherapy (RT) and BSC equally in every two patients (3.2%), except BSC which was applied in 3 (4.8%) of the patients. Death was recorded in 40.9% of the patients, out of whom 3 (12%) died in the first week after the surgery was performed, as a complication of the surgery. The survival rates of our patients are presented in Table 3. As of December 2013, the median overall survival in patients with thymoma was 204 months (95% CI: 116.7–291.3). The 1-, 2-, 5- and 10-year survival rates of patients with thymoma were 85.2, 75.3, 69.9 and 62.8%, respectively. The 1-, and 10-year survival rates were the best in patients in the stage I, the AB HP finding followed by the type A. For patients who were operated on, the 5- and 10-year survival rates were 85.5%, 75.7% and 67.8%, respectively, and in those who were not operated on, the 1-year survival rate was 83%, but only one of them (12.5%) had a 10-year survival rate (Table 3).

Table 3
Survival rate (%) of 62 patients with thymoma according to stage, histopathological (HP) findings and operation

Parameters	1 year	5 year	10 year
All stages	58.2	69.9	62.8
stage I	92.9	92.9	92.9
stage II	94.1	70.6	48.1
stage III	40.0	40.0	40.0
stage IV	72.7	27.3	27.3
HP findings			
type A	86.7	73.3	65.2
type B1	77.8	60.0	60.0
type B2	75.0	75.0	75.0
type B3	87.5	50.0	50.0
type AB	93.7	87.5	72.2
Operation			
yes	85.5	75.7	67.8
no	83.0	16.6	12.5

The comparison of median survival according different patients' characteristics is presented in Table 4. Statistically significantly longer survival was found in women, 240 months (95% CI: 10.8–376.1) compared to men, 108 (95% CI: 0–226.5) (p = 0.036). Patients who had a solitary tumor in the chest had longer survival compared with atypical presentations of the tumor and the metastatic disease (p = 0.015). Patients with non-invasive thymomas had a statistically significant better survival than patients with invasive tumor in fat tissue and metastatic tumors (p = 0.003). The best survival had patients at the stage I of the disease compared to the patients in the stages II, III and IV, as well as the operated patients (p = 0.002). Longer survival was noticed in the patients under the age of 55 (240 months). There were no statistically significant difference when we compared two age groups (p = 0.231), smoking habits (p = 0,246), the presence of myasthenia gravis (p = 0,679), tumor size (p = 0,074), HP findings according to the WHO clasifficiation (p = 0,694) and the extent of resection (p = 0.215) (Table 4).

Table 4
Survival of 62 patients with thymoma, according to the different characteristics

Characteristics	Survival (months), median (95% CI)	p value
Overall survival	204 (116.7–291.3)	
Gender		
male	108 (0–226.5)	0.036
female	240 (10.8–376.1)	
Age		
≤ 55	240 (no CI)	0.231
> 55	168 (88.2–247.8)	
Smoker		
no	204 (125.3–282.7)	0.246
yes	240 (19.3–460.7)	
Myasthenia gravis		
no	240 (71.4–408.6)	0.679
yes	204 (no CI)	
Radiographic finding		
solitary	204 (no CI)	
atypical	168 (64.8–271.2)	0.015
metastatic	17 (0–58.8)	
Tumor size		
> 5 cm	108 (0–240.9)	0.074
< 5 cm	204 (134.5–273.4)	
Tumor Invasiveness		
non-invasive	204 (no CI)	
invasive in fatty tissue	108 (49.3–166.7)	0.003
metastatic	21 (4.7–37.6)	
Masaoka-Koga stage		
I	214.6 (172.1–257.1)*	
II (IIA and IIB)	108 (no CI)	0.001
III	12 (0.191–23.8)	
IV (IVA and IVB)	21 (10.2–31.8)	
WHO histology type		
A	117.8 (81.8–153.9)*	
B (B1, B2, B3)	204 (0–494.6)	0.694
AB	168 (no CI)	
Operation		
no	17 (4.9–29.0)	0.002
yes	204 (124.4–281.5)	
Extent of resection (n=55)		
radical/complete/resection	204 (125.7–282.2)	0.215
palliative/incomplete/resection	54 (0–118.6)	

WHO – World Health Organization.
***Mean (95% confidence interval) instead of median (95% confidence interval).**

Eight (16.0%) of the radically operated patients experienced a recurrence (relapse) of the disease. Seven patients died while one (12.5%) female patient is still alive. She was

in the stage II, with the type A thymoma, smoker. The treatment was continued by chemotherapy and RT, with 10-year survival rate. In other patients, recurrence free survival was 6 months in 4 (50.0%) of the patients, two of whom were in the stage III, both had the type B1 thymoma. All were smokers except one patient, with the 1-year survival rate despite chemotherapy and RT treatment after relapse of the disease. One patient had a relapse after one year, he was a smoker with the preoperative stage II, the type B3 thymoma. The operation was carried out again as well as chemotherapy and RT, with the 5-year survival rate. Two patients had a relapse of the disease after five years. The relapse of the disease occurred in two patients after 5 years, one patient was a smoker with the preoperative stage III, the type B3 thymoma and the 10-year survival rate, while the other patient was a nonsmoker, with the initial stage II of the disease, and the type A thymoma, with the 5-year survival rate. Significant predictors of survival according to the Cox regression analysis are presented in Table 5. Gender and clinical stage were significant predictor of longer survival.

Table 5
Significant predictors of survival, according to the Cox regression analysis

Predictors of survival	*p* value	HR (95% CI)
Gender (male)	0.004	4.129 (1.114–12.025)
Clinical stage		
I	0.002	1 – ref. category
II	0.029	3.709 (1.114–12.025)
III	0.002	14.971 (2.787–80.416)
IV	0.001	7.253 (2.293–22.946)

HR – hazard ratio; CI – confidence interval.

Discussion

According to published data, thymomas are heterogeneous group of infrequent thoracic tumors, with evidence of annual incidence from 1.3 to 3.2 per million [14]. The thymoma incidence is probably related to genetic risk factors because they are more common in Blacks and Asians/Pacific Islanders than in Whites and Hispanic [15]. There is little information about thymoma in the region of the Balkans including our country and Southern Europe too. Thymoma is the most common in the middle age and seventies patients, while in children and young adults it is very rare [16, 17]. Our study is one of the largest about thymoma cases in the Southeast Europe, and so far no similar studies have been conducted in the region. We found that there were more female than male patients (gender ratio 1.2 : 1) with thymoma, mostly in the seventies, but approximately the same number of patients was in the sixth decade of life too, about 30% each, which is confirmed by papers from different regions [5, 14], but there were studies with different findings. Weis et al. [18] gave the results of the study which was focused on 4,221 thymomas diagnosed between 1983 and 2012 with WHO histotype information from the ITMIG database presented with the aim to recognize the most important clinical features and to improve the options for treatment, due to rarity of the disease and lack of information from single centers. According to those results, gender factor is not important (49% male and 51% female patients). The type A and AB patients were much older than type B1-3 patients. Concerning symptoms before the diagnosis in our study, nearly half of the patients (46,7%), came for an examination in the period of the first 6 months from the appearance of symptoms, while the duration of symptoms of one month and more than one year were approximately similar, 26.7 and 25% respectively. Quick establishing diagnosis is most probably due to obvious clinical symptoms which showed the existence of the tumor. Almost one third of the patients in our study were asymptomatic. Coughing dominated in more than one third of patients, followed by general weakness and chest pain in 35.5% of patients, while a small number of patients (4.8%) had dysphagia, and only one patient had both stridor and hoarseness, indicating that in most of the patients the disease was not in an advanced stage. Other complaints included nonspecific symptoms such as weight loss, fever, sweating, as well as hemoptysis and cardiac arrhythmia. Our experience is consistent with literature data. Chest pain, cough and dyspnoea are frequent symptoms of local compression on mediastinal structures, as well as hoarseness due to pressure on recurrent laryngeal nerve or phrenic nerve palsy, and superior vena cava obstruction may be present too. Presentation with pericardial or pleural effusions, as a sign of metastatic disease, is indicative of a poor prognosis. Our research does not differ from the analysis of the other centers in which it has been stated that one third to one half of patients are incidentally diagnosed because they are asymptomatic and one third presents with local symptoms [8]. In 40% of patients there is paraneoplastic syndrome, such as systemic and neurologic syndromes [19, 20]. Above mentioned syndromes myasthenia gravis (MG) is well studied due to its frequency (it appears in 30-50% of patients). On the other hand 10–15% of MG patients are diagnosed with thymoma. Five percent of thymoma patients with MG can have several paraneoplastic syndromes [19]. In contrast, MG was less frequently observed among our patients (12.9%). MG was more frequent among women, and only one patient of them had polyneuropathy. Neurological symptoms existed in just over half of our patients with paraneoplastic syndrome. Comorbidities were recorded in half (48.4%) of the patients, and mainly in women (70%). The most common was arterial hypertension (43.3% of the patients), and it cannot be characterized as a predisponing disease or condition. Other chronic illnesses, such as hyperplasia of the thyroid gland, angina pectoris, and cardiac arrhythmia, were observed in fewer cases, while degenerative spinal diseases and alcoholism were represented in even smaller numbers. Diseases that represent a risk factor for the occurrence of malignant lung disease were present in two patients (6.6%) who had chronic obstructive disease. Secondary malignancies were found in 4 (13.3%) of the patients in our study; these patients had previously been cured of breast cancer, carcinoma of the urinary bladder, skin cancer and uterine cancer. Some studies [21] conducted in single centers have shown that thymoma patients are under a high risk for

cancer (lung, thyroid, prostate, lymphomas, sarcomas and leukemias etc), probably because of genetic risk factors or immune disorders. Besides, environmental risk factors, as well as therapy for thymoma (chemo/radiotherapy) are responsible for occurrence of the disease [22, 23]. The influence of smoking history, secondary malignancies, particularly those caused by viruses as Epstein-Barr Virus (EBV) on the occurrence of thymoma have not been clearly proven jet [24]. In our study, FNAB was performed as the invasive diagnostic procedure in a certain proportion of the patients (12.9%). That was a small number of patients who could not be operated due to extensive disease or because of the presence of comorbidities that represented a risk factor for the operation, which is in line with the literature data [7]. In order to establish diagnosis of thymic tumors clinical, radiologic and HP examinations are necessary. Computed tomography (CT) scan is used if the disease is suspect and to check up the patients during the treatment regimens [25]. Magnetic resonance imaging (MRI) is very useful in preoperative preparation in order to detect possible infiltration of the heart and great vessels [26]. Position emission tomography (PET) scan is used to differ benign from malignant lesions. Cytohistological diagnosis by biopsy is performed only if the tumor is unresectable [27, 28]. Radiographic findings in our research showed that solitary tumors in the chest were more frequent, in two-thirds (61.3%) of the patients. Massive tumors larger than 5 cm were the most commonly present, occurring in more than half of the patients (58.1%). On the contrary, atypical lesions and metastatic process were significantly less present (21% and 17.7% of the patients, respectively). All the patients had pathological findings on radiography. Sometimes it is impossible to detect the disease by X ray due to small size of the tumor localized in the anterior mediastinum, which cannot be visualized. This is the reason why the diagnosis is often established lately, hence radical operation (RO) cannot be implemented on time. Fukui et al. [29] proved that the size of the tumor is one of the crucial prognostic factors in the prediction of better survival. In our analysis it was noted that the noninvasive thymomas were the most common, in half (52.5%) of the patients, while the invasive process in the fatty tissue was observed in 18%, and metastatic disease in 29.5% of the patients. Recent researches proved rising occurrence of metastases in the United States. For example, 17 cases were diagnosed in 1973, while in 2008, 90 cases were registered in the Survellance, Epidemiology and End Results Program (SEER) database. Kaufman and Flores [30] discovered that male population is more susceptible to the disease. This is explained by Engels and Pfeiffer [15] who demonstrated the influence of an occupational exposure. Authors reported the presence of pleural and/or pericardial metastasis in 6.8% of all thymoma cases. Stage of the disease is one of the the main prognostic factors in thymoma patients, as an indicator of operability that represents a therapeutic approach with the highest probability of healing. Kaufman and Flores [30] described that the ability to do radical operation for early stage disease is almost 100%, while in the third and fourth stages of the disease, the radical operation rate fluctuated considerably, depending on the tumor location and degree of invasion, as well as the difference in the strategic approaches to radical operation treatment of the medical centers [30]. Our analysis showed that a majority of the patients, nearly one half, were in the stage I, while a remarkable variability in the proportion of stages, as specified by the Masaoka-Koga staging system, was noticed in the stages II, III and IV. Di Crescenzo et al. [31] have found that roughly 40% of thymomas are discovered while they are in the stage, while 25% are in the stages II and III, 10% at the stage IVa and 1–2% at the stage IVb. Invasion into the mediastinal tissue (in the stages II and III) occurs in 50% of thymomas. Pleural invasion is the most widespread, followed by pulmonary and pericardial invasion. About 30% of these cases involve the innominate vein or superior vena cava and 20% involve the phrenic nerve. Our analysis of HP findings showed that the most frequently present was the the type B1, followed by type A and type AB (equally represented in nearly a third of the patients), and finally the types B3 and B2 were least represented. Margaritora et al. [32] performed an analysis of 317 patients with thymomas, but some of them had a thymic carcinoma too, and discovered that the type B2 tumors were the most often noticed (in 57.5% of the cases), followed by the type B1 (19.2% of the cases) and finally the type AB (9.5% of the cases). Weis at al [18] in multicentre analysis proposed that difference was due to geography; the rate of occurrence of the type A thymoma is roughly the same in Europe as in the United States (15% and 14%, respectively), but markedly lower in Asia (6%). Thymomas of the type AB occur more often in Asia than in Europe or the United States (27%, 23% and 18%, respectively). The frequency of the type B2 thymomas is comparable in Europe (31%) and the United States (32%), but considerably lower in Asia (20%). Type B3 thymomas are found more often in Asia than in Europe or the United States (32%, 15% and 16%, respectively). However, the type B1 thymoma (16–20%) does not appear to vary drastically among different geographic regions. In patients with the type AB when compared to thymomas of the type B1 to B3 (38% of the type B3 thymomas were in the stage III). The median overall survival (OS) (Table 3) in patients with thymoma was 204 months. Overall, almost two thirds of patients with thymoma had 10- years survival rate, and most of these patients were in the stage I, as expected by literature data. Patients in the stage I had the best survival rate in all of these categories (1-,2-,5-,10-years survival) as compared to other stages of the disease, which is in accordance with other center studies. Statistically significantly longer survival was found in women compared to men. There was no statistically significant difference in survival in relation to age, but longer survival was observed in patients under the age of 55. There was no statistically significant difference in survival in smokers compared to non-smokers. There is no precise data in the literature about smoking habits [33]. The presence of MG was not statistically significant in survival, but patients without MG had a better survival rate in comparison with those who had MG. Our results did not show an important connection between immunological disorders and secondary malignancies and survival, but some researches have shown that the types A and AB thymomas have a low association with

MG, whereas the types B1 and B2 are more likely to be associated with MG, and thus may contribute to prognosis. Okumura et al. [34] claimed that the type A thymomas and the type C carcinomas were not found in cases with MG, while the types AB, B1, B2, and B3 were found in 6.8%, 40%, 55.6%, and 10% of the cases, respectively. In previously published studies, the impact of MG on the prognosis for thymoma is disputed. Initial studies seemed to indicate that the presence of MG signalled a poor prognosis. The latest publications appear to show that existence of MG may either not have an effect on the prognosis, or that these thymoma patients might have a more favorable prognosis [35]. The results of a study carried out by Wang et al. [36] suggest that MG has a positive impact on the long-term outcomes of thymoma. Filosso et al. [37] pointed to a link between MG and the early Masaoka stage (Table 6).

Table 6

Staging of thymic epithelial tumors: Masaoka-Koga-based staging system [38, 39], International Thymic Malignancy Interest Group refinements [40] and overall survival and recurrence-free survival (range) [41]

Masaoka-Koga, 1994		International Thymic Malignancy Interest Group, 2011	10-year overall survival	10-year cumulative incidence of recurrence	
				Thymoma	Thymic carcinoma
Stage I	Grossly and microscopically completely encapsulated tumour	– Invasion into but not through the capsule – In the absence of capsule, absence of invasion into surrounding tissues	84% (81–86%)		
Stage IIA	Microscopic transcapsular invasion	– Microscopic transcapsular invasion (< 3 mm)	83% (79–87%)	8% (7–8%)	25% (22–29%)
Stage IIB	Macroscopic invasion into thymic or surrounding fatty tissue, or grossly adherent to but not breaking through the mediastinal pleura or pericardium	– Gross extension into normal thymus or perithymic fat surrounding the tumour (microscopically confirmed) – Adherence to pleura or pericardium, with microscopic confirmation of perithymic invasion			
Stage III	Macroscopic invasion into neighbor organ (i.e. pericardium, great vessel or lung)	– Microscopic invasion of the mediastinal pleura (either partial or penetrating the elastin layer) – Microscopic invasion of the pericardium (either partial in the fibrous layer or penetrating through to the serosal layer) – Microscopically confirmed direct penetration into the outer elastin layer of the visceral pleura or into the lung parenchyma – Invasion into the phrenic or vagus nerves (microscopically confirmed) – Invasion into or penetration through major vascular structures (microscopically confirmed) – Adherence (i.e. fibrous attachment) of lung or adjacent organs only if there is mediastinal pleural or pericardial invasion (microscopically confirmed)	70% (64–75%)	29% (27–31%)	59% (44–76%)
Stage IVA	Pleural or pericardial metastasis	– Microscopically confirmed separate nodules in the visceral or parietal pleural, pericardial or epicardial surfaces	42% (26–58%)	71% (34–100%)	76% (58–100%)
Stage IVB	Lymphogenous or haematogenous metastasis	– Lymphogenous or haematogenous metastasis	53% (32–73%)	57% (24–90%)	54% (37–67%)

Information rewritten: Girard et al. [7].

Symptoms of MG help to detect the disease early, and that is way that the prognosis is better. A statistically significant difference in survival was in patients who had a solitary tumor in the chest in our study, compared to atypical presentations of thymomas and the metastatic disease, and in our analysis, the impact of tumor size was not of statistical significance to survival. But dimension of the tumor mass was approaching the conventional level of significance. This analysis did not differ from those earlier reported in the literature. Patients in our study with non-invasive thymomas had a statistically significant better survival than those with invasive ones in the fat tissue and metastatic tumors. Patients in the stage I had the best survival rate and the difference was statistically significant compared to patients in the stages II, III, and IV. It was confirmed by our work that the Masaoka-Koga stage is the best prognostic predictor of survival [38–42]. Correlation between the WHO classification and stage at diagnosis can best explain clinical features and prognostic factors of thymoma [6, 43, 44]. There was no statistically significant difference in survival in our study in relation to HP findings, although longer survival was registered in patients with the type B thymoma versus the type AB and type A of the tumor. Although it is still debatable, it has been concluded that the stage of the disease is better predictor of its outcome, in spite of the fact that many authors, including Carillo et al. [45] underline that HP findings can also predict the survival rate: starting from the type A to B3, prognostic factors are getting worse. Patients with the early stage of the disease are good candidates for radical operation, but this also depends on patient`s general condition and the presence of potential comorbidities. As for treatment, in the group of patients analyzed in our Clinic, the most common was the operative approach (88.7% of the patients), with the majority of the patients (90.9%) completely operated, while 10.1% of the patients had incomplete resection. In nonoperated patients, other treatment modalities were applied: in 3.2% of the patients, chemotherapy and RT were administered, and 4.8% of the patients received BSC. Thymomas are rare tumors, so that for a long time there were no recommendations or guidelines for the treatment of these diseases, which is an explanation for the small amount of applied neoadjuvant/adjuvant chemotherapy and/or RT, as well as our patient series was analyzed for a period of 20 years since 1993. A statistically significant difference in survival was in patients who were operated compared to those who were not operated in our research, but there were no statistically significant differences in relation to whether the operation was radical compared to palliative one. However, survival was longer in radical operated patients and the results are in agreement with studies conducted in other centers. Sixteen percent of the radically operated patients experience relapse, of whom one woman is still alive. She was initially in the stage II, with the HP type A. Chemotherapy and RT continued after the relapse of the disease and she has 10-year survival rate. In other patients, recurrence free survival was from 6 months in 4 patients to 5 years, with a survival time of 240 months. The survival rate after radical operation for thymoma is great. In the most comprehensive retrospective researches of thymectomy for thymoma, 92% of patients had radical operation. The survival rate at both 5 and 10 years for the stage I thymoma was 100%. Patients in the stage II of the disease had very similar 5-year and 10-year survival rates of 98%. However, the stage III patients exhibited a much lower survival rate compared to the stage I and II patients, with 5-year survival rate of 89% and 10-year survival rate of 78%. Patients in the stage IV manifested similar tendencies, approximately 5-year survival rate of 71% and 10-year survival rates of 47%. This shows that even patients with extensive disease can rich long-term survival rate that are not common in other malignancies [30]. If radical operation cannot be accomplished at the time of surgery (either initially or after neoadjuvant chemotherapy), patients should undergo maximum debulking procedure followed by postoperative chemotherapy/RT [46]. The operational approach has another important role: even in unresectable cases, explorative thoracotomy is recommended for establishing the diagnosis of thymoma, in terms to precisely define diagnosis and prevent the disease dissemination due to invasive diagnostic procedures such as fine needle aspiration biopsy. Due to the fact that usually the nature of this tumor is not aggressive in majority of the cases, even for the patients who were operated, the prolonged follow-up is advisable. The authors are consistent in this statement. Locoregional recurrence is the most frequent one [47–49] but it can often be seen (≤ 92.0%) on the pleura [50, 51]. Weis et al. [18] noticed that the progression of free survival is better in the early stages and in the types A and AB (recurrence rates, 1–2%) compared to the types B1 to B3 thymomas (2–7%). Whenever a relapse occurs, it is advised to retry the operation, followed by adjuvant therapy as RT or chemiotherapy or both, depending on the stage of the disease [52]. Our study analyzed a small heterogeneous group of patients whose age ranges from 21 to 61 years, which had diverse smoking habits. They differed in relation to the presence of comorbidity. All the HP findings and clinical stages of the disease were manifested in these patients. Given this small group of respondents, based on these findings, a general conclusion cannot be drawn about the predisposing factors on the onset of the disease relapse. However, according to conducted multicenter examinations, we can conclude that radicality of the operation, early stage of the disease and A-AB HP findings are the aspects that can influence on better survival [38, 41, 42]. The chemotherapeutic approach in this chemosensitive tumor is quite complex. It can be applied in non-operable patients with the extensive disease or with comorbidities which prevent surgical approach, alone or combined with RT. In patients who are primarily nonoperable, chemotherapy can decrease the disease and thus a patient can become operable. In postoperative approach, chemothetrapy is administrated as adjuvant treatment together or without RT with the intention of preventing recurrence of the disease. Platinum-based regimens are recommended. Some researchers give advantage to anthracycline-based regimens, and carboplatin/paclitaxel options in B3/C thymoma [53]. The patients with the advanced stage of the disease have benefits from postoperative RT (PORT) [54]. Although, findings are contradictory such as those of Boothe et al. [55] who found that pa-

tients with thymic carcinoma and the type A and AB thymomas have better results. When a patient is not considered to be operative, RT is suggested along with chemotherapy [56], where multidisciplinary approach is needed. As thymoma patients have long-term good prognosis, lung parenchyma and heart have to be protected during RT. Recently, there has been an expansion of knowledge related to the molecular biology of thymoma, and this has led both, to the discovery of numerous significant mutations and to the consideration of therapeutic options related to thymoma genetics [57], as it is called personalized medicine. One of the most encouraging and recent treatment strategies, so-called checkpoint blockade, has been shown to block immunosuppression by using inhibitors against particular checkpoint receptors. There are a number of reports of favorable systemic responses to immunotherapy treatment [58]. If multiple studies justify the application of molecular therapy and immunotherapy in patients with thymomas, and these drugs are included in the treatment of these patients, it is likely that longer survival will be achieved with improving the quality of life of the patients, since the therapy is better tolerated and has less adverse effects. Final concluion, according to our results, cannot be made because we had a small and very heterogeneous group of patients with thymoma over a long, two-decade time interval in which treatment approaches were changed and supplemented, until finally guidelines for treating thymoma were established, what is confirmed by other researches as well. Based upon data from retrospective series, treatment guidelines have been declared by the European Society for Medical Oncology (ESMO) [7], National Comprehensive Cancer Network (NCCN), and by the Cancer Care Ontario program [28]. The European Society of Thoracic Surgeons (ESTS) Thymic Group, the Japanese Association for Research in Thymus (JART), the ITMIG and the International Association for the Study of Lung Cancer (IASLC) played a major role in developing of the 8th edition of the tumor-node-metastasis (TNM) classification of thoracic malignancies (Table 7), which has been officially accepted by the Union of the International Cancer Control (UICC) and American Joint Committee of Cancer (AJCC) and globally implemented in 2017, but the AJCC has delayed its implementation until 2018 [59]. The new TNM staging (Table 7) may help to formalize resectability criteria [60].

The retrospective nature is one of the factors which influences the results of our research. In order to make important conclusions, which would improve the treatment of the thymoma patients, it is necessary to conduct the study in the countries of the region, ie the Balkans, as a multicentre study, and with cooperation with worldwide countries and thus to integrate all findings. Further projects should intensify genomic explorations and establish data base for these patients.

Table 7

Proposed Tumour-Node-Metastasis staging (the International Association for the Study of Lung Cancer Prognostic Factors Committee-International Thymic Malignancy Interest Group) [58] and corresponding Masaoka-Koga stage

Tumour stage		**Descriptors**
T1	T1a	Encapsulated or unencapsulated, with or without extension into the mediastinal fat
	T1b	Extension into the mediastinal pleura
T2		Direct invasion of the pericardium (partial or full-thickness)
T3		Direct invasion of the lung, the brachiocephalic vein, the superior vena cava, the chest wall, the phrenic nerve and/or hilar (extrapericardial) pulmonary vessels
T4		Direct invasion of the aorta, arch vessels, the main pulmonary artery, the myocardium, the trachea or the oesophagus
Node		
N0		N0 No nodal involvement
N1		N1 Anterior (perithymic) nodes (IASLC levels 1, 3a, 6 and/or supradiaphragmatic/inferior phrenic/pericardial)
N2		N2 Deep intrathoracic or cervical nodes (IASLC levels 2, 4, 5, 7, 10 and/or internal mammary nodes)
Metastasis		
M0		No metastatic pleural, pericardial or distant sites
M1	M1a	Separate pleural or pericardial nodule(s)
	M1b	Pulmonary intraparenchymal nodule or distant organ metastasis
Stage grouping		**Corresponding Masaoka-Koga stage**
I	T1N0M0	I, IIA, IIB, III
II	T2N0M0	III
IIIA	T3N0M0	III
IIIB	T4N0M0	III
IVA	T any N0,1 M0,1a	IVA, IVB
IVB	T any N0-2 M0-1b	IVB

Information rewritten: Girard et al. [7].

Conclusion

Predictive factors of better survival in patients with thymoma in our study are early stage of the disease, non-invasive thymoma, younger age and operative treatment approaches. There is a need for further analysis of the dimension of the tumor mass as a predictive factor. Timely detection of the disease with providing a timely therapy will prolong the overall survival of these patients, prolong the recurrence free survival and increase the number of cures.

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Biochemical and functional quality assessment of platelet concentrates

Biohemijska i funkcionalna ispitivanja kvaliteta koncentrata trombocita

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Abstract

Background/Aim. Preparation of platelet concentrate (PC) from a unit of whole blood is a method dependent on a number of factors, which alone or together, can have a significant impact on the quality of the final product. Quality of PCs is determined with criteria defined in the Guide to the preparation, use and quality assurance of the Council of European Committee for Blood Transfusion. The aim of the study was to analyze the quality of PCs prepared from buffy coat (BC) and to allocate the factors of improvement and standardization of the quality of PCs. **Methods.** The study included a total of 80 PCs prepared from BC according to the standard procedure in the Blood Transfusion Institute Niš. The quality of PCs was determined according to the product volume and laboratory testing on the first (PC_1), the third (PC_3) and the fifth day (PC_5) of the PC storage. The following parameters were determined: platelets, residual erythrocytes and leukocytes count, gas analysis [partial pressure of oxygen, carbon dioxide (pO_2, pCO_2)], pH, sterility and platelet aggregation triggered by using 3.2, 6.4 and 9.6 μg collagen/mL (impedance aggregometry). **Results.** There were significantly lower platelet count, pO_2, pCO_2 and pH in the PC_3 and PC_5 samples ($p < 0.001$). Except for the fulfillment of the criteria for platelet count, all the other quality parameters were in accordance with recommended criteria. Platelet aggregation for all the concentrations of collagen showed a decrease during the storage period, with statistically significant differences for PC_3 and PC_5 as compared to PC_1 ($p < 0.01$). There was statistically significant decrease in activity of PCs triggered with higher concentrations of collagen (6.4 and 9.6 μg collagen/mL) in comparison with lower concentration of collagen (3.2 μg collagen/mL). **Conclusion.** Platelet count, evaluated biochemical parameters and the platelet function were significantly changed during the storage period. In order to improve the quality of PCs it is important to store the products under proper conditions, change the type of plastic bag for PC storage and use platelet additive solutions (PAS) instead of plasma.

Key words:
blood platelets; reference standards; blood chemical analysis; platelet aggregation; platelet count.

Apstrakt

Uvod/Cilj. Priprema koncentrata trombocita (KT) iz jedinice cele krvi je metoda uslovljena velikim brojem faktora koji pojedinačno ili udruženo mogu imati značajan uticaj na kvalitet konačnog proizvoda. Kvalitet koncentrata trombocita određen je kriterijumima definisanim u Vodiču za pripremu, korišćenje i obezbeđenje kvaliteta Evropskog komiteta za transfuziju krvi Saveta Evrope. Cilj istraživanja bio je da se ispita kvalitet KT dobijenih izdvajanjem iz "buffy coat"-a (BC) i izdvojiti faktore koji doprinose poboljšanju kvaliteta i standardizaciji KT. **Metode.** Ispitivanje je obuhvatilo ukupno 80 KT izdvojenih iz BC prema standardnoj proceduri pripreme u Zavodu za transfuziju krvi u Nišu. Kvalitet KT određivan je na osnovu zapremine produkta i laboratorijskih testiranja prvog (KT_1), trećeg (KT_3) i petog dana skladištenja (KT_5). Određivan je broj trombocita, zaostalih eritrocita i leukocita, parcijalni pritisak kiseonika i ugljendioksida (pO_2, pCO_2), pH, sterilnost i agregacija trombocita izazvana dodavanjem 3,2; 6,4 i 9,6 μg kolagena/mL (impedantna agregometrija). **Rezultati.** Nađen je statistički značajno manji broj trombocita, pO_2, pCO_2 i pH u uzorcima KT_3 i KT_5 ($p < 0,001$). Osim ispu-njenosti kriterijuma za broj trombocita, svi ostali parametri bili su u saglasnosti sa preporukama Vodiča. Agregacija trombocita za sve koncentracije kolagena pokazala je pad tokom skladištenja, sa statistički značajnom razlikom za uzorke KT_3 i KT_5 u odnosu na uzorak KT_1 ($p < 0,01$). Postojala je statistički značajna razlika u smanjenju aktivnosti uzoraka KT aktiviranih primenom visokih koncentracija kolegena (6,4 i 9,6 μg kolagena/mL) u odnosu na nižu koncentraciju kolagena (3,2 μg kolagena/mL). **Zaključak.** Broj trombocita, posmatrani bi-

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ohemijski parametri i funkcija trombocita statistički se značajno menjaju tokom perioda skladištenja. U cilju poboljšanja kvaliteta KT važno je skladištiti KT u propisanim uslovima, promeniti vrstu kese za skladištenje KT i koristiti aditivni rastvor za čuvanje trombocita umesto plazme.

Ključne reči:
trombociti; referentni standardi; krv, hemijske analize; trombociti, agregacija; trombociti, broj.

Introduction

The transfusion of platelet concentrates (PC) is used in the prevention and treatment of bleeding in patients with thrombocytopenia or thrombocytopathias. Other most common indications are hematological diseases, transplantation of bone marrow and other organs, treatment of cardiac surgery patients and the gastrointestinal bleeding. Applications of aggressive medical treatments including chemotherapy increase the rate of applied transfusions of random donor platelets [1, 2]. Transfusion of PCs is intended to bring a sufficient number of platelets in the patient's circulation in order to increase their number and allow normal hemostasis, achieve prevention and treatment of bleeding, and, on the other side, reduce side effects, infections and alloimmunization to the lowest level. Contemporary data show that each year about 1,5 million of PCs in the United States and about 2,9 million of PCs in Europe are transfused [3].

PCs are the blood products obtained from whole blood units [prepared from platelet rich plasma (PRP) or "buffy-coat" (BC – a layer of white blood cell and platelets occurred after red blood cells sedimentation in an unit of whole blood)] or apheresis of platelets obtained from a single procedure of thrombocytapheresis, using automated blood cell separator. Preparation of PCs from an unit of whole blood is a method dependent on a number of factors, which alone or together, can have a significant impact on the quality of the final product. Standardization of PCs is very difficult to execute. The most important factors influencing the quality of PC are the type and quality of blood bags, the characteristics of centrifugation, separation method and storage conditions of the prepared PCs [4, 5]. Today, in all developed countries, including our, most of PCs are prepared by removing the BC layer and their quality is improved especially if blood is collected in “top & bottom” blood bags [6]. Platelets are stored in special plastic bags for storage that allow the transport of oxygen, at a temperature of 22 ± 2 °C, with continuous agitation on a horizontal agitator (approximately 70 cycles per minute) to prevent platelet aggregation and accelerate the transfer of oxygen for up to 5 days. Application of additive solution for platelets (PAS) extends the storage life of concentrate to 7 days, and generally speaking has numerous advantages over the plasma which is normally used as a medium for storage of platelets, both in terms of improving the quality of PCs and their efficiency in patients [4, 7].

The standard therapeutic dose of platelets for adults is prepared from 4–6 units of blood (dose: 1 concentrate/10 kg body weight – BW), and some number of PCs can be merged into one bag by pooling. Pooled platelet concentrate is now commonly prepared by pooling several BC units of the same ABO blood group before centrifugation. Dilution of prepared pool can be performed with plasma or PAS.

Quality of PCs is determined with criteria defined in the Guide to the preparation, use and quality assurance of the Council of European Committee for Blood Transfusion [8]. Mandatory requirements of quality control of PCs prepared from BC are volume of 50–75 mL which contains at least 60×10^9 platelets, less then 0.05×10^9 leukocytes, the number of erythrocytes $0.2–1 \times 10^9$ and pH greater than 6.4. In addition, it is recommended to test PC for the presence of bacteria till the end of storage as well as to test platelet function in PCs, which usually means the examination of platelet aggregation by adding the appropriate agonist. This is essential to assess the *in vitro* function of platelets in respect to different activation pathways [9–11].

The study aimed to analyze the quality of PCs prepared from BC by determination platelet count, gas analysis, biochemical parameters *in vitro*, platelet aggregation and sterility during the whole storage period. On the basis of the results we evaluated if random donor PCs were prepared in accordance with the criteria of the Guide of Council of Europe, and whether we can allocate the factors of improvement and standardization of the quality of PCs.

Methods

The study included a total of 80 PCs prepared from BC according to the standard procedure in the Blood Transfusion Institute Niš. Whole blood from voluntary blood donors of both sexes, non-reactive on the markers of transmissible diseases, with normal clinical and laboratory parameters, who were not taking antiplatelet drugs for last 7 days, was collected in a system of quadruple plastic bags containing 63 mL of CPD anticoagulant solution and 100 mL of additive solution of saline, ademine, glucose and manitol (SAGM) for storage of red blood cells (JiaxingTiahne Pharmaceutical, China). All units of blood in the assay were stored at room temperature (20–24°C) and within a period of 6 hours of collection centrifuged for 15 minutes at 3200 rpm and a temperature of 22°C (centrifuge with a cooling for blood, Heraus, Cryofuga 8500). After that, automatic separation of whole blood units was done on the T-ACE II (Terumo) device, and from each blood unit concentrate of erythrocytes, the plasma unit and platelet-leukocyte layer – “buffy coat” (BC), which was left to stand at room temperature for two hours, were prepared. After that, BCs were centrifuged for 8 minutes at 1100 rpm and a temperature of 22°C, and PCs were transferred in bags that allow storage of platelets for five days. PCs were left a short time on a flat surface (in order to disaggregate platelets), labeled (ISBT 128) and stored

horizontally on the agitator (Helmer PC4200i) until the expiration of the storage.

The quality of the PCs was determined according to the volume of the product and laboratory testing was performed on the first (PC1), the third (PC3) and the fifth day (PC5) of the storage. PCs with a volume less than 20% of the standard volume of the product, chylous, hemolyzed or in any way contaminated were excluded from this investigation. In order to obtain aliquots from samples of PCs, a sterile connection (TSCD Terumo) was used which ensured the integrity of the environment. From each PC sample, the following parameters were determined: hematological analysis – count of platelets, residual erythrocytes and leukocytes on a Abbott Cell-Dyn Rubyanalyser (Abbott Laboratories); gas analysis – pO_2, pCO_2 on the AVL Compact 3 Blood Gas Analyzer (Roche Diagnostics); pH – on the CyberScan pH510 device (Eutech Instruments); sterility – on the Bact / ALERT 3Ddevice (Biomerieux, France): the tested samples were plated in one vial for the presence of aerobic (BPN) and one for anaerobic bacteria (BPA). The bottles were incubated for 7 days at 37°C. The sensor system detects a change in the color of the surface, sensitive to the change of carbon dioxide concentration. If the bacteria grow, concentration of carbon dioxide is increasing, which leads to a color change on the base of the bottle. Platelet aggregation was determined by impedance aggregometry method (Multiplate Platelet Function Analyzer, Roche). The method measures platelet aggregation that is *ex vivo* stimulated by application of various platelet agonist (eg. adenosine diphosphate, arachidonic acid, collagen). Multiplate test cells have two independent measuring units, each of them is composed of two copper, silver-coated electrodes 3.2 mm high and 0.3 mm in diameter. The procedure involves mixing of 150 μL PC with 450 μL of buffer (0.81% NaCl, 0.0067 M PO4, pH 7.2) in a particular test cell. After incubation at 37°C for a period of three minutes 20 μL of the selected agonist is added, e.g. collagen in concentrations of 3.2; 6.4 and 9.6 μg/mL. A blood sample containing added agonist is automatically stirred (800 U/min) using a magnetic stirrer coated with poly-tetra-fluoroethylene (PTFE). Activated platelets adhere to the electrodes and increase the electrical impedance between them, which is registered within 6 minutes, and the increase in impedance is converted into arbitrary units of aggregation [aggregation arbitary units (AU)]. The most important parameters monitored are: area under the aggregation curve (AUC), which is directly dependent on the height of the curve, and shows the overall activity of platelets, the height of the aggregation curve, which shows the degree of platelet aggregation and the maximum slope of the aggregation curve, which indicates the rate of platelet aggregation.

Statistical analysis was performed using Statistical Package for Social Science (SPSS Software GmbH, Germany), version 15.0. The results are presented in tables and figures, using the mean values, standard deviations (SD) and medians (Me).Qualitative characteristics of the investigated variables are given as frequency (n) and the percentage (%). Normality of the distribution of continuous variables according to the size of the sample was examined by Kolmogorov-Smirnov or Shapiro-Wilk test. Statistical significance of the experimental data during the storage period was analyzed using the Wilcoxon Signed Ranks Test and the Paired Samples *t*-test. Statistical significance of the differences between the absolute frequencies of samples was analyzed by the Pearson's χ^2 test or Fisher's exact test. The effect of different concentrations of collagen on the monitored variables changes over time was determined by analysis of variance for repeated measures (RM ANOVA).

Results

The average volume of investigated PCs was 58.75 ± 3.92 mL. The results of hematological analysis are shown in Table 1. Comparing values of the investigated hematological parameters of PCs during storage, it was found significantly lower platelet count in PCs on the Days 3 (PC3) and 5 (PC5) in relation to that on the the Day 1 (PC1).

Conformity of PCs quality with the standard quality criteria for blood products during the storage period is shown in Table 2.

Except for the fulfillment of the criteria for platelet count, all the other hematological parameters were in accordance with recommended criteria. On the first day of storage 91.25% of PCs had the required platelet count, on the third day the percentage was reduced to 83.75%, while on the fifth day only 66.25% of the PCs had more than 60×10^9/L of platelets.

Values of laboratory parameters and gas analysis during the study period are shown in Table 3. There were significantly lower values of pO_2, pCO_2 and pH on the third and fifth day of storage. pH, as a required criterion for testing the quality of PCs met the recommended criterion for all the concentrates (80/80, 100%) during the whole period of storage till the fifth day.

Table 1

Hematological analysis of platelet concentrates (PC) during storage

Blood components	PC_1 mean ± SD (median)	PC_3 mean ± SD (median)	PC_5 mean ± SD (median)
Erythrocytes ($x10^9$)	0.005 ± 0.010 (0.000)	0.005 ± 0.011 (0.000)	0.004 ± 0.015 (0.000)
Platelet ($x10^9$)	62.166 ± 2.416 (62.190)	60.915 ± 2.337*** (61.190)	59.642 ± 2.478*** (60.315)
Leukocytes ($x10^9$)	0.007 ± 0.004 (0.006)	0.008 ± 0.004 (0.007)	0.007 ±0.004 (0.006)
Volume (mL)	58.753 ± 3.919 (58.870)		

PC_1 – PC on the first day; PC_3 – PC on the third day; PC_5 – PC on the fifth day; SD – standard deviation; * – $p < 0.001$ (Wilcoxon Signed Ranks Test).**

Table 2

Conformity of platelet concentrates (PC) quality with standard quality criteria during storage

Parameters	PC_1, n (%)	PC_3, n (%)	PC_5, n (%)
Erytrocytes < 0.2–1 × 10^9	80 (100)	80 (100)	80 (100)
Platelets > 60 × 10^9	73 (91.25)	67 (83.75)	53 (66.25)***
Leucocytes < 0.05 × 10^9	80 (100)	80 (100)	78 (97.50)
Volume > 40mL	80 (100)		

PC_1 – PC on the first day; PC_3 – PC on the third day; PC_5 – PC on the fifth day; * – $p < 0.001$ (χ^2-test).**

Table 3

Laboratory parameters and gas analysis of platelet concentrates (PC) during storage

Parameters	PC_1 mean ± SD (median)	PC_3 mean ± SD (median)	PC_5 mean ± SD (median)
pH	7.191± 0.067 (7.178)	7.181 ± 0.063***(7.171)	7.170 ± 0.062***(7.161)
pO_2	137.056 ± 7.888 (137.50)	130.87 ± 7.593***(130.75)	123.70 ± 6.544***(122.95)
pCO_2	49.259 ± 2.604 (48.950)	42.339 ± 2.739***(42.30)	23.854 ± 3.012***(23.650)
pH > 6.4, n (%)	80 (100)	80 (100)	80 (100)

PC_1 – PC on the first day; PC_3 – PC on the third day; PC_5 – PC on the fifth day; SD – standard deviation; * – $p < 0.001$ (Wilcoxon Signed Ranks Test, Paired Samples *t* test).**

Table 4

Platelet aggregation induced by collagen during storage

Collagen concentration (μg/mL)	PC_1 mean ± SD (median)	PC_3 mean ± SD (median)	PC_5 mean ± SD (median)
3.2	1057.29 ± 98.07 (1056.0)	590.14 ± 80.15***(591.50)	252.46 ± 56.75***(250.00)
6.4	1111.18 ± 111.3 (1133.5)	966.90 ± 127.04**(985.00)	734.18 ± 120.12***(741.00)
9.6	1181.65 ± 119.3 (1215.0)	1094.49 ± 121.89*(1119.0)	956.15 ± 120.16***(986.00)

PC_1 – Platelet concentrates on the first day; PC_3 – Platelet concentrates on the third day; PC_5 – Platelet concentrates on the fifth day; SD – standard deviation; * – $p < 0.001$ (Wilcoxon Signed Ranks Test, Paired Samples *t*-test).**

Table 5

The effects of different collagen concentrations on platelet aggregation after 1st, 3rd, and 5th storage day – the results of generalized linear method for repeated measurements

Parameters	Within participant effects				The effect of different concentration	Interaction concentration× time
	All concentrations	3.2 μg/mL	6.4 μg/mL	9.6 μg/mL		
Platelet aggregation						
p	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Size effect *	0.9695	0.9826	0.9636	0.9327	0.7669	0.9013

***Partial eta squared (ηp^2).**

Platelet aggregation of PC_1, PC_3 and PC_5 triggered with different concentration of collagen is shown in Table 4. It is observed that for all the concentrations of collagen there was a decrease in the aggregation during the storage period, with statistically significant differences for PC_3 and PC_5 as compared to aggregation for PC_1 ($p < 0.01$).

Based on the data in Table 4, as well as a generalized linear model for repeated measurements (Table 5), taking into account the different concentrations of collagen, it was confirmed that during the storage there was a significant decrease in capability of platelets to aggregate regardless of collagen concentrations used ($p < 0.001$).

Also, it was found that these changes were statistically significantly different among the different collagen concentrations (the smallest decrease of aggregation was observed for collagen at the highest concentration, and the highest decrease of platelet activity was at the lowest concentration of collagen, $p < 0.001$).

All PCs had negative results for microbiological control till the end of the storage period.

