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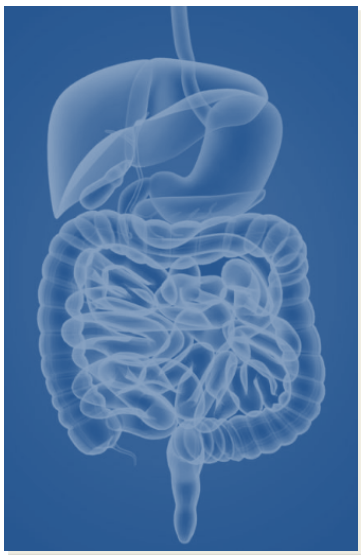
WORLD DIGESTIVE HEALTH DAY

29 May 2022

Colorectal Cancer Prevention: Getting Back on Track



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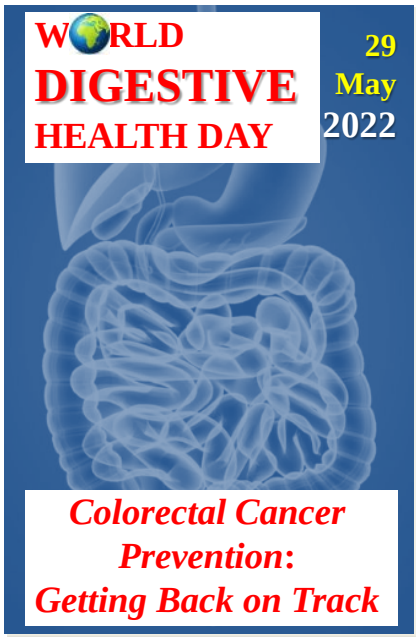

World Digestive Health Day (WDHD) is celebrated every year on May 29. WDHD marks the beginning of a twelve-month-long campaign focused on raising awareness of a particular digestive disease/disorder. The motto of the 2022 campaign is “Colorectal Cancer Prevention: Getting Back on Track”. Colorectal cancer (CRC) is the second leading cause of cancer-related deaths worldwide. The COVID-19 pandemic has disrupted CRC screening programs all around the world. Now more than ever, it is necessary to understand the risk factors for CRC and focus on detecting the disease at an early stage.

Svetski dan zdravlja organa za varenje (SDZOV) obeležava se svake godine 29. maja. SDZOV označava početak dvanaestomesečne kampanje čiji je fokus podizanje svesti o određenoj bolesti/poremećaju digestivnog sistema. Moto kampanje 2022. godine je „Prevencija kolorektalnog karcinoma: povratak na pravi put”. Kolorektalni karcinom (KRK) je drugi vodeći uzrok smrti od karcinoma u svetu. Pandemija COVID-19 je poremetila programe „skrininga” KRK širom sveta. Sada je, više nego ikada, potrebno razumevanje faktora rizika od nastanka KRK i usmerenost na otkrivanje bolesti u ranoj fazi.

ORIGINAL ARTICLES

UDC: 616.132.2-085.8-036
DOI: https://doi.org/10.2298/VSP200810126S

The effects of cardiac rehabilitation on haemodynamic parameters measured by impedance cardiography in patients with coronary artery disease

Uticaj kardiovaskularne rehabilitacije na hemodinamski status bolesnika sa koronarnom bolešću srca procenjen korišćenjem kardiografske impedance

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Abstract

Background/Aim. Well-organized cardiovascular rehabilitation (CVR) reduces cardiovascular burden by influencing cardiovascular risk factors, improving the quality of life, and reducing mortality and hospital readmission. However, its effects on hemodynamic status are largely unknown. The aim of our study was to evaluate the influence of a three-week CVR program on hemodynamic status and to investigate if there is a correlation between physical strain tolerance and hemodynamic parameters measured by impedance cardiography (ICG) before and after the CVR program in patients with coronary artery disease (CAD). **Methods.** Fifty-two patients attended a three-week CVR program. At the beginning and the end of the rehabilitation program, laboratory tests, exercise stress tests (EST), and ICG measurements were taken. **Results.** Patients showed better strain tolerance on the second exercise stress test (EST2) by achieving a higher strain level ($Z = 2.315$; $p = 0.021$) and a longer duration of the test ($Z = 2.305$; $p = 0.021$). There was a strong positive correlation between the level of EST2 and cardiac output (CO) ($r = 0.538$; $p < 0.001$) and stroke volume (SV) ($r = 0.380$; $p = 0.017$) on the second ICG (ICG2). Moreover, there was a strong negative correlation between EST2 level and systemic vascular resistance (SVR) ($r = -0.472$; $p = 0.002$) and SVR index (SSVRI) ($r = -0,407$; $p = 0.010$) on ICG2. There was a strong positive correlation between EST2 duration and CO ($r = 0.517$; $p = 0.001$) as well as between EST2 duration and SV ($r = 0.340$; $p = 0.034$), and a strong negative correlation between EST2 duration and SVR ($r = -0.504$; $p = 0.001$) as well as between EST2 duration and SVRI ($r = -0.448$; $p = 0.004$), according to ICG2. **Conclusion.** Our study showed that a well-designed CVR program can lead to better physical strain tolerance in patients with CAD. Furthermore, CVR led to a significant positive correlation between EST and CO as well as between EST and SV measured by ICG. On the other hand, there was a significant negative correlation between EST and vascular-related parameters according to ICG at the end of the CVR program.

Key words:
cardiography, impedance; coronary artery disease; myocardial infarction; physical exertion; plethysmography, impedance; rehabilitation.

Apstrakt

Uvod/Cilj. Dobro organizovana kardiovaskularna rehabilitacija (KVR) smanjuje kardiovaskularno opterećenje uticajem na faktore rizika od kardiovaskularnih bolesti, poboljšavajući kvalitet života i smanjujući smrtnost i ponovni kardiovaskularni događaj. Međutim, njeni efekti na hemodinamski status bolesnika uglavnom su nepoznati. Cilj rada bio je da se proceni uticaj tronedeljnog programa KVR na hemodinamski status i da se istraži postojanje povezanosti između tolerancije fizičkog napora i hemodinamskih parametara merenih kardiografskom impedancom (*impedance cardiography* – ICG) pre i posle programa KVR kod bolesnika sa oboljenjem koronarnih arterija. **Metode.** Ukupno 52 bolesnika učestvovala su u tronedeljnom programu KVR. Na početku i na kraju programa rehabilitacije vršeni su laboratorijski testovi, testovi fizičkim opterećenjem (TFO) i ICG merenja. **Rezultati.** Bolesnici su pokazali bolju toleranciju fizičkog napora na drugom TFO (TFO2) dostižući viši nivo opterećenja ($Z = 2,315$; $p = 0,021$) i duže trajanje testa ($Z = 2,305$; $p = 0,021$). Postojala je jaka korelacija između nivoa drugog TFO i minutnog volumena (MV) ($r = 0,538$; $p < 0,001$) i udarnog volumena (UV) ($r = 0,380$; $p = 0,017$) na drugoj ICG (ICG2). Takođe, postojala je jaka negativna korelacija između

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nivoa TFO2 i sistemskog vaskularnog otpora (SVO) (r = -0,472; p = 0,002) i indeksa SVO (SVOI) (r = -0,407; p = 0,010) na ICG2. Postojala je jaka pozitivna korelacija između dužine trajanja TFO2 i MV (r = 0,517; p = 0,001), kao i između dužine trajanja TFO2 i UV (r = 0,340; p = 0,034), i jaka negativna koralcija između trajanja TFO2 i SVO (r = -0,504; p = 0,001), kao i između trajanja TFO2 i SVOI (r = -0,448; p = 0,004) na ICG2. **Zaključak.** Naša studija je pokazala da dobro dizajniran program KVR može dovesti do poboljšanja tolerancije fizičkog napora kod bolesnika sa oboljenjem koronarnih arterija. Takođe, KVR je dovela do pozitivne korelacije između TFO i MV kao i TFO i UV procenjenih primenom ICG. Sa druge strane, postojala je značajna negativna korelacija između TFO i vaskularne rezistencije procenjene korišćenjem ICG na kraju programa KVR.

Ključne reči:
kardiografija, impedansna; koronarna bolest; infarkt miokarda; napor, fizički; pletizmografija, impedansna; rehabilitacija.

Introduction

Cardiovascular rehabilitation (CVR) is of great importance in the secondary prevention of cardiovascular diseases (CVD). On the one hand, it promotes recovery from CVD, and on the other, it prevents future cardiovascular events [1]. Well-organized CVR, which includes supervised physical training, education, social support, and lifestyle changes, reduces cardiovascular burden by influencing cardiovascular risk factors, improving the quality of life, and reducing mortality and hospital readmission [2–4].

The influence of CVR on hemodynamic status has been a subject of numerous studies. It can reduce heart rate, blood pressure, and rate-blood pressure product values [5, 6]. On the other hand, it can increase arterial compliance, especially in patients with diabetes mellitus [7]. This is why all relevant guidelines consider CVR as an indispensable part of secondary prevention in patients with CVD [8, 9].

Thoracic impedance or impedance cardiography (ICG) is a simple, non-invasive diagnostic method used for assessing hemodynamic status by monitoring electrical conductivity changes of the thorax [10]. It is usually used in patients with heart failure where it can accurately assess stroke volume (SV) and cardiac output (CO) [11–13]. Other indications include pulmonary hypertension, congenital heart diseases, sleep apnea, arterial hypertension, etc. [14–17].

Using ICG, we can assess CO, SV, systemic vascular resistance (SVR), thoracic fluid content, and many other parameters that can help us both with the CVD diagnostic algorithm and the therapeutic approach.

The aim of our study was to evaluate the influence of a three-week CVR program on hemodynamic status in patients with coronary artery disease (CAD). Furthermore, we tried to investigate whether there was a correlation between physical strain tolerance and ICG parameters before and after the CVR program. To our knowledge, this was the first study that has provided this kind of investigation so far.

Methods

Recruitment and patients

The study involved 52 patients referred to the Institute for Treatment and Rehabilitation “Niška Banja” for a three-week CVR program after surviving myocardial infarction (MI), percutaneous coronary intervention (PCI), or coronary artery bypass grafting (CABG). Patients with an artificial valve, implemented permanent pacemaker, reduced ejection fraction (EF < 50%), significant unsolved stenotic lesions on coronary arteries (stenosis ≥ 70%), atrial fibrillation, ventricular arrhythmia, severe anemia, impaired physical or mental conditions were excluded from the study. Data about ejection fraction, valve disease, and other echocardiographic parameters were extracted from previous medical examinations done after MI/PCI/CABG and before study enrollment.

Study design

The three-week CVR program included supervised and personalized physical training that involved cardiovascular exercise training, bicycle riding, and walking. At the beginning and the end of the CVR program, laboratory tests, exercise stress tests (EST), and ICG measurements were taken.

The tests were done on the Treadmill track using the Bruce protocol. After the initial EST, all patients underwent regular aerobic physical training, which included aerobic exercises, bicycle riding, and walking for 45 min *per* session, two sessions on a daily basis. All patients attended physical training five times a week, three weeks in a row. The intensity of physical activity was limited to the submaximal physical capacity determined at the level when 70–80% of the maximum heart rate was reached on the initial EST.

Exercise stress test

ESTs were done on the treadmill (3017 Full Vision Drive, Newton, Kansas, USA) according to the Bruce protocol. Tests were limited by submaximal heart rate (calculated as 85% from the 220-age equation), symptoms like chest pain, dyspnea, fatigue, dizziness, etc., and electropathological changes on electrocardiogram (ECG), which included horizontal or down-sloping ST-segment depression ≥ 1 mm, complexed ventricular arrhythmia that involved couplets of ventricular premature beats or ventricular tachycardia, patient's request to stop the test, and hypertensive reaction which was defined as a sudden increase of systolic BP to values ≥ 220 mmHg, or decreased in systolic BP > 10 mmHg.

Impedance cardiography

Hemodynamic data were obtained noninvasively using CardioScreen® 2000 (Medis GmbH, Germany). All patients underwent ICG monitoring for 15 min. Measurements were done at the same time, approximately half an hour after morning medications and before breakfast. All measurements were done in a supine position after 15 min of rest. Blood pressure was measured every three minutes, and ICG analyzes were taken every five min. The last recorded values were taken for statistical analysis.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 20; SPSS, Chicago, IL, USA). Frequencies and percentages were used for the description of the patient's characteristics. Numerical data were expressed as mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used to test the normality of data. Statistical significance for nominal data was tested with the χ^2 test and, where appropriate, the Fisher exact test. The Student's *t*-test was used to assess the statistical significance of parametric continuous data, and Mann–Whitney *U*-test and Wilcoxon Signed Ranks Test were for nonparametric continuous variables. The Pearson's correlation was used to determine the correlation between variables. Statistical significance was set to a level of $p < 0.05$.

Results

Baseline characteristics

The study included 52 patients with CAD, 42 (80.8%) men and 10 (19.2%) women. The age structure was similar in both genders (male 56.62 ± 9.80 *vs* female 62.30 ± 9.08; $p = 0.101$). Thirty-nine (75%) patients suffered from arterial hypertension, 47 (90.4%) had lipid disorder or took lipid-lowering drugs, and 21 (40.4%) patients suffered from diabetes mellitus. Thirty-nine (75%) patients were smokers or ex-smokers, and 21 (40.4%) had a positive family anamnesis for CVD. Forty-four (84.6) patients survived MI, 35 (67.3%) underwent PCI, and 13 (25%) patients had CABG (Table 1).

Laboratory measurements

At the beginning and the end of CVR, laboratory parameters were measured (Table 2). Cholesterol, low-density lipoprotein (LDL), glycosylated hemoglobin (HbA1c), and creatinine kinase (CK) were lower at the end of CVR compared to the values at the beginning of CVR but without statistical significance. On the other hand, the values of erythrocytes ($t = 3.859$; $p < 0.001$), hemoglobin ($t = 3.245$; $p = 0.002$), and hematocrit ($t = 3.551$; $p = 0.001$) were significantly higher during the second measurement.

Table 1

Baseline characteristics of patients

Parameters	No	Yes
	n (%)	n (%)
Arterial hypertension	13 (25.0)	39 (75.0)
Hyperlipidemia	5 (9.6)	47 (90.4)
Diabetes mellitus	31 (59.6)	21 (40.4)
Smoking habit	13 (25.0)	39 (75.0)
Heredity	21(40.4)	31 (59.6)
Myocardial infarction	8 (15.4)	44 (84.6)
Coronary artery by-pass surgery	39 (75.0)	13 (25.0)
Percutaneous coronary intervention	17 (32.7)	35 (67.3)

Table 2

Laboratory measurements at the beginning and the end of rehabilitation

Parameters	At the beginning (mean ± SD)	At the end (mean ± SD)	t/*Z	*p*
Cholesterol	4.08 ± 0.93	4.01 ± 0.86	0.683	0.498
LDL	2.42 ± 0.74	2.39 ± 0.77	0.250	0.804
HDL	0.99 ± 0.20	0.94 ± 0.18	2.067	0.044
Triglyceride	1.59 ± 0.72	1.59 ± 0.64	0.770	0.939
Glucose	5.97 ± 1.17	6.22 ± 1.67	0.376*	0.707
HbA1c	5.42 ± 1.42	5.28 ± 1.47	1.374*	0.078
CK	157.60 ± 109.27	142.60 ± 78.77	1.167	0.250
CKMB	17.08 ± 8.45	17.87 ± 9.08	0.596*	0.551
Hematocrit	0.41 ± 0.03	0.42 ± 0.04	3.551	0.001
Erythrocytes	4.74 ± 0.44	4.85 ± 0.45	3.859	< 0.001
Hemoglobin	139.02 ± 12.24	141.35 ± 12.36	3.245	0.002
Creatinine	98.22 ± 19.37	98.44 ± 15.81	0.102	0.919
AUR	341.48 ± 106.03	331.14 ± 104.53	1.065	0.294

***Z* – Wilcoxon Signed Ranks Test; LDL – low-density lipoproteins; HDL – high-density lipoproteins; HbA1c – glycosylated hemoglobin; CK – creatinine kinase; MB – myocardial fraction; AUR – acidum uricum; SD – standard deviation.**

Exercise stress test and impedance cardiography

Additionally, all patients underwent EST and ICG measurements at the beginning and the end of the CVR program (Table 3). Patients showed better strain tolerance on the second EST (EST2) by achieving a higher strain level (Z = 2.315; $p = 0.021$) and longer duration of the test (Z = 2.305; $p = 0.021$) compared to the values of the first EST (EST1). Furthermore, a higher number of patients achieved submaximal heart rate values on the EST2 (Z = 3.153; $p = 0.002$).

According to impedance cardiography, CO and SV were higher at the end of CVR, but without any statistical significance ($t = 0.512$; $p = 0.611$ and Z = 1.349; $p = 0.184$). On the other hand, heart rate, systemic vascular resistance index (SVRI), and stroke systemic vascular resistance index (SSVRI) were lower during the second measurement, but also without statistical significance (Z = 1.068; $p = 0.292$, Z = 0.510; $p = 0.613$ and Z = 0.950; $p = 0.348$).

Correlation between exercise stress test and impedance cardiography

There was no significant correlation between the levels of the EST1 and the parameters of the first ICG measurements (ICG1). On the other hand, there was a strong positive correlation between the levels of EST2 and CO ($r = 0.538$; $p < 0.001$) as well as between the levels of EST2 and SV ($r = 0.380$; $p = 0.017$), according to the second ICG measurements (ICG2). Moreover, there was a strong negative correlation between EST2 levels and SVR ($r = -0.472$; $p = 0.002$) as well as between EST2 levels and SVRI ($r = -0.407$; $p = 0.010$), measured by ICG2 (Table 4).

CO significantly positively ($r = 0.357$; $p = 0.026$) and SVR ($r = -0.409$; $p = 0.010$) and SVRI ($r = -0.356$; $p = 0.026$) significantly negatively correlated with EST1 duration when ICG1 parameters were compared. There was a strong positive correlation between EST2 duration and CO ($r = 0.517$; $p = 0.001$) as well as between EST2 duration and

Table 3

Exercise stress test and impedance cardiography

Parameters	At the beginning (mean ± SD)	At the end (mean ± SD)	*t*/*Z	*p*
Exercise stress test				
level	2.93 ± 1.10	3.24 ± 0.87	2.315*	0.021
duration (min)	7.13 ± 3.30	7.79 ± 2.63	2.305*	0.021
SHR (bpm)	0.43 ± 0.50	0.70 ± 0.46	3.153*	0.002
Impedance cardiography				
CO (L/min)	5.85 ± 1.19	5.91 ± 1.32	0.512	0.611
SV (mL)	90.91 ± 22.91	93.63 ± 22.69	1.349	0.184
ACI (1/100/s²)	77.09 ± 26.53	79.21 ± 27.39	0.679	0.490
VI (1/1000/s)	47.07 ± 15.04	47.84 ± 14.19	0.499	0.620
BP systolic (mmHg)	127.77 ± 9.54	128.77 ± 12.94	0.453	0.666
BP diastolic (mmHg)	75.84 ± 9.34	85.79 ± 8.02	0.041	0.968
HR (bpm)	65.14 ± 9.73	63.84 ± 9.27	1.068	0.292
SVR (dyne·s/cm^5)	1237.93 ± 275.98	1238.40 ± 311.27	0.004	0.997
SVRI (dyne·s/cm^5/m²)	2397.40 ± 450.33	2368.81 ± 502.97	0.510	0.613
SSVRI (dyne·s/cm^5/m²)	156.95 ± 34.58	152.91 ± 36.40	0.950	0.348

***Z* – Wilcoxon Signed Ranks Test; SHR – submaximal heart rate; CO – cardiac output; SV – stroke volume; ACI – acceleration index; VI – velocity index; BP – blood pressure; HR – heart rate; SVR – systemic vascular resistance; SVRI – SVR index; SSVRI – stroke SVRI; SD – standard deviation.**

Table 4

Correlation between the exercise stress test (EST) level and impedance cardiography (ICG) parameters

ICG parameters	EST1 level		EST2 level	
	r	*p*	*r*	*p*
CO (L/min)	0.266	0.102	0.538	< 0.001
SV (mL)	0.177	0.280	0.380	0.017
ACI (1/100/s²)	0.075	0.652	0.265	0.104
VI (1/1000/s)	0.172	0.295	0.284	0.079
BP systolic (mmHg)	0.078	0.635	0.128	0.438
BP diastolic (mmHg)	0.082	0.619	0.008	0.964
HR (bpm)	0.155	0.345	0.187	0.255
SVR (dyne·s/cm^5)	-0.209	0.201	-0.472	0.002
SVRI (dyne·s/cm^5/m²)	-0.163	0.322	-0.407	0.010
SSVRI (dyne·s/cm^5/m²)	-0.031	0.850	-0.236	0.148

For abbreviations see under Table 3.

SV (r = 0.340; p = 0.034), and a strong negative correlation between EST2 duration and SVR (r = -0.504; p = 0.001) as well as between EST2 duration and SVRI (r = -0.448; p = 0.004) measured by ICG2 (Table 5).

Table 5

Correlation between exercise stress test (EST) duration and impedance cardiography (ICG) parameters

ICG parameters	EST1 duration (min)		EST2 duration (min)	
	r	*p*	*r*	*p*
CO (L/min)	0.357	0.026	0.517	0.001
SV (mL)	0.246	0.131	0.340	0.034
ACI (1/100/s²)	0170	0.302	0.226	0.167
VI (1/1000/s)	0.257	0.115	0.264	0.104
BP systolic (mmHg)	-0.075	0.649	0.061	0.714
BP diastolic (mmHg)	-0.040	0.807	-0.056	0.737
HR (bpm)	0.161	0.328	0.225	0.169
SVR (dyne·s/cm^5)	-0.409	0.010	-0.504	0.001
SVRI (dyne·s/cm^5/m²)	-0.356	0.026	-0.448	0.004
SSVRI (dyne·s/cm^5/m²)	-0.182	0.267	-0.246	0.131

For abbreviations see under Table 3.

Discussion

The beneficial effects of CVR are proven in patients with coronary artery disease, severe arterial hypertension, heart failure, heart valve repair, and heart transplantation [18]. A well-designed CVR program reduces cardiovascular mortality and hospital readmission and increases the quality of life in CVD patients [19–21]. This is why relevant Guidelines give recommendations for CVR in patients with CVD [8, 22–27]. Three main postulates of CVR are exercise training, lifestyle changes, and psychological intervention [18]. The main goal of CVR is "to provide the best possible physical, mental, and social conditions, so that the patients may, by their efforts, preserve or resume optimal functioning in their community and through improved health behavior slow or reverse the progression of the disease" [28].

Physical activity as a cornerstone of CVR has a positive effect on CVD regardless of patients' reduced or preserved ejection fraction [8, 29]. In addition, the beneficial effects of exercise-based CVR do not depend on gender or age structure [30, 31]. It increases physical strain tolerance [30] and arterial compliance [7], improves lipid profile [32, 33], reduces the symptoms of depression [34–36], and leads to weight and blood pressure reduction [37, 38]. It can even induce gray matter volume recovery [39]. Our study once again showed that personalized and supervised physical training improves physical strain tolerance. The average strain level was higher, and the duration of the test was significantly longer in the second EST. Additionally, a higher number of patients achieved submaximal heart rate on EST2 compared to EST1.

On the other hand, CVR did not lead to significant changes in lipid profile or any other laboratory parameters except red blood count. It did reduce total cholesterol, LDL, and HbA1c, but without statistical significance. This is probably due to the shorter duration of the CVR program, as our study included patients with three-week CVR. For significant laboratory changes, CVR should last longer, at least eight weeks [31, 32, 40]. For example, in a study with 547 coronary heart disaese (CHD) patients, CVR led to a better lipid profile (total cholesterol, triglycerides, LDL, and HDL). However, the CVR program in this study included 24 exercise sessions scheduled over eight weeks [41]. Furthermore, Vergès et al. [42] showed in their research that CVR could improve response to the hypolipidemic therapy in patients with CHD. However, the average duration of CVR in this study was two months. On the other hand, we noted an improvement in red blood count which can have a positive impact on functional capacity [43].

By using ICG, we tried to investigate the effects of CVR on hemodynamic status in CHD patients. Besides CO and SV, we assessed contractility-related (VI, ACI) and vascular system-related parameters (SVR, SVRI, SSVRI). It is well known that physical activity can decrease blood pressure values through the reduction of vascular resistance [44]. The exact mechanism of how physical activity leads to vascular resistance reduction is unknown, but it probably includes the sympathetic nervous system and renin-angiotensin-aldosterone system [45]. Furthermore, a combination of endurance and resistance training can improve endothelial function and arterial compliance [46, 47]. In our study, exercise-based CVR did lead to SVRI and SSVRI reduction, but without statistical significance (Z = 0.510; p = 0.613 and Z = 0.950; p = 0.348). However, we emphasize the fact that the beneficial effects of exercise training are dose-dependent. It would be interesting to see whether the reduction of vascular-related parameters would be statistically significant if the CVR program lasted eight or twelve weeks.

Beneficial but temporary results of CVR on hemodynamic status are found in patients with stable chronic heart failure [48]. Using ICG, Gielerak et al. [49] showed that CVR could reduce fluid retention in patients with HF. Moreover, exercise training can improve SV and CO [50]. However, in our study, CO and SV were higher at the end of CVR, but without any statistical significance (t = 0.512; p = 0.611 and Z = 1.349; p = 0.184). Other studies that investigated the influence of physical training on SV or CO included patients with reduced ejection fraction or patients with heart failure with preserved EF (HFpEF). Our study included stable patients with preserved EF and without any symptoms and signs of HF.

The next step in our investigation was to assess whether there was a correlation between the parameters of the exercise stress test and ICG. There was no significant correlation between the average level of EST1 and ICG1 parameters. On the other hand, there was a strong positive correlation between the level of EST2 and CO ($r = 0.538$; $p < 0.001$) and between the level of EST2 and SV ($r = 0.380$; $p = 0.017$) according to ICG2. Moreover, there was a strong negative correlation between the EST2 level and SVR ($r = -0.472$; $p = 0.002$) as well as between the EST2 level and SVRI ($r = -0.407$; $p = 0.010$) according to ICG2. CO significantly positively ($r = 0.357$; $p = 0.026$) and SVR ($r = -0.409$; $p = 0.010$) and SVRI ($r = -0.356$; $p = 0.026$) significantly negatively correlated with EST1 duration when ICG1 parameters were compared. There was a strong positive correlation between EST2 duration and CO ($r = 0.517$; $p = 0.001$) as well as between EST2 duration and SV ($r = 0.340$; $p = 0.034$), and a strong negative correlation between EST2 duration and SVR ($r = -0.504$; $p = 0.001$) as well as between EST2 duration and SVRI ($r = -0.448$; $p = 0.004$) according to ICG2.

These results show that CVR has a great influence on hemodynamic parameters. Namely, CVR led to a positive correlation between physical strain tolerance and cardiac and stroke volume and a better negative correlation between physical strain tolerance and peripheral vascular resistance. As far as we know, this is the first study that investigated the effects of CVR on hemodynamic parameters and its correlation with physical strain tolerance in patients with CAD and preserved ejection fraction. An implementation of ICG as a valuable tool in assessing the effects of CVR on the hemodynamic status of CAD patients should be considered.

Study limitations

There are several limitations to this study: 1) the study sample was relatively small. However, almost all studies that involved ICG as a diagnostic tool included 50 patients or less; 2) we did not perform echocardiographic examinations. It would be interesting to see how some ICG parameters (*i.e.*, SV and CO) correlate with some echocardiographic parameters (*i.e.*, left ventricular mass index and cardiac index); 3) our study included the CVR program that lasted for three weeks. We consider that a longer CVR duration (*i.e.*, eight weeks) could have greater beneficial effects on the hemodynamic status of CHD patients.

Conclusion

Our study once again showed that a well-designed CVR program could lead to better physical strain tolerance in patients with CAD. Furthermore, CVR led to a significant positive correlation between EST and cardiac output as well as between EST and stroke volume measured by ICG. On the other hand, there was a significant negative correlation between EST and vascular-related parameters according to ICG at the end of the CVR program.

Conflict of interests

The authors have no conflict of interest to declare regarding the present paper.

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Expression of apoptotic protease-activating factor-1 in adenoid cystic carcinoma of the salivary glands and its clinicopathological relevance

Ekspresija faktora-1 aktivacije proteaza apoptoze u adenoidnom cističnom karcinomu pljuvačnih žlezda i njegov kliničkopatološki značaj

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Abstract

Background/Aim. Apoptotic protease-activating factor-1 (Apaf-1) is a key molecule in the intrinsic or mitochondrial pathway of apoptosis. Some pathological conditions, such as cancer, stroke, and neurodegenerative diseases, are the result of dysregulation in the intrinsic apoptosis pathway. The aim of this study was to analyze the immunohistochemical expression of Apaf-1 in adenoid cystic carcinoma (ACC) cells of the salivary glands and its correlation with clinicopathological parameters of patients (gender, age, localization, histological type, and overall survival). **Methods.** Formalin-fixed, paraffin-embedded tissues of ACC of the salivary glands from 50 male and female patients with an average age of 58 years, were used for the study. We used the technique of tissue microarray (TMA blocks). Sections from the TMA mold, 5 μm thick, were stained with the streptavidin-biotin immunohistochemical technique using primary antibodies specific for Apaf-1 (Leica Biosystems, Newcastle, UK). Stained tissue sections were analyzed by the light microscope (Olympus type BH-2). Based on the data collected, the database was created in SPSS software v. 22.0 (SPSS Inc., Chicago, ILL, USA), which was used for further statistical analysis. The statistical data analysis included methods of descriptive and analytical (inferential) statistics. **Results.** The results of the immunohistochemical analysis of Apaf-1 expression in the samples of patients with ACC of the salivary glands were compared with the clinicopathological parameters of these patients. The immunohistochemical expression of Apaf-1 showed no statistical significance with regard to the patients' gender ($p = 0.552$), age ($p = 0.106$), histological tumor type ($p = 0.654$), and localization of ACC in the salivary glands ($p = 0.486$). There was no statistically significant correlation observed between the overall survival of ACC patients and Apaf-1 expression in tumor cells ($p = 0.340$, Log-Rank test). **Conclusion.** With regard to ACC, Apaf-1 expression is not in correlation with clinicopathological parameters (gender, age, localization, histological tumor type, outcome of the disease, and overall survival). Therefore, we believe Apaf-1 cannot be regarded as an independent prognostic factor for course and outcome of ACC of the salivary glands.

Key words:
sex; apoptosis regulatory proteins; age factor; immunohistochemistry; apoptotic protease-activating factor; carcinoma, adenoid, cystic; survival.

Apstrakt

Uvod/Cilj. Faktor 1 aktivacije proteaza apoptoze (Apaf-1) je ključni molekul u unutrašnjem ili mitohondrijskom putu apoptoze. Različita patološka stanja, kao na primer maligniteti, neurodegenerativne bolesti, moždani udar, povezuju se sa poremećajem unutrašnjeg puta apoptoze. Cilj rada bio je da se analizira imunohistohemijska ekspresija Apaf-1 u ćelijama adenoidnog cističnog karcinoma (ACC) pljuvačnih žlezda i njegova korelacija sa kliničko-patološkim parametrima obolelih (pol, starost, lokalizacija, histološki tip i ukupno preživljavanje). **Metode**. U studiji su korišćeni parafinski uzorci ACC pljuvačnih žlezda 50 obolelih muškaraca i žena, prosečne starosti 58 godina. Korišćena je tehnika mikrotkivnog niza (*tissue microarrays* – TMA *block*). Mikrotkivni preseci, debljine 5 μm, bojeni su streptavidin-biotin imunohistohemijskom tehnikom primenom primarnog antitela specifičnog za Apaf-1 (Leica Biosystems, Newcastle, UK). Preparati su analizirani svetlosnim mikroskopom (Olympwus type BH-2). Na osnovu prikupljenih podataka kreirana je baza podataka u SPSS softveru 22.0 (SPSS Inc., Chicago, ILL, USA) koji je korišćen za dalju statističku analizu. Statistička analiza podataka obuhvatala je metodu deskriptivne i analitičke

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(inferencijalne) statistike. **Rezultati**. Rezultati imunohistohemijske analize ekspresije Apaf-1 u uzorcima bolesnika sa ACC pljuvačnih žlezda upoređeni su sa njihovim kliničko-patološkim parametrima. Ekspresija Apaf-1 nije pokazala statističku značajnost u odnosu na pol bolesnika (p = 0,552), starost (p = 0,106), histološki tip tumora (p = 0,654) i lokalizaciju ACC u pljuvačnim žlezdama (p = 0,486). Nije primećena statistički značajna korelacija između ekspresije Apaf-1 i preživljavanja bolesnika sa ACC (p = 0,340, Log-Rank test). **Zaključak**. U odnosu na ACC, ekspresija Apaf-1 nije u korelaciji sa kliničko-patološkim parametrima bolesnika (pol, starost, lokalizacija, histološki tip i ukupno preživljavanje). Zato verujemo da se Apaf-1 ne može smatrati nezavisnim prognostičkim faktorom za tok i ishod ACC pljuvačnih žlezda.

Ključne reči:
pol; apoptoza, regulatorni proteini; životno doba, faktor; imunohistohemija; faktor-1, proteaza-aktivirajući, apoptotički; karcinom, adenoidni cistični; preživljavanje.

Introduction

According to the latest WHO histological classification [1], salivary gland carcinomas encompass over 20 histological types and comprise 8.5% of all head and neck carcinomas [2]. Being one of the most frequent malignant tumors affecting the salivary glands, adenoid cystic carcinoma (ACC) comprises 3–5% of all head and neck malignancies. While ACC may develop in all salivary glands, it is mainly located in the minor salivary glands, usually in the palate. Slow growth, extensive local invasion, perineural spread, and the late development of distant metastases are characteristic of ACC [3–5]. Microscopically, ACC may show a cribriform, tubular, or solid histopathological pattern, even though all three patterns are present in most cases, with one pattern being dominant [6, 7].

The primary goal of treating ACC patients is to control the disease locally while maintaining normal functioning and preventing distant metastases. Therefore, surgical resection of the tumor is the therapy of choice in most cases. However, given the nature of the lesion, adjuvant therapy is used besides surgical resection. The role of postoperative radiotherapy is still considered controversial due to divergent reports concerning its effects on ACC patients. Moreover, the role of chemotherapy in ACC patients has not been adequately defined yet [8].

In recent years, there has been extensive research into new biological markers in order to foresee the efficacy of these therapies with more confidence. Nowadays, there is a particular interest in the molecules involved in programmed cell death – apoptosis [9, 10]. There are two apoptotic pathways: extrinsic (receptor) and intrinsic (mitochondrial). Apoptotic protease-activating factor-1 (Apaf-1) has an important role in the intrinsic apoptotic pathway. Apaf-1 is a multidomain protein (139 kDa) that contains the amino-terminal domain, the central oligomeric nucleotide-binding domain, and the carboxy-terminal domain. The Apaf-1 protein binds cytochrome c released from mitochondria and initiates conformational changes and the formation of a multiprotein complex – apoptosome, comprised of cytochrome c, Apaf-1, and procaspase-9. This complex proteolytically activates the caspase-9 enzyme, whose role is to activate effector caspases (caspase-3 and -7) in cascade reactions, which then initiate the degradation phase of apoptosis [11–14, 15].

The evaluation of immunohistochemical expression of Apaf-1 in relation to clinicopathological parameters of patients has been conducted on various types of human tumors. Some authors have indicated that lower expression of Apaf-1 can represent the trigger for the malignant transformation of cells and can also be the cause of chemotherapy resistance [16–18].

Therefore, the aim of this study was to analyze the immunohistochemical expression of Apaf-1 in ACC tumor cells of the salivary glands and its correlation with clinicopathological parameters of patients.

Methods

This study included 50 patients diagnosed with ACC of the salivary glands. From the paraffin blocks, from each case, three cylinders of tissue were taken from the tumor area with a donor block with a diameter of 1.2 mm and placed in the three recipient paraffin blocks (TMA blocks) every 62 cores. Control (human myocardium tissue) is also included in the TMA blocks.

Five-micrometer cut sections from TMA blocks were deparaffinized, rehydrated, placed in 3% H_2O_2 for 10 min to block endogenous peroxidase activity, and washed with tap water. Then, they were processed with 0.01 citrate buffer (pH 6.0) and treated in a microwave oven for 20 min at 600 W, and placed in a bath of tap water for 20 min, then in distilled water and TBS buffer (pH 7.6) for 5 min, and placed in diluted normal serum for 10 min. Afterward, the tissue sections were incubated for 1 h with the following rabbit monoclonal primary antibody Apaf-1, Product Code: NCL-APAF1, dilution 1:40, Leica Biosystems, Newcastle, UK.

Streptavidin-biotin method using DAKO's LSAB+ kit (DAKO, Denmark) was applied, with diaminobenzidine (DAB) as the chromogen solution and Mayer's hematoxylin for the counterstain.

All immunostained sections were independently evaluated by two pathologists. The results of immunohistochemical staining Apaf-1 were scored by a semiquantitative technique into 3 grades according to the intensity of the staining: 1+ (weak or negative), 2+ (moderate), and 3+ (strong).

Statistical analysis

Statistical analyses were performed using SPSS software v. 22.0 (SPSS Inc., Chicago, ILL, USA). Descriptive

data for all groups and variables were expressed as mean ± standard deviation (SD) for continuous variables or percent of a group for discrete variables (categorical data). Categorical data were analyzed using the Pearson's χ^2 test. Normal distribution was tested using the Kolmogorov-Smirnov test, and the normal distribution of continuous data was tested with a one-way ANOVA test. Kappa coefficient of agreement was used for the evaluation of correspondence between two pathologists. Overall survival rates were calculated from the day of diagnosis by the Kaplan-Meier method, and differences were evaluated by the Log-Rank test. All reported *p*-values were two-sided. Differences were considered significant when the *p*-value was < 0.05.

Results

This study used the streptavidin-biotin immunohistochemical method in order to investigate the immunohistochemical expression of Apaf-1 in ACC cells of the salivary glands in relation to clinicopathological parameters of the patients. The evaluation was performed on biopsy samples of 50 patients (19 male and 31 female), with a mean age of 58 years (range 28–78 years). Statistical analysis showed a high degree of interobserver concordance between the two pathologists ($\kappa = 0.817$).

In ACC cells, Apaf-1 was expressed in their cytoplasm. In most cases (42%), the intensity of Apaf-1 expression was strong (3+) (Figure 1a). Moderate expression (2+) was observed in 15 (30%) samples (Figure 1b), while 14 (28%) samples showed Apaf-1 expression graded as 1+ (Figure 1c). The differences in the intensity of Apaf-1 expression in ACC cells in the salivary glands were not found statistically significant ($p = 0.423$) (Table 1).

The results of the immunohistochemical analysis of Apaf-1 expression in the samples of patients with ACC of the salivary glands were compared with the clinicopathologi-

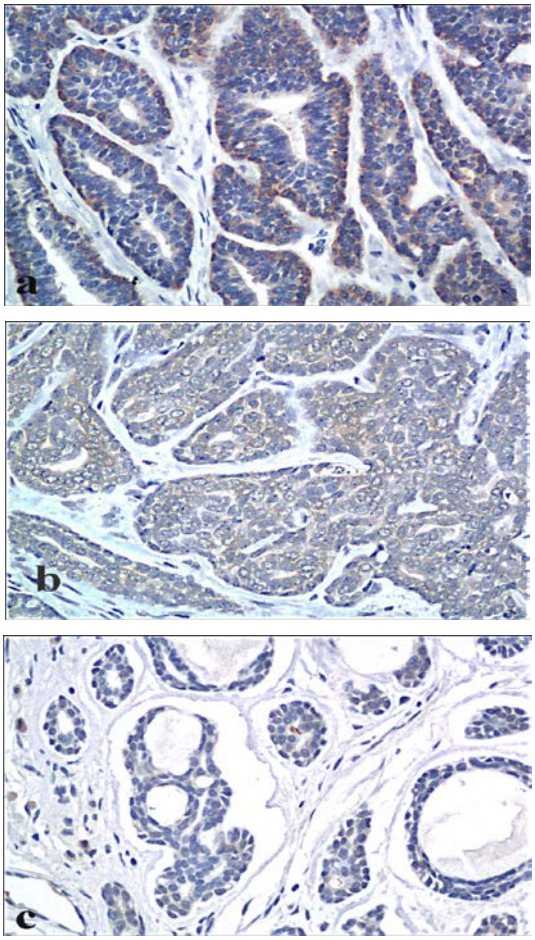

Fig. 1 – Streptovidin-biotin immunohistochemical staining of adenoid cystic carcinoma (ACC) tumor cells in the salivary glands: a) strong (×400), b) moderate (×200), and c) weak (×200) intensity of apoptotic protease-activating factor-1 (Apaf-1) expression.

Table 1

Apoptotic protease-activating factor-1 (Apaf-1) expression in adenoid cystic carcinoma (ACC) of the salivary glands and its correlation with patient's clinicopathological parameters

Patient's characteristics	Apaf-1 expression			Significance (*p*-value)
	+	++	+++	
ACC, n (%)	14 (28.0)	15 (30.0)	21 (42.0)	[a]0.423
Gender, n (%)				
male	7 (36.8%)	5 (26.4%)	7 (36.8%)	[a]0.552
female	7 (22.6%)	10 (32.3%)	14 (45.2%)	
Age (years), mean ± SD	52.50 ± 15.42	57.80 ± 12.5	61.81 ± 10.23	[b]0.106
(median; min-max)	(56.5; 29–78)	(56; 39–78)	(61; 41–75)	
Localization, n (%)				
parotid	1 (25.0)	1 (25.0)	2 50.0)	
submandibular	1 (11.2)	4 (44.4)	4 (44.4)	
hard palate	8 (27.6)	7 (24.1)	14 (48.3)	[a]0.486
minor salivary gland	4 (50.0)	3 (37.5)	1 (12.5)	
Histological type, n (%)				
cribriform	2 (14.3)	5 (35.7)	7 (50.0)	
tubular	6 (33.3)	4 (22.2)	8 (44.4)	[a]0.654
solid	6 (33.3)	6 (33.3)	6 (33.3)	
Outcome, n (%)				
surviving	6 (31.6)	7 (36.8)	6 (31.6)	[a]0.494
deceased	8 (25.8)	8 (25.8)	15 (48.4)	

n – number of patients; SD – standard deviation.

[a]χ^2-test; [b]One-way ANOVA.

cal parameters of these patients (Table 1). The immunohistochemical expression of Apaf-1 showed no statistical significance with regard to the patients' gender (p = 0.552) and age (p = 0.106). In male patients, the grades 3+ and 1+ were equally represented, with 5 patients showing moderate (2+) Apaf-1 expression. The intensity of Apaf-1 expression in female patients was most frequently graded as 3+ (14/31). There were 10 female patients showing moderate (2+) Apaf-1 expression and 7 female patients with weak (1+) Apaf-1 expression.

Concerning the age of the patients, Apaf-1 expression of 1+ or 2+ was observed in the patients whose mean age was 56 years, whereas Apaf-1 expression graded as 3+ was observed in the patients whose mean age was 61 years (Table 1).

Regarding the localization of ACC in the salivary glands, the intensity of Apaf-1 expression was not found statistically significant (p = 0.486) (Table 1). ACC located in the minor salivary glands of the hard palate demonstrated strong Apaf-1 expression (3+) in 48% of cases. There was an approximate number of cases with moderate (24.1%) and weak (27.6%) intensities of Apaf-1 expression. A similar intensity of Apaf-1 expression was observed in ACC of the parotid glands. As opposed to these findings, the tumors located in the submandibular salivary gland showed the same percentage (44.4%) of strong and moderate intensities of Apaf-1 expression, while in 11.2% of cases, Apaf-1 expression was graded as 1+.

No statistically significant correlation was detected between the histological tumor type and Apaf-1 expression (p = 0.654) (Table 1). Apaf-1 expression in tumors with the cribriform pattern was most frequently graded as 3+ (50%). In 35.7% of cases, the intensity of Apaf-1 expression was graded as moderate, and in 14.3% of cases, it was graded as 1+. The percentage of Apaf-1 expression graded as 3+ was somewhat lower in ACC with the tubular pattern (44.4%), while Apaf-1 expression graded as 1+ was present to a higher extent when compared to tumors with the cribriform pattern. When it comes to solid tumors, the three different grades of Apaf-1 expression were equally distributed.

The present study also investigated the significance of evaluating Apaf-1 expression in relation to the outcome of the disease (Table 1). The percentages of different Apaf-1 expression grades were quite similar in patients who were alive at the end of this investigation. Strong immunopositivity (3+) was observed in tumor cells of approximately 50% of deceased patients. The evaluation of Apaf-1 expression was not found statistically significant in relation to surviving and deceased patients (p = 0.494).

There was no statistically significant correlation observed between the overall survival of ACC patients and Apaf-1 expression in tumor cells (p = 0.340, Log-Rank test) (Table 2, Figure 2).

Table 2

Apoptotic protease-activating factor-1 (Apaf-1) expression in adenoid cystic carcinoma (ACC) of the salivary glands in relation to the 13-year survival period

Grade of Apaf-1 expression	Follow-up period (years)								Significance (p-value)
	1	3	5	7	9	10	11	13	
+	92.9	78.6	78.6	71.4	63.5	50.8	16.9	16.9	
++	93.3	93.3	80.0	53.3	53.3	53.3	53.3	26.7	#0.340
+++	90.5	66.7	47.6	47.6	33.3	33.3	22.2	22.2	

All values are expressed as percentages (%) of the patients.
#Log-Rank test.

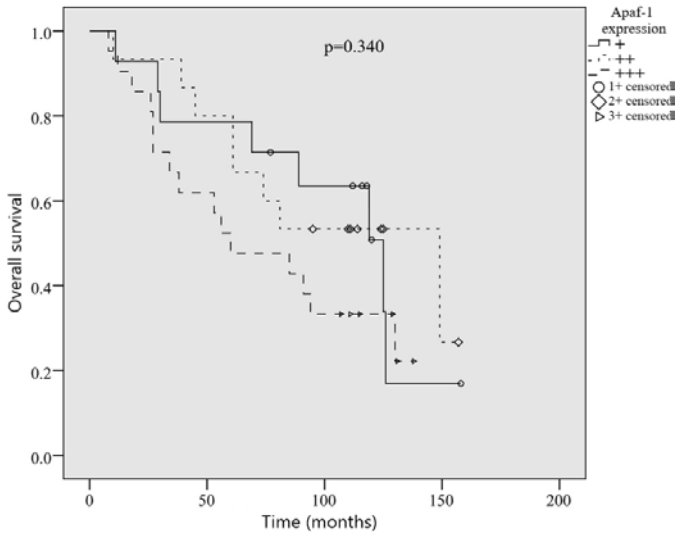

Fig. 2 – Overall survival of patients with adenoma cystic carcinoma (ACC) in relation to apoptotic protease-activating factor-1 (Apaf-1) expression.

The mean survival period of patients with Apaf-1 expression of 1+ was 125 months. In these patients, the 5-year survival rate was 78.6%, the 10-year survival rate was 50.8%, and the 13-year survival rate was 16.9%. The patients with Apaf-1 expression graded as 2+ had the longest mean survival period of 149 months. Their 5-year survival rate was 80%, while their 10- and 13-year survival rates were 53.3% and 26.7%, respectively. The shortest mean survival period of 60 months was detected in the patients with Apaf-1 expression of 3+. Their 5-year survival rate was 65%, whereas their 10- and 13-year survival rates were 45.8% and 22.9%, respectively.

Discussion

Salivary gland carcinoma is characterized by a disbalance of regulatory mechanisms of different cellular pathways, including apoptosis. Apaf-1 is a key effector molecule of the intrinsic apoptotic pathway, and it has been investigated in various human tumors [15–18].

Our findings suggest that ACC cells in the salivary glands show Apaf-1 immunopositivity in most cases. Two independent pathologists evaluated the intensity of cytoplasmic expression in the sections from TMA blocks with a high degree of interobserver concordance. Strong Apaf-1 expression was detected in 42% of cases, while moderate Apaf-1 expression was observed in 30% of cases. Weak or negative Apaf-1 expression was found in 28% of patients.

The evaluation of immunohistochemical expression of the antibody typical of Apaf-1 has been conducted on patients with malignant melanomas. The level of Apaf-1 cytoplasmic expression was determined in the samples from TMA blocks. As opposed to our findings, in 36 patients out of 70 (51.4%) diagnosed with melanoma, Apaf-1 expression was graded as weak. Moderate expression was observed in 21 (30%) patients, while strong expression was found in 7 (10%) patients. Six melanoma patients (8.6%) did not show immunopositivity for this antibody [15]. Significantly reduced expression of Apaf-1 molecules has been observed in colon adenocarcinoma. Out of 529 patients with colon adenocarcinoma, only 129 (22%) reacted positively to the antibody typical of Apaf-1 [17]. Since Apaf-1 expression was graded as strong in 76.9% of cases, Apaf-1 has been characterized as a highly immunopositive marker, which is in line with our findings observed in nevus cells. The loss of Apaf-1 expression can be the trigger for initiating a malignant transformation of melanocytes. Furthermore, it is argued that reduced Apaf-1 expression in melanoma cells, which have demonstrated weak Apaf-1 expression in most cases, can be the cause of chemotherapy resistance [15]. Given the fact that adenomas also showed 100% positivity for Apaf-1 antibody, the authors believe that reduced Apaf-1 expression in adenocarcinoma is in correlation with the malignant alteration of adenoma [17].

Cytoplasmic positivity for Apaf-1 antibody was observed in cervix carcinoma, which is in accordance with our findings. Out of 86 patients diagnosed with cervix carcinoma, 42% showed strong immunopositivity to Apaf-1. Moderate expression was observed in 34% and weak or negative in 24% of patients [16].

The ability of tumor cells to resist apoptotic signals can affect the aggressiveness and, therefore, the prognosis. Several studies have reported the results of the expression, *i.e.*, the activity, of the factor of apoptotic signaling, which affects the apoptotic resistance of tumor cells or cell lines in the *in vitro* conditions or animal models. However, the correlation of these results with clinicopathological parameters and malignancy prognosis is particularly significant [19–21]. The correlation between Apaf-1 expression and clinicopathological parameters of ACC patients was not found statistically significant in our study. Apaf-1 expression was most frequently graded as 1+ or 3+ in 19 male patients. Apaf-1 expression of 3+ was observed in 14 out of 31 female patients. The mean age of the patients with expressions of 1+ and 2+ was 56 years, while in patients with expressions of 3+, it was 61 years. The tumors affecting the minor salivary glands of the hard palate, which was the most common localization in the present study, showed Apaf-1 expression of 3+ in approximately 50% of cases. The correlation between the histological type and Apaf-1 expression was not found statistically significant either. Apaf-1 expression detected in cribriform and tubular tumors was mainly graded as 3+. An equal distribution of the three different grades of Apaf-1 expression was observed in the solid variant of ACC, which is commonly associated with a less favorable prognosis and the formation of distant metastases.

Our findings are in line with the findings reported about melanomas since Apaf-1 expression was not found statistically significant concerning gender, age, histological subtype, and localization [15].

As opposed to our findings, reduced Apaf-1 expression in colorectal carcinoma was in correlation with poor prognostic factors such as depth of invasion, formation of metastases in regional lymph nodes, and histological grading [18]. In patients diagnosed with cervix carcinoma, there was a significant correlation between Apaf-1 expression and nodal status. The patients with strong or moderate Apaf-1 expression had a significantly lower number of metastases in the lymph nodes at the time of surgical intervention when compared to the patients with weak or negative expression [16].

According to the latest reports, the survival rates following surgical removal of ACC is 70% after 5 years and 60% after 10 years [3, 4].

Furthermore, Apaf-1 expression was not statistically significant to the overall survival of ACC patients. In summary, the number of surviving patients with Apaf-1 expression of 1+ and 2+ is approximately the same at the 5- and 10-year follow-ups. A lower survival rate was observed in the patients with Apaf-1 expression of 3+. In these patients, the survival rate was 47.6% after 5 years and 33.3% after 10 years. The mean survival period of ACC patients was the longest in the patients with expression graded as 2+ (149 months), while the shortest mean survival period was observed in the patients with expression graded as 2+ (60 months). Concerning the surviving and deceased patients, Apaf-1 was not found statistically significant. The patients who were alive at the end of the follow-up period had almost equal percentages of different Apaf-1 ex-

pression grades. Apaf-1 expression of 3+ was more common in the deceased patients, *i.e.*, in 15 out of 31 deceased patients.

Our findings are in line with the findings obtained in the studies investigating Apaf-1 and the survival of patients with melanoma [15] and colorectal carcinoma [18]. The 5-year survival rate of patients diagnosed with colorectal carcinoma who had negative Apaf-1 expression was 67.7%, and their 10-year survival rate was 62.3%. The five-year survival rate of patients with positive Apaf-1 expression was 72.7%, and their 10-year survival rate was 67.1%. These findings are in accordance with the findings obtained by Zlobec et al. [17], who investigated Apaf-1 expression in a large series of 1,420 colorectal carcinomas.

Conclusion

With regard to ACC, Apaf-1 expression is not in correlation with clinicopathological parameters of patients (gender, age, localization, histological tumor type, outcome of the disease, and overall survival). Therefore, we believe Apaf-1 expression cannot be regarded as an independent prognostic factor for course and outcome of ACC.

