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ASSESSMENT OF ADHERENCE IN OUTPATIENTS WITH OPEN-ANGLE GLAUCOMA

Jasmina Djordjević-Jocić[1,2], Hristina Jocić[3], Milan Stoiljković[1]

Glaucoma is chronic, progressive optic nerve neuropathy that leads to permanent defects of the visual field. Glaucoma still cannot be cured, however, with proper and correct use of prescribed therapy, it can be managed in a way to slow its progression and consequent loss of vision. Thus, having good adherence to recommended medications is of utmost importance for glaucoma patients.

The study aimed to assess the degree of adherence to prescribed therapy for open-angle glaucoma in outpatients.

In February 2019, one-month research was done at the Glaucoma Department of the Eye Clinic, Clinical Center Niši. It was performed on 77 outpatients using an anonymous, volunteer-based questionnaire consisting of 11 questions related to demographic and socioeconomic characteristics, disease duration, as well as adherence to recommended therapy and reasons for possible non-adherence.

Out of the total outpatient number interviewed, 62.34% stated that they took their therapy as recommended, and 37.66% stated doing it not so regularly. Among those who were not taking therapy regularly, more were patients of older age ($p = 0.00001$; $p < 0.05$). No difference related to gender was found. Patients on multidrug glaucoma therapy were less adherent than those who used only one drug ($p = 0.00034$; $p < 0.05$). Better adherence was found in patients without comorbidities (87.5%) compared to those with some concomitant disease (35.14%), there was a statistically relevant correlation between these two parameters ($p = 0.000002$; $p < 0.005$). The most common reasons for non-adherence were adverse drug effects (100%), very long treatment period (89.66%) and patient's forgetfulness.

Relatively high, but not absolutely adequate degree of adherence is present among open-angle outpatients. Improvement of adherence can be achieved with optimal choice of therapeutic regimen, prescription of drugs with milder adverse effects, patient education about the course of disease and its possible consequences, i.e. blindness, as well as with emphasizing the importance of following recommended pharmacotherapeutic measures.

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Key words: *adherence, open-angle glaucoma, outpatients*

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Introduction

Glaucoma is a chronic, progressive neuropathy of the optic nerve leading to changes in the optic nerve and damage to the visual field. Glaucoma is a multifactorial disease, where elevated intraocular pressure (IOP) is a key factor in its development and the only one that can currently be addressed through medication (1). This is a disease with a growing global prevalence, affecting approximately 60 million people worldwide, of whom 4 million are blind. The estimated number of affected individuals in Serbia is around 100,000 (2). It currently ranks as the second leading cause of blindness worldwide but is the leading cause of preventable blindness. Once diagnosed, glaucoma cannot be cured, but proper therapy can prevent further progression of the disease and vision loss.

Adherence represents the extent to which a patient's behavior aligns with the prescribed recommendations of the prescribing authority. Establishing good patient adherence is a constant challenge but is considered a key component of therapy. Several studies have shown that achieving good patient adherence is more likely if the patient has a good understanding of their disease, recognizes the importance of therapy,

and the treatment regimen is straightforward (3). Additionally, the use of eye drops in glaucoma treatment further complicates the proper use of therapy and reduces adherence in these patients (4).

This scientific study aimed to examine the level of adherence among outpatients with open-angle glaucoma.

Material and Methods

The study was conducted at the Glaucoma Department of the Eye Clinic, Clinical Center Niš, in February 2019, through anonymous and voluntary patient surveys. A total of 77 individuals completed the questionnaire. The questionnaire consisted of 11 questions related to the demographic and socioeconomic characteristics of the participants, the duration of the disease, adherence to the prescribed therapy, and reasons for possible non-adherence. Multiple sources were used to compose the questionnaire, and some questions were specifically designed for this research. The survey was conducted in collaboration with the staff of the Eye Clinic, Clinical Center in Niš.

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Clinical Center Niš, Niš, Serbia (No. 16368/112; approval date: 12 June 2012).

All analyses were performed using the online free calculator Social Science Statistics. Values for continuous variables were presented as the mean with standard deviation, while the frequency was used for categorical variables. Parametric (Student's t-test) and non-parametric (χ^2 test) correlation tests were used to assess the correlations between variables. Statistical significance was determined at the level of $p < 0.05$.