Discussion

Considering that the main objective of the transfusion of blood products is to achieve the highest therapeutic effect

with high safety of the transfusion, it is clear that the evaluation of the quality of prepared blood products is of great importance for efficient transfusion. History of platelet transfusion begins in 1960s with the detection of association between hemorrhagic syndrome and decreased number of platelets in the circulation of patients. Since then there have been many changes in the way of preparation and storage of PCs, with the aim of improving the quality of the product. On the other hand, the quality of PC is not just an accordance with the criteria of quality system, but also the ability of transfused PC to provide *in vivo* hemostatic support. Many studies confirmed that only 66% of transfused platelets circulate freely, and many factors can affect their function, such as infection, the use of antibiotics and anti-inflammatory drugs, as well as previous separation and storage lesions in PCs. According to Gulliksson [12] there are the three most important points in the process of preparation and storage of PCs that are essential for maintaining good quality of the products. First, it is important to prevent or reduce the platelet activation during the blood collection, preparation and storage of PCs. Second, the level of glycolytic activity, anaerobic glucose consumption and lactate production should be maintained at the lowest level. Third, it is important that a certain amount of glucose must be present in tissue during the whole period of storage.

Numerous studies have shown that there are significant changes in PCs during the storage period, both in number and platelet function, and biochemical alterations with consequent changes of intracellular metabolism [13–15]. During storage period platelet count in PCs progressively decreases comparing to their numbers immediately after preparation. There are changes of the morphological distribution and morphological score of platelets, wherein the number of the discoid and spherical shaped platelets decreases, while dendritic and ballooned cells, which have functionally lesser value, are increasing. This investigation showed that the average number of platelets on the first and third day of the storage met the required recommendations (on the first day of storage 91.25% of investigated PCs had a platelet count greater than 60×10^9, and on the third day 83.75% of PCs had the required number of platelets). During storage till the fifth day, platelet count was statistically significantly decreased, while the average number of platelets was below the required value, and only 66.25% of the PCs had a number of platelets greater than 60×10^9.

In the analysis of the leukocyte content in PCs, we obtained less than 0.05×10^9 leukocytes, which indicated normality. This is very important because leukocytes are responsible for a variety of acute and delayed transfusion reactions, primarily an immune-mediated reactions, transmission of the viruses and graft-versus-host disease, but also *in vitro* production of inflammatory cytokines and the development of febrile non-haemolytic transfusion reactions [16, 17]. All the investigated samples of PCs remained sterile for aerobic and anaerobic microorganisms until the end of the storage. This is also of the great importance as the presence of bacteria in PC can lead to sepsis and other transfusion reactions. Data from the literature presents the incidence of bacterial contamination of 1 in 2,000 PCs [18].

pH is an important marker of the quality of PCs *in vitro* since at values below 6.8 platelets become spherical and this change in shape becomes irreversible when pH drops below 6.2 [19, 20]. This investigation showed statistically significant decrease in pH, which appears to be a consequence of a greater permeability of the plastic bag for PC storage to gases, particularly CO_2. These changes in gas concentrations lead to changes in the concentrations of bicarbonate, with a resulting buffering of the system and the change of pH.

Although gas analysis do not belong to the group of manadatory parameters for quality testing, recommendations imply that the level of blood gases should stay at a constant level, ideally as on the first day. A number of studies show that this does not occur in the practice, mostly depending on the kind of the used bag and a temperature of 22°C. Obtained results showed that pO_2 and pCO_2 significantly decreased on the third and the fifth storage day. It is known that during storage of PCs extracellular alterations can cause cellular lesions, implying metabolism variations and function decrease [13, 21].

The focus of this study was functional testing of platelets in PCs. Collagen is known as a very potent platelet agonist which activates several intracellular metabolic systems with different receptors on platelet membranes. Thus, it binds to von Willebrand factor (vWF), creating an adherence bridge between collagen and the platelet glycoprotein Ib receptor. Granule content secretion is triggered by platelet surface receptor agonists [22]. In our study, the results of platelet aggregation showed that after 5 days of storage a reproducible aggregation response could be determined, but there was statisticaly significant decrease in platelet aggregation on the third and fifth day of storage compared to the first day. Also, there was statisticaly significant decrease in activity loss between PCs triggered with higher concentration of collagen (6.4 and 9.6 μg collagen/mL) and with smallest concentration of collagen used (3.2 μg/mL). A possible explanation for these changes might be the changes of pH, increase of lactate concentration as well as the variation in the composition of the platelet membrane.

Considering the changes that have been proven in the PC quality during storage it is important to identify factors that may be important to reduce the level of these changes and to improve the quality of PCs, specially from the third to the fifth day of storage. A large number of investigations on this subject concluded that there are many factors that can be singled out as determinants of functional and biochemical changes in PCs [23–25]. The most important of them are temperature, volume, agitation and the kind of plastic bag used for conservation of PCs. As all the investigated parameters of PC_1 showed a high level of quality, we can conclude that the blood collection process did not significantly affect the platelet quality. Our results lead us to the conclusion that in the conditions under which PCs were prepared and stored, there were the two main factors affecting the quality of PCs. These were the kind of bag for storage of PCs and plasma, as a medium for storage of platelets. The observed gas exchanges are capable of causing platelet lesions, altering their metabolism, which, on the other hand, lead to significant platelet activation and reduction of functional capacity [23]. It is essential

to choose some other type of plastic bags for PC storage which can provide an environment that results in an improved product quality and will permit 5-day storage of PCs with preserved quality. On the other hand, it is known that platelets derive energy from glucose oxidation through glycolysis and β-oxidation of long-chain fatty acids, and during the storage of PC there is an increase of glucose metabolism by the glycolitic process, lactate production is also increased while pH and production of bicarbonate are decreased. Various authors have suggested that instead of plasma should be used platelet additive solutions (PAS) for PCs storage in order to improve their quality. PAS contains acetate as a nutrient medium for platelets, which is a basic substrate for normal platelet metabolism, it reduces the production of lactate and increases the production of hydrogen carbonate, which finally leads to pH stabilization. Additionally, acetate acts as a buffer. The other ingredient of PAS is phosphate, which has a double effect on the metabolism of platelets. In addition to its action as a buffer, a phosphate also stimulates glycolysis, which can lead to a significant drop in pH, but also the production of higher levels of ATP, which allow greater platelet viability. Magnesium and potassium in PAS reduce the activation and aggregation of platelets, maintain the morphological score and decrease in lactate production [26–28]. A number of previous studies have shown that *in vitro* quality of platelets stored in the PAS is a statistically significantly improved compared to platelets that are stored in the plasma, and bearing in mind the other advantages of PAS (reduction of the incidence of allergic and febrile transfusion reactions, ABO-incompatible transfusion of platelets, pathogen inactivation, increased amount of plasma available for fractionation, storage of PCsfor 7 days [29], it is necessary to include PAS in routine transfusion practice for PC preparation and storage.

Conclusion

Platelet count, pO_2, pCO_2, pH and the platelet aggregability were statistically significantly changed during the storage period. In order to improve the quality of PCs it is important to store the products under proper conditions, change the type of plastic bag for PC storage and the use PAS instead plasma.

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The examination of the quality of life changes of patients with urolithiasis regarding different methods of treatment

Ispitivanje promena kvaliteta života bolesnika sa urolitijazom s obzirom na različite metode lečenja

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Abstract

Background/Aim. Urolithiasis is one of the most common urological illnesses with a continual rise in incidence and prevalence in the population. Its pathogenesis is multifactorial; hence, its consequences are serious problems that can significantly impact the quality of life of patients. In the last years, operational modes of urolithiasis treatment had undergone evolution changes towards minimally invasive treatment techniques aimed at improving its efficacy and patients' life quality. The aim of the study was to examine and evaluate the quality of life of the patients with urolithiasis depending on the applied treatment method. **Methods.** This research was designed as a panel study – a combination of a cross-sectional and cohort study. The sample included patients with urolithiasis treated with extracorporeal shock wave lithotripsy (ESWL) or ureteroscopic lithotripsy (Lithoclast). The research was carried during one year period and 100 respondents met the inclusion criteria. They were divided into two equal groups considering the applied method of the stone disintegration: the Lithoclast group (URSL) and the ESWL group. The instrument used for measuring the quality of life was Short Form (SF) 36 questionnaire. It was administrated to the patients immediately before the operation and one month after the operation. **Results.** The statistical analysis of the scores obtained preoperationally on the SF 36 questionnaire revealed the decrease in the quality of life of patients with urolithiasis in almost all dimensions of life. The statistically relevant difference in preoperative SF scores between the two groups of patients was not established except in the domain of the role of physical health and the domain of mental health. In the domain of the role of physical health, the Lithoclast group had a statistically significant higher score than the ESWL group, but in the domain of mental health, the ESWL group had a statistically significant higher score than the Lithoclast group. The postoperative statistical analysis of SF questionnaire and the examination of the impact of the treatment mode on the quality of life showed that the use of the Lithoclast method resulted in the much higher, statistically significant score at SF36 questionnaires regarding several life dimensions than the ESWL method. The application of the ESWL method even resulted in the decrease in the postoperational score for some life dimensions. **Conclusion.** The assessment of the quality of life is an adequate tool for the evaluation of treatment modes in the clinical practice. By using the SF 36 questionnaire in this study, we established that the ureteroscopic lithotripsy (the Lithoclast method) is a method that postoperatively results in much higher and statistically significant improvement of the quality of life of patients with urolithiasis in several health domains than the ESWL method.

Key words:
urolithiasis; lithotripsy; quality of life; surveys and questionnaires; methods; treatment outcome.

Apstrakt

Uvod/Cilj. Urolitijaza je jedno od najčešćih oboljenja u urologiji sa stalnim porastom incidence i prevalence u populaciji. Patogeneza ovog oboljenja je multifaktorijalna, a za posledicu ima veoma ozbiljne probleme koji mogu imati značajan uticaj na kvalitet života ovih bolesnika. Tokom prethodnih godina, hirurški modaliteti lečenja urolitijaze pretrpeli su evolutivne promene u korist minimalno invazivnih tehnika lečenja, postizanja bolje efikasnosti lečenja i poboljšanja kvaliteta života bolesnika. Cilj studije bio je da se ispita i proceni kvalitet života bolesnika sa urolitijazom u odnosu na pimenjenu metodu lečenja. **Metode.** Ispitivanje je dizajnirano kao panel studija (kombinacija studije preseka i kohortne studije). U studiju su bili uključeni bolesnici sa urolitijazom lečeni *extracorporeal shock wave lithotripsy* (ESWL)

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ili ureterorenoskopskom litotripsijom (URSL aparat Lithoclast) u periodu od jedne godine. Sto ispitanika ispunilo je kriterijume za uključivanje u studiju. Ispitanici su podeljeni u dve grupe: Lithoclast grupa (URSL) i ESWL grupa. Kao instrument za merenje kvaliteta života korišćen je *Short Form* (SF) 36 upitnik koji su bolesnici popunjavali neposredno pre i jedan mesec posle opercije. **Rezultati.** Analizom skora SF 36 upitnika preoperativno ustanovljeno je da postoji smanjenje kvaliteta života kod bolesnika sa urolitijazom u gotovo svim dimenzijama života. Nije bilo statistički značajne razlike u preoperativnom SF skoru između dve grupe bolesnika izuzev domena uloge fizičkog zdravlja u kojoj je Lithoclast grupa imala statistički značajno viši skor od ESWL grupe i mentalnog zdravlja u kojoj je ESWL grupa imala statistički značajno viši skor od Lithoclast grupe. Statističkom anlizom SF 36 upitnika postoperativno i ispitivanjem uticaja modaliteta lečenja na kvalitet života, ustanovljeno je da Lithoclast metoda postiže statistički značajno viši skor SF 36 u nekoliko dimenzija života i značajno utiče na poboljšanje kvaliteta života u odnosu na ESWL metodu koja, postoperativno, beleži blagi pad u skoru za neke dimenzije kvaliteta života. **Zaključak.** Procena kvaliteta života dobar je način za procenu modaliteta lečenja u kliničkoj praksi. Primenom SF 36 upitnika u našoj studiji ustanovljeno je da je ureterorenoskopska litotripsija (Lithoclast metoda) modalitet lečenja urolitijaze koja postoperativno daje statistički značajno poboljšanje kvaliteta života u nekoliko domena zdravlja u odnosu na ESWL metodu.

Ključne reči:
urolitijaza; litotripsija; kvalitet života; ankete i upitnici; metodi; lečenje, ishod.

Introduction

Urolithiasis is a common illness resulting in serious health problems that significantly impact the quality of life of patients [1]. This illness represents a group of metabolic and endocrine disorders in the organism that together with changes in the urinary tract lead to the formation of stones and incurrence of urolithiasis. The incidence of urolithiasis in the global population is around 12 % [2]. Albeit it is found in all age groups, the highest incidence is among people in 3rd, 4th, and 5th decade of life. Moreover, it should be underlined that this illness is prone to recidivation. It is assumed that more than 50% of patients experience recidivation during the ten year period. Accordingly, urolithiasis is rightly labeled as „illness for the whole life“ [2]. The occurrence of urolithiasis is three times more common in men than in women [3]. In the clinical practice, urolithiasis is most commonly classified according to the size and anatomic localization of the stone, which decisively impacts the decision on the mode treatment, or more precisely, the selection of the stone disintegration method. Today, indications and application of minimally invasive urological techniques dominant in the treatment of calculus in everyday clinical practice are clearly defined [4, 5]. Extracorporeal Shock Wave Lithotripsy (ESWL) is a method of the stone disintegration by the shock waves formed outside of the patient's body. Subsequently, they are focused on the stone. Currently, it is the most commonly used stone disintegration method [6]. Ureteroscopic lithotripsy is a method that initially introduces a citoscope for the identification of ureter's orificium. Subsequently, a guide is used to introduce an ureteroscope to visualize and disintegrate the stone. For sure, these two methods had increased treatment efficacy and decreased the occurrence of complications. Today, they are primary modes of urolithiasis treatment [7].

According to numerous studies, symptoms related to the existence of urolithiasis, illness complications, chronicity, recidivism, and different treatment modes represent external factors that can significantly impact the quality of life of these patients [5]. Regarding the quality of life, it must be underlined that there is no widely accepted definition of this term nor the golden standard for its measurement [8]. However, the most common definition is the one proposed by the World Health Organization. Accordingly, it is an individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards, and concerns [9]. In the dictionary „Health for Everybody for the 21st century“, the quality of life is defined as a perception of individuals or groups that their needs will be recognized in time and met in order to achieve happiness and fulfillment [10].

The term "the quality of life regarding health" describes the subjective satisfaction of an individual with his or her health status [11]. In this case, the quality of life is a factor for exploration of the impact of the illness and treatment modes on an individual's health by integrating the objective assessment of the health status and subjective perception. Accordingly, the examination of the quality of life is a crucial factor that complements laboratory and diagnostic treatment of patients and contributes to the assessment of the illness flow and treatment mode of life and functioning of individuals. Undoubtedly, the introduction of the term quality of life in the medical sign has enabled the medical practitioners to perceive a patient as a complete person and to prevent the division between the patient's body and his or her personality. Currently, the incidence of chronic illnesses is on the rise. Accordingly, the number of patients who are long-term beneficiaries of health care system services is increasing as well. As a consequence, the interest for exploration of the quality of life has gained prominence among researchers, considering that, evidently, it is a valuable source of information about the flow, the success rate of the treatment modes, and the outcome of the illness. The purpose of this study was to assess the postoperative quality of life of patients with urolithiasis depending on the applied treatment method.

Methods

This research was designed as a panel study – a combination of a cross-sectional and cohort study. The sample in-

cluded patients with urolithiasis of the Urology Clinic of the Clinical Center of Serbia treated with ESWL or ureteroscopic lithotripsy (URSL apparatus Lithoclast), on the basis of the decision of the Calculosis Consilium, following the recommendations of the European Association of Urologists. Our research did not affect the decision of the Consilium on the applied method. The research was carried out between February 1, 2017 and February 1, 2018, and 100 respondents met the inclusion criteria. They were divided into two equal groups considering the applied method of the stone disintegration: the group 1 or Lithoclast Group (URSL) and the group 2 or ESWL group, each with 50 respondents.

The following criteria for inclusion in the study were: the patients with the diagnosis of urolithiasis older than 18 who gave consent to participate in the study and who were members of the group American Society of Anesthesiologist I-III (ASA I-III) classification. The study excluded patients who did not want to participate, patients with ASA score 4 and 5, individuals with heavy injuries and illnesses and heavy infections of the urinary tract, patients with hemorrhagic diathesis, and patients with contraindication (according to the recommendation of the European Association of Urologists) for performing one of those methods. The instrument used for measuring the quality of life was Short Form 36 (SF 36) questionnaire. It was administrated to the patients immediately before the operation and one month after the operation during the control checkup. This study used linguistically and culturally adapted and validated Serbian version of SF 36 questionnaire (Proqualid Patient-reported outcome Quality of life instruments Database SF 36 Health Survey Serbian Version accessed on 20 June 2012) [12]. The patients filled it in independently; however, in the presence of a doctor whose role was to clarify the questions. SF 36 is an instrument for measuring individual perceptions of the overall health condition, the ability of functioning, limitations caused by emotional problems, limitations caused by physical problems, pain, fatigue, and problems in social functioning. The questionnaire consists of 36 questions divided into eight health domains regarding the 4-week period: physical functioning, limitations due to physical health, bodily pain, overall health, social functioning, limitations due to emotional problems, and mental health [13]. Answers in each domain were scored. The scale of answers was represented by numbers from 0 (the worst) to 100 (the best). Thus, the higher value indicated a higher quality of life – better physical functioning, better physical role, absence or lesser bodily pain, improved overall health, higher vitality, better social functioning, improved the emotional role and better mental health. The Cronbach analysis was used to test the reliability of the SF 36 questionnaire. More precisely, it tested the reliability of scales of given groups of the features. The testing confirmed the internal consistency of questions and reliability of the measurement instrument [14]. Descriptive and analytical statistic methods were used to analyze and present the obtained data. Concerning descriptive methods, absolute and relative numbers, measures of central tendency (arithmetic mean, median), and measures of dispersion were used (standard deviation). Also, the following analytical methods were used: tests of difference (χ^2-test, *t*-test, Mann-Whitney *U* test) and correlation analysis. SPSS 21.0 (IBM) program was used for data analysis.

Results

An overview of the basic demographic characteristics of patients divided into groups is shown in Table 1.

The average age of respondents in this study was 50. The youngest respondent was 20 and the oldest 70 years. The sample included 60% of male and 40% of female respondents. Statistically significant differences regarding age and gender were not revealed. However, the data analysis demonstrated statistically significant differences regarding the value of body mass index (BMI). Concerning comorbidity, 46% of respondents did not report accompanying comorbidities, 32% reported hypertension, 11% diabetes, and 11% other accompanying illnesses such as angina pectoris, depression, rheumatism, and disorder of a thyroid gland. The statistically significant differences between the two groups were not noted (Table 2).

The significant distribution of patients by groups existed depending on urological diagnosis preoperatively. Hence, the kidney stones (renal calculi) were prevalent in the ESWL group, whereas ureteral calculus was more dominant in the Lithoclast group.

As shown in Tables 3 and 4, the statistically significant difference was noted regarding the localization of the stone and present symptoms of urolithiasis preoperatively. Calculus of urinary tract was accompanied by intense distress and symptoms requiring an adequate treatment (Table 4). According to the patients, the most unpleasant symptom of urolithiasis is renal colic, as it is very painful for each patient [15].

Table 1

Demographic characteristics of patients with urolithiasis (n = 100)

Characteristics of patients	All patients	Lithoclast group n = 50	ESWL group n = 50	*p*
Age (years), mean ± SD	50	51.04 ± 12.70	50.20 ± 10.30	0.717
BMI (kg/m$^{2)}$, mean ± SD	26.19	25.54 ± 3.15	26.84 ± 3.06	0.039
Gender, m/f	40/60	20/30	20/30	1.000
ASA 1	2	0	2	0.218
ASA 2	89	44	45	
ASA 3	9	6	3	

BMI – body mass index; m/f – male/female; ASA – American Society of Anesthesiologists; ESWL – extracorporeal shock wave lithotripsy; SD – standard deviation.

Table 2

The group distribution of patients by the diagnosis

Diagnosis	Lithoclast group n = 50	ESWL group n = 50	*p*
Calculus renis	19	49	
Calculus ureteris	29	1	< 0.001
Other	2	0	

ESWL – extracorporeal shock wave lithotripsy.

Table 3

Localization of the stone

Localisation	Lithoclast group n = 50	ESWL group n = 50	*p*
Kidney upper pole	13	16	
Kidney lower pole	9	33	
Proximal ureter	9	0	< 0.001
Distal ureter	18	1	
Urinary bladder	1	0	

ESWL – extracorporeal shock wave lithotripy.

Table 4

The preoperative distribution of clinical symptoms of urolithiasis among patients

Symptoms	Lithoclast group n = 50	ESWL group n = 50	*p*
Asymptomatic	6	0	
Nausea	4	3	
Vomiting	2	6	< 0.001
Pain	28	15	
Renal colic	7	26	
Hematuria	1	0	

ESWL – extracorporeal shock wave lithotripsy.

The SF scale of answers was used for the assessment of the quality of life of patients preoperatively and postoperatively. The scale of answers was represented by numbers from 0 (the worst) to 100 (the best). Thus, the higher value indicated a higher quality of life (Table 5).

The statistical analysis of the preoperative SF 36 questionnaire revealed that patients with urolithiasis in both groups hade lower SF 36 score of the quality of life in almost all dimensions or domains of health. The particularly low score was attained in the domain of physical functioning in the Lithoclast group. This score was statistically significantly lower in comparison to the patients of the ESWL group. The low scores were also noted in the following health dimensions: bodily pain, overall health, vitality, emotional role, and mental health. The statistically significant difference in the SF 36 score preoperatively was proven in the domain of mental health as well, as the ESWL group obtained a higher score than the Lithoclast group.

Considering the impact of the treatment mode of urolithiasis on the quality of life of respondents postoperatively, we established statistically significant differences in the patients' quality of life between two groups in several dimensions of health. Ureteroscopic lithotripsy has outperformed the ESWL method in the following dimensions: physical functioning, a role of physical functioning, bodily pain, vitality, social functioning, and mental health. The patients in the ESWL group even experienced a lower SF score (the negative impact on the quality of life) in the following dimensions: mental health, vitality, social functioning, and the role of physical functioning. The exceptionally high statistically significant difference regarding the higher score (better quality of life), the Lithoclast group attained in three health domains: physical functioning, social functioning, and bodily pain.

Discussion

The demographic characteristics of the patients in this study regarding age, gender, BMI, the frequency of urolithiasis, as well as the most common and the most significant symptoms of the illness, correspond to the results of previous studies on urolithiasis. Until recently, only several studies examining the quality of life of patients with urolithiasis had been published [16]. The examination of the quality of life in urology started in 1992, following the recommendation of the American Urological Association to include questionnaires on the patients' quality of life in urological research.

Table 5

Results of the Short Form 36 (SF-36) questionnaire in the patients with urolithiasis

SF-36 domain	The type of performed intervention	Mean	SD	Median	*p*
Physical function					
preop.	Lithoclast	77.3	23.11	85	0.335
	ESWL	74.2	20.19	72.5	
postop.	Lithoclast	66.20	16.43	95	0.015
	ESWL	77–30	22–57	82.50	
Delta Physical function	Lithoclast	8.90	19.07	5.00	0.152
	ESWL	3.10	23.82	0.00	
Role physical					
preop.	Lithoclast	36.00	45.46	0.00	0.028
	ESWL	57.50	45.68	75.00	
postop.	Lithoclast	68.50	45.68	100.00	0.032
	ESWL	51.00	46.00	50.00	
Delta role physical	Lithoclast	33.50	45.63	0.00	< 0.001
	ESWL	- 6.50	38.07	0.00	
Body pain	Lithoclast	53.14	20.57	52.00	0.520
preop.	ESWL	52.88	27.59	31.00	
postop.	Lithoclast	66.20	18.38	67.00	0.002
	ESWL	52.32	23.35	52.00	
Delta Body pain	Lithoclast	13.06	19.84	12.50	0.001
	ESWL	-0.56	19.31	0.00	
General health					
preop.	Lithoclast	59.44	14.76	57.00	0.538
	ESWL	61.48	18.05	57.00	
postop.	Lithoclast	59.06	15.10	53.50	0.766
	ESWL	60.00	16.33	55.00	
Delta General health	Lithoclast	-0.36	7.33	0.00	0.546
	ESWL	-1.48	10.54	0.00	
Vitality					
preop.	Lithoclast	56.50	16.82	52.50	0.260
	ESWL	60.40	17.51	60.00	
postop.	Lithoclast	60.90	13.20	60.00	0.364
	ESWL	58.00	18.18	60.00	
Delta Vitality	Lithoclast	4.40	10.38	2.50	0.009
	ESWL	-2.40	14.79	0.00	
Social functioning					
preop.	Lithoclast	71.00	19.82	75.00	0.331
	ESWL	74.92	20.28	75.00	
postop.	Lithoclast	80.48	17.66	88.00	0.018
	ESWL	71.22	20.54	75.00	
Delta Social functioning	Lithoclast	9.48	18.20	12.00	< 0.001
	ESWL	-3.70	16.89	0.00	
Role emotional					
preop.	Lithoclast	40.68	45.84	0.00	0.059
	ESWL	57.32	46.21	83.50	
postop.	Lithoclast	76.00	43.14	100.00	0.066
	ESWL	60.66	47.00	100.00	
Delta Role emotional	Lithoclast	35.32	48.77	0.00	0.001
	ESWL	3.34	47.77	0.00	
Mental health					
preop.	Lithoclast	64.40	14.21	64.00	0.037
	ESWL	70.72	15.68	76.00	
postop.	Lithoclast	66.00	12.86	64.00	0.792
	ESWL	65.20	17.07	64.00	
Delta mental health	Lithoclast	1.60	10.35	0.00	0.001
	ESWL	-5.52	10.25	-4.00	

SD – standard deviation; preop. – preoperatively; postop. – postoperatively.

Initially, the research focused on patients with prostate and malignant illnesses, whereas the quality of life of patients with urolithiasis had remained unexplored due to a limited number of studies on this issue [16–18].

According to the literature review, the majority of authors analyzes and evaluates the quality of life of individuals regarding the functional ability, degree, and quality of social interaction, mental wellbeing, somatic sensations, and life satisfaction [18].However, the authors commonly argue that although objective assessment of health is important for the quality of life, also a subjective assessment of the health of the patient as well as his or her expectations of the treatment and the treatment outcome should be taken into account.

One of the most challenging aspects of measuring the quality of life is a quantification of all components and domains of health. To enhance the efficacy of measurements of different domains of the quality of life through specific questions, the fundamental measures were developed, namely, measures of psycho-physical condition and measures of the perceptions of sensations [17]. One of the instruments frequently used in practice is SF 36 questionnaire for the examination of the quality of life. In the recent years, several studies have used this questionnaire. The study of Donnally et al. [18] examined the quality of life of patients with utolithiasis. Nine studies on 1,570 patients with urolithiasis also used SF 36 questionnaire as an instrument for measuring the quality of life [19]. In our study SF 36 questionnaire was also used, considering that numerous previous studies had confirmed its psychometric validity for measuring the quality of life [20]. In this study, the statistical analysis of SF 36 scores preoperatively established that patients with urolithiasis in both groups had a lower quality of life in all dimensions or domains of health. In particular, the lowest score of the SF 36 questionnaire was attained pre-operatively in the domain of physical health in the Lithoclast group. This score was statistically significantly lower than the score of patients in the ESWL group. Accordingly, prior to the intervention, patients in the Lithoclast group had much more problems in work and in fulfilling daily activities due to lower physical health. The lower score was also noted in the following dimensions of health: bodily pain, overall health, vitality, emotional role, and mental health. The statistically significant difference in SF score between two groups preoperatively was also proven in the domain of mental health, as the patients from the ESWL group had higher scores than those of the Lithoclast group. More precisely, the patients from the ESWL group reported being nervous and felt less depressed. The results of this study are in line with those of the similar studies carried out abroad [21]. Seven studies on the quality of life of patients with urolithiasis confirmed the decreased quality of life [19]. Although there are variations among studies, it can be argued that patients with urolithiasis have a lower quality of life in comparison to the general population [22–24]. According to the study of Bryant et al. [16], patients with urolithiasis have a significantly lower SF 36 score than the general US population in six out of eight domains of health. This study also confirmed that the SF 36 questionnaire is a valid instrument for assessment of the quality of life. However, a lack of the baseline SF 36 questionnaire for the healthy population in Serbia poses a significant challenge for the researchers, as it is not possible to carry out comparisons. Considering the impact of the urolithiasis treatment mode on the postoperative quality of life of respondents, there is a lack of relevant studies. Moreover, the study results are varied and even conflicting [23, 24]. Conclusion is that in spite of various treatment modes of urolithiasis and their high efficacy, further research in this area is needed to improve the postoperative outcomes of the models and the quality of life of patients [25, 26].

This study demonstrated through the statistical analysis of SF 36 questionnaire, filled in by the patients four weeks after the operation, that there is a statistically significant difference regarding the quality of life of patients postoperatively between two groups of patients (the treatment modes of urolithiasis) in several domains of health. With the higher score on SF 36 questionnaire, ureteroscopic lithotripsy outperformed the ESWL method in the following dimensions: physical functioning, the role of physical functioning, bodily pain, vitality, social functioning, and mental health. Concerning daily clinical practice, it was concluded that patients treated by ureteroscopic lithotripsy had fewer limitations in performing physical activities, fewer problems at work and other activities due to the physical health, reduction or elimination of bodily pain, and fewer problems in social functioning due to emotional and economic difficulties. Moreover, they were more vital and less nervous and depressed, unlike the patients in the ESWL group, who even experienced a lower SF score (the negative impact on the quality of life) in the following dimensions: mental health, vitality, social functioning, and the role of physical functioning. The exceptionally high statistically significant difference regarding higher score (better quality of life) the Lithoclast group attained in three health domains: physical functioning, social functioning, and bodily pain. This study showed that ureteroscopic lithotherapy as a treatment mode of urolithiasis is much more efficient than the ESWL mode. Moreover, it has a more positive impact on the quality of life of patients after the intervention.

Conclusion

The treatment of urolithiasis, its chronicity and the impact on the quality of life of those patients represents a challenging for selecting the right treatment mode. Indications and contraindications for performing the ESWL or ureteroscopy (URSL) are very similar and sometimes even identical, thus, posing a great challenging for the clinical practice. The assessment of the quality of life is a recommended method for the evaluation of treatment modes and it allows their measurement and comparison.The assessment of the quality of life-related to health enables the healthcare practitioners to perceive the patient not only as a carrier of illness but as a personality as well. The SF 36 questionnaire is a highly reliable tool for assessing the quality of life by measuring different dimensions. This study confirmed it as a valid measurement instrument. The study demonstrated that ureteroscopic lithotripsy is a treatment mode of urolithiasis that

postoperatively results in statistically significant enhancement of the quality of life of patients in several health domains in comparison to the ESWL method. Moreover, it provided the basis for further research with aim to establish faster and more optimal decision-making process about efficient and safe treatment methods of urinary calculosis that should, besides healing, also improve the quality of life of patients postoperatively.

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The effects of acutely and subchronically applied DL-methionine on plasma oxidative stress markers and activity of acetylcholinesterase in rat cardiac tissue

Efekti akutno i subhronično primenjenog DL-metionina na markere oksidativnog stresa u plazmi i aktivnost acetilholinesteraze u tkivu srca pacova

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Abstract

Background/Aim. Chronically induced hypermethioninemia leads to hyperhomocysteinemia which causes oxidative stress, atherogenesis, neurodegeneration and cancer. However, little is known about the acute and subchronic effects of DL-methionine (Met). The aim of study was to assess the effects of acutely and subchronically applied Met on oxidative stress parameters in rat plasma [enzymes: catalase (CAT), glutathione peroxidise (GPx), superoxide dismutase (SOD) and index of lipid peroxidation, malondialdehyde (MDA)], and acetylcholinesterase (AChE) activity in rat cardiac tissue. **Methods.** The enzymes activities, as well as MDA concentration were evaluated following acute (n = 8) and subchronic (n = 10) application of Met [i.p. 0.8 mmoL/kg body weight (b.w.) in a single dose in the acute overload or daily during three weeks in the subchronic overload]. The same was done in the control groups following application of physiological solution [i.p. 1 mL 0.9% NaCl (n = 8) in the acute overload and 0.1–0.2 mL 0.9% NaCl, daily during three weeks (n =10) in the subchronic overload]. Tested parameters were evaluated 60 minutes after application in acute experiments and after three weeks of treatment in subchronic experiments. **Results.** There were no difference in homocysteine values between the groups treated with Met for three weeks and the control group. Met administration significantly increased the activity of CAT and GPx after 1 h compared to the control group ($p = 0.008$ for both enzymes), whereas the activity of SOD and MDA concentrations were unchanged. Subchronically applied Met did not affect activity of antioxidant enzymes and MDA level. AChE activity did not show any change in rat cardiac tissue after 1 h, but it was significantly decreased after the subchronic treatment ($p = 0.041$). **Conclusion.** Results of present research indicate that Met differently affects estimated parameters during acute and subchronic application. In the acute treatment Met mobilizes the most part of antioxidant enzymes while during the subchronic treatment these changes seems to be lost. On the contrary, the acute Met overload was not sufficient to influence on the AChE activity, while longer duration of Met loading diminished function of the enzyme. These findings point out that methionine can interfere with antioxidant defense system and cholinergic control of the heart function.

Key words:
oxidative stress; methionine; homocysteine; rats; plasma; enzymes; cholinesterases.

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Apstrakt

Uvod/Cilj. Hronično indukovana hipermetioninemija dovodi do hiperhomocisteinemije koja izaziva oksidativni stress, aterogenezu, neurodegeneraciju i karcinome. Međutim, malo se zna o efektima akutne i subhronične primene DL-metionina (Met). Cilj ovog istraživanja bila je procena efekata akutno i subhronično primenjenog Met na parametre oksidativnog stresa u plazmi pacova [enzime: katalaza (CAT), glutation peroksidaza (GPx), superoksid dismutaza (SOD) i indeks lipidne peroksidacije, malondialdehid (MDA)] i na aktivnost acetilholinesteraze (AChE) u tkivu srca pacova. **Metode.** Aktivnosti enzima, kao i koncentracija MDA mereni su nakon akutne (n = 8) i subhronične (n = 10) primene Met (i.p. 0,8 mmoL/kg u jednoj dozi u akutnom eksperimentu ili svakodnevno tokom tri nedelje u subhroničnom eksperimentu). Isti način tretmana je bio primenjen i u kontrolnoj grupi, ali su životinje bile tretirane fiziološkim rastvorom [i.p. 1 mL 0,9% NaCl (n = 8) u akutnom i 0,1–0,2 mL 0,9% NaCl svakodnevno tokom tri nedelje (n = 10), u subhroničnom eksperimentu]. Testirani parametri su mereni 60 min nakon aplikacije supstanci u akutnim eksperimentima i nakon tri nedelje tretmana u subhroničnim eksperimentima. **Rezultati.** Nije bilo razlike u vrednostima homocisteina između grupe tretirane Met tokom tri nedelje i kontrolne grupe. Primena Met značajno je povećala aktivnost CAT i GPx nakon 1h u poređenju sa kontrolnom grupom (p = 0,008 za oba enzima), dok je aktivnost SOD i koncentracija MDA bila nepromenjena. Subhronično primenjen Met nije uticao na aktivnost antioksidativnih enzima, ni na koncentraciju MDA u plazmi. Aktivnost AChE u srčanom tkivu pacova nije se menjala nakon 1 h, ali je bila značajno smanjena nakon subhroničnog tretmana (p = 0,041). **Zaključak.** Rezultati istraživanja pokazuju da Met različito utiče na ispitivane parametre tokom akutne i hronične primene. Posle akutne primene Met mobiliše veći deo antioksidativnih enzima, dok se tokom subhroničnog tretmana ove promene gube. Nasuprot tome, akutna primena Met ne utiče na aktivnost AChE, dok duže trajanje metioninskog opterećenja smanjuje funkciju ovog enzima. Ovi nalazi ukazuju na to da metionin može da interferira sa antioksidativnim sistemom zaštite i holinergičkom kontrolom funkcije srca.

Ključne reči:
stres, oksidativni; metionin; homocistein; pacovi; plazma; enzimi; holinesteraze.

Introduction

Methionine (Met) is an essential sulfur-containing amino acid. It is the first amino acid that is embedded during the process of protein synthesis. It is considered that its primary role is initiation of translation rather than inclusion in the protein structure because it is usually removed from proteins during their synthesis [1]. Activation of Met involves its conversion to S-adenosylmethionine (SAM), which is a methyl group donor in the methylation process. SAM, *via* S-adenosylhomocysteine translates into homocysteine (Hcy). It is normally metabolized *via* two biochemical pathways – remethylation and transsulfuration. Remethylation converts Hcy back to Met, in the presence of betaine or *via* Met synthase in the presence of folic acid and vitamin B12. Transsulfuration converts Hcy to cysteine and glutathione (GSH) in the presence of vitamin B6. GSH is the main product in fighting against oxidative stress [2]. Transsulfuration is regulated by the balance between prooxidants that favorize it and antioxidants that inhibit it [3].

It is believed that excess of Met in tissues is responsible for aging and a reduced life span. Prolonged Met overload can cause increased levels of hydroperoxide, LDL cholesterol, lipid peroxidation, oxidative stress in the liver and plasma Hcy level, which is angiotoxic, causes endothelial dysfunction, hypertension, and it is an important factor in the development of atherosclerosis [4]. Experimental hyperhomocysteinemia caused by long-term oral administration of Met shows the greatest reduction of vasodilatation after 8 h when Hcy level reaches maximum, and the value of Met normalizes [5]. This demonstrates that Hcy is responsible for endothelial dysfunction through the induction of oxidative stress or increased content of an endogenous inhibitor of NO synthesis, asymmetric dimethylarginine (ADMA), product of NO methylation [5, 6]. Another study has shown that, with an unchanged content of Met, and in the absence of B12, significantly elevated levels of Hcy does not cause endothelial lesions in rats with elevated LDL. It has also been shown that the administration of Met in the absence of B12 increases Hcy level to a lesser extent, but it causes significantly greater endothelial lesions [7]. This might mean that Hcy *per se* is not the culprit, and it does not induce oxidative stress itself but the excess of Met inhibits methylation of Hcy and redirects it to NO, forming ADMA. This is supported by the fact that ADMA elevation is observed only in homocysteinemia caused by Met, but not in chronic homocysteinemia [6].

Some authors suggest that cysteine is the one that causes oxidative stress, and has a higher vascular toxicity than Hcy [8]. Others assume that Hcy masks harmful effects of other substances, such as S-adenosylhomocysteine, formed from excess of Hcy, which inhibits methyltransferase and methylenetetrahydrofolate reductase with subsequent deleterious effects [9].

Despite numerous experiments that indicate possible toxicity of Met, primarily through Hcy, severe hyperhomocysteinemia occurs only after application of Met in a dose of 100 mg/kg during one week, which is seven times more than the necessary daily intake of sulfur-containing amino acids [10, 11]. Met is a precursor of glutathione, endogenous antioxidant, so physiological concentrations of Met are required for detoxication in the liver. One study showed that L-Met alone increased the reduction state of glutathione, as well as the total content of this tripeptide [12]. In investigations performed on thioredoxin and glutathione reductase knockout mouses, it has been shown that Met is an alternative fuel for the redox processes in the hepatocytes [13]. Other authors have demonstrated that Met applied during 3.5 days has protective effects against oxidative stress induced by polymyxin B in

rat kidney tubular cells [14]. Met may also directly neutralize reactive oxygen species (ROS) *via* sulfhydryl group [15].

In addition, there are three main enzymes that fight oxidative stress: catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). They together reduce superoxide radicals and hydrogen peroxide to water [16].

Acetylcholinesterase (AChE) is a serine hydrolase that cleaves and inactivates the neurotransmitter acetylcholine (Ach) [17–19]. Although this enzyme is mostly present in the brain and sceletal muscle, it has a significant role in cholinergic system of the heart.[20]

It has been proven that excess of Ach or inadequate functioning of AChE causes hyperactivity of excitable tissues, muscle weakness and acute subjunctional necrosis of muscle fibers, as a consequence of increased levels of Ca^{2+} and generation of ROS [21].

However, connection between AChE activity and oxidative stress is poorly investigated and still unclear. These informations have been accumulated in recent years and interaction between prooxidant and antioxidant moleculs and this enzyme seems to exist [22, 23]. In a very recent study it has been shown that increased ROS production during sepsis can reduce AChE activity in the diaphragms of rats [23].

As Met is an important factor in antioxidant defense, we aimed to examine the effects of acutely and subchronically applied Met on plasma oxidative stress markers and AChE activity in rat cardiac tissue.

Methods

Physiological assay and experimental protocol

Adult male Wistar albino rats, body weight 250 ± 50 g for acute experiments (n = 16) and around 140 ± 20 g (at the start) for subchronic experiments (n = 20) were used. Rats intended for subchronic experiments were three weeks younger in order to have the same age and approximately the same weight after the three weeks treatment with methionine as the animals from the acute series of experiments, on the day of sacrificing. Animals were raised in strictly controlled conditions (air temperature of 22 ± 1°C, relative humidity 50%, a cycle of brightness: darkness = 12 : 12 h, starting bright period at 8AM), with free access to water and standard food. For acute experiments, the animals were divided into two groups: the control group [0.9% NaCl i.p., pH 7.4; 1 mL/kg i.p.) (n = 8)]; Met group [0.8 mmoL/kg i.p. DL-Met) (n = 8)]. For subchronic experiments, the animals were also divided into two groups, which were given the substance according to the following scheme: the control group [0.9% NaCl, pH 7.4; 0.1–0.2 mL/day i.p., for 3 weeks (n = 10)] and Met group [0.8 mmoL/kg/day i.p. DL-Met, for 3 weeks (n = 10)]. Acute and subchronic experimental protocols were chosen according to literature data [24, 25].

All experimental procedures were done in concordance with Directive of the European Parliament and of the Council (2010/63/EU) and approved by the Ethical Committee of the Faculty of Medicine, University of Belgrade.

Tissue and biochemical analyses

Sixty minutes after administration of tested substances, the rats were anesthetized with ketamine (10 mg/kg) and xylazine (5 mg/kg) and euthanized by decapitation. After sacrificing of rats, venous blood samples were collected for biochemical analyses and hearts were isolated for determination of AChE activity in samples of cardiac tissue homogenate.

In samples of venous blood following biochemical parameteres were measured in the plasma: homocysteine, malondialdehyde (MDA), and activities of enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). These parameters were determined in the control condition, and then in series of experiments.

For determination of AChE activity in samples of cardiac tissue homogenate whole hearts were isolated, rinsed in a phosphate buffer pH 8.0, and homogenized in cold phosphate buffer (pH 8.0). The final tissue concentration was 20 mg tissue per mL of the buffer [26].

Determination of plasma homocysteine level

For this process, blood was collected through a glass funnel and placed in appropriate vacutainers coated by heparin. After the collection, the plasma samples remained at room temperature for 15 minutes and then were centrifuged (15 min × 3000 rpm) and analyzed. At the beginning of and after the experiment, the samples were analyzed using the electrochemiluminescence method (ECL), electrochemiluminescence immunoassay system (ADVIA Centaur XP System, Siemens Healthcare GmbH, Erlangen, Germany); the range of reference values was Hcy < 15 μmol/L. Samples for determination of oxidative stress parameters were frozen (−80°C) until measurement. All measurements were performed on ice.

Determination of catalase activity

Catalase activity was measured by an assay that follows the degradation of H_2O_2 [27]. Suspension of plasma (50 μL) was added to the quartz glass cuvette at room temperature, containing 2.975 mL of 50 mM phosphate buffer solution in 0.4 mM EDTA. The enzyme reaction was initiated by adding 30 μL of 3% H_2O_2. Reduction in the value of the absorbance due to enzymatic degradation of H_2O_2 at 240 nm for 3–5 minutes was monitored. Catalase activity was expressed as U/mL of plasma. One unit (U) of enzyme activity was defined as 1 μmoL of spent H_2O_2/min.

Determination of glutathione peroxidase activity

To measure the glutathione peroxidase activity reaction cocktail was prepared as follows: 8.9 mL of phosphate buffer, pH 7 (50 mM NaH_2PO_4 + 0.40 mM EDTA), 50 μL of 200 mM reduced glutathione (GSH), 1 mg of β-NADPH, and 100 μL of 100 units/mL glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*). The reaction cocktail (3 mL) and plasma sample (0.3 mL) were added in a quartz

glass cuvette (room temperature). Cuvette was placed in a spectrophotometer, and the enzymatic reaction was started by adding 50 μL of 0.042% H_2O_2 (A_{340} = 0.52–0.56). The decline in the value of absorbance (λ = 340 nm) for 15 seconds during 4–5 minutes was monitored. GPx activity was expressed as ΔA/min/mL of plasma [28].

Determination of superoxide dismutase activity

Superoxide dismutase activity was measured according to the method of Misra and Fridovich [29]. Plasma sample (10–30 μL) was added to 3 mL of 0.5 M EDTA-sodium carbonate buffer, pH 10.2. The enzymatic reaction was started by the addition of 100 μL of adrenaline (30 mM in 0.1 M HCl) and the activity was measured at 480 nm during 4 minutes. One unit (U) was defined as the amount of enzyme that inhibits the rate of the oxidation of adrenaline by 50%. The enzyme activity was expressed as U/mL of plasma.