Conflict of interest

The authors declare no conflict of interest. This study received no funding.

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Is the insulin necessary for the struggle against oxidative stress in diabetes mellitus type 2 – a pilot study

Da li je insulin neophodan za borbu protiv oksidativnog stresa u dijabetesu melitusu tip 2 – pilot studija

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Abstract

Background/Aim. Hyperglycaemia has a detrimental effect on the progress of micro/macrovascular complications in patients with diabetes mellitus type 2 (T2DM). Additionally, all known complications in T2DM are coupled with oxidative stress developed from different metabolic pathways. The aim of this study was to estimate the quality of glucoregulation and the degree of oxidative stress in T2DM patients depending on the applied therapeutic protocol and assess their correlation with clinical data and crucial biochemical parameters important for the development of diabetes complications. **Methods.** All included patients were divided into two groups: those treated with oral antidiabetic drugs (OAD) and those treated with oral antidiabetic drugs and insulin (OADINS). Thiobarbituric acid reactive substances (TBARS), total sulfhydryl groups (TSH), the activity of superoxide dismutase (SOD), total nitrites (NOx), vascular endothelial growth factor (VEGF), and activities of matrix metalloproteinase 9 (MMP9) were measured, together with lipid profile and routine biochemical parameters. All subjects were analyzed for demographic characteristics and detailed medical history as well as smoking habits and calculated for body mass index (BMI). **Results.** All patients were uniformly poor glucoregulated and dyslipidemic. SOD activity was decreased, and lipid peroxidation was increased in the OAD group compared to OADINS. Deficient glucoregulation in both the OAD and the OADINS groups did not associate with an oxidative state outcome. In both of these groups, the concentrations of VEGF and MMP9 were significantly higher than in controls. **Conclusion.** The better antioxidative outcome, expressed with a normalized concentration of TBARS, preserved TSH, and normalized SOD activity in T2DM patients treated with OADINS compared to those treated exclusively with OAD, suggests the need for more careful consideration of earlier insulin introduction into T2DM therapy in order to prevent the development of complications.

Key words:
diabetes mellitus, type 2; insulin; oxidative stress.

Apstrakt

Uvod/Cilj. Kod bolesnika sa dijabetesom melitusom tipa 2 (T2DM), hipergikemija podstiče progresiju mikro/makrovaskularnih komplikacija. Dodatno, sve poznate komplikacije u T2DM povezane su sa oksidativnim stresom koji nastaje različitim metaboličkim procesima. Cilj rada bio je da se proceni kvalitet glikoregulacije i stepen oksidativnog stresa kod bolesnika sa T2DM u zavisnosti od primenjenog terapijskog protokola i njihova povezanost sa kliničkim podacima i ključnim biohemijskim parametrima važnim za razvoj dijabetesnih komplikacija. **Metode.** Svi ispitivani bolesnici bili su podeljeni u dve grupe: grupa bolesnika lečenih samo oralnim antidijabetičnim lekovima (OAD) i grupa lečenih OAD i insulinom (OADINS). Praćeni su tiobarbiturna kiselina-reagujuće supstance (TBARS), totalni sulfhidrili (TSH), aktivnost superoksid dizmutaze (SOD), ukupni nitriti (NOx), vaskularni endotelni faktor rasta (VEGF), aktivnost matriksne metaloproteinaze 9 (MMP9), lipidni profil i rutinski biohemijski parametri. Svim ispitanicima su analizirane demografske karakteristike, detaljna medicinska istorija, pušačke navike, a izračunat im je i indeks telesne mase (BMI). **Rezultati.** Svi bolesnici su imali loše regulisanu glikoregulaciju i bili su dislipemični. Pokazana je smanjena aktivnost SOD i povećana lipidna peroksidacija u OAD grupi u poređenju sa OADINS grupom. Loša glikoregulacija u grupama OAD i OADINS nije bila povezana sa rezultatima oksidativnog stanja. I u OAD i u OADINS grupi, koncentracije VEGF i MMP9 bile su značajno više u odnosu na kontrole. **Zaključak.** Bolji

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antioksidativni odgovor registrovan kroz normalizovanu koncentraciju TBARS, očuvan TSH i SOD u granicama normalnih vrednosti kod T2DM bolesnika lečenih OADINS u odnosu na bolesnike lečene samo OAD, upućuju na potrebu za pažljivijim razmatranjem ranijeg uvođenja insulina u terapiju obolelih od T2DM, kako bi se sprečio razvoj komplikacija.

Ključne reči:
dijabetes melitus, tip 2; insulin; stres, oksidativni.

Introduction

Diabetes mellitus (DM) type 2 (T2DM) is a foremost public health problem and one of the health challenges of the XXI century. It includes a heterogeneous group of metabolic dysfunctions characterized by impaired insulin secretion, increased glycemia, and variable degrees of insulin resistance. It precedes by a period of abnormal glucose homeostasis classified as impaired fasting glucose or impaired glucose tolerance [1, 2].

In patients with T2DM, hyperglycaemia has a detrimental effect on the progress of micro/macrovascular complications. Glycaemia variability is positively associated with the development of diabetic retinopathy, neuropathy, and nephropathy, whose manifestations are related to atherosclerosis [3]. All of them, including diabetic neuropathy, are particularly emphasized in the appearance of oxidative stress, which promotes the functional and structural changes of the tissue and organs [4]. Besides endothelial dysfunction and inflammation, oxidative stress participates in all known complications during DM [5].

Oxidative modifications of lipids and proteins have been identified in vascular lesions, confirming the role of oxidative stress in atherogenesis. Underlying mechanisms of provoked oxidative stress during DM include the hexosamine pathway, pathway of polyols, activation of protein kinase C, and expansion of advanced glycation end-products [6]. Published data revealed that intermittent hyperglycaemia rather than chronic hyperglycaemia amplifies the production of reactive oxygen species (ROS) [7]. The complications can be suppressed and slowed by adequate therapeutic regulation of glucose and lipids levels. Therefore, regular monitoring of long-term glucose level control through the concentration of the haemoglobin fraction A1c (HbA1c) represents a gold standard in assessing the quality of the therapy [8].

The concentration of substances that react with thiobarbituric acid (TBA) may realize a significant task as a lipid peroxidation indicator as well as the content of total sulfhydryl groups (TSH), which are indicators of the oxidative protein damage [9]. These parameters are also useful in assessing antidiabetic therapy.

Enzyme activities of superoxide dismutase (SOD) and matrix metalloproteinase 9 (MMP9) in plasma represent the reflection of tissue events during the disease-systemic redox milieu and disturbed tissue structure [10]. The proinflammatory actions of MMP9 in DM have already been documented [11]. MMP9 also influences tissue availability to bound vascular endothelial growth factor (VEGF), which is a prominent factor in the development of diabetic complications [12]. VEGF in the systemic circulation is an indicator of endothelial activity whose integrity and functions are of great importance during therapy of DM [13]. The predictive values of these parameters are particularly significant from the aspect of their correlation with other proatherogenic parameters as well as HbA1c concentration.

This study intended to estimate the quality of glucoregulation and the degree of oxidative stress in T2DM patients depending on the applied therapeutic protocol, with the aim to assess their correlation with clinical data and crucial biochemical parameters important for the development of diabetes complications.

Methods

Patients and clinical protocol

This prospective study was conducted in agreement with ethical principles confirmed in the Helsinki Declaration and the approval from the Ethics Committee of the Military Medical Academy (MMA) from April 11, 2017. The clinical part of the study was carried out at the Clinic of Endocrinology of the MMA, while the laboratory measurements were achieved at the Institute for Medical Research and Central Chemical Laboratory of the MMA. All the participants were aware of the purpose of the investigation, after which they authorized the information approval.

Forty T2DM patients diagnosed according to the criteria of the World Health Organization were included in the study. Patients were divided into two groups based on medical therapy for T2DM: those treated with oral antidiabetic drugs (OAD group; n = 20) and those treated with OAD and insulin (OADINS group; n = 20). Baseline insulin substitution in the OADINS group was applied with the whole average daily amount of 0.5 units *per* kilogram of body weight (U/kg bw). The control group of 20 healthy volunteers consisted of 13 males and 7 females, with an average age of 52.6 ± 7.4 years and an HbA_{1c} level of 5.21 ± 0.43%.

Demographic characteristics and detailed medical history, as well as smoking habits, were analyzed. Smokers were considered those respondents who have regularly smoked more than five cigarettes a day for the past six months. Data about comorbidities, such as cardiovascular events, nephropathy, retinopathy, polyneuropathy, and microangiopathy, have been analyzed as well.

The nephropathy was evaluated based on the estimated glomerular filtration rate (eGFR), dividing the T2DM patients into those with eGFR below 60 mL/min/1.73 m² (kidney disease) and those with eGFR above 60 mL/min/1.73 m² (normal) [14].

The degree of retinopathy was assessed based on ophthalmologist examinations, which were performed with ophthalmoscopy and fluorescence angiography. Based on the ophthalmic examination, T2DM patients were classified as subjects with no diabetic retinopathy or those with nonproliferative diabetic retinopathy.

The neuropathy was assessed by an experienced neurologist based on diabetic neuropathic symptoms and electrophysiological analysis of nerve conduction. Cardiovascular events included diagnosed coronary arterial disease, stroke, or peripheral arterial disease [14].

All subjects were anthropometrically measured for body weight (kg) and height (cm) and then calculated for body mass index (BMI).

Biochemical parameters

The concentrations of fasting plasma glucose, HbA1c, creatinine, urea, cholesterol, low-density lipoprotein (LDL), high-density lipoproteins (HDL), and triglycerides were determined from the blood by standard laboratory methods on the apparatus Siemens ADVIA 1800 Chemistry system in the Central Chemical Laboratory MMA.

The plasma concentration of TSH was measured spectrophotometrically at 412 nm in phosphate buffer (0.2 moL + 2 mmoL EDTA, pH 9) using 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.01 M, Sigma). The results are expressed as μmol/L [15].

Lipid peroxidation in the plasma was measured as the production of substances assayed in the reaction with thiobarbituric acid, as described by Girotti et al. [16]. Reactions were performed with TBA reagent [15% trichloracetic acid + 0.375% TBA (both supplied by Merck, Darmstadt, Germany)] in boiling water for 15 min. TBA acid reactive substances (TBARS) were measured spectrophotometrically at 533 nm. The results are expressed as μmol/L.

Total SOD activity in plasma was measured spectrophotometrically at 480 nm as a percentage of inhibition of epinephrine autooxidation in an alkaline medium. After adding 10 mM epinephrine (Sigma, St. Luis, USA), the analysis was performed kinetically, for 10 min, in the sodium carbonate buffer (50 mM, pH 10.2; Serva, Feinbiochemica, Heidelberg, New York) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St. Luis, USA). The activity of SOD is expressed in international units (U/mL). An international unit is defined as an enzyme activity that inhibits 50% of epinephrine autooxidation [17].

Nitrite/nitrate concentration (NOx) was assayed spectrophotometrically at 492 nm, using the colorimetric method of Griess [18].

VEGF in plasma was measured with commercial Quantikine Immunoassay VEGF (R&D System) with a minimum detection level of 9.0 pg/mL. The results are expressed as pg/mL.

MMP9 in plasma was measured with commercially available Elisa kits (Quantikine Elisa Human MMP-9 (R&D System). The results are expressed as ng/mL.

Statistical analysis

Data were presented as mean ± standard deviation (SD) or percentage (%). Parametric variables between groups were analyzed by independent *t*-test and one-way ANOVA with the Tukey *post hoc* test. Nonparametric data were analyzed by the χ^2 test. Spearman's bivariate testing was applied to establish the correlations between biochemical parameters, expressing them through correlation coefficients (ρ). All statistical tests were performed using the statistical software package GraphPadPrism, version 5.03. Statistical significance was set at $p < 0.05$.

Results

Baseline clinical characteristics of T2DM patients divided into two equal groups according to antidiabetic therapy are presented in Table 1. The study groups were consistent in terms of

Table 1

Baseline clinical characteristics of the study population

Characteristics	OAD (n = 20)	OADINS (n = 20)
Age (years), mean ± SD	60.6 ± 11.1	61.9 ± 12.3
Male gender (%)	65	75
DM duration (years), mean ± SD	13.8 ± 7.8	15.2 ± 10.3
Smokers (%)	25	25
Ex-smokers (%)	15	20
Medical history (%)		
hypertension	90	90
nephropathy	85	70
retinopathy	35	30
neuropathy	35	65*
dyslipidaemia	65	65
cardiovascular event	20	25
Additional therapy (%)		
ACE inhibitors	65	65
Ca- antagonists	50	40
beta-blockers	30	40
ATR 2 antagonists	10	10
aspirin	50	45
statins	45	55
BMI (kg/m^2), mean ± SD	29.35 ± 5.13	26.5 ± 4.4*

DM – diabetes mellitus; OAD – oral antidiabetic drugs; OADINS – OAD and insulin; ACE – angiotensin-converting enzyme; Ca – calcium; ATR – angiotensin receptor; BMI – body mass index; SD – standard deviation.

***$p < 0.05$ – statistical significance compared to the OAD group.**

age, DM duration, smoking habits, applied medications (except insulin), and most of the comorbidities, except neuropathy (Table 1). Namely, in the OADINS group, a significantly higher percentage of neuropathy was recorded compared to the OAD group ($p < 0.05$). Furthermore, it was found that patients of the OAD group had a higher BMI ($p < 0.05$).

Compared to the healthy controls, biochemical parameters of both OAD and OADINS subjects showed significantly increased values of HbA1c ($p < 0.001$), cholesterol ($p < 0.001$), MMP9 ($p < 0.001$), and VEGF ($p < 0.001$). On the other hand, plasma HDL level was reduced ($p < 0.001$). No significant difference was noted between study groups in terms of the above-mentioned parameters (Table 2).

OAD respondents showed significant oxidative protein damage compared to control individuals, evaluated by plasma concentrations of TSH ($p < 0.05$). Simultaneously, plasma TSH levels of OADINS subjects were close to the control values (Table 2).

As we already emphasized, SOD is an antioxidant enzyme that plays an important role in protecting against damage that produces ROS. Therefore, the measurement of SOD activity was of particular importance in our investigation. We found significantly decreased SOD activity in the OAD group compared to the OADINS group ($p < 0.001$). At the same time, there was no difference between OADINS subjects and controls (Figure 1A). Another relevant parameter of oxidative damage is the degree of lipid peroxidation. An increased degree of lipid peroxidation, estimated by plasma concentration of TBARS, was detected in the OAD group compared to the controls and the OADINS group (Figure 1B).

Table 2

Biochemical parameters in the study population and controls

Biochemical parameters	Control (n = 20)	OAD (n = 20)	OADINS (n = 20)
HbA1c (%)	5.51 ± 0.73	8.68 ± 1.71***	8.37 ± 1.90***
Cholesterol (mmol/L)	3.08 ± 0.60	4.87 ± 1.39***	4.89 ± 0.89***
Triglycerides (mmol/L)	1.81 ± 0.33	2.01 ± 0.75	1.84 ± 0.98
LDL (mmol/L)	2.09 ± 0.46	2.90 ± 1.17	2.78 ± 0.73
HDL (mmol/L)	1.96 ± 0.50	1.12 ± 0.24***	1.24 ± 0.42***
Urea (mmol/L)	5.86 ± 1.08	6.37 ± 3.02	6.98 ± 2.33
Creatinine (mmol/L)	69.20 ± 9.83	82.70 ± 39.82	83.4 ± 27.6
TSH (μmol/L)	0.489 ± 0.043	0.40 ± 0.14*	0.45 ± 0.08
NOx (μmol/L)	6.58 ± 1.49	9.66 ± 4.01	7.88 ± 4.18
MMP9 (ng/mL)	42.96 ± 3.23	74.37 ± 9.82***	78.42 ± 10.75***
VEGF (pg/mL)	24.25 ± 4.52	56.04 ± 11.85***	63.43 ± 12.70***

OAD – oral antidiabetic drugs; OADINS – OAD and insulin; HbA1c – haemoglobin A1c; LDL – low-density lipoprotein; HDL – high-density lipoprotein; TSH – total sulfhydryl groups; NOx – nitrite and nitrate; MMP9 – matrix metalloproteinase 9; VEGF – vascular endothelial growth factor.

Data are shown as mean ± standard deviation. The statistical differences between groups were performed by one-way ANOVA with the Tukey *post hoc* test.

***$p < 0.05$; ***$p < 0.001$ – statistical significance compared to the control group.**

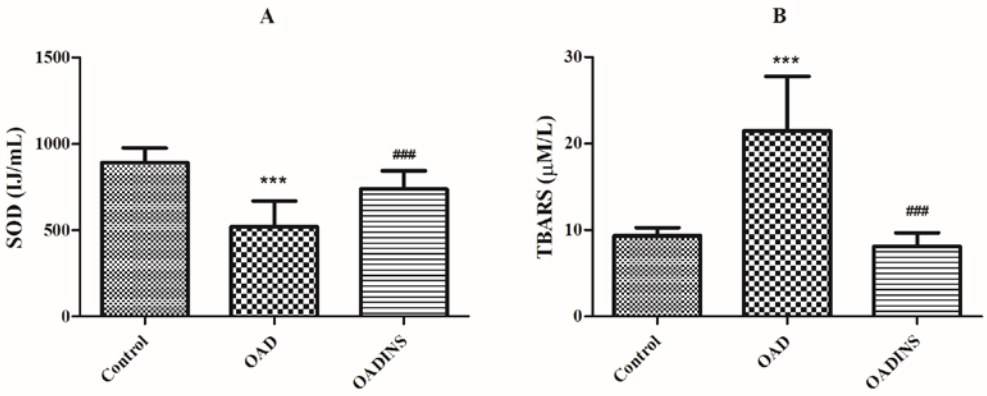

Fig. 1 – A) Superoxide dismutase (SOD) activity and B) thiobarbituric acid reactive species (TBARS) in plasma of the control and type 2 diabetes mellitus (T2DM) patients. Bars in the graph represent the mean ± standard deviation from 20 subjects per group. OAD – oral antidiabetic drugs; OADINS – OAD and insulin.

***$p < 0.001$, statistical significance compared to the control group; ###$p < 0.001$, statistical significance compared to the OAD group.**

Table 3

Spearman's correlation analysis between HbA_{1c} and biochemical parameters in OAD and OADINS groups

Biochemical parameters	OAD ρ	OADINS ρ
Cholesterol (mmol/L)	0.1429	0.2982
Triglycerides (mmol/L)	0.2475	0.2331
LDL (mmol/L)	0.2406	0.1645
HDL (mmol/L)	-0.3469	-0.00565
Urea (mmol/L)	-0.4170	-0.1597
Creatinine (mmol/L)	-0.0572	0.0407
TSH (μmol/L)	0.2851	-0.0659
TBARS (μmol/L)	-0.0376	0.0783
SOD (U/mL)	-0.1414	0.6052**
NOx (μmol/L)	0.0959	-0.0026
MMP9 (ng/mL)	-0.3143	-0.1292
VEGF (pg/mL)	0.0947	-0.1943

OAD – oral antidiabetic drugs; OADINS – OAD and insulin; HbA1c – haemoglobin A1c; LDL – low-density lipoprotein; HDL – high-density lipoprotein; TSH – total sulfhydryl groups; TBARS – thiobarbituric acid reactive species; SOD – superoxide dismutase; NOx – nitrite and nitrate; MMP9 – matrix metalloproteinase 9; VEGF – vascular endothelial growth factor; ρ – coefficient of correlatin.
****$p < 0.01$ – statistical significance compared to the OAD group.**

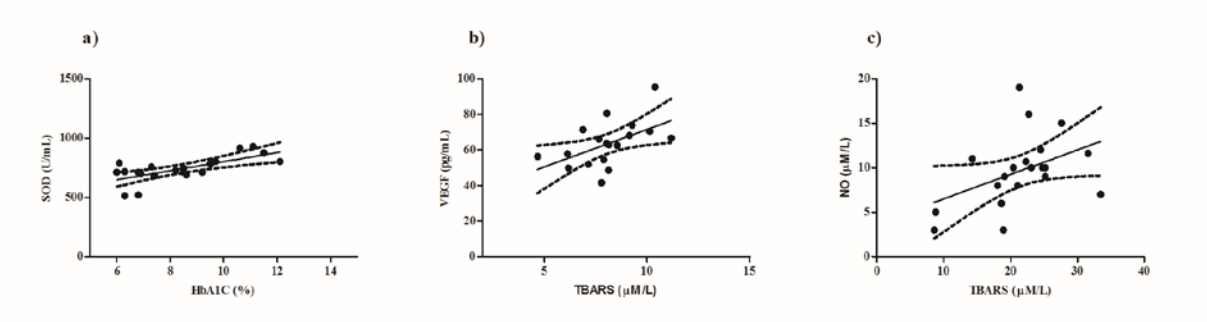

Fig. 2 – Correlation analysis between: a) haemoglobin A1c (HbA1c) (%) and superoxide dismutase (SOD) activity (U/mL) ($\rho = 0.6052$; $p < 0.01$), and b) thiobarbituric acid reactive species (TBARS) (μmol/L) and vascular endothelial growth factor (VEGF) (pg/mL) ($\rho = 0.5356$; $p < 0.05$) in patients treated with oral antidiabetic drugs (OAD) and insulin (OADINS); c) TBARS and total nitrite and nitrate (NOx) ($\rho = 0.4700$; $p < 0.05$) in patients treated only with oral antidiabetic drugs (OAD).

Performing Spearman's correlation analysis among HbA1c (%) and biochemical parameters (Table 3), we found a significant positive correlation between HbA1c and SOD activity in the OADINS group (Figure 2a). In the same group, there was also a positive correlation between TBARS and VEGF (Figure 2b). In the OAD group, a positive correlation was found between TBARS and total nitrite and nitrate (NOx) (Figure 2c).

Discussion

Hyperglycaemia stimulates ROS production from different cellular sources. The electron transport chain in mitochondria is initially a superoxide producer, which leads to the activation of protein kinase C and the formation of advanced glycation end products [19]. ROS-induced mechanisms in the development of T2DM complications are complex. Besides activating protein kinase C, ROS has also been shown to inhibit insulin signal transduction by nuclear kappa factor B, confirming further the association between T2DM and oxidative stress development [20].

As we have already pointed out, HbA1c is the gold standard for monitoring the therapeutic effects in diabetics. Uniformly elevated HbA1c in both OAD and OADINS groups indicate poor glycaemic control regardless of the applied therapy (Table 2). The results of HbA1c follow previously published reports about the frequent occurrence of poor glucose control, which is associated with earlier and more severe complications in T2DM [6, 21].

Deficient glucose control in both OAD and OADINS groups did not associate with an antioxidative outcome. Compared to the OAD group, we found significantly lower lipid peroxidation and elevated SOD in the OADINS group, which indicates a higher antioxidant potential despite persistent poor glucose control (Figure 1). Additionally, a positive correlation between HbA1c and SOD is registered in

the OADINS group (Figure 2a), which is diverse from the published results of Verma et al. [22], who found a negative correlation between SOD and HbA1c in T2DM patients. In the OADINS group, despite the increase in HbA1c, lipid peroxidation reached the control level, and an increase in SOD activity might have been one of the reasons. Elevated SOD could target superoxide, eliminating their destructive effects on the cell membranes and improving antioxidative properties.

The only difference in treatment protocol between the two study groups was insulin, so the question arises about the potentially positive effect of insulin itself on antioxidant status. In the experimental model of DM induced by injection of streptozotocin, it has been proven that insulin may manifest antioxidative potential through the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, which counterbalances endogenous antioxidants against oxidants [23].

In T2DM patients in our study, hypertension, nephropathy, retinopathy, and dyslipidaemia were revealed equally in both OAD and OADINS subjects (Table 1). However, neuropathy was more common in the OADINS group, suggesting this factor was perhaps significant for the introduction of insulin in the therapy. This was later confirmed by an analysis of medical reports. In the pathogenesis of diabetic neuropathy, oxidative stress is persistently active and supported by both inflammation and compromised neural conduction velocity [24]. Thus, the increased antioxidant potential in the OADINS group could have a positive effect on slowing the progression of neuropathy which requires further investigation.

Calculating BMI gives us data on overweight subjects in both OAD and OADINS groups, but BMI was remarkably higher in the OAD group (Table 1). Obesity is a promoting factor for metabolic disturbances, insulin resistance, and the development of metabolic syndrome during T2DM [25, 26]. Together with smoking, being overweight accelerates the development of complications in blood vessels. Several cohort studies with T2DM patients realized that HbA1c and smoking were the strongest predictors regarding the risk of the outcomes and death [27]. It is well documented that compounds from cigarette smoke have the potential to stimulate endothelial NADPH oxidase and promote mitochondrial oxidative stress. Besides the effects on promoting vascular resistance, compounds from cigarette smoke also stimulate the oxidation of LDL, which potentiates its pro-oxidative activity [28]. Oxidized LDL is more effective in activating NADPH oxidase, which stimulates ROS generation [29]. In both OAD and OADINS groups, smokers and ex-smokers were almost half of the subjects (Table 1). We did not obtain differences in LDL and triglyceride levels between the OAD and OADINS group, nor compared to controls, which could be due to the therapeutic usage of antilipemic drugs (Table 2). However, despite the uniform use of antilipemics (Table 1), cholesterol level was elevated in both the OAD and OADINS group compared to controls, while HDL concentrations were reduced (Table 2). In addition, there is a trend of a positive correlation between HbA1c and cholesterol, triglycerides and LDL levels, and a negative correlation between HbA1c and HDL ones (Table 3), confirming that faulty glycaemia control is associated with dysfunctional lipid metabolism. By inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, statins reduce levels of total cholesterol, LDL, and triacylglycerols and concurrently increase HDL level in plasma. Statins are also known for their pro-oxidative potential as they induce metabolic transformation involving CYP450 enzymes, known to potentiate oxidative stress [30]. Assuming the nonsignificant difference in the use of statins in both study groups, this factor could not be the reason for a different antioxidant outcome.

VEGF is an important participant in vascular tissue remodeling during T2DM [31]. In both OAD and OADINS groups, the concentration of VEGF was significantly higher than in controls but with no difference between them (Table 2) and with no correlation with statin use. These results were contrary to expectations given the published data on suppressed angiogenesis and decreased VEGF in T2DM patients using statins [32]. A significant positive correlation between VEGF and TBARS in the OADINS group (Figure 2b) draws attention to the possible indirect relationship of elevated VEGF with the degree of lipid peroxidation in the OADINS group. In the same group, elevated HbA1c level positively correlated with the elevation of SOD activity, indicating a higher degree of antioxidative defence (SOD) in more compromised glycaemia control (Figure 2a).

Tissue changes in both the OAD and OADINS group are also supported by homogenously elevated MMP9 activity compared to controls. Uniformly increased MMP9 activity in both the OAD and OADINS group decreases the possibility of its direct influence on the better antioxidant output in the OADINS group.

We have already mentioned that statins have pro-oxidative potential. About half of the participants in our study were taking statins. Furthermore, the additional therapy of our subjects included other drugs that are known to affect oxidative status – angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and calcium channel antagonists.

Angiotensin II manifests the potential to activate NADPH oxidase and induce ROS production in the mitochondria *via* respiratory chain complexes I and III and a protein kinase C-dependent pathway, improving endothelial function and insulin resistance during T2DM. The increased levels of angiotensin II in diabetic patients may additionally reduce dihydrofolate reductase expression and decrease tetrahydrobiopterin (BH4) recycling from dihydrobiopterin (BH2), thus indirectly influencing endothelial nitric oxide synthase [33, 34]. Hence, ACE inhibitors act as antioxidants. Beta-blockers are also established antioxidants through several different mechanisms, while calcium channel antagonists promote superoxide scavenging, thus increasing the antioxidant capacity of the vessel endothelium [35-37]. Equal frequencies of applied beta-blockers, ACE inhibitors, and calcium channel antagonists in both study groups (Table 1) eliminate them as prominent factors of better antioxidant outcomes in the OADINS group.

In T2DM, glucose intolerance impairs endothelial nitric oxide (NO) synthase (eNOS) activity directly through improved oxidative stress [38]. An excessive amount of superox-

ide anion, which is an inducer of oxidative stress in mitochondria, rapidly induces NO to convert it to highly reactive peroxynitrite. Peroxynitrite expresses prooxidative potential to oxidize lipids, induce cellular injury, and modulate arterial contraction. It also depresses NO bioavailability and additional impairment of endothelium-mediated vasodilation. The enhanced ROS production and consecutive peroxynitrite in T2DM promote vascular inflammation, DNA damage, and vascular aging [39]. Unchanged concentrations of NOx in both OAD and OADINS patients compared to controls and each other indicate a reduced potential for peroxynitrite production. It also directs to higher bioavailability of NO to improve vascular response during T2DM. Mutually, compared with the OAD group, elevated SOD (which removes superoxide), depressed TBARS, and unchanged NOx in the OADINS group suggest a better antioxidant capacity and suppressed nitrosative stress. Since better antioxidant outcomes were not achieved in the OAD group, these quote a significant effect of insulin given in the OADINS group.

Conclusion

The therapeutic approach in T2DM is directional at preserving endothelial function. Obtained results indicate the improvement of antioxidative defence in T2DM patients due to supplemented insulin therapy. Normalized SOD activity and regulated lipid peroxidation upgrade a redox status, which is a promising outcome for restraint of complications in T2DM. The better antioxidative outcome expressed with a normalized concentration of TBARS and SOD activity in T2DM patients treated with insulin besides OAD supports the need for more careful consideration to upstart earlier with insulin in T2DM therapy.

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Oxidative/antioxidative effects of colloidal silver ions and chlorhexidine in saliva and gingival fluid of periodontal patients

Oksidativni/antioksidativni efekti jona srebra i rastvora hlorheksidina u salivi i gingivalnoj tečnosti pacijenata sa parodontopatijom

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Abstract

Background/Aim. Chronic periodontitis is an inflammatory disease. Oxidative stress is an important factor in periodontitis progress, hence examining the antioxidative properties of antiseptics, such as chlorhexidine (CHX) and silver ions solution (SSI), is a beneficial biomarker in estimating the recovery of tissue impairment during periodontal disease treatment. **Methods.** This clinical trial was conducted on the control group referred to healthy volunteers and individuals with periodontal disease, divided into two subgroups: before and after applying antiseptic treatments (CHX or SSI). Measurements of oxidative/antioxidative parameters were addressed to determine thiobarbituric acid products (TBARS) concentration and total superoxide dismutase (tSOD) activity in saliva and gingival crevicular fluid (GCF) of periodontal patients. **Results.** TBARS concentration was increased in saliva before the CHX treatment compared to the periodontal group after the CHX treatment, as well as before both CHX and SSI antiseptic treatment in CGF, compared to controls and periodontal groups after the treatment. Patients before SSI treatment had increased tSOD activity in saliva compared to the control group treated with SSI, as well as compared to patients after the SSI treatment. Additionally, tSOD activity was increased in GCF in patients with periodontitis before antiseptic treatment (CHX, SSI) compared to the control or the group of patients after the appropriate treatment. **Conclusion.** Our results revealed elevated lipid peroxidation in CGF, which reflected the promotion of oxidative stress during periodontal inflammation. The study suggests that antiseptics with antioxidant properties may reduce tissue damage initiated by periodontal disease. Moreover, the determination of oxidative/antioxidative parameters can be important for diagnosing, monitoring, and prognosis of the clinical state of periodontal patients.

Key words:
antioxidants; chlorhexidine; oxidative stress; periodontal diseases; silver.

Apstrakt

Uvod/Cilj. Hronična parodontopatija je inflamatorno oboljenje. Oksidativni stres je veoma važan faktor u razvoju parodontopatije, tako da je praćenje mogućnosti antioksidativnih delovanja antiseptika, kao što su hlorheksidin (CHX) i rastvor jona srebra (SSI), koristan biomarker u proceni oporavka oštećenja tkiva tokom lečenja parodontopatije. **Metode.** Kliničko istraživanje obuhvatilo je kontrolnu grupu (grupu zdravih dobrovoljaca), kao i pacijente sa parodontopatijom, koji su bili podeljeni u dve podgrupe: pre terapije antisepticima (CHX ili SSI) i nakon terapije. Merenje parametara oksidativnog stresa i antioksidativne zaštite obuhvatilo je određivanje koncentracije reaktivnih produkata tiobarbiturne kiseline (TBARS) i aktivnosti ukupne superoksid dizmutaze (tSOD) u salivi i gingivalnoj tečnosti (GCF) pacijenata sa parodontopatijom. **Rezultati.** Koncentracija TBARS bila je povećana u salivi pre CHX tretmana u poređenju sa grupom pacijenata sa paradontopatijom posle CHX tretmana, kao i pre CHX ili SSI antiseptičnih tretmana u GCF u poređenju sa kontrolom i grupama pacijenata sa parodontopatijom nakon tretmana. Kod pacijenata pre SSI tretmana aktivnost tSOD u salivi je bila povećana u poređenju sa kontrolnom grupom tretiranom SSI, kao i grupom pacijenata nakon SSI tretmana. Takođe, aktivnost tSOD bila je povećana u GCF kod pacijenata sa parodontopatijom pre tretmana antisepticima (CHX, SSI) u poređenju sa kontrolnom ili grupom pacijenata posle odgovarajućeg tretmana. **Zaključak.** Prikazani rezultati pokazuju indukciju lipidne peroksidacije u GCF, što dovodi do pokretanja oksidativnog stresa

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u toku zapaljenja kod parodontopatije. Studija ukazuje na to da antiseptici sa antioksidativnim delovanjem mogu redukovati pokrenuto oštećenje tkiva u parodontopatiji. Određivanje oksidativnih/antioksidativnih parametara može biti od značaja u dijagnostici, praćenju i prognozi kliničkog stanja pacijenata sa parodontopatijom.

Ključne reči:
antioksidansi; hlorheksidin; stres, oksidativni; periodontalne bolesti; srebro.

Introduction

Periodontal disease is one of the most common chronic diseases, with a progressively increasing prevalence spread throughout the world [1]. Periodontitis is reflected as an inflammatory disorder that damages tissue through the complex interactions between periodontopathic bacteria and host defense systems [2]. Oxidative stress is an imbalance between prooxidants and antioxidants and has been linked with both onsets of periodontal tissue damage and systemic inflammation. Reactive oxygen species (ROS) comprise oxygen-derived free radicals, such as superoxide anion, hydroxyl radical, nitric oxide, and hydrogen peroxides, created through the bacteria-host mediated pathway. ROS also excite polymorphonuclear leukocytes (PMNL) to produce radicals by the oxidative burst. ROS cause tissue impairment through several mechanisms, which contain lipid peroxidation (LPO), DNA destruction, oxidation of main enzymes, damage of proteins, and release of proinflammatory cytokines by monocytes and macrophages [3]. ROS are extremely toxic to the affected infectious agent as well as to the extracellular structure and can encourage LPO, causing effects on the periodontal tissue [4]. Terminated production of LPO can result in oxidative stress and, therefore, destroy cellular integrity. The LPO causes oxidative stress, and various markers have been used to follow this process. Malondialdehyde (MDA) is the major and much-studied product of polyunsaturated fatty acid peroxidation that can specify the spread of oxidative stress [5]. The most frequently applied test for the measurement of MDA is the assessment of thiobarbituric acid reactive substances (TBARS).

Many studies are dedicated to the role of ROS, products of oxidative stress, and LPO as well as antioxidant defense systems in the pathology of periodontitis [6, 7]. The capacity of total antioxidant status in a sample reflects the full range of antioxidant activity against numerous reactive oxygen and nitrogen radicals (RNS).

The human body has different nonenzymatic and enzymatic antioxidants, which eliminate detrimental ROS and prevent their deleterious actions [2]. Protective antioxidants like superoxide dismutase (SOD), catalase, and glutathione peroxidase function by enzymatic removal of superoxide ions. The SOD is found in all the tissues and cells of aerobic organisms. An alteration in antioxidant enzyme activity was a consequence of scaling and root planing, and it also revealed the role of oxidative stress in periodontal destruction. Novakovic et al. [8] suggested that salivary antioxidants like SOD credibly reveal periodontal reaction and the tissue response to treatment.

Potential inflammatory markers of host response can be found in saliva, gingival crevicular fluid (GCF), and serum samples and can be used as diagnostic markers [9]. Saliva is of major importance in the maintenance of oral health [10]. It is a complex secretion and could be used as a non-invasive diagnostic fluid to measure biomarkers circulated during the initiation and progression of periodontal disease [11, 12]. Saliva contains biochemical systems involved in periodontal tissue reparation, as well as numerous antibacterial, antiviral, and antifungal components, including lysosome, salivary peroxidase, and various antioxidants [13]. Instead, GCF is an exudate originating from serum and can be collected from the gingival sulcus surrounding natural teeth [14]. The flow of this biological fluid is an important element for the status of periodontal tissues, which reflects the cellular response in periodontium by the components of serum and influences from the gingival crevice [15].

A very capable periodontal therapy is the mechanical removal of bacterial biofilm and bacterial toxins from the tooth surfaces, which includes scaling and root planing for patients with chronic periodontitis [16]. Microbes compete for the most crucial part in the progress of periodontitis in which chlorhexidine (CHX) and colloidal silver ions solution (SSI) are strong antiseptics that have been utilized in dentistry for a long time [17, 18]. The CHX is considered a “gold standard” for the adjuvant treatment of periodontal patients. However, it has adverse effects since studies tested SSI as a very strong antiseptic with nontoxic and antibacterial power [19, 20]. Silver in any form is not considered toxic to the immune, cardiovascular, nervous, or reproductive systems. Furthermore, SSI has been used as medical aid and dietary supplement [21].

The aim of this study was to determine oxidative/antioxidative status in saliva and GCF in periodontal patients and to evaluate the diagnostic power of antiseptic treatments with SSI and CHX, especially with regard to the inflammatory process.

Methods

Participants and ethical conditions

This randomized prospective clinical study was conducted at the Periodontology and Implantology Departments of the Dental Clinic, Military Medical Academy in Belgrade, Serbia. Ninety examinees of both sexes participated in the study and they were divided into study and control groups, each of them having two subgroups depending on the treatment time (for the study groups) or the antioxidant used (for the control group): the control group – healthy individuals (with no prior history of periodontal disease), the probing depth in this latter group did not exceed 3 mm [22] (a) treated

by rinsing with a 0.2% solution of chlorhexidine (n = 20), (b) treated by rinsing with a 5 mg/mL colloidal SSI (n = 20); the CHX study group (n = 25) – periodontal patients treated by rinsing with a 0.2% solution of CHX after scaling and root planing of periodontal pockets (a) before the treatment, (b) after the treatment; the colloidal SSI study group (n = 25) – periodontal patients treated with a 5 mg/mL SSI rinsing, in addition to the treatment of periodontal pockets by scaling and root planing (a) before the treatment, (b) after the treatment.

This study was in agreement with the ethical principles of the World Medical Association Declaration of Helsinki (1964). Permission for this study was obtained from the Ethics Committee of the Military Medical Academy, Belgrade, Serbia (project No. 13/03/2014), and the study included only individuals who agreed to participate after reading and signing a free and informed consent form, except those with difficulty in understanding and communicating, with a physical handicap, or both, which could have compromised the sample collection.

Inclusion criteria were: the presence of chronic inflammation (pain, redness, heat, swelling), diagnosed according to bleeding on probing, at least 5 or 6 sites with probing depth ≥ 5 mm, attachment loss ≥ 3 mm, and extensive radiographic bone loss [22].

For all groups, the following items were considered exclusion criteria: infection, cardiovascular and/or neurological illness, renal insufficiency and/or diabetes; pregnancy; smoking; use of antibiotics and/or hormonal or nonhormonal anti-inflammatory drugs 6 months prior to tissue collection.

Clinical examination

Samples of unstimulated saliva and GCF were taken at the beginning of the study. One mL of unstimulated saliva was collected using sterile injectors, centrifuged for 15 min at 3,000 × g to remove cell elements, and then stored at -80 °C. GCF samples were taken using sterile paper points from the area of periodontal pockets depth ≥ 5 mm for 30 sec. Paper points were immediately immersed in 50 μL of buffered physiological solution, vortexed for 10 sec, and centrifuged at 3,000 × g for 5 min to remove plaque and cellular elements. Finally, the paper points were frozen at -80 °C until the time of determining the laboratory parameters [23].

After sampling saliva and GCF in the next few days, mechanical processing was carried out in several sessions of periodontal pockets by quadrants. Periodontal pockets of one group of subjects were washed with aqueous solution SSI (concentrations of 5 mg/mL), while in the other group, they were washed with CHX solution (0.2%). Furthermore, patients could choose one of the two offered antiseptics for rinsing the oral cavity 10 days after starting therapy. On the 30th day after the end of the therapy, the gingival and periodontal parameters were measured again, after which samples of unstimulated saliva and GCF were collected. In the control groups which received one of the antiseptics (CHX, SSI), a sample of unstimulated saliva and GCF was taken from the gingival sulcus.

The nonsurgical periodontal treatment was performed using a method of scaling and root planing per quadrant (in the following four days, one quadrant was processed). After the scaling and root planing, the application of a particular adjuvant antiseptic (CHX or SSI) was carried out by injecting 10 mL into the periodontal pockets. The patient was asked to mouthwash a given amount of antiseptic for 60 sec and not to take food for one hour after the treatment. Depending on the allocated group, each patient was given the same solution for home use for the next 10 days with precise usage instructions. A month after scaling and root planing, including a particular adjuvant antiseptic, all parameters' control measurement was performed to compare the values to the baselines.

Examined parameters

The oxidative/antioxidative status parameters (TBARS, tSOD) were determined in both saliva and GCF of control groups (C_{CHX}, C_{SSI}), as well as periodontal groups before (P_{CHX} before, P_{SSI} before) and after (P_{CHX} after, P_{SSI} after) the treatment. Total protein concentration was estimated with bovine serum albumin as a standard [24].

Lipid peroxidation analyses in both saliva and GCF were measured as TBARS production, assayed in the thiobarbituric acid reaction, and described by Girotti et al. [25]. The results are expressed as μmol TBARS/mg proteins.

The total SOD (tSOD; EC 1.15.1.1) activity was measured by a spectrophotometer as the inhibition of spontaneous autoxidation of epinephrine at 480 nm. The kinetics of sample tSOD activity was followed in a 50 mmoL carbonate buffer on pH 10.2, comprising 0.1 mmoL ethylene diamine tetraacetic acid (EDTA), after the addition of 10 mmoL epinephrine [26]. Data were expressed as U tSOD per mg of proteins.

All drug solutions were prepared on the day of the experiment. In addition, all the chemicals used in this study were of analytical grade.

Statistical analysis

The type of study according to which the research was carried out is a prospective cohort study. In one study, the therapeutic efficacy of oral formulations of CHX on a relatively small sample was tested, and statistically significant effects were obtained by registering a difference of 15% between treatments [27]. Application of *t*-test for independent groups (study power 80% and the probability of type 1 error (α) 0.05) showed that the minimum number of subjects in each group was 20.

After confirming a normal distribution in all groups using the Kolmogorov-Smirnov test, the data were presented as mean ± standard deviation (SD). All data were analyzed statistically by one-way ANOVA using Dunnet's C test. The linear regression analysis was performed to determine the relationship between different parameters using the statistical program GraphPad Prism. Statistical significance was described as $p < 0.05$.

Results

The age of the subjects included in the study is shown in Table 1.

In the saliva of periodontal patients before the CHX or SSI treatment, TBARS concentrations were not significantly different compared to controls (Table 2). In the saliva of the periodontal group after the CHX treatment, the TBARS concentration significantly decreased ($p < 0.01$) compared to the group of patients before CHX treatment (Figure 1A). Contrary to this, TBARS concentration in GCF was significantly higher in all periodontal patients before the treatment (CHX, SSI) compared to that in the controls (Table 3). However, these values in both study groups (CHX and SSI) significantly decreased after the treatment with antiseptics (Figure 2B).

Table 1

Demographic variables of the study population

Group	Number of patients	Age (years) mean values ± SD
P_{CHX}	25	49.47 ± 8.58
P_{SSI}	25	51.64 ± 9.41
C_{CHX}	20	42.05 ± 7.19
C_{SSI}	20	40.95 ± 8.19
Total	90	45.29 ± 9.27

SD – standard deviation; P – periodontal patients; C – healthy individuals (control group); CHX – chlorhexidine treatment; SSI – silver ions solution treatment.

Table 2

Thiobarbituric acid reactive substances (TBARS) concentration (μmoL/mg proteins) and total superoxide dismutase (tSOD) activity (U/mg proteins) in the saliva of healthy individuals (C) with the treatment (T) of chlorhexidine – CHX (C_{CHX}) or silver ions solution – SSI (C_{SSI}), periodontal patients (P) before the T with CHX (P_{CHX} before T) or SSI (P_{SSI} before T), as well as P after the T with CHX (P_{CHX} after T) or SSI (P_{SSI} after T)

Parameters	$P_{CHX\ before\ T}$	$P_{CHX\ after\ T}$	C_{CHX}	$P_{SSI\ before\ T}$	$P_{SSI\ after\ T}$	C_{SSI}
TBARS (μM/mg prot.)	21.3 ± 4.7	16.5 ± 2.3$^{\blacklozenge\blacklozenge}$	17.6 ± 5.2	20.6 ± 4.3	16.2 ± 5.4	17.1 ± 7.9
tSOD (U/mg prot.)	422.4 ± 99.5	339.4 ± 119.2	353.0 ± 113.7	464.1 ± 87.3***	320.1 ± 41.7$^{\blacklozenge\blacklozenge\blacklozenge}$	312.7 ± 93.5

All results are represented as mean ± standard deviation (SD). Labels of statistical significance: * – compared to the control group (C); ♦ – compared to P_{CHX} before T or P_{SSI} before T. Statistical significance was considered at: ♦♦$p < 0.01$, *,♦♦♦$p < 0.001$ (one-way ANOVA, Dunnett's C test).**

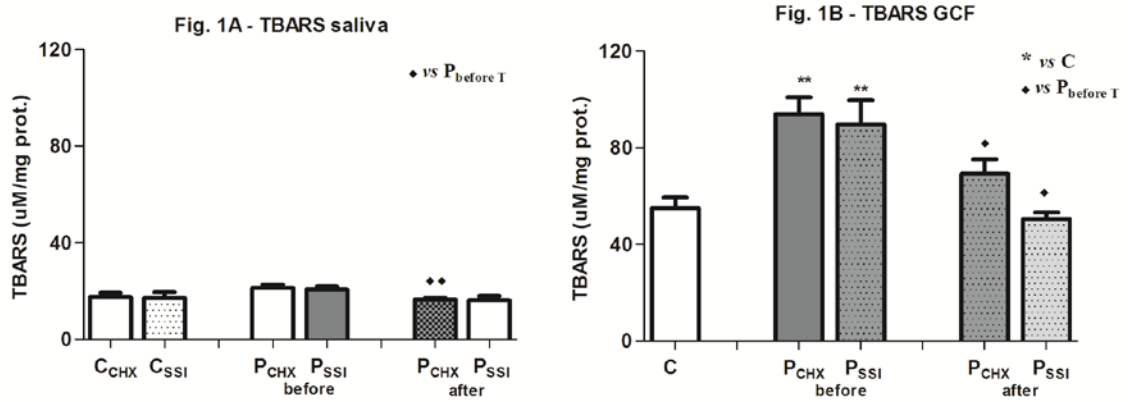

Fig. 1 – A) Thiobarbituric acid products (TBARS) concentration (μmol/mg proteins) in saliva, and B) gingival crevicular fluid (GCF) of the control group (C) with the treatment (T) of chlorhexidine – CHX (C_{CHX}) or silver ions solution – SSI (C_{SSI}), periodontal patients (P) before T with CHX (P_{CHX} before T) or SSI (P_{SSI} before T), as well as P after the treatment with CHX (P_{CHX} after T) or SSI (P_{SSI} after T). Bars in the graph represent mean ± standard deviation (SD). Labels of statistical significance: * – compared to the C before T; ♦ – compared to P_{CHX} before T or P_{SSI} before T treatment; Statistical significance was considered at: *,♦$p < 0.05$, **,♦♦$p < 0.01$, *,♦♦♦$p < 0.001$ (one-way ANOVA, Dunnett's C test).**

Table 3

Thiobarbituric acid reactive substances (TBARS) concentration (μmoL/mg proteins) and total superoxide dismutase (tSOD) activity (U/mg proteins) in the gingival fluid of healthy individuals with the treatment (T) of chlorhexidine – CHX (C_{CHX}) or silver ions solution – SSI (C_{SSI}), periodontal patients (P) before the T with CHX (P_{CHX} before T) or SSI (P_{SSI} before T), as well as P after the T with CHX (P_{CHX} after T) or SSI (P_{SSI} after T)

Parameters	$P_{CHX\ before\ T}$	$P_{CHX\ after}$	C_{CHX}	$P_{SSI\ before}$	$P_{SSI\ after}$	C_{SSI}
TBARS (μM/mg prot.)	93.9 ± 22.6**	69.2 ± 19.1♦	55.2 ± 16.4	89.6 ± 32.1**	50.5 ± 8.8♦	55.2 ± 16.4
tSOD (U/mg prot.)	1210.3 ± 463.5**	881.6 ± 207.6♦	769.9 ± 196.3	1296.0 ± 411.2**	859.6 ± 187.7♦♦	769.9 ± 196.3

All results are represented as mean ± standard deviation (SD). Labels of statistical significance: * – compared to the control group (C); ♦ – compared to P_{CHX} before T or P_{SSI} before T. Statistical significance was considered at: ♦*p* < 0.05, **,♦♦*p* < 0.01 (one-way ANOVA, Dunnett's C test).

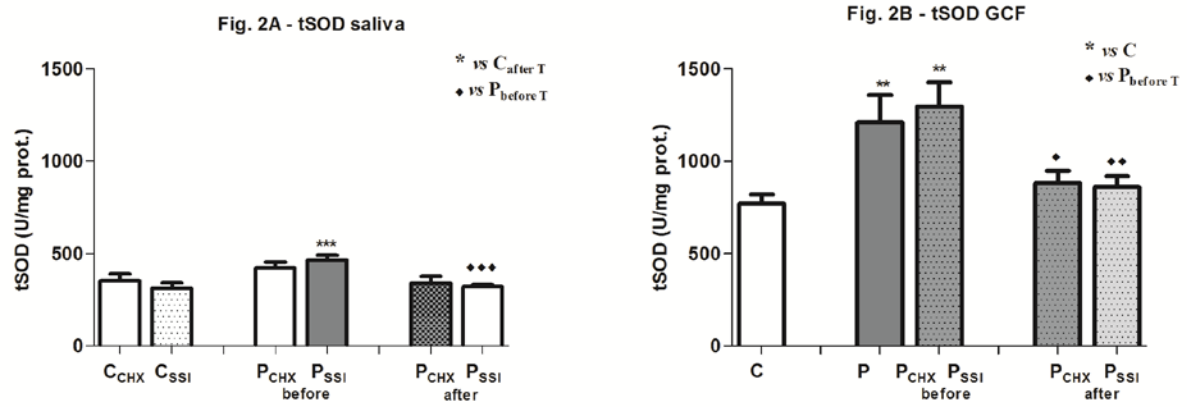

Fig. 2 – A) total superoxide dismutase – SOD activity (tSOD; U/mg proteins) in saliva, and B) gingival crevicular fluid (GCF) of the control group (C) with treatment (T) of chlorhexidine – CHX (C_{CHX}) or silver ions solution – SSI (C_{SSI}), periodontal patients (P) before T with CHX (P_{CHX} before T) or SSI (P_{SSI} before T), as well as P after T with CHX (P_{CHX} after T) or SSI (P_{SSI} after T). Bars in the graph represent mean ± standard deviation (SD). Labels of statistical significance: * – compared to the control group before T; ♦ – compared to P_{CHX} before T or P_{SSI} before T. Statistical significance was considered at: *,♦*p* < 0.05, **,♦♦*p* < 0.01, *,♦♦♦*p* < 0.001 (one-way ANOVA, Dunnett's C test).**

In the group of periodontal patients before the SSI treatment, tSOD activity was significantly higher ($p < 0.001$) in the saliva compared to that in the C group (Figure 2A). On the contrary, tSOD activity decreased in the saliva of patients after the SSI treatment compared to the values before the SSI treatment (Figure 2A; $p < 0.001$). In both periodontal groups before the antiseptic treatment (CHX or SSI), tSOD activity was increased in the GCF (Figure 2B) compared to the control values. The activity of tSOD significantly decreased in GCF after both antiseptic treatments compared to the values before the treatment.

Discussion

Increased TBARS concentration and SOD activity in the saliva and GCF of periodontal patients indicate prominent cell and tissue impairment. In the literature, there is no human study on the oxidative stress biomarkers that appear as an outcome of antiseptic effects in periodontal tissues. Therefore, our study is the first on this issue.

Oxidant/antioxidant balance differs across oral environmental compartments like hard dental tissue, saliva, and GCF [28, 29]. Patients with periodontal disease are more susceptible to an imbalance of antioxidant-oxidative stress conditions [30]. ROS-related tissue destruction should be determined *via* the final product of LPO, such as MDA since ROS have an extremely short life and are not easy to detect.