Results

The percentage of adherence in patients with glaucoma is presented in Figure 1. The percentage of patients who regularly used therapy was 62.34%, while the percentage of those who did not regularly use the prescribed therapy was 37.66%.

The distribution of participants by gender did not show a statistically significant difference between male and female genders, although the number of female participants was higher (46 males, 31 females). The average age of the participants was 62.7 ± 12.73 years. Ten patients (12.99%) completed primary school, 41 patients (53.25%) completed high school, and 26 patients (33.77%) had higher education. The correlation between demographic and socioeconomic characteristics and the degree of adherence is shown in Table 1. We proved that there was no statistically significant correlation between genders and the degree of adherence ($p = 0.32$; $p < 0.05$). The average age of participants who regularly used therapy was 57.4 ± 12.29 years, while the average age of participants who did not regularly take therapy was 71.4 ± 7.52 years. There was a statistically significant correlation between the average age and the degree of adherence ($p = 0.00001$; $p < 0.05$), as well as between the level of education and the degree of adherence ($p = 0.00012$; $p < 0.05$).

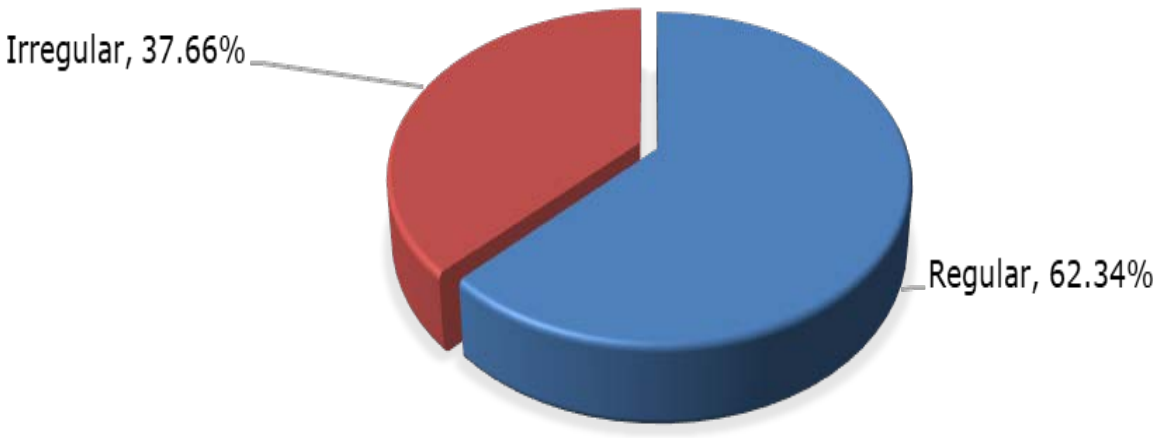

Figure 1: Percentage of patients who regularly use therapy

Table 1. Correlation between demographic and socioeconomic characteristics and degree of adherence

	Sex male/female N (%)	Average age (years)	Education ps/hs/he N (%)
Regular use of therapy	26/21 (56.52/67.74)	57.4 ± 12.29	2/24/22 (20/58.54/84.62)
Irregular use of therapy	20/10 (43.48/32.26)	71.4 ± 7.52	8/17/4 (80/41.46/15.38)
	p = 0.32; p < 0.05	p = 0.00001; p < 0.05	p = 0.00012; p < 0.05

We also showed that there was a statistically significant correlation between regular follow-up visits and regular medication intake (p = 0.01; p < 0.05). All patients who regularly took medication also reported regularly attending follow-up examinations.

Figure 2 shows the reasons for patient non-adherence. The largest number of non-adherent patients cited discomfort and unwanted effects during therapy (100%) as the reasons for their non-adherence, followed by the long duration of therapy (89.66%) and forgetfulness (62.01%). Just over half of the patients said they only used therapy when they experienced symptoms (58.62%), while about half of the patients expressed uncertainty about how long they should continue the therapy (51.72%). Patients less frequently mentioned not understanding how to use therapy (44.83%), thinking that the therapy would not help them (44.83%), not seeing the importantance of regular use was (27.59%), not considering glaucoma a serious disease (24.14%), and not having anyone to help them with the application of therapy (24.14%). The least common reason mentioned by respondents was the belief that the therapy could do more harm than good (3.45%).