Determination of malondialdehyde

For the determination of MDA in plasma sample, thiobarbituric assay was used [30]. In 500 μL of plasma sample, 500 μL of 25% HCl and 500 μL of 1% thiobarbituric acid in 50 mM NaOH were added. The mixture was placed in a boiling water bath for 10 minutes, and then cooled to room temperature. N-butanol (3 mL) for extraction was added and shaken in vortex for 30 seconds. For the successful separation of the phases, centrifugation for 10 minutes at 2000 × g in a Sorvall centrifuge was necessary. Content of MDA was determined spectrophotometrically by measuring the absorbance of the organic phase (upper layer) at 532 nm. The blank probes contained 50 mM NaOH instead of thiobarbituric acid, and were prepared for each sample separately. The value of MDA content was expressed as nmol MDA/mL of plasma, and it was based on the measured values of absorbance and molar absorption coefficient of the complex malondialdehyde-thiobarbituric acid.

Determination of acetylcholinesterase activity in cardiac tissue

The specific activity of AChE in the cardiac tissue was measured *in vitro* by the Ellman method [31]. The method is based on the reaction of a color reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) – DTNB, with the product of hydrolysis of the thiocholine substrate, acetiltioholine iodide (AChI), thiocholine, to give a yellow-colored compound, 5-thio-2- nitrobenzoate, whose intensity is proportional to the activity of AChE. An appropriate amount of a homogenate of the tested tissue (40 μL of the heart homogenate in 580 μL of phosphate buffer pH 8.0) was preincubated for 10 minutes at a temperature of 37°C. After preincubation, 20 μL of color reagent DTNB and 10 μL of AChI substrate were added. The change in absorbance at 412 nm was measured spectrophotometrically (Gilford Instrument, Model 250) for 3 minutes. The blank probe contained all the components of the assay for following AChE activity, except the tissue homogenate. The measurements were performed in duplicate. Specific enzyme activity of AChE in the heart was expressed as ΔA/min/mg of tissue.

Chemicals

All used substances were purchased from Sigma Aldrich (Germany). Substances used in the experiment were *pro* analysis quality.

Statistical analysis

Statistical significance of differences in the activity of the enzymes CAT, GPx, SOD, AChE and concentration of MDA between groups was analyzed by Student's *t*-test for independent samples. Statistical data were analyzed by a computer program "R". Values are presented as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

Results

Determination of total plasma homocysteine level

The homocysteine values in the subchronically methionine treated group were non-significantly different in relation to those in the control group (9.51 ± 0.59 μmol/L vs. 9.98 ± 0.65 μmol/L, respectively).

Plasma catalase activity

Acutely applied Met induced significant increase in CAT activity (78.37 ± 7.79 U/mL) compared to the control group (47.85 ± 4.78 U/mL). Enzyme activity was not changed after 3 weeks of Met administration (163.53 ± 21.33 U/mL) in comparison to the control (132.22 ± 10.37 U/mL) (Figure 1).

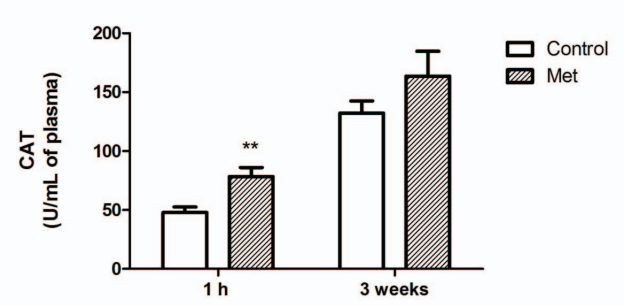

Fig. 1 – The effects of methionine (Met) on catalase (CAT) activity after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM. **$p < 0.01$ compared to the control group.

Plasma glutathione peroxidase activity

The specific activity of GPx in the plasma of rats given physiological solution was 3.21 ± 0.33 U/mL, whereas in the

experimental group was 5.23 ± 0.510 U/mL suggesting that DL-Met increase the activity of this enzyme after 1 h. However, GPx activity remained unchanged after 3 weeks of Met application (10.90 ± 1.41 U/mL vs. 8.81 ± 0.69 U/mL in the control group) (Figure 2).

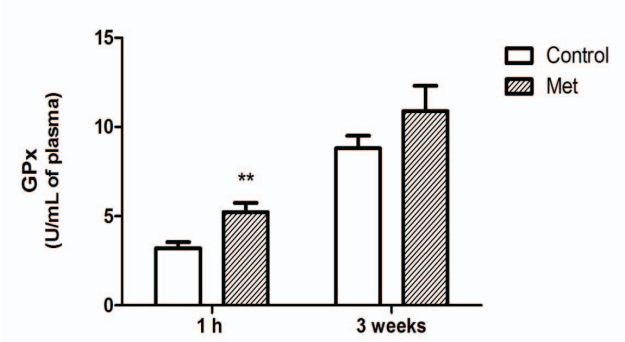

Fig. 2 – The effects of methionine (Met) on glutathione peroxidase (GPx) activity after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM. **$p < 0.01$ compared to the control group.

Plasma superoxide dismutase activity

There was no statistically significant changes in SOD activity in the experimental group (23.47 ± 0.77 U/mL) compared to the control one (24.01 ± 0.84 U/mL) 1 h after Met administration. Also, there was no statistically significant difference in SOD activity between groups in the subchronic experiment. SOD activity was 29.92 ± 0.44 U/mL in the Met treated group and 30.09 ± 0.82 U/mL in the control group (Figure 3).

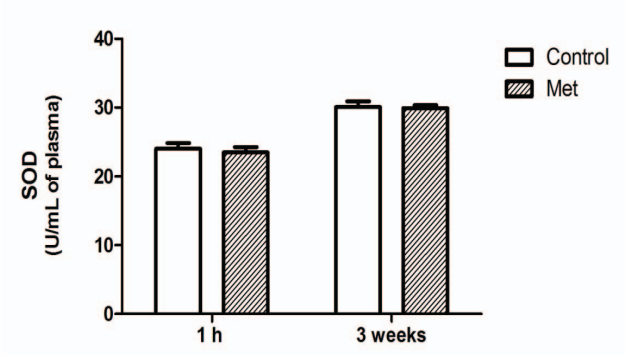

Fig. 3 – The effects of methionine (Met) on superoxide dismutase (SOD) activity after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM.

Plasma malondialdehyde concentration

MDA concentration was not changed either after acutely or subchronically application of Met in relation to the control. After 1 h it was 4.77 ± 0.80 nmol/mL in the experimental group and 4.87 ± 0.43 nmol/mL in the control one, whereas after 3 weeks it was 15.03 ± 1.39 nmol/mL in the experimental group and 12.56 ± 1.38 nmol/mL in the control group (Figure 4).

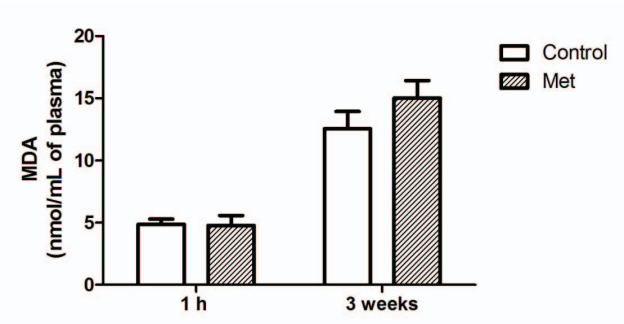

Fig. 4 – The effects of methionine (Met) on malondialdehyde (MDA) concentration after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM.

Cardiac tissue homognate acetylcholinesterase acitivity

The acute application of Met did not induce change in AChE activity in the rat cardiac tissue homogenate (0.043 ± 0.001 ΔA/min/mg of tissue) vs. control (0.049 ± 0.002 ΔA/min/mg of tissue). However, subchronically applied, Met caused the significant decrease of the enzyme activity in the cardiac tissue homogenates of treated group (0.046 ± 0.004 ΔA/min/mg of tissue) in comparison to the control group (0.057 ± 0.002 ΔA/min/mg of tissue) (Figure 5).

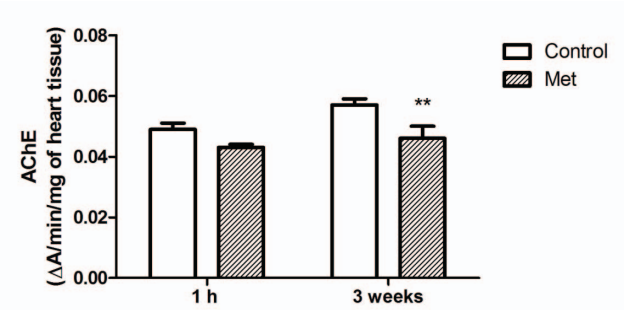

Fig. 5 – The effects of methionine (Met) on acetylcholinesterase (AChE) activity after acute (1 h) and subchronic (3 weeks) application in the cardiac tissue of rats. Values are presented as mean ± SEM. **$p < 0.01$ compared to the control group.

Correlation analysis

A positive correlation was noticed between following values/groups: CAT and SOD values in the control group from the acute experiment (Table 1); Hcy values from the acute experiment and GPx and MDA values from the subchronic experiment in the control group; CAT values in subchronically treated Met group and SOD values in the control group from subchronic experiment; GPx values and MDA values in the control group from the subchronic experiment; SOD values in the control group and CAT values in the subchronically treated Met group in the subchronic experiment (Table 2); CAT values in the control group from the acute experiment and CAT values in the subchronically Met treated group from the subchronic experiment; CAT values in the control group from the acute experiment and SOD values in the control group from the subchronic experiment;

CAT values in the acutely treated Met group and CAT values in the control group from the subchronic experiment; SOD values in the control group from the acute experiment and CAT values in the subchronically Met treated group from the subchronic experiment; SOD values in the control groups from the acute and subchronic experiment (Table 3).

Table 1
Correlation matrix between hemocysteine (Hcy) and parameters of oxidative stress in acutely methionine treated rats

Parameters	Hcy-C	Hcy-M	CAT-C	CAT-M	GPx-C	GPx-M	MDA-C	MDA-M	SOD-C	SOD-M
Hcy-C	/	r = -0.34	r = -0.60	r = 0.60	r = 0.24	r = 0.24	r = -0.45	**r = -0.84**	r = -0.60	r = 0.46
		p = 0.51	*p* = 0.20	*p* = 0.08	*p* = 0.64	*p* = 64	*p* = 0.65	***p* = 0.04**	*p* = 0.20	*p* = 0.36
Hcy-M	r = -0.34	/	r = -0.41	r = -0.62	r = -0.13	r = 0.49	r = -0.21	r = 0.65	r = -0.41	r = -0.72
	p = 0.51		*p* = 0.41	*p* = 0.19	*p* = 0.80	*p* = 0.32	*p* = 0.69	*p* = 0.16	*p* = 0.41	*p* = 0.10
CAT-C	r = -0.60	r = -0.41	/	r = -0.20	r = 0.00	r = -0.63	r = 0.60	r = 0.32	**r = 1.00**	r = 0.2
	p = 0.20	*p* = 0.41		*p* = 0.70	*p* = 1.00	*p* = 0.18	*p* = 0.21	*p* = 0.54	***p* = 0.000**	*p* = 0.70
CAT-M	r = 0.76	r = -0.62	r = -0.20	/	r = 0.00	r = 0.32	r = -0.6	r = -0.63	r = -0.20	r = 0.40
	p = 0.08	*p* = 0.19	*p* = 0.70		*p* = 1.00	*p* = 0.54	*p* = 0.21	*p* = 0.18	*p* = 0.70	*p* = 0.43
GPx-C	r = 0.24	r = -0.13	r = 0.00	r = 0.00	/	r = -0.46	r = 0.38	r = -0.46	r = 0.00	r = 0.77
	p = 0.64	*p* = 0.80	*p* = 1.00	*p* = 1.00		*p* = 0.36	*p* = 0.45	*p* = 0.36	*p* = 1.00	*p* = 0.07
GPx-M	r = 0.24	r = 0.49	r = -0.63	r = 0.32	r = -0.46	/	**r = -0.95**	r = 0.25	r = -0.63	r = -0.63
	p = 0.64	*p* = 0.32	*p* = 0.18	*p* = 0.54	*p* = 0.36		***p* = 0.004**	*p* = 0.63	*p* = 0.18	*p* = 0.18
MDA-C	r = -0.46	r = -0.21	r = 0.60	r = 0.60	r = 0.38	**r = -0.95**	/	r = 0.00	r = 0.60	r = 0.40
	p = 0.36	*p* = 0.69	*p* = 0.21	*p* = 0.21	*p* = 0.45	***p* = 0.004**		*p* = 1.00	*p* = 0.21	*p* = 0.43
MDA-M	**r = -0.84**	r = 0.65	r = 0.32	r = -0.63	r = -0.46	r = 0.25	r = 0.00	/	r = 0.32	r = -0.79
	***p* = 0.04**	*p* = 0.16	*p* = 0.54	*p* = 0.18	*p* = 0.36	*p* = 0.63	*p* = 1.00		*p* = 0.54	*p* = 0.06
SOD-C	r = -0.61	r = -0.41	r=-1.000	r = -0.20	r = 0.00	r = -0.63	r = 0.6	r = 0.32	/	r = 0.2
	p = 0.20	*p* = 0.41	*p* = 0.00	*p* = 0.70	*p* = 1.00	*p* = 0.18	*p* = 0.21	*p* = 0.54		*p* = 0.70
SOD-M	r = 0.46	r = -0.72	r = 0.2	r = 0.40	r = 0.77	r = -0.63	r = 0.40	r = -0.79	r = 0.2	/
	p = 0.36	*p* = 0.10	*p* = 0.70	*p* = 0.43	*p* = 0.07	*p* = 0.18	*p* = 0.43	*p* = 0.06	*p* = 0.70	

C – control goup; M – methionine trated group.
CAT – catalase; GPx – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase.
Pearson correlation coefficient (r): low or no correlation $0 \leq r \leq 0.3$; moderate correlation $0.3 \leq r \leq 0.7$; strong correlation $0.7 \leq r < 1$ (- indicates negative correlation); *p* value less than 0.05 was considered as significant (statistically significant differences are bolded).

Table 2
Correlation matrix between homocysteine (Hcy) and parameters of oxidative stress in subchronically methionine treated rats

Parameters	CAT-C3	CAT-M3	GPx-C3	GPx-M3	MDA-C3	MDA-M3	SOD-C3	SOD-M3
Hcy-C	r = 0.72	r = 0.36	**r = 0.84**	r = 0.49	**r = 0.54**	r = 0.34	r = -0.36	r = 0.00
	p=0.10	*p* = 0.48	***p* = 0.04**	*p* = 0.32	**p=0.04**	*p* = 0.51	*p* = 0.51	*p* = 1.00
Hcy-M	**r = -0.82**	r = -0.65	r = -0.65	r = 0.58	r = -0.65	**r = -0.93**	r = 0.65	r = 0.16
	***p* = 0.047**	*p* = 0.16	*p* = 0.16	*p* = 0.22	*p* = 0.16	***p* = 0.008**	*p* = 0.16	*p* = 0.76
CAT-C3	/	r = 0.25	r = -0.75	r = -0.19	r = 0.75	r = 0.71	r = 0.25	r = 0.00
		p = 0.63	*p* = 0.09	*p* = 0.72	*p* = 0.09	*p* = 0.12	*p* = 0.63	*p* = 1.00
CAT-M3	r = 0.25	/	r = 0.00	**r = -0.96**	r = 0.00	r = 0.71	**r = 1.00**	r = 0.25
	p = 0.63		*p* = 1.00	***p* = 0.003**	*p* = 1.00	*p* = 0.12	***p* = 0.000**	*p* = 0.63
GPx-C3	r = -0.75	r = 0.00	/	r = 0.19	r = **1.00**	r = 0.71	r = 0.00	r = -0.25
	p = 0.09	*p* = 1.00		*p* = 0.72	***p* = 0.000**	*p* = 0.12	*p* = 1.00	*p* = 0.63
GPx-M3	r = -0.19	**r = -0.96**	r = 0.19	/	r = -0.19	r = -0.54	r = **-0.96**	r = -0.19
	p = 0.72	***p* = 0.003**	*p* = 0.72		*p* = 0.72	*p* = 0.27	***p* = 0.003**	*p* = 0.72
MDA-C3	r = -0.75	r = 0.00	**r = 1.00**	r = 0.19	/	r = 0.71	r = 0.00	r = -0.25
	p = 0.09	*p* = 1.00	***p* = 0.000**	*p* = 0.72		*p* = 0.12	*p* = 1.00	*p* = 0.63
MDA-M3	r = -0.71	r = -0.71	r = -0.71	r = -0.54	r = -0.71	/	r = -0.71	r = 0.00
	p = 0.12	*p* = 0.12	*p* = 0.12	*p* = 0.27	*p* = 0.12		*p* = 0.12	*p* = 1.00
SOD-C3	r = -0.25	**r = 1.00**	r = 0.00	**r = -0.96**	r = 0.00	r = -0.71	/	r = 0.25
	p = 0.63	***p* = 0.000**	*p* = 1.00	***p* = 0.003**	*p* = 1.00	*p* = 0.12		*p* = 0.63
SOD-M3	r = 0.00	r = 0.25	r = -0.25	r = -0.19	r = -0.25	r = 0.00	r = 0.25	/
	p = 1.00	*p* = 0.63	*p* = 0.63	*p* = 0.72	*p* = 0.63	*p* = 1.00	*p* = 0.63	

C – control group and M – methionine treated group in the acute experiment; C3 – control group and M3 – methionine treated group in the subchronic experiment.
CAT – catalase; GPx – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase.
Pearson correlation coefficient (r): Low or no correlation $0 \leq r \leq 0.3$; moderate correlation $0.3 \leq r \leq 0.7$; strong correlation $0.7 \leq r < 1$ (- indicates negative correlation); *p* – value less than 0.05 was considered as significant (statistically significant differences are bolded).

Table 3
Correlation matrix for parameters of oxidative stress between acutely and subchonically methionine treated rats

Parameters	CAT-C3	CAT-M3	GPx-C3	GPx-M3	MDA-C3	MDA-M3	SOD-C3	SOD-M3
CAT-C	r = 0.00	**r = 0.95**	r = -0.32	**r = -0.97**	r = 0.32	r = 0.45	**r = 0.95**	r = 0.32
	p = 1.00	***p* = 0.004**	*p* = 0.54	***p* = 0.002**	*p* = 0.54	*p* = 0.37	***p* = 0.004**	*p* = 0.54
CAT-M	**r = 0.95**	r = 0.00	r = -0.63	r = 0.00	r = -0.63	r = 0.45	r = 0.00	r = 0.00
	***p* = 0.004**	*p* = 1.00	*p* = 0.18	*p* = 1.00	*p* = 0.18	*p* = 0.37	*p* = 1.00	*p* = 1.00
GPx-C	r = 0.15	r = 0.15	r = 0.46	r = 0.10	r = 0.46	r = 0.43	r = 0.15	r = 0.15
	p = 0.74	*p* = 0.74	*p* = 0.36	*p* = 0.86	*p* = 0.36	*p* = 0.40	*p* = 0.77	*p* = 0.77
GPx-M	r = 0.00	r = -0.75	r = 0.25	r = 0.57	r = 0.25	r = -0.71	r = -0.75	r = 0.00
	p = 1.00	*p* = 0.09	*p* = 0.63	*p* = 0.23	*p* = 0.63	*p* = 0.12	*p* = 0.09	*p* = 1.00
MDA-C	r = -0.32	r = 0.63	r = 0.00	r = -0.48	r = 0.00	r = 0.45	r = 0.63	r = 0.00
	p = 0.54	*p* = 0.18	*p* = 1.00	*p* = 0.33	*p* = 1.00	*p* = 0.37	*p* = 0.18	*p* = 1.00
MDA-M	r = -0.75	r = 0.00	r = -1,000	r = -0.19	r = -1,000	r = -0.71	r = 0.00	r = 0.25
	p = 0.09	*p* = 1.00	*p* = 0.00	*p* = 0.72	*p* = 0.00	*p* = 0.12	*p* = 1.00	*p* = 0.63
SOD-C	r = 0.00	**r = 0.95**	r = -0.32	r = -0.97	r = -0.32	r = 0.45	**r = 0.95**	r = 0.32
	p = 1.00	***p* = 0.004**	*p* = 0.54	*p* = 0.002	*p* = 0.54	*p* = 0.37	***p* = 0.004**	*p* = 0.54
SOD-M	r = 0.63	r = 0.47	r = 0.79	r = -0.24	r = 0.79	r = 0.89	r = 0.47	r = 0.00
	p = 0.18	*p* = 0.34	*p* = 0.06	*p* = 0.64	*p* = 0.06	*p* = 0.02	*p* = 0.34	*p* = 1.00

C – control group and M – methionine treated group in the acute experiment; C3 – control group and M3 – methionine treated group in the subchronic experiment.
CAT – catalase; GPx – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase.
Pearson correlation coefficient (r): Low or no correlation $0 \leq r \leq 0.3$; moderate correlation $0.3 \leq r \leq 0.7$; strong correlation $0.7 \leq r < 1$ (- indicates negative correlation); *p* – value less than 0.05 was considered as significant (statistically significant differences are bolded).

Discussion

Present investigation aimed to assess the influence of acute and subchronic Met treatment on plasma oxidative stress markers and AChE activity in rat cardiac tissue.

Absence of increase in Hcy values in the group subchronically treated with Met may be consequence of duration of methionine loading and/or applied dose. In our previous study we showed that rats treated for 4 weeks with diets enriched in methionine (with or without deficiency in B vitamins) had increased Hcy levels especially in conditions of deficit in vitamin B complex [32]. Furthermore, some human studies pointed out that methionine overload can increase Hcy levels in only 33% of cases [33]. In that sense it is possible that time of exposure to methionine and its concentration were insufficient to cause elevation in Hcy levels in the present study.

In this study it was found that acutely applied Met increased activities of CAT and GPx, whereas did not significantly altered activity of SOD and MDA level in the rat plasma after 1 h.

The liver is particularly sensitive to prolonged administration of Met, and some authors claim that after chronic application, level of MDA in the liver is increased, which could be due to increased levels of iron [34, 35]. However, other experiments have shown that after 1 h MDA in the liver is significantly decreased [36]. The same authors have shown that after 1 h, CAT activity in the liver is increased *in vitro* and decreased *in vivo*, which means that there is a possibility that CAT is released from the liver and its activity could be increased in plasma. Previously may explain the increase in CAT activity in the plasma observed in our study. We obtained that SOD activity in the plasma was unchanged. This result is in agreement with the results of Costa et al. [36]. Some authors suggest that 2–3 h after the application of Met concentration of MDA in plasma is not changed and that is noticed only after 8 h, which corresponds to a maximum concentration of Hcy [37].

A lot of research has been done in order to investigate the origin and mechanism of Hcy toxicity, as well as the connection between Met and Hcy on one side and oxidative stress and vascular diseases on the other side.

In one study it is shown that Met has a protective effect in atherosclerotic lesions by increasing the activity of antioxidant enzymes in the heart up to 24 h from the application and then this effect begin to decrease [38]. It is also shown that Met increases GPx activity at the level of mRNA, while the activities of CAT and SOD are regulated by post-transcriptional or post-translational modification. GPx and CAT were significantly increased, and SOD was unchanged, which is consistent with our results. In that study, it was demonstrated that a significant reduction of MDA in the heart coincides with a maximum of GPx activity after 24 h.[38] In another study it was shown that chronic application of Met caused an increase of MDA level and GPx activity in the heart as a response to increased level of ROS (due to elevated Hcy), not as a direct effect of Met [39].

Tests conducted on the rat hippocampus showed no change in the level of MDA 1 h after giving Met [40], which is in accordance with the obtained values in the plasma in our study. On the other hand, the same study indicates an increase in MDA in hippocampus after 3 h [40], which may be the result of increased concentrations of iron, lipid content and low activity of antioxidants in basal conditions [41]. Other autors demonstrated that acutely given Met increased SOD and GPx activity 1h after application, but decreased CAT activity in the rat brain cortex [42]. In another experiment SOD activity in the rat brain remained unchanged, which is consistent with the results obtained in the heart in our study.

No data were found in the literature about the impact of Met on the AChE activity in the heart tissue. However, our recent published study investigated the effects of Hcy or Hcy-thiolactone on the plasma oxidative stress and AChE activity in the rat cardiac tissue 1 h after application [24]. Statistically significant reduction of the AChE activity was found, and the same was observed in this study after subchronic Met overload, contrary to the acute treatment that did not give any change. Although stress-induced increased sympathetic action is inevitable and can cause a weaker function of AChE, there is a significant activity of the enzyme in most of the sympathetic ganglions in the rat (including, among others, the heart). On this way, sympathetic preoccupation may also be associated with an increased activity of AChE [43].

Moreover, it is known that excess of AchE has protective effects in the heart only in conditions related to the oxidative stress such as inflammation, hypoxemia, ischemia [44–46]. Reduction of AChE in these cases could be a compensatory mechanism. Taking into consideration that subchronically given Met did not induce oxidative stress, but can induce increase of CRP level [47], we were not able to assume that reduction of the enzyme activity was a direct effect of Met. However, Met could also provoke oxidative stress in the heart *via* high level of AChE. Because of that, further studies are needed to differentiate whether this is consequence of elevated Met, Hcy or some other products levels obtained from Met cycle after 3 weeks. Besides, some authors claimed that maximum concentration of Met in the plasma was achieved after 15 min of application [4], and it would be also preferable to examine such effects.

Conclusion

The present study showed that acutely applied Met has some beneficial effects; it protects from oxidative stress, through increasing activities of two antioxidant enzymes in the plasma – CAT and GPx. Nevertheless, after 3 weeks of the treatment these changes seems to be lost. On the other hand, given in both manners, acutely and subchronically, Met did not influence on lipid peroxidation process.

Acute Met overload was not sufficient to influence on activity of AchE in the rat heart, while longer duration of Met loading diminished function of the enzyme. These findings point out that Met can interfere with antioxidant defense system and cholinergic control of the heart function. More detailed examinations are needed to determine the effects of this amino acid and its possible therapeutic options.

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Conflict of interest

None.

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Twin pregnancies conceived by assisted reproduction – Early prediction of preterm birth

Blizanačke trudnoće nastale tehnikom asistirane reprodukcije – rano predviđanje prevremenog porođaja

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Abstract

Background/Aim. Twins conceived by assisted reproduction techniques (ART) are the most susceptible for perinatal complications. The aim of this study was to examine the role of prenatal noninvasive fetal screening of the first and second trimester in prediction of delivery time of ART conceived twins. **Methods.** Prospective cohort study of all ART conceived twin pregnancies was conducted at the Clinic for Obstetrics and Gynecology, Clinical Center of Serbia, during the period from January 1, 2016 to December 31, 2017. In the 12th gestational week (GW) twins crown-rump lenght (CRL) and thickness nuchal translucency (NT) were measured ultrasonographically. Moreover, serum levels of beta subunit of human chorionic gonadotropin (β hCG) and pregnancy-associated plasma protein A (PAPPA) were assessed. In the 17th GW twins biparietal diameter (BPD), abdominal circumference (AC) and femur length (FL) were determined ultrasonografically. Additionally, β hCG, alpha-fetoprotein (AFP), estriol (E3) and inhibin (INH) were measured in mothers serum. The GW of delivery was noted for each pregnancy. **Results.** Study included 100 pregnant women with mean age 35.44 ± 5.82. In the examined sample of ART conceived twins significantly more (51%) were delivered in term (≥ 35 GW) ($p = 0.001$). Delivery time correlated negatively with NT and first trimester β hCG serum levels, while it correlated positively with FL of the smaller twin, second trimester β hCG, AFP and E3 concentrations. According to obtained model for prediction of delivery time in ART conceived twin pregnancies based on first trimester diagnostic tests the significant predictors were PAPPA and β hCG in the 12th GW as well as NT of the first larger twin. Nevertheless, reliability (sensitivity 50%–75%, specificity 30%–40%) of these diagnostic tests was moderate. **Conclusion.** Prenatal noninvasive fetal screening of the first and second trimester (ultrasonography and laboratory testing) can be used for prediction of delivery time of ART conceived twins.

Key words:
premature births; prognosis; reproductive techniques, assisted; twins; ultrasonography.

Apstrakt

Uvod/Cilj. Blizanci nastali tehnikama asistirane reprodukcije (ART) su najosetljiviji na pojavu perinatalnih komplikacija. Cilj rada bio je da se ispita uloga prenatalnog neinvazivnog fetalnog skrininga prvog i drugog trimestra u predviđanju vremena porođaja blizanaca nastalih metodama ART. **Metode.** Prospektivna kohortna studija kojom su obuhvaćene sve blizanačke trudnoće nastale ART metodama sprovedena je na Klinici za ginekologiju i akušerstvo Kliničkog centra Srbije u periodu od 01.01.2016. do 31.12.2017. U 12. gestacionoj nedelji (GN) ultrasonografski su blizancima izmereni razmak teme-trtica (CRL) i nuhalno zadebljanje (NT). Takođe, određeni su serumski nivoi beta podjedinice humanog horionskog gonadotropina (β hCG) i plazma proteina A povezanog sa trudnoćom (PAPPA). U 17. GN ultrasonografski su blizancima izmereni biparietalni dijametar (BPD), obim abdomena (AC) i dužine femura (FL). Pored toga, β hCG, alfa fetoprotein (AFP), estriol (E3) i inhibin (INH) su izmereni u serumu majki. Za svaku trudnoću zabeležena je GN porođaja. **Rezultati.** Studija je obuhvatila 100 trudnica koje su u proseku imale 35,44 ± 5,82 godina života. U ispitivanom uzorku blizanaca, nastalih tehnikama ART, značajno više (51%) je bilo porođeno u terminu (≥ 35 GN) ($p = 0,001$). Vreme porođaja je negativno korelisalo sa NT i nivoima serumskog β hCG u prvom trimestru, dok je pozitivno korelisalo sa FL manjeg blizanca, kao i koncentracijama β hCG, AFP i E3 u drugom trimestru. Prema dobijenom modelu za predviđanje vremena porođaja blizanaca nastalih tehnikom ART, na osnovu dijagnostičkih testova prvog i drugog trimestra, značajni prediktori bili su PAPPA i β hCG u 12. GN, kao i NT prvog većeg blizanca. Ipak, pouzdanost (senzitivnost 50%–75%, specifičnost 30%–40%) tih dijagnostičkih testova bila je umerena. **Zaključak.**

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Prenatalni neinvazivni fetalni skrining prvog i drugog trimestra (ultrasonografija i laboratorijsko testiranje) može se koristiti za predviđanje vremena porođaja blizanaca nastalih metodama ART.

Ključne reči:
porođaj, prevremeni; prognoza; reprodukcija, asistirana, tehnike; blizanci; ultrasonografija.

Introduction

Application of assisted reproduction technologies (ART) as infertility treatment, especially in-vitro fertilisation (IVF), intracytoplasmic sperm injection (ICSI) and oocyte donation (DO), has lead to increase in the number of twin pregnancies 1, 2.

Despite advances in obstetrical and neonatal care, it is well kown that twin pregnancies still carry a high risk for numerous gestational complications and adverse pregnancy outcomes [1–3]. Twin pregnancies are associated with low birth weight of fetuses, caesarean section, prematurity and associated perinatal morbidity and mortality. Literature data show that in twin pregnancies there is also an increased risk of gestational hypertensive disorders of the mother [2, 3]. Nevertheless, there are conflicting reports on whether conception by ART further increases these risks when compared to spontaneously conceived pregnancies [4, 5]. Pregnancies concieved by ART are found to be more often complicated with preterm delivery and fetal low birth weigh, placental abruption, gestational diabetes and hypertension [2–4]. Consequently, ART conceived twins are the most susceptible for various perinatal complications among which the most severe is premature pregnancy ending [6].

One of the most important issues in perinatology is early and noninvasive prediction of adverse pregnacy outcomes [1, 6]. Therefore, the aim of this study was to examine the role of prenatal noninvasive fetal screening of the first and second trimester in prediction of delivery time of ART conceived twins.

Methods

A prospective cohort study of all pregnant women with ART conceived twin pregnancies was conducted at the Clinic for Gynecology and Obstetrics, Clinical Center of Serbia in Belgrade, during the period from January 1st 2016, to December 31, 2017.

Exclusion criteria for this study were miscarriage before 8th gestational week (GW), genetic disorders of twins, monochorionicity and monoamnionicity of twins and existence of chronic ilnesses in mothers that could influence the pregnancy course and outcome. The study was approved by the Institutional Review Board. All patients signed a written informed consent form.

Already during the first examination for pregnancy confirmation, a complete history regarding mothers age, chronic ilnesses, previous parity and ART type (IVF, ICS, DO) was taken from each patient. Investigated women were regularly checked-up at least once per trimester in our Clinic.

In the first trimester, during the 12th gestational week, for each twin, the crown-rump lenght (CRL) and the thickness of nuchal translucency (NT) were measured and chorionicity determined ultrasonographically. Moreover, biochemical markers that are incorporated in Double test screening such as beta subunit of human chronic gonadotropin (β hCG) and pregnancy-associated plasma protein A (PAPPA) were assessed. In the 17th GW, ultrasound examination for both twins determined their biometric parameters such as biparietal diameter (BPD), abdominal circumference (AC) and femur length (FL). Additionally, in the second trimester we evaluated markers used for Triple testing i.e. β hCG, alpha fetoprotein (AFP), estriol (E3) and inhibin (INH).

Monitoring of twins was performed by ultrasound examinations. Ultrasound biometry and monitoring were performed through ACCUVIX device (Samsung Medison, Seoul, Sought Korea), with 3.75 MHz abdominal and vaginal probe.

For laboratory diagnostic tests 5 to 10 mL of blood was drawn by venipuncture into nonheparinized tubes and centrifuged for 15 min. After serum separation concentrations of investigated biochemical markers were measured using BRAHMS KRYPTOR analyzer and applying fluorocytometric immunoassay with SsdwLab 5 software. The measured serum concentrations (IU/L) of biomarkers were then converted into multiples of median (MoM) and adjusted for GW for easier comparison and analysis. The standard referral range of all tested pregnancy markers, most widely used in literature and adopted in this study as well, was from 0.5 to 2 MoM.

All complications of pregnancy were registered regularly throughout pregnancy. The GW in which delivery occurred as well as the delivary type [vaginal or cesarean section (SC)] were noted for each pregnancy. Time of delivery was classified as miscarriage (before 12 GW), early preterm (before 25th GW), late preterm (before 35 GW) and term delivery (in and after 35th GW).

Upon delivery, all data collected throughout pregnancy were correlated with delivery time and analyzed by methods of descriptive statistics (number, percent, mean, standard deviation) and analytical statistics using the SPSS 20 software. Correlations of biochemical parameters and occurrence of hypertension were tested using Spearman's correlation analysis. Significance of differences between categories of assessed parameters was examined by χ^2 test.

Enter method of binary logistic regression was applied to construct models for prediction of delivery time of ART conceived twins based on investigated ultrasonographic and biochemical markers of the first and second pregnancy trimester. All models were adjusted for potential confoundings (mothers' age, and parity).

Finaly, study authors performed the Receiver Operating Characteristics (ROC) analysis to set the cut-off values of ul-

trasonographic and biochemical parameters of the first and second trimester that could imply the delivery time in ART conceived twin pregnancies.

Results

Study included 100 pregnat women with mean age 35.44 ± 5.82 (range 21 to 45). Eighty eight percent of investigated women did not have previous deliveries, while 29% of them had previous miscarriages and/or abortions. Significantly more (59%) women had no gestational illnesses and pregnancy complications ($p = 0.001$). The most common pregnancy complication was gestational hypertension (21% of mothers). In our sample there were no significant differences in frequency of different ART types (53% had only IVF, while 42% had IVF/ICSI; $p = 0.259$). Still, only five women had DO.

In the examined sample of ART conceived twins significantly more (51%) were delivered in term (after 35 GW) ($p = 0.001$). We registered 8 cases of miscarriage before 12th GW, 8 early preterm births before 24th GW, while 33 twins were born between 24th and 33th GW ($p = 0.001$). Majority of twins were liveborn (86% of first larger and 87% of second smaller twins). Delivery was mostly by SC ($p = 0.001$).

Diagnostic parameters (ultrasonografy and laboratory) of the first and second pregnancy trimester are shown in Table 1. Majority of twins had all values in the refferal range for GW.

Correlations of delivery time with general patients data and the first trimester diagnostic testing are presented in Table 2, while correlations with the second trimester ultrasound and laboratory findings are presented in Table 3.

Delivery time correlated positively with mothers age and negatively with previous parity. Out of all assessed ultrasonographic fetal measures in the first trimester only the thickness of NT for both twins was negatively correlated with the delivery time. Moreover, FL of the smaller second twin measured ultrasonographically in the second trimester correlated positively with the delivery time, but was not correlated with the exact GW of delivery.

When laboratory analyses were assessed it was determined that β hCG serum levels in the first trimester correlated positively with gestational week of delivery. Contrary, β hCG as well as AFP concentrations in the second trimester correlated negatively with the exact GW of delivery. Serum levels of E3 in the second trimester correlated positively with term delivery.

We obtained a significant model for prediction of delivery time in ART conceived twin pregnancies based on the first trimester diagnostic tests – ultrasound and laboratory (B = 0.041; Wald = 0.140; Nagelkerke $R^2 = 0.628$; $\chi^2 = 38.664$; $p = 0.001$; explained variance = 73.0%). The significant predictors were PAPPA and β hCG in 12th GW as well as NT of the first larger twin. Mothers age and previous parity were confoundings in this model.

ART twins term delivery = 3.054 – 1.142 × PAPPA 12 GW– 1.263 × β hCG 12 GW – 0.940 × NT gemellus I – 2.128 x previous parity + 0.104 x mothers age

Table 1
Ultrasound and laboratory diagnostic findings of first and second trimester

Parameters	Min–Max	Mean ± SD
I trimester-12 gestational weeks		
CRL twin I	17.00–87.00	58.51 ± 11.21
NT twin I	1.40–3.70	2.21 ± 0.54
CRL twin II	16.00–85.90	60.69 ± 11.88
NT twin II	1.40–3.70	2.21 ± 0.52
PAPPA	0.40–4.10	1.33 ± 0.72
Beta-hCG	0.65–2.56	1.21 ± 0.57
II trimester-17 gestational weeks		
AC twin I	112.00–126.00	117.46 ± 4.72
AC twin II	112.00–126.00	117.17 ± 4.42
BPD twin I	31.00–39.00	34.83 ± 2.67
BPD twin II	31.00–39.00	34.84 ± 2.60
FL twin I	2.00–29.00	20.73 ± 7.64
FL twin II	2.00–29.00	20.77 ± 7.64
Beta-hCG	0.51–2.80	1.30 ± 0.64
Estriol	0.51–2.80	1.31 ± 0.69
Alpha fetoprotein	0.51–2.80	1.32 ± 0.70
Inhibin	0.51–2.80	1.22 ± 0.66

CRL – crown-rump lenght; NT – nuchal translucence; HCG – human chorionic gonadotropin; AC – abdominal circumference; BPD – biparietal diameter; FL – femur lenght; PAPPA – pregnancy-associated plasma protein A; min – minimum; max –maximum; SD – standard deviation.

Table 2

Correlation of delivery time with general patients data and first trimester diagnostic testing

Parameters	Term delivery	Delivery time		Gestational week (GW)	
		twin I	twin II	twin I	twin II
Mothers age					
Ro	**0.208**	**0.273**	**0.273**	**0.280**	**0.279**
p	**0.038**	**0.006**	**0.006**	**0.005**	**0.005**
Parity					
Ro	**-0.256**	**-0.348**	**-0.348**	**-0.355**	**-0.355**
p	**0.010**	**0.001**	**0.001**	**0.001**	**0.001**
ART type					
Ro	0.009	-0.084	-0.084	-0.131	-0.132
p	0.926	0.408	0.408	0.194	0.191
CRL twin I					
Ro	-0.031	0.020	0.020	-0.022	-0.024
p	0.760	0.840	0.840	0.827	0.816
NT twin I					
Ro	**-0.288**	**-0.354**	**-0.354**	**-0.373**	**-0.373**
p	**0.004**	**0.001**	**0.001**	**0.001**	**0.001**
CRL twin I					
Ro	-0.106	-0.056	-0.056	-0.054	-0.054
p	0.294	0.580	0.580	0.590	0.592
NT twin II					
Ro	**-0.218**	**-0.248**	**-0.248**	**-0.230**	**-0.231**
p	**0.047**	**0.013**	**0.013**	**0.021**	**0.021**
PAPPA					
Ro	0.041	0.023	0.023	-0.002	-0.003
p	0.683	0.823	0.823	0.982	0.977
Beta-hCG					
Ro	**0.253**	**0.264**	**0.264**	**0.234**	**0.235**
p	**0.011**	**0.008**	**0.008**	**0.019**	**0.019**

ART – assisted reproduction technique; term delivery (yes/no) – before or after 35 GWs; delivery time – before 12 GWs, from 12 to 25, from 25 to 35, 35 and more GWs; CRL – crown-rump lenght; NT – nuchal translucence; HCG – human chorionic gonadotropin; PAPPA – pregnancy associated plazma protein A; ART type – *in vitro* fetrilisation (IVF), intracytoplasmic sperm injection (ICSI), oocyte donation (DO); Ro – coefficient of correlation.
Nore: Statisticaly significant values are bolded.

Table 3

Correlation of delivery time with second trimester diagnostic testing

Parameters	Term delivery	Delivery time		Gestational week (GW)	
		twin I	twin II	twin II	twin II
AC twin I					
Ro	0.027	0.045	0.045	-0.002	-0.002
p	0.798	0.673	0.673	0.982	0.986
AC twin II					
Ro	0.002	-0.023	-0.023	0.040	0.040
p	0.997	0.826	0.826	0.706	0.706
BPD twin I					
Ro	0.003	-0.014	-0.014	-0.036	-0.035
p	0.981	0.898	0.898	0.735	0.743
BPD twin II					
Ro	0.117	0.116	0.116	0.027	0.026
p	0.266	0.269	0.269	0.798	0.803
FL twin I					
Ro	0.134	0.153	0.153	0.081	0.080
p	0.202	0.145	0.145	0.444	0.447

Table 3 – *continued*

Parameters	Term delivery	Delivery time		Gestational week (GW)	
		twin I	twin II	twin II	twin II
FL twin II					
Ro	**0.219**	**0.222**	**0.222**	0.121	0.121
p	**0.036**	**0.033**	**0.033**	0.249	0.252
Beta HCG					
Ro	-0.135	-0.194	-0.194	**-0.221**	**-0.220**
p	0.200	0.064	0.064	**0.035**	**0.035**
Estriol (E3)					
Ro	**0.215**	0.184	0.184	0.099	0.098
p	**0.039**	0.079	0.079	0.349	0.354
Alpha feto protein (AFP)					
Ro	-0.166	-0.201	-0.201	**-0.245**	**-0.246**
p	0.114	0.055	0.055	**0.019**	**0.018**
Inhibin					
Ro	0.077	0.066	0.066	0.101	0.101
p	0.467	0.531	0.531	0.337	0.338

Term delivery (yes/no) – before or after 35 GWs; delivery time – before 12 GWs, from 12 to 25, from 25 to 35, 35 and more GWs; AC – abdominal circumference; BPD – biparietal diameter; FL – femur lenght; HCG – human chorionic gonadotropin.
Ro – coefficient of correlation.
Nore: Statisticaly significant values are bolded.

Table 4
ROC analysis of potential predictors for assisted reproduction technique (ART) conceived twins delivery time

Parameters	Area under the curve	*p*	Cut-off value	Sensitivity (%)	Specificity (%)
CRL twin I	0.440	0.322	56.50	53.00	32.70
NT twin I	0.381	**0.051**	1.95	53.00	30.30
CRL twin II	0.378	**0.045**	57.50	60.80	32.80
NT twin II	0.433	0.268	1.86	60.80	30.00
AC twin I	0.516	0.798	115.00	62.70	45.00
AC twin II	0.500	0.997	115.00	64.70	41.50
BPD twin I	0.501	0.981	33.50	68.60	44.30
BPD twin II	0.576	0.210	22.00	63.00	42.50
FL twin I	0.567	0.270	33.50	70.60	45.10
FL twin II	0.626	**0.039**	22.00	68.60	46.30
PAPPA 12 GW	0.546	0.446	0.81	74.50	40.10
Beta-hCG 12 GW	0.630	**0.033**	0.81	76.50	41.50
Beta-hCG 17 GW	0.503	0.956	0.82	64.70	31.70
Estriol (E3) 17 GW	0.491	0.884	0.85	55.00	30.20
AFP 17 GW	0.404	0.114	0.87	54.90	41.50
Inhibin 17 GW	0.544	0.465	0.80	64.70	40.90

CRL – crown-rump lenght; NT – nuchal translucence; HCG – human chorionic gonadotropin; AC – abdominal circumference; BPD – biparietal diameter; FL – femur lenght; HCG – human chorionic gonadotropin; PAPPA – pregnancy associated plazma protein A; AFP – alpha fetoprotein; GW – gestational week.
Note: Statistically significant values are bolded.

We did not manage to obtain the significant binary logistic regression for prediction of delivery time in ART conceived twin pregnancies based on the second trimester diagnostic tests ($\chi^2 = 22.355$; $p = 0.217$).