Membrane fluidity is a crucial factor that determines cellular communication, membrane elasticity, and biological transport of proteins and lipids. In the presence of reducing equivalents, microsomal membrane fluidity is declined, and the membrane becomes rigid and vulnerable to oxidative injury. Lipid peroxidation has been implicated in the pathogenesis of periodontal disease [7]. ROS can attack polyunsaturated fatty acids and form various LPO products such as MDA, which is one of the most frequently used indicators of LPO and may be a potential biomarker indicating oxidative stress. An increase in salivary levels of MDA is an important indicator of periodontal tissue destruction and of great importance as it provides an impression on the common periodontal health. Similar to our previous study, Tsai et al. [31] also reported that LPO significantly correlated with clinical parameters of periodontal disease. Our previous study revealed comparable results as we obtained lower clinical parameters during periodontal treatment with both antiseptics [20].

In saliva samples, no difference in TBARS concentration was found among patients and healthy controls (Figure 1A). The GCF appears to be a more reliable source for identifying periodontal disease. If a disruption of the balance between ROS and antioxidants occurs, it may play a role in the progress of oral inflammatory diseases, as increased TBARS concentration in GCF evokes an enhancement in LPO level in the periodontium and the oral environment in periodontitis (Figure 1B).

There are several explanations for higher TBARS concentrations in GCF compared to the saliva of periodontal patients (Tables 2 and 3). Some investigations proposed that elevated GCF flow was associated with enlarged PMNL levels, which sequentially contributed to peroxidase improvement through the activity of myeloperoxidase. Secondly, enhancement in TBARS concentration in GCF may partially be a sign of intensified LPO in the periodontium itself, contrary to increased amounts of bacterial products. TBARS levels in GCF, which were higher than those in saliva in our study, defined that a local increase in the LPO process was more prominent in the periodontal region and was more significant than the systemic increase.

Development of oxidative stress in GCF of patients with periodontal disease is characterized by ROS overproduction, depletion of reducing equivalent sourced, and oxidative injury of biomolecules accompanied by lipids [32]. All these results correspond to those in our study. In addition, we revealed the improved reduction of TBARS level in saliva after the CHX treatment (22%), as well as after both antiseptics in GCF of periodontal patients, which suggests that applied antiseptics had beneficial effects in lowering lipid components and preventing oxidative stress improvement in chronic periodontitis (Figures 1A and 1B).

The antioxidative defense system performs a notable part as a scavenger of ROS in the process of their removal. Changes in the activities of antioxidative enzymes may indicate an increase in reactive oxygen and the regular function disturbance of antioxidative defense systems. The enzymatic antioxidant activities were significantly higher in these patients, which suggests that the scavenging of disproportionately generated LPO products at the inflammatory sites possibly induces an increased enzymatic antioxidant activity [33]. In addition to diminished energy production, the products of anaerobic glucose metabolism lead to the formation of ROS, which disrupts the deterioration and periodontal tissue destruction. Total SOD activity, as an indicator of oxidative stress, was higher in periodontal patients in saliva in relation to the activity of SOD in the control group, indicating that the periodontal environment induced the synthesis of SOD (Figure 2A). In accordance with our results, Novakovic et al. [8] also found that patients with periodontitis had higher tSOD in saliva compared to controls. These findings suggest that an increase in SOD activity may be accompanied by an early inflammatory syndrome, while its improvement arises in response to pathological progression. The reduction of tSOD activity was noticed only in the P_{SSI} group (32%) after the treatment ($p < 0.001$) compared to the periodontal group before the SSI treatment in saliva (Figure 2A).

Furthermore, our study observed higher tSOD activity in GCF of periodontal patients compared to controls (Table 3). The human periodontal ligament acquires the SOD, which proposes biological protection against ROS. Bacterial lipopolysaccharides also stimulate superoxide release from gingival fibroblasts, suggesting that the induction of SOD may correspond to an important protective mechanism of the fibroblasts in inflammation. Increased SOD activity in inflamed gingiva may reveal increased superoxide radical generation by PMNL occupying the diseased sites. Antiseptics therapy can reduce the disease-causing microbes associated with oral plaque, which may assist in inflammatory reduction. The reason for reduced tSOD activity (Figure 2) in treated groups (CHX, SSI) may be attributed to the lack of the substrate, such as superoxide anion, which reacts easily with nitrogen monoxide to form harmful peroxynitrite anion and this reaction, involved in the acetylation of amino acids, is accomplished by gram-negative anaerobes [34].

Our results explained that antioxidants like SOD in GCF of periodontal patients before the antiseptic treatment credibly expose periodontal response and the tissue response to treatment. In our study, we revealed a positive Spearman correlation between tSOD activity before and after CHX treatment ($r = 0.7400$, $p < 0.01$), as well as between tSOD activity before antiseptic treatments in GCF of periodontal patients. This positive correlation analysis could be just a result of the preserved antioxidative defense system.

The efficacy of two different antiseptics in decreasing the inflammatory impact on the periodontal tissues subsequently improves clinical parameters and systemic biochemical oxidative stress and inflammatory markers. The current study proposed that GCF has better diagnostic potential than saliva. TBARS may be a better marker for gingival inflammation rather than periodontitis. The major changes in oxidant/antioxidant status were registered in periodontal groups when increased TBARS concentration was accompanied by increased tSOD activity.

Conclusion

This is the first paper that achieved the antioxidative effects of antiseptics with SSI on periodontal patients. Utilized antiseptics with antioxidant capacity can reduce periodontal disease initiated by tissue damage, which is of remarkable clinical application in dentistry. We showed that the assessment of saliva, as well as GCF oxidative status, might represent a useful method for evaluating the applied antiseptics in patients with chronic periodontitis.

Conflict of interest

No conflict of interest exists for any of the authors of this article.

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Seroreactivity against *Helicobacter pylori* VacA, 50 kDa, and 30 kDa along with alarm features may improve the diagnostic approach to uninvestigated dyspepsia – a pilot study

Seroreaktivnost protiv *Helicobacter pylori* VacA, 50 kDa i 30 kDa zajedno sa simptomima i znacima alarma može unaprediti dijagnostički pristup neistraženoj dispepsiji – pilot studija

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Abstract

Background/Aim. Alarm features (AF) are of limited utility in predicting endoscopic findings, and the majority of patients with uninvestigated dyspepsia will have no organic pathology identified at upper gastrointestinal endoscopy. In our previous study, we highlighted seroreactivity against *Helicobacter pylori* (HP) antigens VacA, 50 kDa, and 30 kDa as biomarkers for gastric cancer, peptic ulcers, and functional dyspepsia. We designed and conducted this pilot study in order to compare the diagnostic utility of seroreactivity against HP VacA, 50 kDa, and 30 kDa with AF and investigate the possibility and adequacy of its synchronous application. **Method.** A careful history and physical examination with special attention to AF, esophagogastroduodenoscopy with biopsy, abdominal ultrasound or computer tomography, complete blood count (CBC) and blood biochemistry, a Western Blot IgG against HP antigens VacA, 50 kDa, and 30 kDa, were performed in 123 patients with dyspepsia: 31 with gastric cancer, 31 with duodenal ulcer, 31 with gastric ulcer, and 30 with gastritis and functional dyspepsia. AF *vs* various combinations of seroreactivity against HP VacA, 50 kDa, and 30 kDa in patients with functional dyspepsia and others were analyzed in this study. Synchronous and alternative seroreactivity against VacA, 50 kDa, and 30 kDa, along with/without AF in patients with functional dyspepsia and other groups of patients were also analyzed. **Results.** VacA and 50 kDa seropositivity or AF had excellent case-findings clinical utility index for investigating dyspepsia. The absence of AF and seroreactivity against VacA either with: 50 kDa or 30 kDa seropositivity or 50 kDa and 30 kDa seropositivity had an excellent screening clinical utility index for investigating dyspepsia. **Conclusion.** Seroreactivity against HP antigens VacA, 50 kDa, and 30 kDa might improve our approach to patients in investigating dyspepsia if used along with AF.

Key words:
antigens; biomarkers; duodenal neoplasms; duodenal ulcers; gastroscopy; helicobacter pylori; stomach neoplasms; stomach ulcer.

Apstrakt

Uvod/Cilj. Simptomi i znaci alarma (SZA) su od ograničene koristi u predikciji endoskopskih nalaza i većina pacijenata sa neistraženom dispepsijom neće imati nalaz organske patologije tokom gornje gastrointestinalne endoskopije. U našoj prethodnoj studiji istakli smo seroreaktivnost protiv *Helicobacter pylori* (HP) antigena VacA, 50 kDa i 30 kDa kao biomarkere karcinoma želuca, peptičkih ulkusa i funkcionalne dispepsije. Ova pilot studija je dizajnirana i sprovedena sa ciljem da se uporedi dijagnostička korist seroreaktivnosti protiv HP VacA, 50 kDa i 30 kDa sa onom od SZA i da se istraži mogućnost i adekvatnost njihovog zajedničkog korišćenja. **Metode.** Od 123 bolesnika sa dispepsijom među kojima je bilo 31 sa karcinomom želuca, 31 sa ulkusom duodenuma, 31 sa ulkusom želuca i 30 sa gastritisom i funkcionalnom dispepsijom uzeta je pažljiva anamneza i obavljen fizikalni pregled sa posebnim osvrtom na SZA. Svim bolesnicima je urađena ezofagogastroduodenoskopija sa biopsijom, ultrazvuk abdomena ili kompjuterizovana tomografija, kompletna krvna slika (KKS) i biohemijske analize, *Western*

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Blot IgG prema HP antigenima VacA, 50 kDa i 30 kDa. Analizirana je pojava SZA naspram različitih kombinacija seroreaktivnosti protiv HP VacA, 50 kDa i 30 kDa kod bolesnika sa funkcionalnom dispepsijom i ostalih bolesnika. Analizirana je sinhrona i alternativna seroreaktivnost protiv VacA, 50 kDa i 30 kDa sa/bez SZA kod funkcionalne dispepsije i drugih grupa. **Rezultati.** Seropozitivnost VacA i 50 kDa ili SZA imaju odličan dijagnostički klinički indeks korisnosti kod neistražene dispepsije. Odsustvo SZA i seroreaktivnosti protiv VacA bilo sa 50 kDa ili 30 kDa seropozitivnosti ili 50 kDa i 30 kDa seropozitivnosti imaju odličan klinički indeks korisnosti za skrining kod neistražene dispepsije. **Zaključak.** Seroreaktivnost protiv HP antigena VacA, 50 kDa i 30 kDa može unaprediti naš pristup bolesnicima sa neistraženom dispepsijom ukoliko se koristi zajedno sa SZA.

Ključne reči:
antigeni; biološki pokazatelji; duodenum, neoplazme; duodenum, ulkus; gastroskopija; helicobacter pylori; želudac, neoplazme; želudac, ulkus.

Introduction

Dyspepsia is a complex condition comprising chronic and recurrent symptoms related to the upper gastrointestinal tract. The cardinal symptoms are epigastric pain and discomfort, including postprandial fullness and early satiety, which may overlap with heartburn and regurgitation. Around 25–40% of adults in the general population have dyspepsia, and dyspepsia accounts for 2–5% of all consultations in primary care [1]. Although several guidelines have been published [1–6], the management of patients with uninvestigated dyspepsia is still controversial [2]. Individual dyspeptic symptoms or subgroups of symptoms, such as predominant epigastric pain (ulcer-like) or discomfort (dysmotility-like), poorly predict the presence of underlying organic lesions. A systematic review found that neither primary care doctors nor gastroenterologists could distinguish patients with organic lesions from those with functional dyspepsia (FD) on the basis of symptom evaluation. Dyspeptic symptoms can have several organic causes, but in many patients, no obvious cause is identified. Extragastrointestinal causes, such as hepatobiliary and pancreatic diseases, are infrequent but important and should always be considered. However, most cases of dyspepsia can be ascribed to one of four causes – gastro-oesophageal reflux disease with or without oesophagitis, peptic ulcer disease, malignancy, and FD [3]. Traditionally, clinicians are alerted to the possibility of malignancy by the history and physical examination; alarm features (AF) on the clinical evaluation that include chronic gastrointestinal bleeding, progressive unintentional weight loss, progressive difficulty swallowing, persistent vomiting, iron deficiency anemia, or epigastric mass. AF, also called alert features, red flags, or warning signs, are specific features thought to be associated with serious gastroenterological diseases such as underlying malignancy or significant pathologies such as a stricture or ulcer.

Current guidelines recommend that, in the initial evaluation of patients with dyspepsia, the decision to perform endoscopy should be based on older age and AF because it is generally believed that these factors indicate a higher probability of malignancy being present [4].

Although AF are used to help prioritize access to upper gastrointestinal endoscopy, they are of limited utility in predicting endoscopic findings, and the majority of patients with dyspepsia will have no organic pathology identified at upper gastrointestinal endoscopy. This has led to the investigation of alternative diagnostic approaches, including whether a capsaicin pill or combined serum biomarkers can accurately identify patients with FD. However, there is insufficient evidence to support either of these approaches at present [5].

Our previous publications suggested that Western Blot IgG against *Helicobacter (H) pylori* antigens VacA, 50 kDa, and 30 kDa may be a biomarker for a specific outcome of an infection encompassing gastric cancer (GC), duodenal and gastric ulcer (DU and GU, respectively), and FD, as the most important cause of uninvestigated dyspepsia [5, 6].

Therefore, we designed and conducted an additional pilot analysis on the material of our previous study in order to investigate the diagnostic value of seroreactivity against *H. pylori* VacA, 50 kDa, and 30 kDa *versus* and along with AF, as a non-invasive predictor for GC and peptic ulcers (PU) as well FD.

Methods

The study was conducted and performed in 2009 at the Clinic for Gastroenterology and Hepatology, Institute of Pathology and Institute of Microbiology of the Military Medical Academy (MMA), Belgrade, Serbia. We selected and enrolled patients with upper dyspeptic symptoms, different underlying diseases [gastric cancer (GC), duodenal ulcer (DU), gastric ulcer (GU), and gastritis), and actual *H. pylori* infection confirmed by histopathological examination and anti-*H. pylori* IgG positive Vira Blot.

We have taken a careful history from all patients and performed a physical examination, abdominal ultrasonography (US) or computed tomography (CT), esophagogastroduodenoscopy (EGDS), complete blood count (CBC), and liver and renal chemistry. Inclusion criteria were as follows: 1) presence of upper dyspepsia symptoms; 2) previously untreated patients due to *H. pylori* infection; 3) without proton pump inhibitors and H_2 blockers use in the last two weeks; 4) absence of malignancy except for GC; 5) absence of any immunological disorder; 6) informed consent of the patients for: EGDS and biopsy, taking the blood samples for analyses, participation in the study; endoscopic and histopathological diagnosis of one of the following diseases: GC, DU, GU, gastritis; 8) confirmed histopathological diagnosis of *H. pylori* infection; 9) Western blot (ViraBlot) IgG positive for *H. pylori* infection.

EGDS was performed in all our patients in the endoscopy section using Olympus (GIFQ165, SN: 2207997,

Olympus Corporation, Tokyo) forward-viewing EGD under local application of Xylocaine spray. A minimum of four gastric mucosal tissue biopsies (2 each from the antrum and corpus) were taken, including additional biopsies from every endoscopically visible lesion. All patients were examined for findings suggestive of endoscopic gastritis, such as erythema, hyperemia, atrophy, and mucosal nodularity according to the criteria of the Sydney grading system, and for gastric tumor, duodenal and gastric ulcer [7, 8]. All the obtained biopsies were collected, placed on filter paper, fixed in 10% neutral formalin, and sent for preparation of formalin-fixed, paraffin-embedded tissue blocks. Three-micrometer-thick sections were prepared. One set of tissue sections was stained with hematoxylin and eosin and the other with Giemsa stain for histopathological examination, including detection of *H. pylori* in the gastric mucosa. The biopsies were evaluated for the intensity of mononuclear inflammatory cellular infiltrates, inflammatory activity (neutrophilic infiltrations), glandular atrophy, metaplasia, and *H. pylori* colonization [8]. Additionally, the cases were graded according to the Houston-updated Sydney system [7], which was graded according to the intensity of mononuclear inflammatory cellular infiltrates within the lamina propria: absent inflammation (Grade 0), mild inflammation (Grade 1), moderate inflammation (Grade 2), and severe inflammation (Grade 3). Grading was done for activity, atrophy, intestinal metaplasia, and degree of *H. pylori* colonization. Additional immunohistochemistry staining was performed in the case of a tumor.

The blood samples were obtained from all of them and frozen at -20 °C degrees. Using the Western Blot detection system (ViraBlot), IgG anti Vacuolating cytotoxin A (VacA) 87 kDa, Cytotoxin associated with gene A (CagA) 136 kDa, urease B 66 kDa (UreB 66), heat shock protein 60 kDa (Hsp60), flagellin 55 kDa (Fla 55), 50 kDa, 30 kDa, urease A 26 kDa (UreA 26), and 24 kDa *H. pylori* antigens were identified. *H. pylori* antigens of ViraBlot represent a combination of German patient isolates of highly antigenic *H. pylori* strains. Bands for diagnosing *H. pylori* infection are divided into highly specific as CagA 136 kDa, VacA 87 kDa, 30 kDa, UreA 26 kDa, 24 kDa, and less specific as Hsp 60 kDa and 50 kDa. According to the manufacturer guidelines for use, the test was negative if: there were no bands or if there were nonspecific bands, one or more UreB 66 kDa, Hsp 66 kDa, Fla 55 kDa, 50 kDa. The test was suspected positive: if one clear of a specific band of 30 kDa, UreaA 26 kDa, 24 kDa was present. The test was positive if: at least one band of the following two specific CagA 136 kDa or VacA 87 kDa or at least one clear band of 30 kDa, Urea A 26, 24 kDa or one clear band of 30 kDa, UreA 26 kDa, 24 kDa and one clear band of Hsp60 kDa, 50 kDa was present.

All patients included in our cross-sectional study were classified and analyzed in several ways. In the first analysis we compared frequency of AF and six in previously work selected synchronous/alternative combinations [3] of VacA seroreactivity, 50 and 30 kDa seroreactivity: VacA+ or 50 kDa-; VacA+ or (50 kDa- 30 kDa-); VacA- 50 kDa+; VacA- 30 kDa+; VacA- 50 kDa+ 30 kDa+; VacA- (50 kDa+ or 30 kDa+) in GC, DU and GU and FD, and the fraction correct (FC) in all groups.

In the second analysis, the frequency and FC of the following seroreactivity combinations and AF were investigated in GC, DU, GU, and FD groups and in all patients: I. VacA+ or AF+ either with 50 kDa- or (50 kDa-, 30 kDa-); II. VacA- and AF- either with (50 kDa+ or 30 kDa+), 50 kDa+, 30 kDa+, (50 kDa+, 30 kDa+). They were also compared with the features of the AF group alone.

Statistical analysis

Complete statistical data analysis was done with the statistical software package SPSS Statistics 18.

Most of the variables were presented as a frequency of certain categories; hence, the *t*-test of proportion or cross-tabulation analysis (odds ratio, confidence intervals) was done to calculate the statistical significance of differences between groups.

In the case of continuous data, variables were presented as median, minimal, and maximal values (range).

Accuracy and discriminative ability of the seroreactivity combinations against *H. pylori* VacA, 50 kDa and 30 kDa, and AF, in predicting the outcome of infection was estimated with Sensitivity (Se), Specificity (Sp), Positive Predictive Value (PPV), Negative Predictive Value (NPV), FC and Clinical Utility Index (CUI) in the form of Case-finding Utility or Positive Clinical Utility Index (CUI+) and Screening Utility or Negative Clinical Utility Index (CUI -). CUI + = Se × PPV and CUI Ve- = Sp × NPV represent important indices for clinicians, estimating together the accuracy and discriminative ability of the test [9, 10].

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

Results

Four groups of patients with GC, DU, GU, and upper FD were comparable in gender and age (Table 1). The pa-

Table 1

Demographic and clinical characteristics of the patients

Parameters	Groups				Total (n = 123)
	GC (n = 31)	DU (n = 31)	GU (n = 31)	FD (n = 30)	
Gender, n					
male	10	13	12	13	48
female	21	18	19	17	75
Age (years), median (range)	65 (40–85)	54 (21–87)	67.5 (34–81)	63.5 (21–80)	63 (21–87)

GC – gastric cancer; DU – duodenal ulcer; GU – gastric ulcer; FD – functional dyspepsia.

tients with GCr were represented with advanced GC and none with early GC.

At least one of the AF was present in 81 of 123 participants, with an FC of 71% (Table 2). The GC group had the highest FC (98%) and the lowest FD (60%).

VacA+ 50 kDa- had higher FC (74%), with better results in GU and DU, but lower FC in GC and FD compared with AF.

VacA+ or (50 kDa- 30 kDa-) had a slightly higher FC (72%), higher in DU, GU, and FD, but lower in GC compared with AF.

VacA- (50 kDa+ or 30 kDa+) had the same FC as AF, and in the group with FD, lower in the GC group but higher in DU and GU groups compared with AF.

VacA- 50 kDa+ had higher FC (74%) than AF, lower in GC, and FD, but higher in DU and GU compared with AF.

VacA- 30 kDa+ had a higher total FC (73%), lower in GC and FD, but higher in DU and GU compared with AF.

VacA- (50 kDa+ 30 kDa+) had the highest total FC (76%), slightly lower FC in GC, lower in FD, and higher in DU and GU compared with the results of AF.

There is no significant difference in total FC between groups with different criteria and AF (Table 2).

AF alternatively with VacA+ or 50 kDa, were present in all GC patients, FC (100%), in almost all of DU (97%) and GU (97%), but undesirable in more FD (FC = 40%) (Table 3). Total FC was high (84%), significantly higher than in AF alone ($p = 0.015$). AF approach missed indicating EGDS in 23/93 (25%) patients with GC or PU, with the performance of unnecessary EGDS in 12/31 (39%) patients with FD. The combined approach failed to indicate EGDS in only 2% of patients with a serious disease (no one with cancer, two with PU) but indicated 18/31 (60%) unnecessary EGDS in FD. If we analyzed all unnecessary endoscopies indicated with the combined approach, we would find 7 patients with gastric atrophy of different degrees. Reasons for EGDS would be AF and VacA+ in 4 patients, AF and 50 kDa- in 1, VacA+ in 2, and AF in only 1 patient. After correcting the number of unnecessary EGDS with findings of gastric atrophy, 11 (37%) EGDS were left as "true unnecessary". It is lower than in the AF approach (40%). Total FC with this correction raised to 89%, and, therefore, to a highly statistically significant difference compared with the AF approach alone (71%) ($p = 0.0008$).

VacA+ or (50 kDa- 30 kDa-) or AF+ is present in all patients with GC and 97% of PU but also in 87% of FD. Due to a very high percent of positivity in FD, FC was only 77%, and the difference compared with AF was not significant nor with/without correction for atrophy in FD.

VacA- and (50 kDa or 30 kDa+), along with AF-, were absent in GC and present in 3% and 6% of DU and GU and 47% of FD. FC was 85% and it was significantly better than in AF ($p < 0.01$), and with the correction of atrophy findings in FD, the difference was also statistically significant. With this approach, we will improve proper indication in GC by 3% and will miss proper indication in DU by 3% (39% in

Table 2

The number of confirmatory results regarding alarm feature and seroreactivity against *Helicobacter pylori* VacA, 50 kDa, and 30 kDa in four groups of patients

Parameters	Number (%) of confirmatory results (fraction correct)				
	GC (n = 31)	DU (n = 31)	GU (n = 31)	FD (n = 30)	Total (n = 123)
Alarm feature	30 (97)	19 (61)	20 (65)	12 (60)	81 (71)
VacA+ 50 kDa-	24 (77)	25 (81)	26 (84)	14 (53)	89 (74)
VacA + or (50 kDa- 30 kDa-)	24 (77)	20 (65)	24 (77)	10 (67)	78 (72)
VacA- 50 kDa+	6 (81)	5 (84)	6 (81)	15 (50)	32 (74)
VacA- 30 kDa+	5 (84)	10 (65)	4 (87)	16 (53)	35 (73)
VacA- (50 kDa+ or 30 kDa+)	8 (74)	10 (67)	6 (81)	18 (60)	42 (71)
VacA- (50 kDa + 30 kDa+)	2 (94)	5 (84)	3 (90)	11 (37)	21 (76)

GC – gastric cancer; DU – duodenal ulcer; GU – gastric ulcer; FD – functional dyspepsia.

Table 3

The number of confirmatory results regarding alarm features (AF) alone and/or seroreactivity against *Helicobacter pylori* VacA, 50 kDa, and 30 kDa in four groups of patients

Parameters		Number (%) of confirmatory results (fraction correct)				
		GC (n = 31)	DU (n = 31)	GU (n = 31)	FD (n = 30)	Total (n = 123)
AF		30 (97)	19 (61)	20 (65)	12 (60)	81 (71)
AF or	VacA+ 50 kDa-	31 (100)	30 (97)	30 (97)	18 (40)	103 (84)*
AF or	VacA + or (50 kDa- 30 kDa-)	31 (100)	30 (97)	30 (97)	26 (13)	95 (77)
AF- and	VacA- 50 kDa+	0 (100)	1 (97)	1 (97)	10 (33)	101 (82)*
AF- and	VacA- 30 kDa+	0 (100)	5 (84)	2 (94)	14 (47)	100 (82)
AF- and	VacA- (50 kDa+ or 30 kDa+)	0 (100)	1 (97)	2 (94)	14 (47)	104 (85)**
AF- and	VacA- (50 kDa+ 30 kDa+)	0 (100)	1 (97)	1 (97)	14 (47)	105 (85)**

GC – gastric cancer; DU – duodenal ulcer; GU – gastric ulcer; FD – functional dyspepsia.
AF *vs* other AF and/or seroreactivity combinations: * $p < 0.05$; ** $p < 0.01$.

AF) and in GU by 6% (35% in AF) with failure to help us in FD by 53% (60% for AF).

VacA-, along with 50 kDa+ and AF- was absent in the group with GC and present in 3% of groups with GU and DU. It was also present in 33% in FD. FC was 82% and it was significantly better than in FD, but with a correction for atrophy findings in FD, significance disappeared.

VacA- and 30 kDa+ AF- were absent from the GC group but present in 16% of DU, 6% with GU, and 47% of FD, making FC 82%, which was not significantly different from AF with atrophy correction.

VacA- (50 kDa-, 30 kDa-) AF- was completely absent in GC, present in 3% of DU and GU, and in 47% of FD. FC was 85%. It was statistically significant ($p < 0.01$) better than AF, being significantly different even after correction for atrophy in FD ($p < 0.05$).

The four combinations, which were significantly better than AF, were analyzed for their diagnostic characteristics (Se, Sp, PPV and NPV, CUI+ and CUI-) (Table 4).

Alarm features alone had moderate sensitivity, low specificity, high PPV, low NPV, satisfactory CUI+ (0.63), and very poor CUI- (0.26), making overall utility poor. After correction for atrophy findings in FD CUI+ become good 0.67 (0.58–0.77), and overall utility satisfactory (FC 75%).

AF or VacA+ 50 kDa- had high sensitivity (98%), very low specificity, high PPV and NPV, excellent CUI+ (0.82), very poor CUI- (0.34), with overall utility satisfactory (FC 84%). After correction for atrophy in FD, Sp rised to a modest 63%, CUI- (0.57) to the satisfactory and overall utility at good test (FC 89%).

AF- along with VacA- (50 kDa or 30 kDa+) had low sensitivity, but high specificity, PPV and NPV, poor CUI+ (0.38) but excellent CUI- (0.82), with overall utility as satisfactory (FC 85%). After correction for atrophy findings in FD, Sp rose to 64%, CUI+ became satisfactory (0.57), and overall utility became good (FC 89%).

AF- VacA- 50 kDa+ had very low sensitivity, but high Sp, PPV and NPV, very poor CUI+ (0.28), good CUI- (0.8) and overall utility satisfactory (FC 82%). After correction for atrophy in FD, Se rose to 40%, CUI- became excellent (0.82), and overall utility became good (FC 84%).

AF- VacA- (50 kDa+ 30 kDa+) had low sensitivity, high Sp, PPV and NPV, poor CUI+ (0.41), and excellent CUI- (0.83), making overall utility satisfactory (FC 85%) (Table 4). After correction for atrophy in FD, overall utility became good (FC 86%).

Discussion

The approach to the patient with uninvestigated dyspepsia based on the AF represents a standard with unsatisfactory results. AF approach has high Sp and NPV but low Se and PPV in diagnosing gastroesophageal malignancy. There was a positive correlation between AF and the diagnosis of advanced gastreoesophageal cancer, with the exception of gastrointestinal bleeding signs [11, 12].

The value of individual AF varies widely. Dysphagia, significant weight loss, and age ≥ 55 were found to be significant predictors for GC, but the value of other accepted AF was more limited. Fast track endoscopy in patients with AF suspected of upper gastric malignancy results in a significant yield of cancer (~ 4%) and serious benign diseases such as severe oesophagitis, peptic stricture, or PU [13]. In Western countries, 40–60% of patients with dyspepsia have normal findings of upper gastrointestinal endoscopy, with findings of oesophageal and gastric cancer in less than 1%. A meta-analysis of the value of AF in the Asian population finds overall malignancy detection of 1.3%, with calculated all organic disease detection of 26.4%, and among them PU 11.9% and oesophageal disease 5.5%. AF yield moderate diagnostic accuracy for predicting malignancy, with an area of 0.74 under curve [13, 14]. A Scandinavian study reported significant endoscopic findings in 32–40% of patients with AF (erosive oesophagitis 15–23%, PU 13–16%, erosive duodenitis 2%, gastric neoplasia 1–2%) [15]. Defining serious endoscopic findings (SEF) as gastric malignancy, PU, stricture, and erosive oesophagitis in 650 patients, Abdeljawad et al. [16] reported only 10.2% of patients had SEFs. AF were more

Table 4

Diagnostics characteristics of alarm feature (AF), and AF or VacA+ 50 kDa-, AF along with VacA- and 50 kDa+, 50 kDa or 30 kDa+, 50 kDa+ 30 kDa+

Parameters	Diagnostics characteristics						
	Sn	Sp	PPV	NPV	CUI+	CUI-	FC
AF	74 (65–89)	66 (43–78)	85 (77–93)	43 (28–58)	0.63 (0.59–0.73)	0.26 (0.12–0.39)	71
AF or VacA+ 50kDa-	98 (95–100)	40 (25–58)	84 (77–99)	86 (67–100)	0.82** (0.75–0.89)	0.34 (0.15–0.54)	84 •
AF- VacA-50kDa+	33 (16–50)	98 (95–100)	83 (62–100)	82 (75–98)	0.28 (0.02–0.54)	0.8* (0.75–0.85)	82•
VacA- (50kDa+30kDa+)	47 (29–65)	98 (95–100)	88 (71–100)	85 (78–92)	0.41 (0.17–0.65)	0.83** (0.79–0.88)	85••
VacA- (50kDa+ or 30kDa+)	47 (28–64)	97 (93–100)	83 (64–100)	85 (78–92)	0.38 (0.15–0.62)	0.82** (0.77–0.87)	85•

Sn – sensitivity; Sp – specificity; PPV – positive predictive value; NPV – negative predictive value; CUI+ – clinical utility index positive; CUI- – clinical utility index negative; FC – fraction correct.

A qualitative interpretation of the clinical utility index: excellent (E) => 0.81; good (G) => 0.64; satisfactory/adequate (SA) => 0.49; poor (P) => 0.36; very poor (VP) < 0.36.

***good clinical utility index; ** excellent clinical utility index; • overall utility satisfactory/adequate; •• overall utility good.**

frequent in patients with SEFs (12.6% *vs* 5.4%). A Nigerian study, considering oesophagitis, gastritis, duodenitis, PU, and gastric polyp or cancer as SEFs, did not find an association between AF and SEFs. Sp, Se, and PPV and NPV of AF are 65%, 49%, 71%, and 41%, respectively [17]. A large multicenter database study encompassing 3,815 participants did not find an effective prediction of age and alarm symptoms for upper endoscopy finding, with a Se of 87% and Sp of 26% [18]. A large Chinese retrospective analysis of more than 100,000 participants reported the presence of AF in only 52% of patients with malignancy, and in patients with AF, only 14.8% were found to have gastric malignancy. The pooled Se and Sp of the AF were 96.6% and 13.4%, respectively [19]. German analysis of 215 patients with GC and AF did not find any association of AF with the stage of the disease, duration of dyspeptic symptoms, age threshold of 45 years, and AF [20].

Our pilot study in a small population with an exceptionally high percentage of SEFs in 75% of participants (Table 1) and a small percentage of non-significant findings in 25% had Se, Sp, and PPV and NPV of AF 74%, 66%, 85%, and 43%, respectively, with clinical utility indices in the range of poor to borderline with a satisfactory range for case-finding (0.63) and very poor for screening (0.26) (Table 4). When we compared FC of the AF group with FC of the investigated seroreactivity combinations, regardless of the numerically higher value of FC in 5/6 seroreactivity combinations, the difference was not significant (Table 2).

Upgrading AF with seroreactivity combinations leads to a significant increase of FC in four out of six AF and seroreactivity against *H. pylori* VacA, 50 kDa, and 30 kDa combinations. AF or VacA seropositivity and 50 kDa seronegativity had significantly better FC than AF alone and yielded a case-finding utility index in the range of excellent (0.82) and overall utility in the range of satisfactory. The absence of AF along with VacA seronegativity and 1) 50 kDa or 30 kDa seropositivity and 2) 50 kDa and 30 kDa seropositivity reached FC significantly higher compared to AF ($p < 0.01$). The absence of alarm features along with VacA seronegativity and 50 kDa seropositivity yielded significantly higher FC than AF alone. AF- VacA- (50 kDa or 30 kDa+) and AF- VacA- (50 kDa and 30 kDa+) had excellent screening utility indices with satisfactory overall utility and good overall utility, respectively. AF- VacA- 50 kDa+ had a good screening utility index and a satisfactory overall utility (Tables 3 and 4).

In our study, we considered the presence of any of the AF as a positive finding without analyzing the specific value of each of them. A very interesting finding of our study was the fact that adding seroreactivity combinations to AF did not overlap with the disadvantages of alarming features but rather upgraded them. In patients with SEFs, particularly for PU peptic ulcers, seroreactivity combination indicated an alert only in patients without AF leading to an increase in test sensitivity. On the other hand, the absence of AF in the FD group was straightened with the presence of a "benign" seroreactivity combination and without significant deterioration of specificity.

The fact that AF did not have such high sensitivity is important, particularly for PU and early GC, which had a significantly better prognosis than the advanced disease. The survival of early GC undergoing curative endoscopic submucosal dissection was 5 years in 92.6% [21] with a significant decrease and an advancing stage of the disease both in Japan [Ia (91.5%), Ib (83.6%), II (70.6%), IIIa (53.6%), IIIb (34.8%), and IV (16.4%) [22]] and Sweden (31–44% in noncardia cancer and 21–43% in cardia cancer) [23]. An Italian report is notably interesting for finding alarm features in only 41.3% of 92 patients ≤ 45 years with GC [24].

Our approach is original for combining alarm features with serology, but it is not the first attempt to use a non-invasive approach for investigating dyspepsia. Low IgG titer against *H. pylori* was associated with GC and incorporated in GC screening ABC method [25]. Low IgG titer against *H. pylori* and anti-*H. pylori* IgG < IgA ratio was also associated with GC [26, 27]. A test with oral capsaicin (75 mg) in 224 patients without AF, investigating dyspepsia yielded a Se of the test between 0.51–0.59, with a Sp of 0.84–0.89 and the PPV for the diagnosis of FD of 70–71%. FD patients had significantly higher median delta symptom scores as compared to inflammatory bowel disease, PU, irritable bowel syndrome, and patients classified with "other disease". Patients with gastroesophageal reflux disease had significantly lower symptom scores when FD was not concomitantly diagnosed compared to the time when FD was present [28]. The combination of pepsinogen, gastrin-17, and anti-*H. pylori* antibodies serological assays (panel test) are a non-invasive tool for the diagnosis of atrophic gastritis. Twenty studies with a total of 4,241 subjects assessed the performance of the serum panel test for the diagnosis of atrophic gastritis regardless of the site in the stomach. The summary Se was 74.7% and the Sp was 95.6%. With a prevalence of atrophic gastritis of 27% (median prevalence across the studies), the NPV was 91% [29]. None of these non-invasive methods has been incorporated into clinical practice until now.

In our study, as in most other studies, we had only locally advanced GC without patients with early GC. Subtle but significant mucosal abnormalities, which usually are not present with AF, could be the target of "upgraded" alarm features within our pilot study and investigated seroreactivity combination in further studies.

AF and/or seroreactivity combination against *H. pylori* VacA, 50 kDa, and 30 kDa may improve the proportion of diagnosed PU playing the role of a "red flag" in patients without AF without a significant increase of unnecessary endoscopies in patients without AF and FD. In our study, AF had a high Se for advanced GC, and even though AF in combination with seroreactivity had maximal FC, it was not possible to reach a statistically significant difference. Therefore, it would be very interesting to test this approach in patients with early GC who, in the majority of cases, have no AF.

Our study has several limitations. First, this is a pilot study in *H. pylori*-infected individuals with a small number of participants and a different proportion of organic disease in comparison with the general population. We do not analyze the specific value of AF separately and use the German

ViraBlot test with *H. pylori* isolates from the German population. Studies about seroreactivity to different *H. pylori* antigens in specific outcomes of infection gave us significant but very variegated results regarding the value of specific *H. pylori* antigens [30–37]. In that sense, local validation of each specific *H. pylori* antigen could be considered.

Hence, the results of our pilot study justify conducting a large prospective study in patients with uninvestigated dyspepsia infected with *H. pylori*, eradication of *H. pylori* in treatment-naïve patients, considering the value of each AF separately and its upgrading with seroreactivity against *H. pylori* antigens VacA, 50 kDa, and 30 kDa in Serbia.

Conclusion

Seroreactivity against *H. pylori* antigens VacA, 50 kDa, and 30 kDa might improve our approach to patients investigating dyspepsia if we used it along with AF. Results of our pilot study demand confirmation and further exploration in a larger, well-designed study.

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Assessment of volemia status using ultrasound examination of the *inferior vena cava* and spectroscopic bioimpedance in hemodialysis patients

Procena statusa volemije ultrazvučnim pregledom donje šuplje vene i spektroskopskom bioimpendancom kod bolesnika na hemodijalizi

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Abstract

Background/Aim. Hypervolemia is an important risk factor for the development of cardiovascular morbidity and mortality in patients treated with regular hemodialysis. There is still no reliable method for assessing the status of volemia in these patients. The aim of the study was to assess the status of volemia in patients treated with regular hemodialysis by measuring the parameters of the *inferior vena cava* (IVC) and bioimpedance. **Methods.** The effect of hemodialysis treatment on ultrasound parameters of the IVC, as well as on the parameters measured by bioimpedance, was examined before and after hemodialysis. The values of the N-terminal prohormone of brain natriuretic peptide (NT-proBNP) were measured both before and after hemodialysis. Forty-five patients were involved in this non-interventional cross-section study, including the patients treated with standard bicarbonate dialysis. According to the interdialytic yield, the patients were divided into three groups: I (up to 2,000 mL), II (2,000–3,000 mL), and III (over 3,000 mL). **Results.** The values of the IVC parameters and the parameters measured with bioimpedance were significantly lower after treatment with hemodialysis ($p < 0.005$). The third group of patients had a significantly higher total fluid volume in the body compared to the group I, as well as a significantly greater volume of extracellular fluid ($p < 0.005$). The significantly lower values of NT-proBNP in all groups ($p < 0.005$) were detected after hemodialysis. After treatment with hemodialysis, a positive correlation was observed between the concentration of NT-proBNP in the serum and the extracellular/intracellular water ratio. However, the correlation between NT-proBNP concentration and total fluid measured by bioimpedance spectroscopy did not reach statistical significance. **Conclusion.** Measurement of the IVC ultrasound parameters and volemia parameters using bioimpedance significantly contributes to the assessment of the status of volemia. Nevertheless, it cannot be used as a separate parameter, only in combination with all other methods.

Key words:
plasma volume; renal dialysis; spectrophotometry; vena cava, inferior; ultrasonography.

Apstrakt

Uvod/Cilj. Hipervolemija je značajan faktor rizika od razvoja kardiovaskularnog morbiditeta i moratliteta kod bolesnika koji se leče redovnom hemodijalizom. Još uvek ne postoji suverena metoda za procenu statusa volemije kod tih bolesnika. Cilj istraživanja bio je da se merenjem parametara donje šuplje vene i merenjem bioimpedance proceni status volemije kod bolesnika koji se leče redovnom hemodijalizom. **Metode.** Ispitivan je uticaj tretmana hemodijalizom na ultrazvučne parametre donje šuplje vene, kao i na parametre merene spektroskopskom bioimpedancom (SB) pre i posle hemodijalize. Merene su i vrednosti N-terminalnog prohormona moždanog natriuretskog peptida (NT-proBNP) pre i posle hemodijalize. U neinterventnu studiju preseka bilo je uključeno 45 bolesnika koji se leče standardnom bikarbonatnom dijalizom. Prema intradijaliznom prinosu bolesnici su bili podeljeni u tri grupe: I (do 2 000 mL), II (2 000 – 3 000 mL), III (preko 3 000 mL). **Rezultati.**

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Vrednosti parametara donje šuplje vene i parametara izmerenih SB-om bili su značajno niži nakon tretmana hemodijalizom ($p < 0.005$). Treća grupa bolesnika imala je značajno veću ukupnu zapreminu tečnosti u organizmu pre hemodijalize u poređenju sa I grupom, kao i značajno veću zapreminu vanćelijske tečnosti ($p < 0,005$). Nakon hemodijalize detektovane su značajno niže vrednosti NT-proBNP-a u svim grupama ($p < 0,005$). Posle tretmana hemodijalizom, zabeležena je pozitivna korelacija između koncentracije NT-proBNP-a u serumu i odnosa ekstracelularne/intracelularne tečnosti; međutim korelacija između koncentracije NT-proBNP-a i ukupne tečnosti izmerene putem SB nije dostigla statističku značajnost. **Zaključak.** Merenje ultrazvučnih parametara donje šuplje vene i parametara volemije SB-om u značajnoj meri doprinosi proceni statusa volemije, ali se ne može koristiti kao odvojeni parametar, već u kombinaciji sa svim drugim metodama.

Ključne reči:
plazma, volumen; hemodijaliza; spektometrija; v. cava inferior; ultrasonografija.

Introduction

Hypervolemia is an important risk factor for the development of cardiovascular morbidity and mortality, as well as adverse outcomes in patients treated with regular hemodialysis [1]. The assessment of volemia status in these patients is performed by clinical examination, lung radiography, ultrasound examination of the lungs, inferior vena cava, and the heart, measurement of volemia using bioimpedance spectroscopy (BIS), and monitoring of N-terminal prohormone of brain natriuretic peptide (NT-proBNP) in the serum [1].

The goals of hemodialysis are to eliminate excess fluid, achieve adequate dry weight and depuration of the organism from uremic toxins, and regulate electrolyte imbalance. Dry weight is the weight obtained at the end of a regular dialysis session, below which the patients will most likely develop symptomatic hypotension [2].

Symptomatic hypotension is associated with ultrafiltration rate during hemodialysis treatment and the rate at which the removal is performed. Inadequate dry weight assessment leads to chronic volume overload. Removing too much excess fluid (below the dry weight) causes hypovolemia and vertigo, headache, pain, and cramps in the muscles, and a decrease in perfusion of vital organs occurs. In cases where the elimination of fluid is not sufficient (weight above the dry weight), a chronic hypervolemic condition and complications occur, such as hypertension, left ventricular hypertrophy, diastolic dysfunction, congestive heart failure, and edema of the lungs [3].

Radiography of the heart and lungs is highly specific but with low sensitivity for the conditions of hypervolemia. The disadvantage of this diagnostic method is that it sometimes takes several hours for the radiographic changes of hypervolemia to occur. In addition, in 20% to 40% of patients, radiographic changes of hypervolemia are absent [4]. What is important when assessing the status of volemia is the echocardiographic examination of the heart to assess the structure and function of the left ventricle, as well as the disorder of left ventricular systolic and diastolic function. This examination may indicate the condition of hypervolemia, but it does not give us information that the patient is in normovolemia [5–7].

Ultrasound of the lungs is used to estimate the volume of fluid in the extravascular lung section and the severity of the degree of hypervolemia [8]. This method is useful in detecting pulmonary congestion, even before the manifested clinical picture, and it correlates well with systolic cardiac function. The disadvantage of this method is that it may not always correlate with hypervolemia [9].

The results of numerous studies indicate that hyper- or hypovolemia can be determined in a dialysis patient using ultrasound measurement of the diameter of the *inferior vena cava* (IVC) [10, 11]. However, the recommended values of the IVC diameter (IVCd) that would correlate with optimal body weight are not generally accepted because of individual variations, as well as due to significant subjectivity in the measurement. In their study, Munizt Pazeli et al. [12] concluded that ultrasound of the IVC could be performed by nephrologists who have little experience in ultrasound, and the findings were potentially useful for dry weight assessment in patients on dialysis. The IVCd, IVC index (IVCi), and IVC collapsibility index (IVCci) are measured using ultrasound examination of IVC. The normal diameter of the IVC is 15–20 mm, and it varies depending on the breathing cycle. In the inspirium, the diameter decreases and amounts to 0–15 mm, and in the expirium, it increases and amounts to 15–20 mm. The anterior-posterior diameter of the IVC was measured subxiphoidally at a distance of 2–3 cm from the right atrium during spontaneous breathing, forced inspiration, and forced expiration. The IVCi is the ratio of the IVCd and the body surface (BS) of the patient: IVCd/BS (mm/m^2). The normal IVCi is 8.0–11.5 mm/m^2 (euvolemia). The IVCi below 8 mm/m^2 indicates hypovolemia, and over 11.5 mm/m^2 is considered hypervolemia. In order to calculate the IVCci, it is necessary to measure its diameter in inspirium (IVCinsp) and expirium (IVCexp). The IVCci is calculated using the formula: IVCci = [(IVCexp- IVCinsp)/ IVCexp] × 100%. If the IVCci is 50% to 75%, we are talking about euvolemia, if it is below 50%, it is hypervolemia, and over 75%, it is hypovolemia [13, 14].

Additionally, the concentration of NT-proBNP in plasma, which has a significant prognostic value for cardiovascular mortality in hemodialysis patients, was used in assessing the status of volemia in these

patients [15,16]. NT-proBNP is often increased in patients with chronic renal failure [17,18]. When examining patients with asymptomatic chronic renal failure who have not yet started dialysis treatments, an increased level of NT-proBNP was observed in more than half of the patients [18]. However, there is no clear cut-off in the literature for the concentration of NT-proBNP that would distinguish cardiac from renal failure (cut-off values range from 5,000 to 7,000 pg/mL). NT-proBNP is not only specific for hypervolemia but also for nutritional status, systolic and diastolic left ventricular dysfunction [19].

Precise assessment of the status of volemia in patients on dialysis also includes the analysis of BIS. Bioimpedance represents the total resistance of tissue and fluid to the flow of micro-amperage alternating current through the body [20]. Two methods work on the principle of BIS. One is the so-called "whole body BIS" where the electrodes are placed on the wrist and ankle on the same side of the body, and using appropriate mathematical and physiological tissue models, we obtain data on the total amount of water in the body (TBW) and the amount of water in tissue cells [20, 21]. The second method of BIS is a segmental BIS, which separately measures the amount of water in the extremities and the trunk. It can be done with eight electrodes; it is very precise, but the examination lasts for a longer time, and in practice, it has not shown an advantage in relation to the whole body BIS [22, 23].

A shift in the application of BIS to determine the condition of volemia in hemodialysis patients was provided by two studies published in 2009. These studies have shown that: 1) hypervolemia prior to dialysis treatment greater than 15% of normal extracellular fluid (approximately 2.5 L in an average person of 70 kg) correlated with a twice higher risk of fatal outcome during the 3.5 year follow-up period, compared to those patients in whom this predialysis hypervolemia was lower [24]; 2) reduction of this "critical" hypervolemia can improve arterial blood pressure values and reduce therapy in hemodialysis patients [25].

The aim of this study was to investigate the degree of association between the status of volemia assessed clinically (interdialytic yield), with IVC measurement, BIS, and determination of NT-proBNP, and the values of the parameters measured by BIS. Moreover, we determined the influence of hemodialysis treatment on the concentration of NT-proBNP in the serum of the patients. We also examined the influence of the degree of ultrafiltration on the values of the parameters of ultrasound examination of IVC, as well as the degree of correlation between NT-proBNP in the serum and the parameters of ultrasound examination of the inferior vena cava before and after hemodialysis.

Methods

Patients

A cross-sectional study (non-interventional study) included 45 patients treated with regular hemodialysis for more than three months. According to the rate of targeted ultrafiltration, the patients were divided into three groups. The first group consisted of patients with a yield of up to 2,000 mL (n = 14), the second group consisted of patients with a yield of 2,000 mL to 3,000 mL (n = 12), and the third group of patients had a yield of over 3,000 mL (n = 19). Patients were dialyzed using low-flux and high-flux synthetic dialyzers, 12 hrs a week, using a bicarbonate ultrapure hemodialysis solution, and the machines type Fresenius 4008, 508S and Gambro AKA20US and Gambro Artis. The study was conducted at the Center for Nephrology and Dialysis of the Clinical Center of Kragujevac with respect to the Helsinki Declaration and Good Clinical Practice.

Biochemical analyses

The following examination parameters were determined in all patients: complete blood count (CBC), the status of iron in the organism (serum iron concentration, transferrin saturation, serum ferritin concentration), parameters of secondary hyperparathyroidism (serum calcium concentration, serum phosphate concentration), solubility produc, serum alkaline phosphatase concentration, serum intact parathyroid hormone concentration), microinflammatory parameters (C-reactive protein), nutritional status parameters (concentration of total protein and serum albumin). The concentrations of NT-proBNP in the serum were assessed 15 min before the start of hemodialysis and 15 min after the shutdown of hemodialysis. The adequacy of hemodialysis was assessed on the basis of Kt/V index (dialysis dose, i.e., product of urea clearance during dialysis (K) and time of dialysis procedure (t) divided by urea distribution volume, V) and URR index (urea reduction ratio) [26]. Laboratory parameters were sampled in accordance with a laboratory test protocol for patients who were treated in the chronic hemodialysis program; there was no need for additional blood sampling.

BIS was performed by Body Composition Monitor (BCM) module (BCM; manufacturer Fresenius Medical Care, Software version: 3.2); the patient's hydration status was assessed 15 min before the start of hemodialysis and 15 min after the shutdown of hemodialysis. The electrodes were placed on the wrist of one hand and the foot on the same side of the body of the patient in a lying position. This BIS device uses a multi-frequency current range (50 different frequencies from 5 to 1,000 kHz) and provides data on the total body water (TBW), the extracellular fluid (ECF), the intracellular fluid (ICF), and the quantification of the excess volume of ECF. The results are displayed on the monitor after 2 min of measurement and stored on a card for each patient individually.

Ultrasound examination of the IVC

Ultrasound examination was performed on the LOGIQ P5 ultrasound apparatus using a 3.5 MHz probe.

The anterior-posterior diameter of the IVC (IVCd) was measured in its proximal during spontaneous breathing and during inspiration (IVCinsp) and expiration (IVCexp), 30 min before and after the shutdown of hemodialysis, subxyphoidally at a distance of 2–3 cm from the right atrium of the heart. Based on the measured IVC parameters, the IVCi and IVCci were determined.

Statistical analysis

The data were processed in the statistical software SPSS 20.0 for Windows (IBM SPSS Statistic for Windows, Version 20.0, USA). The results are presented as the mean ± standard deviation (SD) for numerical and the frequency for attribute data. The following tests were used for statistical analysis of the obtained data: the Kolmogorov-Smirnov test (estimation of the normality and distribution of data), the Student's *t*-test, the Mann-Whitney *U* test, the single-factor analysis of variance – ANOVA, the Kruskal-Walis test, the univariate and multivariate logistic regression analyses, the Spearman's and Pearson's correlation coefficient. The significance threshold was 0.05.

Results

Table 1 shows general patient data that include the analysis of demographic and clinical characteristics. The values of the measured biochemical parameters are also shown.

Figure 1 shows the percentage of patients according to the rate of ultrafiltration.

Table 1

General patient data including demographic and clinical characteristics (values of the measured biochemical parameters are also shown)

Variable	Values
Gender (female), n (%)	15 (33.7)
Age (years)	64.91 ± 10.82
Length of HD treatment (years)	7.02 ± 6.48
Body weight index (kg/m^2)	26.17 ± 4.80
Ultrafiltration (L/HD)	2.67 ± 1.19
Hypervolemia measured using BCM (L)	2.71 ± 1.35
Systolic arterial blood pressure before HD (mm Hg)	130.49 ± 18.47
Diastolic arterial blood pressure before HD (mm Hg)	77.75 ± 8.43
Medium arterial blood pressure before HD (mm Hg)	104.45 ± 12.81
Pulse arterial blood pressure before HD (mm Hg)	52.47 ± 13.76
Erythrocytes (x10^{12}/L)	3.27 ± 0.45
Leukocytes (x10^9/L)	6.54 ± 2.09
Hemoglobin (g/L)	101.89 ± 13.10
Sodium (mmol/L)	137.49 ± 2.98
Potassium (mmol/L)	5.33 ± 0.67
Magnesium (mmol/L)	1.22 ± 0.29
Calcium (mmol/L)	2.28 ± 0.80
Phosphorus (mmol/L)	1.33 ± 0.50
Iron (umol/L)	8.43 ± 3.14
TIBC (mmol/L)	33.6 ± 6.22
Ferritin (ng/mL)	794.40 ± 286.31
Transferrin saturation	0.23 ± 0.06
Uric acid (umol/L)	364.00 ± 52.74
Total protein (g/L)	63.33 ± 5.35
Albumins (g/L)	36.24 ± 4.76
CRP (mg/L)	6.98 ± 9.60
Parathormone (pg/mL)	261.64 ± 395.91
Urea (mmol/L)	20.78 ± 4.32
Creatinine (umol/L)	743.07 ± 206.46
URR index	63.31 ± 13.88
Kt/V	1.22 ± 0.61

HD – hemodialysis; BCM – Body Composition System; TIBC – total iron binding capacity; CRP – C-reactive protein; URR – urea reduction ratio; Kt/V – dialysis adequacy index (K = clearance of urea; t – duration of dialysis; V – total body water). All values are expressed as mean ± standard deviation, except gender given as number (percentage).

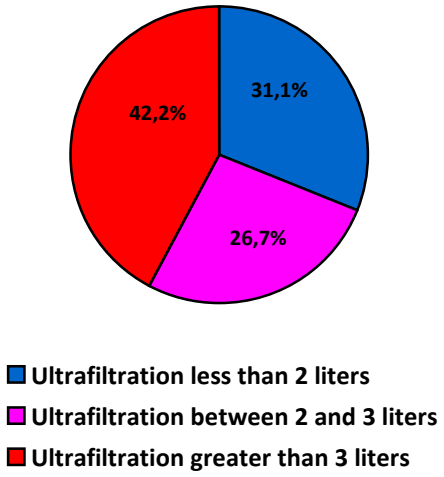

Fig. 1 – The percentage of patients according to the rate of ultrafiltration.

Influence of the rate of ultrafiltration on the examined parameters used to assess volemia

By comparing the mean values of ECF between the three groups, it was found that they were not equal. Namely, the third group (ultrafiltration greater than 3,000 mL) had significantly higher ECF values than the first group ($p = 0.017$) and the second group ($p = 0.025$). A statistically significant difference in comparison of TBW was recorded only between the first and the third group of patients ($p = 0.046$) (Table 2).

Mean values of extracellular fluid in examined patient groups

Statistically significant differences were not detected between the groups when the values of NT-proBNP, IVCd, IVCi, IVC insp, IVC exp, IVCci, and the ECF/ICF ratio were compared. The mean values of ECF are shown in Figure 2.

Table 2

Influence of the rate of ultrafiltration on the examined parameters used to assess volemia

Variable	Ultrafiltration (L)			*p*
	0 – 2 (n = 14)	2 – 3 (n = 12)	> 3 (n = 19)	
NT-proBNP (pg/mL)	5,188 (3,968–14,070)	7,536 (6,612 – 24,625)	11,949 (5,675–19,361)	0.309
IVCd (mm)	17.51 ± 3.47	18.73 ± 3.51	17.75 ± 3.09	0.615
IVCi (mm)	9.4 ± 1.84	10.70 ± 2.22	9.17 ± 1.71	0.119
IVCinsp (mm)	1.1 ± 0.38	1.17 ± 0.41	1.26 ± 0.38	0.681
IVCexp (mm)	2.25 ± 0.45	2.36 ± 0.41	2.93 ± 3.17	0.608
IVC collapsibility	50.02 ± 11.53	51.34 ± 11.41	45.0 ± 10.07	0.238
ICF (L)	17.90 (17.10–9.00)	15.05 (12.55–18.60)	18.80 (16.60–21.00)	0.105
ECF (L)	17.65 (16.30–18.80)	16.65 (15.25–19.40)	19.80 (18.35–21.60)	*0.020*
OH (L)	2.24 ± 1.08	2.8 ± 1.55	2.97 ± 1.37	0.282
TBW (L)	35.60 (34.10–38.10)	30.75 (27.80–38.15)	38.90 (35.15–42.60)	0.046
ECF/ICF index	1.0 ± 0.12	1.08 ± 0.18	1.08 ± 0.14	0.299

NT-proBNP – N-terminal prohormone of brain natriuretic peptide; OH – hypervolemia; TBW – total body water; IVC – *inferior vena cava*; d – diameter; i – index; insp – inspirium; exp – expirium; ICF – intracellular fluid; ECF – extracellular fluid.
Values are given as median (25th percentile – 75th percentile) or mean value ± standard deviation.

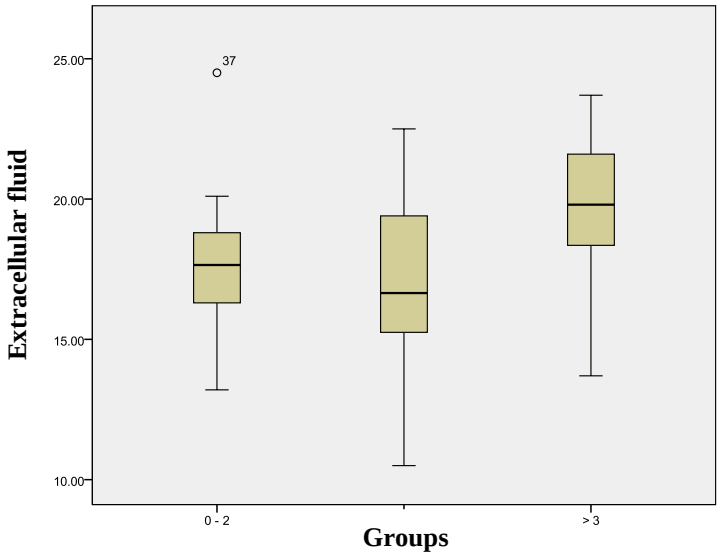

Fig. 2 – Mean values of extracellular fluid in examined patient groups formed according to the rate of ultrafiltration (in liters).

Comparison of the examined parameters before and after hemodialysis

The values of laboratory findings, ultrasound (IVCd, IVCi, IVCci), and volemia parameters used to assess the clinical condition of patients were statistically significantly lower after hemodialysis, except in the measurement of IVC values ($p = 0.990$). The values of IVCci 30 min after hemodialysis were statistically significantly higher compared to the values of this parameter before hemodialysis (48.15 ± 11.38 *vs* 55.45 ± 15.07, respectively; $p = 0.008$). This result suggests that the patients switched from the state of hypervolemia to the state of euvolemia after hemodialysis. This was corroborated by the result of weight changes before and after hemodialysis. The results of these comparisons are given in Table 3.

The degree of correlation of serum NT-proBNP values with other before-hemodialysis parameters

A positive correlation was detected between serum NT-proBNP with ultrasound parameters VCId, VCIi, VCInsp, VCIexp, VCIci, and changes in body weight. NT-proBNP was also positively correlated with the ECF/ICF index and weight change. Persons with higher values of NT-proBNP lose more body fluid. NT-proBNP was in a negative correlation with the VCI collapsibility index, which means that higher NT-proBNP values are in correlation with a lower VCI collapsibility index. That is, the patients in hypervolemia have a high NT-proBNP and low VCI collapsibility index (Table 4).

The degree of correlation of serum NT-proBNP values with other parameters after hemodialysis

Correlations of NT-proBNP with ultrasound parameters and volemia condition parameters after dialysis are shown in Table 5.

It can be seen that after hemodialysis, NT-proBNP was positively correlated with volemia, body weight, and ECF/ICF index. However, a negative correlation was recorded between NT-proBNP and IVCci, which was on the border of statistical significance ($p = 0.060$).

Table 3

Comparison of the examined parameters before and after hemodialysis

Variable	Before hemodialysis	After hemodialysis	*p*
NT-proBNP (pg/mL)	12.982 ± 10.865	8.837 ± 9.355	< 0.0005
IVCd (mm)	18.07 ± 3.35	14.70 ± 4.29	< 0.0005
IVCi (mm)	9.61 ± 1.97	7.10 ± 2.85	< 0.0005
IVCinsp (mm)	1.20 ± 0.39	0.87 ± 0.34	< 0.0005
IVCexp (mm)	2.60 ± 2.15	2.03 ± 0.59	0.002
IVC collapsibility	48.15 ± 11.38	55.45 ± 15.07	0.008
ICF (L)	18.30 ± 4.41	18.21 ± 4.02	0.990
ECF (L)	18.65 ± 3.74	16.51 ± 3.36	< 0.0005
OH (L)	2.71 ± 1.35	0.27 ± 1.38	< 0.0005
TBW (L)	36.65 ± 6.88	34.30 ± 6.21	< 0.0005
ECF/ICF index	1.06 ± 0.15	0.90 ± 0.20	< 0.0005
Body weight (kg)	76.53 ± 15.18	73.08 ± 14.88	0.0001

Explanations for abbreviations are given below Table 2.
Values are given as mean value ± standard deviation.

Table 4

The degree of correlation of serum NT-proBNP values with other parameters before hemodialysis

Variable 1	Variable 2	R	*p*
NT-proBNP	VCId	0.471	0.001
NT-proBNP	VDIi	0.369	0.018
NT-proBNP	VCIinsp	0.484	0.001
NT-proBNP	VCIexp	0.393	0.008
NT-proBNP	VCI collapsibility	-0.400	0.006
NT-proBNP	ICF	-0.182	0.236
NT-proBNP	ECF	0.261	0.084
NT-proBNP	OH	0.452	0.002
NT-proBNP	TBW	0.084	0.584
NT-proBNP	ECF/ICF	0.499	< 0.0005
NT-proBNP	Ultrafiltration	0.230	0.134
NT-proBNP	Body weight	-0.031	0.840
NT-proBNP	Change in body weight	0.427	0.004

Explanations for abbreviations are given below Table 2.

Table 5
The degree of correlation of serum NT-proBNP values with other parameters after hemodialysis

Variable 1	Variable 2	R	*p*
NT-proBNP	VCId	0.107	0.501
NT-proBNP	VDIi	-0.061	0.707
NT-proBNP	VCIinsp	0.109	0.491
NT-proBNP	VCIexp	0.152	0.336
NT-proBNP	VCI collapsibility	-0.287	*0.060*
NT-proBNP	ICF	-0.137	0.370
NT-proBNP	ECF	0.242	0.109
NT-proBNP	OH	0.373	*0.012*
NT-proBNP	TBW	0.046	0.766
NT-proBNP	ECF/ICF	0.425	*0.004*
NT-proBNP	Ultrafiltration	0.175	0.255
NT-proBNP	Body weight	0.739	< 0.0005
NT-proBNP	Change in body weight	0.249	0.103

Explanations for abbreviations are given below Table 2.

Discussion

The results of many studies suggest that hyper- or hypovolemia in patients on hemodialysis can be determined by ultrasound measurement of the diameter of the IVC [27, 28]. It is usually stated in the literature that the IVCd indexed to the body surface (IVCi) greater than 11.5 mm/m^2 correlates with mean pressure in the right atrium greater than 7 mmHg or with significant hypervolemia, while this index smaller than 8 mm/m^2 correlates with significant circulatory hypovolemia. However, this index is not sufficiently accurate, as other factors such as heart rate, arterial pressure, and antihypertensive drug therapy have an effect on it.

The results of our study show that there was no statistically significant difference in the measurement of the IVCd during normal breathing, inspirium, and expirium, among the three groups of patients with different degrees of ultrafiltration. A statistically significant difference was observed by comparing the IVCd before and 30 min after hemodialysis. After hemodialysis, the IVCd significantly decreases.

In the available literature, it is stated that the values of NT-proBNP positively correlate with the risk of mortality in patients on dialysis [29]. However, one measurement of the NT-proBNP value cannot be a reliable parameter for the assessment of the condition of volemia because it depends on both the volemia and the degree of myocardial damage. However, serial measurement of this marker may be a good indicator of the condition of volemia measured by bioimpedance [29]. In our study, we measured the values of NT-proBNP before and after hemodialysis. Our results show that the measured values of NT-proBNP before hemodialysis positively correlate with the IVC diameter. Moreover, the higher NT-proBNP values before hemodialysis were positively correlated with ECF/ICF and body fluid changes. However, only a positive correlation between the value of this marker and the condition of volemia was recorded by measuring the value of NT-proBNP after hemodialysis. The results of our study are similar to the results of a study in which the cut-off for the value of NT-proBNP was determined (from 5,000 to 7,700 pg/mL) as an indicator of hypervolemia [30]. In their cross-section study, Velasco et al. [30] described an excellent correlation between the average volemia assessed by BCM before hemodialysis and the levels of NT-proBNP measured before hemodialysis in patients aged up to 72 years. In the study by Paunic et al. [31], normovolemic patients (also estimated by BIS measurement) have NT-proBNP up to 4,700 pg/mL, and hypervolemic have this value above 5,800 pg/mL.

A study by David et al. [29] showed a significant correlation between serum NT-proBNP and the ratio of extracellular water and body weight only in hemodialysis patients with systolic left ventricular dysfunction but not in those without systolic dysfunction. Although the level of NT-proBNP can be increased with the increase in volume load, the summary of currently available evidence gives the impression that this marker has a limited role in assessing the state of hydration in the dialysis population. The use of this cardiac biomarker in assessing the condition of volemia in patients on hemodialysis has not yet entered the standard procedure due to the high cost and poor specificity of the tests, as well as due to the lack of a clear criterion for the normal range of NT-pBNP values in hemodialysis patients [32]. Consistent with the above study results, the results of our study cannot provide a clear cut-off value of NT-proBNP (range 5,188 to 7,536 pg/mL).

Our results show that hemodialysis treatment has a statistically significant effect on the values of parameters for assessing the state of volemia measured by BIS. The values of the parameters measured by BIS after hemodialysis treatment are highly statistically significantly lower compared to the values before hemodialysis. Our results correlate with the results of the study conducted by Chamney et al. [33].

Furthermore, our results show that the parameters obtained by BIS provide objective, qualitative, and useful data on the state of volemia in patients treated with hemodialysis. The results obtained by this method correlate with the biohumoral cardiac marker NT-proBNP. Similar results were obtained in the study by Velasco et al. [30].

Conclusion

Neither IVC diameter, BIS nor NT/proBNP measurement can be used as a stand-alone test for monitoring the condition of hydration of patients on hemodialysis. The results obtained by these methods must be interpreted individually and adapted for each patient. Only in this way could these parameters add value to the clinical judgment of an individual patient's hemodialysis optimal body weight.

Conflict of interest

None declared.

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Cognitive ability and motor performances in the elderly

Kognitivne sposobnosti i motoričke performanse starijih osoba

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Abstract

Background/Aim. Aging entails a wide range of cognitive processes that are not independent of one another. It leads to changes in physical-motor characteristics and sometimes to disability. The aim of this study was to examine the association between multiple cognitive performances in elderly subjects and their physical-motor abilities. **Method.** The study included 98 elderly participants (60+) (16 males and 82 females). Cognitive abilities were assessed by the Montreal Cognitive Assessment (MoCA)/Serbian version, and physical measures were assessed by the Senior Fitness Test with its five subtests, supplemented by the Walking Speed Test. **Results.** Several MoCA items demonstrated relatively low variability, i.e., they proved to be too easy for most of the participants. The participants exhibited the lowest performance on the memory relating to other domains, followed by executive functions, visuospatial skills, attention, concentration, and working memory domains, with the highest performance on temporal and spatial orientation relating to other domains. Executive functions and language correlated most significantly with physical strength. Agility and dynamic balance, lower- and upper-body strength, and aerobic endurance correlated moderately and positively. **Conclusion.** This study underlines the positive correlation between physical fitness and cognitive level in the elderly and emphasizes the importance of physical fitness for cognitive functions, especially those of executive type in elderly subjects. Clinicians should consider the association between cognitive function and physical-motor performances when dealing with functioning improvement in the elderly. The importance of designing the most efficient exercise programs to achieve maximal somatic and cognitive effects is emphasized.

Key words:
aging; cognition; exercises; aged; physical fitness.

Apstrakt

Uvod/Cilj. Proces starenja podrazumeva promene na širokom spektru kognitivnih procesa koji nisu nezavisni jedni od drugih. On, takođe, dovodi do promena u fizičko-motoričkim karakteristikama, a ponekad i do invaliditeta. Cilj istraživanja bio je da se ispita povezanost između više kognitivnih performansi kod starijih ispitanika i njihovih fizičko-motoričkih sposobnosti. **Metode.** U istraživanju je učestvovalo ukupno 98 starijih ispitanika (60+) (16 muškog i 82 ženskog pola). Kognitivne sposobnosti procenjene su Montrealskom skalom kognicije (*Montreal Cognitive Assessment* – MoCA)/srpska verzija, a mere fizičkih sposobnosti su procenjene *Senior Fitness* testom koji se sastoji od pet subtestova, dopunjenih testom brzine hoda. **Rezultati.** Na nekoliko subtestova MoCA rezultati su ukazali na relativno malu varijabilnost, tj. pokazalo se da su previše jednostavni za većinu ispitanika. Ispitanici su pokazali najslabije rezultate u funkcionisanju memorije u odnosu na druge domene, a zatim slede izvršne funkcije, vizuelno prostorne veštine, pažnja, koncentracija i radna memorija, sa najvišim performansama na vremenskoj i prostornoj orijentaciji u odnosu na druge domene. Izvršne funkcije i jezik su najznačajnije korelirali sa fizičkom snagom. Spretnost i dinamična ravnoteža, snaga donjih i gornjih ekstremiteta i aerobna izdržljivost su korelirali umereno i pozitivno. **Zaključak.** Studija ukazuje na pozitivne korelacije između fizičko-motoričkih sposobnosti i kognitivnog nivoa kod starijih osoba i naglašava značaj fizičke spremnosti za kognitivno funkcionisanje, a naročito u domenu izvršnih funkcija kod njih. Kliničari bi trebalo da imaju u vidu povezanost između kognitivnih funkcija i fizičko-motoričkih performansi, kada se bave poboljšanjem funkcionisanja starijih osoba. Ukazuje se na važnost dizajniranja najefikasnijih programa vežbanja za postizanje maksimalnih somatskih i kognitivnih efekata.

Ključne reči:
starenje; saznanje; vežbanje; starije osobe; sposobnost, fizička.

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Introduction

Aging is accompanied by a decline in a wide range of cognitive and physical processes, including psychomotor speed, working memory, executive functions, memory, linguistic abilities, and general knowledge [1]. The decline in cognition during aging is gradual and, in general, is not statistically significant until after 60 years of age [1]. Processes observed in cognitive aging are not independent of one another. Processing speed is reduced in more complex tasks such as noticing and responding to sudden situational changes. According to actual theories, slowing effects increase with age, hence older adults are disproportionately more affected if compared to younger adults as tasks increase in complexity [2].

Age-related or pathological cognitive changes are known to affect abilities in instrumental activities of daily living, i.e., those with executive demands. Performance on cognitive tests tends to show subtle declines well before everyday functioning is affected and could, therefore, be a useful clinical predictor [3].

Aging is a multifactorial process leading to changes in skeletal muscle quantity and quality, which causes muscle weakness and can lead to disability as an outcome in the aging population. Several mechanisms may be involved in the onset and progression of muscle mass loss (sarcopenia), such as protein synthesis, proteolysis, neuromuscular integrity, and muscle fat content [4]. The rate of muscle loss has been established to range from 1% to 2% per year past the age of 50, as a result of which 25% of people over the age of 70 years and 40% of those over the age of 80 years are sarcopenic [5].

The mechanisms accounting for a decline in muscle strength can be attributed to a combination of "neural" and "muscular" factors. It is known that muscle strength is not solely dependent on muscle size. Some relatively recent studies indicate that the decline in muscle strength is much more rapid than the concomitant loss of muscle mass [6]. In addition, advancing age is associated with a reduction of spinal excitability [7], altered motor unit discharge properties, and reduced motor unit size and numbers [8]. Some findings indicate that aging is associated with widespread qualitative and quantitative changes in the motor cortex and spinal cord, reducing the ability to modulate the activity of motor networks when required and reducing cortical plasticity [9]. Collectively, these changes are likely to contribute to age-related reductions in motor performance, although the exact relationship to strength loss is yet to be determined [6].

Sarcopenia is a loss of muscle mass and can be regarded as an early marker of physical and cognitive decline [10]. The relationship between sarcopenia and dynapenia (loss of muscle strength) with cognitive decline and dysfunction is not well-defined. It is widely accepted that motor neuron dysfunction can lead to decrements in muscle mass and strength, but a reverse association is not confirmed. However, studies are indicating that muscle strength, walking ability, and balance are significant predictors of cognitive performance in healthy older adults [11].

It has been confirmed that a decline in cognitive performance results in gait and balance deterioration [12]. Moreover, there are suggestions that there is a motor gait phenotype associated with a decline in cognitive performance, which could be used to improve the prediction of dementia, even before the prodromal stage [12].

There is an association between gait slowing and cognitive impairment [13]. It is supported by a shared neural substrate that includes a smaller right hippocampus. This finding underscores the value of long-term gait slowing as an early indicator of dementia risk [14]. It has been found that upper-body flexibility and agility/dynamic balance correlate with cognitive functioning [15]. These studies accentuate the relation between physical and cognitive status in the elderly. It is still not clear whether they are independently affected by some mutual factors or connected by a causal relationship.

The aim of the study was to examine the association between multiple cognitive performances in elderly subjects and their physical abilities measured by a variety of tests.

Methods

Sample

The sample consisted of 98 participants ranging in age from 61 to 85 years [mean = 68.50, standard deviation (SD) = 4.57], 16 males and 82 females. Before taking part in the research, the participants were introduced to the nature of the research; they also gave their informed consent for participation.

The following inclusion criteria were used: the absence of serious previous diseases and damage to the central and peripheral nervous system; participant's ability to cooperate during examination and testing; blood pressure within the normal range, with or without therapy (controlled hypertension); the absence of severe diseases of the heart, lung, liver, kidney, and other organs; the absence of/or well-controlled diabetes mellitus.

The exclusion criteria were: previous stroke or other severe neurological brain diseases; diagnosis of dementia (the presence of damage to at least two cognitive areas accompanied by everyday functional impairments); decompensated cardiomyopathy; uncontrolled arterial hypertension; the presence of malignant disease; hepatic, renal, or pulmonary insufficiency; uncontrolled diabetes mellitus with hypo- and hyperglycemia, and quarterly glycosylated hemoglobin (HbA1c) above 7%.

Instruments and measures

Cognitive measures

The Montreal Cognitive Assessment (MoCA) [16] is a screening instrument for detecting mild cognitive dysfunction [17]. The MoCA consists of a variety of tasks measuring different cognitive domains: executive functions, attention and concentration, memory, language, visuoconstructional skills, conceptual thinking, calculation, and orientation. The instrument contains the following subtests: alternating trail

making, digit span forward and backward, serial 7 subtraction, learning and delayed recall of the 5-word list; naming, sentence repetition, verbal fluency test, clock drawing test, abstract reasoning, and orientation in time and space. For this research, we used the Serbian version of the MoCA [18]. The administration time is approximately 10 min. The total score is obtained by summing scores for individual items and adding one point for the individuals with 12-year formal education or less, for a possible maximum of 30 points. The final total score of 26 and above is considered normal. The battery demonstrates good psychometric properties and a six-factor structure [19].

Physical measures

Six tests were used in order to comprehensively assess participants' overall physical fitness – five subtests from the Senior Fitness Test (SFT) [20], supplemented by the walking speed test [21]. The tests used in this study serve as indicators of the lower limb muscle strength and upper-body strength and assess overall aerobic endurance, the elasticity of soft tissue, and functional mobility. They indicate whether the person is in the zone of risk of physical incapacity in the near future. The following six physical measures were used: 1) Up and Go test [20] (SFT subtest). The participant's task is to stand up from a chair, walk 2.5 m, and then get back in the sitting position. The score is the time (measured in seconds) needed to perform the task. Performance in this test reflects agility and dynamic balance; 2) Chair Stand Test [20] (SFT subtest). This test is designed for the assessment of lower-limb muscle strength. It requires participants to repeatedly stand up from and sit down on a chair for 30 sec, and the number of stands is recorded; 3) Arm Curl Test [20] (SFT subtest). The test assesses upper-body strength. It requires the participant to repeatedly lift a weight of 3.5 kg for men and 2.5 kg for women for 30 sec. The score is expressed as the number of flexions performed in 30 seconds; 4) Chair Sit and Reach Test [20] (SFT subtest). The test measures lower-body flexibility and elasticity of the soft tissues of the lower extremities. The test is performed from a seated position on a chair with an extended leg and arms reaching towards the toes. The performance is a distance (in cm) that remains between the fingers of the arm and the toes at a maximum reach; 5) Back Scratch Test [20] (SFT subtest). Performance in the test is indicative of upper-body flexibility. The test assesses how close the hands can be brought together behind the back. The score is expressed as the distance (in cm) between the middle fingers of each hand; 6) Walking Speed Test [21]. This test measures the participants' aerobic endurance. The participant is required to walk 4 m, and the time needed to pass the route is measured.

Procedure

The local Ethical Committee of the University Clinical Center Zvezdara approved the study, and all participants provided a signed informed consent before the assessments. The study was conducted in accordance with the Declaration of Helsinki postulates. The research was conducted at the Gerontology Center of Belgrade at Daily Centers and Clubs for Aged People, located in the territory of Belgrade, as well as at the Department of Geriatrics of the Internal Medicine Clinic of the University Clinical Center Zvezdara, Belgrade, where outpatients were tested. Testing procedures were completed in a single day. The test schedule included taking anamnestic data, then the MoCA test, and physical testing at the end.

Results

Sample characteristics

Descriptive statistics related to the participants' age and their sociodemographic structure are presented in Table 1. Male and female subsamples did not differ in terms of their age [t (96) = 0.119, p = 0.119]. Regarding their educational status, only three participants completed primary school, while 47 of them finished secondary school and received a

Table 1

Socio-demographic characteristics of the sample

Variable	Male (n = 16)	Female (n = 82)	Total (n = 98)
Age (years), min-max	65–81	61–85	61–85
(mean ± SD)	(68.63 ± 5.15)	(68.48 ± 4.49)	(68.50 ± 4.57)
Education (n)			
elementary school	0	3	3
high school	4	44	48
higher education	2	12	14
university education	10	23	33
Marital status (n)			
married	14	33	47
single	1	3	4
divorced	1	14	15
widow/er	0	32	32
Financial status (n)			
below average	3	16	19
average	2	30	32
above average	11	36	47

SD – standard deviation; n – number of participants.

university degree. According to marital status, 47 participants were married, while the rest were widowed, divorced, or single. Only 19 participants assessed their financial status as below average.

Cognitive domain

Table 2 presents descriptive statistics for cognitive measures, i.e., MoCA's individual items and total score. Several items have demonstrated relatively low variability, i.e., they proved to be too easy for most of the participants (drawing cube, clock's contour, naming all items, forward digit span, vigilance, orientation, and subtraction 1), and consequentially the majority of these items showed low or non-existent correlations with the overall performance on MoCA. However, the instrument showed satisfactory reliability.

In line with the recommendations given by the MoCA authors and empirical evidence of the instruments, six-factor solution scores for six cognitive domains were calculated: executive functions – derived from the alternating trail making, verbal fluency, and abstraction items; language – derived from the naming, sentence repetition items and verbal fluency; visuospatial skills – calculated from drawing items; memory – derived from delayed recall items; attention, concentration, and working memory – calculated from forward and backward digit spans, vigilance, and subtraction items; and temporal and spatial orientation – derived from the orientation items [16, 19]. Table 3 displays descriptive statistics for scores of the aforementioned cognitive domains.

Due to the high non-normality of scores in the analyses to follow, nonparametric tests were used. The Friedman's test pointed to the differential performances in the tests of six cognitive domains [χ^2 (5) = 249.97, $p < 0.001$]. Namely, *post-hoc* tests (Wilcoxon signed-rank test) (Table 4) revealed that the participants demonstrated the lowest performance in memory in relation to other domains, followed by executive functions, visuospatial skills, and attention, concentration, and working memory domains while compared to others the greatest performance was recorded in temporal and spatial orientation.

Rank correlations between six cognitive domains are presented in Table 5. Executive functions and language correlated most significantly since both subscales include verbal fluency items. Language and visuospatial skills demonstrated a similar pattern of correlations, i.e., both measures correlat-

Table 2

Descriptive statistics for MoCA's individual items and total score

Items	Mean ± SD	Min–Max	*r*
Alternating Trail Making	0.51 ± 0.50	0–1	0.210*
Visuoconstructional Skills (Cube)	0.91 ± 0.29	0–1	0.129
Visuoconstructional Skills (Clock): Contour	0.98 ± 0.14	0–1	0.226*
Visuoconstructional Skills (Clock): Numbers	0.74 ± 0.44	0–1	0.242*
Visuoconstructional Skills (Clock): Hands	0.54 ± 0.50	0–1	0.296**
Naming 1	0.99 ± 0.10	0–1	-0.066
Naming 2	0.94 ± 0.24	0–1	0.184
Naming 3	0.99 ± 0.10	0–1	0.063
Forward Digit Span	0.92 ± 0.28	0–1	0.196
Backward Digit Span	0.76 ± 0.43	0–1	0.359**
Vigilance	0.97 ± 0.17	0–1	0.053
Subtraction 1	0.99 ± 0.10	0–1	0.030
Subtraction 2	0.85 ± 0.36	0–1	0.361**
Subtraction 3	0.84 ± 0.37	0–1	0.351**
Subtraction 4	0.78 ± 0.42	0–1	0.432**
Subtraction 5	0.78 ± 0.42	0–1	0.448**
Sentence repetition 1	0.48 ± 0.50	0–1	0.248*
Sentence repetition 2	0.83 ± 0.38	0–1	0.334**
Verbal fluency	0.70 ± 0.46	0–1	0.336**
Abstraction 1	0.82 ± 0.39	0–1	0.351**
Abstraction 2	0.53 ± 0.50	0–1	0.334**
Delayed recall – word 1	0.46 ± 0.50	0–1	0.384**
Delayed recall– word 2	0.42 ± 0.50	0–1	0.498**
Delayed recall– word 3	0.46 ± 0.50	0–1	0.358**
Delayed recall– word 4	0.46 ± 0.50	0–1	0.274**
Delayed recall– word 5	0.64 ± 0.48	0–1	0.473**
Orientation: date	0.96 ± 0.20	0–1	0.144
Orientation: day	0.97 ± 0.17	0–1	0.203*
Orientation: month	0.96 ± 0.20	0–1	0.258*
Orientation: year	0.99 ± 0.10	0–1	0.095
Orientation: place	0.97 ± 0.17	0–1	0.278**
Orientation: city	0.99 ± 0.10	0–1	0.159
MoCA total score	230.95 ± 30.18	15–30	α = 0.693

MoCA – Montreal Cognitive Assessment; *r* – item-total correlations; α – internal consistency of the instrument (Cronbach's alpha).
***p* < 0.05; *******p* < 0.01.**

ed with executive functions, attention, concentration, and working memory and were not related to the memory domain, while only language correlated with the temporal and spatial orientation. Attention, concentration, and working memory correlated with all other domains, including memory, which, on the other hand, achieved the only significant relation with the aforementioned domain. All six domains correlated with the MoCA total score.

Physical-motor domain

Table 6 displays descriptive statistics for six physical measures used in the study. The distribution of participants' scores appeared to be far from symmetrical and substantially skewed in the Chair Stand test and the Back Scratch test. The Kolmogorov-Smirnov test of normality of distribution of scores differs in the Chair Sit and Reach and the Back Scratch tests.

Table 3

Descriptive statistics for six cognitive domains

Domain	Mean	SD	Min	Max	Sk	Ku	K-S
EF	0.64	0.24	0.25	1	-0.107	-0.951	2.124**
LANG	0.82	0.14	0.50	1	-0.0199	-0.811	2.159**
VSS	0.79	0.19	0.25	1	-0.446	-0.573	2.309**
MEM	0.49	0.31	0.00	1	-0.172	-1.013	1.890**
ACWM	0.86	0.19	0.25	1	-1.258	0.665	2.737**
TSO	0.97	0.09	0.33	1	-4.962	32.549	4.876**

EF – executive functions; LANG – language; VSS – visuospatial skills; MEM – memory; ACWM – attention, concentration, and working memory; TSO – temporal and spatial orientation; SD – standard deviation; Sk – skewness; Ku – kurtosis; K-S – Kolmogorov-Smirnov test of normality of distribution of scores.
***p* < 0.05; ***p* < 0.01.**

Table 4

Differences in performances in six cognitive domains

Domain	LANG	VSS	MEM	ACWM	TSO
EF	-6.919**	-4.996**	-3.792**	-6.566**	-7.845**
LANG		-1.261	-7.481**	-2.670**	-7.120**
VSS			-6.979**	-3.168**	-6.629**
MEM				-7.793**	-8.224**
ACWM					-5.173**

EF – executive functions; LANG – language; VSS – visuospatial skills; MEM – memory; ACWM – attention, concentration, and working memory; TSO – temporal and spatial orientation.
The numbers presented in Table are Wilcoxon signed-rank test statistics.
***p* < 0.05; ***p* < 0.01.**

Table 5

Correlations between six cognitive domains and Montreal Cognitive Assessment (MoCA) total score

Domain	LANG	VSS	MEM	ACWM	TSO	MoCA
EF	0.541**	0.269**	0.144	0.337**	0.246*	0.519**
LANG		0.276**	0.084	0.304**	0.355**	0.480**
VSS			0.151	0.360**	0.016	0.470**
MEM				0.246*	0.129	0.648**
ACWM					0.274**	0.535**
TSO						0.429**

EF – executive functions; LANG – language; VSS – visuospatial skills; MEM – memory; ACWM – attention, concentration, and working memory; TSO – temporal and spatial orientation.
***p* < 0.05; ***p* < 0.01.**

Table 6

Descriptive statistics for physical measures

Test	Mean	SD	Min	Max	Sk	Ku	K-S
Up and Go	9.75	1.57	7	15	0.569	0.423	0.863
Chair Stand	14.57	4.41	8	42	2.579	14.167	1.168
Arm Curl	21.42	5.13	12	39	0.795	1.086	1.125
Chair Sit and Reach	2.74	7.57	-17	20	-0.149	0.430	1.534*
Back Scratch	-2.30	7.40	-25.0	11.0	-1.051	1.108	2.215**
Walking Speed	4.46	0.74	2.51	6.85	0.369	0.504	0.622

SD – standard deviation; Sk – skewness; Ku – kurtosis; K-S – Kolmogorov-Smirnov test of normality of distribution of scores.
***p* < 0.05; ***p* < 0.01.**

Before the correlation analysis, values of the variables for which lower values indicate better performance (time measures) were recorded so that the higher values for all the variables indicate better test performance. The correlation analysis showed that performances in the tests of agility and dynamic balance (Up and Go test), lower- (Chair Stand test) and upper-body strength (Arm Curl test), and aerobic endurance (Walking Speed test) all correlated moderately and positively. On the other hand, the tests of lower- (Chair Sit and Reach test) and upper-body (Back Scratch test) flexibility demonstrated positive intercorrelation, while they did not correlate with strength, agility, and endurance measures. More precisely, the Back Scratch test did not correlate with any other measure, while the Chair Sit and Reach test achieved relatively low correlation only with the Up and Go test (Table 7).

To gain an insight into the latent structure of physical properties, six physical measures were subjected to factor analysis. Factors were extracted using the maximum likelihood method, and the factors were Promax-rotated. Both the Kaiser-Guttman criterion and scree plot suggested the retention of two latent dimensions. Retained factors accounted for 39.03% of the variance of performance in physical tests. The pattern matrix is presented in Table 8.

The first factor is defined by the Walking Speed, Up and Go, Arm Curl, and Chair Stand tests, i.e., aerobic endurance, agility, dynamic balance, and lower- and upper-body strength. The second factor, on the other hand, has appeared to be defined by lower- and upper-body flexibility tests, i.e., Back Scratch and Chair Sit and Reach tests. In line with the primary loadings, the first factor was named physical strength, while the second was named physical flexibility. Two factors were shown to be fairly independent demonstrating a trivial relationship (r = 0.139).

Relationship between cognitive and physical measures

Table 9 displays the correlations between cognitive domains and physical measures.

The executive functions correlated with the physical strength factor mostly due to its relationship with dynamic balance and agility (Up and Go test) and aerobic endurance indicators (Walking Speed test). On the other hand, memory, attention, concentration, and working memory, and temporal and spatial domains were not related to any physical measure. However, language functions correlated with physical flexibility but not with physical strength. Lastly, visuospatial skills demonstrated a negative correlation with the lower-

Table 7

Correlations between six physical measures

Test	Chair Stand Test	Arm Curl Test	Chair Sit and Reach Test	Back Scratch Test	Walking Speed Test
Up and Go Test	0.459**	0.349**	0.225*	0.128	0.531**
Chair Stand Test		0.425**	0.008	-0.121	0.340**
Arm Curl Test			-0.014	-0.067	0.382**
Chair Sit and Reach Test				0.396**	0.105
Back Scratch Test					0.000

***p* < 0.05; ****p* < 0.01.**

Table 8

Pattern matrix for physical measures (Maximum likelihood extraction, Promax rotation)

Test	Factors	
	1	2
Walking Speed	0.717	0.005
Up and Go	0.714	0.217
Arm Curl	0.556	-0.180
Chair Stand	0.346	-0.129
Back Scratch	-0.178	0.678
Chair Sit and Reach	0.055	0.565

Table 9

Correlations between cognitive domains and physical measures and domains

Domain	Up and Go test	Chair Stand test	Arm Curl test	Chair Sit and Reach test	Back Scratch test	Walking Speed test	Physical strength	Physical flexibility
EF	0.217*	-0.013	0.155	0.069	-0.026	0.229*	0.255*	0.058
LANG	0.073	-0.104	-0.021	0.131	0.191	0.047	0.048	0.223*
VSS	0.059	-0.239*	-0.117	0.083	0.109	0.146	0.047	0.147
MEM	0.076	-0.088	-0.057	0.098	0.046	0.034	0.040	0.107
ACWM	0.107	0.007	0.171	0.034	0.023	0.175	0.160	0.044
TSO	-0.015	-0.097	-0.043	0.107	-0.036	-0.075	-0.030	0.046
MoCA	0.125	-0.102	-0.030	0.253*	0.152	0.109	0.127	0.261**

EF – executive functions; LANG – language; VSS – visuospatial skills; MEM – memory; ACWM – attention, concentration, and working memory; TSO – temporal and spatial orientation; MoCA – Montreal Cognitive Assessment total score.

***p* < 0.05; ****p* < 0.01.**

body muscle strength measure (Chair Stand test), and this domain did not correlate with any other physical measure.

Discussion

This study compared data obtained by cognitive and motor tests' performance in individuals over 60 years of age in various levels of physical fitness. Our sample had a substantial predominance of females, thus, sex differences are difficult to assess due to a small number of male participants. Moreover, the predominance of female participants could affect the predictions due to potentially different test results in females and males.

Cognition assessed with the MoCA showed that several items have relatively low variability, i.e., they proved to be too easy for most of the participants (for example, drawing a cube, clock's contour, naming all the items, forward digit span, etc., that show the "ceiling" effect). Vocabulary and forward span are known to be relatively resistant to age-related decline [17]. Statistical analysis showed differences in six MoCA cognitive domains [16, 19] with high significance. Namely, the participants demonstrated the worst performance in memory relative to other domains, followed by the executive functions, visuospatial skills and attention, concentration, and working memory domains, while having the finest performance in temporal and spatial orientation in relation to other domains. Memory is prone to age changes, as it is affected by multiple processes, including speed, working memory, executive functions, and sensory decline. Generally, it is a very demanding cognitive process. According to the compensation-related utilization of neural circuits hypothesis, while performing tasks under lower cognitive demands, older adults engage greater volumes of cortical tissue compared to younger adults, which aids in successful performance [22]. However, under higher demands, older adults have already exhausted their compensatory circuits and reached a resource ceiling, resulting in poorer task performance. In contrast to this, younger adults are able to engage these compensatory circuits to meet the increased cognitive demands [22]. Failures in retrieving previously learned material are an important feature of memory aging [1]. However, some studies report that not all forms of human memory are equally affected by the advancing age [23]. Declarative memory domains such as semantic and episodic memory are differently affected by aging [24]. These findings follow the known vulnerability of recent declarative memory in aging that has several scenarios of decline [25]. While positive age gradients have been found for semantic memory [26], episodic memory is considered to be the form of long-term memory that displays the largest degree of age-related decline [27]. The opinion that implicit memory remains stable during normal aging is widely accepted, but some studies report that priming, as an indicator of implicit memory in older adults, is significantly reduced compared to young adults [28]. Compared to other cognitive tests such as the Mini-Mental State Examination, the MoCA presents a higher episodic memory demand (five words instead of three) [29]. In the study conducted by Cecato et al. [30], the authors found that episodic memory (word recall) was one of the subtests that discriminated participants with mild cognitive impairment (MCI) against cognitively healthy subjects.

The next worst performance was in the executive functions subtest. Executive functions are operations that include task definition, planning, execution, monitoring, updating, mental set-shifting, and the inhibition of prepotent responses, as well as the verification of accomplishment [31]. Age-related differences are observed in the tasks that rely heavily on executive functions in goal maintenance. Compared to younger adults, older adults have more difficulty as the number of relations that must be integrated while performing reasoning tasks increases [1]. Some studies suggest that brain aging affects executive functions through reductions of structural and functional connectivity [32]. In addition, many theoretical frameworks of cognitive aging emphasize the age vulnerability of the prefrontal cortex and its connections with the basal ganglia, especially the *striatum* [33]. One should always be cautious in interpreting cognitive tests as they have common, domain-specific, and test-specific factors [34].

In our subjects, all tests done for physical fitness, i.e., agility and dynamic balance (Up and Go test), lower body strength (Chair Stand test), upper body strength (Arm Curl test), and aerobic endurance (Walking Speed test) correlated moderately and positively. This is in line with the conclusions of the population-based cross-sectional study in Madeira, Portugal, that strength, flexibility, and especially aerobic endurance are crucial for maintaining or improving balance and mobility [35]. Thereby, it seems to be an indicator of a common factor. Statistical analysis showed two factors. The first one was defined by the Walking Speed, Up and Go, Arm Curl, and Chair Stand tests, i.e., aerobic endurance, agility, dynamic balance, lower- and upper-body strength, and the second factor was defined by lower- and upper-body flexibility tests, i.e., Back Scratch and Chair Sit and Reach tests. The first factor was named physical strength and the second physical flexibility.

Our results show that physical strength is an indicator of the lower-extremity function, which is consistent with findings that patients with MCI and Alzheimer's disease have slower walking speed if compared with cognitively healthy persons [36]. Another study conducted among community-dwelling adults indicated that slower the Timed Up and Go test (TUG) time is independently associated with poorer performance in global cognition, executive function, and memory tests and slower processing speed. This highlights that the TUG is more than just a simple mobility task [37]. Another study designed to determine normative values of the TUG in community-dwelling older adults based on cognitive status, gender, and age groups, showed that, generally, older adults with diagnosed MCI took a longer time to accomplish TUG [38]. There are suggestions that differences in lower-extremity function across the cognitive aging spectrum may be explained by atrophy of a neural network, including the dorsolateral prefrontal cortex, cingulate gyrus, parietal association areas, basal ganglia, and medial temporal lobes, particularly the hippocampus [39].

We found that physical fitness correlated with cognitive abilities in the domain of executive functions. The physical strength factors were connected with executive functions. Our findings are in line with the results of a North American study that recruited 56 older adults (60+ years) that found an association between mobility and multiple executive function processes [40]. Higher mobility and physical ability are desired for maintaining executive function capability. In the above-mentioned study, mobility was assessed via gait speed, TUG, chair stand, and as a composite physical performance score, and executive functions were assessed with the Trail Making Test, semantic fluency, and phonemic fluency [40]. The connection and correlation between executive functions and walking performances could be explained by the imaging study that showed an association between higher activity in brain regions involved in complex cognitive functions (including the prefrontal cortex and the hippocampus) and increasing complexity of gait [14].

Executive functions in our research correlated with physical strength mostly due to its relationship with dynamic balance and agility (Up and Go test) and walking speed. This can be explained by suggestions about the shared neural basis for fast-paced walking and executive functions in older adults without dementia [41].

In our analysis, both executive functions and language domains included phonemic fluency testing. Verbal fluency in phonemic format is mainly dorsolateral prefrontal function and hence more vulnerable than naming, which is widely spread in the dominant cortex [31]. Language and visuospatial skills correlated with the executive functions, attention, concentration, and working memory and were not related to the memory domain. Only language correlated with temporal and spatial orientation domains. According to the number of authors, a connection between orientation and language can be established. The hippocampus may be responsible for scene construction, drawing on information stored in many regions of the brain but allowing for vivid mental construction and reconstruction of events [42]. This structure contains the so-called “grid cells”, which have spatial functions. Grid cells modulate different levels of spatial resolution, from more detailed to a broader view. This function allows zooming, enabling us to locate ourselves in the surrounding environment [43]. There is a possibility that the generative and recursive nature of language is derived from spatiotemporal imagination. Generativity, defined as a self-contained system from which users draw an independent ability to create, generate, implement or produce new content unique to that system without additional help or input from the system’s original creators, in turn, is grounded in the hippocampal mechanisms for establishing the awareness of location and orientation in space. The generativity of language, then, is not so much a property of the language itself as of the underlying thoughts that we use to convey language [43]. Besides, the findings of Piai et al. [44] reveal that the hippocampal complex contributes to language in an active fashion, relating incoming words to stored semantic knowledge, a necessary process in generating the meaning of a sentence [44]. Linguistic abilities are mainly stable in time and can be considered a measure of premorbid intellectual level [45]. Attention, concentration, and working memory correlated with all other domains, including the memory domain. This is probably because working memory is a system that allows temporary storage and manipulation of information during cognitive tasks and has been linked to activations in many neuroanatomical locations, including frontoparietal networks, occipital cortices, and the cerebellum [46]. This system plays a central role in the human ability to complete a range of everyday activities such as mathematical problem solving, workplace performance, and some other vital activities [47]. Attentional abilities are the general factor included in all other neuropsychological functions, so these findings are to be expected as many complex neuropsychological tests include executive functioning among other factors [17].

The executive functions of the MoCA domain correlated with the physical strength factor. Memory, attention, concentration, and working memory, and temporal and spatial domains were not related to any of the physical measures. These higher cortical functions mostly rely on attentional factors [45]. Language functions domain correlated with physical flexibility but not with physical strength. Lastly, the visuospatial skills domain showed a negative correlation with lower-body muscle strength measures (Chair Stand test) and was independent of other physical measures. A possible connection between visuospatial skills and lower-body muscle strength can be found through testosterone action. Some findings are suggesting that testosterone supplementation improved the strength in the elderly [48] and that there was a positive influence of testosterone on visuospatial skills in the elderly [49]. However, these findings are not in accordance with ours. It is difficult to interpret these findings, but it accentuates the need to explore various effects of physical training on different cognitive abilities.

A comparative study with similar measures to ours was conducted in Japan on 1,552 cognitively non-impaired older participants [50]. They used the Japanese version of the MoCA and handgrip strength, leg strength, sit-to-stand rate, gait speed, and one-leg stand time as physical fitness measures. Each of these five physical fitness measures was positively associated with the MoCA score in multiple linear regression analyses. This study did not include the correlation of subsets of neuropsychological functions and measures of physical fitness.

Our findings underline the positive correlation between physical fitness and cognitive level in the elderly. The latest longitudinal study in Sweden, with a span of 44 years, found that in a population-based sample of women exercising during midlife dementia, the risk was reduced by nearly 90% [51]. High-level fitness contributed more than mid- or low-level fitness. This study and some previous ones pave the way for the development of vascular risk factors control through exercise and other means [52]. Another issue is the immediate effect of fitness on the cognitive level. A specially developed cognitive enhancement fitness program in one session exerted measurable effects on short-term memory (forward digit/word span test) and serum levels of brain-derived neurotrophic factor (BDNF) in healthy middle-aged women [53].

Limitations

A possible limitation of this study is uneven gender representation in our sample. There was limited availability of male elderly subjects who exercise, so the intersex comparison could not have been done, and the results might be biased.

Conclusion

Our study clearly showed the connections between physical fitness (motor performances) and cognitive functions in elderly subjects, especially those of executive type, and emphasizes the need for larger involvement of the general population in physical activities. Further research should be conducted in the form of large population studies on both males and females, encroaching on multiple risk factors and health-enhancing measures. Clinicians need to consider the association between executive function and physical performance when aiming at functional improvement in the elderly population.

Conflict of interest

No potential conflict of interest was reported by the authors.

Acknowledgement

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Gastrointestinal symptoms in COVID-19 patients

Gastrointestinalni simptomi bolesnika sa COVID-19

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Abstract

Background/Aim. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a global threat and a huge problem for our community. There are so many open questions. The aim of this study was to establish the frequency of gastrointestinal (GI) symptoms in hospitalized patients with infection caused by this virus (coronavirus disease-19 – COVID-19), but also to compare if patients with GI symptoms have a higher computed tomography (CT) scan severity score of interstitial pneumonia (IP) compared to patients with COVID-19 without GI symptoms. **Methods**. Our database comprised 322 patients with COVID-19 who were divided into two groups, patients with and without GI symptoms. All information was taken from anamnestic data and patients' history, followed by statistical analysis. **Results.** Thorax CT scans of 206 patients (63.9%) were described as bilateral IP, of which 76 CT scans (36.9%) were described by radiologists as the peak of infection. Moreover, 130 patients (40.4%) had GI symptoms, and even 58 out of 130 patients (44.6%) reported GI symptoms as the first manifestation of COVID-19 infection. The most commonly reported one was the lack of appetite (73 patients or 56.15%). Furthermore, 65 (50%) patients reported diarrhea, 25 (19.2%) patients reported nausea and vomiting, and 9 (6.9%) patients reported abdominal pain. In addition, among patients with bilateral IP and GI tract symptoms, 31 (40.79%) of them did not have a higher CT scan severity score at the peak of the disease compared to the patients without GI symptoms (45 of them or 59.2%), (p = 0.704). **Conclusion.** GI symptoms often are the first manifestation of COVID-19. Therefore, every patient with newly formed digestive tract symptoms should be tested for COVID-19. On the other hand, GI symptoms do not indicate COVID-19 patients will have a severe form of IP.

Key words:
covid-19; diagnosis; diarrhea; feeding and eating disorders; sars-cov-2; severity of illness index; signs and symptoms, digestive; tomography, x-ray computed.

Apstrakt

Uvod/Cilj. Teški akutni respiratorni sindrom korona virus 2 (SARS-CoV-2) je globalni problem i pretnja, i još uvek ima puno otvorenih pitanja. Cilj ispitivanja bio je da se utvrdi učestalost gastrointestinalnih (GI) simptoma kod hospitalizovanih bolesnika sa infekcijom prouzrokovanom SARS-CoV-2 (*coronavirus disease*-19 – COVID-19), kao i da se utvrdi da li ti bolesnici imaju viši skor intersticijalne pneumonije (IP) na pregledu pluća kompjuterizovanom tomografijom (KT) u odnosu na bolesnike sa COVID-19 bez GI tegoba. **Metode.** Ispitivanje je obuhvatilo 322 hospitalizovana bolesnika sa COVID-19 koji su bili podeljeni u dve grupe: grupu sa GI simptomima i grupu bez GI simptoma. Svi podaci su dobijeni anamnestički i iz istorije bolesti bolesnika, a nakon toga su statistički obrađeni. **Rezultati**. Od 322 bolesnika, 206 (63.9%) je imalo opisanu obostranu IP na KT pregledu grudnog koša, a 76 bolesnika (36.9%) imalo je opisanu IP u vrhuncu bolesti. Takođe, 130 bolesnika (40.4%) prijavilo je GI simptome, čak 58 od 130 bolesnika (44.6%) je anamnestički navelo da su GI simptomi bili prva manifestacija COVID-19 infekcije. Najčešći simptom bio je gubitak apetita [kod 73 bolesnika (56.15%)]. Od 130 bolesnika sa GI simptomima, 65 (50%) je prijavilo dijareju, 25 (19.23%) mučninu i povraćanje, a 9 (6.9%) bolesnika prijavilo je bol u trbuhu. Bolesnici sa obostranom IP i GI simptomima [31 (40.8%)] na vrhuncu bolesti nisu imali veću zahvaćenost plućnog parenhima IP na pregledu grudnog koša KT, u poređenju sa bolesnicima bez GI simptoma [45 (59.21%)], (p = 0.704). **Zaključak**. GI simptomi su često prva manifestacija COVID-19. Stoga,

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kod svih bolesnika sa novonastalim digestivnim simptomima treba proveriti postojanje COVID-19. S druge strane, prisustvo GI simptoma ne ukazuje na to da će bolesnici sa COVID-19 imati teži oblik IP.

Ključne reči:
covid-19; dijagnoza; dijareja; ishrana, poremećaji; sars-cov-2; bolest, indeks težine; znaci i simptomi, digestivni; tomografija, kompjuterizovana, rendgenska.