ROC analysis of ultrasonografic diagnostic tests is presented on Figure 1 while on the Figure 2 we presented laboratory tests of the first and second trimester. Newly established cut-off values with their sensitivity and specificity for potential ultrasonographic and laboratory predictors of twins delivery time are presented in Table 4. The significant predictors were β hCG in 12th GW, NT of the first larger twin in 12th GW as well as CRL in 12th GW and FL in 17th GW of the second smaller twin.

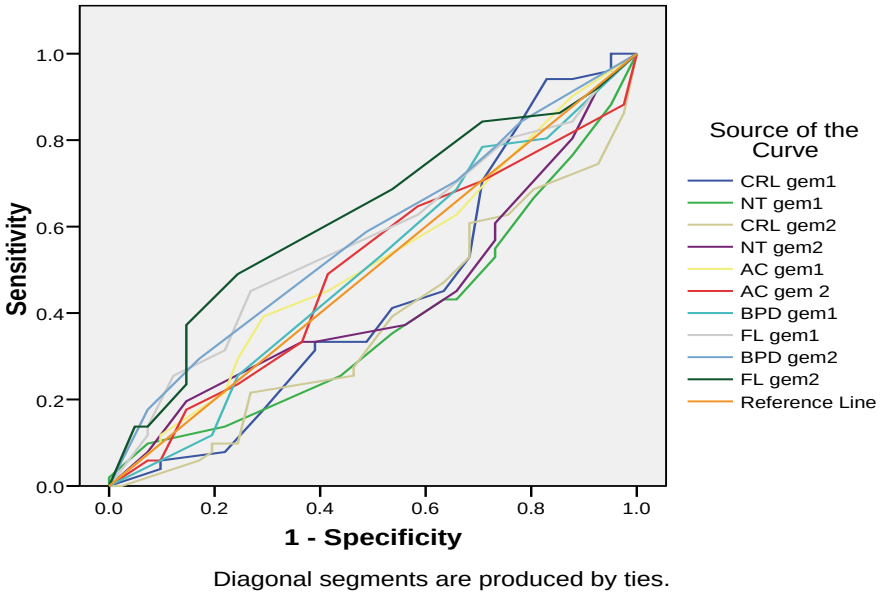

Fig. 1 – Ultrasonographic predictors for assisted reproduction technique (ART) conceived twins delivery time.
CRL – crown-rump length; NT – nuchal translucence; AC – abdominal circumference; BDP – biparietal diameter; FL – femur length.

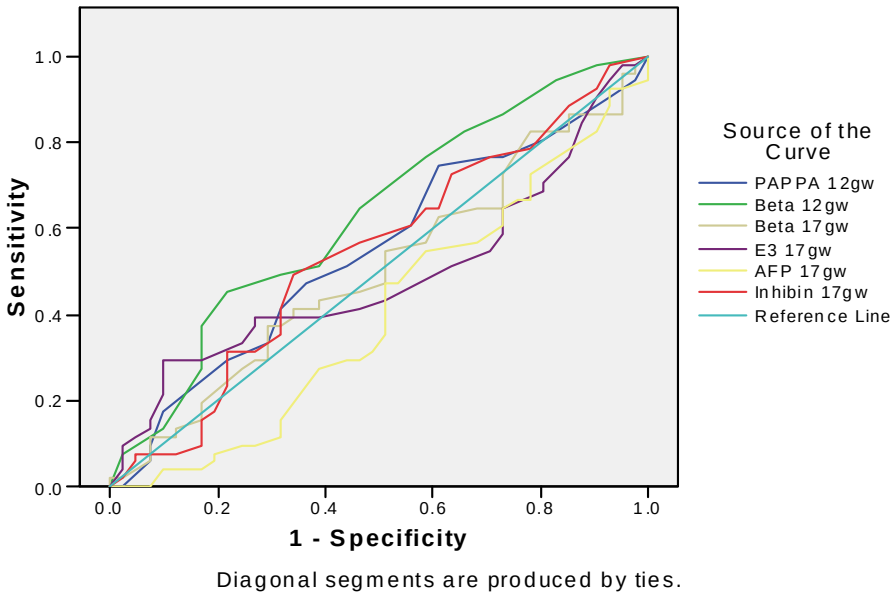

Fig. 2 – Laboratory predictors for assisted reproduction technique (ART) conceived twins delivery time.
PAPPA – pregnancy-associated plasma protein A; GW – gestational week; E_3 – estriol; AFP – alpha fetoprotein.

Discussion

Twin pregnancies, in general, are at higher risk of numerous pregnancy complications including miscarriage or preterm birth caused by a development of two fetuses instead of one [1, 2]. Adverse obstetric outcome after ART might be caused by the very procedure [4]. Moreover, ART conceived twins can be at even higher risk of pregnancy complications and adverse obstetric outcomes due to maternal age, pre-existing maternal medical conditions such as polycystic ovary syndrome or thyroid diseases that are associated with miscarriages and aneuploidies [7–9].

Studies show that the mean gestational age at delivery was significantly lower in the ART group compared with the spontaneous conception group regardless of the cerclage application [3]. ART conceived twins are at significantly increased risks of preterm birth both early and late, althouhg according to majority of studes ART twins were mostly de-

livered moderately preterm (32–34 GW) [2, 10]. However, the rate of preterm birth of ART conceived twins in several investigations was similar to that of spontaneously conceived ones or that very preterm deliveries even occurred more frequently in spontaneous twin pregnancies [4]. In our study, similarly, more women delivered at term than preterm (51% vs. 49%, respectively), while late preterm birth was predominant time of delivery of preterm twins (33%). Moreover, although somewhat unexpectedly, older mothers delivered at term more often. A possible explanation could be that older women who had ART as infertility treatment are more concerened for the pregnancy sucess and therefore comply better to their therapy and have more regular check-ups that could have prevented preterm labor.

Consequently, ART twin pregnancies require additional antenatal care [7]. Potential early prediction of adverse outcomes could enable adequate and timely management of pregnancy complications allowing preventing premature pregnancy ending [11]. Prenatal noninvasive fetal screening in patients with ART conceived twin pregnancies is essential part of appropriate control of these high-risk pregnancies. It is performed at the time of standard fetal screening for chromosomal anomalies (Double, Triple or Q test) by ultrasound examination of both fetuses and the analysis of mothers biomarkers (hormones) serum levels has been established during the first (11 to 14 GWs) and/or second (16 to 18 gestational weeks) pregnancy trimester. Literature data show that regular and thorough check-ups of twins can show, apart from potential risk of aneuploidies, also the risk of adverse pregnancy outcomes [11].

Data from current literature are still contradictory regarding the strength of association between pregnancy biochemical markers and twin pregnancy outcomes [12, 13]. Biochemical markers are associated with a variety of external factors, such as patient's and partner's age, demographic data, course and outcome of previous pregnancies, especially premature births, spontaneous abortion, perinatal morbidity and mortality, type of performed ART (IVF, ICSI, DO), number of previous unsuccessful ART attempts, the course of current pregnancy and the presence of symptoms that indicate imminent abortion or premature delivery [12, 13]. There is still an on going debate how could serum biomarkers be used in the prediction of pregnancy course and outcome. It has been found that in the case of twin pregnancies the values of biomarkers depend on the horionicity as well as the gestational age [6]. Studies that evaluated serum β hCG levels for prediction of twins preterm birth found that reliability and predictive performance of high β hCG levels were low [11, 14]. Similar findings were reported for PAPPA serum levels (senisitivity mostly below 50%). Nevertheless, specificity of PAPPA as well as the first and second trimester β hCG serum concentrations were significantly higher reaching above 90% in some cases for prediction of twins premature birth [11, 14]. Although several studies found a negative correlation between elevated maternal serum AFP levels and twins gestational age at birth, predictive ability of elevated maternal AFP levels was poor (sensitivity of 30%, specificity of 88%) [11, 14].

The role of ultrasound in control of ART conceived twin pregnancies is essential as it for diagnosing chorionicity and placentation, discordinant growth, twin-to-twin transfusion syndrome [15]. Both early the first and second trimester sonographic examinations are investigated in recent studies for predicting adverse perinatal outcomes [16]. It is postulated that fetuses suffering from reduced oxygenation and/or nutrient limitation caused by progressed pregnancy complications during early pregnancy tend to be smaller when their biometrical measurements are evaluated [17, 18]. Several studies have examined the relationship between the parameters obtained during ultrasonographic surveillance of twins with the course, delivery time and type as well as the outcome of both dichorionic and monochorionic twin pregnancies [5, 19]. According to literature data significant correlations of parameters measured and determined during the ultrasound screening such as intertwin differences in CRL and NT, chorionicity, amniotic fluid quantity, AC of both fetuses in the second trimester with the pregnancy outcome and numerous gestational complications are commonly found [15, 18]. According to some investigations abnormal measurements of CRL in the first trimester were found to be associated with early fetal loss [10, 20]. A meta-analysis showed that twin pregnancies with CRL discordance $\geq 10\%$ were at significantly higher risk of fetal loss and premature delivery after 24th until 34th GW, but not before 24 weeks of gestation [21]. Only few studies have assessed NT in predicting preterm birth in twin gestations. Still, they reported sensitivity of 75% and specificity of 94% in prediction of preterm birth of twins [11, 22]. Intertwin AC discordances were proven by different authors implying on adverse obstetric outcomes. A single biometric assessment of twins at 16 weeks could adequately predict subsequent pregnancy complications and premature pregnancy ending in almost 50% of cases [23, 24]. On the other hand, high reliability of biometrical measurements of the first and second trimester was not confirmed in all available studeis. Moreover, cut-off values of ultrasonographically determined twins biomety vary among studies. Further studies on this matter are still needed [20, 21].

In our study we found that out of assessed ultrasonographic fetal measures the thickness of NT of both twins and femur lenght of the smaller second twin in the second trimester could be used for prediction of preterm birth of ART conceived twins. When laboratory analyses were assessed β hCG serum levels measured in the first and the second trimester, as well as PAPPA and AFP concentrations could imply on GW of ART concieved twins delivery. We obtained a significant model for prediction of delivery time in ART concieved twin pregnancies based on the first trimester diagnostic tests – ultrasound and laboratory. Moreover, we set the cut-off levels for ultrasonografic and laboratory diagnostic tests of the first and second trimester. Performed ROC anlysis showed that sensitivity was better than specificity for prediction of delivery time of ART conceived twins. Nevertheless, reliability of these diagnostic testst in our study was not high (sensitivity 50% to 75%, specificity 30 to 40%), similarly as in the literature.

Conclusion

It can be seen that in the examined sample of one hundred ART concieved twins significantly more (51%) were delivered in term (after 35 GW), while more than 80% of them were liveborn. Based on the results of our study it can be concluded that prenatal noninvasive fetal screening of the first and second trimester (ultrasonography and laboratory testing) can be used for prediction of delivery time of ART conceived twins. The significant predictors were PAPPA and β hCG of the first trimester, larger twin NT as well as smaller twin CRL and FL. Nevertheless, reliability of these diagnostic testst in our study was moderate. Therefore, further studies are needed to more thoroughly investigate how could we potentially increase the reliability of these predictors as well as to test the obtained prediction model for delivery time of ART conceived twins based on ultrasonography and laboratory diagnostic tests of the first and second trimester.

Conflict of interest statement

Authors declare no conflict of interest. This study received no funding.

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Application of questionnaires in the assessment of clinical severity of chronic rhinosinusitis

Primena upitnika u proceni kliničke ispoljenosti hroničnog rinosinuzitisa

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Abstract

Background/Aim. Diagnosis of chronic rhinosinusitis (CRS) is based on clinical symptoms, and confirmed with endoscopic findings and computed tomography (CT) scans of paranasal sinuses. However, the results of numerous studies have shown that the symptoms that patients report are not in correlation with the degree of the disease spread obtained by radiological findings. The aim of our study was to examine is there a correlation between the degree of symptoms intensity of the non-polypous and polypous form of CRS and the degree of the disease spread, obtained on the basis of radiological diagnostics. **Methods.** A total of 60 patients, of which 30 patients with CRS without nasal polyps (CRSsNP) and 30 with CRS with nasal polyps (CRSwNP), were included in this cross-sectional study. Symptoms were evaluated using two questionnaires: Sino-Nasal Outcome Test 22 (SNOT-22) and Visual Analogue Score (VAS). The Lund Mackay CT score was used as a radiological parameter of the disease expansion. In addition, each of the subjects was examined for sensitivity to standard inhalation allergens. **Results.** In patients with CRSsNP, there were statistically significant positive correlations between the Lund Mackay CT score and the SNOT-22 score (r = 0.578, p = 0.001) and between the Lund Mackay CT score and the VAS (r = 0.408, p = 0.025). We found no correlation between the both questionnaire scores and the Lund Mackay score in CRSwNP patients. In patients with CRSwNP, a statistically significant difference was found in the values of SNOT-22 between patients with and without sensitivity to inhalation allergens, with higher values of the score in patients with allergy (p = 0.039). **Conclusion.** There is a positive correlation between the severity of the symptoms and the radiological findings only in patients with CRSsNP, which suggests that application of these questionnaires would be possible only in the case of this clinical entity.

Key words:
rhinitis; sinusitis; chornic disease; surveys and questionnaires; signs and symptoms; diagnosis.

Apstrakt

Uvod/Cilj. Dijagnoza hroničnog rinosinuzitisa (HRS) se postavlja na osnovu kliničkih simptoma, a potvrđuje na osnovu endoskopskog nalaza i snimaka kompjuterizovane tomografije (KT) paranazalnih sinusa. Međutim, rezultati brojnih studija su pokazali da simptomi koje bolesnici navode nisu u korelaciji sa stepenom proširenosti bolesti koji se dobija radiološkim pretragama. Cilj naše studije je bio da se ispita da li postoji korelacija između stepena izraženosti simptoma nepolipozne i polipozne forme HRS i stepena proširenosti bolesti, dobijenim na osnovu radiološke dijagnostike. **Metode.** U ovu studiju preseka bilo je uključeno ukupno 60 bolesnika, od toga 30 bolesnika sa nepolipoznom i 30 sa polipoznom formom HRS. Simptomi su procenjivani primenom dva upitnika: *Sino-nasal Outcome Test*-om – 22 (SNOT-22) i *Visual Analogue Score*-om (VAS). Kao radiološki parameter proširenosti bolesti korišćen je *Lund Mackay* KT skor. Pored toga, svakom od ispitanika je ispitivana senzitivnost na standardne inhalacione alergene. **Rezultati.** Kod nepolipozne forme HRS postoje statistički značajne pozitivne korelacije između Lund Mackay KT skora i vrednosti SNOT-22 skora (r = 0,578; p = 0,001), kao i između Lund Mackay KT skora i VAS (r = 0,408, p = 0.025). Kod polipozne forme HRS nije pokazana statistički značajna korelacija između Lund Mackay KT skora i vrednosti oba upitnika. Kod bolesnika sa polipoznom formom bolesti je uočena statistički značajna razlika u vrednostima SNOT-22 upitnika između bolesnika sa i bez preosetljivosti na inhalacione alergene, pri čemu su veće vrednosti skora bile kod bolesnika sa alergijom (p = 0,039). **Zaključak.** Dobijeni rezultati su pokazali da postoji pozitivna statistička

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povezanost između težine simptoma i radioloških nalaza samo u slučaju nepolipozne forme HRS, što govori u prilog tome da bi opravdanost za eventualnu primenu ovi upitnici imali samo u slučaju ovog kliničnog entiteta.

Ključne reči:
rinitis; sinusitis; hronična bolest; ankete i upitnici; znaci i simptomi; dijagnoza.

Introduction

According to the criteria published in the European Position Paper on Rhinosinusitis and Nasal Polyps, EPOS 2012, chronic rhinosinusitis (CRS) is defined as a disease characterized by the presence of at least two symptoms which persist for at least 12 weeks, and one of the symptoms should be either nasal blockage or nasal discharge: anterior/ posterior nasal drip, while the facial pain/pressure and reduction or loss of smell may or may not be present [1, 2]. There are two forms of this disease: chronic rhinosinusitis without nasal polyps (CRSsNP) and chronic rhinosinusitis with nasal polyps (CRSwNP) [1, 2]. These two forms have different histological finding, based on the dominant cellular infiltrate, as well as immunological one on the basis of the dominant profile of cytokines and chemokines [3, 4]. According to the presence of nasal symptoms, there is no clear difference between these two forms of the disease [5]. The prevalence of CRS is increasing, and the cost of its treating represents a major economic burden [6, 7]. Also, the poor quality of life of CRS patients should not be ignored. The diagnosis of the disease, as well as the decision on its further treatment, is based on the already mentioned clinical symptoms. However, additional diagnostic procedures, such as endoscopic examination and/or computed tomography (CT) of paranasal sinuses are required for the final diagnosis. The presence of CRS symptoms and the degree of the disease spread, estimated on the basis of endoscopy and CT scan, often do not correlate, so Stankiewicz and Chow [8, 9] have shown in their studies that 53% of patients with a clinical diagnosis of CRS, based on the present symptoms, have had no disease presentation on CT or the finding has been minimal. CT is certainly a diagnostic gold standard, but it is not routinely used because of the high dose of radiation and the cost of the procedure. It is only used in cases of unsuccessful medical treatment, preparation for surgical treatment and in the case of threatening complications of CRS [2]. Nowadays, questionnaires are used to obtain information on the quality of life and the severity of the disease based on clinical symptoms [10, 11]. However, the diagnosis of CRS, estimating the extent of the disease spread and the decision about the necessary therapy based on clinical symptoms only are not reliable.

The aim of this study was to examine whether symptoms of CRS, assessed by questionnaires, correlate with the degree of CRS spread, estimated on CT scans of paranasal sinuses, and to demonstrate whether it is possible to apply these questionnaires as a part of standard diagnostic procedures.

Methods

This study was conducted in the Department of Otorynolaringology of the tertiary care hospital Military Medical Academy (MMA) in Belgrade, Serbia. The protocol of investigation was approved by the Ethics Committee of MMA and written informed consent was obtained from all participants. Sixty (n = 60) patients who met the criteria for the diagnosis of CRS and were candidates for functional endoscopic sinus surgery (FESS), after an unsuccessful medical treatment, were included in the study. Thirty patients had CRS without nasal polyps (CRSsNP) and the other thirty had CRS with nasal polyps (CRSwNP). Patients were diagnosed with CRS according to the EPOS 2012 criteria [2]. Anterior and posterior rhinoscopy was performed in all patients, followed by an endoscopic examination after which the patients were divided into two groups: CRSsNP and CRSwNP. Exclusion criteria included: presence of systemic diseases involving the nasal cavity (Vegener's granulomatosis, Churg – Strauss syndrome, sarcoidosis, etc.), the presence of fungal rhinosinusitis, the use of antihistamines, corticosteroids and antibiotics at least three weeks before the surgery, previous endoscopic sinus surgery (ESS) or other surgery in the nasal area and paranasal cavities, patients under the age of 18, and pregnancy.

Before their surgical treatment, patients were asked to fill out two questionares about the severity of their symptoms. The first questionnaire was the Sino-Nasal Outcome Test 22 (SNOT-22), which offers the answers for 22 symptoms: the need to blow nose, sneezing, runny nose, nasal obstruction, loss of smell or taste, cough, post-nasal discharge, thick nasal discharge, ear fullness, dizziness, ear pain, facial pain/pressure, difficulty falling asleep, waking up at night, lack of a good night's sleep, waking up tired, fatigue, reduced productivity, reduced concentration, frustration/restlessness/irritation, sadness, embarrassed. Patients circled the numbers depending on the severity of the symptoms : 0 – "no problem," 1 – "very mild problem," 2 – "mild or slight problem," 3 – "moderate problem," 4 – "severe problem," and 5 – "extremely severe problem." The maximum score of this questionnaire is 110.

The Visual Analogue Score (VAS) questionnaire contains answers offered for 18 symptoms: headache, nasal obstruction, nasal discharge, postnasal discharge, impaired sense of smell, facial fullness, dental pain, facial pain/pressure, epiphora, cough, epistaxis, deposition of dried secretion in the nose, general health condition, fatigue, fever, nausea, vomiting, diarrhea. Patients evaluated the symptoms by placing a vertical hatch mark on a scale of 0 to 10 cm depending on the severity of their symptoms. Depending on where on the scale the hatch mark was placed the values of these symptoms ranged between 0 (without symptoms) and 10 (the worst symptom). Ten out of these 18 symptoms were included in the final evaluation score: headache, nasal obstruction, nasal discharge, postnasal discharge, impaired sense of smell, facial fullness, facial pain/pressure, cough, fatigue, nausea. The maximum score of the questionnaire was

100. Both questionnaires also had questions about the patient's age and gender.

As a gold standard for estimating the degree of the CRS spread, we used CT scans of paranasal cavities in coronal, axial and sagittal planes. The CT findings were estimated by the Lund-Mackay score [12]. Each of the paranasal sinuses on both sides of the face were estimated as follows: 0 – a complete lucency in the sinus, 1 – a partial opacity and 2 – a complete opacity. The score was determined bilaterally for the anterior etmoidal cells, posterior etmoidal cells, maxillary, sphenoidal and frontal sinus. An additional bilateral score was included for the ostiomeatal complex: 0 – not occluded, 2 – occluded. The maximum value of the Lund Mackay CT score was 24.

Each of the patients was tested on hypersensitivity to standard inhalation allergens, based on which it was assessed whether the presence/absence of sensitivity to allergens had an effect on the severity of symptoms and/or the degree of CRS spread. We used the standard battery with fifteen respiratory allergens for skin prick tests (Soluprick® SQ, Hørsholm, Denmark).

Using statistical analysis, it was examined whether there was a difference between the scores of the symptoms (SNOT-22 and VAS) and Lund Mackay CT scores between CRSsNP and CRSwNP. Comparison of Lund Mackay, questionnaire scores and individual symptom severity between CRSwNP and CRSsNP patients was performed using the Mann–Whitney test. Pearson's correlation test was used to estimate the correlation between the questionnaire score values and the values of individual symptoms derived from tests and the Lund Mackay CT scores. The p values < 0.05 were cosidered statistically significant. All data was processed in the SPSS 20.0 software package.

Results

In the group of patients with CRSsNP, there were 12 (40%) men and 18 women (60%), while in the CRSwNP group there were 18 (60%) men and 12 (40%) women. The average age of respondents with CRSsNP was 36.77 ± 10.41, and of those with CRSwNP was 49.90 ± 13.28 (Table 1).

In patients with CRSsNP, the average Lund Mackay CT score was 6.57 ± 1.04. The average intensity of symptoms obtained using SNOT-22 was 52.60 ± 19.36, while this value in the case of VAS questionnaire was 45.53 ± 17.04. The average Lund Mackay CT score in CRSwNP patients was 18.10 ± 4.26, while the average value of SNOT-22 questionnaire was 47.77 ± 19.56 and that of VAS was 48.27 ± 16.08. We found no significant differences between CRSsNP and CRSwNP regarding the total SNOT-22 and VAS score (Table 2).

Table 1

Demographic data of patients included in the study

Type of sinusitis	Age (years)			Gender, n (%)	
	min	max	mean ± SD	male	female
CRSsNP (n = 30)	19	63	36.77 ±10.41	12 (40)	18 (60)
CRSwNP (n = 30)	23	77	49.90 ± 13.28	18 (60)	12 (40)

CRSsNP – chronic sinusitis without nasal polyps; CRSwNP – chronic sinusitis with nasal polyps; SD – standard deviaton.

Table 2

Average values of Lund Mackay CT score, SNOT-22 and VAS questionnaires in patients with CRSsNP and CRSwNP (n= 30)

Questionnaire/Test	CRSsNP (n = 30)			CRSwNP (n = 30)			
	min	max	mean ± SD	min	max	mean ± SD	
Lund Mackay CT score	5	9	6.57 ± 1.040	12	24	18.10	4.26
SNOT-22	11	86	52.60 ± 19.36	18	96	47.77	19.56
VAS	15	82	45.53 ± 17.04	16	86	48.27	16.08

CT – computed tomography; SNOT-22 – Sino-Nasal-Outcome Test 22; VAS – Visual Analogue Score; CRSsNP – chronic rhinosinusitis without nasal polyps; CRSwNP – chronic rhinosinusitis with nasal polyps; SD – standard devation.

Table 3

Correlation between questionnaires and individual symptoms and Lund Mackay CT score in patients with CRSsNP

Questionnaire/Symptom	Pearson's correlation coefficient	Sig. (2-tailed)
SNOT- 22	0.578	0.001
VAS	0.408	0.025
Nasal obstruction	0.437	0.016
Facial fullness	0.421	0.021

CT – computed tomography; CRSsNP – chronic rhinosinusitis without nasal polyps; SNOT-22 – Sino-Nasal Outcome Test 22; VAS – Visual Analogue Score.

Pearson's correlation analysis confirmed that there was a statistically significant positive correlation between Lund Mackay CT score and the scores of SNOT-22 (r = 0.578, p = 0.001) and VAS (r = 0.408, p = 0.025) questionnaires in patients with CRSsNP (Figures 1 and 2). Also, in these patients, there was a statistically confirmed correlation between Lund Mackay CT score and individual symptoms: nasal obstruction (r = 0.437, p = 0.016) and facial fullness (r = 0.421, p = 0.021) (Table 3).

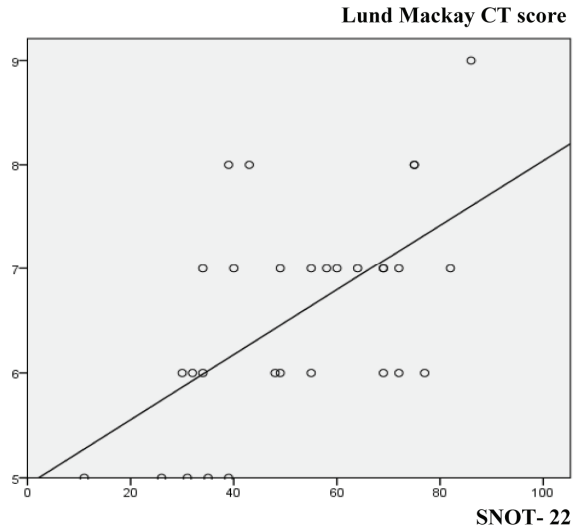

Fig. 1 – Correlation between Sino-Nasal Outcome Test 22 (SNOT- 22) questionnaire and Lund Mackay computed tomography (CT) score in patients with CRSsNP.

In patients with CRSwNP, there was a poor correlation between Lund Mackay CT score and the total questionnaire scores (SNOT-22, VAS), but Lund Mackay CT score correlated with individual symptoms that entered in VAS questionnaire: nasal obstruction (r = 0.391, p = 0.033) and smell impairment (r = 0.466, p = 0.009) (Table 4).

Table 4
Correlation between individual symptoms and Lund Mackay CT score in patients with CRSwNP

Symptom	Pearson's correlation coefficient	Sig. (2-tailed)
Nasal obstruction	0.391	0.033
Impaired sense of smell	0.466	0.009

CT – computed tomography; CRswNP – chornic rhinosinousitis with nasal polyps.

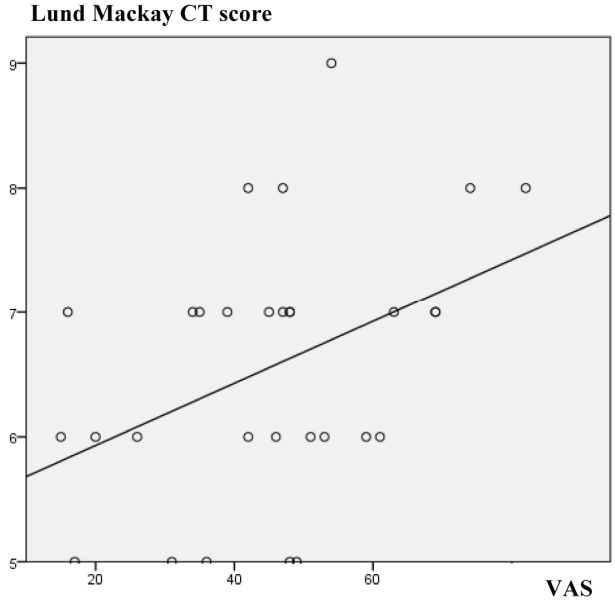

Fig. 2 – Correlation between Visual Analogne Score (VAS) questionnaire and Lund Mackay computed tomography (CT) score in patients with chronic rhinosinusitis without nasal polyps (CRSsNP).

In patients with CRSwNP, the average Lund Mackay CT score was significantly higher in comparison to patients with CRSsNP (p = 0.000) (Table 5).

Using the Mann-Whitney test, the average values of the individual symptoms that entered the final VAS score were compared between CRSwNP and CRSsNP. It was shown that there was a statistically significant difference in the values of individual symptoms between these two forms of CRS. Headache (p = 0.037), fatigue (p = 0.033) and nausea (p = 0.001) were significantly higher in CRSsNP, whereas nasal obstruction (p = 0.000), nasal discharge (p = 0.003) and impaired sense of smell (p = 0,000) were higher in patients with CRSwNP (Table 6).

In patients with CRSsNP, there was no statistically significant difference in the values of SNOT-22, VAS and Lund Mackay CT scores between non-allergic and allergic patients (Table 7). However, the average value of the SNOT-22 score was significantly higher in allergic CRSwNP patients comparing to non-allergic ones (p = 0.039). In the values of VAS and Lund Mackay, no difference was found between the groups of subjects with and without hypersensitivity to inhalation allergens (Table 7).

Table 5
Differences in SNOT-22 and VAS questionnaire values and Lund Mackay CT scores between CRSsNP and CRSwNP

Statstical parameters	Lund Mackay CT score	SNOT- 22	VAS
Mann-Whitney U	0.000	376.500	423.500
Wilcoxon W	465.000	841.500	888.500
Z	-6.692	-1.087	-0.392
Asymp. Sig. (2-tailed)	0.000	0.277	0.695

SNOT-22 – Sino-Nasal Outcome Test 22; VAS – Visual Analogne Score; CRSsNP – chronic rhinosinusitis without nasal polyps; CRSwNP – chronic rhinosinusitis with nasal polyps.
CT – computed tomography.

Table 6
Differences of individual symptom scores between CRSsNP and CRSwNP

Symptoms	CRSsNP (sum of ranks)	CRSwNP (sum of ranks)	*p*
Headache	1053.00	777.00	0.037
Nasal obstruction	674.00	1156.00	0.000
Nasal discharge	713.50	1116.50	0.003
Postnasal discharge	856.50	973.50	0.381
Facial fullness	862.50	967.50	0.431
Impaired sense of smell	665.50	1164.50	0.000
Facial pain/pressure	1023.50	806.50	0.101
Cough	938.00	892.00	0.730
Fatigue	1057.50	772.50	0.033
Nausea	1115.00	715.00	0.001

CRSsNP – chronic rhinosinusitis without nasal polyps; CRSwNP – chronic rhinoisusitis with nasal polyps.

Table 7
Differences of SNOT-22, VAS and Lund Mackay CT score between patients with and without allergen sensitivity in patients with CRSsNP and CRSwNP

Statistical parameter	CRSsNP			CRSwNP		
	Lund Mackay CT score	SNOT- 22	VAS	Lund Mackay CT score	SNOT- 22	VAS
Mann-Whitney *U*	93.500	106.000	109.500	79.000	38.500	57.000
Wilcoxon *W*	246.500	259.000	200.500	107.000	314.500	333.000
Z	-0.743	-0.189	-0.042	-0.074	-2.060	-1.153
Asymp. Sig. (2-tailed)	0.458	0.850	0.967	0.941	0.039	0.249
Exact Sig. [2*(1-tailed Sig.)]	0.483	0.869	0.967	0.962	0.037	0.266

SNOT-22 – Sino-Nasal Outcome Test 22; VAS – Visual Analogne Score; CT – computed tomography; CRSsNP – chronic rhinosinusitis without nasal polyps; CRSwNP – chronic rhinosinusitis with nasal polyps.

Discussion

CRS is a heterogeneous disease, based on both etiopathogenesis and its clinical characteristics. The diagnostic procedures used are not consistent all around the world, which additionally complicates the recognition and treatment of the disease.

The aim of this study was to examine which symptoms of CRS correlate with Lund Mackay CT score depending on the disease form: polypous or non-polypous one. It was noticed that there were no differences in the total SNOT-22 and VAS symptom scores between these two forms of the disease. However, there was a statistically significant difference in the values of the Lund Mackay CT score between the CRSsNP and CRSwNP patients. Our results are in accordance with previous ones that patients with CRSwNP have higher endoscopic and CT scores than the patients with CRSsNP [13].

In patients with CRSsNP, both questionnaires (SNOT 22 and VAS) were in correlation with the values of the Lund Mackay CT score, indicating that these questionnaires can be used for assessment the severity of this form of the disease. In subjects suffering from CRSwNP, we found no similar results and our findings could confirm results of previously published studies in which subjective symptoms do not often correlate with the severity of CRS [14–18].

In our study, headache, fatigue and nausea were shown to be significantly more noticeable in patients with CRSsNP, while nasal obstruction, nasal discharge and impaired sense of smell were of higher intensity in CRSwNP patients. Bannerji et al. [19] had previously demonstrated that patients with CRSwNP have higher values of nasal obstruction and hyposmia, while CRSsNP patients often suffer from pressure in the face and headache. It is important to understand that the way in which patients experience their symptoms depends on their mental status. One of the key symptoms in CRS patients is headache. Therefore, it can be assumed that presence of headache was the main reason why the value of SNOT 22 was lower in CRSwNP than in CRSsNP, although the degree of the disease spread was greater in patients with CRSwNP. The results of numerous studies show a statistically significantly lower presence of headache as a symptom of CRSwNP when compared to CRSsNP [5, 18–20]. In a study conducted by Drake-Lee et al. [21] it has been shown that only 35% of patients with nasal polyposis complained of headache. In a Stammberger and Wolf [22] study, it has been explained that the presence of pain in CRS is the result of local release of substance P in the nasal mucosa as well as its cen-

tral release into *dura mater*. Substance P is the main neurotransmitter for pain, and it is released in the nasal mucosa after stimulation of the so-called polymodal receptors. They are positioned along the entire nasal mucosa and can react to mechanical, thermal and chemical stimuli. Stammberger and Wolf [22] state that in cases of the edematous mucous membrane, there is contact of adjacent mucosal surfaces, primarily on the level of the ostiomeatal complex, which leads to the local release of substance P. In the nasal mucosa, substance P have a role of strong inflammatory mediator, leading to increased blood vessel permeability, plasma extravasation, relaxation of smooth muscle fibers, and glandular hypersecretion. All these effects lead to an increase of mucous membrane edema and an increase in the sensation of pain. On the other hand, there is a belief that due to the lack of local innervation, nasal polyps are painless inflammatory structures that can be greatly increased before they cause any discomfort in patients [23]. Lately, more and more studies have shown that headache in CRSwNP is caused by other diseases such as migraine and tension headache [24, 25]. The International Headache Association, in its etiological classification, does not even include CRS but only the acute rhinosinusitis [26], although according to EPOS guidelines, headache is one of the diagnostic criteria. The results of our study indicate a lower intensity of headache in patients with CRSwNP corraborating with previous points. The explanation for this may be the fact that polyps as painless structures, gradually occupy space in the nasal cavities and thus do not allow edematous mucous membranes to contact, causing the effect of an "airbag" that does not allow the local release of substance P. However, further experimental studies are necessary to confirm our findings.

In patients with CRSwNP, there was a statistically significant difference in the values of the SNOT-22 questionnaire between patients with and without hypersensitivity to inhalation allergens in favor of patients with allergy. The connection between allergic rhinitis and nasal polyposis has long been known and according to the results in the EPOS 2012 guidelines it ranges between 10% and 64% [2]. Allergic rhinitis is considered as a factor that is more often associated with CRSwNP, which is explained with the existence of Th2 immune response in both diseases, or with similar profile of inflammatory mediators in allergic rhinitis and nasal polyposis. Pathohistologically, CRSwNP is characterized by an edematous stroma with albumin precipitation, forming of pseudocysts, and subepithelial and perivascular infiltration of inflammatory cells, primarily eosinophils [27]. The presence of edema in patients with allergic rhinitis leads to additional disturbance in ventilation and drainage of paranasal sinuses and creates a precondition for enhanced local inflammatory response, and thus increases the intensity of nasal symptoms.

Conclusion

The results of our investigation showed that the values of the intensity of symptoms obtained by using the SNOT-22 and VAS questionnaires correlate with the radiological indicators of severity of disease such as Lund Mackay CT score only in patients with CRSsNP, whereas no correlation was observed in subjects with CRSwNP. In patients with CRSsNP, symptoms that correlated with Lund Mackay CT score were nasal obstruction and facial fullness, while in the CRSwNP patients these symptoms were nasal obstruction and impaired sense of smell. The results presented here could have practical significance in diagnosis, evaluation of the quality of life, and in assessment of medical and surgical treatment efficacy in patients with CRS, although the possible application of these questionnaires would be only in patients with CRSsNP.

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Vascular endothelial growth factor as a potential prognostic factor for T3N0 rectal cancer

Vaskularni endotelni faktor rasta kao potencijalni prognostički faktor kod T3N0 stadijuma karcinoma rektuma

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Abstract

Background/Aim. Rectal cancer still presents a major health problem. Although a surgery is the mainstay of the rectal cancer treatment, there is now widespread agreement that combined modality therapy is often indicated. Around 20% of T3N0 rectal cancer patients develop distant or local relapse of the disease. There is a need for prognostic biomarkers that could help us determine the subgroup of patients with a high risk for recurrence. The aim of this study was to determine the prognostic potential of vascular endothelial growth factor (VEGF) in patients with T3N0 rectal carcinoma. **Methods.** This retrospective study included 163 selected T3N0 rectal cancer patients, operated on the Department for Colorectal Surgery of the Clinic for Digestive Surger (First Surgical Clinic), Clinical Centre of Serbia, Belgrade. VEGF expression was immunohistochemically assessed. Oncological outcome was analyzed using data from prospectively designed data base. Parameters of interest were: distant metastases, the disease free and overall survival. Survival and time to recurrence were evaluated using Kaplan Meier`s method and the factors were compared with the long-rank test. **Results.** There were 102 men and 61 women. The median age was 62 years (age range, 31–88 years). Median follow-up interval was 81 months (range, 4–177 months). During the follow-up period 6 patients developed local recurrence, in 31 patients distant metastases occurred. Three factors were found to be associated with distant metastases: VEGF expression, mucinous adenocarcinoma and tumor differentiation ($p < 0.05$). In patients with positive VEGF expression, the disease free survival and overall survival were significantly worse than in negative ones (65% and 59%, respectively) (log-rank test, $p < 0.05$). **Conclusion.** High VEGF expression in T3N0 rectal carcinomas together with some standard histopathological tumor features can give us enough information to identify subgroup of patients with high risk for recurrence and poorer prognosis.

Key words:
biomarkers; prognosis; rectal neoplasms; recurrence; vascular endothelial growth factor.

Apstrakt

Uvod/Cilj. Karcinom rektuma još uvek predstavlja veliki zdravstveni problem. Iako je hirurško lečenje primarno, široko je prihvaćena činjenica da je kombinovana terapija često indikovana u tretmanu ove bolesti. Lokalni ili distalni recidiv javlja se kod oko 20% bolesnika sa karcinomom rektuma T3NO stadijuma. Danas postoji potreba za prognostičkim biomarkerima uz čiju pomoć se mogu predvideti bolesnici sa visokim rizikom od recidiva bolesti. Cilj ove studije bio je da se ispita prognostički potencijal vaskularnog endotelnog faktora rasta (VEGF) kod bolesnika sa T3N0 staadijumom karcinoma rektuma. **Metode.** Retrospektivnom studijom bila su obuhvaćena 163 bolesnika sa T3N0 stadijumom karcinomom rektuma, operisana na III Odeljenju Klinike za digestivnu hirurgiju (Prva hirurška klinika), Kliničkog centra Srbije u Beogradu. Imunohistohemijski je ispitivana ekspresija VEGF. Podaci su prikupljani u prospektivno dizajniranoj bazi podataka. Kao parametri od interesa definisani su pojava udaljenih metastaza i preživljavanje. Preživljavanje i vreme do recidiva bolesti procenjivano je na osnovu Kaplan-Meier-ove metode i *log-rank* testa. **Reziltati.** U studiju su bila uključena 102 muškarca i 61 žena. Prosečna starost ispitanika bila je 62 godine (31–88 godina), a postoperativno praćenje iznosilo je u proseku 81 mesec (4–177 meseci). Kod šest bolesnika je dijagnostiko-

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van lokalni, a kod 31 udaljeni recidiv bolesti. Tri faktora su pokazala značajnu povezanost sa udaljenim metastazama: ekspresija VEGF, mucinozni adenokarcinomi i diferencijacija tumora. Kod bolesnika sa pozitivnom ekspresijom VEGF preživljavanje je bilo lošije u odnosu na bolesnike sa negativnom ekspresijom VEGF (65% i 59%, redom; *log-rank* test $p < 0,05$). **Zaključak.** Povišena ekspresija VEGF kod T3N0 stadijuma karcinoma rektuma, zajedno sa standardnim histopatološkim karakteristikama tumora, može dati dovoljno informacija za definisanje bolesnika sa visokim rizikom od pojave recidiva bolesti i lošijom prognozom.

Ključne reči:
biomarkeri; prognoza; rektum, neoplazme; recidiv; faktori rasta endotela krvnih sudova.

Introduction

Colorectal cancer (CRC) is the major health problem of both developed and some developing countries. The incidence of rectal cancer (RC) in the European Union is ~ 125,000 per year, i.e. ~35% of the total CRC incidence [1].

Biological behavior of RC is in a way different from the colon tumors and its treatment modalities are specific. Besides the need for individualized and meticulous preoperative staging there is sometimes a problem in choosing the optimal mode of treatment. Surgery is still the mainstay of treatment, but neoadjuvant therapy proved to be effective in cases of locally advanced RC [2]. Current problem presents a group of RC patients in T3N0M0 stage. These patients may not benefit from aggressive neoadjuvant and adjuvant approach [3]. Nevertheless, we still have around 20% of patients in this group who develop distant or local relapse of the disease [4, 5]. There is a need for predictive and prognostic markers that could help us determine the subgroup of patients with high risk of relapse [6–8].

Among others, vascular endothelial growth factor (VEGF) has a significant role in angiogenesis, tumor proliferation and metastatic potential. As such it could be used as valuable prognostic tool [9].

The aim of this study was to determine the prognostic potential of VEGF in patients with T3N0 RC in the absence of neoadjuvant treatment.

Methods

This retrospective analysis included patients curatively operated for RC between January 2003 and December 2013. All patients were operated by the same surgical team, on the Department for Colorectal Surgery of the Clinic for Digestive Surgery (First Surgical Clinic), Clinical Centre of Serbia. The patient selection criteria were as follows: without any preoperative therapy; histopathologically confirmed T3N0 rectal adenocarcinoma; no evidence of distant metastases; R0 resections; available to provide follow-up information at least once. Patients deceased within 30 days from operation were excluded from the study. After careful reviewing medical and pathologic records, 163 consecutive patients with T3N0M0 RC were selected. Principles of the standard total mesorectal excision (TME) surgery were uniformly applied. RC was defined as adenocarcinoma located within 15 cm from the anal verge. TME was performed for most patients with mid and distal RC. For upper third RCs partial mesorectal excision was performed with minimal distal clearance of 5 cm. In cases where the restorative prosedure was not possible (cases with external anal sphincter involvement, voluminous tumors with intraoperative perforation, etc.) abdominoperineal excision (APR) or Hartmann's procedure was performed. Pathologic stage and pathologic grade were classified according to the 6th edition of the Union for International Cancer Control (UICC) classification. Oncological outcome of selected patients was analyzed using data from prospectively designed data base. Parameters of interest were: distant metastases, disease free (DFS) and overall survival (OS). Local recurrence (LR) was defined as any histological, morphological or clinical evidence of recurrence of RC within the pelvis, either alone or in association with distant metastases. Distant metastases were defined as the disease recurrence detected in organs excluding the pelvis.

OS was defined as the time from the date of surgery to the date of death or the date of last follow-up of patient who were still alive. DFS was defined as the time from the date of surgery to the date of the diagnosed recurrence. Patients who died without evidence of LR or distant recurrence were censored at the date of death, and patients alive without evidence of LR or distant recurrence were censored at the date of last follow-up.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks from selected patients were used for tissue microarray (TMA) construction. The immunohistochemical detection of VEGF was performed on fresh 3 μm paraffin sections from the TMA block. Slides with 3-μm-thick sections from TMA tissue blocks were dried in a 60°C oven for one hour. The sections were placed in a Bond Max Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Germany) according to the following protocol. First, tissues were deparaffinized and pretreated with the Epitope Retrieval Solution 2 at 100°C for 20 min. After washing steps, peroxidase blocking was carried out for 5 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). Slides were again washed and then incubated with the primary antibodies (VEGF, Clone VG1, DAKO, Cat. No M7273, dilution 1 : 50) for 15 min. Subsequently, tissues were first incubated with Post Primary Reagent for 8 min and then with Polymer for 8 min. After washing, sections were developed with DAB-chromogen for 10 min, and counterstained with hematoxylin for 8 min. Omission of the primary antibody was used as a negative control.

Immunohistochemical evaluation

Within tumor cells immunoreactive VEGF protein was detected primarly in the cytoplasm. The evaluation of staining of all TMAs were scored semi-quantitatively by two experienced pathologist blinded to the clinical data. The percentage of positive cells were assessed as follows: 0 – 0% of positive cells; 1 – < 5% of positive cells; 2 – 5%–50% of positive cells; and 3 – > 50% of positive cells. The intensity of staining was scored as: 0 – negative; 1 –weak; 2 – intermediate; and 3 – strong. The final score for the immunoreactions was defined as the sum of both parameters, and grouped as: 0 – negative; 1– weak; 2 – moderate, and 3 – strong. For statistical purposes, only the moderate and strong immunoreactions were considered as positive ones [10].