Introduction

As it is known, the first human cases of coronavirus disease-19 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were observed in December 2019 in Wuhan, a city in China [1]. The causative agent of those types of pneumonia was investigated, and it was discovered that there was a new type of human-infecting Betacoronavirus named SARS-CoV-2 [2]. The Chinese government declared that the outbreak of the SARS-CoV-2 virus first started in the Huanan seafood market in Wuhan [3]. After only one month, the World Health Organization (WHO) announced that SARS-CoV-2 became a Public Health Emergency of International Concern [4]. Now, it is obvious that this outbreak caused the 21st-century pandemic [5]. As COVID-19 typically causes viral pneumonia, it is easy to conclude that the route of transmission is mainly through contact with respiratory droplets. The most commonly reported symptoms of COVID-19 were fever, cough, and fatigue, as this virus predominantly attacks the respiratory tract of the human host [6]. Other less common but equally clinically important symptoms include gastrointestinal (GI) tract symptoms such as diarrhea, abdominal pain, vomiting, and loss of appetite [6]. In radiology, the computed tomography (CT) scan stage of interstitial pneumonia (IP), described as the peak of infection, is defined by "consolidation, which refers to an area of homogeneous increase in lung parenchymal attenuation that obscures the margins of vessels and airway walls" [7].

We conducted a retrospective cohort analysis of GI symptoms seen in COVID-19 patients and compared whether those symptoms correlate with the severity of the disease.

Methods

This study was carried out at the University Hospital Medical Center "Bežanijska Kosa", in Belgrade, Serbia, including 324 patients hospitalized either with IP or with positive reverse transcription-polymerase chain reaction (RT-PCR) test on SARS-CoV-2, in the COVID-19 unit, from June 2020 to August 2020. Two patients were excluded because of missing data, making the total number of patients in our database 322. The database contains patients' age, dates of admission to the hospital, dates of hospital discharge, RT-PCR test results of SARS-CoV-2 and SARS-CoV-2 serology testing, oxygen support, CT scan severity scores (0 to 25), CT scan stage of the disease, and GI symptoms. Data on comorbidities were also added [arterial hypertension (AH), diabetes mellitus (DM) type 2, asthma and chronic obstructive pulmonary disease, and history of malignant diseases]. Weight and height were obtained from 101 patients for calculating and calculated to body mass index (BMI). The presence of GI symptoms was obtained on admission, and it included diarrhea, nausea/vomiting, abdominal pain, and lack of appetite. All the information was taken from the patient's history (electronic medical records) and anamnestic data, from admission through discharge.

Statistical analysis was performed using Python 3.7, and descriptive statistics were used to analyze the data. Categorical variables were expressed as counts with proportions. Patients were divided into groups depending on the presentation of GI symptoms and their respective peak CT scan severity scores, including also their age and BMI, which were compared using the *t*-test, while the χ^2 test was used for correlation between gender and the presence of GI symptoms (the level of significance was $p = 0.05$). Further, the Spearman's correlation was used to compare patients' BMI and their respective CT scan severity score of IP at the peak of the disease (the level of significance was $p = 0.05$).

Ethical approval for the study was obtained from the Ethics Committee of the University Hospital Medical Center "Bežanijska Kosa" (approval number 5197/2, from 19.08.2020).

Results

The study included 322 patients; the youngest was 21 years old, while the oldest was 86 (Figure 1). The average patient age was 57. Total hospital stay length ranged from 2 to 56 days, with an average length of stay of approximately 12 days. The BMI of 101 patients was in the range between

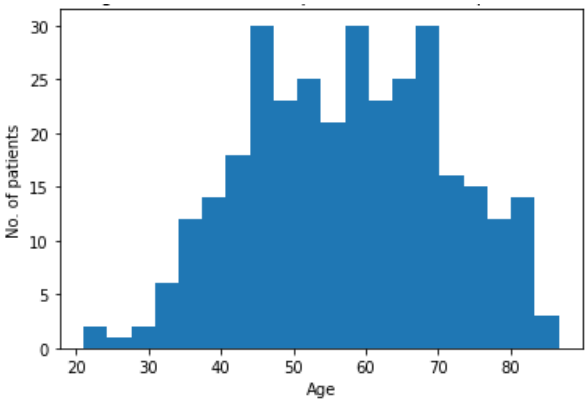

Fig. 1 – Age (years) distribution of coronavirus disease-19 (COVID-19) patients included in this study.

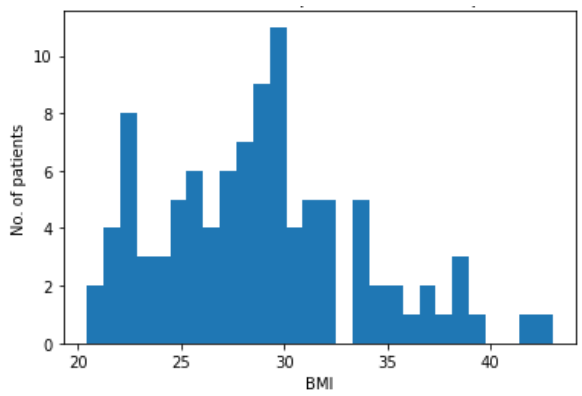

Fig. 2 – Body mass index [BMI (kg/m^2)] distribution of coronavirus disease-19 (COVID-19) patients included in this study.

20 kg/m^2 and 42 kg/m^2, with the average value being 28 kg/m^2 (Figure 2). RT-PCR SARS-CoV-2 test was done in 318 out of 322 patients (98.75%); 214 patients had a positive test result (67.3%). During the hospital stay, additional serological testing was performed: 67 patients (20.8%) were tested for IgM and IgG SARS-CoV-2 antibodies, 19 (28.35%) of them had both IgM and IgG antibodies, 1 of them had only IgM antibodies, 16 (23.9%) of them had IgG antibodies, and 31 (46.3%) had no antibodies. Furthermore, 15 out of 322 patients (4.65%) tested negative with RT-PCR SARS CoV-2 but had positive serology. Chest CT scans of 206 patients (63.9%) were described as bilateral IP (Figure 3), out of which 76 CT scans (36.9%) were described by radiologists as the peak of infection. The oxygen supply was required in 240 patients (74.5%). As mentioned above, data on comorbidities were also included in this database. In addition, 156 patients (48.4%) had AH, 22 patients (6.8%) had asthma or chronic obstructive pulmonary disease, 16 (4.9%) had DM type 2, and 48 patients (14.9%) had a history of malignant diseases. Moreover, 130 patients (40.4%) had GI symptoms, and even 58 out of 130 patients (44.6%) reported GI symptoms as the first manifestation of COVID-19. The most commonly reported one was the lack of appetite (73 patients or 56.15%). Furthermore, 65 out of 130 patients (50%) reported diarrhea, 25 out of 130 patients (19.2%) reported nausea and vomiting, and 9 out of 130 patients (6.9%) reported abdominal pain. Additionally, lack of appetite was reported in 73 of our patients (22.7%), while out of 322 patients, 65 (20.2%) reported diarrhea, 25 (7.8%) reported nausea and vomiting, and 9 (2.8%) reported abdominal pain. In addition, we tested the correlation between age, gender, and BMI and the presence of GI tract symptoms and concluded there was no correlation (p = 0.947, p = 0.187, and p = 0.321, respectively). We also tested the correlation of CT scan severity score of bilateral IP at the peak of infection in patients with (31 of them or 40.8%) and without (45 of them or 59.2%) GI tract symptoms (Figure 4). We concluded that there is no statistically significant difference between those two groups. Namely, patients with bilateral IP and GI tract symptoms did not have a higher CT scan severity score at the peak of the disease compared to the patients without GI symptoms (p = 0.704). In addition, the correlation between CT scan severity score of bilateral IP at the peak of infection and BMI was tested, but there was no correlation (p = 0.796).

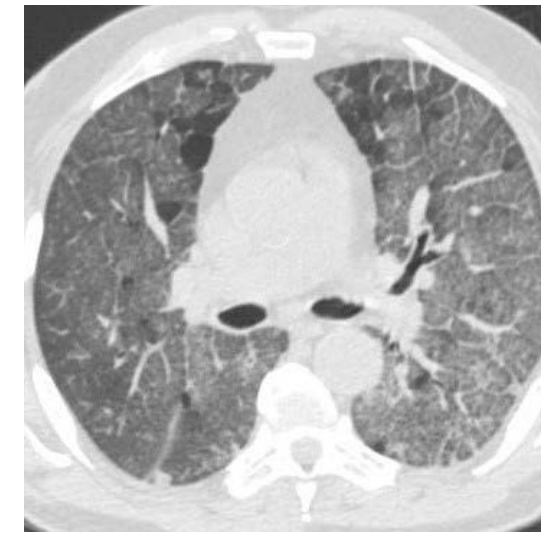

Fig. 3 – Computed tomography (CT) scan of bilateral interstitial pneumonia (CT scan severity score 25).

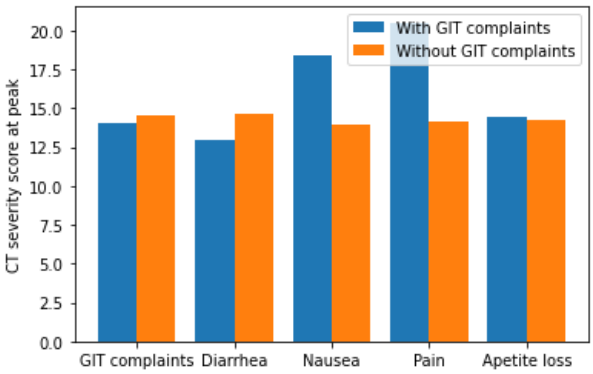

Fig. 4 – Interstitial pneumonia computed tomography (CT) scan severity scores at peak of the disease in coronavirus disease-19 (COVID-19) patients with and without gastrointestinal tract (GIT) symptoms.

Discussion

The age of patients with COVID-19 infection in our study was between 21 and 86 compared to studies in which the median age of patients was between 34 and 59 [8, 9]. The result we obtained had a strong background. Advanced age has been clearly identified as a major risk factor for the development of severe COVID-19 that requires hospital treatment [10]. On the other hand, there was no correlation between a patient's age or gender and the presence of GI symptoms in our study (p = 0.947, p = 0.187, respectively).

Furthermore, this viral infection will more likely infect patients with comorbidities, especially cardiovascular diseases (CD) and DM [11]. It is obvious that there is, as well, a correlation between obesity and COVID-19 disease severity and mortality. As already mentioned, patients with certain comorbidities will have a more severe clinical

manifestation of this viral infection, and obese patients are more prone to CD and DM type 2. In obese patients, excessive adipose tissue stimulates adipocytes to release pro-inflammatory mediators predisposing the body to a pro-inflammatory state and oxidative stress [12]. One more reason for obese patients to have the severe form of COVID-19 is that angiotensin-converting enzyme 2 (ACE2) is expressed in adipocytes [13]. It has been shown that the receptor for ACE2 is crucial for the virus to enter a cell [14]. In our study, there was no correlation between BMI and the presence of GI tract symptoms (p = 0.321), even though BMI ranged from 20 kg/m^2 to 42 kg/m^2, with an average BMI of 28 kg/m^2, and it is found in the literature that BMI equal or higher than 25 kg/m^2 [15] is considered overweight. In comparison to one study in the United States of America (USA), the most common comorbidities were AH, obesity, and DM [16]. In our study, AH is the leader of comorbidities as 48.4% of our patients had it, and there were only 4.9% of patients with DM type 2.

Compared to a study that concluded that approximately 75% of patients had bilateral pneumonia, our result of 63.9% of patients with bilateral IP was almost equal [11]. Moreover, radiology findings depended on the patients' age, disease progression, immunity status, patients' comorbidities, and initial management of disease presentation [17]. In some radiology studies, pneumonia seen on CT scans of patients with COVID-19 infection was described as unusual. All of them showed multifocal patchy "ground-glass" opacities on the periphery of the lungs [18]. In other words, a typical CT scan finding is bilateral IP with "ground glass" opacities and consolidation [8, 19]. By reviewing the literature, we confirmed exactly what we had concluded during our COVID-19 clinical practice. After 0 to 2 days of onset of the disease symptoms, 56% of patients had a normal CT scan finding [20]; afterward, the lesions on the CT scan gradually progressed as an extension of the density of lung opacities [21]. However, chest CT scans are very important in diagnosing COVID-19 as a patient can have a nasopharyngeal swab test for SARS-CoV-2 negative by PCR test, but CT scanning could detect bilateral COVID-19 IP even if the patient did not have clinical symptoms [22].

One study reviewed 15 articles on GI symptoms in COVID-19. The study concluded that the frequency of GI symptoms varied from 3% to 39.6% [14]. Like every other virus infections, this one can also have different clinical manifestations. First of all, we need to emphasize that a significant number of patients in our study had GI symptoms as the first manifestation of COVID-19 (44.6%), so every physician must suspect COVID-19 when a patient at risk presents with only GI symptoms. In all reviewed articles, the predominantly reported symptom was diarrhea [14], opposite to our result that showed lack of appetite as the most frequent one. On the other hand, two doctors from Wuhan got the same result as in this study, with 81 out of 103 patients (79%) with a lack of appetite, following diarrhea, vomiting, and abdominal pain, respectively [23]. Furthermore, there is a report of COVID-19 hospitalized patients in Wuhan, with 3% of them having diarrhea and all of them with a mild clinical picture. In other words, none of the patients needed intensive care [8]. Another report indicated a higher prevalence of diarrhea in patients hospitalized in intensive care units [24]. In the same study, nausea was reported in 10%, vomiting in 4%, and abdominal pain in 2% of the patients [24]. They also reported that patients hospitalized in intensive care units are more likely to have abdominal pain than patients hospitalized in non-intensive care units [24]. This also cannot be correlated because all of our patients were hospitalized in a non-intensive care unit. Although in our study, out of all GI symptoms, the most prevalent was the lack of appetite, diarrhea was also not negligible because 50% of patients reported it. This is a similar result as in one report from the USA, specifically New York, stating that mild diarrhea could reach a prevalence of more than 50% of patients admitted with COVID-19 [5].

We come to a question – how does SARS-CoV-2 infect the GI tract, causing mainly diarrhea? Angiotensin-converting enzyme 2 (ACE2) receptor is crucial for the virus to enter a cell [14]. These receptors are identified in alveolar cells type 2 in the lungs but also in the glandular cells of the stomach and epithelial cells (enterocytes) in the ileum and colon [14, 25, 26]. When SARS-CoV-2 enters the GI tract, specifically enterocytes, it leads to malabsorption, unbalanced intestinal secretion, and activated enteric nervous system, which results in diarrhea [25]. It must be mentioned that the same mechanism for entering GI cells was seen in the previous SARS-CoV epidemic, more precisely via the ACE2 receptor [27].

Furthermore, there is a study about the correlation between GI tract symptoms and severity of the disease, and it shows that almost 23% of patients with GI tract symptoms had a severe clinical picture, and there was also a statistically significant number of patients with GI tract symptoms that needed mechanical ventilation [28]. Nevertheless, there was no difference in inflammatory markers in COVID-19 patients with and without GI tract symptoms [28].

Despite all of these presented facts, we did not found any correlation between radiological findings and GI symptoms, nor between radiological findings and BMI. As we already mentioned, a chest CT scan was done on 206 patients, and radiologists described them with CT scan severity score and stage. CT scan severity score range was 0 to 25, and stages were initial, progressive, peak, late, and resolution. Most of our patients, more precisely 76 of them, were at the peak of bilateral IP with a wide range of CT scan severity scores during hospitalization. Thus, we tested the correlation of CT scan severity score of bilateral IP at the peak in patients with and without GI tract symptoms. We concluded that there was no statistically significant difference between those two groups of patients. In other words, patients with bilateral IP and GI tract symptoms did not have a higher CT scan severity score at the peak of the disease compared to the patients without GI symptoms (p = 0.704). The average CT scan severity score at the peak of the disease in patients with GI symptoms was 14, and in patients without GI symptoms was 14.53. Although we found no difference between radiological findings and GI symptoms,

there is a study about the correlation in patients with and without diarrhea. They also did not find any difference in the laboratory and radiological findings, but they indicated COVID-19 patients with diarrhea had headaches, fatigue, cough, nausea, and vomiting more frequently than patients without diarrhea [29]. On the other hand, it was expected that the obese patients would have a more severe CT scan score of bilateral IP at the peak of the disease, but the results were different. We found no correlation between BMI and radiological findings on a CT scan of the thorax at the peak of the disease ($p = 0.796$).

In all reviewed literature, as well as in this study, all data about GI symptoms are taken from anamnestic data from patients that were hospitalized. Because of that, there is a gap because patients with a mild clinical picture that were not hospitalized were not included and could influence the results [30]. Furthermore, one study concluded that patients without GI symptoms were with a milder clinical picture, and they spent fewer days in hospital than those with GI symptoms. They believe this is due to viral replication in the GI tract causing a more severe clinical picture or because patients with GI symptoms get respiratory symptoms later on and are admitted to a hospital afterward [31]. Discussing viral replication in the GI tract, it is important to emphasize the potential route of transmission through the GI tract (the fecal-oral route). Therewith, stool samples are not normally used for the diagnosis of COVID-19. The priority is still the nasopharyngeal swab which undergoes RT-PCR. It is now known that SARS-CoV-2 RNA is present in patients' stool [5]. Moreover, it is reported that stool samples remained positive for SARS-CoV-2 RNA longer compared to nasopharyngeal swabs [32]. Furthermore, one report showed a COVID-19 patient with more than one nasopharyngeal swab tested negative for SARS-CoV-2 but still positive for SARS-CoV-2 in stool [33]. Compared to previous SARS studies, when the SARS-CoV epidemic started, 16–73% of patients had diarrhea, usually within the first week after the infection [34]. SARS-CoV could also be found in the stool, but active viral replications were shown in histopathological biopsy findings of both small and large intestines as well [35]. Knowing these facts, there is, obviously, a way for fecal-oral transmission of this virus, but also, this mode of transmission may occur after viral clearance from the respiratory tract [22]. If this is true, then the nasopharyngeal swab should be replaced with a rectal swab for testing on SARS-CoV-2 before hospital discharge [36, 37].

The autopsies are crucial for confirming the relationship between COVID-19 and GI tract. There is an autopsy report about an elderly man with COVID-19 infection, and the autopsy revealed segmental dilatation and stenosis in the small intestine, but there is no proof that these are mainly due to COVID-19 infection [25].

Conclusion

Digestive symptoms are often seen as the first manifestation of SARS-CoV-2 infection. Therefore, every patient with newly formed GI tract symptoms, especially the immunocompromised patients, should be tested for COVID-19 in the context of the ongoing global pandemic. As it is known, COVID-19 is a systemic disease, and GI symptoms seen in this infection ratify this fact. On the other hand, GI symptoms do not indicate the patient will have a severe form of IP.

Conflict of interest

The authors declare no conflict of interest.

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Mortality of COVID-19 pneumonia during anticancer treatment in lung cancer patients

Stopa mortaliteta od COVID-19 pneumonije u toku onkološkog lečenja bolesnika sa karcinomom bronha

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Abstract

Background/Aim. The coronavirus disease 2019 (COVID-19) pandemic has multiple impacts on the management of cancer patients. Treatment of malignancies, including chemotherapy, targeted therapy, immunotherapy, and radiotherapy, can suppress the immune system and lead to the development of severe complications of COVID-19. The aim of this study was to determine the mortality of lung cancer (LC) patients in whom the COVID-19 was confirmed during active antitumor treatment. **Methods.** This retrospective study was conducted at the Institute for Pulmonary Diseases of Vojvodina, Sremska Kamenica, Serbia. All patients included in the study underwent active anticancer treatment at the time of diagnosis of COVID-19. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection was determined by a polymerase chain reaction (PCR) test. Patient data were collected using the institutional database and the observed period was from November 20, 2020, to June 5, 2021. Statistical analysis of the derived patient data used multivariate and univariate testing. **Results.** Out of 828 observed COVID-19 hospitalized patients, 81 were LC patients on active antitumor treatment. Patients were predominantly male (67.9%), smokers (55.6%), and with an average age of 66.5 years (range 43–83). The majority of patients (50.6%) had the Eastern Cooperative Oncology Group Performance Status (ECOG PS) 1, and 83.9% had at least one comorbidity. The most common comorbidities were arterial hypertension (66.7%), chronic obstructive pulmonary disease (COPD) (28.4%), and diabetes mellitus (21%). Obesity, congestive heart failure, and other cardiovascular diseases were present in 11%, 6.2%, and 7.4% of patients, respectively. The most common was adenocarcinoma (33.3%), followed by squamous (30.9%) and small-cell LC (24.7%). Predominantly, 63% of the patients were in stage III of the disease, and 33.3% were in stage IV. Metastases were most commonly present in the contralateral lung/pleura (14.8%), brain (6.2%), bone (3.7%), and liver (3.7%). Systemic anticancer therapy was applied in 37 out of 81 patients (45.6%), chest radiotherapy in 35 (43.2%), concurrent chemoradiotherapy in 1 (1.2%), and other types of radiotherapy in 8 (9.87%) patients. The most common forms of systemic therapy were chemotherapy (35.8%), immunotherapy (7.4%), and targeted therapy (2.4%). The most common chemotherapy was a cisplatin-based regiment applied in 34.6% of patients. The mortality from COVID-19 was 19.8%. The statistical significance in relation to the type of treatment was not observed. Statistical significance was observed between mortality and the ECOG PS ($p = 0.011$). **Conclusion.** LC patients are dependent on antitumor treatment and, at the same time, highly susceptible to potential infection. In this study, we did not find statistically significant differences in mortality related to the type of antitumor treatment in COVID-19 positive LC patients. Further detailed research on a larger scale is needed in order to explore the effects of SARS-CoV-2 on cancer patients. All possible methods of protection against SARS-CoV-2 virus should be performed in order to minimize the risk of infection in all but especially in immunocompromised cancer patients.

Key words:
antineoplastic agents; comorbidity; covid-19; lung neoplasms; mortality; neoplasms staging.

Apstrakt

Uvod/Cilj. Pandemija *coronavirus disease* 2019 (COVID-19) ima višestruki uticaj na lečenje bolesnika obolelih od karcinoma. Lečenje malignih bolesti, uključujući hemioterapiju, ciljanu terapiju, imunoterapiju i radioterapiju može delovati supresivno na imunski sistem i dovesti do razvoja teških komplikacija COVID-19. Cilj ovog rada bio je da se utvrdi mortalitet bolesnika sa karcinomom bronha, kod kojih je prisustvo COVID-19 potvrđeno tokom aktivnog antitumorskog lečenja. **Metode.** Ova retrospektivna studija je sprovedena na Institutu za plućne bolesti Vojvodine u

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Sremskoj Kamenici, Srbija. Kod svih bolesnika sa karcinomom bronha obuhvaćenih studijom, u toku aktivnog antitumorskog lečenja je potvrđeno prisustvo COVID-19, testiranjem nazofaringealnog brisa lančanom reakcijom polimeraze. Podaci o bolesnicima prikupljeni su korišćenjem institucionalne baze podataka za period od 20. novembra 2020. do 5. juna 2021. Statistička analiza podataka je urađena korišćenjem multivarijatnog i univarijatnog testiranja. **Rezultati.** Od ukupno 828 hospitalizovanih bolesnika sa COVID-19, bilo je 81 sa karcinomom bronha u toku aktivnog antitumorskog lečenja. Bolesnici, tačnije njih 55 (67,9%), bili su muškarci, pušači (55,6%), prosečnog životnog doba od 66,47 godina (u rasponu od 43–83 godine). Najveći broj bolesnika (50,6%) imao je the *Eastern Cooperative Oncology Group Performance Status* (ECOG PS) 1, a 83,9% je imalo najmanje jedan komorbiditet. Najčešći komorbiditeti bili su arterijska hipertenzija (66,7%), hronična opstruktivna bolest pluća - HOBP (28,4%) i dijabetes (21%), dok je gojaznost, kongestivnu srčanu insuficijenciju i druge kardiovaskularne bolesti imalo 11%, 6,2% i 7,4%, redom. Najčešći je bio adenokarcinom (33,3%), zatim skvamozni karcinom (30,9%) i mikrocelularni karcinom bronha (24,7%). Većina bolesnika (63%) bila je u stadijumu III, dok je 33,3% bolesnika bilo u stadijumu IV. Metastaze su najčešće bile prisutne u kontralateralnom plućnom krilu/pleuri (14,8%), mozgu (6,2%), a u kostima i jetri su bile prisutne jednako (3,7%). Samostalna sistemska terapija primenjena je kod 37 od 81 (45,6%) bolesnika, radioterapija grudnog koša kod 35 (43,2%), konkurentna hemioradioterapija kod jednog (1,2%) i drugi vidovi radioterapije kod 8 (9,87%) bolesnika. Najčešći oblici sistemske terapije bili su hemioterapija kod 29 od 81 (35,8%) bolesnika, imunoterapija kod 6 (7,4%) i ciljana terapija kod 2 (2,4%) bolesnika. Najčešće (34,6%) su primenjivani protokoli na bazi cisplatina. Ustanovljena stopa mortaliteta od COVID-19 iznosila je 19,8%, bez statistički značajne razlike u odnosu na vrstu lečenja (p = 0,973). Utvrđena je statistička značajnost uticaja ECOG PS na porast mortaliteta (p = 0,011). **Zaključak.** Bolesnici sa karcinomom bronha su zavisni od antitumorskog lečenja, ali su istovremeno populacija koja je osetljiva na COVID-19. U našem istraživanju nisu pronađene razlike u mortalitetu u odnosu na vrstu antitumorskog lečenja. Potrebna su dalja istraživanja kako bi se bolje razumeli efekti infekcije SARS-CoV-2 na bolesnike sa karcinomom. Takođe, potrebno je sprovesti sve moguće metode zaštite od infekcije SARS-CoV-2, kako bi se rizik od infekcije sveo na minimum kod svih osoba, a posebno kod imunokompromitovanih bolesnika sa karcinomom.

Ključne reči:
antineoplastici; komorbiditeti; covid-19; pluća, neoplazme; mortalitet; neoplazme, određivanje stadijuma.

Introduction

Since December 2019, when a new virus appeared in Wuhan, China, the health care system around the globe has undergone significant changes. It is now well known that coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), can occur in the form of asymptomatic infection but also as severe viral pneumonia, acute respiratory distress syndrome and lead to death [1, 2].

Patients with associated diseases, such as chronic obstructive pulmonary disease (COPD), diabetes mellitus, and cardiovascular disease, may have a more severe form of COVID-19 and develop numerous complications. Therefore, their treatment more often requires hospitalization and respiratory support – from oxygen therapy to mechanical ventilation [3].

Cancer patients are an extremely sensitive subgroup in the COVID-19 pandemic. The pathway of patients with malignancy represents a major challenge for oncologists, from diagnosis to treatment and palliative care [1]. Several problems emerged in access to cancer patients during the COVID-19 pandemic. One of them is the possible overlap of symptoms between pneumonia caused by the SARS-CoV-2 virus and lung cancer (LC) such as shortness of breath, cough, and fatigue. Exposure of patients to infection during visits to health care facilities is another expected problem. Patients with LC are most often elderly smokers with already mentioned associated diseases. These factors increase the risk of COVID-19 [1, 4, 5].

Radiologically, COVID-19 can be presented in the same way as radiation pneumonitis, immunotherapy-associated pneumonitis, or other infections, which further complicates the assessment of patients with LC. In this situation, bronchoscopy plays an important role in the differential diagnosis, but it is relatively contraindicated in suspicion of COVID-19 [1].

Immunosuppressive antitumor drugs may increase the risk of viral infection, affecting neutrophil function and humoral immunity [6]. Most LC patients are current or former smokers, which can lead to further complications in possible COVID-19 [6, 7]. Multifactorial anemia and hypoproteinemia, often presented in these patients, further increase susceptibility to infectious pathogens [8]. As a result of previous thoracic surgery or radiation therapy, damage to the alveolar architecture and/or malignant airway obstruction may occur, which is another possible contributing factor to more severe infections [9].

In the last two years, the topic of SARS CoV2 infection has probably been the most current field of research in medicine. Several large meta-data research that analyzed COVID-19 mortality in patients with malignancies demonstrated an increased risk of death ranging from 1.66 to 3.16 [10–12]. Management of cancer patients undergoing active antitumor treatment is a particularly important issue. Immunosuppressive factors and susceptibility to SARS-CoV-2 infection differ between certain types of cancer and the type and duration of treatment. Kuderer et al. [13] demonstrated that patients who were in remission or without evidence of active disease had a lower risk of death from COVID-19 than patients with active diseases treated with some of the antitumor forms of treatment.

On the other hand, this area of interest has a lot of controversial results. Among recent research, a study by Wang et al. [14] showed that the use of radiotherapy, immunotherapy, or hormone therapy one month before SARS-CoV-2 infection was not associated with an increased risk of mortality among cancer patients.

The effect of chemotherapy on the outcome of cancer patients with COVID-19 may be different. Several studies have reported an increase in the mortality rate from COVID-19 in cancer patients previously treated with chemotherapy [15–17]. However, recent large observational studies have shown that there is no evidence of COVID-19 higher mortality rate associated with active chemotherapy [13, 18, 19].

An important factor in considering the outcome of treatment of patients with malignant disease is the possible negative impact of delays in the application of an anticancer treatment which allows the progression of cancer and certainly requires precise guidelines and recommendations.

The aim of this study was to determine the mortality of LC patients in whom the COVID-19 was confirmed during active antitumor treatment.

Methods

This retrospective study included data from LC patients diagnosed with COVID-19. This trial was conducted at the Institute of Lung Diseases of Vojvodina, Sremska Kamenica, Serbia, in the observed period from November 20, 2020, to June 5, 2021.

Inclusion criteria were the following: confirmed SARS-CoV-2 infection, radiologically confirmed pneumonia, cytologically/histologically confirmed LC, LC patients receiving at least one cycle of active antitumor treatment (chemotherapy, immunological or targeted therapy), and/or application of at least 2 radiotherapy fractions. COVID-19 was determined by reverse transcription polymerase chain reaction (RT-PCR) test for SARS-CoV-2 according to institutional guidelines based on the recommendations of the National Ministry of Health [20].

Patients were classified as active if less than 30 days had passed since the last dose of chemotherapy or immunotherapy, if targeted therapy was being actively taken, or if the time since the last fraction of radiotherapy was less than two weeks.

Data on demographic and clinical characteristics of patients, type of cancer, type of specific antitumor treatment, laboratory and radiological data, and treatment outcome were collected using the institutional database. COVID-19 treatment was performed according to institutional protocols based on the recommendations of the National Ministry of Health [21].

Results

Study population

In the period from November 20, 2020, to June 5, 2021, a total of 828 patients with COVID-19 were hospitalized at the Institute for Lung Diseases of Vojvodina (COVID-19 Clinical Department) in Sremska Kamenica, Serbia. Of these patients, 81 patients were on active cancer therapy and met the criteria for inclusion in this study.

The clinical characteristics of examined patients in this study corresponded to the average demographic data of patients with LC. Demographic and treatment characteristics of LC patients with COVID-19 are shown in Table 1.

The majority of patients were in stage III (63%), while the metastatic stage was present in 33.3% of patients (Table 1). The most common localizations of metastases

Table 1

Demographic and treatment characteristics of lung cancer (LC) patients with coronavirus disease 2019 (COVID-19) (n = 81)

Characteristics	Values
Age (years)	66.47 (43–83)
40–49	1 (1.2)
50–59	10 (12.3)
60–69	42 (51.9)
70–79	24 (29.7)
80–89	4 (4.9)
Gender	
male	55 (67.9)
female	26 (32.1)
Smoking status	
current	45 (55.6)
former	31 (38.3)
non-smoker	5 (6.2)
ECOG PS	
1	41 (50.6)
2	26 (32.1)
3	14 (17.3)
Comorbidities	
no	13 (16.0)
hypertension	54 (66.7)
COPD	23 (28.4)
diabetes mellitus	17 (21.0)
obesity	9 (11.1)
congestive heart failure	5 (6.2)
other cardiovascular diseases	6 (7.4)
others	8 (9.8)
Pathology	
adenocarcinoma	27 (33.3)
SqCC	25 (30.9)
SCLC	20 (24.7)
others	9 (11.1)
Stage	
I	1 (1.2)
II	2 (2.5)
III	51 (63.0)
IV	27 (33.3)
Metastasis-location	
lung/pleural	12 (14.8)
brain	5 (6.2)
bone	3 (3.7)
liver	3 (3.7)
others	4 (4.9)

All variables are expressed as numbers (%) except age, which is expressed as median (range).
COPD – chronic obstructive pulmonary disease; SqCC – squamous cell carcinoma; SCLC – small-cell LC; ECOG PS – Eastern Cooperative Oncology Group Performance Status.

were in the contralateral lung/pleura (14.8%), brain (6.2%), bone (3.7%), and liver (3.7%), and the median number of metastatic sites was 1 (range 1–3).

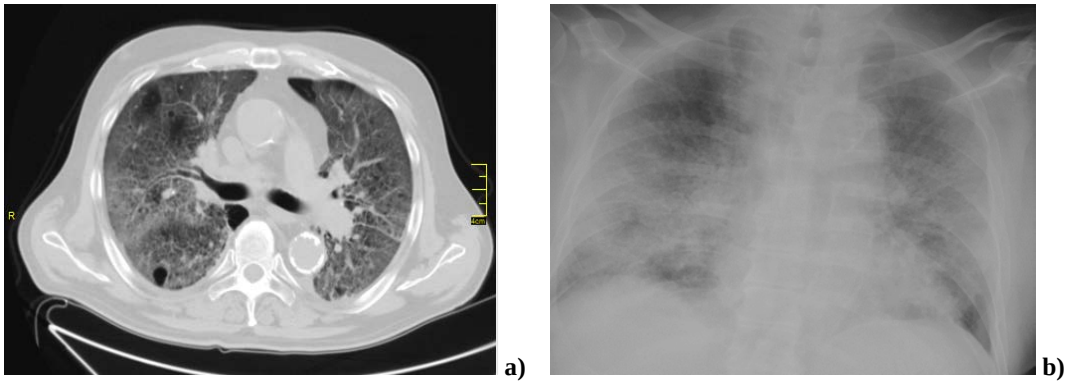
All included patients were treated with anticancer therapy, systemic therapy, and/or radiotherapy at the time of diagnosis of COVID-19 (Table 2). Regarding the type of therapy, out of 81 patients, 37 (45.6%) received systemic therapy, thoracic radiotherapy was applied in 35 (43.2%) patients, concurrent chemo-radiotherapy in one (1.2%) patient, and non-thoracic radiotherapy in 8 (9.87%) patients. The types of applied systemic therapy are shown in Table 2. In the observed study group, no patient with COVID-19 was treated with the combination of chemo- and immunotherapy. Non-thoracic radiotherapy was applied as palliative brain radiotherapy (whole-brain radiation therapy – WBRT or prophylactic cranial radiation – PCI). Chemotherapy was most often used as first-line chemotherapy (24.7%), followed by second-line chemotherapy (12.3%), neoadjuvant and adjuvant chemotherapy (2.5% and 1.2%, respectively). The most commonly used was cisplatin-based protocols in 34.6% of LC patients.

We observed high variability of computed tomography (CT) and radiological findings (Figures 1 and 2). On chest X-rays, the highest number of patients had bilateral pneumonia (33/81), and on CT examination, most patients had chest multilobar pneumonia (52/81). Overall, multilobar

Table 2

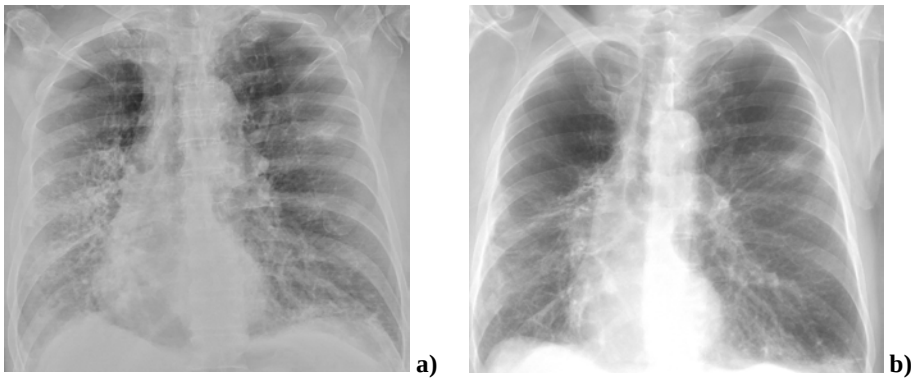
Anticancer treatment of lung cancer (LC) patients with coronavirus disease 2019 (COVID-19) (n = 81)

Treatment type	Number (%) of patients
Chemotherapy	29 (35.8)
Radiotherapy	43 (53.1)
Targeted therapy	2 (2.5)
Immunotherapy	6 (7.4)
Combined chemoradiotherapy	1 (1.2)



a) b)

Fig. 1 – a) Computed tomography (CT) findings and b) Radiological findings in patients with lung cancer (LC) at the time of diagnosis of coronavirus disease 2019 (COVID-19): bilateral pneumonia in a 71-year-old male with stage IV non-small-cell LC treated with first-line cisplatin-based chemotherapy.



a) b)

Fig. 2 – a) and b) Radiological findings in patients with lung cancer (LC) at the time of diagnosis of coronavirus disease 2019 (COVID-19): radiological regression of bilateral pneumonia in a 62-year-old male with stage III squamous cell carcinoma treated with chest radiotherapy.

pneumonia was the most common radiological presentation in 64.2% of patients.

Respiratory failure type 1 was registered in 76 (93.8%) patients and type 2 in 5 (6.2%) patients. Oxygen therapy was administered to all patients: in 69 (85.2%) patients with the regular cannula and in 9 (11.1%) with a high-flow nasal cannula. Non-invasive ventilation was performed in 2 (2.5%) patients, and one (1.2%) patient was treated in the Intensive Care Unit with invasive mechanical ventilation (Table 3).

Overall, 64 (79.0%) patients were treated with antibiotics, while antiviral drugs were administered to 3 (3.7%) patients. Anticoagulant therapy with nadroparin was introduced to 76 (93.8%) examined patients. In two (2.5%) patients, thromboembolic events were diagnosed during hospitalization (Table 3). Corticosteroid therapy was performed in 77 (95%) patients, while tocilizumab was not administered to any patients. Renal replacement therapy or extracorporeal membrane oxygenation has not been administered. Finally, our results showed that 16 (19.8%) patients with LC died from the COVID-19, while 65 (80.2%) patients survived the infection (Table 3).

Table 3

Outcomes of lung cancer (LC) patients with coronavirus disease 2019 (COVID-19) (n = 81)

Parameter	Number (%) of patients
Evolution	
alive	65 (80.2)
died	16 (19.8)
Antibiotics	64 (79.0)
Antiviral treatment	3 (3.7)
Corticosteroids	77 (95.1)
Anticoagulants	76 (93.8)
Oxygen support	
regular cannula	69 (85.2)
high-flow nasal cannula	9 (11.1)
NIV	2 (2.5)
IMV	1 (1.2)

NIV – noninvasive ventilation; IMV – invasive mechanical ventilation.

The mortality rate in a series of our patients was 19.8% (16/81 patients). Univariate analysis showed a statistically significant association between the ECOG PS and mortality rate ($p = 0.011$) (Table 4). The highest number of deaths was observed in patients treated with radiotherapy – 8 (9.8%) patients, followed by chemotherapy – 5 (6.17%) patients. We did not observe a statistically significant correlation between the type of antitumor therapy and mortality ($p = 0.973$) (Table 4).

Table 4

Predictive factors of mortality in lung cancer patients with coronavirus disease 2019 (COVID-19) (n = 81)

Variables	*p*
Treatment type	0.973
Stage	0.890
ECOG PS	0.011

ECOG PS – Eastern Cooperative Oncology Group Performance Status.

Discussion

One of the most important challenges for oncologists during the COVID-19 pandemic is the possible effect of applied specific anticancer treatment on morbidity and mortality of SARS-CoV2 infected cancer patients. This study highlights the importance of treating patients with LC, despite their high susceptibility to COVID-19.

Clinical characteristics and results of treatment in 81 patients with LC hospitalized due to confirmed SARS-CoV2 infection presented in our study showed that the majority of patients were men (67.9%), smokers (55.6%), and, in average, 66.47 years old (range 43–83). The largest number of patients had the ECOG PS 1 (50.6%). Most patients (83.9%) had at least one comorbidity (arterial hypertension in 66.7%, COPD in 8.4%, and diabetes mellitus in 21% of patients). According to the histopathological LC type, the most common were adenocarcinoma (33.3%), followed by squamous cell carcinoma (30.9%), and small-cell LC (24.7%). Regarding the stage of the disease, 63% of the patients were in stage III, while 33.3% were in stage IV at the time of the confirmed COVID-19. Metastases were most often localized in the contralateral lungs and pleura (14.8%), brain (6.2%), and the bones and liver (3.7% each). This result is comparable to the average demographic data of LC patients in the world [22].

In a study by Luo et al. [23] from the Memorial Sloan Kettering Cancer Center (MSKCC), which included 102 patients with diagnosed LC and COVID-19, 52% were women. The mean age was 68 years (range 31–91), 64% were smokers, and 72% had active or metastatic LC. The global registry for patients with LC and confirmed COVID-19 TERAVOLT (Thoracic Cancers International COVID-19 Collaboration) initially included a total of 200 patients from 21 countries [24] and is expanded to 1,012 patients [25]. The patients were predominantly men (70.5%), current or former smokers (81.1%), with NSCLC (non-small-cell LC) (75.5%) in the metastatic stage (73.5%) with comorbidities (83.8%) [24, 25].

The mortality rate in our study was 19.8%, which corresponds with most of the available results. Most of the available data suggest that mortality from COVID-19 in patients with malignancy is 25% to 30%, but not for all types of cancer equally [9, 16, 26, 27]. The results indicate that patients with LC and hematological malignancies have the highest risk of death from COVID-19 [28, 29]. According to data from Wuhan, China. [16], active anticancer treatment administered 14 days before SARS-CoV-2 infection in 28 cancer patients, statistically significantly increased the risk of severe COVID-19 outcomes (HR 4.079, $p = 0.037$), with a mortality rate of 28.6%. Dai et al. [30] presented the treatment results of immunotherapy-treated LC patients infected with SARS CoV2. Although the study had a small number of patients, the mortality rate was 66.7%. Luo et al. [23] observed a mortality rate of 25% and revealed that pulmonary comorbidities such as COPD or smoking history were most associated with poor disease outcomes. However, according to their results, anticancer treatment, radiotherapy, or surgery, as well as the presence of the active and metastatic

disease, did not affect the most severe outcome of COVID-19. However, some studies have shown significantly lower mortality in this population. Calles et al. [28] published the results of a study of 23 LC patients who received systemic anticancer therapy where the mortality rate of COVID-19 was 2.1%, with no difference concerning the type of oncological treatment.

All patients included in this study were treated with systemic anticancer therapy or radiotherapy at the time of diagnosis of COVID-19. The highest number of deaths was in patients treated with radiotherapy – 8 (9.8%) patients, followed by patients receiving chemotherapy – 5 (6.17%) patients, but no statistically significant correlation between the type of oncology therapy and mortality was observed ($p = 0.973$).

Results of the impact of the type of oncology treatment on the outcome of COVID-19 are diverse, and the approach to patients with a diagnosed malignant disease has changed under the influence of the ongoing pandemic. The results of the TERAVOLT study showed an increased risk of death from COVID-19 for patients with LC treated with chemotherapy (hazard ratio-HR 1.71) but not in patients treated with immunotherapy or targeted therapy (HR 1.04) [24, 25]. A retrospective study of Memorial Sloan Kettering conducted on LC patients and COVID-19 showed that, although mortality was 25%, anti-cancer therapy had no effect on poor outcomes [23]. Lee et al. [31] published the results of a large cohort study involving 800 patients. They reached a mortality rate of 27% in 281 patients with active cancer treated with chemotherapy one month before COVID-19. As the mortality rate in patients not treated with chemotherapy was 29%, the authors concluded that chemotherapy was not a significant risk factor for mortality from COVID-19.

These results suggest that anticancer treatment does not increase mortality from COVID-19. The study included patients with different types of cancer, a wide range of anticancer treatments, as well as the impact of comorbidities, which may be limiting factors in interpreting the results.

According to an analysis conducted by Luo et al. [23], which included only patients with LC, immunotherapy associated with smoking status was an independent predictor of poor outcomes of COVID-19. The study from the Gustave Roussy Center in Paris included 137 cancer patients with COVID-19. Among those patients, 12 (10.1%) had LC and, in univariate analysis, chemotherapy treatment in the last 3 months significantly increased the risk of clinical deterioration with HR 2.60 ($p = 0.006$), while chemotherapy or immunotherapy were without the effect [32].

In our study, univariate analysis showed a statistically significant correlation between mortality and the ECOG PS ($p = 0.011$). It is known that clinic characteristics of the patient certainly affect the outcome of the disease, and the ECOG PS is only one of them. In the study by Kuderer et al. [13], an increased risk of severe complications in patients with COVID-19 was associated with an ECOG higher than 2. In the largest multicenter retrospective cohort study from China, which included 13,077 patients with COVID-19, 23 were with LC. In this subgroup of patients, the mortality rate was 39%. This study also confirmed that the ECOG PS $\geq$ 2, metastatic stage of disease, and older age were associated with an increase in mortality rate [12]. In the already mentioned study by Barlesi et al. [32], conducted by the Gustave Roussy Center, multivariate analysis confirmed a statistically significant effect of the ECOG PS on the poor outcome with HR 3.9 ($p = 0.008$).

Treatment of patients with LC during the COVID-19 pandemic should include careful and regular monitoring of clinical and radiological changes, more so than in patients with other types of malignancies. It is recommended that cancer patients be tested for SARS-CoV-2 at the beginning of treatment and during therapy in case of clinical suspicion of COVID-19 [33].

Our study has several limitations. This trial was conducted in a single institution as a retrospective study with a limited follow-up period. A possible underestimation of the actual incidence of COVID-19 in our study population may be due to false-negative RT-PCR tests, as well as the inability to display patients diagnosed outside of our hospital system. We did not include patients with an active disease without specific cancer therapy (*eg*, patients receiving the best supportive care) in the definition of an "at-risk" population. The impact of COVID-19 on these patients is an important area for future research.

Conclusion

The COVID-19 pandemic affected all aspects of the management of LC patients. The diagnostic and therapeutic approach to patients with LC has been changed due to incoherent and insufficient data. The biological and clinical aggressiveness of this type of cancer generally does not allow discontinuation or delay of specific anticancer treatment. Careful monitoring of the clinical condition and radiological status of LC patients on active treatment during a pandemic is highly recommended. Research on a larger number of patients is needed to make recommendations for optimal measures to reduce patient exposure to COVID-19, best treatment strategies, and safe implementation of anticancer procedures.

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Phenolic profile, antioxidant, and antiproliferative activities of *Convolvulus aucheri* Choisy

Fenolni profil, antioksidantno i antiproliferativno delovanje *Convolvulus aucheri* Choisy

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Abstract

Background/Aim. It is known that some members of the genus *Convolvulus (C)* L. are commonly used in Turkish folk medicine. These species are powerful in curing toothache and joint pains. Due to the limited information on the biological activities of *C. aucheri,* a species almost exclusively occurring in Turkey, we aimed to investigate the antioxidant and cytotoxic effects of three extracts obtained from the plant, as well as to characterize their phenolic profile. **Methods.** The antioxidant activity of the extracts was determined by using ABTS, NO, FRAP, phosphomolybdenum, and metal chelating assays. In addition, the bioactive compounds found in the extracts, such as total phenolics, flavonoids, and saponins, were determined. Cytotoxicity was assessed by using the CellTiter-Glo assay on HeLa and H1299 cancer cells. **Results.** The methanol extract of *C. aucheri* demonstrated the highest antioxidant activity as well as the highest phenolic, flavonoid, and saponin content. The high-performance liquid chromatography (HPLC) analysis showed that the major phenolic compounds in the extract were chlorogenic acid, (+)-catechin, rosmarinic acid, and rutin. The methanolic extract obtained from the aerial parts of *C. aucheri* was found to interfere with the viability of HeLa cells, with an IC_{50} value of 14.22 μg/mL being recorded. **Conclusion.** Our results showed that *C. aucheri* could be a good candidate as a novel and alternative natural antioxidant and antitumor source.

Key words:
antioxidants; convolvulus; chromatography, high-pressure liquid; phytotherapy; turkey.

Apstrakt

Uvod/Cilj. Poznato je da se neke biljne vrste roda *Convolvulus (C)* L. često koriste u turskoj narodnoj medicini kao snažni agensi u lečenju zubobolje i kod bolova u zglobovima. Zbog ograničenih informacija o biološkim aktivnostima *C. aucheri*, vrste koja se gotovo isključivo javlja u Turskoj, cilj rada bio je ispitivanje antioksidativnog potencijala, kao i citotoksičnih svojstava tri različita ekstrakta, uz karakterizaciju njihovog fenolnog profila. **Metode.** Antioksidativna aktivnost ekstrakta određena je korišćenjem ABTS, NO, FRAP, fosfomolibdenskog i testa heliranja metala. Pored toga, u ekstraktima su identifikavana i određena sledeća bioaktivna jedinjenja: ukupni fenolni, flavonoidi i saponini. Citotoksičnost je procenjena primenom *CellTiter-Glo* testa na kancerskim HeLa i H1299 ćelijskim linijama. **Rezultati.** Metanolni ekstrakt *C. aucheri* ispoljio je najveću antioksidativnu aktivnost, a u njemu je utvrđen i najveći sadržaj fenola, flavonoida i saponina. Tečnom hromatografijom pod visokim pritiskom (HPLC) utvrđeno je da su glavna jedinjenja u ovom ekstraktu hlorogenska kiselina, (+)-katehin, ruzmarinska kiselina i rutin. Otkriveno je da metanolni ekstrakt dobijen iz nadzemnih delova *C. aucheri* smanjuje vitalnost HeLa ćelija, a određena IC_{50} vrednost bila je 14,22 μg/mL. **Zaključak.** Naši rezultati su pokazali da bi *C. aucheri* mogao biti dobar novi i alternativni izvor prirodnih antioksidanasa i jedinjenja sa citotoksičnim efektom.

Ključne reči:
antioksidansi; convolvulus; hromatografija, tečna, pod visokim pritiskom; fitoterapija; turska.

Introduction

Natural products, especially plant derivatives, are a potential supply for novel biologically active compounds [1]. The herbal products have become very popular, especially because of the negative effects of synthetic drugs [2]. Natural resources with their rich polyphenolic contents and different types of secondary metabolites could be potential antioxidant

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and anticancer agents. Antioxidants are able to prevent or postpone the oxidative injury, which causes several diseases, by intervening with the formation of free radicals or defusing them whereafter they are produced. In most cases, natural compounds are more effective and more reliable than synthetic antioxidants. For this reason, there is a growing interest in the identification of natural components that are useful for humans [3].

The genus *Convolvulus (C)* L. belongs to the Convolvulaceae family, including 250 taxa, generally recognized as bindweeds. With respect to recent research, this genus is represented by 39 taxa (three of them hybrids) in Turkey [4]. Some taxa of this genus are used in the treatment of toothache and also as anthelminthic, laxative, and cholagogue in Turkish folk medicine [5]. *C. aucheri* Choisy is located in a narrow area in the southern part of Turkey**.** The observations made in field studies and the fact that *C. aucheri* could be collected from only one area contributed to evaluating the threat category of this species as Vulnerable (VU) according to the IUCN (International Union for Conservation of Nature) [6].

Extracts of various members of *Convolvulus* have been demonstrated to have antioxidant, anticancer, and antinociceptive activities [7–9]. Although some studies have been performed on the phytochemicals and biological activities of *C. arvensis*, *C. pluricaulis*, and *C. fatmensis* [7, 10, 11], only one study has been performed with *C. aucheri* [12]. Therefore, the aim of this research was to reveal the antioxidant potentials and total bioactive compounds (phenolic, flavonoid, and saponin) of *C. aucheri*, as well as their cytotoxic effect on HeLa and H1299 cancer cells. In this research, the phenolic components of *C. aucheri*, which were collected from Turkey, were also determined *via* reversed-phase high-performance liquid chromatography (RP-HPLC).

Methods

Plant material and extraction

The individuals of *C. aucheri* were collected in the flowering stage from Hatay-Turkey at NATO Radar Station in Kisecik, serpentine slopes, ca 885 m. Taxonomic identification of the plant was confirmed by the senior taxonomist Dr. Candan Aykurt in the Department of Biology, Akdeniz University, Antalya-Turkey. The voucher specimen was deposited at the Akdeniz University Herbarium (Voucher no: C. Aykurt 2665).

The plants were air-dried, and their aerial parts were powderized. Each of the powdered plants (10 g) was separately extracted with 100 mL of methanol, acetone, and petroleum benzine in a shaker water bath for 6 hrs at 55 °C. The extracts were filtered and vaporized using a rotary evaporator, and the methanol: water (70 : 30, v/v) extract was lyophilized due to the residual water content. The highest extraction yield was obtained with methanol (36.11%). The yields of the acetone and petroleum benzine extracts were 21.25% and 10.64%, respectively. The crude extracts were kept at +4 °C until needed.

Total phenolic content (Folin–Ciocalteu assay)

The total phenolic content of the extracts was analyzed via the Folin-Ciocalteu method, in which gallic acid was used as a standard [13]. One mL of extract solution was added to 46 mL of distilled water and 1 mL of Folin-Ciocalteu reagent and was mixed properly. After 3 min, the mixture was mixed with 3 mL of sodium carbonate (2%) and shaken intermittently for 2 hrs. The absorbance was measured at 760 nm, and the total phenolic constituent was determined as gallic acid equivalents (mg GAEs/g).