Statistical analysis

Statistical data analysis was performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). Univariate Cox regression model was applied to identify factors affecting distant metastases. A multivariate analysis using the Cox proportional hazards model was performed to investigate the independence of the risk factors identified as significant in the univariate analysis.

Survival was analyzed using Kaplan Meier`s test and the factors were compared with the *log-rank* test. All *p*-values less than 0.05 were considered statistically significant.

Results

Clinical and pathological characteristics of the patients are presented in Table 1. There were 102 (62.6%) men and 61 (37.4) women. Their median age was 62 years (range, 31–88 years). The average number of lymph nodes examined was 22 (range, 4–65). The location of the tumor was in average 9.4 ± 4.2 cm measured from the anal verge. Tumor size (measured as the largest tumor diameter) was 5.7 cm (range, 2–16 cm). Median follow-up interval was 81 months (range, 4–177 months). During the follow-up period 6 patients developed LR, in 31 patients we discovered distant metastases. The 5-year LR and distant metastases rate were 4% and 20%, respectively.

All potential risk factors for distant metastases, including clinicopathologic features and biomarker (VEGF) were evaluated by univariate Cox regression model. Among ten potential prognostic factors only the histological subtype of the tumor, tumor grade, lymphovascular invasion and VEGF expression exhibited correlation with distant metastases (Table 2). Patients with distant metastases had significantly higher VEGF expression, mucinous subtype of adenocarcinoma, poorly differentiated tumors and lymphovascular invasion than patient without metastases ($p < 0.05$). To establish independent risk factors, four variables were identified as significant in univariate analysis. Additionally, they were tested in a multivariate Cox proportional hazards model. It revealed three factors to be associated with distant metastases: VEGF expression, mucinous adenocarcinoma and tumor differentiation (Table 3).

In patients with positive VEGF expression, DFS (Figure 1) and OS (Figure 2) were significantly worse than in negative ones (65% and 59%, respectively; *log-rank* test, $p < 0.05$).

Table 1
Clinicopathological characteristics of the patients

Variables	Number (%)
Sex	
male	102 (62.6)
female	61 (37.4)
Age (years)	
< 60	68 (41.7)
> 60	95 (58.3)
Distance from the anal verge (cm)	
< 5	30 (18.4)
5–10	75 (46.0)
> 10	58 (35.6)
Tumor size (cm)	
< 5	65 (39.9)
> 5	98 (60.1)
Type of operation	
abdominal resection	151 (92.6)
abdominoperineal resection	7 (4.2)
hartmann	5 (3.2)
Histological type	
adenocarcinoma	133 (81.6)
mucinous adenocarcinoma	30 (18.4)
Grade	
well differented	133 (81.6)
moderately differented	24 (14.7)
poorly differented	6 (3.7)
pT stage	
pT3a	29 (17.8)
pT3b	75 (46.0)
pT3c	44 (27.0)
pT3d	15 (9.2)
No of lymph nodes examined	
< 12	24 (14.7)
> 12	139 (85.3
Lymphovascular invasion	
positive	69 (42.3)
negative	94 (57.7)
VEGF expression	
positive	90 (55.2)
negative	73 (44.8)
Total	163 (100)

pT – primary tumor; VEGF – vascular endothelial growth factor.

Table 2

Univariate Cox-regression analysis of potential prognostic factors for distant metastases

Variables	Number of patients (n = 163)	Univariate analysis		
		hazard ratio	95% CI	*p*
Sex				
male	102	1.39	0.69–2.82	0.361
female	61			
Age (years)				
< 60	68	1.00	0.97–1.03	0.905
> 60	95			
Distance from the anal verge (cm)				
< 5	30	0.99	0.61–1.60	0.966
5–10	75			
> 10	58			
Tumor size (cm)				
< 5	65	0.92	0.45–1.87	0.811
> 5	98			
Histological type				
adenocarcinoma	133	3.40	1.65–7.03	0.001
mucinous adenocarcinoma	30			
Grade				
well differented	133	3.57	2.26–5.64	0.001
moderately differented	24			
poorly differented	6			
pT stage				
pT3a	29	1.17	0.78–1.75	0.462
pT3b	75			
pT3c	44			
pT3d	15			
No of lymph nodes examined				
< 12	24	0.66	0.27–1.62	0.367
> 12	139			
Lympho-vascular invasion				
positive	69	2.26	1.10–4.66	0.027
negative	94			
VEGF expression				
positive	90	6.35	2.22–18.17	0.001
negative	73			

pT – primary tumor; CI – confidence interval; VEGF – vascular endothelial growth factor.

Table 3

Multivariate Cox-regression analysis of potential prognostic factors for distant metastases

Variables	Number of patients (n = 163)	Multivariate analysis		
		hazard ratio	95% CI	*p*
Histological type				
adenocarcinoma	133	3.46	1.60-7.52	0.002
mucinous adenocarcinoma	30			
Grade				
well differented	133	3.02	1.79-5.08	0.001
moderately differented	24			
poorly differented	6			
Lympho-vascular invasion				
positive	69	2.02	0.93-4.42	0.076
negative	94			
VEGF expression				
positive	90	5.51	1.88-16.19	0.002
negative	73			

CI – confidence interval; VEGF – vascular endothelial growth factor.

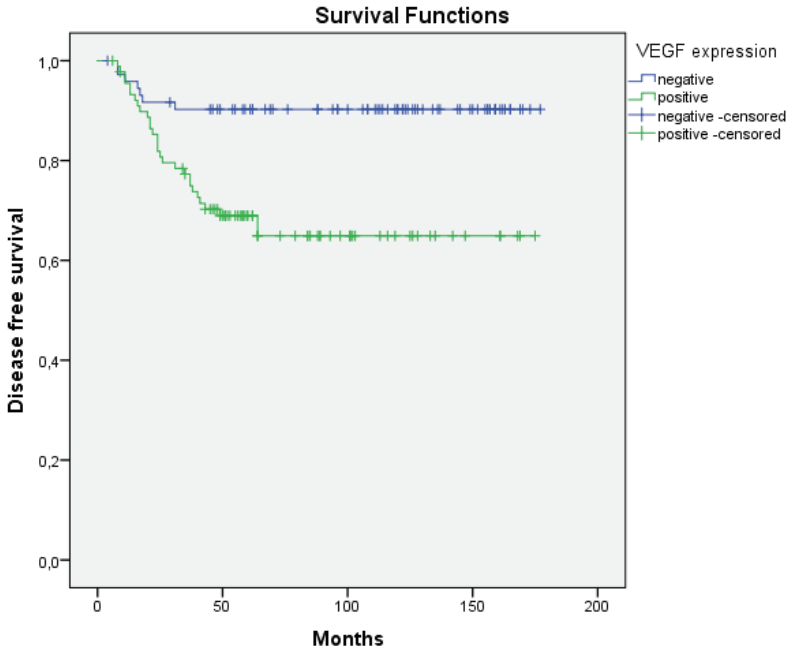

Fig. 1 – Kaplan-Meier`s curves of the disease-free survival of the patients according to the vascular endothelial growth factor (VEGF) expression.

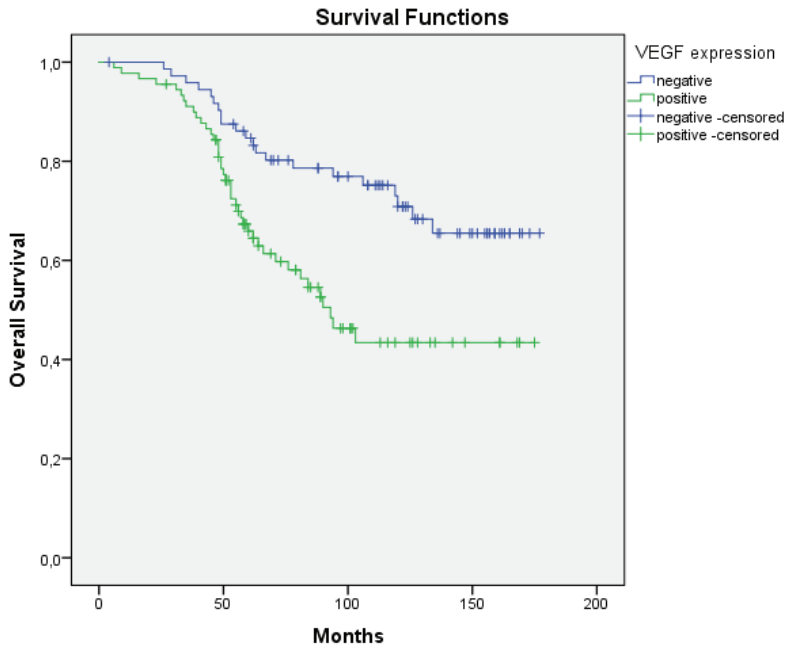

Fig. 2 – Kaplan-Meier`s curves of the overall survival of the patients according to the vascular endothelial growth factor (VEGF) expression.

Discussion

RC still presents major health problem. Developments in the field of surgery (introduction of TME) and neoadjuvant treatment led to the significant reduction in the percentage of LR and better quality of life of the affected patients [11, 12]. Local control and survival rates have been significantly improved. Major trials have reported that TME alone can reduce the local recurrence from 15%–20% to 4%–7% and improve the survival rate to 80%–85% for patients with the stage II disease [13].

Another fact is that screening programs, now widely implemented, have significant impact on the structure of operated patients. We have ever cases of early RC where we can expect favorable treatment results, but at a cost. Neoadjuvant treatment and TME surgery have certain downsides.

Namely, functional deficits, morbidity and mortality are inevitable in certain percent. Preoperative therapy can potentiate stated downsides [3, 8, 14].

Being aware of mentioned facts, we can conclude that certain population of patients would benefit from omitting chemoradiotherapy.

Improved preoperative staging can help us determine where it is safe to proceed only with surgical treatment of early RC. High-resolution magnetic resonance imeging (MRI) examination proved to be an excellent diagnostic tool which can help us in the decision where to omit neaodjuvant treatment [15]. T1 and T2 carcinomas can safely be treated with surgery alone [1]. But the population of patients with the T3N0 stage, circumferential resection margin (CRM) negative tumors can also benefit from this approach. There is no official consensus, or published guidelines, that clearly state that T3N0 RC deserves no surgery, but nowadays the majority of experts agree that surgery alone is sufficient treatment for this population of patients [1, 16].

Therefore, it is imperative to identify group of potential risk factors after TME in the cases of RC stages as the T3N0 disease in order to help further individualized strategy for those patients. There is a number of traditional clinical prognostic and risk factors [5, 17], but with no consensus reached which additional combination of biological factors would be most useful. In the mentioned group of patients, the risk of relapse is about 20% which makes standard adjuvant treatment unnecessary. There are attempts to select subgroup of the stage II patients using additional biomarkers where we should consider additional therapy.

In those efforts, a number of markers were investigated, among them one of more promising ones is VEGF [18, 19]. Angiogenesis is a key moment in tumor growth and in the development of metastatic potential and can be, in that context used, relatively reliable prognostic factor in patients with certain solid tumors. VEGF is 45kDa glycoprotein with the central role in tumor angiogenesis, influencing other proangiogenic factors and their inhibition suppresses tumor growth. There are different VEGF protein isoforms having subunit polypeptides of 121, 145, 165, 189 or 206 amino acid residues. $VEGF_{165}$ is the predominant molecular species, but transcripts encoding $VEGF_{189}$ are also commonly found in cells expressing the VEGF gene. $VEGF_{145}$ is the major splice variant in several tumor cell lines originating from the female reproductive organs. In contrast, $VEGF_{206}$ is a rare form. The splice variants differ in their bioavailability. It would be interesting to investigate prognostic and predictive value of each individual isoform in CRC patients, which would be a topic for further research [9, 10].

VEGF expression together with some standard histopathological tumor features could give us enough information to identify subgroup of the high risk stage II RCs.

Our study confirmed well known facts that mucinous component and poor differentiation of the tumor means unfavorable prognosis for RC patients in terms of the development of distant metastases. However, real biological aggressiveness of these tumors is often difficult to assess. In this group of tumors we can find signet ring cell carcinomas as well as those with partial mucin production. (i.e. mucinous and mixed neuroendocrine carcinomas) [20, 21]. Histological grade of the tumor on the other hand, can be subjective with considerable inter observer variations [20].

In our study, in 55.2% analyzed samples, higher VEGF expression was found and was associated with the development of the distant metastases. Five year DFS in VEGF positive patients was 65% and in VEGF negative group 90%. OS was also significantly affected when comparing VEGF positive and negative cases (59% and 80% respectively). There are studies that reached similar conclusion [22–24], but majority of studies found significant association of VEGF expression and more advanced stages of the disease (stage III and IV) [18, 19]. High VEGF expression is associated with poor prognosis and advanced stage of the disease, but its real prognostic significance in patients with CRC is still unclear, especially in the early stage. One of the reasons for this can be pathologist bias, nonstandardized staining protocols and scoring systems as well as the differences in analyzed materials (stage of the disease, number of participants...).

Finally, despite reaching statistically significant results, we must state that our study had limitations (stage of the disease, only RC patients and retrospective nature).

Conclusion

VEGF can be potentially used as prognostic factor in patients with T3N0 RCs. Combined with standard clinical and pathological prognostic factors it can help us identify the group of patients with poor prognosis who can be candidates for adjuvant treatment and more aggressive follow-up protocol. Nevertheless, more prospective multicentric studies are needed in order to finally establish the real role of VEGF as a prognostic factor in patients with early RC.

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Intraoperative imprint cytology of sentinel lymph nodes in breast cancer patients: comparation with frozen section

Intraoperativni citološki otisak sentinelnih limfnih čvorova kod bolesnica sa karcinomom dojke: poređenje sa ledenim rezovima

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Abstract

Background/Aim. Sentinel lymph node (SLN) biopsy has been established as the standard of care for axillary staging in patients with invasive breast carcinoma and clinically negative lymph nodes. Intraoperative assessment of sentinel lymph nodes might be done by frozen section (FS), touch imprint cytology (TIC) and one step nucleic acid amplification. The aim of this study was to review our institution's results with SLN biopsy using TIC and FS technique as intraoperative diagnostic tool for breast cancer patients. **Methods.** SLNs from 101 patients were examined intraoperatively by frozen hematoxylin-eosin (H&E) stain and by touch imprint cytology. Results of TIC were compared with FS and permanent histology sections. **Results.** The total number of dissected SLNs was 163 with a mean of 1.6 (1–4) per patient. The permanent H&E identified 19 (19%) patients with a sentinel lymph node metastasis and 82 (81%) patients with tumor-free sentinel nodes. The sensitivity/specificity rates were 73.7%/99.3%, respectively for TIC and 84.2%/100%, respectively for FS. Relevant positive/negative predictive values were 93.3%/96.6%, respectively for TIC and 100%/97.9%, respectively for FS. **Conclusion.** Our experience with TIC and FS for the intraoperative evaluation of SLNs is similar to the findings from previously reported studies. We detected the high specificity for both methods, but TIC technique appeared to be less sensitive than FS in detecting SLN metastases in breast cancer patients. TIC could be recommended as reasonable alternative to frozen section due to its simplicity and low cost.

Key words:
sentinel lymph node biopsy; breast neoplasms; intraoperative period; diagnostic techniques and procedures.

Apstrakt

Uvod/Cilj. Biopsija limfnog čvora stražara (engl. *sentinel lymph node* – SLN) je standardna procedura za intraoperativnu procenu statusa aksilarnih limfnih čvorova kod bolesnica sa karcinomom dojke koje imaju klinički negativne limfne čvorove pazušne jame. Pregled SLN se intraoperativno izvodi tehnikama ledenih rezova (engl. *frozen section* – FS), citološke evaluacije otiska (engl. *touch imprint cytology* – TIC) i metodom amplifikacije nukleinskih kiselina u jednom koraku. Cilj studije bio je da se uporede i procene pouzdanost i tačnost tehnike FS i TIC kao metoda za pregled SLN kod bolesnica sa karcinomom dojke. **Metode.** SLN, dobijeni od 101 bolesnice, intraoperativno su pregledani na FS bojenim hematoksilin-eozin (HE) bojenjem i citološkom analizom razmaza dobijenim otiskom limfnih čvorova. Rezultati citološke analize poređeni su sa FS nalazom i trajnim histološkim preparatima. **Rezultati.** Ukupan broj analiziranih SLN iznosio je 163, u proseku 1,6 (1–4) po bolesnici. Na definitivnim, parafinskim preparatima, metastaze u SLN ustanovljene su kod 19 (19%) bolesnica, dok kod 82 (81%) bolesnice u limfnim čvorovima nije bilo tumora. Senzitivnost i specifičnost za TIC iznosila je 73,7% i 99,3%, dok je za tehniku FS senzitivnost bila 84,2%, a specifičnost 100%. Pozitivna/negativna prediktivna vrednost za TIC je iznosila 93,3%/96,6%, a za metodu FS 100%/97,9%. **Zaključak.** Naše iskustvo sa metodama TIC i FS u intraoperativnoj proceni statusa SLN kod bolesnica sa karcinomom dojke slično je rezultatima ranije objavljenih studija. Utvrđena je visoka specifičnost

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za obe metode, ali je senzitivnost tehnike TIC u detekciji metastaza u SLN nešto niža u odnosu na metodu FS. TIC metoda intraoperativnog pregleda SLN može predstavljati pouzdanu alternativu metodi FS zbog jednostavnosti i niske cene.

Ključne reči:
limfni čvorovi, stražarski; biopsija; dojka, neoplazme; intraoperativni period; dijagnostičke tehnike i procedure.

Introduction

Axillary lymph node (ALN) status is an important prognostic factor and determinant of treatment for patients with breast carcinoma. Sentinel lymph node (SLN) biopsy has been established as the standard of care in assessing the axilla in patients with invasive breast carcinoma and clinically negative lymph nodes [1]. It is a minimally invasive procedure that accurately evaluates the status of the axilla and can obtain the same prognostic information derived from axillary lymph node dissection (ALND) with significantly less morbidity [1,2]. SLN is the first node receiving lymphatic drainage directly from the primary tumor. Thus, it is the node most likely to be the site of initial lymphatic metastasis. Currently, there is level 1 of evidence that documents that SLN biopsy is as accurate as ALND for breast cancer staging. If SLN is negative, it is predicted that the rest of the ALNs will also be negative [3]. Conversely, if SLN is positive the rest of the ALNs might also contain metastatic tumor deposits [1,4–6].

SLN biopsies are performed in highly equipped institutions by lympho-scintigraphy scan or blue dye mapping of SLN or combined technique [1,4].

Intraoperative evaluation of SLNs is enabled by various techniques such as frozen section (FS), touch imprint cytology (TIC) and one step nucleic acid amplification (OSNA) [4]. FS examination is the most common method for intraoperative diagnostics of SLN, but disadvantages are loss of valuable tissue for definitive histological examination, considerable time consumption, technical difficulty in preparation of fatty tissue, specific instrumentation and costs [5–7]. TIC is rapid, inexpensive, easy, widely available method with maximum tissue preservation that allows clear cytological details, but it requires well educated pathologist in terms of breast cytology. Immunohistochemical (IHC) staining for cytokeratines is not routinely used in intraoperative evaluation. ICH is very accurate technique, but is a time consuming and expensive method that requires a special equipment [1,6–9].

All intraoperative diagnostic techniques are followed by routine examination of paraffin-embedded and hematoxylin-eosin (H&E) stained sections, which is a reference standard, after which the definite staging of axillary lymph nodes is performed [1,3,8,10].

Both FS and intraoperative cytology imprints have a wide variety in sensitivity rates [4,10,11].

The purpose of this study was to determine our institution's experience using both FS and TIC techniques for intraoperative detection of metastases in SLN biopsy and comparation with standard permanent section examination.

Methods

Prospective study was performed at the Oncology Institute of Vojvodina, Sremska Kamenica, Serbia during 2014, 2015 and 2016. Study included 101 patients with histologically confirmed breast cancer and clinically negative axillary lymph nodes treated operatively with SLN biopsy. Neoadjuvant chemotherapy was an excluding factor for the study. Detection of SLN was performed by combined method: preoperative application of 1 mL methylene blue dye and 1 mL (1mCi) of radioactive isotope (Tc99 nanocolloid).

SLN were identified successfully in all patients and were sent immediately for pathological examination. Fresh lymph nodes larger than 3 mm were bisected along long axis and each surface was touched on glass slide. The imprint samples were air-dried and fixed in 95% alcohol, than stained by May-Grünwald-Giemsa Quick-stain (Bio-Optica, Italy) and analyzed under microscope. Fresh cut lymph nodes were then frozen, cut at 5 μm, 3–5 sections *per* slide, stained in standard H&E stain and microscopically analyzed. Slides were analyzed by experienced pathologist and reported to surgeon intraoperatively.

The decision of performing ALND was made based on results of FS of SLN. The SLNs specimens were then placed in cassette, fixed in 10% buffered formalin for routine processing and standard pathological examination.

Breast cancer tumor staging was performed based on tumor size, status of axillary lymph nodes and metastases, determining pathological (p)TNM – tumor-nodus-metastasis status of breast cancer according to the American Joint Committee on Cancer (AJCC) [12]. Assessment of breast cancer differentiation was performed by modified Bloom-Richardson score [13].

The results of FS and TIC were compared with definitive postoperative histopathology results of SLNs and analyzed using Statistical Package for Social Sciences (SPSS), version 18 (SPSS Inc. Chicago, USA).

Results

Breast surgery with SLN biopsy was made in 101 female patients with breast cancer and clinically negative axillary lymph nodes. Sentinel lymph node was successfully obtained in all patients (100%) and 163 lymph nodes were recieved for pathological analysis. The patients ranged in age from 29 to 82 (mean age 58.2). None of the 10 patients with non invasive extensive high-grade *in situ* carcinoma had SLN metastases. Invasive breast tumors were classified as pTis (n = 10; 9.9%), pT1 (n = 58; 58.4%) and pT2 (n = 33; 32.7%). Most frequent type of the tumor in analyzed group was ductal invasive carcinoma (n = 69; 68.3%). Primary tumor grading using Bloom-Richardson Grading System found that 28.7% patients had grade 1, 43.6% had grade 2 and 27.7% grade 3 of breast carcinoma. Tumor size varied from 3 mm up to 40 mm and 4 cases were multifocal carcinomas. Patients and tumor characteristics are summarized in Table 1.

Table 1

Patient and tumor characteristics

Characteristics	Values
Age (years), mean (range)	58.19 (29–82)
Side of the tumor, n (%)	
left	44 (43.56)
right	57 (56.44)
Surgical procedure, n (%)	
quandrantectomy	97 (96.0)
mastectomy	4 (4.0)
Tumor stage, n (%)	
pTis	10 (9.90)
pT1	58 (57.42)
pT1a	4 (3.96)
pT1b	12 (11.88)
pT1c	42 (41.58)
pT2	33 (32.67)
Histologic tumor type, n (%)	
ductal invasive carcinoma	69 (68.32)
lobular invasive carcinoma	8 (7.92)
in situ carcinoma	10 (9.90)
other types	14 (13.86)
Histologic grade, n (%)	
1	29 (28.71%)
2	44 (43.56%)
3	28 (27.72%)

Average number of lymph nodes *per* patient was 1.6 (1 to 4). A total number of 163 SLN was examined by TIC, FS and permanent histopathological section methods. TIC was positive for SLN metastases in 15 cases (Figure 1) and negative for metastases in 148 cases. FS detected metastases in 16 SLN (Figure 2) and 147 were negative.

Metastatic deposits > 2 mm were marked as micrometastases and ≤ 2 mm as macrometastases. Permanent histology sections, considered a gold standard in diagnostics of metastatic deposits, showed metastases in 19 SLN. Macrometastases were present in 17 SLN and micrometastases in 2 examined lymph nodes.

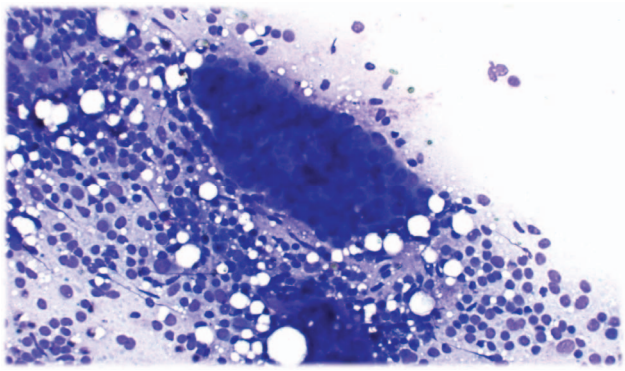

Fig. 1 – Clusters of tumor cells in touch imprint cytology smears in lymph node with breast cancer metastasis (May-Grünwald-Giemsa, ×400).

There was discordance between TIC and histopathology reports in 6 SLN. Five cases of negative TIC turned out positive for metastases in histopathology, and one of the positive TIC was found negative in histopathology. Two cases of false negative in TIC were micrometastases and other 3 were macrometastases. In total 163 SLN examined, 14 were positive for metastases on both TIC and permanent sections, and 143 were negative after analyzing with both methods.

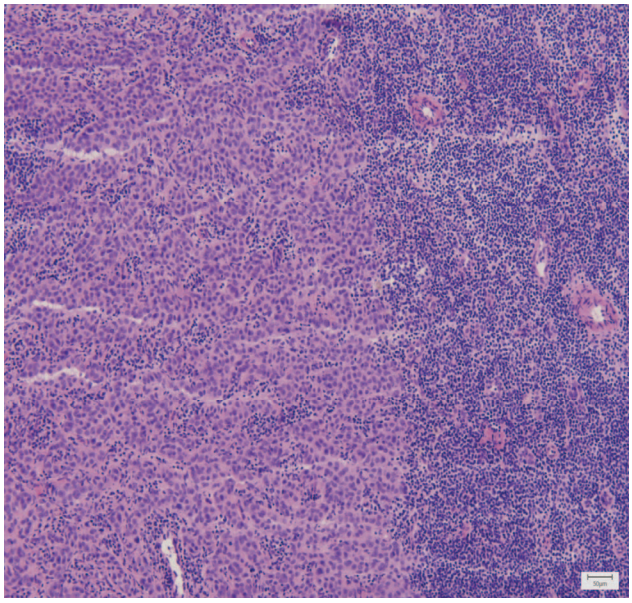

Fig. 2 – Metastasis of breast carcinoma in sentinel lymph node on frozen section (hematoxylin-eosin, ×10).

Intraoperatively, FS technique found 16 positive cases and 147 negative cases for nodal metastases. Permanent sections and FS showed discordance in 3 false negative cases, two for micrometastases and one for macrometastases (Table 2).

Based on examination of 163 SLN acquired from 101 patients in our study, the sensitivity for metastases detected by TIC was (14/19) 73.9%, specificity was (143/144) 99.3%, positive predictive value was (14/15) 93.3% and negative predictive value was (143/148) 96.6%. Overall accuracy for TIC in detecting SLN metastases was (157/163) 96.3%.

The sensitivity of FS from our study was (16/19) 84.2%, specificity was (144/144) 100%, positive predictive value was (16/16) 100% and negative predictive value was (144/147) 97.9%. Overall accuracy of FS for detection of nodal metastases was (160/163) 98.1%.

Discussion

Sentinel lymph node biopsy is a worldwide accepted concept for patients with breast carcinoma. Therefore, intraoperative detection of SLN is an imperative. However, because of the lack of equipment such as special infrastructure for preparation, storage and handling of radioactive technetium 99-label colloid or ineffective purchase of methylene blue dye, many facilities still use ALND [6].

Table 2
Comparation of the results found in permanent histology sectons, FS and TIC in 101 examined breast cancer patients

Sentinel lymph node	Permanent histology sections (n)	Frosen section (n)	Touch imprint cytology smears (n)		
Positive for metastases	19	16	15	True	14
				False	1
Negative for metastases	144	147	148	True	143
				False	5

FS – frozen section; TIC – touch imprint cytology.

Frozen section may provide information on the size of metastasis, but it causes loss of tissue for permanent sections, it is time consuming and expensive technique requiring a cryostat as well as skilled professionals. Touch imprint cytology requires less effort, it is faster, saves tissue for permanent sections, but pathologist needs to be trained for reporting cytology samples. Intraoperative cytology provides rapid results with minimal artifacts. However, number of examined cells in cytology samples is smaller [4, 11].

Numerous studies comparing FS and TIC in intraoperative evaluation of SNLs have demonstrated significant variation in sensitivity of 44–100% for FS and 34–95% for TIC [4, 10]. However, the variations of the methodology involved in the intraoperative as well as permanent section histopathologic evaluation make it very difficult to reliably compare different studies.

Tew et al. [10] reviewed 31 studies comparing TIC and FS in the literature and overall sensitivity of TIC was 63%, with a pool sensitivity of 81% for macrometastases and 22% for micrometastases. A similar meta-analysis reporting on FS examination found an overall sensitivity of 78%, with 94% for macro- and 40% for micrometastases [11].

In comparison of TIC and FS, although there was higher sensitivity of FS, no statistically significant difference between these two methods was found in the most of the studies [7, 11, 13–16]. The lower sensitivity of TIC is usually caused by inadequate sampling, and might be overcome when the number of slides during TIC is increased. This can improve sensitivity of the method without losing tissue for permanent histological examination [7, 15, 17].

High specificity for both FS and intraoperative cytology approach, indicates that the false positive rates of these techniques are close to zero [10, 18]. Higher false negative rates for both methods of intraoperative examination of SLNs are seen in low nuclear grade metastatic tumors and particularly lobular carcinomas, since these tumor cells are small and poorly cohesive [11].

Our study showed 73.7% sensitivity and 99.3% specificity for TIC. The case of false positive imprint was due to misinterpretation of epithelioid histiocytes. Germinal center lymphocytes or activated endothelial cells could also rarely be mistaken for tumor cells [15]. The omission of micrometastases is the major cause of false negative intraoperative diagnoses. In accordance to literature data, our study showed two false negative imprints as the result of micrometastases. Reasons for false negative result is smaller number of examined cells comparing to FS and unrecognized individual tumors cells in well differentiated carcinomas [11, 18].

The use of intraoperative immunohistochemistry with cytokeratins could minimize the intraoperative false negative rates. Such protocols are now available for using on either FS or cytology imprints. However, turnaround time for such protocols is 16–20 minutes, and it prolongs the time of the surgery and costs of the diagnostics and thus it is not a standardized procedure [14, 15, 17]. Recently, intraoperative ultrarapid IHC has been investigated for its feasibility, validity, and effectiveness in comparison with FS. Ultrarapid cytokeratin IHC significantly enhanced intraoperative detection of metastasis in SLNs without increased time for assessment. This technique is currently not widely available and requires specialized expertise [7, 9]. Immunohistochemistry is a standard procedure if there is a suspicious presence of metastatic cells during permanent section examination.

The clinical prognostic significance of micrometastases in SLN remains controversial, and some authors consider micrometastases to behave similarly to macrometastases [1, 17, 18]. Several studies have questioned the clinical and pathologic significance of finding micrometastases in SLN, particularly in intraoperative consultations [19, 20].

Currently, the standard practice has been to offer completion axillary lymph node dissection in patients who are found to have positive SLN for metastatic carcinoma either during the primary surgical procedure or in permanent histopathology report [20–22]. However, the recently reported results from the American College of Surgeons Clinical Oncology Group (ACOSOG) Z0011 trial found that there was no statistically significant benefit from ALND for women who had clinically negative axilla but the SLN was positive [3]. Recommendations from recent studies advise that axillary lymph node dissection can be omitted in patients with one or two positive sentinel nodes when conventionally whole-breast radiation therapy is planned [21, 22]. Therefore, the role of intraoperative assessment of SLN in breast cancer seems to be in evolution.

Conclusion

Our experience with TIC and FS for the intraoperative evaluation of SLNs is similar to the findings from previously reported studies. We detected the high specificity for both methods, but TIC technique appeared to be less sensitive than FS in detecting SLN metastases.

TIC results can be obtained with reasonable accuracy within a short time frame, permitting intraoperative decisions regarding management of the axilla in the breast cancer patients. Therefore TIC could be recommended as an alternative to FS in view of its simplicity and low cost.

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Prognostic parameters in recurrent colorectal cancer: A role of control or restaging by FDG-PET/CT

Prognostički parametri u rekurentnom kolorektalnom karcinomu: uloga kontrole ili ponovnog određivanja stepena bolesti FDG-PET/CT-om

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Abstract

Background/Aim. Colorectal cancer ranks the third most frequent cancer in the world. Approximately 40% of the disease recurs after surgical resection. Determination of predictive parameters for recurrence may help in stratification of patients and contribute to patient management. There are still very few studies which sought factors to predict the recurrence of colorectal cancer. The aim of this study was to examine the predefined risk factors in metastatic development and evaluate clinical significance of 18F-fluorodeoxyglucose (FDG) uptake. **Methods.** The study was conducted with 56 patients for whom FDG-PET/CT (FDG-positron emission tomography/computed tomography) was requested for the suspicious recurrence or metastasis by routine conventional screening tests. Thirty three patients in whom recurrence/metastases were established with final histopathologic diagnosis formed the malignant group, and 23 patients with no recurrence/metastases formed the benign group. Risk factors [age, serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (Ca 19-9) levels, the maximum standardized uptake volume (SUVmax), tumor size (TS), CT/magnetic resonance imaging (MRI) findings, sex, primary tumor localization, lymphovascular invasion, perineural invasion (PNI), initial neoadjuvant therapy, lymph node initial metastasis (ILNM) excision, stage, tumor differentiation] were compared between these groups. **Results.** CEA, Ca 19-9, SUVmax, TS, PNI, ILNM, FDG uptake pattern, pattern of lesions on CT and tumor differentiation were found statistically significant by univariate analysis. After multivariate analysis, SUVmax and ILNM remained as the main risk parameters impacting recurrence/metastases. Mean SUVmax was 7.25 in the benign group, while it was 11.7 in the malignant group (p = 0.019). ILNM was present in 66.5% of patients in the malignant group, and in 30.5% of patients in the benign group (p = 0.015). For an estimated cut-off value of 6.3 and 12.5, respectively on ROC curve, the calculated specificities were 61% and 87%, respectively. **Conclusion.** ILNM and SUVmax are the main risk factors for recurrence of colorectal cancer and the patients with these factors must be followed up carefully. FDG-PET/CT is very sensitive for the detection of recurrence/metastases of colorectal cancer and SUVmax appears to improve its specificity.

Key words:
colorectal neoplasms; neoplasm staging; prognosis; radiopharmaceuticals; recurrence; sensitivity and specificity; tomography, emission-computed; tomography, x-ray computed.

Apstrakt

Uvod/Cilj. Kolorektalni karcinom se svrstava u treći najčešći karcinom na svetu. Kod približno 40% obolelih bolest se vraća posle hirurške resekcije. Određivanje prediktivnih parametara za relaps može pomoći u stratifikaciji i doprineti vođenju bolesnika. Još uvek je nedovoljan broj studija koje istražuju prediktivne faktore za relaps kolorektalnog karcinoma. Cilj rada bio je da se ispitaju prethodno definisani faktori rizika od razvoja metastaza i proceni klinički značaj preuzimanja 18F-fluorodeoksiglukoze (FDG). **Metode.** Studijom je bilo obuhvaćeno 56 bolesnika kojima je bilo potrebno uraditi FDG-PET/CT (FDG-pozitron emisionu tomografiju/kompjute-rizovanu tomografiju), zbog sumnje na relaps ili metastaze postevljene rutinskim testovima. Od 33 bolesnika kojima su finalnom patohistološkom dijagnozom utvrđeni relaps ili metastaze formirana je grupa sa malignitetima, dok je druga grupa ispitanika bila sa benignim promenama. Između te dve grupe ispitanika poređeni su sledeći faktori rizika: životno doba, serumski nivoi karcinoembrionskog antigena (CEA) i karbohidratni antigen 19-9 (CA 19-9), vrednost maksimalnog standardizovanog preuzimanja (SUVmax), veličina tumora, nalaz CT/magnetna rezonanca

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(MR), pol, primarna lokalizacija tumora, limfovaskularna invazija, perineuralna invazija (PNI), inicijalna neoadjuvantna terapija, inicijalna ekscizija metastatskih limfnih čvorova (ILNM), stadijum i diferencijacija tumora. **Rezultati.** Univarijantnom analizom utvrđena je statistička značajnost za CEA, Ca 19-9, SUVmax, veličinu tumora, PNI, ILNM, obrazac preuzimanja FDG, obrazac lezije na CT-u i diferencijacija tumora. Multivarijantnom analizom su SUVmax i ILNM utvrđeni kao glavni parametri rizika koji utiču na metastaze ili relaps. Srednji SUVmax iznosio je 7,25 u grupi bolesnika sa benignim promenama, a 11,7 u grupi sa malignitetima (p = 0,019). U grupi sa malignitetima ILNM je bio prisutan kod 66,5% ispitanika, a u grupi sa benignim promenama kod 30,5% ispitanika (p = 0,015). Za procenjeni *cut-off* od 6,3 i 12,5 na *Receiver Operating Characteristic* (ROC) liniji, izračunata specifičnost iznosila je 61% i 87%, redom. **Zaključak.** Glavni faktori rizika od relapsa kolorektalnog carcinoma su ILNM i SUVmax, zbog čega bolesnici sa tim faktorima rizika moraju biti pažljivo praćeni. Za otkrivanje relapsa ili metastaza kolorektalnog karcinoma FDG-PET/CT je veoma senzitivan test, a SUVmax poboljšava njegovu specifičnost.

Ključne reči:
kolorektalne neoplazme; neoplazme, određivanje stadijuma; prognoza; radiofarmaci; recidiv; osetljivost i specifičnost; tomografija, kompjuterizovana, emisiona; tomografija, kompjuterizovana, rendgenska.

Introduction

Colorectal cancer (CRC) ranks the third most frequent cancer and it was the fourth most frequent cause of cancer-related death in the world. Approximately 40% of the disease recurs after surgical resection of the primary tumor in two years [1]. There are some well-known predefined clinicopathological risk factors for recurrent/metastatic CRC. These are age, serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 levels, tumor depth (invasion), the maximum standardized uptake value (SUVmax) on FDG-PET/CD (18F-fluoro-deoxyglucose – positron emission tomography/computed tomography), tumor size (TS), CT/magnetic resonance imaging (MRI) findings, sex, primary tumor localization (PTL), lymphovascular invasion (LVI), perineural invasion (PNI), initial neoadjuvant therapy (INAT), initial lymph node metastasis (ILNM) excision at primary surgery (ILNM), stage, type of surgery, localization of metastasis (organ), cytogenetic factors, tumor differentiation (DIF). Detecting the recurrence is mandatory for convenient therapy. Different laboratory and imaging tests are handled to identify recurrent and/or metastatic CRC. Most guidelines recommend thoracoabdominal CT, routine serial carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (Ca 19-9) assays to monitor the disease [2].

CEA is expressed by a lot of epithelial tumors and its serum levels may rise in non-malignant disorders [3]. Nearly 70% of patients with CRC display an elevated CEA level at the time of diagnosis. This fact made it a routine monitoring marker for the disease recurrence [4]. Unfortunately, latest meta-analysis studies have declared conflicts about its utility in the detection of the recurrent disease. They are stating roundly sensitivities of 65% and specificities of 90% that can be considered poor for a biomarker [5]. Ca 19-9 assays have also a poor performance. It has been reported that Ca 19-9 was expressed only in 20%–40% of metastatic CRC [6].

Imaging has the key role in postoperative assessment of the metastatic disease. Molecular imaging with FDG-PET combined with CT is the most recent modality for this purpose [7]. The main limitation of CT and other morphological imaging techniques evaluating the recurrence of all types of cancers like CRC is size of the lesion and/or distortion of normal anatomic structures. FDG-PET/CT accomplishes this deficit by the capability to show recurrent CRC as in many other cancers, through pathologically increased tumor metabolism before the appearance of morphological changes [8]. As a glucose analogue, FDG reflects increased glucose consumption of cancer cells and a great majority of CRC are FDG-avid. FDG-PET/CT has been used for primary staging, evaluation of treatment response and restaging in CRC just like in many other cancers. It is more sensitive than conventional tests in patients with suspected recurrence and/or metastasis. But it has some intrinsic limitations. Inflammatory pathologies, fibrosis or edema following irradiation and/or surgery may cause increased FDG uptake [9, 10].

There are still very few studies which sought factors to predict the recurrence of CRC. The aim of this study was to examine the predefined risk factors in metastatic development and evaluate clinical significance of uptake on FDG-PET/CT during the follow-up after primary curative surgery and/or chemoradiotherapy for recurrence in patients with CRC.

Methods

This retrospective cohort study involved 56 patients treated at the Department of Nuclear Medicine and Department of General Surgery of a tertiary health care hospital between 2009 and 2016. Inclusion criteria were as follows: histopathologically established diagnosis of CRC by surgical specimen after primary surgery, pathological FDG uptake on control (evaluation of treatment response) or restaging by FDG-PET/CT requested for the suspicious recurrence or metastases by routine conventional screening tests in the follow-up, confirmation of all these abnormal uptakes by colonoscopy or histopathology. All cases were treated by surgery and/or chemoradiotherapy. The neoadjuvant chemotherapy was administered to the patients 6 weeks before the primary surgery and consisted of 5-fluorouracil. The files of the patients were retrieved from the archive and looked over retrospectively.

We evaluated the lesions on FDG-PET/CT in 56 patients. Indications for FDG-PET/CT were suspicion of recurrence/metastases (32 patients) and treatment response monitoring (24 patients). Elevated CEA and/or Ca 19-9 levels raised the suspicion of recurrence in 11 cases, conventional imaging in 21. All FDG uptakes were confirmed by colonoscopic

findings or histopathologically. The reference range of Ca 19–9 was 0–35 U/mL; normal range of CEA was < 2.5 ng/mL for nonsmokers, < 5 ng/mL for smokers. Tumors were staged by the seventh edition of the American Joint Committee on Cancer Classification. Predefined risk factors for recurrence were age, serum CEA and Ca 19–9 levels, SUVmax, TS, CT/MRI findings, sex, PTL, LVI, PNI, INAT, ILNM, stage, FDG uptake pattern (FDGP), pattern of lesions on CT (CTP), DIF. PTL was classified as distal rectum (4 cm), middle rectum (5–9 cm), rectosigmoid region, sigmoid (descending) colon and cecum-transverse/right colon. DIF was defined as low grade, moderate differentiation, high grade and mucinous component. FDGP was heterogeneous, diffuse or focal. CTP was soft tissue mass, wall thickening or hypodense lesion. Thirty three patients in whom recurrence/metastases were established with final histopathological diagnosis formed the malignant group, and 23 patients with no recurrence/metastases formed the benign group. The above-mentioned parameters were compared between these two groups.

FDG-PET/CT imaging protocol

Patients were hungry at least for 6 hours and their blood glucose levels were obliged to be below 150 mg/dL before the injection of an activity of 370–555 MBq of FDG calculated according to patient's body weight. Images were acquired one hour later with an integrated PET/CT scanner (Discovery 690-GE Healthcare). Unenhanced low dose CT and PET emission data were obtained from mid-thigh to the vertex of the skull in supine position with the arms raised over head. CT data were collected by an automated dose modulation of 120 kVp (maximal 100 mA) with the collimation of 64 × 0.625 mm, field of view (FOV) of 50 cm, noise index of 20%, reconstruction to images of 0.625 mm transverse pixel size and 3.75 mm slice thickness. PET data acquisition was performed in 3D mode with the scanning period of 2 min per bed position and an axial FOV of 153 mm. The emission data were corrected in a standardized way consisting of random, scatter and attenuation. Iterative reconstruction was done in a matrix size of 256 × 256 by Fourier rebinning and VUE Point FX [3D] with 3 iterations, 18 subsets).

Two nuclear medicine specialists unaware of patient history interpreted FDG-PET/CT images visually. Focally or heterogeneously increased FDG uptake, diffuse or heterogeneously increased FDG uptake and/or soft tissue mass on CT component, hypodense or nodular lesion on CT with or without FDG uptake, diffuse uptake accompanied by wall thickening, consolidation or ambiguous lesions on CT with or without uptake were supposed as pathologic. SUVmax corrected for body weight were computed by a standard protocol on a dedicated Workstation from the activity at the most intense voxel in three-dimensional tumor region from the transaxial whole-body images on attenuation-corrected PET/CT images. The corresponding CT scan of lesions was framed as a guideline if their boundaries were difficult to demarcate for the determination of SUVmax.

Statistical analysis

The whole data were analyzed by IBM Corporation Released 2013; IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY : IBM Corporation. Number and percentage values were used for the description of categorical data; mean, median, standard deviation (SD), minimum (min) and maximum (max) values for continuous data. Student's *t*-test (age) and Mann-Whitney *U* test (serum CEA and Ca19–9 levels, SUVmax, TS) were performed for categorical variables; Fisher's exact test (CT/MRI findings) and χ^2 test (sex, PTL, LVI, PNI, INAT, ILNM, stage, FDGP, CTP, DIF) for continuous variables in the univariate analysis. The parameters which were found statistically significant in univariate analysis were processed with multivariate analysis. The variables having a value of $p < 0.05$ were accepted as statistically significant. Reciever operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of SUVmax on recurrent disease. Informed consent was deemed as a retrospective study using records, documents and data of patients referred to our clinic for the test. The study was approved by Our Institutional Review Board Committee.