Total flavonoid content

The total flavonoid content of the extracts was determined by the aluminum colorimetric method [14]. One mL extract was mixed with 1 mL of 2% aluminum trichloride ($AlCl_3$) in methanol. After a 10 min incubation at 25 °C, the absorbance was read at 415 nm. The total flavonoid content was expressed as quercetin equivalent (mg QE/g).

Total saponin content

Total saponin content was determined by the vanillin-sulphuric acid method [15]. The extracts were mixed with the same amount of vanillin (8%, w/v) and twice the amount of sulphuric acid (72%, w/v). The mixture was incubated at 60 °C for 10 min, followed by cooling in an ice-water bath for 15 min. Absorbance was read at 535 nm. The total saponin content was expressed as quillaja equivalents (mg QAE/g).

Phenolic compound analyses

Phenolic compounds of *C. aucheri* were analyzed by RP-HPLC (Shimadzu, Japan) as described by Caponio et al. [16] with some modifications. Separation was performed at 30 °C by using a reversed-phase column (Agilent Eclipse XDB C-18, 250 mm × 4.6 mm, 5 μm) using the mixture of two solvents (A: the acetic acid solution 3% and B: methanol) as a mobile phase. Gradient conditions were particularized at a flow rate of 0.8 mL/min as follows: 93% A + 7% B for 0–20 min, 72% A + 28% B in 20–28 min, 75% A + 25% B in 28–35 min, 70% A + 30% B in 35–60 min, 67% A + 33% B in 60–62 min, 58% A + 42% B in 62–70 min, 50% A + 50% B in 70–73 min, 30% A + 70% B in 73–75 min, 20% A + 80% B in 75–80 min, 0% A + 100% B in 80–81 min, 93% A + 7% B in 81–90 min. Phenolic compounds in the methanolic extract of *C. aucheri* were expressed as μg/g extract and analyzed with a diode-array detector (DAD) at 280 nm (for the phenolic acids) and 320 and 360 nm (for the flavones and flavonols, respectively). The quantitative analysis was made by comparing the standards used (gallic acid, protocatechuic acid, catechin, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, epicatechin, syringic acid, vanillin, *p*-coumaric acid, ferulic acid, sinapinic acid, benzoic acid, *o*-coumaric acid, rutin, naringin, hesperidin, rosmarinic acid, eriodictyol, cinnamic acid, quercetin, luteolin, kaempferol, apigenin). The identification of each target compound was based on a

combination of retention time and spectral matching. The HPLC method was validated as to linearity, accuracy, and sensitivity. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using signal to noise ratio method.

Antioxidant activity assays

Ferric reducing antioxidant power (FRAP) assay was applied as described by Zengin et al. [17] with some modifications. Extract solutions were added to the FRAP reagent, which was mixed in advance [acetate buffer – 0.3 M, TPTZ (2,4,6-tripyridyl-s-triazine) – 10 mM, $FeCI_3$ – 20 mM]. After measuring the absorbances at 593 nm, FRAP activity was expressed as Trolox (mg TEs/g extract) equivalents.

Total antioxidant capacity (Phosphomolybdenum method)

The phosphomolybdenum method was used to evaluate the total antioxidant capacity of the extracts. Briefly, different extract solutions were mixed with the reagent solution (0.6 M H_2SO_4, 28 mM Na_3PO_4, and 4 mM $(NH_4)_2MoO_4$) and incubated for 90 min at 95 °C. The absorbance values were determined at 695 nm wavelength [18]. Total antioxidant capacity was expressed as Trolox equivalent (mmoL TE/g extract).

Metal chelating activity

Extract solutions at different concentrations were added to $FeCl_2$ (0.05 mL, 2 mM). The reaction that started directly after adding 5 mM of ferrozine was measured at 562 nm after 10 min left at room temperature. Metal chelating activity was expressed as ethylenediaminetetraacetic acid (EDTA) equivalent (mg EDTAE/g extract) [17].

Nitric oxide (NO) scavenging activity

NO was produced from sodium nitroprusside (SNP), which was measured, as described by Balakrishnan et al. [19], by using the Griess reaction. The mixture containing SNP (5 mM) in phosphate-buffered saline (PBS) (pH 7.3) was prepared with the extracts in PBS at different concentrations and incubated for 3 hrs at 25 °C. The absorbance value was determined at 546 nm wavelength. Ascorbic acid was used as a positive control. The results were indicated as IC_{50}.

ABTS [*2,2 azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)*] *radical scavenging activity*

The scavenging activity towards ABTS radical was analyzed as described by Re et al. [20] with some modifications. Freshly prepared and diluted ABTS solution was mixed with the various solvent extracts of *C. aucheri*, and the absorbances were read after 30 min at 734 nm. The results were indicated as IC_{50}.

Cell culture and cell antiproliferation capacity

HeLa (as human cervix adenocarcinoma cell line) and H1299 (as non-small cell lung adenocarcinoma cell line) cells were used and cultured in RPMI 1640 medium in a CO_2 incubator. After being seeded into 96-well plates (2×10^3 cells/well) and a 24 hrs incubation, the medium was removed from the well, leaving the adherent cells. The cells were applied with extract for 24, 48, and 72 hrs in the range of 3.125–100 µg/mL. After the time was up, cytotoxicity was determined using the CellTiter-Glo assay. Viability was calculated using the background-corrected absorbance as follows: Viability (%) = Abs of experiment well / Abs of control well x 100.

Statistical analysis

Statistical analysis was performed using the software SPSS version 22.0 program. Statistical significance was determined using the one-way ANOVA. Multiple group comparisons were analyzed with Tukey's multiple comparison test. Data were expressed as mean ± standard deviation (SD) and *p*-value of < 0.05 was considered statistically significant.

Results

Total bioactive compounds

C. aucheri collected from its single location in Turkey was extracted using methanol, acetone, and petroleum benzine. Then, the total phenolic, flavonoid, and saponin contents (TPC, TFC, and TSC, respectively) of these extracts were investigated with spectrophotometric methods, and the results are presented in Table 1. According to the obtained

Table 1

Total phenolic, flavonoid, and saponin contents of *Convolvulus aucheri* extracts (mean ± SD)

Solvent	TPC[x]	TFC[y]	TSC[z]
Petroleum benzine	22.06 ± 0.05[c]	05.04 ± 0.01[c]	11.08 ± 0.03[c]
Acetone	40.05 ± 0.07[b]	10.35 ± 0.02[b]	37.12 ± 0.06[b]
Methanol	87.64 ± 1.12[a]	51.76 ± 0.06[a]	71.30 ± 1.06[a]

TPC – [x]total phenolic content expressed as gallic acid equivalents (mg GAEs/g); TFC – [y]total flavonoid content expressed as quercetin equivalents (mg QEs/g); TSC – [z]total saponin content expressed as quillaja equivalents (mg QAEs/g); SD – standard deviation.
***p* < 0.05 compared with: [a] – petroleum benzine and acetone; [b] – petroleum benzine and methanol; [c] – acetone and methanol.**

data, the total phenolic content (87.64 mg GAEs/g), total flavonoid content (51.76 mg QEs/g), and total saponin content (71.30 mg QAEs/g) were detected to be at the highest in the methanol extract of the plant.

HPLC analysis

Because the methanol extract had higher total phenolic content than the others, it was used in RP-HPLC analysis by using 24 standard compounds (Figure 1) in order to elucidate the phenolic profile of *C. aucheri*. Protocatechuic acid, (+)-catechin, chlorogenic acid, caffeic acid, ferulic acid, rutin, rosmarinic acid, eriodictyol, quercetin, and kaempferol were detected in the extract in varying amounts (Figure 2). Chlorogenic acid (22,690 μg/g) was found as the main component of the extract in the present study, followed by (+)-catechin (8,630 μg/g), rosmarinic acid (4,370 μg/g), and rutin (1,810 μg/g) (Table 2).

Antioxidant activity

The outcomes of free radical scavenging assays (ABTS and NO), phosphomolybdenum, metal chelating, and ferric reducing power assays are presented in Table 3. In both ABTS and NO assays, there were significant differences ($p < 0.05$) between the various extracts (methanol, acetone, and petroleum benzine). In the current study, the highest scavenging activity values obtained from the NO (76.03

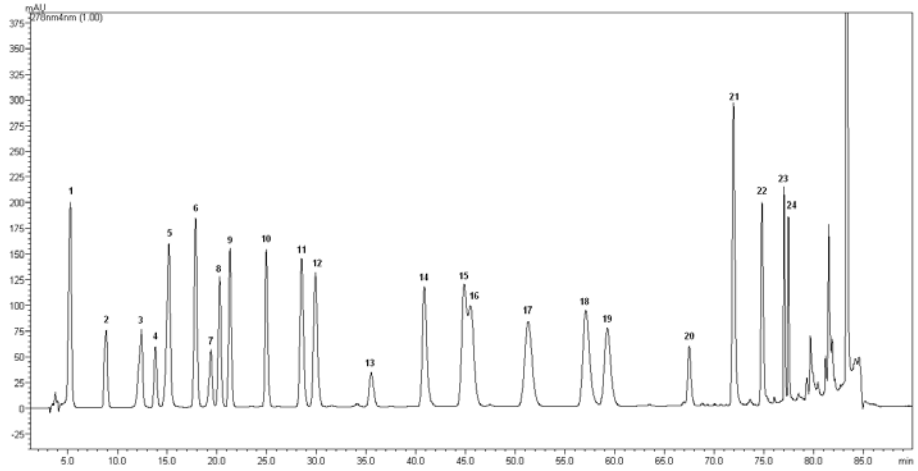

Fig. 1 – High-performance lipid chromatography (HPLC) chromatogram of standards: 1 – gallic acid; 2 – protocatechuic acid; 3 – catechin; 4 – *p*-hydroxy benzoic acid; 5 – chlorogenic acid; 6 – caffeic acid; 7 – epicatechin; 8 – syringic acid; 9 – vanilin; 10 – *p*-coumaric acid; 11 – ferulic acid; 12 – sinapinic acid; 13 – benzoic acid; 14 – *o*-coumaric acid; 15 – rutin; 16 – naringin; 17 – hesperidin; 18 – rosmarinic acid; 19 – eriodictiol; 20 – cinnamic acid; 21 – quercetin; 22 – luteolin; 23 – kaempferol; 24 – apigenin.

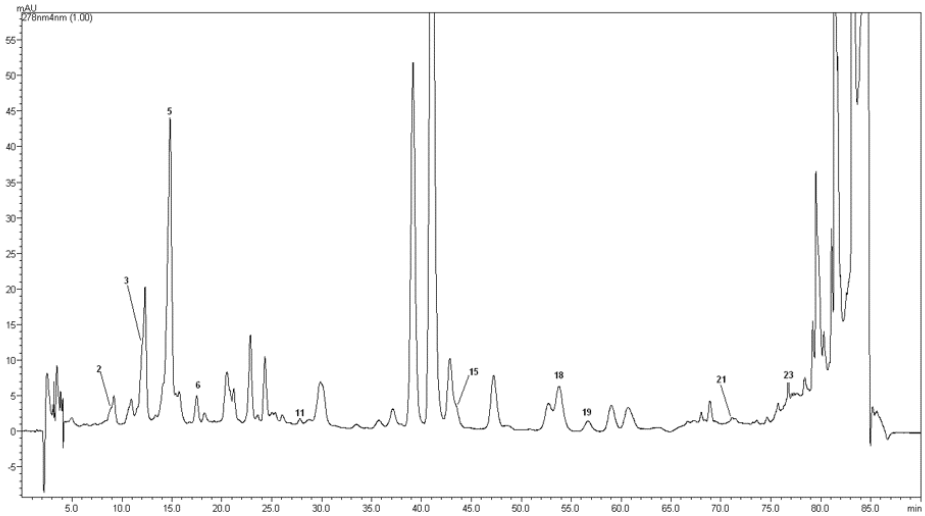

Fig. 2 – High-performance lipid chromatography (HPLC) chromatogram of *Convolvulus aucheri* extract where: 2 – protocatechuic acid; 3 – catechin; 5 – chlorogenic acid; 6 – caffeic acid; 11 – ferulic acid; 15 – rutin; 18 – rosmarinic acid; 19 – eriodictiol; 21 – quercetin; 23 – kaempferol.

Table 2

Phenolic compounds of *Convolvulus aucheri* and high-performance lipid chromatography (HPLC) validation results

Identified phenolic compounds	RT (min)	UV_{max} (nm)	LOD (μg/mL)	LOQ (μg/mL)	Linearity range (μg/mL)	μg/g extract (mean ± SD)
Protocatechuic acid	9.13	280	0.031	0.102	0.263–207.11	850.0 ± 6.0
(+)-Catechin	12.22	280	0.020	0.096	0.258–205.45	8,630.0 ± 176.0
Chlorogenic acid	15.00	320	0.010	0.362	0.250–203.57	22,690.0 ± 490.0
Caffeic acid	17.32	280	0.019	0.043	0.260–209.20	780.0 ± 5.3
Ferulic acid	28.11	320	0.021	0.031	0.275–255.17	310.0 ± 3.5
Rutin	43.23	360	0.050	0.166	0.411–386.33	1,810.0 ± 24.0
Rosmarinic acid	54.00	320	0.016	0.041	0.302–295.02	4,370.0 ± 96.0
Eriodictyol	56.71	280	0.025	0.095	0.256–233.14	550.0 ± 4.0
Quercetin	71.21	320	0.048	0.159	0.432–398.32	290.0 ± 3.0
Kaempferol	77.12	320	0.030	0.106	0.288–261.53	780.0 ± 5.0

RT – retention time; LOD – limit of detection; LOQ – limit of quantification; SD – standard deviation.

Table 3

Antioxidant activities of *Convolvulus aucheri* extracts (mean ± SD)

Solvent	ABTS (IC_{50} μg/mL)	NO (IC_{50} μg/mL)	FRAP assay (mg TEs/g)	Phosphomolyb-denum assay (mmoL TEs/g)	Metal chelating activity (mg EDTAEs/g)
Petroleum benzine	113.65 ± 2.08[a]	128.02 ± 2.77[a]	41.33 ± 0.18[a]	0.33 ± 0.01[a]	4.97 ± 0.01[a]
Acetone	75.13 ± 1.01[b]	94.13 ± 1.72[b]	59.30 ± 0.06[b]	1.38 ± 0.05[b]	16.41 ± 0.03[b]
Methanol	45.64 ± 0.09[c]	76.03 ± 1.05[c]	80.41 ± 1.80[c]	2.45 ± 0.07[c]	25.35 ± 0.06[c]
Ascorbic acid	6.53 ± 0.01[d]	15.06 ± 0.03[d]	nt	nt	nt

ABTS – 2,2 azino-bis (3-ethilbenzothiazoline-6 sulfonic acid); NO – nitric oxide; FRAP – ferric reducing antioxidant power; TEs – Trolox equivalents; EDTA – ethylenediaminetetraacetic acid; EDTAEs – EDTA equivalents; nt – not tested; SD – standard deviation.
***p* < 0.05 compared with: [a] – acetone, methanol, and ascorbic acid; [b] – petroleum benzine, methanol, and ascorbic acid; [c] – petroleum benzine, acetone, and ascorbic acid; [d] – petroleum benzine, acetone, and methanol.**

μg/mL) and ABTS (45.64 μg/mL) assays were detected by methanol extracts. The total antioxidant capacity of extracts was examined by the phosphomolybdenum method, which measures phenolic and nonphenolic compounds related to their reductive activity. The methanolic extracts of *C. aucheri* demonstrated the most powerful total antioxidant activity at 2.45 mmol TEs/g (Table 3) ($p < 0.05$). *C. aucheri* methanolic extracts showed the strongest FRAP activity with 80.41 mg TEs/g, whereas the lowest activity was found in petroleum benzine extracts at 41.33 mg TEs/g. Similar to ABTS and NO assays, *C. aucheri* demonstrated great FRAP activity in its methanolic extract. In conformity with outcomes of other antioxidant tests, effective chelation power was again determined in the methanol extract with 25.35 mg EDTAEs/g.

Cytotoxic activity

In order to evaluate the cytotoxic activity of the *C. aucheri*, the methanol extract was used due to its higher total bioactive compounds and antioxidant activity than other extracts. The cytotoxic activity of the methanolic extracts on the growth of HeLa and H1299 cells was determined using the CellTiter-Glo assay. A decrease in viability in cancer cell lines was observed in a concentration-dependent manner ($p < 0.05$) (Figures 3 and 4). The IC_{50} values (μg/mL) that cause

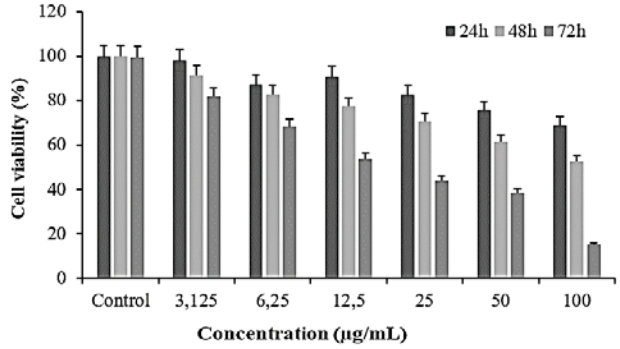

Fig. 3 – Concentration and time-dependent inhibitory effects of *Convolvulus aucheri* extract on cell viability in HeLa cells. Data are presented as mean ± standard deviation.

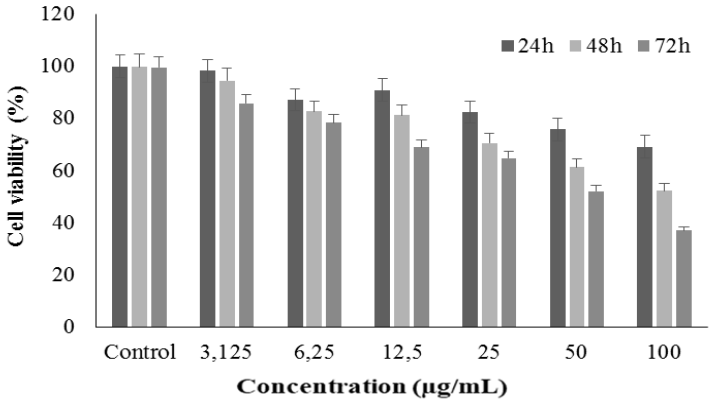

Fig. 4 – Concentration and time-dependent inhibitory effects of *Convolvulus aucheri* extract on cell viability in H1299 cells. Data are presented as mean ± standard deviation.

50% cell death were calculated at 14.22 µg/mL and 64.45 µg/mL on HeLa and H1299 cells, respectively.

The total phenolic content (87.64 mg GAEs/g), total flavonoid content (51.76 mg QEs/g), and total saponin content (71.30 mg QAEs/g) were detected to be at the highest in the methanol extract of the plant.

Discussion

Thanks to many beneficial effects of the secondary metabolites, the plants have been the center of interest for many years [21]. Alkaloids, flavonoids, coumarins, sterols, saponins, and tannins have been isolated from plants of the genus *Convolvulus* L. [22, 23]. The total phenolic and flavonoid content of *C. galaticus* was reported earlier at 84.689 mg GAEs/g and 48.760 mg CEs/g, respectively [24]. TPC of *C. hystrix* was reported at 14.6 mg GAEs/g for ethyl acetate extract [25]. Elzaawely and Tawata [10] found the total phenolic and flavonoid content of *C. arvensis* leaves at 244.6 mg GAE/g and 174.4 mg RE/g, respectively. According to these results, *C. aucheri* methanolic extract used in the study had more abundant (87.64 mg GAEs/g) total phenolic contents than *C. galaticus* and *C. hystrix*. Despite having various amounts of bioactive compounds as a result of using different taxa, solvents, and growing conditions, it would not be surprising to say that most of these *Convolvulus* taxa could be a significant source of phenolic compounds.

The phenolic profile of *C. galaticus* methanolic extracts was obtained by using a liquid chromatography-tandem mass spectrometry analysis [24]. The authors reported that 7 phenolic and flavonoid compounds were detected in the extracts. Methanol extracts of *C. galaticus* contained from the lowest to the highest amount of epigallocatechin (0.094 µg/g), *p*-coumaric acid (4.01 µg/g), vanillic acid (6.264 µg/g), kaempferol (14.832 µg/g), coumarin (15.382 µg/g), caffeic acid (157.432 µg/g), and rutin (286.9 µg/g). Similarly, *C. arvensis* methanol extract demonstrated an abundant amount of phenolic compounds, including *p*-coumaric acid (54.50 µg/g), *p*-hydroxybenzoic acid (47.82 µg/g), and syringic acid (22.50 µg/g) [26]. The phenolic and flavonoid compounds of these extracts mentioned above are quite different from those found in the current study. The amount of caffeic acid (780.0 µg/g), kaempferol (780.0 µg/g), and rutin (1,810.0 µg/g) was remarkably higher in *C. aucheri* methanolic extract than in methanolic extract of *C. galaticus*. Chlorogenic acid (22,690 µg/g) was the major phenolic in *C. aucheri.* Other major components in the extract were (+)-catechin (8,630 µg/g), rosmarinic acid (4,370 µg/g), and rutin (1,810 µg/g). The major components exhibit a broad spectrum of biological activities, including antioxidant, anticancer, and antimicrobial [27–29]. It has been demonstrated that the amounts of chlorogenic acid found in the extract are tightly correlated to metal chelating activity [30]. In accordance with the literature, we can conclude that the major polyphenolic compounds such as chlorogenic acid, (+) catechin, rosmarinic acid, rutin, protocatechuic acid, and kaempferol present within the methanolic extract of *C. aucheri* are responsible for its antioxidant activity. The results showed that major phytochemicals in *C. aucheri* were polar compounds extracted by methanol, which were determined to have the most potent antioxidant activity, including phenolics and saponins.

Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce reactive oxygen species (ROS). Some antioxidants stop or eliminate the side effects of ROS and prevent some disorders, such as cancer, cardiovascular diseases, diabetes, infections, and ischemia [31]. The use of only one method does not reflect the antioxidant activity of plant extracts due to the complicated structure of bioactive secondary metabolites. Hence, chiefly five methods were used in order to detect the antioxidant activity of *C. aucheri* with different solvents. Health-damaging lipid peroxidation may be induced by ferrous ions, which have a significant mission as catalysts in the production of OH radicals. Therefore, the metal ion chelating ability of some phytochemicals is an important activity in eliminating the free radicals. The chelating capacities of the extracts were estimated by Ferrozine assay. The obtained results showed that the methanolic extract has the highest capacity to chelate ferrous ions. Al-Rifai et al. [32] determined the antibacterial and antioxidant activities of the ethanol extracts of two *Convolvulus* species namely, *C.*

austroaegyptiacus and *C. pilosellifolius*, and reported that the DPPH radical scavenging activity of *C. austroaegyptiacus* (IC_{50}: 21.81 μg/mL) was lower than that of *C. pilosellifolius* (IC_{50}: 17.62 μg/mL). Thakral et al. [33] reported that the IC_{50} values obtained from the DPPH and NO assays in *C. arvensis* methanolic extracts were determined at 131.03 and 130.12 μg/mL, respectively. The researchers found that phenolic compounds such as phenolic acids, flavonoids, and tannins, which are found in *C. arvensis*, could be the responsible compounds for antioxidant activity [33, 34]. In the current research, the antioxidant activity of the studied extracts exhibited a satisfactory correlation with their total phenolic, flavonoid, and saponin contents. Our results display that these bioactive compounds are consistent with the assessed antioxidant activity tests. The antioxidant activities of the extracts changed in the same order as the total bioactive compounds (methanol>acetone>petroleum benzine). In this context, the bioactive compounds could be considered major contributors to the antioxidant effect of *C. aucheri* extracts.

Cervical cancer is the third deadliest cancer for women in the emerging countries [35]. Plant origin products show hopeful resources of antitumor substances with lower adverse effects as compared to synthetic medications [36]. In previous studies, researchers demonstrated that different solvent extracts of *C. arvensis* had superior cytotoxic potential on lymphoblastic leukemia, Jurkat cells, rhabdomyosarcoma tumor cells, and HeLa cells [9, 37, 38]. Kaur and Kalia [39] reported that *C. arvensis* ethanolic extract had prominent inhibition on Colo-205 and IMR-32 cell viability by 73% and 85%, respectively. In another study, researchers showed that the *C. arvensis* extract could be regarded as a hopeful anticancer agent, with over 50% prevention of cancer progress [40]. As cancer cells grow, they secrete chemicals that induce new blood vessel growth, which is called angiogenesis. The extracts of *C. arvensis* were reported to have the capability to block angiogenesis in cancer [41]. In addition, the stimulating effect of this plant on the immune system was also revealed [42]. Polyphenols and saponins have been proved as anticancer agents [43, 44]. It is reported that the anticancer effect of *C. arvensis* may be caused by these phytochemicals. In terms of the presence of phenolics in the *C. aucheri*, our results are in good agreement with the literature. In this study, kaempferol in the extract was determined at 780.0 μg/g. It is known that kaempferol, which is a flavonoid, exhibits antitumor properties through the induction of G2/M phase cell cycle arrest and apoptosis on HeLa cells [45].

Conclusion

For the first time, this research notifies the antioxidant, antiproliferative potentials, and HPLC profile of *C. aucheri*. The methanol extract of *C. aucheri* was determined to have different phenolic compounds as identified by HPLC. It can be proposed that the obtained biological activity of the *C. aucheri* extract can be ascribed to the existence of these phenolic compounds. Our results showed that *C. aucheri* could be accepted as a novel and alternative natural antioxidant and antitumor source. For this reason, this plant may shed light on the design of the new drug or food additive formulations. On the other hand, such studies are valuable from the aspect of revealing the contents of traditional plants. Although this is the first study carried out on this plant, further *in-vitro* and *in-vivo* studies are necessary in order to better understand its potential.

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Conflict of interest

The authors declare no conflict of interest.

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Novel protocol for selection of SARS-CoV-2 convalescent plasma donors

Novi protokol za izbor davaoca plazme nakon SARS-CoV-2 infekcije

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Abstract

Background/Aim. Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) 2019 infection represents a global problem. At this moment, in October 2020, there is no vaccine or efficient treatment for infected patients. Treatment with blood plasma rich with anti-SARS-CoV-2 specific antibodies might be a safe, and effective therapy for COVID-19 patients. **Methods.** A total of 768 patients were analyzed in this study, whose samples were collected in a time interval from May 1, 2020, till August 15, 2020. Patients were enrolled in the study from COVID-19 hospitals and out-clinics. In-house ELISA tests were developed to measure the concentration of anti-S1S2 spike and anti-nucleoprotein (np) (IgG, IgA, IgM) SARS-CoV-2 antibodies. Blood convalescent plasma was selectively collected from recovered patients according to specific antibodies concentration. **Results**. The highest concentrations of anti-S1S2 spike or anti-np specific IgG antibodies were detected in patients with the moderate/heavy clinical form of the infection. An extremely high concentration of anti-S1S2 spike IgG and anti-np IgG was demonstrated in 3% and 6% of patients who recovered from severe COVID-19, respectively. Of tested hospitalized patients, 63% and 51% had modest levels of anti-S1S2 spike and anti-np, respectively. After 60 days, in our selected donors, concentrations of anti-S1S2 spike IgG and anti-np IgG antibodies increased in 67% and 58% of donors, respectively. **Conclusion.** In-house developed ELISA tests enable a novel protocol for selecting convalescent blood plasma donors recovered from SARS-CoV-2 infection.

Key words:
antibody specificity; clinical protocols; covid-19 serotherapy; enzyme-linked immunosorbent assay; SARS-CoV-2; plasma; tissue donors.

Apstrakt

Uvod/Cilj. Infekcija *Severe Acute Respiratory Syndrome Corona Virus 2* (SARS-CoV-2) u 2019. godini predstavlja globalni problem. U ovom trenutku (oktobar 2020. godine), ne postoji vakcina, niti efikasan tretman zaraženih bolesnika. Primena krvne plazme bogate antitelima specifičnim za SARS-CoV-2 može da bude sigurna i efikasna terapija za bolesnike sa COVID-19. **Metode.** Ispitivanjem je obuhvaćeno ukupno 768 bolesnika čiji su uzorci krvne plazme bili prikupljeni u vremenskom intervalu od 1. maja 2020. do 15. avgusta 2020. godine. Bolesnici, uključeni u studiju, su bili iz COVID-19 bolnica i ambulanti. *In-house* ELISA testovi su razvijeni za merenje koncentracije antitela na S1S2 *spike* i antitela na nukleoprotein (anti-np) (IgG, IgA, IgM) SARS-CoV-2. Krvna plazma rekonvalescenata je selektivno sakupljana prema koncentraciji specifičnih antitela. **Rezultati.** Najviše koncentracije anti S1S2 *spike* ili anti-np IgG specifičnih antitela detektovane su kod bolesnika sa srednje teškom/teškom kliničkom formom infekcije. Ekstremno visoke koncetracije anti S1S2 *spike* IgG i anti-np IgG nađene su kod 3%, odnosno 6% bolesnika oporavljenih od teškog oblika COVID-19. Od ispitanih hospitalizovanih bolesnika, 63% i 51% su imali minimalne vrednosti anti S1S2 *spike* i anti np antitela, redom. Nakon 60 dana, u plazmi izabranih donora koncentracija anti S1S2 *spike* IgG i anti-np antitela porasla je kod 67%, odnosno 58% donora, redom. **Zaključak.** *In-house* razvijeni ELISA testovi omogućavaju novi protokol za odabir davaoca krvne plazme oporavljenih od SARS-CoV-2 infekcije.

Ključne reči:
antitela, specifičnost; protokoli, klinički; covid-19 seroterapija; elisa; SARS-CoV-2; plazma; tkivo, davaoci.

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Introduction

To date (October 2020), there are no drugs approved by the US Food and Drug Administration (FDA) for treating patients with coronavirus disease 2019 (COVID-19). Current clinical research includes measures for infection prevention and control and, also, supportive care, including oxygen and mechanical ventilation support when necessary. A myriad of drugs that have been approved for other indications are used, as well as a variety of new drugs whose effects are being studied in several hundreds of clinical trials that are underway worldwide.

Scientists around the world are working tirelessly, and information about the mechanisms of transmission, the clinical spectrum of the disease, new diagnostics, and strategies for prevention and therapy is spreading rapidly. In general, there are many unknowns regarding the virus-host interaction, the development of the epidemic, the possibilities and success of treatment, and any research in this field is extremely important.

Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) is spread primarily by respiratory droplets - close face-to-face contact. The infection can be spread by asymptomatic, presymptomatic, and symptomatic carriers. The average time from exposure to the virus to the onset of symptoms is 5 days, and 97.5% of people develop symptoms within 11.5 days. The most common symptoms are high fever, dry cough, and difficulty breathing. Radiographic and laboratory abnormalities, such as lymphopenia and elevated lactate dehydrogenase, are common but nonspecific. The diagnosis is made by detecting SARS-CoV-2 by polymerase chain reaction (PCR), even though false-negative test results can occur in 20% to 67% of patients, depending on the quality and timing of the test. The disease may have an asymptomatic or fulminant course, characterized by sepsis and acute respiratory failure. Approximately 5% of patients with COVID-19, i.e., 20% of those hospitalized, have serious symptoms that require intensive care. More than 75% of patients hospitalized with COVID-19 require additional oxygen, which includes the best therapeutic measures for the treatment of acute hypoxic respiratory failure [1].

In current trials, antiviral therapy, immune modulators, and anticoagulants are being tested. The death rate from COVID-19 varies significantly depending on age, ranging from 0.3 deaths per 1,000 patients (age 5 to 17 years) to 304.9 deaths per 1,000 cases in patients aged 85 years or older in the United States of America (USA). Among patients hospitalized in the intensive care unit, the mortality rate is up to 40%. At least 120 SARS-CoV-2 vaccines are being prepared. Until an effective vaccine is available, the primary methods for reducing the spread are face masks, social distancing, as well as the application of all therapeutic modalities [1].

Since some 10% of patients fail to cope with this disease despite the applied measures, doctors initiated collecting and therapeutic application of convalescent plasma from recovered COVID-19 patients after 14 days from the end of the disease. The idea is old, and it is based on the use of plasma rich in IgG antibodies, which would facilitate the fight, support immunity, and alleviate the clinical picture. Four to six or eight weeks after infection, there should be enough antibodies in the patient's blood to neutralize the virus and theoretically limit the infection [1].

Plasma donors can be convalescents who have had a confirmed positive PCR test for the virus, no symptoms for 14 days, negative PCR test at the time of donation, and have a high titer of specific neutralizing IgG antibodies. In most cases, it is a period of one month [2].

Treatment with plasma from patients recovered from viral infections was first reported during the 1918 influenza pandemic. The first report of 5 critically ill patients with COVID-19 treated with convalescent plasma containing neutralizing antibodies showed an improvement in clinical status in all participants. This was reflected as a combination of changes in body temperature, assessment of organ involvement, oxygen partial pressure, viral load, serum antibody titers, routine blood biochemical index, acute respiratory distress syndrome (ARDS), ventilation, and extracorporeal membrane oxygenation monitored before and after administration of convalescent plasma [3].

However, a subsequent multicenter, randomized clinical trial in China of 103 patients with severe COVID-19 found no statistical significance in the time to clinical improvement within 28 days in patients receiving convalescent plasma compared to standard treatment (51.9% compared to 43.1%, respectively) [4]. Since the study was discontinued, this limited the possibility of detecting a clinically important difference. Alternative approaches include the use of convalescent hyperimmune globulin produced from plasma and monoclonal antibodies directed to SARS-CoV-2 [5, 6].

The virulence of a particular virus is often considered related to the immune response it encounters in the human body. For COVID-19, the immune response is divided into two phases. The initial phase is thought to involve the development of a specific immune response needed to eliminate the virus and stop the disease from progressing. It is, therefore, important to provide treatments that have previously stimulated an immune response, such as antibodies and immunomodulators [2, 6]; there may be a loss of 20% of its own antibodies that will resume a few days later. The immune response, however, weakens with age, making the elderly particularly vulnerable. If the immune response is weak or damaged due to other complications such as cardiovascular disease and diabetes, the virus multiplies and can lead to tissue damage. The second phase of the immune response leads to the damage of the cells causing pneumonia. Such infection is very dangerous since pneumonia causes respiratory disorders, making it difficult for individuals to breathe on their own. Different therapies are tested in different phases of the disease, making it important to identify the exact phase of the patient’s disease before starting the treatment [1].

The World Health Organization warns that there is no evidence that the presence of antibodies means that you are protected from reinfection with COVID-19. The level of immunity and how long the immunity lasts are still unknown. Ongoing studies will eventually reveal more data on this.

Due to all of the above, any research related to COVID-19 therapy is crucial, including the collection and administration of convalescents' plasma to patients.

Patients with a resolved viral infection will develop an immune response with polyclonal antibodies to various viral antigens, and some of these polyclonal antibodies, if the patient has them in high titers, will probably neutralize the virus and prevent more severe forms of the infection. Doctors are trying to use this fact in a therapeutic sense, and that is why the idea of collecting plasma (rich in antibodies) from the convalescents after COVID-19 came about [1–13].

The potential danger of using such plasma in terms of side effects in recipients, including but not limited to allergic reactions, acute lung injury, and circulatory overload in patients with cardiac disorders, must also be mentioned here [14–16].

Our institution also worked on testing the antibody titers in patients who overcame COVID-19 and developed its own protocol for collecting plasma by a manual technique using a system of multiple bags. Since plasma transfusion is a routine medical procedure, no new medical approvals are required to perform it. In fact, the same basic concept was used to treat several Ebola patients with convalescent serum during the 2014–2015 epidemic [17].

Methods

Specific antibody detection

Specific anti-S1S2 SARS-CoV-2 antibodies and anti-nucleoprotein (np) SARS-CoV-2 antibodies were quantified with a home-based ELISA test. Specific SARS-CoV-2 S1S2 and SARS-CoV-2 np (Sino Biological, EU) antigens were coated on polystyrene microwells (0.5 ug/mL, coat buff, 100 uL/well, overnight, 4 ºC). After 3 washing cycles (phosphate buffer saline – PBS, 0.01% Tween) microwells were blocked (PBS, 1% bovine serum albumin – BSA, 1 h, room temperature – RT), and after further washing cycles, patient samples were incubated in duplicates (1/100 diluted, 100 uL/well, 3 h RT, shaking, 60 revolutions – rot/min). Secondary antibodies were incubated after washing cycles (goat anti-human IgG, IgA, or IgM Southern Biotech, USA, 100 uL/well, 1.5 h RT, shaking, 60 rot/min). Final washing cycles were followed with substrate incubation (TMB solution, Siemens, EU, 100 uL/well, 15 min RT, dark), and after stopping (Stop solution, Siemens, EU, 50 μL/well) optical density of each sample was determined at 450 nm (Synergy HT, EU spectrophotometer). The concentration of every sample was expressed in equivalent units (EU)/mL and determined from the standard curves obtained with monoclonal antibodies specific for S1S2 or np (Sino Biological, EU). Equivalent unit is the concentration of tested antibodies from a patient's plasma sample that had the same, i.e. equivalent value of optical units in the ELISA test as the monoclonal antibody specific for the S1S2 domain and the monoclonal antibody specific for the SARS-CoV-2 nucleoprotein.

Patients

A total number of 768 patients, whose samples were collected in the time interval from May 1, 2020, till August 15, 2020, were analyzed in this study. The first group were recovered patients from COVID-19 hospitals (n = 457) and the second group were patients from COVID-19 out-clinics (n = 311). Serum samples frozen from volunteer healthy donors were used as a negative control (n = 160). All control negative samples were collected in the period April – August 2019, a time period long before any signs of the SARS-CoV-2 pandemic.

Clinical data collection

Data were collected after fulfilling the electronic questionnaire (www.covidmirage.com).

Convalescent plasma collection

Plasma collection was performed in patients who had undergone COVID-19, had specific anti-S1S2 SARS-CoV-2 antibodies and np SARS-CoV-2 antibodies present in the circulation, and wanted to donate their plasma to treat other people. Four to six or eight weeks after infection, there should be enough antibodies in the patient's blood to neutralize the virus and theoretically limit the infection [1]. Before collecting plasma, each donor was tested for markers of transfusion-transmitted infections (hepatitis B, hepatitis C, HIV 1/2, and syphilis) by ELISA tests and PCR technique, checked for the titer of specific anti-S1S2 SARS-CoV-2 antibodies and np SARS-CoV-2 antibodies and their blood group was determined (ABO and Rhesus factor). The procedure consists of placing a venous catheter (Cell Connect CC1, Fresenius Medical Care, Germany) into the cubital vein (most often), attaching a multiple bag system (TH, Jiaxing Tianhe Pharmaceutical Co., Ltd, China) containing 63 mL of anticoagulant-preservative CPD/SAG-M solution – composition: citric acid – 0.299 g; sodium citrate – 2.63 g; monobasic sodium phosphate – 0.222 g and dextrose – 2.55 g in a primary bag while accompanying (satellite) bag contained 100 mL of optimal SAGM additive solution (containing: NaCl – 877 mg; adenine – 16.9 mg; dextrose – 900 mg and mannitol – 525 mg), intended for resuspension of concentrated erythrocytes. After that, 450 mL of blood is taken from a voluntary donor, centrifugated (Jouan, Thermo Scientific France) at a speed of 3,500 rpm for 10 min at a temperature of 4 ± 2 °C, and separated into components, i.e., cell suspension and plasma, manually. During the centrifugation time, the donor is given via *iv* catheter 0.9% NaCl solution to maintain cannula patency and volume recovery. In the further procedure, the cellular elements of the blood are returned to the donor after centrifugation, and after that, the procedure of taking another unit of whole blood is repeated. An average of 611.47 (310–680) mL of convalescent plasma was thus collected from each donor, and the procedure took an average of 90 min. Plasma was frozen at -60 °C within 6 h maximum and stored in freezers at -40 ± 5 °C (Fiocchetti, Frigoriferi Scientifici, Italy) with a shelf life of three years. Whole blood (450 ± 45 mL) for the preparation of plasma units was taken from donors (aged 25 to 55 years), unreactive to markers of transfusion-transmitted diseases (hepatitis

B and C, AIDS and lues), performed by ELISA tests and PCR technique, with orderly clinical and laboratory findings.

Statistical analysis

A comparison of antibody concentration between investigated groups was performed with the Mann Whitney test (Figure 1). Data analysis was performed with the software package StatGraph Prism 6.

Results

Average anti-SARS-CoV-2 antibody concentration in investigated groups

As previously explained, we have quantified specific anti-S1S2 spike and anti-np antibodies in COVID-19 patients with heavy or mild clinical picture treated during 3–5 months. All our hospitalized patients demonstrated heavy (but not critical) clinical presentation, with an average of 11 days of hospital treatment. As expected, sera from hospitalized patients contained significantly more anti-S1S2 spike-specific IgG (Figure 1A) and anti-np IgG antibodies (Figure 1B) compared to the group treated out of hospital facilities. Similarly, the group with severe clinical form presented significantly more anti-S1S2 spike specific IgA (Figure 1C) and anti-np IgA antibodies (Figure 1D) compared to the less severe COVID-19 group. Interestingly, the group with severe COVID-19 symptoms demonstrated a significantly higher average concentration of anti-S1S2 spike specific IgM (Figure 1C) and anti-np IgM antibodies (Figure 1D) compared to another group.

Selection of convalescent plasma donors according to the concentration of SARS-CoV-2 specific antibodies

Widely accepted criteria for selection of convalescent plasma donors are levels of specific anti-SARS-CoV-2 IgG antibodies, most frequently antibodies to envelope antigens. In our group of hospitalized patients, 225 had detectable anti-

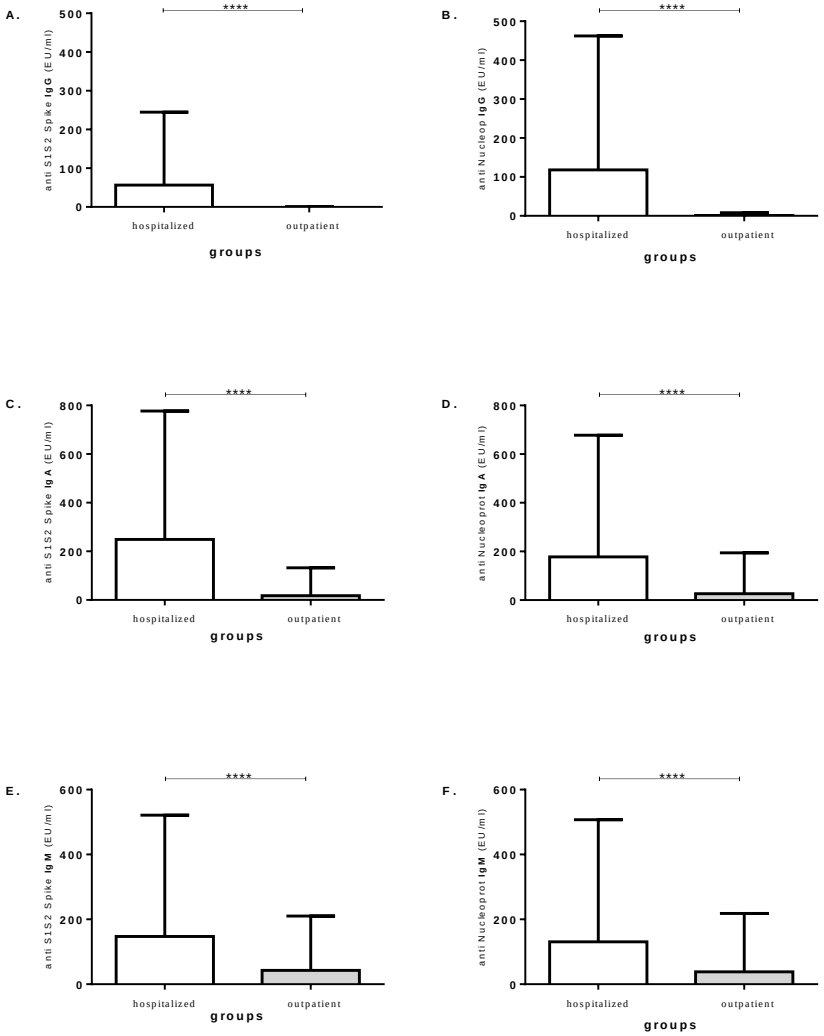

Fig. 1 – Average concentration of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) specific antibodies in investigated patients. All values are expressed as mean ± standard deviation (**$p < 0.0001$, Mann Whitney test). EU – equivalent units.**

S1S2 spike specific IgG antibodies, while 202 had detectable anti-np IgG antibodies. The stratification according to the concentration of specific potential anti-SARS-CoV-2 antibodies in plasma donors are shown is Table 1. Patients that demonstrated a level of specific anti-SARS-CoV-2 antibodies of IgG class in hundreds or thousands EU/mL were selected as convalescent plasma donors. Several patients with a high concentration of specific anti-SARS-CoV-2 antibodies had to be excluded as potential donors according to medical indications (cardiovascular disease, hemophilia).

Time-related concentration change of SARS-CoV-2 specific antibodies in samples of convalescent plasma donors

Until the end of August, we have selected and collected plasma rich in specific anti-SARS-CoV-2 antibodies from 12 people (12/225, 5.3% of patients cured of severe COVID-19). All of these patients had at least two points of specific antibody measurements, with a time interval of no less than 60 days. Interestingly, the concentration of anti-S1S2 spike IgG antibodies increased in 67% of our plasma donors, parallel with the increase of anti-np IgG antibodies in 58% of donors (Table 2). The concentration of anti-S1S2 spike and anti-np of IgA class increased in 50% of all donors. Interestingly, while anti-S1S2 spike IgM concentration decreased in donors, the concentration of anti-np IgM antibodies again was increased in our plasma donors.

Discussion

Convalescent plasma has a strong historical advantage and good biological value. Although this therapeutic approach is promising, it has not yet been shown to be safe in the treatment of COVID-19. Data after transfusion of ABO-compatible human convalescent plasma COVID-19 to 5,000 hospitalized adults with severe or life-threatening COVID-19, of which 66% were in the intensive care unit, were analyzed. The incidence of all serious adverse events, including mortality (0.3%), in the first 4 hours after transfusion was < 1%. Of the 36 reported adverse reactions, 25 were convalescent plasma related, including mortality (n = 4), circulatory overload associated with transfusion (n = 7), acute lung injury associated with transfusion (n = 11), and severe allergic reaction after transfusion (n = 3). However, physicians estimate that only 2 of 36 reactions are definitely associated with convalescent plasma transfusion. The mortality rate after the 7th day was 14.9%. Given the lethal nature of COVID-19 and the large population of critically ill patients included in these analyzes, the mortality rate does not appear to be too high. These early indicators suggest that convalescent plasma transfusion is safe in hospitalized patients with COVID-19 [13–16].

Table 1

Identification of potential convalescent plasma donors according to concentration of specific anti severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) antibodies

Ab conc. (EU/mL)	Patients, n (%)	
	anti-S1S2 spike IgG	anti-nucleoprotein IgG
> 1,000	5 (3)	10 (6)
> 100	19 (8)	31 (15)
> 10	59 (26)	57 (28)
5–10	142 (63)	104 (51)

Ab conc. − antibody concentrations; EU – equivalent units.

Table 2

Time related concentration change of severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) specific antibodies in samples of convalescent plasma donors

Patient's ID, 1st sample	Patient's ID, 2nd sample (after 60 days)	Anti-S1S2 spike Ab			Anti-nucleoprotein Ab		
		IgG	IgA	IgM	IgG	IgA	IgM
617	1,009	▲	▲	▼	▼	▼	▼
666	1,010	▲	▲	▼	▲	▲	▲
778	1,011	▲	▲	▲	▲	▲	▲
664	1,012	▲	▲	▼	▲	▲	▲
12	723	▲	▼	▲	▼	▼	▼
956	1,182	▼	▼	▼	▲	▼	▼
256	1,090	▼	▼	▼	▼	▼	▼
950	1,249	▼	▼	▲	▼	▼	▲
1,030	1,275	▲	▲	▲	▲	▲	▲
367	1,042	▲	▼	▼	▼	▼	▼
119	1,288	▼	▼	▼	▲	▲	▲
1,065	1,287	▲	▲	▲	▲	▲	▲
Increase		67%	50%	42%	58%	50%	58%

Ab – antibody; EU – equivalent units; ID – identity of a patient (donor) indicated by the original protocol number (e.g., donor number one gave the first sample under number 617, and under number 1009, donated plasma for the second time).

In addition to the antiviral mechanisms of neutralizing antibodies, the immunomodulatory effects of plasma components may be beneficial. Several small and large studies have shown the effects of convalescent plasma on the treatment of severe viral illness [diphtheria, scarlet fever, pertussis, poliomyelitis, measles, mumps, flu, Ebola, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome (MERS)] as fever, nausea, allergic reactions, blood-borne pathogens transmission, and some serious adverse events such as transfusion-related acute lung injury (TRALI), transfusion-associated circulatory overload (TACO), and antibody-dependent enhancement (ADE) [13–16, 18–20].

In June 2020, the USA Department of Defence began an action to collect plasma units from patients who had fully recovered from COVID-19 in order to support the development of effective treatment. The goal is to collect 10,000 units of this plasma until September 10, 2020 [19].

Until October 2020, there were no licensed vaccines or targeted therapies against the virus itself. Plasma with anti-SARS-CoV-2 antibodies, obtained from recovered individuals with confirmed COVID-19 infection, has been primarily collected using apheresis devices and stored in blood banks in some countries to be administered to patients with COVID-19 in order to reduce the need for intensive care and a lower mortality rate. Therefore, it is necessary to point out some important issues related to convalescent plasma and its use in the treatment of patients as a form of anti-viral therapy. The protective effect can last for weeks and months. After the donor's assessment, 200–600 mL of plasma can be collected with apheresis devices (used in the world and in our country). The donation interval may vary among countries. Hence, there is the necessity of testing antibody titer values. Although limited published studies are not prospective or random until vaccination or targeted antiviral therapy is approved, plasma therapy appears to be a safe and likely effective treatment for critically ill patients with COVID-19. It can also be used for prophylactic purposes, but the safety and efficacy of this approach should be tested in randomized clinical trials, and a conclusion reached [20].

Eligibility criteria for plasma donors may vary from country to country but certainly include, above all, a safe procedure, health and antibody titers checks, and consent to the procedure [20–22]. The antibody titer will vary according to the duration between the time of collection and the onset of infection. According to literature data, the titer ranges from 1: 1,000 [20] to 1: 160 and 1: 640 [22]. In previous studies, it has been observed that seroconversion occurs between 8 and 21 days after the onset of symptoms. In clinical trials, one plasma unit was given initially (200 mL) and repeated after 12 h. The duration of antibody efficacy is not known, but it is estimated that it will last for weeks to several months [20–24]. On August 23, 2020, the FDA issued an approval for the emergency use of convalescent plasma COVID-19 for the treatment of hospitalized patients with COVID-19. There are insufficient data to recommend for or against the use of convalescent plasma for the treatment of COVID-19. Available data suggest that serious adverse reactions after administration of COVID-19 convalescent plasma are rare and consistent with the risks associated with plasma infusions in other indications. The long-term risks of treatment with convalescent plasma COVID-19 and whether its use reduces the immune response to SARS-CoV-2, making patients more susceptible to reinfection, have not been assessed. Convalescent plasma should not be considered the standard of care for the treatment of patients with COVID-19 [25].

Prospective, well-controlled, adequately initiated randomized trials are needed to determine whether convalescent plasma is effective and safe for the treatment of COVID-19.

All this confirms the fact that it is necessary to collect and store certain quantities of convalescent plasma in order to provide reserves, which encouraged us to provide plasma reserves for the treatment of COVID-19 patients with modest funds and without additional costs.

Conclusion

In-house developed ELISA tests enable a novel protocol for the selection of convalescent blood plasma donors. According to our data, it is necessary to recruit and test a large number of patients who have recovered from severe COVID-19 in order to have a sufficient number of appropriate convalescent plasma donors.

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CASE REPORTS

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The retrograde technique for recanalization of chronically occluded coronary arteries: case series report

Tehnika retrogradnog pristupa kod rekanalizacije hronično okludiranih koronarnih arterija: prikaz serije slučajeva

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Abstract

Introduction. Chronic total occlusion (CTO) of the coronary artery still represents one of the most challenging lesion subsets in the field of interventional cardiology. Considering the complexity and increased risk posed by the retrograde approach, it is most often performed after a failed antegrade approach. **Case report.** We present a series of cases describing the retrograde approach as a special technique for treating CTO of the coronary artery. All cases had some special characteristics that are part of a dedicated portfolio in every catheterization lab today. In our series of cases, all three percutaneous coronary interventions (PCI) with a different strategies of the retrograde approach and supported with rotational atherectomy or intravascular ultrasound finished with successful recanalization of CTO. **Conclusion.** In cases where there is the presence of "interventional" collaterals, as well as when the antegrade approach is very difficult, the retrograde approach can increase the success rate of procedures. The retrograde approach requires a long learning curve as well as very skilled and experienced operators who are able to perform the procedure independently.

Key words:
coronary occlusion; endovascular procedures; methods; percutaneous coronary intervention.