Results

Mean age of the patient population was 58.2 ± 11.1 (30–89) years; 27 (48.2%) of them were males, and 29 (51.8%) females. PTL was distal rectum (11%), middle rectum (18%), rectosigmoid region (27%), sigmoid colon (16%) and cecum-transverse/right colon (28%). Mean serum Ca 19-9 and CEA levels, SUVmax, and TS were: 229.5 U/ml (median, range: 8.5, 0.1–5,548), 6.56 ng/mL (median, range: 2.19, 0.3–71), 9.9 ± 6.3 and 34.7 ± 19.7 mm, respectively. The incidence of LVI, PNI, ILNM were 62.5%, 37.5%, 52%, respectively. 55.5% of the patients were treated by INAT. 11% of the cases were at stage I, 27% at stage II, 37% at stage III, 25% at stage IV. 28.5% of the patients had heterogeneous FDG uptake, 25% diffuse uptake, 37.5% focal uptake and 10% no uptake. Soft tissue mass was seen in 50% of the cases, wall thickening in 34%, hypodense lesion in 16% on CT as CTP. 25% of the tumors were low grade ones, 57% moderately differentiated, 11% were high grade tumors and 7% with mucinous component.

CEA, Ca 19-9, SUVmax, TS, PNI, ILNM, FDGP, CTP and DIF were found statistically significant after the procession of all potential risk factors by univariate analysis. Univariate analysis of predefined potential risk factors (except PTL, FDGP, CTP, DIF) impacting on metastases/recurrence, their mean values and percentages between the benign group and malignant group are shown in Table 1. These factors (LVI was included instead of FDGP) entered multivariate analysis, and SUVmax and ILNM remained as the main risk parameters impacting metastases/recurrence (Table 2). Mean SUVmax was 7.25 in the benign group, while it was 11.7 in malignant group. There was a statistical difference according to SUVmax values between benign and malignant groups ($p = 0.019$). A box-plot graph shows the distribution of SUVmax in benign conditions versus recurrent/metastatic disease (Figure 1).

Table 1

Univariate analysis of some predefined potential risk factors impacting on metastases/recurrence, their mean values and percentages between the benign and malignant groups

Variable	Groups		
	malignant	benign	*p*-value
Mean age (years), mean ± SD	59 ± 11		0.711
Sex, %			
male	45.5	52.2	0.621
female	54.5	47.8	
Serum Ca 19.9 (U/mL), mean (median)	382 (11)	9.7 (7)	0.047
Serum CEA (ng/mL), mean (median)	9.42 (3.5)	2.45 (1.7)	0.009
SUVmax, mean ± SD	11.7 ± 6.2	7.25 ± 5.57	0.019
Mean tumor size (mm, ± SD)	39 ± 21.7	28.5 ± 14.7	0.038
CT/MRI findings, %			
positive	84.8	69.6	0.200
negative	15.2	30.4	
LVI, %			
present	72.7	52.2	0.058
absent	27.3	47.8	
PNI, %			
present	48.5	21.7	0.042
absent	51.5	78.3	
INAT, %			
yes	54.5	56.5	0.884
no	45.5	43.5	
ILNM, %			
present	66.5	30.5	0.015
absent	33.3	69.6	
Stage, %			
I	6.1	17.4	0.253
II	21.2	34.8	
III	45.5	26.1	
IV	27.3	21.7	

Ca – carbohydrate antigen; CEA – carcinoembryonic antigen; SUVmax – maximum standardized uptake value; CT – computed tomography; MRI – magnetic resonance imaging; LVI – lymphovascular invasion; PNI – perineural invasion; INAT – initial neoadjuvant therapy; ILNM – initial lymph node metastasis; SD – standard deviation.

Table 2

Multivariate logistic regression analysis of risk factors influencing recurrence in patients with colorectal cancer

Variable	B	Odds ratio	CI	*p*-value
SUVmax	0.136	1.146	1.022–1.284	0.019
ILNM	1.532	4.626	1.351–15.834	0.015

SUVmax – maximum standardized uptake value; ILNM – initial lymph node metastasis; CI – confidence interval.

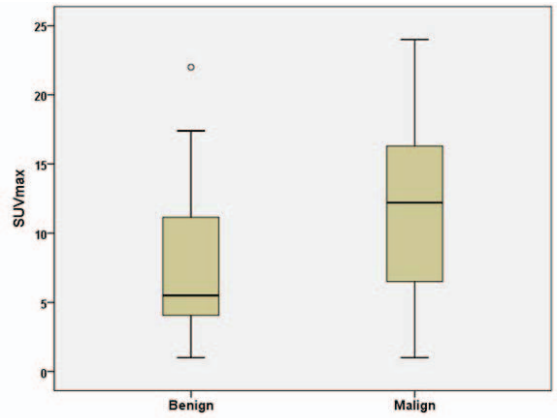

Fig. 1 – Box-plot graph of the distribution of maximum standard uptake value (SUVmax) in the benign conditions versus recurrent/metastatic disease.

ILNM was present in 66.5% of malignant group, 30.5% in benign group and there was a statistical significance between them ($p = 0.015$). A bar graph depicts the presence of ILNM in benign and malignant groups (Figure 2).

There was not a statistically significant difference between the malignant and benign groups according to PTL ($p = 0.944$). FDGP, CTP and DIF were statistically meaningful in the univariate analysis between the malignant and benign groups ($p = 0.014$, $p = 0.006$ and $p = 0.037$, respectively). Focal FDG uptake was present in 81% of recurrence whereas diffuse uptake was seen in 64% of the patients in the benign group. Soft tissue mass on CT was the main pattern (78.5%) in the malignant group, while wall thickening was present in 68.5% of the patients in the benign group. High grade and mucinous component were a clear risk factor for recurrence/metastases. ROC curve for SUVmax was drawn (Fig-

ure 3). AUC (area under curve) was 0.717 [confidence interval (CI): 0.581–0.854] (p = 0.006). Sensitivities and specifities for chosen cut-off values were represented in Table 3.

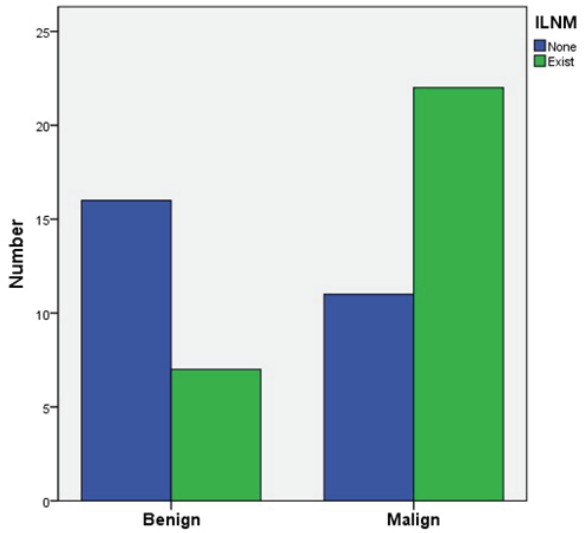

Fig. 2 – Bar graph of initial lymph node metastasis (ILNM) in the benign and malignant groups.

Recurrence and/or metastases developed in 59% of the patients (Figure 4). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of FDG-PET/CT for the detection of recurrence and/or metastases were 91%, 56.5%, 75% and 81%, respectively. FDG-PET/CT results and final histopathologic diagnosis are shown in Table 4.

FDG-PET/CT was true positive in 45% of the patients with normal Ca 19-9 and/or CEA levels and true negative in 12% of cases with elevated Ca 19-9 and/or CEA levels according to histopathologic confirmation or colonoscopy findings. In the follow-up, CT or MRI detected suspicious malignancy in 50% of the patients (28/56) and further examination with FDG-PET/CT was true negative in 32% of these cases (9/28) according to histopathology.

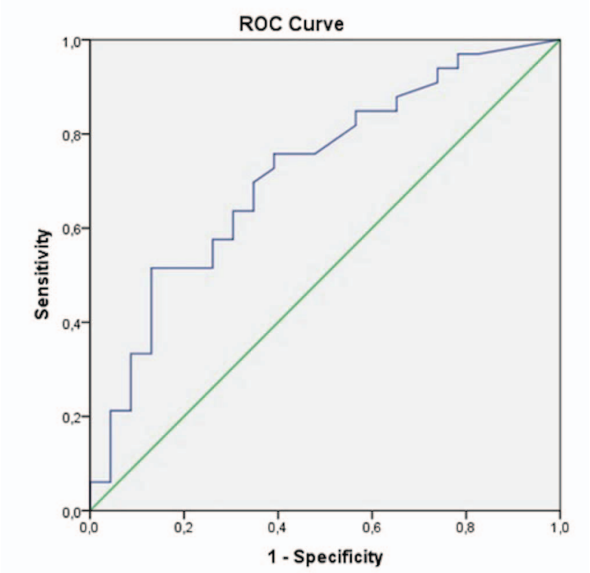

Fig. 3 – Recieved operating characteristic (ROC) curve for the maximum standardized uptake value (SUVmax).

Table 3

Cut-off values, related sensitivities and specificities of SUVmax for recurrence

Cut-off values	Sensitivity (%)	Specificity (%)	Area under curve (95% confidence interval)
12.5	51	87	0.717 (0.581–0.854)
6.3	76	61	

SUVmax – maximum standardized uptake value.

Table 4

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of FDG-PET/CT according to final histopathologic diagnosis

Histopathologic diagnosis	FDG-PET/CT results						
	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Total (n)
Malignant (n)	TP = 30	FN = 3					33
Benign (n)	FP = 10	TN = 13					23
Total (n)	40	16	91%	56.5%	75%	81%	56

TP – true positive; FP – false positive; FN – false negative; TN – true negative; FDG – 18F-fluoro-deoxyglucose; PET – positron emission tomography; CT – computed tomography.

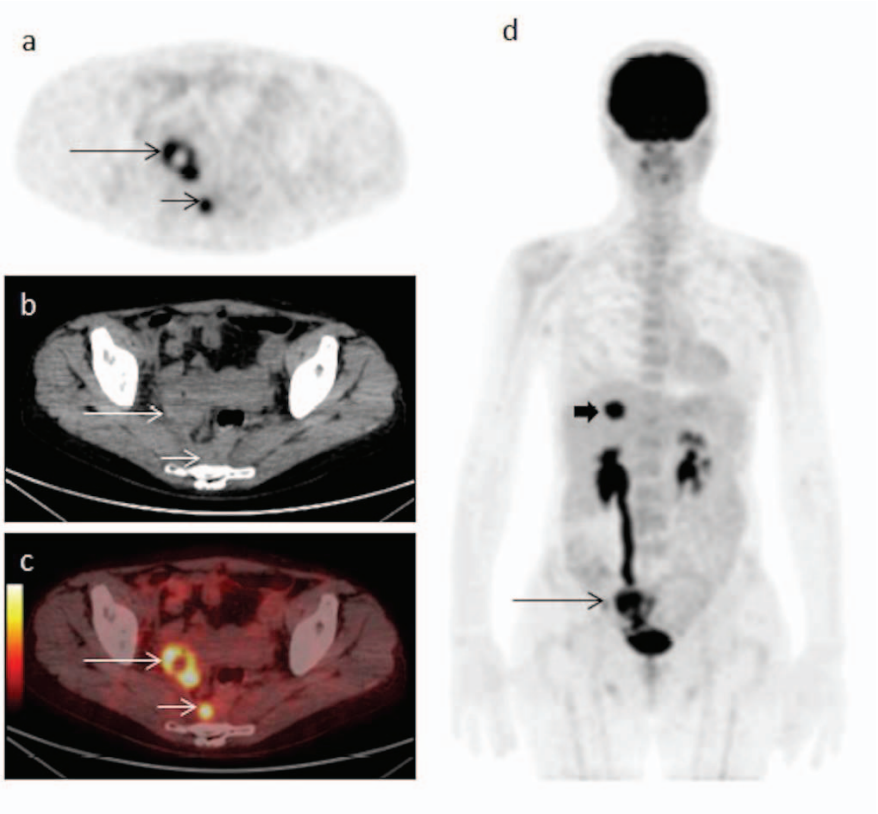

Fig. 4 – A female patient aged 67 years with rectal cancer was operated and treated by chemoradiotherapy. Her axial PET (A), CT (B), fusion (C) and maximum intensity projection (MIP) (D) images on FDG-PET/CT exhibited circular FDG uptake in the rectum (long arrow) with a SUVmax of 10.1 and TLG of 154 accompanied by wall thickening on the CT component. Besides, there was focal FDG uptake in the presacral area (short arrow) which was considered as metastatic lymph node (SUVmax: 9.6). These uptakes raised the suspicion of a probable recurrence and histopathology confirmed both of them as malignant. In whole body MIP images there was a metastatic foci in the liver showing FDG uptake (thick arrow) (SUVmax: 8.8).
PET – positron emission tomography; CT – computed tomography; FDG – 18F-fluoro-deoxyglucose; SUVmax – maximum standardized uptake value; TLG – total lesion glycolysis.

Discussion

Recurrent disease is seen in 30%–50% of patients with CRC after curative resection [11]. The recurrence rate was 59% in our study and it is higher than those described in literature. The most frequently encountered location of recurrence occurs in the area of surgery [12] and our findings were in agreement with this. Primary aim of follow-up surveillance is to identify recurrences at the earliest moment for an immediate cure. Most of the relapsed cases are not operable at the time of diagnosis and 1/3 of the patients with isolated locoregional or distant metastases survive 5 years [13]. Determination of predictive parameters for recurrence may help in stratification of patients and contribute to patient management with intense follow-up. FDG-PET/CT finding may be a prognostic factor and change treatment planning in CRC [14].

Mean age of CRC patients fluctuates around 60 years and younger ages are accepted as a risk factor for recurrence in literature [11]. Average age of our patient population was 58 years and this is in accordance with previous studies. But we observed that age is not a risk factor in our study. Many articles explained stage and LVI as having association with recurrent CRC. Kobayashi et al. [15] evaluated stage of the disease in 5,230 consecutive patients and found advanced stage a risk factor. Ryuk et al. [1] found high postoperative Ca 19-9 level, LVI, ILNM and advanced stage as risk factors for recurrence. Interestingly, we did not identify stage and LVI as statistically significant in the univariate analysis ($p = 0.253$ and $p = 0.058$, respectively). This is possibly due to undersampling or inconvenient data for statistics and not important clinically. PTL is a risk factor in many papers and recurrence in right colon is more incident [16]. However, PTL was not a meaningful prognosticator in our study and this is contrary to previous reports.

High serum CEA and Ca 19-9 levels assayed at follow-up after curative resection are prognostic factors for CRC [17]. They were also risk factors in our study ($p = 0.009$ and $p = 0.047$, respectively). But FDG-PET/CT yielded true positive results at a rate of 45% in patients whose Ca 19-9 and/or CEA levels were normal while it was true negative just in 12% of the cases with elevated Ca 19-9 and/or CEA levels according to histopathological confirmation. The relationship of recurrence with the use of neoadjuvant therapy is still unclear [18]. It was not a significant parameter in our study. Tsai

et al. [19] determined PNI as the most important factor in their study on 778 patients. Tsai et al. [20] showed that DIF, ILNM, LVI, PNI were risk factors in their study on 259 patients. Our findings are in agreement with them, except for LVI.

It has been reported that FDG-PET/CT is more accurate than CT or MRI for establishing recurrence in several studies. Odalovic et al. [21] found FDG-PET/CT more sensitive and specific than MRI. Scott et al. [22] showed that FDG-PET/CT detected 45 additional lesions in a multicenter prospective trial conducted on 93 patients. Detection of a lesion on CT/MRI was not a risk factor ($p = 0.200$) and FDG-PET/CT was more sensitive than CT/MRI findings at the follow-up in our study. It was true negative in 32% (9/28) of the cases on whose CT or MRI were seen lesions which were suspicious of malignancy according to histopathology. TS and DIF (undifferentiated high grade tumors and mucinous component) are clear risk factors for recurrence/metastases [23]. It is well-known that malignant lesions usually appear as focal FDG uptake with a soft tissue mass on FDG-PET/CT whereas diffuse uptake accompanied by wall thickening on CT component is mostly the main pattern in benign conditions [24]. Recurrences tend to occur in large and high grade tumors with usually focal FDG uptake accompanied by a soft tissue mass on CT component. FDGP, CTP and DIF were statistically meaningful in the univariate analysis between the malignant and benign groups in the study ($p = 0.014$, $p = 0.006$ and $p = 0.037$, respectively). ILNM is a strong predictor for recurrence in almost every study and it was the cutest factor together with SUVmax in the univariate analysis ($p = 0.008$ and $p = 0.006$, respectively). At the same time, they came out from the multivariate analysis as the only predictors impacting recurrence/metastases amongst all risk factors ($p = 0.015$ and $p = 0.019$, respectively). All our results are in line with these ones.

The use of FDG-PET/CT in the follow-up of CRC is controversial. Recent data recommend no indication except inconclusive CT with suspicion of distant metastases or in the presence of negative CT and serial CEA increase [12]. Many studies declared that FDG-PET/CT is very sensitive, but not so specific for the detection of recurrence of CRC. It has some limitations. FDG is accumulated in cancer cells at a relatively higher rate during glucose metabolism. However, cancer cells are not the only metabolically hyperactive ones. Inflammatory, infectious and some non-neoplastic diseases can have increased FDG accumulation causing a low specificity for CRC [25] as it was also seen in our study. The benign pathologies in our study consisted of granulation tissue, fibrin and inflammation, fibrosis, pyelonephritis, ulceration of colonic mucosa, fibrosis and inflammation, polyps, secondary changes to radiotherapy or operation. Lots of benign conditions like ours and physiologic FDG uptakes exhibiting focal or diffuse FDG accumulations in gastrointestinal tract can be seen in patients with CRC during the follow-up and confused with true pathologic lesions. It is essential to distinguish them by colonoscopic biopsy. Previously some quantitative parameters based on volume-of-interest FDG uptake were introduced to augment its diagnostic accuracy in several cancers. SUVmax is the first one. Determination of a cut-off level of SUVmax which differentiates between benign conditions and recurrence would certainly be helpful in CRC.

We investigated the value of SUVmax for the discrimination between the benign and malign conditions. Gade et al. [7] found a lower mean SUVmax of 8.6, Marcus et al. [26] of 7.3 in recurrent CRC when compared to our mean SUVmax of 12.7. Shamim et al. [27] found a significant increase of mean SUVmax in recurrence (11.8 for recurrence versus 3.7 for benign conditions) in a study on 32 patients with CRC. These values were 11.7 for the recurrence against 7.2 for the benign group in our study and this difference was statistically significant. Our results revealed that SUVmax was very helpful in the differentiation of the recurrent disease from benign conditions and it improved the diagnostic accuracy of FDG-PET/CT. For an estimated cut- off value of 6.3 and 12.5 on ROC curve, the calculated specificities were 61% and 87%, respectively. According to our findings, SUVmax was very beneficial for increasing the specificity when compared with that of FDG-PET/CT alone (56.5%).

Several studies reported that neighboring organ invasion and depth of tumor infiltration were significant prognostic factors for postoperative recurrence and survival rate in patients with CRC undergoing curative resection [23]. Although the depth of wall invasion by the primary tumor is an important prognostic factor, we could not research it due to lack of sufficient data and this was a limitation in our study. Small patient number and study design were also inevitable limitations. Ideally, prospective studies with large numbers are needed. There was a slight selection bias for our patient population. Lack of some other risk factors (type of surgery, localization of metastasis, especially cytogenetic factors) affecting recurrence/metastases are the other limitations.

Conclusion

ILNM and high SUVmax values on the control or restaging FDG-PET/CT are the main risk factor in recurrent CRC and patients with these risk factors must be followed up carefully. FDG-PET/CT is very sensitive for the detection of recurrence/metastases and SUVmax appears to improve its specificity.

Conflict of Interest

No conflict of interest was declared by the authors.

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Comorbidity as a factor of prognosis in patients with locoregionally advanced, inoperable squamocellular head and neck cancers

Komorbiditet kao prognostički faktor kod bolesnika sa lokoregionalno uznapredovalim, inoperabilnim planocelularnim karcinomima glave i vrata

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Abstract

Background/Aim. Patients (pts) with tumors often have other diseases or conditions in addition to their index cancer which are generally referred to as comorbidities. Due to the fact that well known risk factors for development of squamocellular head and neck cancers (HNSCC) such as tobacco and alcohol abuse can also damage other important organs, pts with this type of cancer are suitable for analyzing the influence of comorbid conditions on prognosis of the disease. The aim of our work was to assess the prevalence of comorbidities, most frequent conditions and their prognostic impact on overall survival in this particular population. **Methods.** Between July 2002 and January 2007 in the Institute for Oncology and Radiology of Serbia, 100 pts with locoregionally advanced, inoperable HNSCC were initially treated with neoadjuvant chemotherapy regimen, cytarabine-5 fluorouracil-cisplatinum. Median age of pts was 55 years, most of them (91%) were males with median number of applied chemo cycles being 4. Data on comorbidities were collected in prospective manner from various sources prior to the treatment. For grading of the severity of comorbid conditions, the Adult Comorbidity Evaluation 27 (ACE-27) comorbidity index was used with four degree scale (0–3). The average follow-up of pts was 15 months with range from 3–59 months. **Results.** Comorbidities were present in 69 (69%) pts, and 31 (39%) pts had no comorbidities prior to the treatment. Among pts with comorbid conditions prevailed alcoholics, active and former (71%), pts with chronic lung diseases (25%) and cardiovascular diseases (18%). Overall comorbidity score was defined according to the highest ranked single ailment, except in the case where two or more grade 2 ailments occured in different organ systems in which case the overall comorbidity score was designated as grade 3. Median overall survival for the whole group was 12 months. Median ACE-27 score was grade 1 (range 0–3) which was observed in 43 (43%) pts. Pts without comorbidities survived significantly longer than those with any kind of comorbidity ($p = 0.0089$), and the same was observed comparing survival of pts without comorbidities and those with comorbidity index 2 and 3 taken together ($p = 0.0047$). Results of other intergroup comparisons were of no statistic significance. **Conclusion.** Comorbidity is important prognostic variable in patients with locoregionally advanced HNSCC and should be properly assessed prior to therapy.

Key words:
carcinoma, squamous cell; head and neck neoplasms; comorbidity; prognosis; survival.

Apstrakt

Uvod/Cilj. Kod bolesnika sa malignim bolestima često se javljaju druga oboljenja ili stanja, nevezana za sam tumor koja se zajednički nazivaju komorbiditetom. Zahvaljujući činjenici da poznati faktori rizika od nastanka planocelularnih karcinoma glave i vrata, kao što su zloupotreba duvana i alkohola, mogu dovesti do oštećenja drugih važnih organa, bolesnici sa ovom vrstom tumora pogodni su za analizu uticaja komorbidnih stanja na prognozu maligne bolesti. Cilj rada bio je procena prevalence komorbiditeta, i najčešćih komorbidnih stanja kao i njihovog uticaja na prognozu preživljavanja u ovoj populaciji bolesnika. **Metode.** U periodu od jula 2002. godine do januara 2007. Godine, 100 bolesnika sa lokoregionalno uznapredovalim, inoperabilnim, planocelularnim karcinomima glave i vrata lečeno je na Institutu za onkologiju i radiologiju Srbije neoadjuvantnom hemioterapijom u sastavu: citarabin-5 fluorouracil-cispaltin. Medijana starosne dobi obolelih bila je 55 godina. Ispitanici su bili najvećim delom muškarci (91%), sa prosečno 4 primenjena hemioterapijska ciklusa. Podaci o komorbidnim stanjima prikupljani su prospektivno korišćenjem različitih izvora podataka i to pre početka samog lečenja. Za procenu ozbiljnosti komorbidnih stanja korišćen je ACE-27 komorbidni indeks (*Adult Comorbidity Evaluation* 27) sa četvorostepenom

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skalom (0–3). Prosečno vreme praćenja bolesnika iznosilo je 15 meseci, sa rasponom od 3 do 59 meseci. **Rezultati.** Komorbiditet je bio prisutan kod 69 (69%) bolesnika, dok 31 (31%) bolesnik nije imao komorbidnih stanja pre početka lečenja. Najčešća komorbidna stanja odnosila su se na alkoholizam, aktivni ili raniji (71%), zatim hronične plućne bolesti (25%), kao i kardiovaskularne bolesti (18%). Ukupni komorbidni zbir bio je određen na osnovu najviše rangiranog opserviranog komorbidnog stanja, osim u slučaju gde su opsrevirana dva ili više oboljenja gradusa 2, u različitim organima, u kom slučaju je ukupni komorbidni zbir iznosio 3. Prosečno preživljavanje za celu grupu bolesnika iznosilo je 12 meseci. Srednji ACE-27 skor iznosio je 1 sa rasponom od 0 do 3 i bio je prisutan kod 43 (43%) bolesnika. Bolesnici bez komorbiditeta imali su značajno duže preživljavanje u odnosu na one sa bilo kojom vrstom komorbiditeta ($p = 0.0089$), a isto je bilo zapaženo poređenjem preživljavanja kod bolesnika bez komorbiditeta u odnosu na bolesnike sa komorbiditetima gradusa 2 i 3, uzeto zbirno ($p = 0.0047$). Rezultati ostalih poređenja između grupa nisu bili statistički značajni. **Zaključak.** Komorbiditet je važan prognostički parametar kod bolesnika sa lokoregionalno uznapredovalim planocelularnim karcinomima glave i vrata, pa se savetuje odgovarajuća procena pre početka lečenja.

Ključne reči:
karcinom, planocelularni; glava i vrat, neoplazme; komorbiditet; prognoza; preživljavanje.

Introduction

Head and neck squamous cell carcinomas (HNSCC) include a wide range of malignant tumors that originate in the different structures of this region of the body. This tumor type is the sixth most common malignancy worldwide, accounting for about 6% of all cancer cases and responsible for an estimated 1–2% of all cancer deaths. Oral cavity and laryngeal cancers are the most frequent globally, with age-adjusted standardized incidence rate of 3, 9 and 2.3 per 100,000, respectively [1, 2]. Similar situation is in Serbia with HNSCC incidence rate of 7% and larynx cancer as the most common entity [3].

Although not leaders in the field of oncology, HNSCC are important because their presentation can cause aesthetic alterations of the face and neck with disturbance of vital functions such as a breathing, swallowing, phonation and hearing. Common risk factors for the development of HNSCC include male gender, advanced age, smoking habits, alcohol consumption and human papillomavirus (HPV) infection. Most of the cases are still attributed to heavy smoking and alcohol abuse, and majority of them (60%) initially present with locoregional disease where therapy consists of surgery, radiotherapy or their combinations while inoperable patients are offered chemoradiation as a standard treatment. When therapeutic goal is organ preservation, reliable option might be neoadjuvant chemotherapy which is followed by definitive irradiation [4].

Besides, HNSCC, tobacco and alcohol use is also associated with the development of other medical conditions ranging from cardiovascular to psychiatric disorders. So, many middle-aged patients with head and neck cancers present with a variety of coincident diseases which are known as comorbidities [5]. From practical point of view, comorbidity in oncology could be defined as any co-existing medical disorder unrelated to the index cancer. The concept of comorbidity and its prognostic importance was first developed from early works of Alvin Feinstein [6] who first had studied the influence of comorbid conditions in patients with diabetes mellitus. Although not a feature of cancer itself, comorbidity is a relevant attribute of the patient and may adversely affect his/her quality of life and survival [7–9]. It is common among patients with HNSCC and incidence of moderate and serious conditions in this particular population is about 25% portending higher mortality risk [10, 11]. So, careful pre-therapy assessment of comorbidity should help in treatment decision making and proper utilization of health care resources.

Our study focused on the impact of patient conditions classified using the Adult Comorbidity Evaluation 27 (ACE-27) index in population with locoregionally advanced, inoperable head and neck cancers treated initially with neoadjuvant chemotherapy. Our aim was to evaluate the prevalence of comorbidities, most frequent conditions among them and their prognostic influence on overall survival in this group of patients.

Methods

This study represents the single institution experience. The research was conducted in the Institute for Oncology and Radiology of Serbia, Belgrade, on the sample of 100 patients with locoregionally advanced, inoperable HNSCC, during the period from July 1, 2002 – January 1, 2007. All of patients were initially treated with neoadjuvant chemotherapy. The study was approved by the institutional Ethics Committee.

Before treatment, the patients were presented to multidisciplinary oncology team for proper decision making.

In this prospective, randomized, clinical study, patients received neoadjuvant chemotherapy with 3 drugs. Randomisation was performed electronically, and the first group received cytosine-arabinoside in a dose of 500 mg/ m^2 (D1), 5 fluorouracil in a dose of 750 mg/m^2 (D1-5) given as a 4-hour infusion, and cisplatin in a dose of 120 mg/m^2 (D1). The second group received the same regimen with 5 fluorouracil given continuously (120 hours). All drugs were applied intravenously.

The aims of the study were to assess the overall survival and efficacy of therapeutic regimens. Surveillance period was from July 1, 2002 – January 1, 2007.

Inclusion criteria were: histologically confirmed squamocellular carcinoma, locoregionally advanced, inoperable disease, patients' age 18–75 years, performance status 0–2 [Eastern Cooperative Oncology Group (ECOG) scale], ex-

pected survival more than 3 months, the presence of at least one measurable lesion, the absence of previous chemotherapy and preserved hematological, renal and hepatic functions.

Maximal number of cycles was 6 and assessment was done before every odd cycle and four weeks after completion of the 6-th cycle.

Depending of response, patients proceeded with definitive radiotherapy with TD 60–70 Gy or surgery and radiotherapy. In case of progression, patients were given palliative chemotherapy or symptomatic treatment.

Data on comorbidity were collected prospectively from previous medical reports and opinions, discharge lists from various hospitals, reports from retirement commissions, and reports from penitentiary and detention institutions.

The instrument to measure the severity of comorbidity was ACE-27 index, a 27-item index developed through modification of the Kaplan-Feinstein Comorbidity index (KFI) which classified specific diseases and conditions into four groups: none, mild, moderate and severe according to severity of the organ decompensation. Overall comorbidity score was defined according to the highest ranked single ailment, except in the case where two or more grade 2 (moderate) ailments occured in different organ systems. In this situation, the overall comorbidity score was designated as grade 3 (severe).

The analyzed comorbid conditions had to be present before starting chemotherapy in order to avoid false conclusions concerning possible toxicity of therapy itself. Survival was calculated from the first day of chemoapplication until death or loss from surveillance, and data about death were obtained from hospital or communal death registries.

The Kaplan – Meier method and Log-rank test were used for survival analysis. The Cox proportional hazards regression was used to investigate the independent effects of ACE-27 scoring on survival.

Results

Within a period of 54 months we analyzed 100 patients with a poor prognosis HNSCC of whom 91 were men and 9 women. The median age for the whole group was 55 years (range 37–75 years).

Tumors were dominantly located in the hypopharynx (46%), then oropharynx (30%) and larynx (20%) while epipharyngeal and sinonasal tumors much less occurred (4%). Most tumors were locally advanced either T3 (21%) or T4 (68%) and regarding the nodal neck metastases most patients had high nodal volume disease – N3 (62%), while bilateral neck metastases – N2C were found in 38 (38%) patients. Tumor tissue in over two thirds (72%) of the patients was of moderately differentiated histological grade (grade 2).

Chemoregimen with continuous 5 fluorouracil infusion was given to 59 (59%) patients while 41 (41%) of them received short infusion (4 hours). The median number of given cycles was 4 (range 1–6).

The response rate [complete response (CR) + CR + partial response (PR)] with therapy was achieved in 45 (45%) patients and 44 (44%) patients (44%) progressed during the chemotherapy. Disease control rate [CR + PR + stable disease (SD)] was observed in 56 (56%) patients (Table 1).

Table 1
Characteristics of patients, disease and treatment regimens

Characteristics of patients	Values
Number (%) of patients	100 (100)
Age (years), median range	55 (37–75)
Gender, n (%)	
male	91 (91)
female	9 (9)
Localisation of tumours, n (%)	
epipharynx	3 (3)
oropharynx	30 (30)
hypopharynx	46 (46)
larynx	20 (20)
cavum nasi	1 (1)
TNM category (T), n (%)	
T1	1 (1)
T2	10 (10)
T3	21 (21)
T4	68 (68)
TNM category (N) , n (%)	
N2c	38 (38)
N3	62 (62)
Histological grade, n (%)	
1	8 (8)
2	72 (72)
3	20 (20)
Therapeutic regimens, n (%)	
Car 500-5FU-CDDP (short infusion - 4h)	41 (41)
Car 500-5FU-CDDP (continuous infusion)	59 (59)
Number of cycles, median (range)	4 (1–6)

TNM – tumor (T), node (N), metastasis (M); Car – cytosine arabinoside; 5FU – 5 fluorouracil; CDDP – cisdiaminodiplatin; n (%) – number (percentage) of patients.

The median time of surveillance was 12 months. Efficacy of the treatment is presented in Table 2.

Table 2
Efficacy of treatment

Maximal therapeutic response	Patients, n (%)
CR	4 (4)
PR	41 (41)
SD	11 (11)
PD	44 (44)
DCR (CR+PR+SD)	56 (56)

CR – complete remission; PR – partial remission; SD – stable disease; PD – progressive disease; DCR – disease control rate.

About one third (31%) of the patients was without comorbid conditions while 69 (69%) patients had some kind of concomitant disorders. The most frequent among them was alcoholism (active and previous) which was present in 49 (71%) patients. The next category of comorbid conditions belongs to the chronic pulmonary diseases and was noted in 25 (36%) patients, and the third were cardiovascular diseases present in 18 (26%) patients with arterial hypertension as a leading condition. Among gastrointestinal disorders, chronic hepatic disease was present in 9 (13%) patients, and diabetes mellitus was present in 10 (14%) patients. The other comorbid conditions were encountered much less frequent (Table 3).

Table 3
The most prevalent comorbid conditions in the study

Comorbidities	Patients, n (%)
Alcoholism	49 (71)
active	10 (14)
former	39 (57)
Chronic pulmonary diseases (obstructive/restrictive)	25 (36)
mild	20 (29)
moderate	5 (7)
Cardiovascular diseases	18 (26)
arterial hypertension	9 (13)
DVT	4 (6)
rhythm disturbances	4 (6)
myocardial infarction	1 (1)
Gastrointestinal diseases	16 (23)
chronic liver disease	9 (13)
stomach ulcer	5 (7)
chronic pancreatitis	2 (3)
Diabetes	10 (14)
Cerebrovascular diseases	3 (4)
Neuromuscular diseases	2 (3)
Mental problems	1 (1)

DVT – deep venous thrombosis.

The comorbidity was measured using ACE-27 index with most frequently observed score being grade I (mild) in 43 % of patients, while grades II (moderate) and III (severe) were present in 20% and 6% of them, respectively (Table 4).

Table 4
ACE-27 comorbidity score

ACE-27 score	Values
Median (range)	1 (0-3)
None, n (%)	31 (31)
Mild, n (%)	43 (43)
Moderate, n (%)	20 (20)
Severe, n (%)	6 (6)

ACE – Adult Comorbidity Evaluation; n (%) – number (percentage) of patients.

Median overall survival for the whole group was 12 months (Figures 1–3).

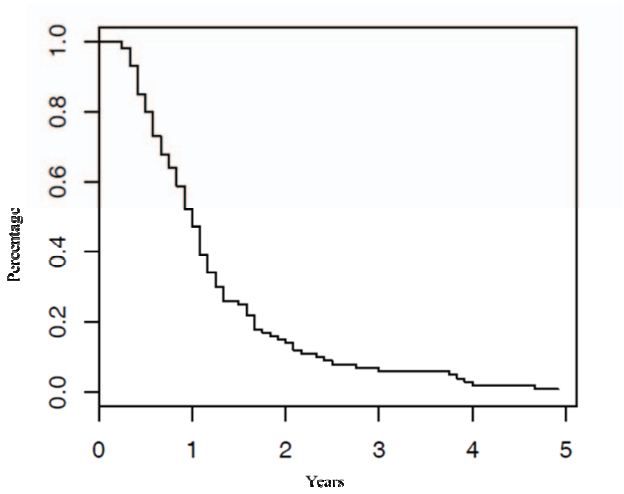

Fig. 1 – Overall survival.

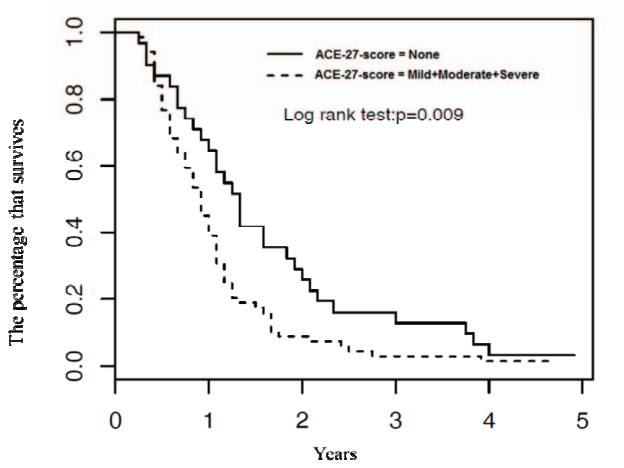

Fig. 2 – Overall survival (OS) and presence/absence of comorbidity according to ACE-27 index.

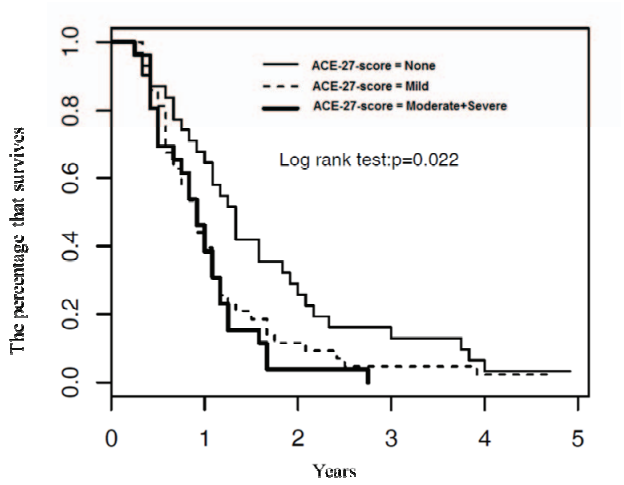

Fig. 3 – Overall survival and categories of ACE-27 score.

Patients without comorbidities survived significantly longer (16 months vs. 11 months) in comparison with their counterparts who had any kind of a comorbid condition ($p = 0.0089$).

Comparing the different pairs of comorbidity categories, there was no significant difference in overall survival

between patients without comorbidities and those with mild ones. The same situation was between those with mild comorbidities and patients with moderate and severe conditions taken together. However, patients without comorbidities survived longer in comparison to those with moderate and severe conditions taken together ($p = 0.00474$) (Tables 5 and 6).

Table 5
Overall survival (OS) and relation to ACE-27 score

ACE-27 score	OS (months), median (95% CI)
None	16 (12-24)
Mild	11 (8-13)
Moderate + severe	111 (8-14)

ACE – Adult Comorbidity Evaluation; CI – confidence interval.
***Log-Rank test: $\chi^2_2 = 7.65$; $p = 0.02181$.**

Table 6
Overall survival between pairs of ACE-27 score categories

Pairs	Log-rank test	
	χ^2_1	p
None vs. Mild	$\chi^2_1 = 4.199$	$p = 0.04044$
None vs. Moderate + severe	$\chi^2_1 = 7.975$	$p = 0.00474$
Mild vs. Moderate + severe	$\chi^2_1 = 0.416$	$p = 0.51872$

ACE – Adult Comorbidity Evaluation,
***Bonferroni correction: 0.05/3 = 0.0167.**

Discussion

Being the most respected clinical skill in the past centuries, medical prognosis regained its importance in the present era of personalized medicine.

The influence of comorbidity on survival in patients with cancer may be direct in relation with heavier disease burden, or indirect through the choice and timing of proper anticancer treatment thus many recent studies show the clinical significance of comorbid conditions in oncology [12–14].

Because of toxic effects of tobacco and alcohol, patients with head and neck cancers are especially likely to have various comorbid health conditions thus serving as a good model for investigating the relationship between comorbidity and survival. Indeed, in the past 15 years many authors have confirmed strong prognostic impact of associate illnesses in head and neck oncology [10, 15].

Our study is unique in a way that it dealt exclusively with patients who had advanced, inoperable cancers initially treated with neoadjuvant chemotherapy. Most of them were predictably males. As one may expect, majority of patients had their primaries in hypopharynx, oropharynx and larynx with N3 nodal stage, which is in accordance with most recent epidemiological data [1].

The median time of surveillance in our series was 15 months with range of 3–59 months which is much longer than in the pivotal work of Piccirillo [5]. Such a follow-up can provide a fair amount of information.

In our study alcoholism (former and active) prevailed, being present in 71% of patients with comorbid conditions which could be explained by the fact that alcohol abuse may lead to addiction but also it is the causative factor in most of HNSCC cases. The other reason might be the specific separation and inclusion of this condition in the ACE-27 scoring index. The second most prevalent group of conditions were pulmonary diseases observed in 36% of our population, while cardiovascular diseases with preponderance of the arterial hypertension were on the third place (26%). Of note was the absence of angina pectoris among our patients for which we do not have a plausible explanation. So, alcoholism aside our data are in concordance with results of Piccirillo and Vlahiotis [11]. The most common score of comorbidity in our patients was grade 1 (mild) present in 43% of cases, while grades 2 (moderate) and 3 (severe) were present in about quarter of patients. In Datema et al. [16] large study, results were partly different with smaller percentage of mild comorbidity (17%), while moderate (13,5%) and severe grades (6%) were much more in accordance with our results. However, among patients in that study the vast majority (74%) had the localized or locoregional disease (TNM stages I and II) where prognosis was better and presence of comorbid ailments was not so prominent.

In our series, 31% of patients were without any kind of comorbid diseases, and quite predictably they survived significantly longer compared with their counterparts with some kind of comorbidity ($p = 0.0089$). Similar results although on far larger scale were obtained by Land et al. [17] and Patnaik et al. [18] analyzing more than 60,000 women with breast cancer where those without comorbidities had much better overall survival. Another statistically significant finding in our study referred to the fact that patients without comorbidities fare better in comparison to those with moderate and severe conditions taken together ($p = 0.00474$). In statistical sense there was no difference in survival between patients with severe comorbidities and those with mild or moderate comorbidities. Although somehow paradoxical, this fact could be explained by the very small number of our patients with severe conditions (6%) which is not sufficient for any firm conclusion regarding the survival of this sole category. So, the only way to overcome this problem is to conduct large clinical studies properly designed to measure and explore the impact of various categories of comorbidity on survival of the patients with HNSCC.

The main limitations of our study are that it reflects the experience of patients at only one large academic institution, analyzing the only one end point – overall survival. This is important because the patients treated in academic centers are in general younger, healthier and with smaller tumors. On the other hand, dealing exclusively with survival, other pertinent end points as quality of life may be missed.

Although it is well known fact that the relative impact of comorbidity tends to be greater for cancers with better prognosis, we demonstrated that even in poor prognostic population this prognostic variable still holds its importance.

Conclusion

The presence of comorbid illnesses is a frequent finding in patients with HNSCC. The most prevalent conditions in our study are alcoholism, chronic pulmonary disorders and cardiovascular disorders. Patients without comorbidities survive significantly longer in comparison to patients with some kind of associated ailment, particularly of moderate and severe intensity. So, proper measurement of comorbid conditions in patients with HNSCC before treatment is strongly recommended.

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Steep keratometry and central pachymetry after corneal collagen cross-linking procedure in patients with keratoconus

Strma keratometrija i centralna pahimetrija nakon kornealne kolagen *cross-linking* procedure kod bolesnika sa keratokonusom

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Abstract

Background/Aim. Keratoconus is a disorder of the eye which results in progressive thinning of the cornea. The cross-linking procedure (CXL) is applied in the treatment of initial progredient forms of keratoconus. It is aiming at increasing biomechanical stability of corneal stromal tissue to slow down or stop progressing the ectatic disorder. The aim of the study was to examine the effect of CXL procedure on values of steep keratometry (K2) and central pachymetry (CCT) six months after the intervention in keratoconus-affected patients. **Methods.** Clinical prospective cohort study included 30 eyes of 29 patients suffering from keratoconus. All patients were examined on Allegro-Oculyzer in order to diagnose and follow up keratoconus, thus obtaining corneal topography parameters and parameters important for this study: K2 and CCT, preoperatively and six months postoperatively. The CXL procedure was carried out by following the modified Dresden Protocol. **Results.** K2 mean value was 49.01 ± 3.99 diopter (Dpt) preoperatively and 48.06 ± 4.46 Dpt six months postoperatively. K2 decreased six months postoperatively by 0.95 Dpt, proportionally in all patients. Student's paired sample *t* test showed that average decrease of K2 ($\bar{d}$ = 0.95 Dpt) was highly statistically significant (t = 3.381; $p < 0.01$). CCT mean value was 480.17 ± 36.62 μm preoperatively and 444.37 ± 45.01 μm six months postoperatively. CCT decreased six months postoperatively by 35.8 μm, proportionally in all patients. Student's paired sample *t* test showed that average decrease of CCT ($\bar{d}$ = 35.8 μm) was highly statistically important (t = 6.40; $p < 0.001$)). **Conclusion.** Application of CXL procedure in the treatment of keratoconus with confirmed progression highly reduces steep keratometry and central pachymetry six months postoperatively. By steep keratometry reducing effect the CXL procedure is efficient in the treatment of keratoconus, especially its initial stages.

Key words:
keratoconus; corneal topography; corneal pachymetry; collagen.

Apstrakt

Uvod/Cilj. Keratokonus je oboljenje oka koje uzrokuje progresivno tanjenje kornee. “Cross-linking” (CXL) procedura primenjuje se u lečenju početnih progredijentnih formi keratokonusa. Ona ima za cilj da pojača biomehaničku stabilnost tkiva strome rožnjače čime usporava, odnosno zaustavlja progresiju ektatičnog procesa. Cilj rada bio je ispitivanje uticaja CXL procedure na vrednosti strme keratometrije (K2) i centralne pahimetrije (CCT) šest meseci posle izvedene intervencije kod bolesnika obolelih od keratokonusa. **Metode.** U kliničku prospektivnu kohortnu studiju bilo je uključeno 30 očiju (29 bolesnika) obolelih od keratokonusa. U cilju dijagnostike keratokonusa i daljeg praćenja, svakom bolesniku obavljen je pregled na Allegro Oculyzer-u kojim su dobijeni parametri kornealne topografije i za studiju bitni parametri: K2 i CCT, preoperativno i šest meseci postoperativno. CXL procedura izvedena je po modifikovanom Drezdenskom protokolu. **Rezultati.** Srednja vrednost K2 preoperativno iznosila je 49,01 ± 3,99 dioptrija (Dpt), dok je

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šest meseci postoperativno bila 48,06 ± 4,46 Dpt. Prosečno kod svih bolesnika šest meseci postoperativno došlo je do smanjenja vrednosti K2 za 0,95 Dpt. Student-ov *t*-test vezanog uzorka pokazao je da za prosečno smanjenje vrednosti K2, d = 0,95 Dpt, postoji visoko statistički značajna razlika ($t = 3,381$; $p < 0,01$). Srednja vrednost CCT preoperativno bila je 480,17 ± 36,62 μm, dok je 6 meseci postoperativno iznoila 444,37 ± 45,01 μm. Prosečno, kod svih pacijenata 6 meseci postoperativno došlo je do smanjenja vrednosti CCT za 35,8 μm. Student-ov *t* test vezanog uzorka pokazao je da za prosečno smanjenje vrednosti CCT od d = 35,8 μm postoji vrlo visoka statistička značajnost ($t = 6,40$; $p < 0,001$). **Zaključak.** CXL procedura u lečenju keratokonusa sa dokazanom progresijom, dovodi do značajnog smanjenja strme keratometrije i značajnog smanjenja centralne pahimetrije šest meseci postoperativno. Efektom smanjenja strme keratometrije CXL procedura je efikasna u lečenju keratokonusa, posebno njegovih početnih stadijuma.

Ključne reči:
keratokonus; kornealna topografija; kornea, pahimetrija; kolagen.

Introduction

Keratoconus is a non-inflammatory ectatic corneal disease which is characterized by biomechanical weakness of stromal tissue causing progressive corneal thinning, resulting in irregular conical corneal shape. Keratoconus is featured with central and paracentral stromal thinning, apical protrusion and irregular astigmatism. This disease is mostly bilateral, with 1 : 2,000 prevalence, and it affects young working people with deterioration of visual acuity caused by irregular astigmatism. Etiology of the disease is unclear, and heredity exists only in 10% of cases [1]. There are different therapeutic options. Rigid contact lenses or implantation of intrastromal corneal ring segments can be applied in initial stages of the disease. Keratoplasty is performed in terminal stages due to extreme corneal steepening and scarring to achieve visual rehabilitation [2].

Corneal collagen cross-linking (CXL) procedure with the use of riboflavin and ultraviolet-A (UVA) irradiation is a new surgical method in keratoconus treatment. Structural abnormalities in stromal collagen are the cause of deformity and thinning of cornea suffering from keratoconus. CXL procedure directly strikes these abnormalities by using UVA irradiation and photosensitizer riboflavin, thus creating new covalent bonds (cross-links) between collagen fibers aiming at improving rigidness and biomechanical stability of cornea. This procedure helps stopping further progression of the ectatic process [3], clinically manifested with decreasing of steep keratometry (K2) and therefore improving of visual acuity.

This study is aiming at examining the impact of CXL procedure on values of K2 and central pachymetry (CCT) in patients with keratoconus 6 months after the intervention.

Methods

A clinical, prospective cohort study was carried out. It included 30 eyes of 29 patients (19 males, 10 females) suffering from keratoconus. In one patient both eyes were operated on. The average age of patients was 32 (32.40 ± 12.24) years.

All patients were examined on Allegro-Oculyzer (Wavelight, Germany) in order to diagnose keratoconus, thus also providing corneal topography parameters: K2 and CCT. Complete ophthalmologic examination of all patients was performed (automatic refractokeratometry, best corrected visual acuity, ocular tonometry, eye fundus observation). After diagnosing keratoconus and confirming the disease progression (increased K2 values in comparison to previous examinations), respecting a condition that central corneal thickness should not be below 400 μm after corneal epithelium removal in order to avoid endothelial cell damage during the intervention, all eyes diseased underwent the CXL procedure.

The CXL procedure was carried out by following the modified Dresden Protocol [4]. In a sterile environment of the operating room, after applying a topical anesthetic (Benoxi® Unimed Pharma eye drops – sol. oxybuprocaine 4 mg/mL), corneal epithelium was removed within a 9 mm wide circular zone with hokey knife, rotating brush or excimer laser. A 0.1% riboflavin solution (10 mg riboflavin-5-phosphate in 10 mL dextran-T-500 20% solution) was applied topically every 2 minutes during 30 minutes. Central pachymetry was checked with Reichert iPac handheld pachymeter to be over 400 μm. Cornea was UVA irradiated (365 nm, 3.0 mW/cm^2) with UV lamp (UV-X 1000 IROC Innocross AG, Swiss) during the course of a 30 minute exposure. Riboflavin solution was applied to the cornea every 2 minutes during irradiation. At the end of the procedure, a combination of topical steroid and antibiotic drops (sol. tobramycin 0.3% + sol. dexamethason 0.1%; Tobradex®, Alcon) was administered, then followed by a bandage contact lens application which was removed the fifth postoperative day. Every patient was dispensing Tobradex® drops three times a day and Hylocomod® drops (sol. sodium hyaluronate 0.1%, Ursapharm) eight to ten times a day during a month after the intervention.

All the patients were examined on Allegro-Oculyzer six months after the intervention in order to provide corneal topography parameters: K2 and CCT. Complete ophthalmologic checkup of all patients was also performed.

Statistical data were processed with methods of descriptive and inferential statistics: mean, standard deviation, maximum and minimum range, mode and median for descriptive statistics, and Student *t*-test for analytical statistics.

Results

Parameters important for the study are presented in Table 1. K2 mean value was 49.01 ± 3.99 diopters (Dpt) preoperatively and 48.06 ± 4.46 Dpt six months postoperatively. K2 therefore decreased six months postoperatively by 0.95

Dpt proportionally in all patients. Student's paired sample *t*-test showed that average decrease of K2 (d = 0.95 Dpt) was highly statistically significant ($t = 3.38$; $p < 0.01$). CCT mean value was 480.17 ± 36.62 μm preoperatively, and 444.37 ± 45.01 μm six months postoperatively. CCT therefore decreased six months postoperatively by 35.8 μm, proportionally in all patients. Student's paired sample *t* test showed that average decrease of CCT (d = 35.8 μm) was highly statistically significant ($t = 6.40$; $p < 0.001$).

Figure 1 shows corneal topography parameters of patient number 15 before the CXL procedure, while Figure 2 shows corneal topography parameters of the same patient six months after the intervention. It can be observed that patient's K2 decreased by 1 Dpt and CCT decreased by 48 μm six months after the CXL procedure.

In our research there were not any particular complications in the course of the six month follow up. Yet there were a couple of things to consider when talking about early postoperative period. Firstly, there was a prolonged reepithelization in the patient whose epithelium was removed with rotational brush. Also, if we are talking about the other two methods of epithelium removal, in all the patients reepithelization was as expected and with mild discomfort.

Table 1

Parameters important for the treatment of keratoconus

Patient No	Gender	Age (years)	Epithelium removal method			Steep K (Dpt)		CCT (μm)	
			rotational brush	hokey knife	excimer laser	Preop.	6 months postop.	Preop.	6 months postop.
1	m	29	+			54.1	54.9	456	401
2	f	21		+		45.7	45.3	469	427
3	m	31			+	60.4	59.8	471	423
4	m	23		+		42.9	40.9	495	467
5	m	19		+		49.1	48.3	489	434
6	m	19		+		53.7	55.8	497	438
7	m	41		+		50.9	49.2	545	532
8	f	30		+		50.8	48.0	472	400
9	f	28			+	49.8	48.7	478	447
10	f	26		+		54.8	52.8	470	458
11r	f	29			+	51.5	50.2	490	442
12l	f	29			+	50.6	51.7	496	488
13	f	22		+		51.6	49.4	450	417
14	m	42		+		45.4	44.6	524	478
15	m	22			+	49.3	48.3	492	444
16	m	27		+		42.7	42.8	464	445
17	m	37		+		47.7	45.8	462	396
18	m	39		+		44.5	43.5	518	478
19	m	56		+		46.0	45.7	467	447
20	m	16		+		43.5	42.2	441	415
21	m	19		+		51.6	53.9	427	411
22	m	19		+		44.8	44.5	457	446
23	m	21		+		44.3	42.8	541	493
24	f	47		+		47.1	46.9	455	431
25	m	63		+		47.7	47.1	464	495
26	m	39		+		49.1	48.3	552	537
27	f	47		+		51.0	51.6	412	408
28	f	48		+		51.3	47.0	447	305
29	m	47		+		50.8	49.4	446	440
30	f	36		+		47.6	42.5	558	488
mean ± SD		32.40 ± 12.24				49.01± 3.99	48.06 ± 4.46	480.17 ± 36.62	444.37 ± 45.01

m – male; f – female; Steep K – steep keratometry; CCT – central pachymetry.

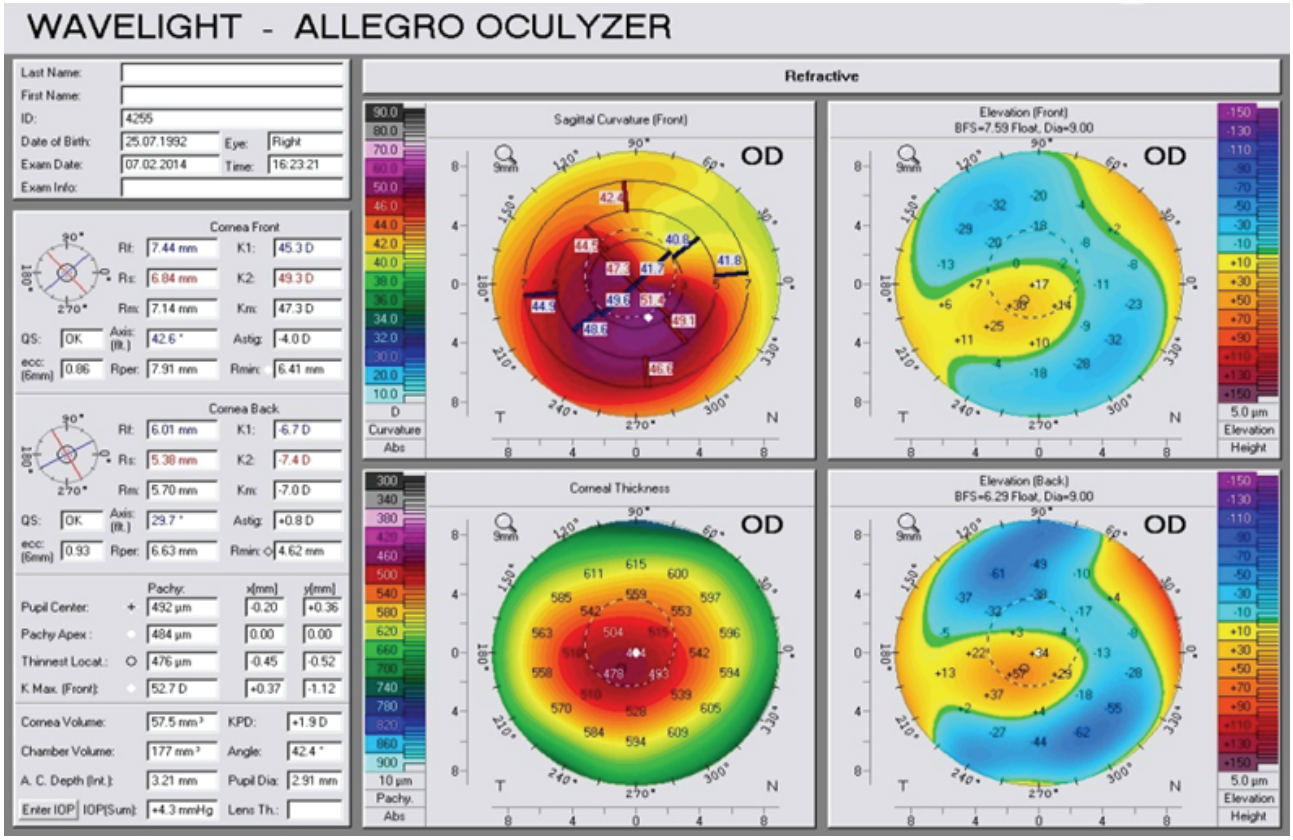

Fig. 1 – Corneal topography parameters before cross-linking (CXL) procedure (patient number 15).

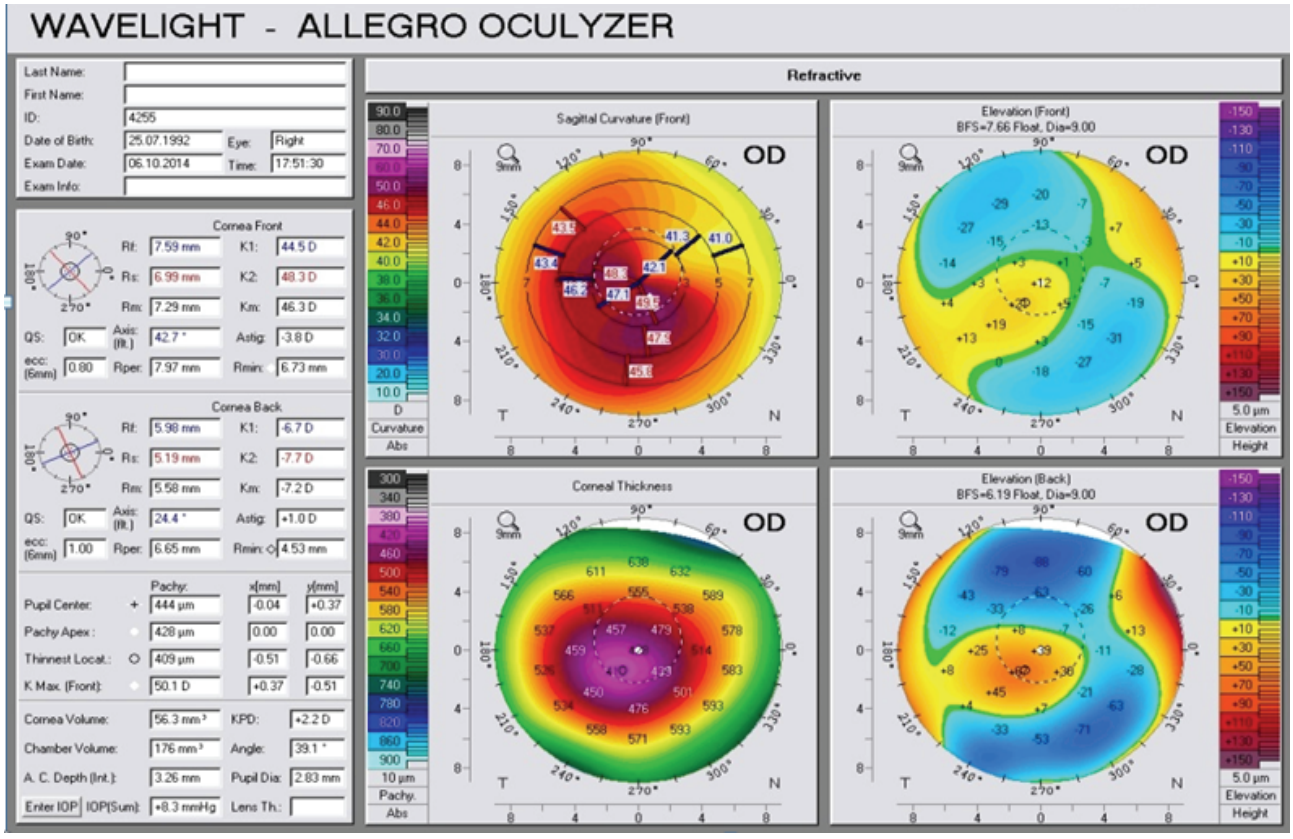

Fig. 2 – Corneal topography parameters 6 months after cross-linking (CXL) procedure (patient number 15).

Discussion

Our study showed that six months after the CXL procedure there was an average 0.95 Dpt reduction of K2 value. The present study also showed that six months after the CXL procedure there was an average 35.8 μm reduction of CCT value.

A study of Hersh et al. [5] showed that during the first month after the CXL procedure there was a rise of steep keratometry, while six months past the intervention there was a decrease of steep keratometry for 0.8 Dpt (from 52.9 Dpt preoperatively to 52.1 Dpt 6 months postoperatively). Koller et al. [6] indicated in their study, which included 151 eyes operated by the CXL procedure, that one year after the intervention there was a corneal flattening (change in maximum K value) for more than 1 Dpt in 37.7% of eyes and for more than 2 Dpt in 13% of eyes.

The study of Greenstein et al. [7] showed that there was corneal thinning within the first three months after the CXL procedure, while after six months preoperative pachymetric values resumed. In their study mean value of corneal thickness at the apex was 458.2 μm preoperatively, 437.8 μm one month postoperatively, 428.3 μm three months postoperatively and 446.3 μm six months postoperatively. The study of Sharma et al. [8] showed that central corneal thickness decreased by mean 22.7 μm six months after the CXL procedure.

The aim of the CXL procedure is to slow down or stop progressing the ectatic disorder on a keratoconus-affected cornea. This is clinically demonstrated by reducing K2 value and therefore reducing astigmatism and improving visual acuity.

Our study confirmed that by reduction in the value of K2, the CXL procedure is effective to stop or slow down further progression of the ectatic process. Our study also showed that the CXL procedure leads to a reduction in the value of CCT. Exact causes of corneal thinning are still unknown. They could be anatomic and structural changes in corneal collagen fibrils such as compression of collagen fibrils [9], and keratocyte apoptosis [10].

There were no complications in our study during the six month follow up. However, it is stated in the literature that the most serious complication of the CXL procedure (in 2.9% of patients) is endothelial loss leading to persistent corneal edema [11, 12]. We did not have this case because we followed the principle to leave central pachymetry greater than 400 μm after removing epithelium, which we checked with handheld pachymeter. As possible complications after the CXL procedure, other authors state sterile infiltrates in 7.6% of eyes and central stromal scars in 2.8% [13].

Conclusion

Application of the CXL procedure in the treatment of keratoconus with confirmed progression highly reduces steep keratometry and central pachymetry six months postoperatively. By steep keratometry reducing effect the CXL procedure is efficient in the treatment of keratoconus, especially its initial stages.

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Laparoscopic colorectal resection: short-term outcomes after 60 procedures – A single center initial experience

Laparoskopska hirurgija kolona i rektuma – naša iskustva nakon 60 učinjenih procedura

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Abstract

Background/Aim. Laparoscopic colorectal surgery is now widely accepted surgical method in the treatment of malignant and benign colorectal diseases. It is getting constantly more supporters due to its positive effects on enhanced patient recovery. The aim of this study was to determine the safety of minimally invasive approach as well as perioperative data, oncologic results and postoperative data. **Methods.** Prospective observational cohort clinical study was carried out at the Department for Colorectal and Pelvic Oncologic Surgery, First Surgical University Hospital, Clinical Center of Serbia, Belgrade. We analyzed demographics records concerning the type of surgery, clinicopathological features and oncological data for all operated patients. Records on early postoperative follow-up were also evaluated. **Results.** Laparoscopic colorectal resection was performed in 60 patients. Mean age of patients was 65 (29–87) years. Majority of patients were man, 37 (62%) of them. The most common indication was colorectal cancer (43 patients, 71.6%); 12 (20%) patients were operated due to the colorectal polyps unfitted for colonoscopic resection and 5 (8.3%) were operated due to Crohn's disease. Average number of lymph node harvested in patients with colorectal carcinoma was 22.5 (6–52). We achieved negative resection margins in all patients operated due to carcinoma. Mean duration of hospital stay was 5 (4–12) days. Postoperative complications were encountered in 5 (8.3%) patients. Overall mortality rate was 1.7% (1 patient died due to thromboembolism). **Conclusion.** This study showed that initiation of laparoscopic colorectal resection is feasible and safe with short hospital stay, adequate oncologic resection and number of lymph node harvested.

Key words:
colonic neoplasms; digestive system surgical procedures; laparoscopy; postoperative complications; rectal neoplasms.

Apstrakt

Uvod/Cilj. Laparoskopske operacije kolona i rektuma su široko prihvaćene i koriste se u tretmanu malignih i benignih oboljenja. Zbog značajno bržeg oporavka bolesnika sve veći broj hirurga uči i podržava ovu metodu. Cilj rada bio je da se utvrdi bezbednost minimalno invazivnog pristupa, kao i procena periopeativnih rezultata, onkoloških rezultata i postoperativnih podataka. **Metode.** Prospektivna opservaciona kohortna klinička studija je sprovedena na IV Odeljenju za kolorektalnu i pelvičnu onkološku hirurgiju Klinike za digestivnu hirurgiju – Prve hirurške Kliničkog Centra Srbije. Kod svih operisanih bolesnika analizirani su demografski podaci, patohistološke karakteristike tumora, vrsta hirurške intervencije, kao i rane postoperativne komplikacije. **Rezultati.** Laparoskopska kolorektalna resekcija je učinjena kod 60 bolesnika koji su imali maligne i benigne lezije. Njihova prosečna starost je iznosila 65 (29–87) godina. Operisano je 37 (62%) muškaraca i 23 (38%) žena. Zbog kolorektalnog karcinoma operisana su 43 (71,6%), zbog polipa 12 (20%) i Kronove bolesti 5 (8,3%) bolesnika. Kod bolesnika sa kolorektalnim karcinomom, prosečno je odstranjeno 22,5 (6–52) limfnih nodusa. Negativne hirurške margine su postignute kod svih bolesnika sa karcinomima. Dužina hospitalizacije je iznosila 5 (4–12) dana. Postoperativne komplikacije su zabeležene kod 5 (8,3%) bolesnika. Zabeležen je jedan smrtni ishod (1,7%) zbog tromboembolije. **Zaključak.** Ova studija je pokazala da se laparoskopska kolorektalna hirurgija može

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bezbedno izvoditi, uz nizak procenat postoperativnih komplikacija, kratko vreme boravka u bolnici, uz adekvatnu hirušku resekciju i broj odstranjenih limfnih nodusa.

Ključne reči:
kolon, neoplazme; hirurgija digestivnog sistema, procedure; laparoskopija; postoperativne komplikacije; rektum, neoplazme.

Introduction

Laparoscopic colorectal surgery is developing strongly, and is becoming the mainstay treatment option for colorectal cancer and benign colorectal diseases in developed countries. It has been recognized as a first treatment option for colorectal cancer according to some leading surgical associations. The first colorectal laparoscopic resection was reported by Jacobs et al. [1] in 1991, while Watanabe et al. [2] were first to report results of laparoscopic colorectal resection for colon cancer in 1993 [2].

In the development of the procedure laparoscopy was reserved for smaller, early cancers. For instance, starting in 1996, health insurance in Japan covered expanses of laparoscopic colorectal resection only for early stages cancer. With the advance of surgical technique and followed with technological innovations, laparoscopy was introduced for larger and advanced tumors, and currently is being recognized as equally effective to open colorectal resection even for this indication [3, 4]. According to Japan's National Registry for Colorectal Cancer, 40,000 colorectal resections are being performed yearly, which compeers number of open procedures. The trends are similar in Europe; for example, in Great Britain in 2012, 40% of colorectal resections were performed laparoscopically, comparing to only 5% in 2005 [5].

The reasons beyond the drawbacks in the development of laparoscopic colorectal surgery were technique difficulties, lack of clinical evidence, learning curve and fear of tumor spreading during laparoscopy. Current evidence, however, strongly suggest that there are no statistically significant differences between open and laparoscopic surgery regarding the incidence of tumor local recurrence, distant metastases or disease free survival [6–9].

Proper surgical training, as well as prior experience with open procedures must back up the initiation of laparoscopic colorectal surgery. The goal of this study was to present the initial experience of single institution with the special emphasis on safety ie. early complication rate analysis [5].

Methods

This prospective observational cohort clinical study was conducted at the Department for Polorectal and pelvic Sncologic surgery, First Surgical University Hospital, Clinical Center of Serbia, Belgrade starting from January 2015 till January 2018.

The study included 60 patients in whom laparoscopic colorectal resection was performed for benign and malignant colorectal diseases. The database was created and tracked prospectively and included: demographic data, records about surgical intervention, and in colorectal carcinoma cases, histological report which included TNM tumor stage, number of lymph nodes harvested and surgical margins analysis. For the purpose of this study 30 days follow-up data were analyzed with the intent of early postoperative complications evaluation.

Primary aim was safety of minimally invasive (MI) approach, while secondary aims were perioperative data (duration, blood lose), oncological results (number of lymph nodes) and postoperative data (excluding complications).

Prior to surgery all patients underwent diagnostic protocol, which included colonoscopy, rigid rectoscopy, abdominal and pelvic computed tomography and pelvic magnetic resonance imaging (MRI) scan for rectal carcinoma. The preoperative radiographic tumor stage was given for all patients with colorectal cancer, regarding the locoregional tumor status and presence of distant metastases.

All patients were properly informed about the surgical intervention and signed informed consent.

Preoperative bowel preparation was performed using the polyethylene glycol solutions. Prophylactic antibiotics and low molecular weight heparin were routinely employed.

Surgical technique

The patients were placed supine, with head down position. The peritoneal cavity was accessed with open Hasson approach and the carbon dioxide was insufflated, maintaining the intraabdominal pressure of 10–12 mmHg. In the case or colorectal cancer surgical resection was performed according to The American Joint Committee on Cancer (AJCC) recommendations [10].

In the case of right colectomy, extracorporeal hand sewn anastomosis was performed. In the case of left colon or rectal cancer intracorporeal anastomosis was performed using "double stapler technique".

All surgical specimens underwent detailed histopathological examination.

Postoperatively nasogastric tube was kept for couple of hours (until patients were full awake); peroral intake of clear fluids was initiated at the day of surgery, followed by soft food diet on the first postoperative day. Abdominal drain was extracted on the second postoperative day. First regular clinical check-up was conducted 30 days after surgery, earlier in case if patients reported any kind of digestive symptomatology. Operative morbidities were defined as complications that lead to prolonged hospitalization or any type of other medical intervention including reoperation, induced by operative treatment.

Morbidity was reported according to the National Cancer Institute Common Toxicity Criteria: grade I of postoperative complications – asymptomatic or mild symptoms (clini-

cal or diagnostic observations only); grade II – moderate (minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living - ADL); grade III – severe or medically significant but not immediately life threatening (hospitalization or prolongation of existing hospitalization indicated; disabling; limiting self-care ADL); grade IV – life-threatening consequences (urgent intervention indicated), and grade V – death [11].

Results

At the Department for Colorectal and Pelvic Oncological Surgery, 60 patients underwent laparoscopic colorectal resection in the observed period due to malignant and benign lesions. Mean age of patients was 65 (29–87) years. Majority of patients were man, 37 (62%) of them. The most common indication was colorectal cancer – 43 (71.6%), 12 (20%) patients were operated due to the endoscopically unresectable colonic polyp resection and 5 (8.3%) were operated due to Crohn's disease.

Detailed number and type of the surgical procedures performed is shown in Table 1.

Table 1
Types of laparoscopic procedures performed

Procedure	Patient n (%)
Low anterior resection	12 (20)
Low anterior resection + loop ileostomy	3 (5)
Right hemicolectomy	29 (48.3)
High anterior resection	8 (13.3)
Left hemicolectomy	8 (13.3)
Total	60 (100)

In patients with colorectal cancer, average number of lymph nodes harvested was 22.5 (6–52). Results of the histopathological analysis are shown in Table 2.

Table 2
Histopathologic characteristics of patients with colorectal cancer (n = 37)

Characteristics	Grade n (%)
T stage	
in situ carcinoma	5 (13.5)
1	6 (16.2)
2	8 (21.6)
3	16 (43.2)
4	2 (5.4)
N stage	
0	28 (75.7)
1	6 (16.2)
2	5 (13.5)
Number of retrieved lymph nodes, median (range)	22.5 (6–52)

T – tumor; N – node

Mean duration of the procedure was 182 min (range 120–270 min). The duration of the procedure was influenced by the learning curve, since the mean duration of the last 10 procedures was 155 min. Mean duration of ileocecal resection and right hemicolectomy was 169 min (range 120–252 min), for left hemicolectomy and high anterior resection 202 min (156–246 min) and for low anterior resection with or without ileostomy 232 min (192–270 min).

Among the 7 (7/67, 10.4%) patients who underwent laparoscopic conversion to open surgery, five conversions were performed due to huge body mass index (BMI), one because of the bowel distension caused by intestinal occlusion and one due to the peritoneal dissemination.

Mean duration of hospital stay was 5 (4–2) days.

Postoperative complications were encountered in 5 (8.3%) patients. Three patients were conservatively treated due to the postoperative bowel paresis (Grade III), one patient was reoperated due to colonic ischemia (Grade IV) and one patient suffered a myocardial infarction followed with massive mesentery thrombosis. This patient was reoperated and died on the 30th postoperative day due to another myocardial infarction (Grade V). Overall, mortality rate in this study population was 1.7%.

Discussion

This clinical study was performed in order to present initial experience in performing laparoscopic colorectal resections in the high volume center, specialized in colorectal cancer and pelvic oncology surgery, with a high number of oncological procedures performed by open surgery. Primary endpoint was safety of MI approach. Secondary aims were perioperative data (duration), oncologic results (number of lymph nodes) and postoperative data (excluding complications).

In this study 7 patients underwent conversion to open procedure (7/60), or 11.6% of overall procedures number, which is comparable with the literature results, especially those analyzing learning curve [12–14]. If we analyze the number of conversions to open surgery per year, there is a significant drop (3 conversions in first and second year, one conversion in third year). Five conversions were performed due to huge body mass index (BMI), one because of the bowel distension caused by intestinal occlusion and one due to the peritoneal dissemination. This can also be partially explained by a learning curve. It is now a standpoint that patients with high BMI and visceral adiposity have the biggest advantage with MI surgical treatment. However, one must observe that those patients are being operated only by the experienced (high volume) surgeons [15].

Incidence of complications is not statistically different when results of open surgery are compared with laparoscopic surgery [16, 17]. In this study early postoperative complications were observed in 5 (8.3%) patients. These results partly coincide with the ones reported in huge surgical series with MI colorectal resections such as study by Juo et al. [18] who have reported 19.8% of complications on 116,261 operated patients, or Kang et al. [19] who reported 24.1% complications rate on 43,165 patients. In the aforementioned studies mortality rates were 0.4 and 0.49%, respectively.

In our study population we did not encountered pulmonary complications, which coincide with the results of Owen et al. [20] who found significantly less pulmonary complications in patients who were treated laparoscopically opposed to the open surgery.

Average length of hospitalization was 5 (4–12) days, which is comparable with other studies with laparoscopic colorectal resections where average hospital stay duration is reported in range from 4 to 9.7 days [19–22]. The longest hospital stay (12 days) was observed in a patient who had ischemic damage of the colon postoperatively. The hospital stay for patients with postoperative intestinal paresis was 9–10 days.

Mean duration of the procedure was 182 min (range 120–270 min), which is in concordance with the other clinical studies, where mean duration was reported to range between 159 and 297 min [23, 24]. One important remark when it comes to the mean operative time should be taken into consideration. The procedures for the rectal cancer are more complex, and time consuming than those for the right or left colon. In our study mean operative time was longer for procedures conducted on the rectum than those conducted on the right colon. The operative time was, as expected, influenced with the learning curve, and was significantly shorter in last 10 procedures. Having this in mind, we should approach the results of study by Prakash et al. [22] who have reported the mean operative time of 297 min, but for rectosigmoidal cancer, and with the results from initial learning curve included. The shortest mean operative time was reported by Kiran et al. [24], 146 minutes, but their study included resection of the right and left colon.

Our study showed that laparoscopic colorectal resection is not inferior to the open procedure when it comes to oncologic issue. According to AJCC, one needs to harvest minimally 12 lymph nodes to have the proper tumor staging [25]. Average number of harvested lymph nodes in our study was 22.5 (ranging 6–25), which makes it sufficient enough.

The study limitations are small number of patients and a short follow-up interval. Another important limitation is absence of the control group, presumably in this case, patients with similar characteristic who were treated with open surgery.

Conclusion

This study showed that initiation of laparoscopic colorectal resection is feasible with low rate of postoperative complications, short hospital stay, adequate oncologic resection and number of lymph node harvested.

This is the reason why MI becomes a standard in the surgical treatment of colonic and rectal diseases.

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CASE REPORTS

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Aerobic physical exercise and prolactin levels in blood during breastfeeding in woman with Hashimoto's thyroiditis – A case report

Aerobno fizičko vežbanje i nivoi prolaktina u krvi tokom dojenja kod žene sa Hašimoto tireoiditisom

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Abstract

Introduction. The World Health Organization (WHO) exclusively recommends breastfeeding for the first six months of the newborn life. Many factors affect milk production. Physical exercise can significantly affect prolactin secretion in the blood. **Case report.** A respondent in this study was a primipara (33 years old) diagnosed with Hashimoto's thyroiditis and a singleton pregnancy. During pregnancy and after the childbirth, she continued with light to moderate physical exercise. During the first six months after the childbirth, the light to moderate intensity aerobic exercise had no negative impact on the blood level of prolactin and growth and development of the child. **Conclusion.** In this case study, light to moderate intensity aerobic exercise had no negative impact on the level of prolactin in the blood during the first six months after the childbirth in a woman with Hashimoto's thyroiditis.

Key words:
breast feeding; hypoparathyroidism; prolactin; exercise.

Apstrakt

Uvod. Svetska zdravstvena organizacija (SZO) preporučuje isključivo dojenje tokom prvih šest meseci nakon rođenja novorođenčeta. Mnogobrojni su faktori koji utiču na proizvodnju mleka. Fizičko vežbanje može značajno uticati na lučenje prolaktina u krvi. **Prikaz slučaja.** Ispitanica u ovoj studiji bila je prvorotka (33 godine) sa dijagnostikovanim Hašimoto tireoiditisom i jednoplodnom trudnoćom. U trudnoći i nakon porođaja nastavila je sa kontinuiranim vežbanjem lakog do umerenog intenziteta. Ustanovljeno je da ovakvo vežbanje, tokom prvih šest meseci nakon porođaja, nije imalo negativni uticaj na nivo prolaktina u krvi i rast i razvoj odojčeta. **Zaključak.** U ovoj studiji slučaja, aerobno vežbanje lakog do umerenog intenziteta nije imalo negativnog uticaja na nivo prolaktina u krvi tokom prvih šest meseci nakon porođaja kod žene sa Hašimoto tireoiditisom.

Ključne reči:
dojenje; hipotireoidizam; prolaktin; vežbanje.

Introduction

Breastfeeding is considered the most optimal nutrition of the newborn in the first months of his/her life [1]. The two major world organizations (World Health Organization – WHO and the United Nations Children's Fund – UNICEF) exclusively recommend breastfeeding during the first six months of the child's life, and possibly longer [1–3].

Due to insufficient information about the importance of natural nutrition, many women stop breastfeeding much earlier and switch to the artificial nutrition of their newborns [1]. In the world, the number of exclusively breastfeeding children up to the age of six months is 38%, while in Serbia this number was only 13.7% in 2010 [1].

Many factors have an effect on the secretion of breast milk. The main reasons why a mother can get into a state of losing milk are sudden weight loss, irregular diet, stress, or exposing to great physical effort [4–7]. Due to the justified fear of losing milk, many women give up recreational exercise after delivery [3, 8]. However, the ability to breastfeed primarily depends on the health status of the woman.

Maintaining proper lactation is significantly influenced by the functioning of the thyroid gland [9]. Any thyroid disorder may have a negative impact on breastfeeding, especially if the woman is exposed to a high physical burden [9]. In addi-

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tion, increased physical effort can lead to thyroid hormone disorder [10, 11].

Earlier studies were less concerned with the impact of high intensity exercise on the possibility of successful breast feeding in women athletes or in women with some chronic illness [6, 9, 12]. Some studies point out that high intensity exercise did not have a negative impact on breast feeding [12, 13,]. It is assumed that the examined women compensated for increased energy consumption due to exercises with higher intake of nutrients, thus excluding the possibility of loss of lactation. Besides, these studies point out that the research was performed on a small number of women who were previously physically active, so the results cannot relate to the general population [13].

Although it is known that the ability to breast feed is very important, as well as the number of feedings *per* day, it is very important that a woman who exercises after a delivery does that in a proper and safe manner [6, 7, 11, 14]. In this case study, a six-month physical exercise of a woman who has recently given birth was monitored. The significance of the study is reflected in the continuous moderate intensity aerobic exercise that continued after delivery, as well as the effect of exercise on lactation in the subject with diagnosed thyroid disorder.

Due to insufficiently studied impact of exercise training during the breast feeding period, there is fear of an early loss of milk in some women [5, 15]. Additionally, there are even less number of studies on the impact of high-intensity exercise in female athletes or women with certain chronic disease [9, 12, 13].

Case report

A respondent was a female athlete (33 years old) diagnosed with Hashimoto's thyroiditis and a singleton pregnancy. Her physical parameters were: height 176 cm, body weight 58.7 kg (before the pregnancy), 68.7 kg (before delivery) and 62.9 kg (after delivery). She recreationally competed in triathlon and marathon for many years. During pregnancy and after the childbirth, she continued with light to moderate intensity running. Hormone therapy in pregnancy was increased from 25 μg to 50 μg of levothyroxine sodium (Tivoral®, Galenika, serbia).

The research covered the period of six months after the childbirth. Before pregnancy and a week after the childbirth, changes in physical status were observed (Table 1). Volume of training was recorded on a daily basis (Figure 1). Analyses of the physical status and prolactin level in the blood were performed at the end of every month during the study period (Table 1). Two weeks after the childbirth, she started running. The number of trainings (3–7 times *per* week) depended on: leisure time, care of the newborn, housework and other obligations. The volume of training (5 km, 7 km or 10 km *per* day) was controlled by fitness monitor (Garmin Forerunner 310 XT). Regular checks of hormones (prolactin and thyroid hormones) and other parameters of the blood (iron, glucose, cortisol, leukocytes and feremia) were carried out in laboratory conditions.

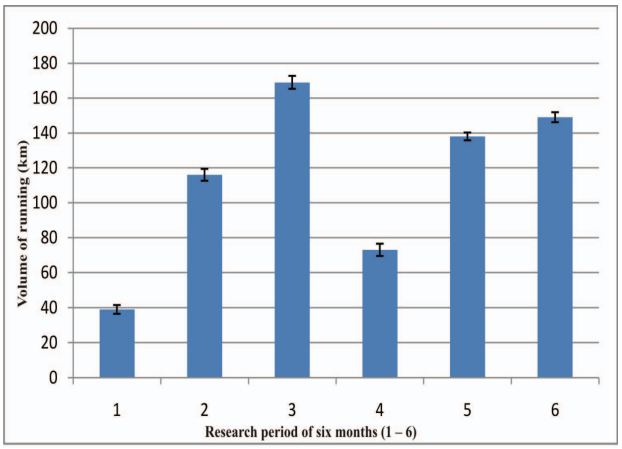

Fig. 1 – Mean values and standard deviation of the monthly volume of training during the first six months after delivery.

During the research period (2nd to 5th month), the respondent reported health problems with symptoms: dry skin, itchy elbows, as well as the whole body itching that was more intense at night. During this period, there was a feeling of "empty breast" and lower secretion of milk. In the analysis of the health condition, tests showed negative results to: *Candida*, parasites in the stool and *Helicobacter pylori* (IgA and IgG).

Table 1
Morphological status and prolactin values in blood of respondents measured during pregnancy and postpartum

Period of measurement	Parameters			
	body weight (kg)	BMI (kg/m^2)	body fat (%)	prolactin (102–496 μU/mL)
40th week of pregnancy	68.7	22.3	25.5	2,798
After delivery				
*	62.9	20.5	20.3	4,095
1	61	19.8	18.3	1,130
2	55	17.9	14.5	823.5
3	56.9	18.5	16.2	934.7
4	56.9	18.5	16.9	616.1
5	57	18.5	16.8	699.3
6	56.7	18.5	16.9	907.8

*** one week after delivery; 1–6 – measurement at the end of the month during the study period.**
BMI – body mass index.

Table 2

Values of thyroid hormones measured during the first six months after delivery

Thyroid hormones	1	Reference values	2	Reference values	3	Reference values	4	Reference values
TSH	3.76	(0.27–4.20) mU/L	< 0.01	(0.35–4.94) mU/L	12.67	(0.27–4.20) mU/L	/	/
T3	/	/	2.5	(1.0–2.7) nmol/L	/	/	/	/
T4	17.21	(12–22) pmol/L	129	(60–160) nmol/L	/	/	9.1	(9.1–19.1) pmol/L

*** 1–4 – number of measuring.**
TSH – thyroid stimulating hormone; T3 – triiodothyronine; T4 – thyroxine.

Ultrasound of the upper abdomen showed no pathological changes in internal organs. A blood test showed: optimal values of serum iron 10.6 μmol/L (reference range, 6.6–26.0 μmol/L), glucose 4.2 (reference range, 4.1–6.1 mmol/L) and evening cortisol 122.30 (reference range, 64–327 nmol/L), low values of leukocytes 3.5 × 10^9/L (reference range, 4.0–10.0 × 10^9/L) and high values of morning cortisol 837.9 nmol/L (reference range, 171–536 nmol/L). This blood variable was measured by calorimetric method. Low thyroid-stimulating hormone (TSH) level was measured (Table 2). Pruritus was diagnosed but therapy treatment was not determined. During this period, the respondent continued with the usual training.

Without specific therapy treatment, itching did not disappear. With a sudden loss of weight (7.9 kg), the following symptoms appeared: a sense of cardiac palpitations (observed: blood pressure – 110/60 mmHg; pulse rate – 80/min), a common nervousness, headache, insomnia and increased hunger. Ultrasonography of the thyroid gland showed normal shape and size of the thyroid gland, of slightly inhomogeneous echotexture, mediocre CD signal, without focal changes, dimensions: right lobe – 13×16×47 mm, left lobe – 14×15×46 mm. Hyperthyroidism was diagnosed. Hormone therapy was reduced to 25 μg of levothyroxine sodium. By applying a certain treatment after 3–4 weeks there have been the following changes: increased appetite, weight gain, feeling of abdominal fullness and bloating. Hormone therapy was increased to 50 μg of levothyroxine sodium. After 10 days, the itching disappeared completely.

During the study period, the respondent exclusively breast fed her child. In the period of six months, a menstrual cycle was not established. The respondent did not change diet during pregnancy, as well as after the childbirth, except in the low milk supply. In the days of so-called "lactation crisis" (the fourth month after pregnancy), the respondent continued running, but with the reduced scope and at a lower intensity. After a while, she completely stopped running for a period of two weeks. During the period of complete rest, she increased fluid intake (about 2 L) and the caloric value of the food in the course of the day. The respondent pointed out that, during the period of relactation, she rested with the baby during the day. The number of breastfeedings was increased to about 10–12 times during 24 hours. When necessary, she used a manual breast pump.

During this period of six months after the childbirth, decline in the value of the hormone prolactin below the lower reference value has not been recorded. After the re-establishment of sufficient quantities of milk for breastfeeding, the respondent increased training volume and continued with regular light to moderate intensity running.

Growth and development of the child was monitored through regular medical checks. The smallest increase in the baby's body weight was noted in the period of the mother's health problem (the third and fourth month after the birth). During the period of six months, the baby doubled body weight at birth (from 3,060 g to 7,000 g) and grew 16 cm (from 48 cm to 64 cm).

Discussion

For the physically active women with a thyroid disorder who want to breastfeed successfully, it is necessary to control the thyroid hormones regularly, as well as to adapt the hormone therapy to the body needs [9]. Increased physical activity can cause a disorder of thyroid hormones [10, 11]. In addition, due to the increased energy consumption, it is essentially to stay nourished and hydrated properly [13, 14]. Studies have shown that continuous moderate aerobic exercise has no adverse effects on the quantity and composition of breast milk, but a certain amount of lactic acid is confirmed which may be a cause of sour flavor and rejection of baby breastfeeding [16, 17]. Some studies show that there was no significant difference in the nutritional value of milk among physically active and sedentary women [4, 6, 8, 13].

Besides, studies that tracked changes in the level of prolactin in the blood in physically active women have shown that moderate aerobic exercise has no harmful effects on the secretion of this important hormone responsible for successful lactation [4, 5, 13, 15]. Disrupting secretion of breast milk may be caused by rapid weight loss due to malnutrition or increased physical effort and stress [4–7]. During pregnancy, prolactin level in the blood of a pregnant woman gradually increases and reaches the maximum value just before giving birth, while this value decreases after the established lactation [11, 18].