Apstrakt

Uvod. Hronične totalne okluzije (HTO) koronarnih arterija i dalje predstavljaju neke od najizazovnijih lezija na polju interventne kardiologije. S obzirom na složenost i povećani rizik koji nosi sa sobom retrogradni pristup, HTO se najčešće izvodi nakon neuspelog anterogradnog pristupa. **Prikaz bolesnika.** Prikazana je serija slučajeva sa opisom retrogradnog pristupa kao specijalne tehnike lečenja koronarnih arterija putem HTO. Svi slučajevi su imali neke posebne karakteristike koje su danas deo svakodnevog portofolija u svakoj sali za kateterizaciju srca. Sve tri prikazane perkutane koronarne intervencije izvršene različitim strategijama retrogradnog pristupa uz podršku rotablatora ili intravaskularnog ultrazvuka okončane su uspešnom rekanalizacijom HTO. **Zaključak.** U slučaju kada postoje „interventne" kolaterale, kao i kada je anterogradni pristup veoma težak, retrogradni pristup može povećati uspešnost procedure. Retrogradni pristup zahteva dugotrajno učenje, kao i veoma iskusne operatore koji su sposobni da samostalno izvode ovakve procedure.

Ključne reči:
koronarna okluzija; endovaskularne procedure; metodi; perkutana koronarna intervencija.

Introduction

For many years, percutaneous treatment of chronic total occlusions (CTO) of the coronary arteries has been a clinical and technical challenge for interventional cardiologists. Successful recanalization rates are increasing primarily due to the constant development of techniques and technological advancements for percutaneous coronary interventions (PCI), along with the growing experience of operators [1, 2]. Many retrospective and prospective registries show better survival, improved left ventricular function, reduced risk of malignant arrhythmias, as well as coronary artery bypass graft surgery (CABG) in procedural success groups [3, 4]. Recent randomized clinical studies suggest a better quality of life in patients

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with successful recanalization of an occluded blood vessel compared to patients on optimal medical therapy (OMT) [5–8]. Among the various techniques for PCI CTO, the retrograde approach with different strategy types is considered the most complex. The retrograde approach should be considered in occlusions with "interventional" collaterals (i.e., collaterals deemed to be negotiable by the operator depending on his/her experience), diseased landing zone, bifurcation at distal cap, and/or proximal cap ambiguity [9, 10].

We presented a complex retrograde technique as the first strategy choice according to the indication in every single case, combined with a contemporary armamentarium of available devices (guiding catheter extension, rotablator of intravascular imaging) to achieve a successful and optimal result. All cases were performed at the Cardiology Department of the University Clinical Center of Serbia (UCCS).

Case report

Case 1

A 69-year-old male had a posterior myocardial infarction in April 2019 as the first manifestation of coronary heart disease. He generally complained of typical anginal symptoms with minimal physical exertion. Stress echocardiography (SEHO) test was not done. Echocardiographic examination showed a left ventricle of normal dimensions with hypokinetic inferolateral wall and preserved systolic function; ejection fraction (EF) was 50%. Apart from hypertension and a positive family history of cardiovascular disease (CVD), the patient had no other risk factors.

During index hospitalization, primary PCI was attempted, in which a single-vessel coronary disease, a calcified subocclusive lesion about 20 mm long in the proximal segment of the dominant circumflex (Cx) artery, intermediate stenosis in the medial segment of the left anterior descending (LAD), and minor right coronary artery (RCA) were observed. Furthermore, catheter guide EBU 3.5/6F was placed in the left main (LM) shaft through the right radial approach. After a challenging placement of the Sion® blue (Asahi Intecc Co., Japan) coronary wire in the distal segment of Cx artery, a 2.5 x 20 mm semi-compliant balloon was placed at the lesion site after being supported by a GuideZilla 6F extension catheter (Boston Scientific, Marlborough, MA). Due to the inadequate expansion of the semi-compliant balloon, an attempt was made to place the non-compliant (NC) balloon without any success (Figure 1).

In May of the same year, PCI of the same lesion was attempted by the femoral approach 6F. The same catheter guide and coronary wire were placed, after which a 3 x 20 NC balloon predilatation was performed. A larger dissection was formed, and the stent could not be placed due to the deviating angle and the existing extensive calcifications (Figure 2). It was proposed to present the patients to the Heart Team, which met in June at the UCCS. The council made the decision to do the first fractional flow reserve (FFR) for the lesion on the LAD, and if the lesion is functionally significant, the patient will be offered surgical revascularization of the myocardium. Otherwise, trying PCI Cx again using a rotablator was suggested.

In the same month, the EBU 3.5/7F guide catheter was placed by right radial access, and the flow reserve was measured at 0.84. With the support of the Corsair microcatheter (Asahi Intecc Co., Japan), Gaia 2 (Asahi Intecc Co., Japan) still has not undergone occlusion in the proximal Cx artery segment with developed ipsilateral collaterals (CC 1-2). Further intervention was abandoned (Figure 3).

A month later, in December 2019, a femoral approach with an EBU 3.5/7F guide catheter was set up for a fourth PCI attempt at the same center. After the placement of the temporary PM, the coronary arteries of BMW (Abbott Vascular) as well as Fielder XT (Asahi Intec Co., Japan) did not undergo occlusion, and further intervention was abandoned (Figure 4).

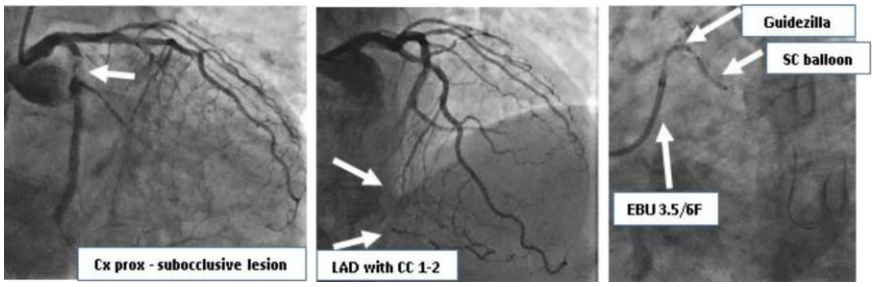

Fig. 1 – Failed recanalization attempt during the index hospitalization.

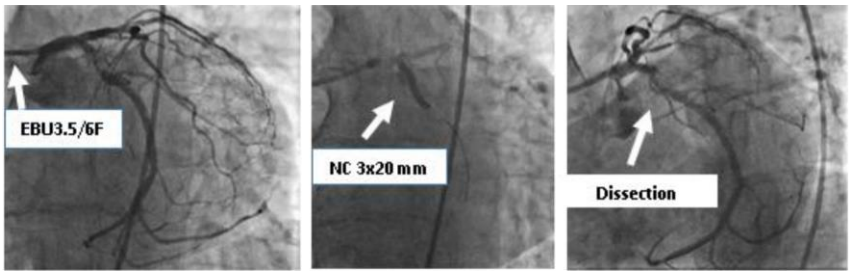

Fig. 2 – Second failed attempt.

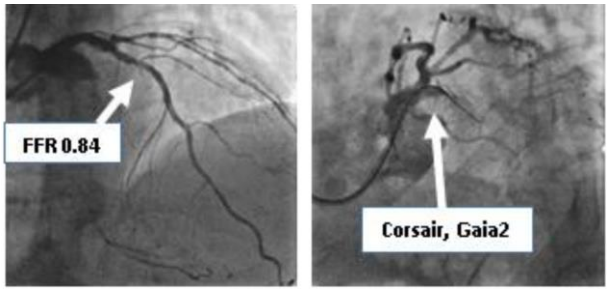

Fig. 3 – Functional assessment of the significance of left anterior descending (LAD) artery stenosis and the third unsuccessful antegrade recanalization attempt.

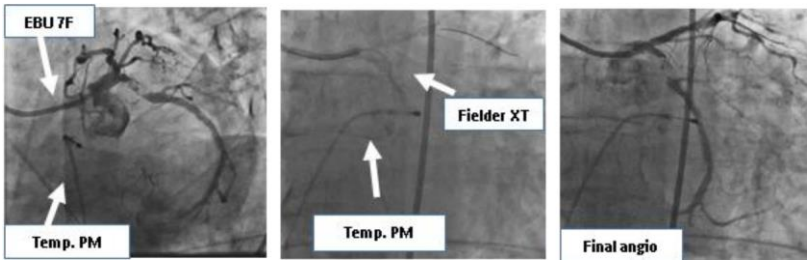

Fig. 4 – Fourth recanalization attempt in the same percutaneous coronary intervention (PCI) center.

It was concluded that the fifth attempt would be in a dedicated center.

In July 2020, the intervention began with a left femoral approach, 7F. The right femoral artery was not palpable, as was the right radial artery. Due to the pronounced calcifications of the left radial and ulnar arteries, placement of the introducer was impossible (Figure 5).

After the placement of EBU 3.5/7F, with the support of the Corsair coronary microcatheter Fielder XT, Gaia 3, and Confianza pro (Asahi Intecc Co., Japan) did not undergo occlusion. After the evaluation of interventional ipsilateral collaterals, a retrograde approach was attempted (Figure 6). The Asahi Sion® black (Asahi Intecc Co., Japan) wire supported by a Corsair microcatheter passed through the septal collat-

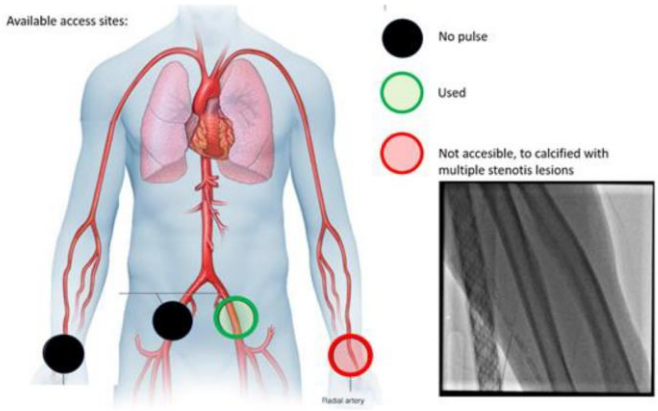

Fig. 5 – Available vascular approaches.

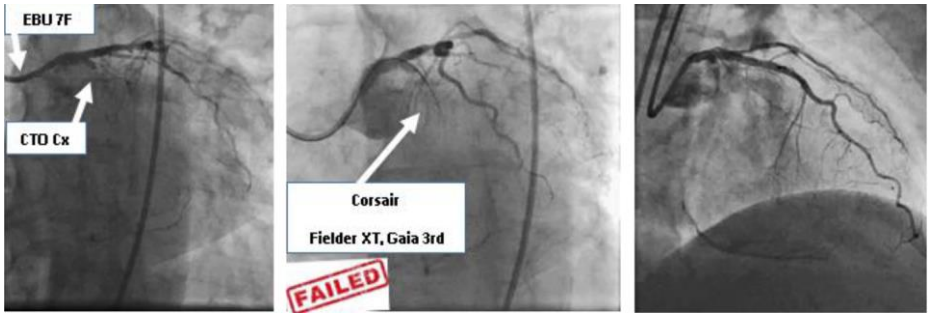

Fig. 6 – Unsuccessful attempt at antegrade recanalization in a dedicated center.

erals into the Cx and without resistance through the distal occlusion cap all the way to the LM. Corsair remained "stuck" in the collateral being intervened. Since the Sion black wire could not be placed in the catheter guide after several attempts, a "rendezvous" in the proximal segment of the Cx artery was attempted with a Finecross® microcatheter being placed antegradely unsuccessfully. Finecross® (Terumo Interventional Systems, Tokyo, Japan) then replaced the Corsair as a retrograde catheter and placed it over the lesion into a catheter guide (Figure 7). Then a "rendezvous technique" [11] retrograde coronary BMW wire was placed with the support of a retrograde Finecross® microcatheter into an antegrade Corsair microcatheter (Figures 8 and 9).

After placing the Rota wire in the distal segment of the Cx artery, a rotational atherectomy with burr 1.75 mm was performed, followed by NC balloon predilatation 3 x 15 mm and placement of drug-eluting stents (3 x 30 mm and 3 x 25 mm) in the distal and proximal segment of the Cx artery with proximal optimization with NC balloon 3.5 x 15 mm, without significant residual stenosis (Figure 9).

Case 2

A 65-year-old female complained of typical anginal symptoms with moderate physical exertion. A positive SEHO test showed inferolateral hypokinesia. Echocardiographic examination showed a left ventricle of normal dimensions with preserved systolic function; EF was 65%. Furthermore, this was a long-term cardiac patient with a previous myocardial infarction in 2016. So far, three unsuccessful attempts have been made to recanalize RCA.

The fourth attempt to recanalize CTO RCA started with a bifemoral approach. Angiographically, single-vessel coronary heart disease has been previously verified, with occlusion more than 5 cm long from the RCA ostium. The posterolateral branch did not show retrograde collaterals, and the impression was gained that it was occluded from its ostial segment (Figure 10). The Corsair microcatheter was placed practically to the distal occlusion cap via LCA intervention collaterals, after which Sion® black was replaced with Gaia 3 coronary wire. Subsequently, a reverse controlled

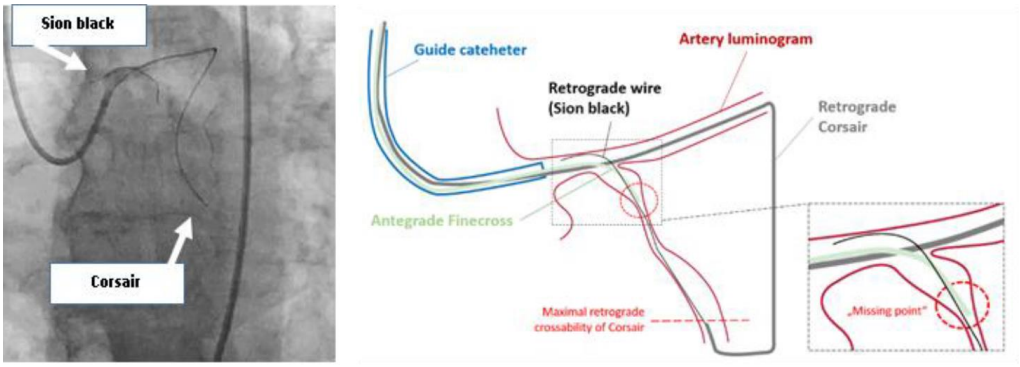

Fig. 7 – Attempt a "rendezvous" technique with a stuck Corsair.

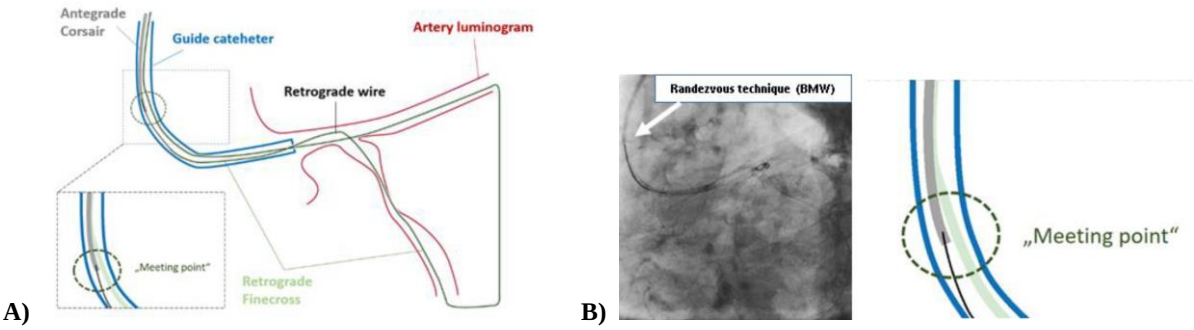

Fig. 8 – A) and B): "Rendezvous" technique – retrograde BMW wire was placed with a retrograde Finecross microcatheter into an antegrade Corsair microcatheter.

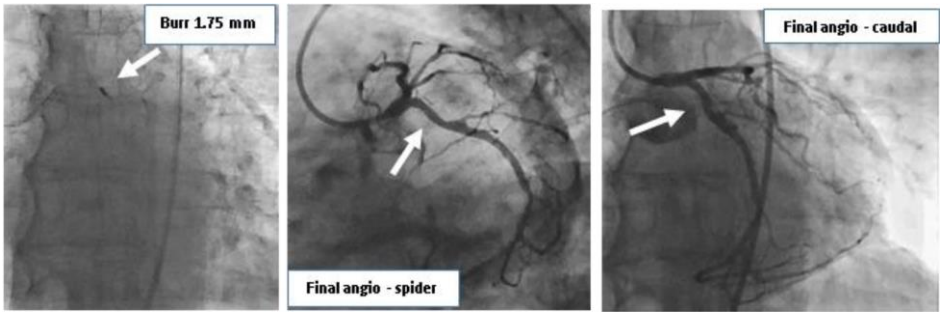

Fig. 9 – Final result after rotational atherectomy.

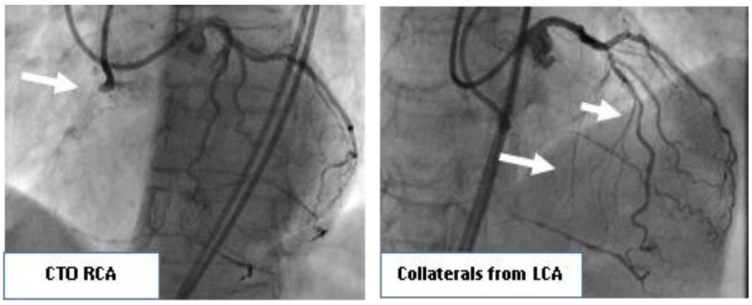

Fig. 10 – Single vessel coronary heart disease with long chronic total occlusion (CTO) of the right coronary artery (RCA) from ostium.

antegrade and retrograde tracking (CART) technique was performed with the help of the Guidezilla TM extension catheter (Figure 11). Gaia 3 retrograde wire was placed in an anterograde extension catheter. Afterward, externalization was performed with RG3 (Asahi Intecc Co., Japan), and 3 drug-eluting stents were placed after appropriate predilatation. Due to the lack of adequate flow in the distal segment of the artery, intravascular ultrasound (IVUS) optimization was performed, followed by additional angioplasty. Thrombolysis in myocardial infarction (TIMI 3) coronary flow was obtained (Figure 12).

Case 3

A 64-year-old female complained of typical anginal discomfort with greater physical exertion. A SEHO test was performed, which showed hypokinesia in inferolateral, and the test was evaluated as positive. Echocardiographic examination showed a ventricle of normal dimensions with preserved systolic function; EF was 60%. The patients was treated due to hypertension and hyperlipidemia as risk factors for CVD. Two years ago, PCI Cx was performed with the implantation of a single stent with drug release.

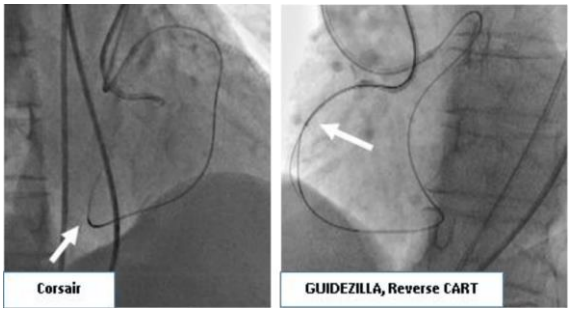

Fig. 11 – Reverse controlled antegrade and retrograde tracking (CART) technique with the support of the Guidezilla extension catheter.

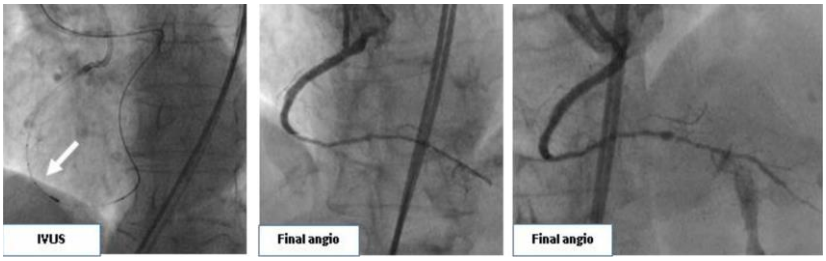

Fig. 12 – Final angio result after intravascular ultrasound (IVUS) optimization.

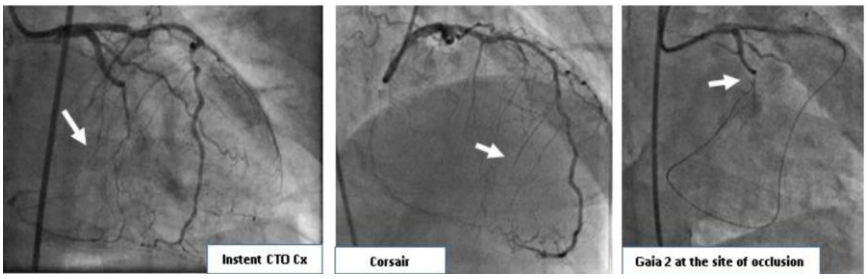

Fig. 13 – Single vessel coronary disease with no stump instent occlusion.

Diagnostic coronary angiography revealed single-vessel coronary disease, with no stump occlusion at the site of a previously implanted stent in the Cx artery, about 15 mm long. The intervention began with a femoral approach, 6F. Corsair was placed retrogradely, overcoming collaterals and Gaia 2 wire, which underwent occlusion with the support of microcatheters, and was placed in the proximal Cx artery (Figure 13). The Fielder XT antegrade wire was then placed, which, with a slight return of the Corsair microcatheter, was placed in the distal segment of the Cx artery parallel with the BMW retrograde wire (which replaced the Gaia 2 wire after the microcatheter underwent occlusion). After adequate predilatation, two drug-releasing stents were implanted, after which TIMI 3 flow was obtained in the distal segment of the Cx artery without significant residual stenosis (Figure 14).

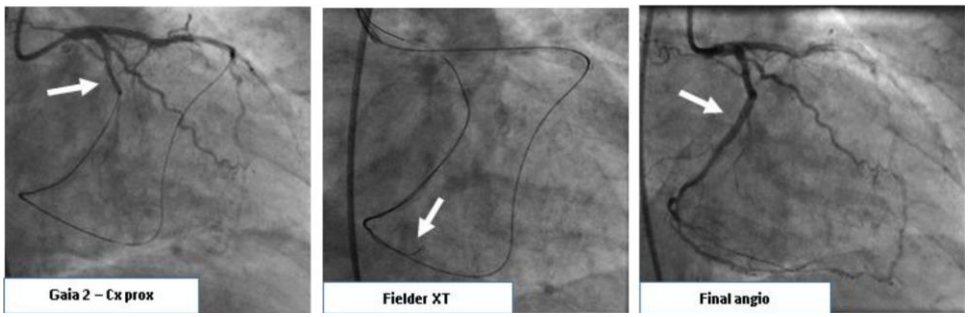

Fig. 14 – Final angio result after Gaia 2 crossing occlusion with support of the Corsair.

Discussion

We presented a series of three cases of recanalization of chronically occluded arteries using the retrograde approach, supported by rotational atherectomy (RA), IVUS as well as various techniques within the retrograde approach.

The first case is a clear demonstration of a technically very complex case that requires a highly flexible and experienced operator. The most aggressive wires, such as Gaia third or Confianza pro 12 (Asahi Intecc Co., Japan), could not cross very complex and calcified lesions. The operator quickly switched to the ipsilateral retrograde technique with soft polymeric wire (Sion black, Asahi Intecc Co., Japan), which crossed occlusion within a few seconds, allowing further calcified lesion modification using RA. The application of RA is considered safe after unsuccessful results for predilatation of calcified lesions in CTO and is considered equally successful in non-CTO procedures. This approach made it possible to finish the procedure with optimal results and minimal risk after four previous unsuccessful attempts.

The second case demonstrated the usage of the guiding catheter extension to facilitate the “reverse CART technique“, which is becoming a standard approach nowadays. Certainly, the most ideal option of the retrograde approach is the "true-to-true lumen" technique, which is possible when there are short uncalcified occlusions. In most cases, successful retrograde recanalizations end in reverse controlled antegrade and retrograde subintimal "tracking" (reverse CART technique). Without a doubt, this technique is the most used. A balloon positioned on the antegrade wire creates a subintimal space for the retrograde wire to advance and make a connection between the antegrade and retrograde space. It usually starts with a smaller balloon (2 mm), and in case of failure, larger balloons are used. For retrograde wire, a very controllable wire is most often used, which also has the power to make this connection (for example, the Gaia wire family). Furthermore, this case showed that after successful recanalization and stent implantation, lack of flow should be assessed by the IVUS. IVUS demonstrated significant mid-stent compression and a very diseased distal vessel. These findings allowed further stent deployment optimization and distal balloon dilatation with excellent TIMI 3 flow. Randomized studies have shown that IVUS improves the outcome of PCI CTO in terms of major adverse cardiac events (MACE) and stent thrombosis [12], most likely due to better optimization of the implanted stent. In the arena of retrograde approach, IVUS can also be helpful in two cases: the passage of a retrograde wire and reverse controlled antegrade and retrograde tracking (CART) technique. When passing a retrograde wire, IVUS can be useful in bifurcation “blunt stump“ occlusions as well as ostial occlusions, especially the LAD and Cx arteries, to avoid dissection of the main trunk of the left coronary artery and closure of the second branch [13].

In-stent CTOs represent about 12% of all PCI CTOs, and these procedures are more complex than in unstented blood vessels [14]. In the third case, the proximal cap of the occlusion was ambiguous, with a small brunch originating at that exact level. The occlusion was positioned at least 10 mm proximally to the proximal edge of the previously implanted stent. In such cases, an antegrade approach is possible with IVUS guided antegrade puncture (with IVUS probe in the side brunch if possible) or by the analyses of the index procedure and possibly available computed tomography (CT) angiography. In this case, the operator correctly started with a retrograde approach using septal interventional collateral, which allowed a very easy crossing of the occlusion body with standard Gaia second wire (Asahi Intecc Co., Japan) since the distal cap is usually softer than the proximal one and that proximal vessel was a relatively big target.

From 2009 until the present time, we have estimated that roughly 300 procedures were performed with the retrograde approach in Serbia [15*].

[*] **Note:** We would like to underline that the first retrograde procedure was performed in 2009 by prof. George Sianos from Greece as a guest operator in Belgrade, and during the same year, prof. Siniša Stojković did the first retrograde recanalization of the right coronary artery at the UCCS.

The retrograde approach should not be used as the first choice technique and is usually reserved for situations after an unsuccessful attempt to recanalize using the antegrade approach. As shown in our series of cases, the retrograde technique can be used as the first choice in certain cases, especially there where "interventional" collaterals are observed and when anterograde recanalization seems challenging due to the complex coronary anatomy of the occluded coronary vessel [16].

Conclusion

In cases where there is the presence of "interventional" collaterals, as well as when the antegrade approach is very difficult, the retrograde approach can increase the success rate of procedures. The retrograde approach requires a long learning curve as well as very skilled and experienced operators who are able to perform the procedure independently.

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CASE REPORT

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Complication after the reconstruction of the old patellar tendon rupture

Komplikacija nakon rekonstrukcije zastarele rupture ligamenta čašice

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Abstract

Introduction. Chronic patellar tendon rupture (PTR) occurs rarely; its frequency and prevalence are unknown. There are very little data on the late patellar tendon reconstruction in rheumatoid arthritis and its complications. **Case report.** We presented a surgical repair of a PTR with early postoperative rupture of the contralateral patellar tendon for a 21-year-old woman with a past medical history of juvenile rheumatoid arthritis (treated with corticosteroids) who sustained initial injury 11 months prior to the presentation. The contralateral side was used for autograft harvesting. We used bone-tendon-bone (BTB) autograft and allografts followed by double-wire loop reinforcement and immediate postoperative mobilization. The patient was followed for 2 years, and the function of both knees was restored completely, with a full active range of motion. In this case, reconstruction of an 11-month-old chronic PTR (with complete resorption of the tendon and completely separated infrapatellar pads, complicated by the contralateral PTR) with BTB autograft and allografts and double wire loop reinforcement gave an excellent functional result. Two years after the surgical treatment, the extensor function of both knees was completely restored with a full range of movements. The patient reported satisfying outcomes and was able to return to all pre-injury activities without the assistance of orthopedic devices. **Conclusion.** This case report highlights the importance of the early diagnosis and describes operative techniques used in chronic PTR repair and treatments of the early postoperative complications such as rupture of the contralateral tendon.

Key words:
early diagnosis; orthopedic procedures; patellar ligament; postoperative complications; reconstructive surgical procedures; rupture.

Apstrakt

Uvod. Hronične rupture patelarne tetive (RPT) su retke, a učestalost i prevalenca su nepoznati. Podaci o kasnoj rekonstrukciji patelarne tetive kod reumatoidnog artritisa i njenim komplikacijama su oskudni. **Prikaz bolesnika.** Prikazali smo operativnu tehniku rekonstrukcije jedanaest meseci stare rupture ligamenta patele 21-godišnje bolesnice sa juvenilnim reumatoidnim artritisom i ranom postoperativnom komplikacijom – rupturom kontralateralnog ligamenta patele sa kog je uzet kost-tetiva-kost autograft. Koristili smo kost-tetiva-kost autograft i alograftove sa duplim žičanim ojačanjima uz postoperativnu mobilizaciju. Bolesnica je praćena u periodu od 2 godine pri čemu je funkcija oba kolena kompletno rekonstruisana, sa punim obimom pokreta. U ovom slučaju, rekonstrukcija 11 meseci stare, hronične RPT (sa kompletnom resorpcijom patelarne tetive i kompletnom separacijom masnog jastučeta kolena, komplikovanom kontralateralnom RPT) sa kost-tetiva-kost autograftom i alograftom i sa duplim žičanim ojačanjima, dala je odličan funkcionalni rezultat. Dve godine nakon operativnog lečenja, funkcija oba kolena (oba ekstenzorna aparata) je u potpunosti obnovljena sa punim aktivnim obimom pokreta i bolesnica nema strah dok hoda stepenicama, niti koristi ortopedska pomagala. **Zaključak.** Ovaj prikaz bolesnika ističe značaj rane dijagnoze, opisuje operativne tehnike korišćene u oporavku hronične RPT, kao i lečenje ranih postoperativnih komplikacija kao što je ruptura kontralateralnog ligamenta patele.

Ključne reči:
dijagnoza, rana; ortopedske procedure; patela, ligament; postoperativne komplikacije; hirurgija, rekonstruktivna, procedure; ruptura.

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Introduction

Chronic PTR (patellar tendon rupture) is a rare injury, and its frequency and prevalence have not been determined yet [1]. There is no consensual decision about criteria that defines PTR as a chronic condition, but the daily activities of patients with this lesion are significantly limited [2, 3]. Diagnosis is based on clinical findings, with the palpation of a tendon defect at the point of rupture and proximal migration of a patellar bone. Ultrasound and magnetic resonance imaging (MRI) are useful in recognizing this lesion, preoperative preparation, and establishing the associated injuries [4].

The operative treatment of the unrecognized PTRs is a surgically challenging procedure with unpredictable postoperative results based on many studies that tried to describe the surgical technique for this type of injury. There is no gold standard in the surgical treatment of these injuries [5]. Described techniques include the use of autografts [6–9] or allografts [10–13] and synthetic materials such as Dacron or Ligament Augmentation and Reconstruction System (LARS) [14–17]. The literature regarding chronic PTR in patients with rheumatoid arthritis is scarce [18–20], and common postoperative complications are infections, patellar fractures, and quadriceps muscle atrophy [5].

We presented a rare form of injury and an early postoperative complication. To our knowledge, such a postoperative complication and its surgical treatment are not reported in the treatment of chronic PTR in patients with juvenile rheumatoid arthritis. We presented an operative technique for the reconstruction of chronic PTR with the postoperative rupture of the contralateral patellar tendon from which bone-patellar tendon-bone autograft was harvested.

Case report

A 21-year-old female suffered a left knee injury after falling from the staircases. The patient felt severe knee pain, as if something “snapped” in her joint, and noticed that her knee was swollen. She has been suffering from juvenile rheumatoid arthritis for many years and has been treated with oral corticosteroids. The patient went to the Emergency Room (ER), where a knee X-ray was taken (Figure 1); she was diagnosed with a knee distortion and contusion. As a result, her knee was put in cast immobilization. Afterward, she underwent a course of physical therapy for 11 months. The passive range of motion was 0 to 130 degrees, with a knee extensor lag. The Lachman, McMurray, and Apley test, and *valgus* and *varus* stress tests were negative. The left knee (MRI) showed a complete PTR with a tendon almost completely resorbed by the surrounding tissue and infrapatellar pads completely separated (Figure 2). The patient underwent surgery under spinal anesthesia with a tourniquet applied. Firstly, a bone-patellar tendon-bone autograft was taken from the contralateral knee, similar to the graft in an anterior cruciate ligament (ACL) reconstruction. Simultaneously, two cadaveric bone-tendon-bone (BTB) allografts of appropriate

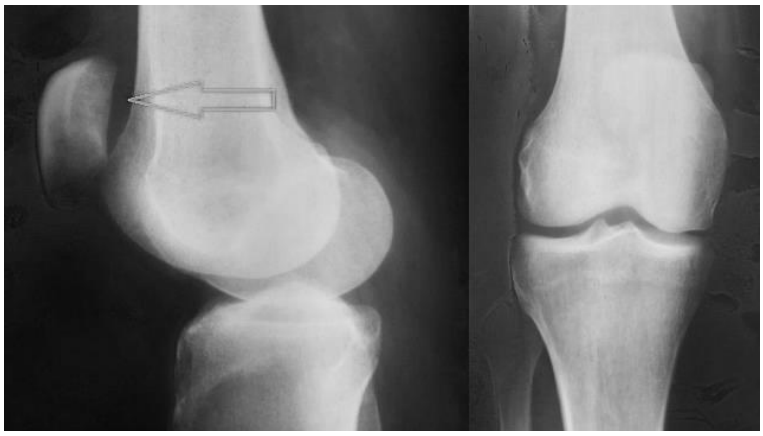


Fig. 1 – The X-ray imaging of the right knee after the injury: patella alta (arrow).

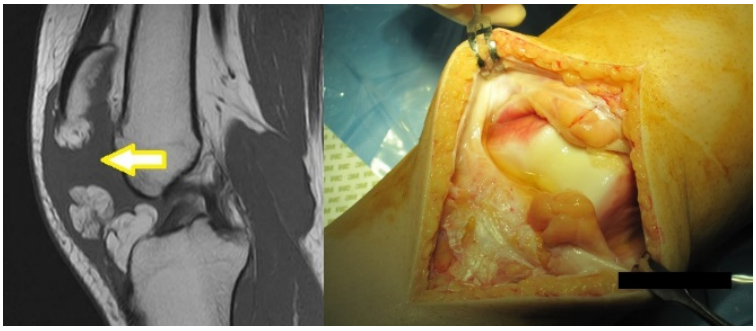


Fig. 2 – Magnetic resonance imaging (MRI) of the right knee: complete resorption of the patellar tendon by the surrounding tissue and separation of the infrapatellar pads (yellow arrow).

dimensions were taken from the bone bank of the Orthopedic Clinic in Novi Sad. A 15-cm-long skin incision was made on the anterior side of the left knee. After the surgical debridement, a 25-mm-long and 8-mm-wide bone trough was created in the tibial tubercle. Then, using a tibial ACL guide, a bone tunnel was made (also 25-mm-long and 8-mm-wide) in the central part of the patella, from its superior to its inferior pole (Figure 3). Afterwards, tibial and patellar bone tunnels of the same dimensions were made on both sides of the initially made tunnel. BTB autograft was set on the central position, press fit into the tibial trough, and stabilized with a 3.5 mm cortical screw; then, a prepared patellar graft was inserted into the centrally bored patellar tunnel, and also stabilized with a 3.5 mm cortical screw. Two cadaveric BTB allografts were set and stabilized using the same procedure; they were prepared to have dimensions identical to those of the contralateral knee autograft. Two metal wires were fastened around the patellar basis with a screw secured to the tibial tubercle to protect the patellar grafts (Figure 4). After tightening the wire loop, the passive knee range of motion was evaluated. A drain was inserted in the knee, and the wound was closed in layers.

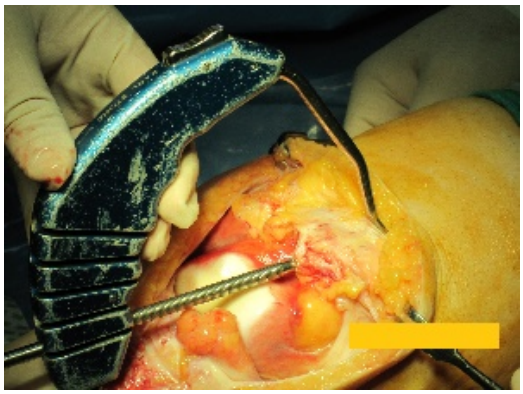


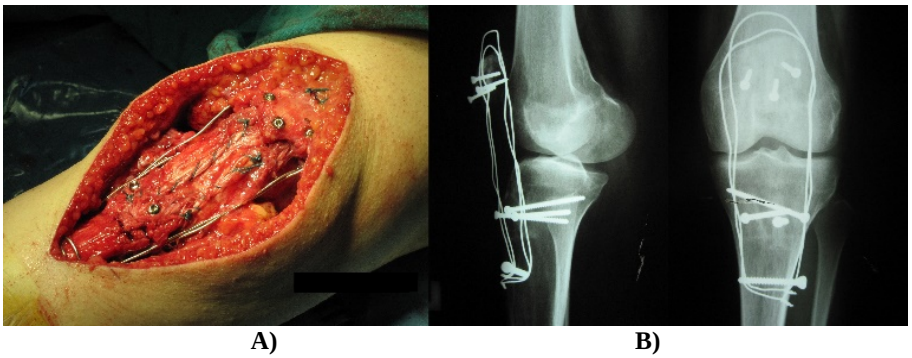
Fig. 3 – Tibial anterior cruciate ligament (ACL) guide for the drilling of the bone tunnels in the patella (from its superior to its inferior pole).

Postoperatively, a continuous passive motion (CPM) machine was used from day 1, and the patient was placed in passive flexion of 90 degrees for 6 weeks. On postoperative day 5, the patient slipped on her way to the bathroom and felt as if something "snapped" in her right knee, which was considerably swollen. A diagnosis of PTR of the right knee was made immediately, from which the BTB autograft was taken during the first procedure. Patella alta was found on the X-ray of the right knee, which was an indication of urgent surgical treatment.

The patient underwent a new procedure the day after this complication. The surgery was performed under spinal anesthesia with the use of a tourniquet. The same skin incision made for the BTB autograft was used to expose the point of rupture of the right patellar tendon. After the surgical debridement, the bone incisions on the tibia and patella (25-mm-long and 8-mm-wide), from which the autograft was taken, were covered with press fit of the prepared cadaveric allograft of the same dimensions and stabilized with two cortical 3.5 mm screws on patella and tibia, respectively. The degenerated remnants of the patellar tendon were sutured using Krakow stitches (Figure 5). Two metal wires were fastened around the patellar base with a cortical screw secured to the tibial tubercle to protect the patellar graft and newly sutured patellar tendon. After tightening the wire loop, the passive right knee range of motion was evaluated. A drain was inserted in the knee, and the wound was closed in layers.

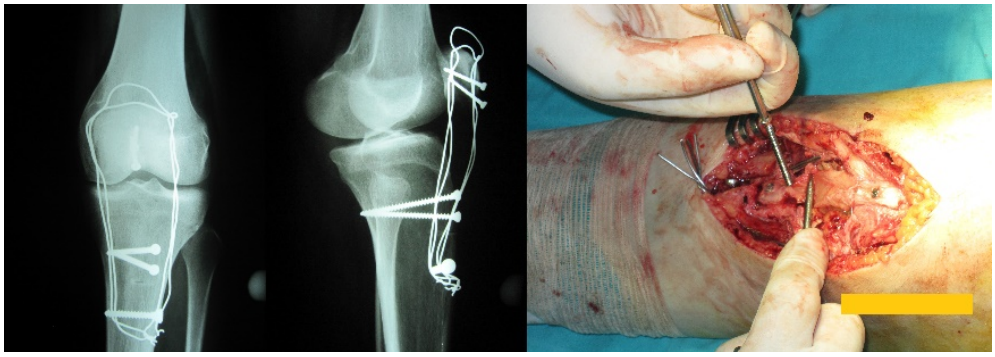
A CPM machine was used from the first postoperative day; the patient was placed in passive flexion of 90 degrees for the first 6 weeks. Simultaneous quadriceps strengthening and active extension exercises were performed during the physical treatment. While walking in the postoperative period, the patient had tutor orthosis on both knees in the full extension, and she was able to fully regain the appropriate footing. At three months of follow-up, the active range of motion on both knees was 0 to 130 degrees. Osteosynthetic materials were removed from her knees after a year and a half.

At two years of follow-up, the patient had a full range of motion on both knees, including both flexion and extension with the restored quadriceps strength and good results of isokinetic muscle testings (PrimaDOC multi-joint isokinetic dynamometer, Easytech, Italy), was able to perform knee bends, and was walking normally without any external support.



A) **B)**

Fig. 4 – A) Two cadaveric bone-tendon-bone (BTB) allografts and an autograft from the contralateral knee, with the cortical screws on the patella and tibial tubercle; B) two metal wires fastened around the patellar basis with a screw secured to the tibial tubercle to protect the patellar grafts.



A) **B)**

Fig. 5 – A) The bone incisions on the tibia and patella (25-mm-long and 8-mm-wide), from which the autograft was taken, were covered with press fit of the prepared cadaveric allograft of the same dimensions and stabilized with two cortical 3.5 mm screws on patella and tibia, respectively; B) Then, the degenerated remains of the patellar tendon were sutured using Krakow stitches, and two metal wires were fastened around the patellar basis with a cortical screw secured to the tibial tubercle to protect the patellar graft and sutured patellar tendon.

Discussion

Among all injuries of the knee extensor mechanism, approximately 3–6% are related to the PTR. Between 1.5% and 2% of all tendon injuries are related to the PTR [21, 22]. They are most common in young men under 40 (male/female ratio is 6:1), while the most frequent mechanism of the PTR is an eccentric overload of the knee extensor mechanism with a planted foot and flexed knee [23].

There are no clear data about the frequency of the chronic PTR or a general agreement about its definition, even though Siwek and Rao [24] consider lesions older than two weeks as chronic PTR. Bushnell et al. [25] reported that as much as 28% of PTRs may be unrecognized and undiagnosed. Ultrasound and MR imaging are not only useful in recognizing these injuries but also preoperatively and in diagnosing the associated lesions [4]. In young patients, chronic injuries are usually associated with systemic diseases (chronic renal failure, systemic lupus erythematosus, rheumatoid arthritis), corticosteroid administration, and treatments with fluoroquinolones [19, 20, 26].

The aggravating circumstances in the treatment of chronic knee lesions are a significant tendon retraction with patella alta, bad quality of the remaining fibers of the ligament, and quadriceps muscle atrophy. An end-to-end suture is usually not possible, thus synthetic fibers, allographs, and autografts are used in the surgical treatment [1, 2, 18, 26].

The use of artificial synthetic materials in the reconstruction of the patellar tendon started in the 1980s, but it was accompanied by complications, such as reactive synovitis and ruptures [27, 28]. The application of LARS was not adequately and timely tested, and due to the small number of randomized studies with LARS, its effects on the process of regeneration of the patellar tendon are still not known. In literature, only midterm studies about LARS augmentation could be found and are usually related to the older patients, so we decided to use a combination of autograft and BTB allografts [5, 17].

The use of BTB allografts is the gold standard that has been successfully applied in the treatment of knee ligament injuries. Milankov et al. [29] reported the use of contralateral BTB autograft for chronic patellar tendon rupture reconstruction, which resulted in an excellent knee extensor mechanism reconstruction.

Due to the long period between the injury and the surgical treatment of about 11 months, our patient had radiographic findings of a patellar tendon completely resorbed by the surrounding tissue and infrapatellar pads completely separated and partially resorbed. Therefore, it was necessary to use both an allograft and a contralateral BTB autograft which is used as a benchmark to correctly prepare allografts (in order to have a correct dimension of the graft and match it to the contralateral side). Burks and Edelson [11] were the first to use BTB allografts in the patellar tendon reconstruction. One bone plug was secured to the tendon insertion at the tibial tubercle with screws, while the other was secured to the patella using the “zuggurtung” technique.

Thus, using a contralateral BTB autograft, we were able to accurately reconstruct the extensor mechanism of the injured knee, which proved to be almost identical to the contralateral knee, with a strong autograft fixation. The main disadvantage of this technique is taking a graft from the non-injured knee, although Shelbourne and Urch [30] proved that taking BTB autografts from the non-injured knee does not affect its function. Long-term treatment with steroids in patients with rheumatoid arthritis may have a role in weakening the patellar tendon. However, due to the lack of studies dealing with complications after taking the autografts from the uninjured knee from patients with a history of corticosteroid treatment, we had a conversation with the patient in order to weigh out the risk and benefits of this procedure. As a result, we decided to proceed with this procedure in order to anatomically reconstruct the injured knee as previously described [19, 20, 26]. The adequate length and tension of the grafts are essential; if these parameters are not correct, the overly tensed graft will cause a defect in the knee flexion, while in-

sufficient tension of the graft will cause a defect in its extension. Palencia et al. [18] described a postoperative persistence of patella alta, and, therefore, it is crucial to determine patellar indexes preoperatively, such as Insall-Salvati Index or Blackburn Peel Index. A correct reconstruction of patellar length is possible only with the ideal size of the graft. That was one of the reasons, together with the resorption of the injured tendon by the surrounding tissue, to apply both allografts and a contralateral autograft because the slightest mistake in assessing the length of a tendon significantly reduces the postoperative range of movements of the knee.

PTR of the "donor" knee was also treated with the application of BTB allografts, double fire loops, and suturing of the ruptured tendon because it was an acute lesion. The double fire loop technique reduces tension on the repaired patellar tendon and contributes to the better regeneration and early mobilization of the injured knee, while the osteosynthetic material is removed after the complete functional restoration of the joint [31]. Our patient was on the long-term corticosteroid therapy with degenerative alterations on her tendon, and our opinion was that end-to-end sutures with no allografts would not be sufficient.

Postoperative immobilization is applied from 6 to 8 weeks [10, 16], or even longer in some cases [32], which may lead to consecutive knee contractures and the need for manipulation under anesthesia [8]. In literature, different types of postoperative external fixation are described, such as reinforced tendon repairs [24, 33–35], mostly with a single wire [6, 8, 13, 14, 36], with immediate CPM treatment in order to avoid quadriceps muscle atrophy and knee contractures. In our case, we used multiple wires described by Casey and Tietjens [31], which are mechanically stronger than a single circumferential loop and allow immediate mobilization without the use of postoperative casting or any other type of postoperative immobilization.

At eighteen months postoperatively, we removed the wires due to their breaking. Some authors [8, 13, 14] suggest the wire removal 6 to 10 weeks after the operation, but Casey and Tietjens [31] recommend the wire removal be postponed at least six weeks after the surgery. It allows a patient to regain the full range of motion in the injured knee, so that repaired tendon can strengthen sufficiently before the wire removal.

Conclusion

In this case, reconstruction of an 11-month-old chronic PTR (with complete resorption of the tendon and completely separated infrapatellar pads, complicated by the contralateral PTR) with BTB autograft and allografts and double wire loop reinforcement gave an excellent functional result. Two years after the surgical treatment, the extensor function of both knees was completely restored with a full range of movements. The patient reported satisfying outcomes and was able to return to all pre-injury activities without the assistance of orthopedic devices. Although additional surgery was needed to remove double wire loop reinforcement, it enabled an early mobilization and more secure healing of the repaired patellar tendons.

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CASE REPORT

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Nonsyndromic impacted triple supernumerary teeth in the maxilla, including single dens in dente malformation: a case report with a two-year follow-up

Tri prekobrojna zuba u gornjoj vilici i razvojna anomalija „zub u zubu" kod pacijenta bez sindroma

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Abstract

Introduction. Mesiodens is a midline supernumerary tooth, mostly located in the maxillary arch. We presented a rare case of a patient with nonsyndromic impacted triple supernumerary teeth precluding the eruption of central maxillary incisors. **Case report.** In addition to providing a detailed 3D picture of the patient's dentition, cone-beam computed tomography (CBCT) scan analysis revealed dens in dente malformation of one of the supernumeraries. Following surgical removal of all supernumerary teeth, the maxillary central incisors erupted spontaneously after a two-year period. **Conclusion.** To the best of our knowledge, no similar case report has been published in scientific literature. This case report highlights the importance of CBCT for proper diagnosis of supernumerary teeth as well as additional anomalies.

Key words:
cone-beam computed tomography; congenital abnormalities; dens in dente; oral surgical procedures; tooth, supernumerary.

Apstrakt

Uvod. Meziodens je prekobrojni zub koji se najčešće javlja u središnjoj liniji gornje vilice. Prikazan je redak slučaj pacijenta bez sindroma kod koga je izostalo nicanje centralnih sekutića gornje vilice kao posledica prisustva tri prekobrojna zuba. **Prikaz bolesnika.** Analizom snimka kompjuterizovane tomografije konusnog zraka (KTKZ) je pored trodimenzionalnog prikaza prekobrojnih zuba, na jednom od tri prekobrojna zuba dijagnostikovana i udružena razvojna anomalija tipa „zub u zubu". Dve godine nakon hirurškog vađenja prekobrojnih zuba došlo je do spontanog nicanja centralnih sekutića gornje vilice. **Zaključak.** Prema našim saznanjima ovakav ili sličan slučaj do sada nije objavljen u literaturi. Istaknut je značaj KTKZ za pravilnu dijagnozu prekobrojnih zuba kao i drugih anomalija.

Ključne reči:
kompjuterizovana tomografija konusnog zraka; anomalije; zub u zubu; hirurgija, oralna, procedure; zub, prekobrojni.

Introduction

Developmental dental anomaly manifesting as supernumerary teeth is diagnosed if the number of deciduous and permanent teeth exceeds 20 and 32, respectively [1]. Most supernumerary teeth are isolated cases, although some may be hereditary or be associated with certain syndromes [2]. Although supernumerary teeth are relatively common in patients with certain syndromes, they are much rarer in the healthy population, where they typically present in the frontal maxillary region. Supernumerary tooth interposed between central maxillary incisors is known as mesiodens and may occur as single, multiple, unilateral, or bilateral. Mesiodens is classified into four main types, namely conical, tuberculate, supplemental, and odontoma [3]. Mesiodens can also assume the vertical, slanted, horizontal, or inverted position. The aforementioned characteristics determine the likelihood of complications associated with mesiodens, as the presence of supernumerary teeth can be asymptomatic. The most frequent mesiodens-induced complication is the disturbance of central incisor eruption.

Although mesiodens etiology is presently unknown, several theories have emerged to explain its occurrence. According to the tooth dichotomy theory, supernumerary teeth are a result of the dichotomy of the (either primary or permanent) tooth bud. Hyper-proliferation of the dental lamina

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or unresolved dental lamina fragments are other possible causes of supernumerary tooth formation [4]. Dens in dente is a dental malformation characterized by enamel enfolding into the dentine, usually involving the crown and occasionally invading the root. The invagination most frequently involves the maxillary lateral incisors, followed by supernumerary teeth [5]. It is not uncommon to find the contemporaneous presence of mesiodens with the dens in dente tooth malformation [6, 7].

We presented a normally developing boy presenting with three impacted supernumerary teeth in the maxillary incisive region. One of the supernumerary teeth additionally displayed dens in dente malformation.

Case report

In April 2018, a 9-year-old boy presented at the Department of Oral Surgery, University Clinic for Dentistry of Vojvodina, Novi Sad, Serbia, showing delayed eruption of permanent central incisors as the chief complaint. Clinical assessment revealed the presence of deciduous central incisors along with the normal eruption of permanent lateral incisors. Panoramic radiograph (Figure 1) and cone-beam computed tomography (CBCT) scans (Figure 2) were obtained, revealing three tuberculate supernumerary teeth in the incisive maxillary region. Pretreatment tridimensional reconstruction obtained from the CBCT showed retained primary central incisors and

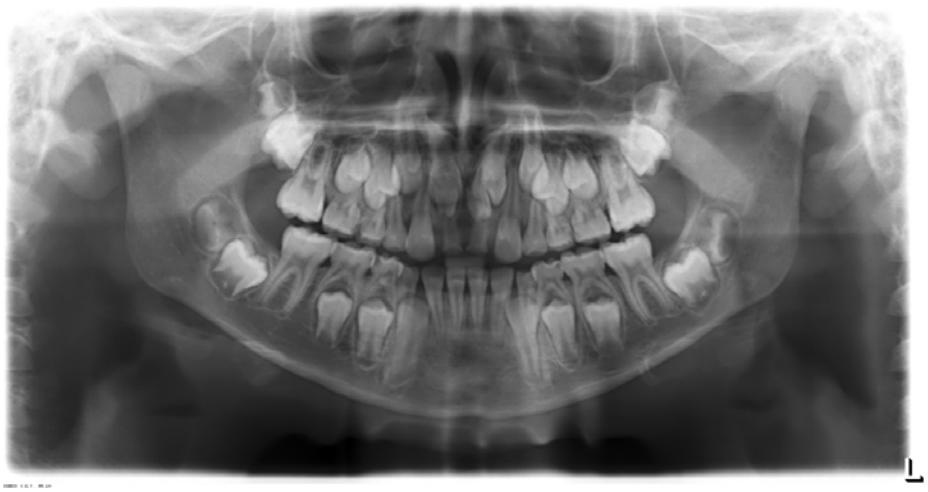

Fig. 1 – Panoramic radiograph showing impacted permanent central incisors and presence of supernumerary teeth.