In this case study, although prolactin levels were above the upper limit of the reference value during the study period of six months, short-term loss of milk probably can be considered the cause of the changes in the thyroid gland, as well as unadjusted training activities [6, 9, 12, 13, 15].

Changing hormone therapy, increased caloric intake and proper hydration had a positive impact on overcoming

minor problems related to breast feeding [5, 9, 14]. Sudden weight loss had no negative impact on the level of prolactin in the blood. Although some studies note that production of breast milk largely depends on the mother's level of nourishment/body mass index, the above mentioned case can be considered a situation where the body reacts to protect lactation in women with a small percentage of fat in the body [6, 13, 15]. With the increased physical activity and energy deficit, the body responds in the form of increase of the hormone responsible for the maintenance of lactation [15]. Some elite female athletes doing aerobic sports state that despite minor problems they successfully breastfed [13, 12]. Although the impact of frequent breast feedings on the level of prolactin in the blood has not been fully confirmed, it is considered that the number of breastfeedings during the day can have a significant impact on milk production [13, 4].

While increased physical activity has a positive effect on weight loss after childbirth, gradual weight loss has not negative impact on the level of prolactin in the blood during lactation [4, 6]. It is very important to continue with the controlled and proper exercise during pregnancy and after delivery [11, 14]. Reduced cortisol and glucose levels in the blood during the lactation period are expected [7]. In this case, regular blood analysis showed normal levels of blood glucose, reduced values of leukocytes and increased values of the morning cortisol.

Conclusion

In a woman with Hashimoto's thyroiditis light to moderate intensity aerobic exercise had no negative impact on the blood level of prolactin during the first six months after the childbirth.

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Malignant postpartal gestational trophoblastic neoplasm: A rare appearance of equal ultrasonography and operative finding in uterine placental site trophoblastic tumor and choriocarcinoma

Maligna postpartalna gestaciona trofoblastna neoplazma: retka pojava sličnog ultrazvučnog i operativnog nalaza kod uterusnog trofoblastnastnog tumora posteljičnog ležišta i horiokarcinoma

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Abstract

Introducton. Frequency of malignant gestational trophoblastic neoplasms (GTN) is estimated at 1.03 cases in 1,000 deliveries with 5 fold greater risk in patients younger than 20 and older than 40 years. Serum value of human chorionic gonadotropin is the most relevant parameter in diagnosis of GTN. In placental site trophoblastic tumor (PSTT), serum levels of chorionic gonadotropin do not have the same significance as they do in other malignant GTN. Definite diagnosis of PSTT is almost always confirmed by immunohistochemistry. **Case report.** In the course of just a few months (August 2016 to January 2017) in the Clinic for Obstetrics and Gynecology "Narodni front" in Belgrade, two GTN patients were admitted and treated, with almost equal ultrasonography (pictures), operative findings and postoperative outcome. Due to histopathological and immunohistochemical examinations two different types of malignant GTN were confirmed. The first patient (admitted in August 2016), 26 years old, was admitted for uterine bleeding 11 months after vaginal delivery and histopathological examination confirmed PSTT. The second patient (admitted in January 2017), 27 years old, was admitted 4 months after vaginal delivery because of uterine bleeding. Histopathological examination confirmed choriocarcinoma. **Conclusion.** Considering the fact that malignant GTN can appear in different types, with different ultrasonography pictures, this report is significant because two distinctly different malignant GTN entities could appear with equal clinical manifestations and equal ultrasound pictures even when they may have very different course of the disease treatment and outcome. Such cases need correct diagnosis which may be reached only after immunohistochemical analysis. The ultrasound patterns, both in gray scale, color flow, and Doppler values, were almost equal in both cases and guided the diagnostic procedures to the final treatment, even regardless of their very different histopathology.

Key words:
trophoblastic tumor, placental site; choriocarcinoma; diagnosis; diagnosis, differential; gynecologic surgical procedures; drug therapy; treatment outcome.

Apstrakt

Uvod. Učestalost malignih gestacionih trofoblastnih neoplazmi (GTN) se procenjuje na 1,03 slučaja na 1 000 porođaja, sa rizikom koji se upetostručava kod bolesnica mlađih od 20 i starijih od 40 godina. Serumska vrednost humanog horionskog gonadotropina je najrelevantiji parametar za dijagnozu GTN. Kod trofoblastnog tumora (PSTT), vrednosti serumskog horionskog gonadotropina nemaju isti značaj kao kod drugih malignih GTN. Konačna dijagnoza PSTT se gotovo uvek potvrđuje imunohistohemijom. **Prikaz bolesnika.** U nekoliko meseci (od avgusta 2016. do januara 2017), na Ginekološko-akušerskoj klinici (GAK) "Narodni front" u Beogradu, dve bolesnice sa GTN su hospitalizovane i lečene, sa gotovo istom ultrazvučnom slikom, operativnim nalazom i postoperativnim ishodom. Nakon patohistološkog i imunohistohemijskog pregleda potvrđeno je da se radi o dva različita tipa maligne GTN. Kod prve bolesnice (primljene u avgustu 2016), stare 26 godina, hospitalizovane zbog krvavljenja iz uterusa 11 meseci nakon vaginalnog porođaja, patohistološki nalaz je potvrdio PSTT. Druga bolesnica (primljena u januaru 2017) u 27. godini života je hospitalizovana četiri meseca nakon vaginalnog porođaja zbog krvavljenja iz uterusa. Patohistološki je

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ustanovljen horiokarcinom. **Zaključak.** Uzimajući u obzir činjenicu da se različite maligne GTN manifestuju u različitim kliničkim formama, sa različitim ultrazvučnim slikama, ovaj rad je značajan jer jasno pokazuje da se dve signifikantno različite maligne GTN mogu prikazati sa gotovo identičnim kliničkim znacima, ultrazvučnim nalazima, čak i kada imaju različit tok bolesti i ishod. Ovakvi slučajevi zahtevaju preciznu dijagnozu koja se može uraditi samo pomoću imunohistohemije. Ultrazvučna slika u sivoj skali, kolor Doppler protoci, gustina vaskularne šare u tkivu, bili su gotovo isti u oba slučaja i usmeravali su na nepohodne dijagnostičke postupke do konačne terapije, bez obzira na njihovu veoma različitu patohistologiju.

Ključne reči:
trofoblastni tumor, posteljičnog ležišta; horiokarcinom; dijagnoza; dijagnoza, diferencijalna; hirurgija, ginekološka, procedure; lečenje lekovima; lečenje; ishod.

Introduction

Frequency of malignant gestational trophoblastic neoplasms (GTN) is estimated at 1,03 cases in 1,000 deliveries with 5 fold greater risk in patients younger than 20 and older than 40 years [1]. One of possible explanations for this increased risk could be in abnormal gametogenesis and atresia of follicles [2]. According to Royal College of Obstetrics and Gynecologist (RCOG), Grade C recommendation, ultrasound is of relative value in diagnostics of malignant trophoblastic disease [3]. In order to increase the precision of the diagnosis we followed the guidelines for GTN, repeating ultrasound scans, monitoring of hot spots and the drop in blood flow resistance index (RI) in the field of trophoblastic invasion where neoangiogenesis is detectable by color Doppler flow mapping.

Serum value of human chorionic gonadotropin is the most relevant parameter in diagnosis of GTN. It depends on secretion of syncytiotrophoblast which is a hormone active component of trophoblast, and most important marker in monitoring the effect of the treatment and outcome of the disease [4]. In placental site trophoblastic tumor (PSTT), serum levels of chorionic gonadotropin do not have the same significance as they do in other malignant GTN, and they may be negative, making this analysis unuseful. Because of this, it may be necessary to determine serum human placental lactogene (HPL), and ultrasound gains in importance. Definite diagnosis of PSTT is almost always confirmed by immunohistochemistry (IHC).

Transvaginal ultrasound remains a powerful tool for basic evaluation of uterine disease in patients affected by malignant GTN, especially in GTN patients with pathological ultrasound finding, or GTN patients on chemotherapy without initial ultrasound pathological findings during the follow-up, when serum chorionic gonadotropin has increasing rate [5].

Case report

In the course of just a few months (August 2016 to January 2017) in the Clinic for Obstetrics and Gynecology "Narodni front" in Belgrade, two GTN patients were admitted and treated, with almost equal ultrasonography (pictures), operative findings and postoperative outcome. Due to histopathological and immunohistochemical examination two different types of malignant GTN were confirmed.

The first patient (admitted in August 2016), 26 years old, was admitted for uterine bleeding 11 months after vaginal delivery. Chorionic gonadotropin level at the time of admittance was less than 1 mIU/mL. Ultrasound scans showed a circular hyperechogenic field in the myometrium of the fundal region of the uterus (15 × 16 mm) in close connected with endometrium (Figures 1A and 1B). This field was hypervascularized, with RI 0,40. Dilation and curettage (D&C) were performed and histopathology was inconclusive – suspicious of choriocarcinoma. X-ray examination of lungs and cranium was done to out rule metastatic development. Chemotherapy administrated included methotrexate + folinic acid (FA). IHC was done because of unclear histopathological finding and negative serum chorionic gonadotropin and this analysis confirmed PSTT.

Since chemotherapy is not the treatment of choice for PSTT, a total laparoscopic hysterectomy (TLH) with preservation of both ovaries was performed (Figure 1C). During surgery the observed uterus looked completely normal, but when the specimen was dissected for histology, the GTN focus in myometrium connected to endometrium was readily seen (Figure 1D). After surgery, histopathological examination confirmed PSTT. In the follow-up chorionic gonadotropin levels stayed negative.

The second patient (admitted in January 2017), 27 years old, was admitted 4 months after vaginal delivery because of uterine bleeding. Transvaginal ultrasound showed a circular hyperechogenic field in the myometrium (20 × 18 mm) on the left side of the fundal region, close connected to the endometrium edge. This field was with evident hypervascularization and had low vascular RI 0,3 (Figures 2A, B). The initial chorionic gonadotropin value was 44.718 mIU/mL. D&C were performed and histopathology showed residual tissue. After two days, the chorionic gonadotropin levels decreased (3.058 mIU/mL). Still after 7 days the chorionic gonadotropin level began to increase (4.110 mIU/mL) and an ultrasound finding, indicative for trophoblastic invasion was present. Another D&C was performed. Preliminary histopathology examination result was PSTT. Because of two different histopathological diagnoses and increasing serum chorionic gonadotropin level, IHC examination was performed on the histology specimens and choriocarcinoma was found as the definite diagnosis. The patient immediately received chemotherapy (methotrexate and FA). In the follow-up, two weeks before the next course of the chemotherapy, serum chorionic gonadotropin level increased and reached 60.800 mIU/mL. Repeated chemotherapy led to a drop of chorionic gonadotropin to 9.813 mIU/mL. Still the ultrasound picture persisted and 4 weeks after the second chemotherapy course the chorionic gonadotropin level again increased to 48.178

mIU/mL. The patient was advised to take a third course of the chemotherapy which she refused. Based upon the course of the disease and her response, the decision was made to perform a TLH with conservation of both ovaries (Figure 2). Similar to the previous case the uterus, during surgery looked normal (Figure 2C), but when dissected, a macroscopic finding of specific GTN focus was seen in myometrium close connected to endometrial tissue (Figure 2D). Histological examination confirmed choriocarcinoma. After surgery, the patient received one final course of the chemotherapy (methotrexate and FA). After 5 weeks serum chorionic gonadotropin level was negative.

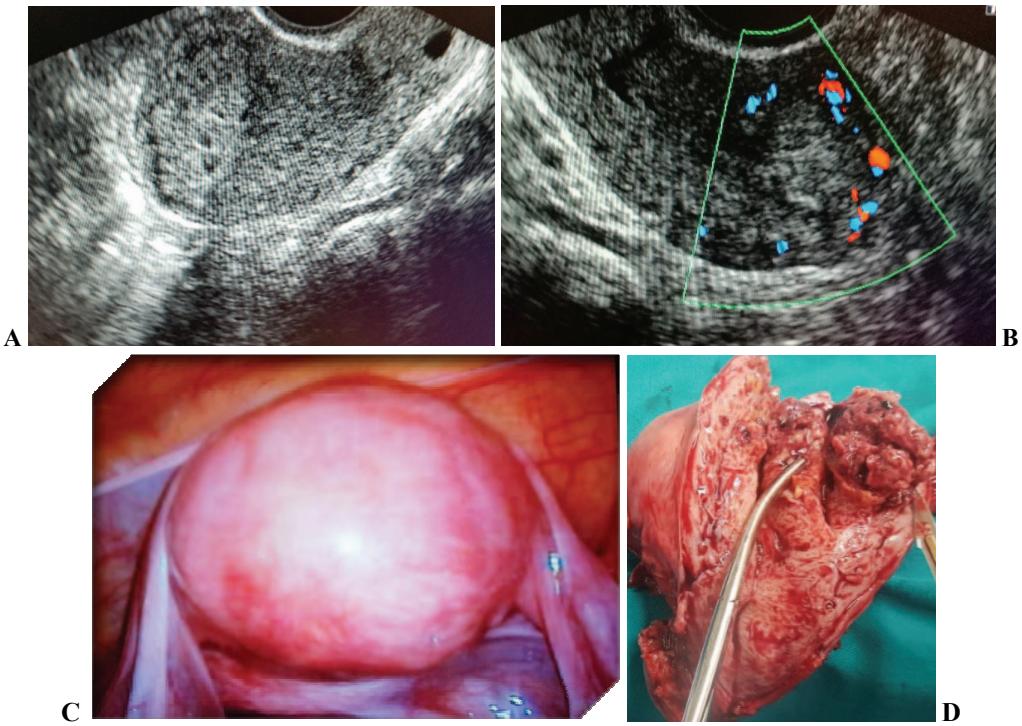

Fig. 1 – A) Uterus: transverse section; B) Gestational trophoblastic neoplasms (GTN) - Color Doppler flow; C) Uterus: laporoscopic view; D) Disected uterus with GTN.

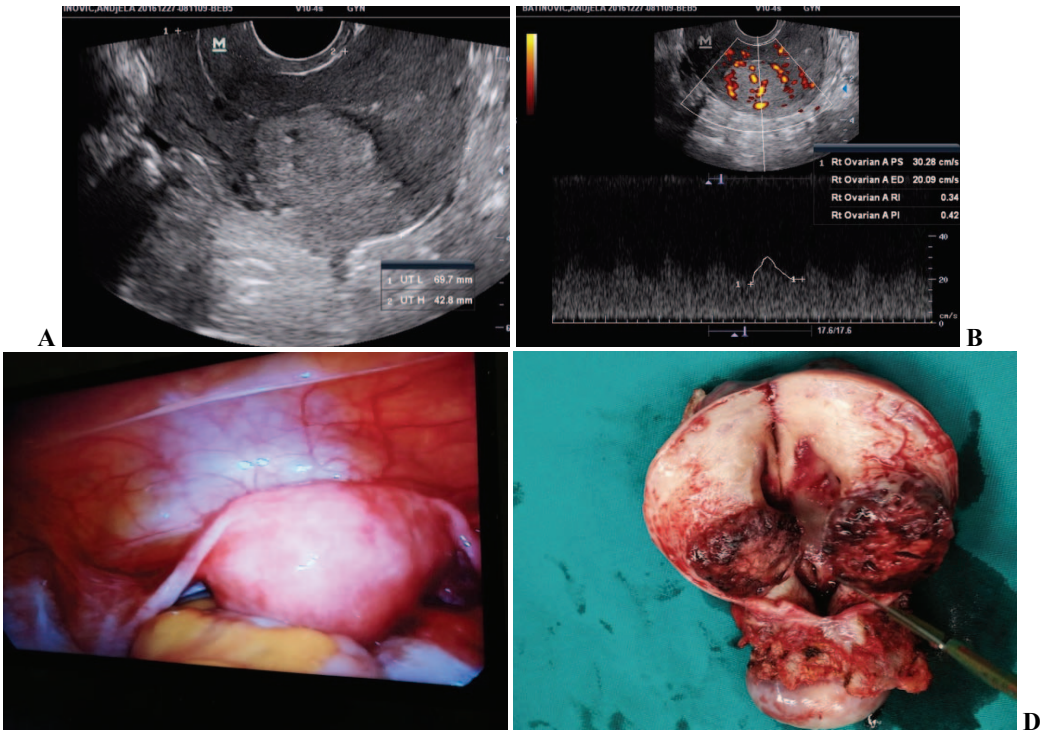

Fig. 2 – A) Uterus with gestational trophoblastic neoplasm (GTN): ultrosound finding; B) GTN: color Doppler flow; C) Uterus: loparoscopic view; D) Disected uterus with GTN.

Discussion

There is not a specific ultrasonographic model for each GTN. Considering the fact that malignant GTN can appear in different types, with different ultrasonography pictures, this report is significant because two distinctly different malignant GTN entities could appear with equal clinical manifestations and equal ultrasound pictures even when they may have very different course, treatment and outcome of the disease. Such cases need correct diagnosis which may be reached only after immunohistochemical analysis. Immunohistochemistry is not standard method for GTN, except for the diagnosis of PSTT. The ultrasound patterns, both in gray scale, color flow, and Doppler values, were almost equal in both cases and guided the diagnostic procedures to the final treatment regardless of their very different histopathology.

In specific GTN cases ultrasonography, as well as histopathology could be of great value in reaching final decision for operative treatment and therapy regimes specially in patients of reproductive age [6, 7].

Hysterectomy, unfortunately, remains an important adjunct in the treatment of the selected subset of patients [8, 9], even as these patients have an imperative in preserving fertility.

Conclusion

Considering the fact that malignant GTN can appear in different types, with different ultrasonography pictures, this report is significant because two distinctly different malignant GTN entities could appear with equal clinical manifestations and equal ultrasound pictures even when they may have very different course of the disease, its treatment and outcome. Such cases need correct diagnosis which may be reached only after immunohistochemical analysis. The ultrasound patterns, both in gray scale, color flow, and Doppler values, were almost equal in both cases and guided the diagnostic procedures to the final treatment, even regardless of their very different histopathology.

Disclosure statement

The authors declare no conflicts of interest.

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CASE REPORT

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Unusual site for metastatic renal cell carcinoma – A case report

Neuobičajena lokalizacija metastaza karcinoma renalnih ćelija

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Abstract

Introduction. The most common malignant tumor of the kidney is renal cell carcinoma (> 90%). The metastasis of the renal cell carcinoma in lingual base is very rare. **Case report.** Patient, 74 years old, renal cell carcinoma underwent radical nephrectomy with evacuation of regional lymph nodes and without any further therapy. After two years of disease-free period, patient appeared with the symptom of difficult swallowing. Ultrasound showed tumor spreading in number of organs. Histopathological analysis of tissue from lingual base confirmed metastatic tumor. **Conclusion.** After radical surgical treatment of the malignant disease, it is important to examine multiple organ systems. Appropriate approach to the diagnostics and evaluation the entire condition of the patient in this stage of the disease, especially lingual base can relieve patients discomfort and provide prolonged survival, even if the prognosis is unfavourable.

Key words:
carcinoma, renal cell; neoplasm metastasis; diagnosis; treatment outcome.

Apstrakt

Uvod. Karcinom bubrežnih ćelija (*renal cell carcinoma)* je najčešći maligni tumor bubrega (90%). Metastaza karcinoma bubrežnih ćelija u bazu jezika je izuzetno retka. **Prikaz bolesnika.** Bolesniku starosti 74 godine dijagnostikovan je tumor bubrega. Izvršena je radikalna nefrektomija sa evakuacijom regionalnih limfnih čvorova, bez primene dodatnog terapijskog protokola. Nakon perioda bez bolesti (*disease free period*) u trajanju od dve godine, bolesnik se javio sa simptomom otežanog gutanja. Ultrazvučnim pregledom utvrđeno je prisustvo metastaza u brojnim organima, dok je biopsijom dokazana metastaza i u bazu jezika. **Zaključak.** Pored primene radikalne hiruške terapije, veliku pažnju treba posvetiti redovnoj sistematskoj kontroli bolesnika sa dokazanom/operisanom malignom bolešću. Rano prepoznavanje i dijagnostikovanje metastaza, naročito u bazu jezika omogućava olakšanje tegoba bolesnika, kao i mogućnost za produženje perioda preživljavanja, bez obzira na nepovoljnu prognozu kod postojanja raširene bolesti.

Ključne reči:
karcinom, bubrežnih ćelija; neoplazme, metastaze; dijagnoza; lečenje, ishod.

Introduction

Renal cell carcinoma (RCC) is neoplasia that originates from the epithelium of the renal tubules [1].

Both genders are affected, but it is more common in men from industrialized regions. Incidence of RCC increases after 40 years and declines after 75 years old. Tobacco smoking is major risk factor beside exposure to arsenic compounds and other cancerogenes (organic solvents, pesticides, etc). Haematuria, pain and weight loss are first simptoms of the disease. Grossly, tumour is surrounded with capsule, within cortex of the kidney. The average size is about 7 cm, with intense yellow-golden color on cut surface (lipid content in cells), with areas of cystic degeneration, focuses of necrosis, haemorrhagia and calcifications [1]. Surgical procedure in smaller tumours is more often nephron-sparing treatment.

Most frequently histological variants of RCC are: clear cell, papillary, chromophobe, medullary, mucinous. Histologically, clear cell RCC may have several architectural structures: solitary, alveolar, acinar. Tumour cells are clear in appearance with eosinophilic cytoplasma and scanty vascular stroma. Sometimes it shows tubular or pseudopapillary pattern. Nuclei are uniform, round in shape, with finely distribution of chromatin [1].

Immunohistochemistry could be of a great help in distiction of tumour tissue that is positive for low molecular we-

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ight cytokeratines (CK8/18), Cam 5.2, vimentine, RCC and epithelial membrane antigen (EMA) [1]. After the stage itself, most important prognostic parameter in nuclear grade. According to the Fuhrman grading system, Grade 1 has small hyperchromatic nuclei, without nucleoli. Grade 2 has finely granular chromatin, but inconspicious nucleoli. For nuclear grade 3, nucleoli are easily recognizable and grade 4 is caracterized by nuclear pleomorphism, hyperchromasia and single to multiple macronucleoli [1]. About 50% of RCC are recognised at stage 1 or 2, less than 5% are stage 4. Hematogenous metastases are most common to the lung, also lymphatic and retrograde metastasis. Prognosis is most accurately predicted by stage and nuclear grade [1].

Usual sites of metastatic RCC are: lung, bone, liver and brain, even after 10 years [2, 3]. Ultrasound is the first diagnostic tool to determine the stage of the disease combined with abdominal computed tomography (CT) [1]. The metastasis of RCC in lingual base is very rare.

Case report

In April 2014, a 74 years old patient was admitted to our center with signs of painless haematuria. After diagnostic procedures he went radical nephrectomy of the left kidney, with removing preaortical and paraaortical lymph nodes. Pathology report confirmed well demarcated tumor 6.5 × 5.5 × 4 cm sized, situated at apical pole of the kidney. Tumor tissue is clear cell carcinoma composed of clear and granular cells with hiperchromatic nuclei and visible nucleoli (Figure 1).

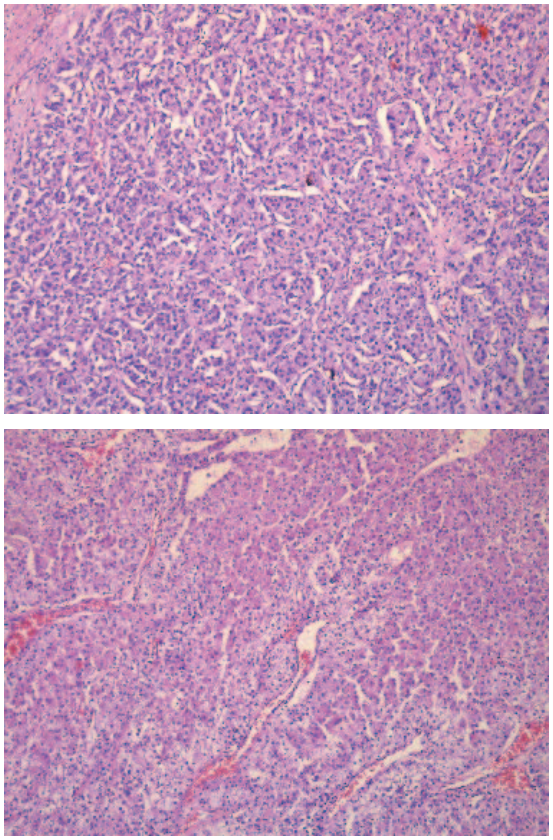

Fig. 1 – Tumor tissue of renal cell carcinoma (RCC) – solid and alveolar pattern (HE, ×100).

Nuclear grade by Fuhrman was 2. Foci of necrosis and haemorrhage were present. Margines were free from tumour. Tumour cells reacted positively for: RCC (Figure 2), CD10, alpha methylacyl-COA-racemase (AMACR), but negative for cytokeratin 7 (CK7). Hilar region contained 2 positive lymph nodes (Figure 3), but 4 of them were negative.

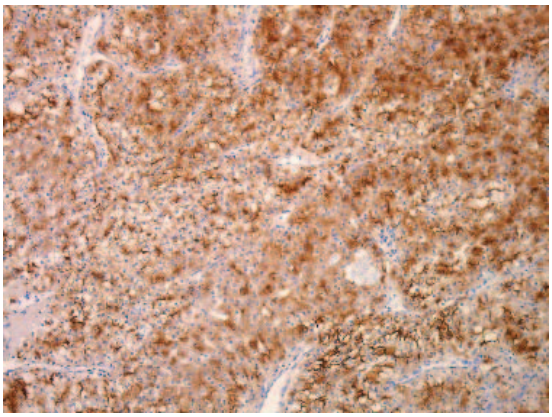

Fig. 2 – Renal cell carcinoma (RCC): immunostaining in tumour tissue (RCC imunohistochemistry, ×100).

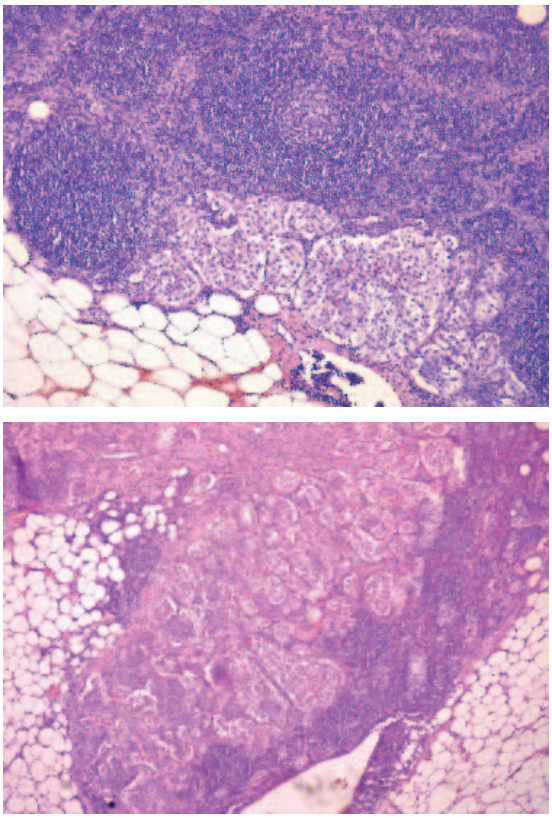

Fig. 3 – Metastases in lymph nodes (HE, ×40).

The group of preaortal and paraaortal lymph nodes contained 1 positive lymph node, and 3 negative nodes – T1b N1 M0 [4]. The patient was not given any other treatment but surgical (nephrectomy). He did not come at any scheduled medical examination, but two years later, in March 2016, the patient referred to the Otorhynolaryngology Department complaining to difficulties in swallowing that lasts for several months.

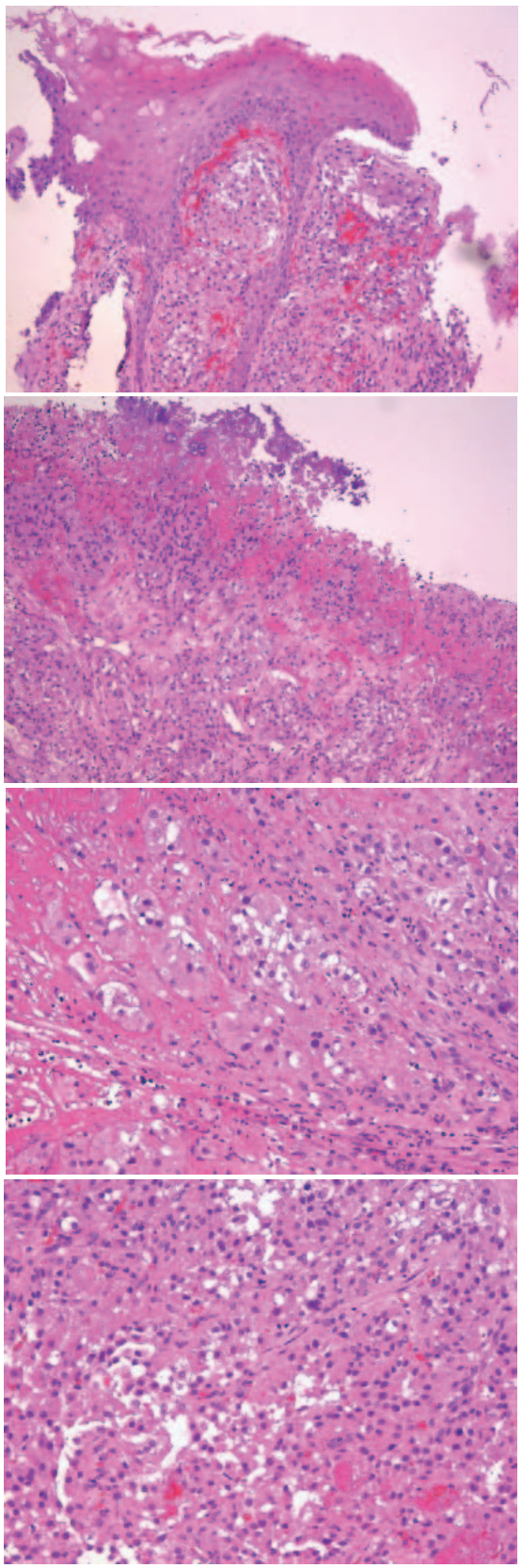

Fig. 4 – Metastasis in lingual base mucosa: HE, ×100 (a, b); HE, x 200 (c, d).

He underwent CT of the chest and abdomen with contrast that revealed bilateral metastatic deposits in lungs and also nectrotizing formations in mediastinum, metastatic deposits in thyreoidea, liver, both iliac bones and a lingual base tumour. Palliative operative treatment of a mass in lingual base was done and the current pathology report resulted by tumour tissue composed of large clear cells with eosinophilic cytoplasma in ulcerated mucosa (Figure 4), that expresses positivity for RCC (Figure 5) and represent metastasis of the previously diagnosed kidney tumour. Although, metastasectomy is the method of choice but inoperable cases like this, generalised metastatic disease was radiologically proven. The following control physical examination was scheduled for a month, but the patient died 20 days after the hospital release.

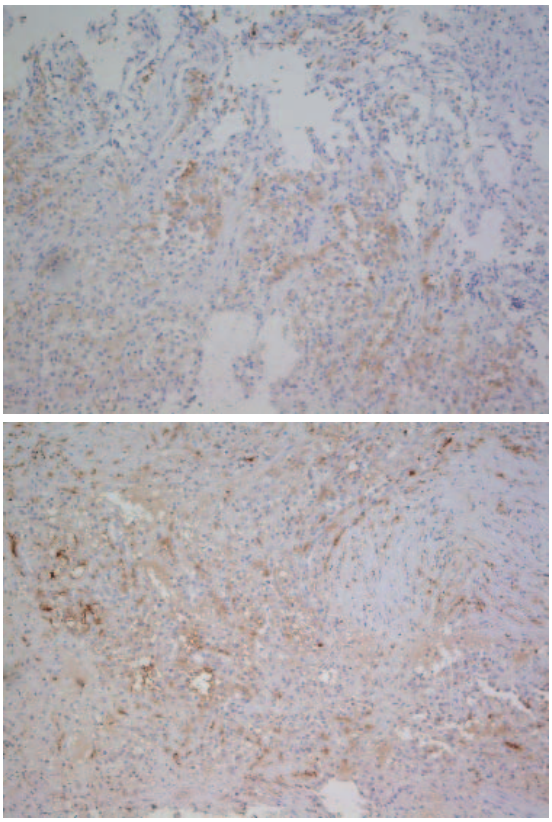

Fig. 5 – Renal cell carcinoma (RCC) positive expression in metastasis (RCC imunohistochemistry, ×100)

Discussion

Lingual base is the site of many benign tumors and pseudotumors. The most common malignant tumor in oral cavity is planocellular carcinoma (90%). The lingual base itself is in 50% the most common site for primary planocellular carcinoma. In majority cases lingual tumors show exophytic growth with ulceration on surface. With progressive growth tumour become indurated and develop raised and everted margins. Even clinically small tumors infiltrate deeply into the muscle. Infiltration of the lingual musculature may cause pain, dysphagia and dysphonia [5]. About 1% of all oral cancers are metastases of primary tumors elsewhere in the body, and they are located in the soft tissues (gingiva and tongue), or bones (mandible or maxilla). Any malignant tumor can metastase in oral cavity, but the most common reported are malignancies of lung, liver, prostate and breast.

The soft tissue metastases manifest as haemorrhagic tumors with ulceration, rapidly evolving. Despite the fact that oral tumors often give symptoms that can easily been seen, many of them represent advanced disease, that require mutilant treatment and implicate a poor prognosis. In most patients with oral metastasis, the primary tumor has already been diagnosed and treated. Sometimes, existance of oral metastasis leads to detection of metastases elsewhere in the body, so the treatment is restricted (palliative). Prognosis is poor, and median survival is less than 6 months [2, 6–8].

Lingual base is common localisation for primary planocellular carcinoma, but is unusual site for metastatic renal cell carcinoma. Oral metastasis represent spreading of the malignant tumor which already had metastazed in distant localisations. However, metastases to the oral cavity are in 30% of cases the first indication of an otherwise occult malignancy.

Conclusion

After radical surgical treatment of malignant tumors, it is important to examine multiple organ systems. Appropriate approach to the diagnostic and evaluation the entire condition of the patient in this stage of the disease, especially lingual base can relieve patients discomfort and provide prolonged survival, even if the prognosis is unfavourable.

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CASE REPORT

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Rare case of dural arteriovenous fistula presenting by spontaneous acute subdural hematoma – A case report and review of literature

Redak slučaj prezentacije duralne arteriovenske fistule putem spontanog akutnog subduralnog hematoma

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Abstract

Introduction. Dural arteriovenous fistulas represent pathological acquired bonds between the meningeal blood vessels (arteries) and drainage veins associated to them. These fistulas can vary in clinical presentations, from being asymptomatic to causing serious neurological deficits, depending mostly on the localization and size. Only one fourth of dural fistulas present themselves with intracranial bleeding. This hemorrhage is most frequently localized in subarachnoid space, occasionally intracerebrally, and seldom beneath the dura mater, *ie* subdurally. **Case report.** We presented a rare case of a patient with spontaneous acute subdural hematoma. After the initial treatment and consequent imaging methods, a diagnosis of a dural arteriovenous fistula was established. After the craniotomy for hematoma evacuation, the patient underwent an uneventful endovascular treatment. Despite the rarity of non-traumatic acute subdural hematoma caused by dural arteriovenous fistula, one should not overlook the possible pathogenesis and etiology in patients with spontaneous acute subdural hematoma. Even with the absence of the symptoms and signs of subdural bleeding, dural arteriovenous fistula, as a cause of it, should not be immediately ruled out. **Conclusion.** Despite the rarity of non-traumatic acute subdural hematoma being caused by dural arteriovenous fistulas, one should not immediately overlook the possible pathogenesis and etiology. Cautious approach is needed when treating such diseases even in the absence of typical symptoms.

Key words:
central nervous system vascular malformation; hematoma, subdural; diagnosis; neurosurgical procedures; treatment outcome.

Apstrakt

Uvod. Duralne arteriovenske fistule predstavljaju patologiju koju odlikuju veze između meningealnih krvnih sudova i njima pripadajućih drenažnih vena. Klinička prezentacija ovih fistula je različita i najviše zavisi od lokalizacije i veličine istih. Mogu biti asimptomatske, a mogu se ispoljavati kao ozbiljni neurološki deficit. Samo jedna četvrtina ovih malformacija se prezentuje kao intrakranijalno krvarenje, najčešće lokalizovano subarahnoidalno, potom intracerebralno, a retko ispod tvrde moždane opne, tj. subduralno. **Prikaz bolesnika.** U radu je prikazan bolesnik sa spontanim akutnim subduralnim hematomom. Nakon inicijalnog tretmana i posledičnih dijagnostičkih metoda, utvrđeno je postojanje duralne arteriovenske fistule. Nakon kraniotomije i evakuacije hematoma bolesnik je podvrgnut endovaskularnom tretmanu bez posledica. Uprkos retkoj pojavi netraumatskog akutnog subduralnog hematoma uzrokovanog duralnom arteriovenskom fistulom, ne bi trebalo prevideti moguću patogenezu i etiologiju ove bolesti. Čak i prilikom izostanka tipičnih simptoma subduralnog krvarenja, ne treba isključiti mogućnost nastanka istog zbog postojanja duralne arteriovenske fistule. **Zaključak.** Iako je pojava netraumatskog akutnog subduralnog hematoma uzrokovanog duralnom arteriovenskom fistulom retka, potencijalna patogeneza i etiologija se ne sme odmah isključiti. Oprezan pristup je nužan tokom lečenja ove vrste bolesti, čak i kod izostanka tipičnih simptoma.

Ključne reči:
centralni nervni sistem, vaskularne malformacije; hematom, subduralni; dijagnoza; neurohirurške procedure; lečenje, ishod.

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Introduction

Dural arteriovenous fistulas (DAVF) are usually defined as pathologically acquired bonds between the meningeal blood vessels (arteries) and drainage veins associated to them [1]. Depending mostly on the localization and size, these fistulas can vary in clinical presentation, from being asymptomatic to causing serious neurological deficits [2]. In 24% of the cases they present themselves with intracranial hemorrhage, most often as subarachnoid or intracerebral hemorrhage [1–3]. An acute subdural hematoma (aSDH) caused by a DAVF occurs rarely [1–3]. There have been only a few reports of aSDH caused by DAVF in the literature [2, 4–7].

Case report

A 66-year-old previously healthy female presented with alteration of consciousness which followed the previous loss of it. Initially the patient was unconscious, and upon awakening displayed symptoms of confusion and drowsiness. She was brought to our emergency clinic for neurological care. On the admission, the patient was confused, opening eyes to voice, obeying commands, Glasgow Coma Score (GCS) was 13. She had no signs of motor or cranial nerve deficits, and her pupils were isochoric and photo-reactive, while the meningeal signs were absent. Initial computed tomography (CT) head scan showed an aSDH on the left convexity of cranium with mass effect (Figure 1). With no confirmed traumatic brain injury (TBI), CT angiography (CTA) was performed showing early opacification located parietooccipitally on the left side (Figure 2). Patient underwent trauma flap craniotomy, and the aSDH was evacuated. During the postoperative hospitalization patient's GCS score was 15 with no neurological deficits, while the postoperative CT scan showed adequate aSDH evacuation (Figure 3).

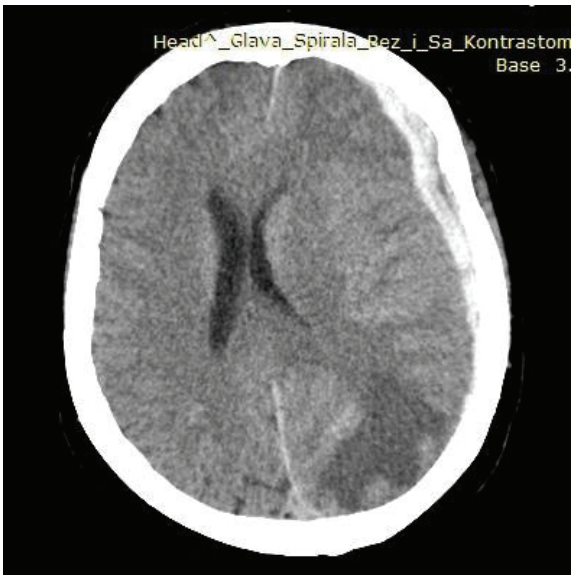

Fig. 1 – Computed tomography (CT) axial head scan depicting a hyperdense lesion of acute subdural hematoma (aSDH) on the left convexity of cranium with mass effect.

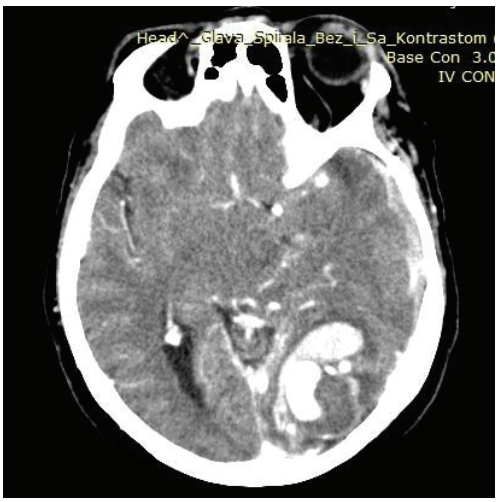

Fig. 2 – Computed tomography angiography (CTA) presenting early opacification located parietooccipitally on the left side.

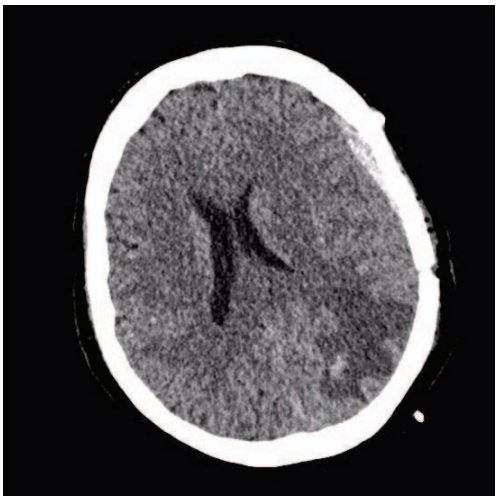

Fig. 3 – Post-operative computed tomography (CT) scan showing adequate hematoma evacuation.

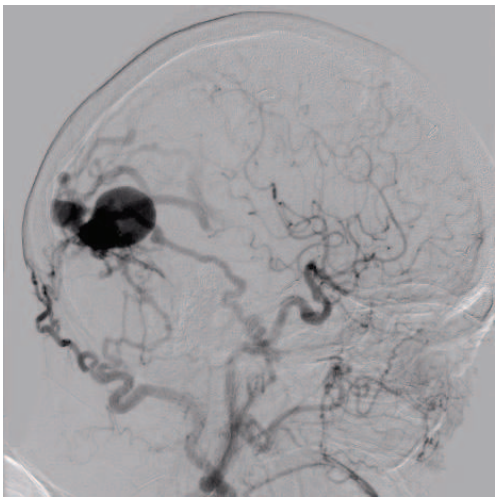

Fig. 4 – Digital subtraction angiography (DSA) showing opacification of the left external carotid artery as well as of the right occipital artery draining via drainage veins into sagittal, transversal and straight sinus.

Digital subtraction angiography (DSA) performed postoperatively (Figure 4) demonstrated opacification of the left external carotid artery as well as opacification of the right occipital artery draining, via bulging drainage veins into sagittal, transversal and straight sinus, thus confirming an intracranial DAVF (Figure 1). After the initial hematoma evacuation and postoperative follow-up, endovascular treatment, embolization of the DAVF with Onyx, was the method of choice for the patient. The patient was uneventful since the discharge period.

Discussion

Dural arteriovenous fistulas make around 10–15% of all neurosurgical arteriovenous lesions [1]. Most often they are fed by external carotid artery or, less frequently, by the internal carotid and vertebral arteries (meningeal arteries), while being drained through dural or leptomeningeal veins [1, 2]. The clinical course of DAVF varies. Depending mostly on the localization and size, presentation can range from being asymptomatic or mildly symptomatic (eg. headache, tinnitus) to causing serious progressive neurological deficits [1, 3].

Only 24% of dural fistulas present themselves with intracranial bleeding, and that hemorrhage is most commonly localized in subarachnoid space, occasionally intracerebrally, and very rarely beneath the dura mater, *ie* subdurally [1, 6]. Therefore, a case of a non-traumatic acute SDH caused by a DAVF can be considered a very rare pathology [1, 2, 4–7]. Current literature explains the occurrence of aSDH by the rupture of the bulged draining vein which was being overflown with blood from the feeding meningeal artery [1, 2, 5]. At the moment of writing this paper, endovascular treatment with Onyx is considered to be the first line of strategy whereas the operative treatment should be performed only if consecutive endovascular interventions fail [1].

Leptomeningeal retrograde venous drainage present in CTA imaging increases the risk of intracranial bleeding from DAVF, and this is why the venous part of the fistulas are of greatest importance for the occurrence of hemorrhage [1, 2, 6].

Conclusion

Despite the rarity of non-traumatic aSDH caused by DAVF, one should not immediately overlook the possible pathogenesis and etiology in patients with spontaneous aSDH. Even with the absence of the symptoms and signs that subdural bleeding is caused by DAVF, it should not be immediately ruled out.

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Tabele

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

Ilustracije

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

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

Skraćenice i akronimi

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.

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