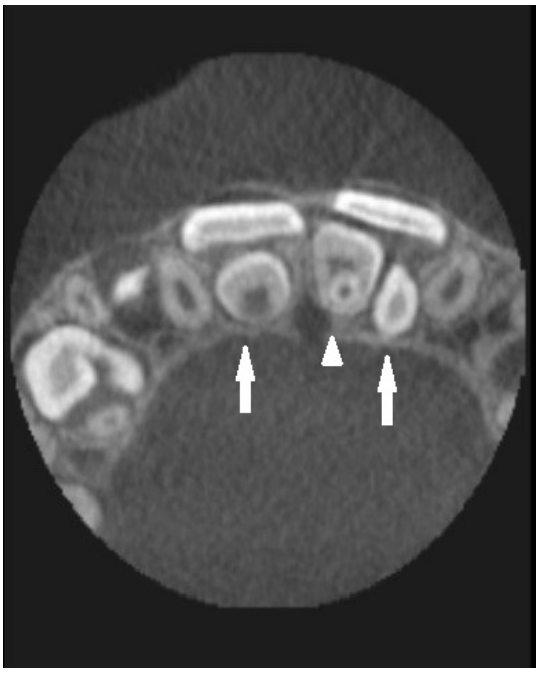

Fig. 2 – Axial cone-beam computed tomography (CBCT) scan showing impacted mesiodentes positioned palatally (indicated by arrows) and mesiodens involving dens in dente malformation (marked by arrowhead).

impaction of permanent maxillary central incisors, with adequate space for permanent upper central incisors to erupt (Figure 3). The sagittal scans further revealed the presence of the dens in dente malformation in one of the supernumerary teeth (Figure 4). Permanent central incisors assumed a vertical position, with incomplete root formation.

The treatment involved extraction of two primary central incisors as well as all three supernumerary teeth under local infiltration anesthesia. At the 18-month postoperative follow-up, spontaneous eruption of maxillary right permanent central incisors was noted (Figure 5). At the two-year postoperative follow-up, both maxillary permanent central incisors had erupted spontaneously (Figure 6), without evidence of additional supernumerary teeth on the repeated panoramic radiograph (Figure 7).

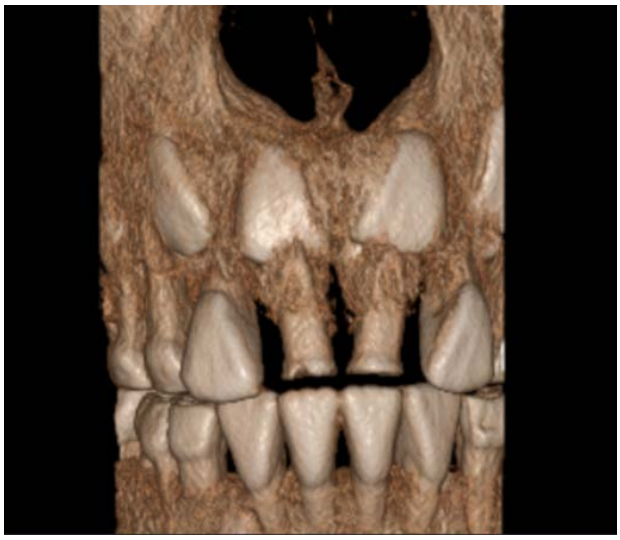

Fig. 3 – Pre-treatment tridimensional reconstruction obtained from cone-beam computed tomography (CBCT) showing retained primary central incisors and impaction of permanent maxillary central incisors.

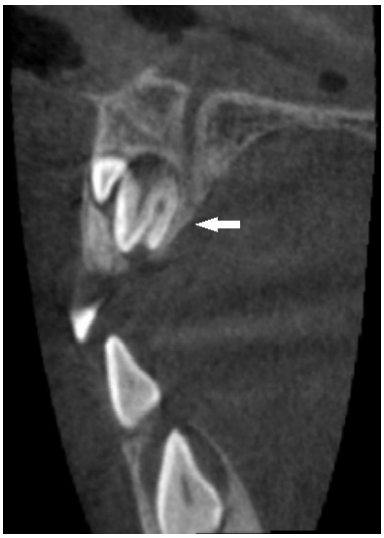

Fig. 4 – Sagittal cone-beam computed tomography (CBCT) scan revealing that one of the supernumerary teeth is associated with dens in dente tooth malformation (indicated by arrow).

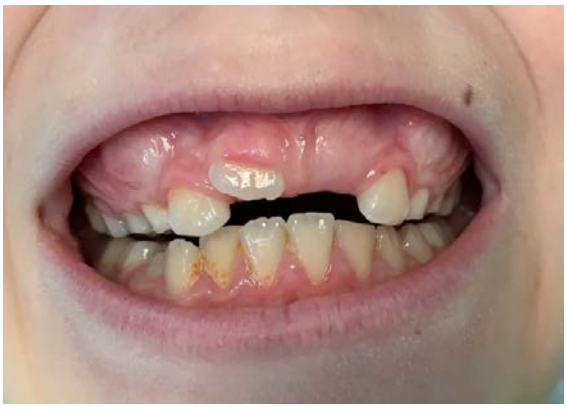

Fig. 5 – Intraoral frontal view 18 months postoperatively.

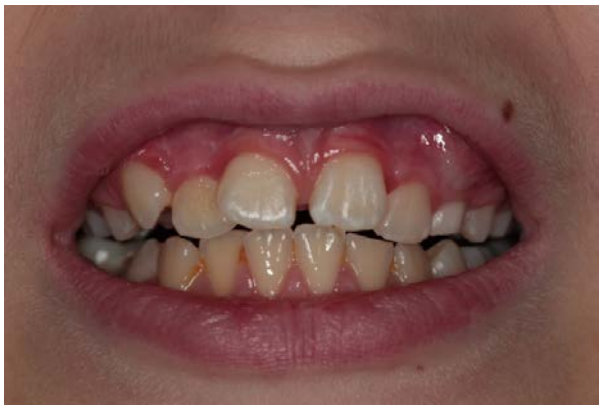

Fig. 6 – Intraoral frontal view 24 months postoperatively.

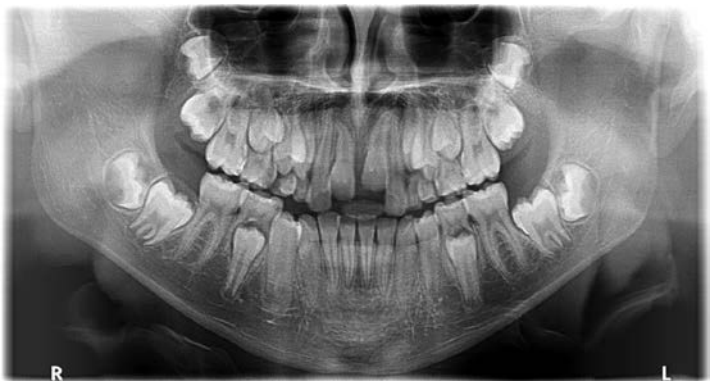

Fig. 7 – Panoramic radiograph taken at two-year follow-up showing spontaneous eruption of permanent central incisors.

Discussion

Mesiodens should be suspected whenever patients present with a delayed central permanent incisor eruption. Clinical practice shows that parents tend to seek orthodontic treatment for children in whom incisors have failed to erupt much sooner than for any other orthodontic anomaly [8]. Timely mesiodens diagnosis is crucial for determining the most optimal course of treatment and for prevention of complications, such as failed central incisor eruption, delayed eruption, ectopic eruption, midline diastema, resorption of adjacent teeth, and the emergence of a follicular cyst around the supernumerary tooth [9]. In the patient who was the subject of this report, failed central incisor eruption was the primary reason for suspecting mesiodens.

Mesiodens is the most common form of supernumerary dentition [10] and tends to present as a solitary tooth, while the incidence rapidly decreases with the number of additional teeth [11]. Impacted mesiodens is typically diagnosed through a periapical, panoramic, or axial radiograph. Diagnosis may also involve analysis of CBCT scans, which provide a 3D representation of the mesiodens and the surrounding dentition. This is particularly important in patients affected by multiple supernumerary teeth, which are difficult to differentiate in 2D images due to superimposition, thus failing to provide sufficient information for determining the best therapy mode. Empirical evidence indicates that when CBCT scans are analyzed, a higher prevalence of mesiodens is reported, especially in cases involving three or more supernumerary teeth [11]. Among the various mesiodens forms, conical is the most common, while tuberculate is the least frequent [12]. According to some authors, tuberculate mesiodens usually remains impacted and thus interferes with the eruption of permanent central incisors [13]. In the case presented here, all three supernumeraries were tuberculate.

Concurrent presence of supernumerary teeth with other developmental dental anomalies is rare, with a few reported cases of singular or double mesiodens accompanied by dens invaginatus [7, 14]. In the case discussed here, only one of the three supernumerary teeth in the midline exhibited this dental malformation.

During a surgical extraction, placing an orthodontic element is advised by some authors, while others recommend leaving permanent incisors to erupt spontaneously. In most cases, central incisors erupt within 6−36 months following the obstruction removal [15]. Available space in the dental arch and the patient's age are the main factors determining the likelihood of spontaneous incisor eruption. In the case presented in this report, the permanent central incisor roots were still developing, which, along with adequate space, typically results in spontaneous eruption upon obstruction removal [16]. Following surgical removal of supernumerary teeth in this patient, both maxillary central incisors erupted spontaneously after two years. In such cases, follow-up evaluation is essential, given several reported cases of emergence of new supernumerary teeth subsequent to surgical intervention [17].

Conclusion

In this specific case, CBCT scan analysis was instrumental in determining the number of supernumerary teeth, as well as their position and shape. As a result, the most optimal therapeutic approach could be initiated in a timely manner, ultimately leading to spontaneous eruption of the central incisors. Although the presence of dens in dente malformation in one of the three impacted supernumerary teeth is not pertinent to the therapeutic result achieved here, it is a phenomenon that, to the best of the authors' knowledge, has not been previously described in the literature in the numerical ratio reported here.

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CASE REPORT

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Transhepatic venous access for hemodialysis – a single-center experience

Transhepatički venski pristup za hemodijalizu – iskustvo jednog centra

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Abstract

Introduction. A percutaneous transhepatic approach has been used to place tunneled catheters for hemodialysis in the *inferior vena cava*. This route through the suprahepatic vein could be used to place a tunnelled catheter for permanent hemodialysis without complications and with an excellent permeability rate. **Case report.** From 2011 to 2020 at the Military Medical Academy, we treated 4 patients with the transhepatic central venous catheter for hemodialysis. All of them had exhausted approaches during the period of hemodialysis. Arterio-venous fistulas thrombosed on the arms, subclavian vein thrombosis bilaterally or *superior* and *inferior vena cava* thrombosis, as well as bilateral iliac and femoral vein thrombosis were present as complications of longterm hemodialysis through femoral catheters. Peritoneal dialysis was not possible. One patient needed a scroll catheter since the hemodialysis did not have a good outcome, and one patient needed a thrombolysis of catheter due to its malfunction. The other two patients have been on hemodialysis without complications for 300 and 1,650 days, respectively. **Conclusion.** Transhepatic venous access under ultrasound and radioscopic guidance is a simple and safe method. It is an acceptable alternative for permanent hemodialysis catheters when other venous accesses are exhausted and when it is performed by a well-trained team.

Key words:
catheters, indwelling; catheterization, central venous; dialysis, renal; liver circulation; radiology, interventional.

Apstrakt

Uvod. Za postavljanje tuneliziranih katetera za hemodijalizu u donju šuplju venu koristi se perkutani transhepatični pristup. Ovaj pristup preko suprahepatičnog dela donje šuplje vene mogao bi da se koristi za postavljanje tuneliziranog katetera za trajnu hemodijalizu, sa minimalnim rizikom od pojave komplikacija i sa odličnom funkcionalnošću. **Prikaz bolesnika.** U periodu od 2011. do 2020. godine, u Vojnomedicinskoj akademiji u Beogradu kod 4 bolesnika bio je postavljen transhepatični kateter za hemodijalizu. Svi bolesnici su imali iscrpljene vaskularne pristupe tokom dugogodišnjeg perioda hemodijalize. Kao posledica dugotrajnih hemodijaliza preko femoralnih katetera kod njih su bile prisutne trombozirane arteriovenske fistule na gornjim ekstremitetima, bilteralna tromboza supklavijske vene, tromboza gornje i donje šuplje vene, kao i bilateralna tromboza ilijačne i femoralne vene. Peritoneumska dijaliza nije bila moguća. Kod jednog bolesnika je bilo potrebno uraditi repoziciju katetera, a kod drugog smo uradili trombolizu katetera zbog malfunkcije. Druga dva bolesnika imala su uspešne hemodijalize bez pojave komplikacija u trajanju od 300, odnosno 1 650 dijaliznih dana. **Zaključak.** Perkutani transhepatični venski pristup vođen ultrazvukom i radioskopskom kontrolom je sigurna metoda i prihvatljiva je alternativa za plasiranje tuneliziranih hemodijaliznih katetera ukoliko su iscrpljeni drugi dijalizni pristupi i kada ih izvodi dobro obučen tim.

Ključne reči:
kateteri, trajni; kateterizacija, centralna, venska; hemodijaliza; jetra, cirkulacija; radiologija, interventna.

Introduction

Problems related to hemodialysis access are a significant cause of morbidity and mortality in patients with end-stage renal disease. Primary arterio-venous (AV) fistulas are recommended with venous transposition if necessary. AV grafts are used when autogenous access is not feasible, and tunneled dialysis catheters are recommended for long-term

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use only when all other options have been exhausted [1]. Complications of vascular access are the most common cause of hospitalization for patients with end-stage renal disease [2, 3].

Within the period 1997–2009 in Serbia, the incidence of patients on renal replacement therapy increased from 108 to 179 per million population (pmp), prevalence from 435 to 699 pmp, while the mortality rate fell from 20.7% to 16.7% [4]. In the United States, by 2011 and beyond, the drive to improve the quality of care for hemodialysis patients has identified vascular access issues as a key contributor to outcomes [5].

Transhepatic venous access was first described in 1994 by Po et al. [6]. A percutaneous transhepatic approach has been used to place tunneled catheters for hemodialysis in the inferior *vena cava*. The outcome of this procedure has been reported in two series [7, 8], constituting a total of 57 catheters in 23 patients. When all vascular approaches were used, transhepatic and translumbar vascular access was recommended as a vascular approach [7, 8]. The transhepatic route through the right hepatic vein could be used to place a tunnelled catheter for permanent hemodialysis with an excellent permeability rate [9].

Case report

In a period from 2011 to 2020 at the Military Medical Academy in Belgrade, we treated 4 patients with the transhepatic central venous catheter for hemodialysis. Our patients were women aged 65–76 years. On the chronic program of hemodialysis before placing the transhepatic catheter, they were 6–15 years. All of them had exhausted approaches during the period of hemodialysis. Arterio-venous fistulas had been thrombosed on the arms, with a worn-out ability to create new AV fistulas at the extremities after multiple interventions and reinterventions.

In the period before placing the transhepatic catheter, they had been dialyzed on transfemoral, subclavian, or jugular permanent catheters. All patients had repeated infections of femoral catheters. Central catheters were placed in the femoral vein bilaterally, but they had to be removed due to thrombosis or infection. Before making a decision to place a transhepatic catheter, we had diagnosed the following in all patients: subclavian vein thrombosis bilaterally, *vena cava superior* (VCS) thrombosis, *vena cava inferior* (VCI) thrombosis, and bilateral iliac vein thrombosis (Figures 1 and 2). In the meantime, an attempt was made with peritoneal

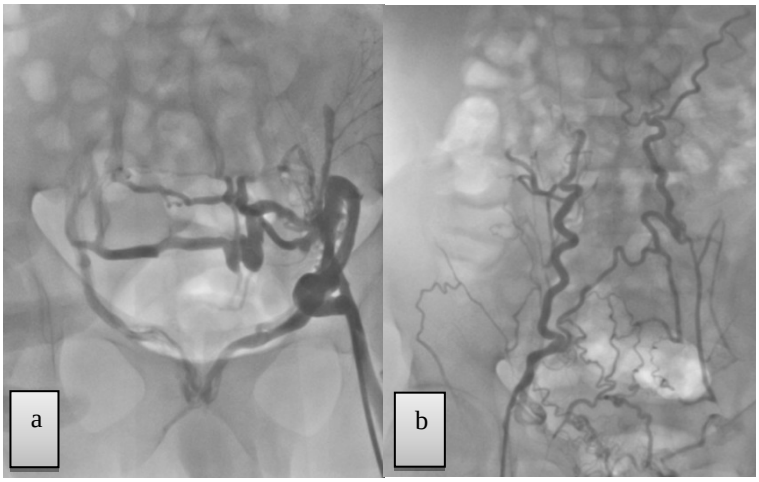

Fig. 1 – Thrombosed (a) left, and (b) right iliac and femoral vein, with thrombosis of *vena cava inferior*.

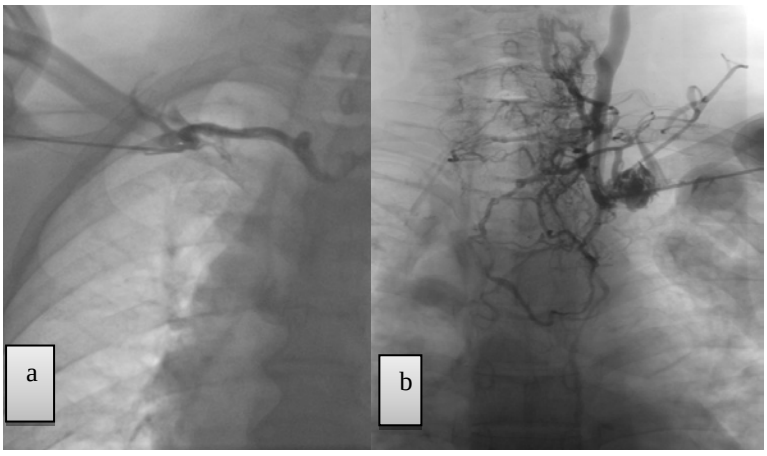

Fig. 2 – Thrombosed (a) right jugular and (b) left subclavian vein with thrombosis of *vena cava superior*.

dialysis, but perivisceral adhesion prevented a good outcome. After consultation among vascular surgeons, nephrologists, and radiologists, we decided to place the transhepatic catheter in the *inferior vena cava* for hemodialysis.

Technique

For the planned procedure, a liver punction kit and a tunneled catheter were provided (Figure 3). In the first step, by using ultrasound, we detected the right hepatic vein between the eighth or ninth intercostal space in the right midaxillary line. After mapping, the right hepatic vein was punctured with a needle from the system. The entire procedure has been followed by X-ray monitoring, also. The guide wire was placed through the right hepatic vein into the *inferior vena cava* (Figure 4). After punction and introduction wire, we approached implementation of the central tunneled catheter step-by-step using standard technique (dilatation, introducer sheet, catheter, making a subcutaneous tunnel, final check function, and position). All procedures were done under X-ray control step-by-step: wire transducer, dilatators, sheet (Figure 5). The catheter was placed with the top in the right atrium. At the end of the procedure, the catheter was tunneled and performed on the front abdominal wall and fixed with skin sutures.

Fig. 3 – Merit Mak Medical system for liver punction (6 Fr, 20 cm) and Arrow 15 Fr Tip to Cuff 27 cm catheter for hemodialysis.

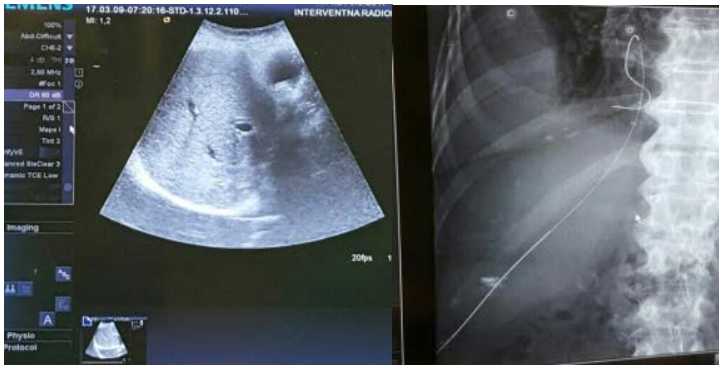

Fig. 4 – Ultrasound maping right hepatic vein; guide wire in right atrium.

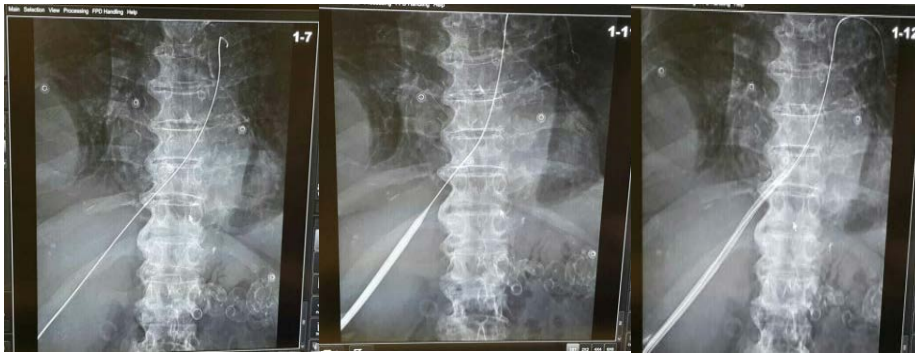

Fig. 5 – Dilatation (left), introducer sheet (center), catheter (right).

Discussion

The transhepatic pathway is a life-saving alternative in patients with the worn-out features of classic vascular access, and a kidney transplant certainly has no alternative. Creating and establishing a reliable route for hemodialysis is still a challenge. In the literature, we can find a small number of papers with case reports and case series addressing the current issues [9–13]. Only four series described the outcome of placing transhepatic catheters for hemodialysis [7, 8, 14, 15]. In the Smith et al. [8] series of 16 patients and 21 catheter placements, the complication rate was 29%, including one death from massive intraperitoneal hemorrhage. In our study, we did not have massive bleeding or death due to immediate complications. Although the average duration of dialysis via this route in the two series was 24 and 138 days, respectively, one patient was dialyzed for 599 days. We had 300–1,650 dialysis days in our series. Complications of this access could be acute: wire embolism, subcutaneous hematoma, catheter misplacement; long-term: air embolism, catheter embolism, catheter occlusion, central venous thrombosis, and stenosis; catheter-related infection and specific for the transhepatic route: massive intraperitoneal hemorrhage, perihepatic hematoma, hepatic arterial injury [8, 14]. We had one catheter malposition that was resolved by repositioning in the angio room. The repositioning was done under scope control, where the catheter tip was moved more distally, having been previously in contact with the atrial wall. One catheter thrombosis was successfully resolved using thrombolytics. Alteplase thrombolysis was performed in a patient whose catheter was thrombosed after two months (Table 1). Transhepatic dialysis catheter placement has a high rate of procedural success but also a higher rate of complications compared with traditional access sites. Immediate catheter failures occur most often due to migration, which can be minimized by placing the catheter tip in the mid or even upper right atrium to avoid caudal migration into the hepatic veins from respiratory motion [16], which we also used.

In our experience, one patient needed repositioning because hemodialysis did not have a good outcome, and one patient underwent catheter thrombolysis after two months. The other two patients have been on hemodialysis without complications for 300 and 1,650 days, respectively. There was no infection, but the number of hospital days in patients with a transhepatic Hickman catheter was increased.

The Hemodialysis Reliable Outflow (HeRO) Graft is a permanent, fully subcutaneous vascular access system for catheter-dependent patients and patients dialysing with failing arteriovenous fistulas or arteriovenous grafts due to outflow stenosis [17]. The HeRO system is another option and a possibility for patients with no contraindications for its placement, where there is no significant local obstruction and limitation of a technical nature, as well as where the price is not a limiting factor. At that time, it was not possible to implant a HeRo graft in our institution due to technical problems. Performing an arterio-arterial prosthetic loop (AAPL) on the upper or lower extremity is another option; it is well described but associated with significant complications [18]. The femoral vein transposition and saphenous vein loop grafts were not possible due to iliac vein thrombosis, which all patients had as a result of the long-term presence of femoral dialysis catheters, frequent punctures, and infections.

Conclusion

Arterio-venous fistula remains the gold standard for the vascular approach for hemodialysis. As a last resort, a transhepatic catheter could be used to extend the time on hemodialysis. Transhepatic venous access under ultrasound and radioscopic guidance is a safe method if you are adequately staffed and technically equipped. It is an acceptable alternative for permanent hemodialysis catheters when other venous accesses are exhausted and when it is performed by a well-trained team.

Table 1

Characteristics of patients undergoing transhepatic venous access

Patient	Gender	Age (years)	Duration on hemodialysis before transhepatic access (years)	Duration of transhepatic access use (days)	Complications/ intervention
1	Female	65	8	1,650	Hemathoma, malposition
2	Female	69	6	959	Malposition/ reposition
3	Female	71	15	465	Catheter thrombosis/ thrombolysis
4	Female	76	11	300	Malposition

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CASE REPORT

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The olfactory bulb – gateway for SARS-Cov-2?

Olfaktorni bulbus – ulaz za SARS-Cov-2?

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Abstract

Introduction. Anosmia and ageusia are one of the most common and characteristic symptoms of severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) infection, with a frequency of almost 50% in patients in Western countries. Hypotheses proposing that the virus potentially affects the central nervous system (CNS) are on the rise. One hypothesis suggests that the virus enters via nasal mucosa and then enters the olfactory bulb via cribriform plate, with further dissemination to the CNS. **Case report.** A 34-year-old female patient experienced the loss of the sense of smell and taste about two months before testing positive for SARS-Cov-2. Coronavirus disease 2019 (COVID-19) presented with minor pneumonia and worsening anosmia and ageusia. After treatment, the patient recovered well, but anosmia and ageusia appeared again, varying in intensity, and since February 2021, they have become persistent. The case was evaluated by an otorhinolaryngologist, pulmonologist, and finally, a neurologist. In the meantime, the patient tested negative for SARS-Cov-2 and received two doses of the Sputnik V vaccine. Brain magnetic resonance imaging (MRI) was performed, and it clearly showed severe bilateral olfactory bulb atrophy. The patient has had anosmia and ageusia up to this day, and future MRI follow-up is planned. **Conclusion.** Loss of sense of smell and taste may be a predictor of further CNS dissemination of the virus and possible neurological complications (which is still a subject of consideration). The olfactory bulb could be a gateway to COVID-19 intrusion into the CNS, and its atrophy could be an indicator of the process. Further investigation on this topic is required, including a wide application of MRI, in order to come to definite conclusions.

Key words:
ageusia; anosmia; atrophy; covid-19; olfactory bulb; magnetic resonance imaging.

Apstrakt

Uvod. Gubitak čula mirisa i ukusa spadaju u najčešće i najtipičnije simptome infekcije izazvane virusom SARS-Cov-2 (*severe acute respiratory syndrome coronavirus 2)*, sa učestalošću od skoro 50% kod obolelih u zemljama Zapada. Javlja se sve više hipoteza o potencijalnim infekcijama centralnog nervnog sistema (CNS) tim virusom. Pretpostavlja se da virus ulazi u organizam preko nosne sluzokože, pa zatim putem *laminae cribrosae* ulazi u olfaktorni bulbus, sa daljom diseminacijom u CNS. **Prikaz bolesnika.** Bolesnica, stara 34 godine, žalila se na gubitak čula mirisa i ukusa oko dva meseca pre nego što je test pokazao da je bila pozitivna na SARS-Cov-2. Bolest koju izaziva SARS-Cov-2 (COVID-19) se ispoljila blagom pneumonijom i smanjenom osetljivosti čula mirisa i ukusa. Nakon lečenja, bolesnica se oporavila, ali su se gubitak čula mirisa i ukusa ponovo pojavili, sa varijacijama u intenzitetu, da bi od februara 2021. postali uporni. Bolesnica je praćena od strane otorinolaringologa, pulmologa i, na kraju, neurologa. U međuvremenu, test je pokazao da je bolesnica bila negativna na SARS-Cov-2, posle čega je primila dve doze vakcine Sputnik V. Urađena je magnetna rezonanca (MR) mozga, koja je jasno pokazala izraženu obostranu atrofiju olfaktornog bulbusa. Bolesnica do danas ima gubitak čula mirisa i ukusa i planirano je dalje praćenje MR-om. **Zaključak.** Gubitak čula mirisa i ukusa mogu biti prediktori dalje CNS diseminacije virusa i potencijalnih neuroloških komplikacija (što je još uvek predmet razmatranja). Olfaktorni bulbus bi mogao predstavljati mesto ulaska virusa u CNS, a atrofija bulbusa bi mogla biti indikator tog procesa. Za definitivne zaključke potrebna su dalja istraživanja na tu temu, uključujući i širu primenu snimanja MR-om.

Ključne reči:
čulo ukusa, poremećaji; čulo mirisa, poremećaji; atrofija; covid-19; bulbus olfactorius; magnetska rezonanca, snimanje.

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Introduction

Anosmia and ageusia are one of the most common and characteristic symptoms of severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) infection, with the frequency of almost 50% in patients in Western countries [1, 2]. This symptom is not always present and cannot be the only diagnostic criteria for coronavirus disease 2019 (COVID-19). However, as the pandemic progresses, more and more hypotheses suggest that the virus potentially affects the central nervous system (CNS). The nasal mucosa is most commonly mentioned as the gateway for the virus intrusion into the brain. Angiotensin-converting enzyme-2 (ACE-2) and transmembrane serine protease-2 (TMPRSS2) receptors are considered essential for SARS-Cov-2 entrance into the host cells [3]. ACE-2 expression is highest in the pulmonary tissue, which explains pneumonia as the dominant manifestation, but it is also high in the nasal mucosa, as well as in some other tissues (liver, kidney, brain). It is presumed that the virus enters via nasal mucosa and then continues via cribriform plate to enter the olfactory bulb, with further dissemination to the CNS [4]. Of course, other hypotheses (like the hypercoagulable-vascular theory) are also considered, and those hypotheses do not exclude each other [5].

Case report

A 34-year-old female patient has been treated for anosmia and ageusia since July 2020. The loss of sense of taste and smell began suddenly and lasted for about a week. She did not have any other health issues. As the patient was aware that those symptoms are frequent in COVID-19, she went to a general practitioner and took a polymerase chain reaction (PCR) test on SARS-Cov-2, which was negative. One month later, she tested herself for SARS-Cov-2 antibodies; the test was also negative, and the patient concluded that her problems were due to allergic rhinitis, to which she inclined for the most of her life. Besides this, she suffered from hypothyreosis and had chronic problems with the cervical spine.

In September 2020, she had the same problems, which lasted only for several days, and she did not consult any doctor.

In the first half of November 2020, she felt minor itching in the throat. The next morning it was accompanied by uncomfortable pain in her hips, which she described as if it were spreading from the ovaries to the lower back. The next day, she felt the loss of sense of smell and taste again, and this time it was complete (previously, it was just partial loss). She took the PCR test again, and now, she tested positive for SARS-Cov-2. For the next five days, low back pain worsened, while throat itching disappeared. She felt disoriented and had memory difficulties. Anosmia and ageusia recovered slowly and became almost normal by the end of the month. She did not have a high fever, cough, or any other symptom. A chest X-ray was performed, which showed minor bilateral pneumonia. Amoxicillin/clavulanic acid was prescribed, and the patient felt better soon.

By the beginning of January 2021, she began to feel the loss of the sense of smell and taste again, and this time, it lasted for a whole month.

In March 2021, she got the Sputnik V vaccine and was revaccinated properly. Three weeks after the second dose of the vaccine, she began losing the sense of smell and taste again, and from that time on up to this day, she never recovered again.

The patient started to investigate her problem, starting with an otorhinolaryngologist. The doctor suspected allergic rhinitis and prescribed mometasone-furoate spray locally as well as a spray for moisturizing and repairing the nasal mucosa based on D-panthenol and vitamins E and A (Rinopanteina®, DMG IT, Italy), olfactory "training", and abundant nasal douching. Consultation with an endocrinologist and pulmonologist was requested.

The pulmonologist performed a control chest X-ray, which showed a complete reduction of the inflammatory process, with slight residual features in both lungs basally. He prescribed salbutamol (Ventolin® spray, GlaxoSmithKline).

In June 2021, the patient had a control examination with the otorhinolaryngologist. Nasal endoscopy, which showed a severe deviation of the nasal septum without any other pathological findings, was performed. The otorhinolaryngologist proposed the same therapy and 'training' and planned olfactometry in three months.

At the end of June 2021, magnetic resonance imaging (MRI) of the brain was performed. It revealed bilateral olfactory bulb atrophy, as well as slightly more voluminous extracerebral cerebrospinal fluid spaces for the average age.

A neurologist was consulted for the first time, and together with a neuroradiologist, a joint analysis of the MRI findings was done, together with additional measurements.

The measurement of the volume of the olfactory bulbs was performed with the standard methodology (Duprez TP and Rombaux P) [6], using T2 weighted fast spin-echo sequence (T2W FSE) in coronal view with 2 mm thick slices, which is optimal for displaying the anatomy of the olfactory bulb and the surrounding regions and allows volumetric evaluation. This sequence is added to the standard view of endocranium, which has to be performed in order to exclude potential traumatic or other lesions of the brain, which were excluded in our patient.

Performed measurements revealed a reduced volume of olfactory bulbs (about 18 mm^3 on the right side, and 20 mm^3 on the left side) (Figure 1); the depth of the olfactory sulcus on the right side was slightly above the limit value, and on the left side it was reduced (8.34 mm on the right side, 7.60 mm on the left side) (Figure 2). Olfactory bulb volume below 40 mm^3 and depth of olfactory sulcus $\leq$ 8 mm is considered reduced in the referent literature**.**

At this moment, the patient still shows no improvement regarding the sense of smell and taste. She is COVID-19 negative and has no other symptoms. Symptomatic therapy of vitamin B was added, and a control MRI is planned.

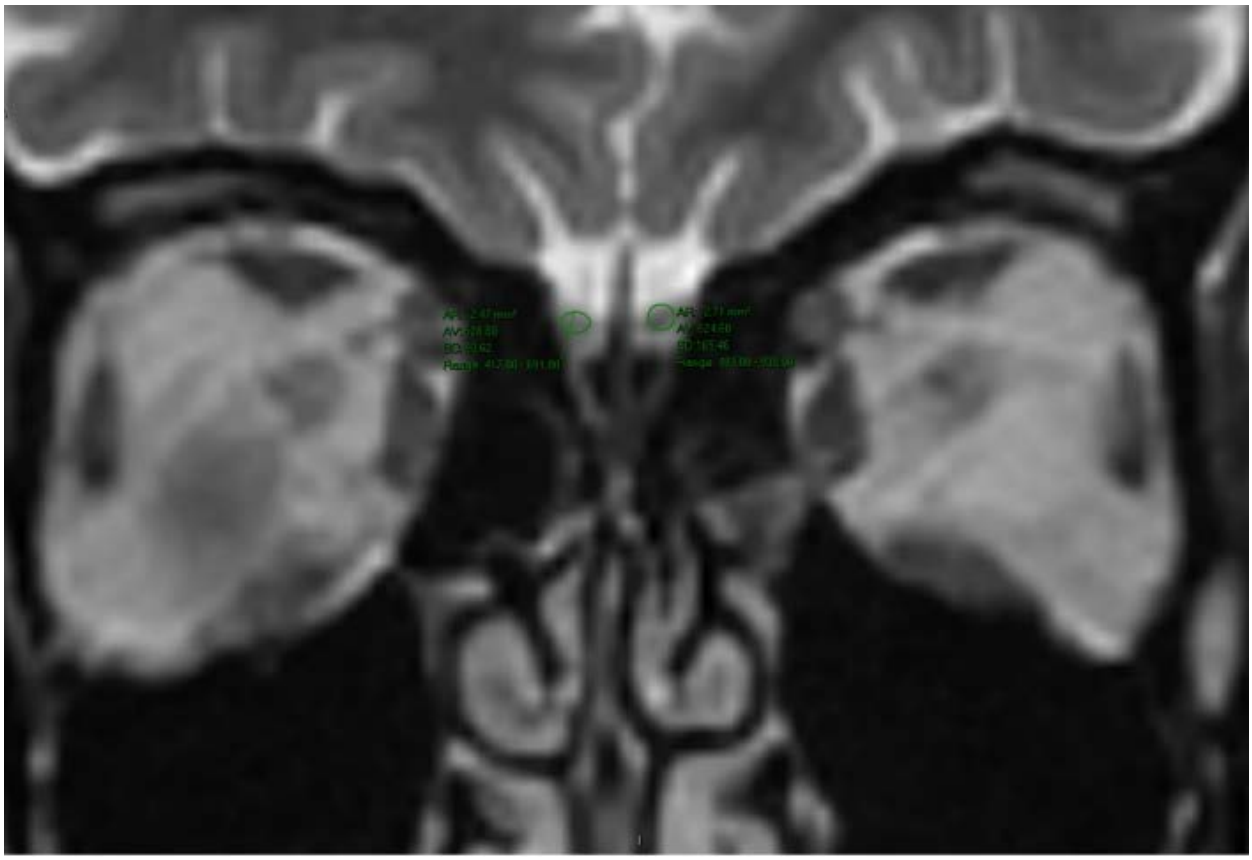

Fig. 1 ─ Coronal T2W thin-sliced tomogram with measurement of the volume of the olfactory bulb which shows signs of volume reduction.

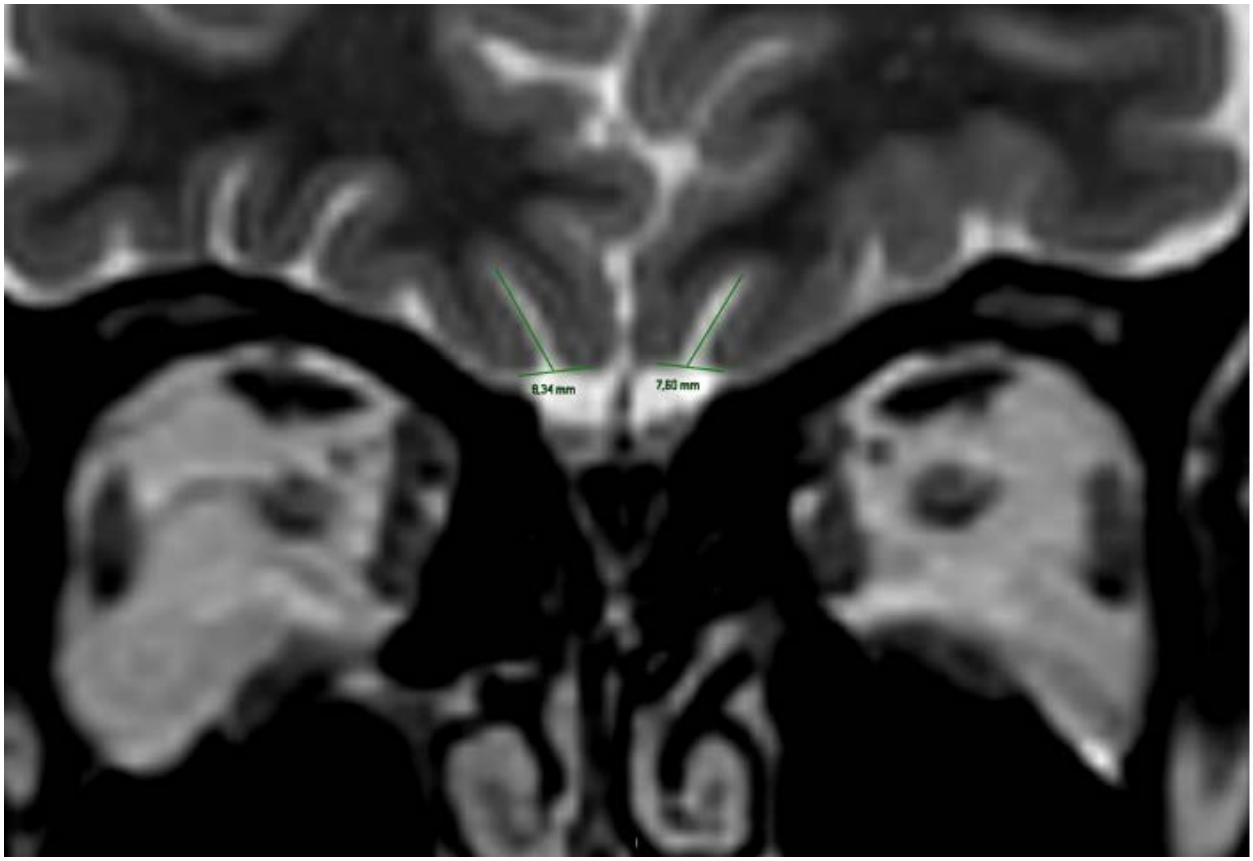

Fig. 2 ─ Coronal T2W thin-sliced tomogram with measurement of the depth of the olfactory sulcus which is reduced.

Discussion

From the beginning of the COVID-19 pandemic in November/December 2019, many controversies and dilemmas were caused over the origin of the virus, its influence on different organs, and means of transmission [7]. Loss (or weakness) of the sense of smell and taste was one of the first and most characteristic symptoms noticed in COVID-19. The whole situation around the COVID-19 pandemic included a variety of extraordinary measures aimed at preserving social and economic order and saving human lives. In these circumstances, planned, randomized, multicentric studies with pre-planned diagnostics still lack. Along with it, many hypotheses evolved as we obtained more and more knowledge about SARS-Cov-2.

Neurological manifestations of COVID-19 are becoming an increasingly interesting aspect of this new disease. Data on this are still very far from being systemized, and studies from different parts of the world show very different results [8]. What we do know is that the two main means of affecting the brain are either via the direct impact of the virus, which causes neurodegenerative processes (mainly atrophy), or through the brain's vascular system with pro-thrombotic state and consequent infarctions.

Nasal mucosa, olfactory bulb, and frontal lobe, in general, had been considered the gateway for SARS-Cov-2 to the brain from the beginning. Anosmia and ageusia, as very common initial symptoms, are supposed to be the result of the effect of the virus on these structures. Some studies mention damage to the nasal mucosa as a primary lesion, but that does not explain ageusia. Most other studies point to the olfactory bulb as the primary target that causes anosmia and ageusia and try to explain the mechanisms of the virus entering the brain. Almost all studies indicate the importance of expression of ACE-2 in particular tissues as indicators of organotropism of the virus [9, 10]. Expression of ACE-2 is particularly high in brain tissue. SARS-CoV-2 uses its spike protein to bind with ACE-2, and this link is formed with the aid of TMPRSS2. TMPRSS2 is a protease present on the surface of the target cell, which plays an important role in the virus entry pathway as it cleaves a specific point of the spike protein, thus allowing a connection between the C-terminal domain (CTD) of pico protein and ACE-2. Recent studies have shown that another transmembrane protease, TMPRSS4, is able to perform the same function as TMPRSS2, hence being an alternative protease for SARS-CoV-2. In addition to transmembrane proteases, there is also the intracellular protease known as cathepsin-L, which can also be responsible for the entry of the virus [10].

Many authors tried to determine exactly which part of the olfactory system is attacked by the virus and understand the potential further invasion of the nervous system [10]. Furthermore, a question was raised about the pathophysiologic origin of the anosmia and subsequent mechanism of recovery. Gupta et al. [9] researched the significance of ACE-2 and TMPRSS2 expression towards particular types of olfactory mucosa cells. The results showed that sustentacular cells (SUS), olfactory stem cells (OSC), and Bowman's gland cells (BGS) are particularly sensitive to binding with spike protein of the virus and that they are most probably responsible for anosmia. The study of De Melo et al. [11], performed on hamsters, is in accordance with these conclusions. It is known that some viruses can penetrate the olfactory bulb in such a manner and later disseminate in the brain, but whether SARS-Cov-2 has the same mechanism is still debated. Butowt and von Bartheld [12] propose the following four possible mechanisms of anosmia: local nasal obstruction due to congestion, destruction of olfactory receptor neurons, infiltration of the olfactory bulb, and damage of supportive cells, which they consider the most probable explanation (although our case shows otherwise). These authors also point out a much higher prevalence of anosmia in patients from the Western hemisphere compared to East Asian patients, for which we still have no valid explanation. Bilinska et al. [13] consider that the transfer of the virus from SUS cells to olfactory neurons cannot yet be proven, but, as it is known, the olfactory receptor neurons are enwrapped with SUS cells, and that kind of transfer is possible via exosomes, which was proven for the herpes virus.

Many studies tried to find out morphological evidence of the direct effect of SARS-Cov-2 on the brain. However, neuroimaging of the brain, especially the olfactory region, is not regularly performed on patients with anosmia and ageusia; hence we can usually find case reports and series of cases rather than studies. Chiu et al.[14] reported a patient with a loss of sense of smell and taste as an initial symptom of COVID-19. They performed an MRI of the brain, which showed olfactory bulb atrophy. Coincidentally, the patient had already measured the olfactory bulb due to diagnosed prolactinoma. Therefore, baseline dimensions of the bulb were already known. The duration of anosmia in this patient was two months. In their paper, Shor et al. [15] focused on olfactory bulb atrophy primarily from a radiological perspective and dealt with possible misinterpretation of MRI findings. On the contrary, Galougahi et al. [16] present us a case of a patient with clear unilateral anosmia during COVID-19 infection that showed no abnormalities of the olfactory bulb on MRI. The case report and analysis of Liang et al. [17] gives us data of a patient with COVID-19 and anosmia which lasted long after the patient was PCR tested negative several times, and emphasizes that MRI olfactometry should be performed minimum one month after the symptom onset if we want to document any changes on MRI. According to them, olfactory bulb atrophy is almost always asymmetric, and we should not check only for anatomical changes but also for functional decrease. Las Casas Lima et al. [18] give us a systematic review of numerous papers presenting morphological changes in the olfactory bulb connected to COVID-19 anosmia and ageusia. They also emphasize the importance of ACE-2 and TMPRSS 2 receptors in the olfactory epithelium cells. Their main hypothesis is that anosmia is caused due to damage to non-neuronal cells, which then affects the normal olfactory metabolism. MRI studies display the connection between a

decrease of the neuronal epithelium and the olfactory bulb atrophy. Damage to other cells, which are not neuronal, explains why the average recuperation lasts several weeks. This damage can be worsened by an enhanced immune response, leading to additional damage to neuronal and stem cells, inciting long-lasting or permanent loss of sense of smell. Tsivgoulis et al. [19] conducted a study on 8 patients who survived COVID-19 infection and had a persistent loss of sense of smell and taste for longer than 40 days. All of them presented with olfactory bulb atrophy (mild to moderate) but also thickness and edema of the nasal mucosa. Xydakis et al. [20], in their review article, tried to comprehend all the knowledge we have obtained on COVID-19 until now. The replication of the virus in neurons and glia have not been proven. Neurotropic characteristics of SARS-CoV-2 are not understood completely. However, malfunctioning of the olfactory tract can be understood as a focal neurological deficit in patients with COVID-19. Collected information indicates inflammation in the olfactory epithelium, olfactory bulb, or both. The mechanisms of olfactory dysfunction are hard to understand because of the dissimilarity of clinical features. That kind of dissimilarity indicates that SARS-CoV-2 infection can cause impairment of the olfactory function at various levels and by the means of different pathophysiological mechanisms which do not exclude each other. The factors which affect different kinds of recovery are unknown. Multiple hypotheses are trying to explain olfactory bulb dysfunction. One of them considers the sterile immune reaction triggered by active replication of the virus, where the virus plays the initial triggering but secondary role. There are many other conditions responsible for the invasion of the olfactory bulb cells by the virus**.** One of those conditions – the neurotropism of the virus – can be emphasized.

Future efforts on this topic are needed, with broader use of structural and functional olfactory system MRI, which should be done during the acute phase of COVID-19. They could contribute to a better understanding of this problem [20]. The recovery of the sense of smell occurs mostly during the first 4–6 months. Renaud et al. [21] studied the dynamics of recovery of the sense in patients with COVID-19 (half of the patients were assessed by olfactometry, and half by their subjective assessment). The recovery went gradually, and after one year, 96.1% of the patients recovered their sense of smell completely. There is evidence that the olfactory bulb and olfactory tract could be the focus of some neurodegenerative diseases. Thomann et al. [22] investigated olfactory bulb volumes in patients with mild cognitive disorder and patients with probable Alzheimer's disease (AD), along with healthy people as a control group. The olfactory bulb atrophy was most prominent in patients with AD, with also a significant reduction of medial temporal lobe gray matter density. This study implies that the atrophy of these two regions could be connected and could constitute predictor markers of AD. It is widely accepted that the so-called non-motor symptoms of Parkinson's disease (PD) can precede the presentation of the disease itself. The common symptom of PD which precedes motor symptoms is anosmia. Post-mortem studies showed a high prevalence of olfactory bulb atrophy in such patients but also in other neural structures responsible for the functioning of the sense of smell (anterior olfactory nucleus, piriform cortex, amygdaloid cortex, entorhinal complex, and the hippocampus [23]). A meta-analysis of Wattendorf et al. [24] points out the significance of MRI detection of olfactory bulb atrophy as an early symptom of PD.

Regarding the impact of vaccination on anosmia, Lechien et al. [25] point out that such complications are rare and are occasionally recorded after AstraZeneca's and Pfizer-BioNTech's vaccines. They explain such complications either by post-vaccinal inflammatory reaction of the olfactory neuroepithelium or by an immune reaction to the asymptomatic presence of the virus in the neuroepithelium after the vaccination (the prevalence of asymptomatic carriers of the virus ranges from 17.9% to 51%). The absence of nasal congestion makes the former hypothesis unlikely, whilst repeated negative nasal swabs question the latter, hence further investigations are needed. Farsalinos et al. [26] propose a hypothesis according to which the spike protein, expressed locally after vaccination, interacts with alpha-7 nicotinic acetylcholine receptors (nAChRs) in macrophages. Cholinergic pathway disorder triggers cytokine production, which could be transferred via neural pathways to distant regions. Such an inflammatory reflex produces neural signals, which are transferred via vagal nerve to the brain stem, and further to distant tissues by efferent neural pathways. The existence of such neuroimmune interaction may explain the inflammatory response in distant neural regions, as is the olfactory epithelium.

Persistent anosmia can severely damage the quality of life in patients recovering from COVID-19. Although we cannot still efficiently cure anosmia, the neural plasticity of the olfactory system offers treatment options in terms of stimulating the sense of smell. In their article, Sorokowska et al. [27] state that the regenerative capabilities of olfactory pathways vary from changes in membrane excitability and change in synaptic efficiency to neurogenesis and apoptosis. Studies that dealt with this olfactory training show promising results. On their model on mice, Liu et al. [28] prove that primarily sensory populations of neurons and their projections can retain plasticity in the adult age, which offers the basis for the mechanisms of learning and olfactory 'training'. Finally, Zhang et al. [29] present the protocol for future study, which will deal with the efficiency and safety of the olfactory 'training' for the patients with COVID-19 anosmia.

Although anosmia and ageusia began before the patient tested positive for COVID-19, the most probable cause is the virus, as real-time (RT) PCR testing of SARS-Cov-2 has only moderate sensitivity [30]. The impairment is severe and long-lasting, and the prognosis is uncertain. The prevalence of this kind of COVID-19 consequences is also uncertain, as MRI is rarely routinely done in COVID-19 patients. Possible pathological findings on patients' brains are practically nonexistent, as an autopsy on COVID-19 patients in Serbia is practically prohibited.

Conclusion

Loss of sense of smell and taste is a very common symptom of COVID-19. It may be a predictor of further CNS dissemination of the virus and possible neurological complications (which is still a subject of consideration). The olfactory bulb could be a gateway to COVID-19 intrusion into CNS, and its atrophy could be an indicator of the process. Further investigation on this topic is required, including the wide application of MRI, in order to come to definite conclusions.

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C O R R I G E N D A

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1. In the original article titled "**Root canal treatment from patients' perspective: knowledge, awareness, and expectations**" by **Muhammad Qasim*, Omair Anjum†, Gotam Das‡, Fariha Naz*, Saima Razaq Khan§, Abdul Razzaq Ahmed‡, Saurabh Chaturvedi‡**, published in the April 2022 print issue of the Vojnosanitetski pregled (Vojnosanit Pregl 2022; 79(4): 325–329); https://doi.org/10.2298/VSP200825112Q), there are errors in affiliations.

   Instead of the existing affiliations:

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   there should be:

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2. In the original article titled **"How and when do we use continuous renal replacement therapy for acute kidney injury in Serbia? – The multicentric survey"** by **Violeta Knežević, Dejan Ćelić, Tijana Azaševac, Sonja Golubović, Vesna Sladojević, Nataša Nestorov, Djoko Maksić, Radomir Naumović, Tatjana Lazarević, Vojislava Nešković**, published in the April 2022 print issue of the Vojnosanitetski Pregled (Vojnosanit Pregl 2022; 79(4): 330–336; DOI: https://doi.org/10.2298/VSP191231110K), there is an error in the e-mail of the corresponding author.

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Tabele

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

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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