Table 2 shows the correlation between the duration of the disease and the degree of adherence. The average duration of the disease in participants who regularly took therapy was 6.71 ± 3.63 years, while the average duration of the disease in participants who did not regularly take therapy was 10.97 ± 4.54 years. There was a statistically significant correlation between the duration of the disease and the degree of adherence (p = 0.000014; p < 0.05).

Table 3 presents the correlation between associated diseases and the degree of adherence. Patients with associated diseases, whether ophthalmological or systemic, had a significantly lower degree of adherence (35.14%) compared to patients without associated diseases (87.5%). There was a statistically significant correlation between these two parameters (p = 0.000002; p < 0.005).

Table 4 displays the correlation between the number of medications used in therapy and the degree of adherence. Patients who used three or four drugs in therapy had a significantly lower degree of adherence (three drugs—42.86%, four drugs—30.77%) compared to patients who used one or two drugs in therapy (one drug—81.81%, two drugs—88%). There was a statistically significant correlation between the number of drugs used in therapy and adherence (p = 0.00034; p < 0.05).

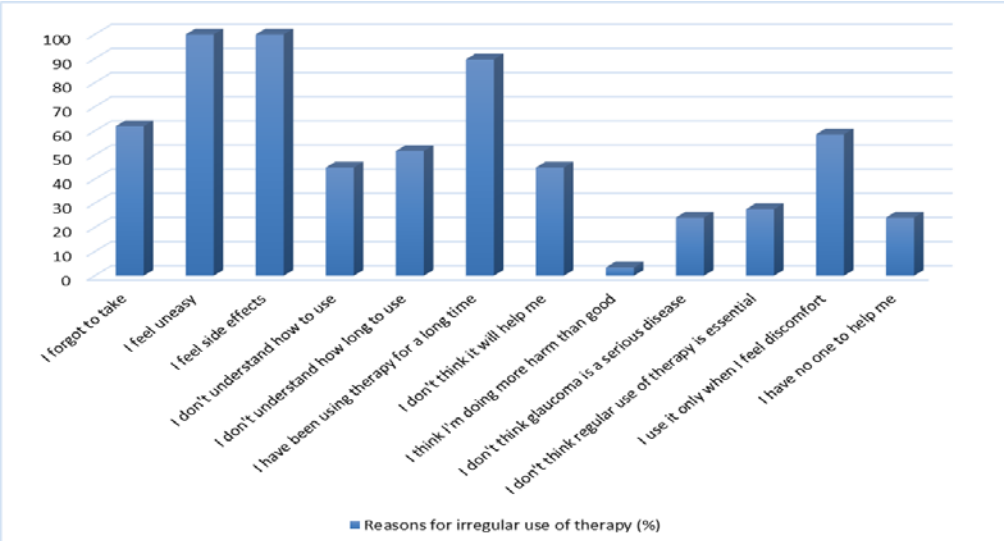

Figure 2. Reasons for irregular use of therapy (%)

Table 2. Correlation between the duration of the disease and the degree of adherence

	Average duration of disease in years
Regular use of therapy	6.71 ± 3.63
Irregular use of therapy	10.97 ± 4.54
p = 0.000014; p < 0.05	

Table 3. Correlation between comorbidities and degree of adherence

	With associated diseases N (%)	**Without associated diseases N (%)**
Regular use of therapy	13 (35.14)	35 (87.5)
Irregular use of therapy	24 (64.86)	5 (12.5)
p = 0.000002; p < 0.005		

Table 4. Correlation between the number of drugs used in therapy and the degree of adherence

	1 drug N (%)	**2 drugs N (%)**	**3 drugs N (%)**	**4 drugs N (%)**
Regular use of therapy	9 (81.81)	22 (88)	12 (42.86)	4 (30.77)
Irregular use of therapy	2 (18.19)	3 (12)	16 (57.14)	9 (69.23)
p = 0.00034; p < 0.05				

Discussion

Patient non-adherence continues to be a significant problem in the treatment of open-angle glaucoma, despite advancements in finding medications and therapeutic regimens that are easier to follow with fewer side effects. The percentage of non-adherent patients in our study was 37.66%. Various studies have shown different rates of non-adherence, with most ranging from 23% (5) to 27.3% (6). A study conducted in the United Kingdom revealed that 77% of respondents claimed to regularly and correctly take their medication. Still, only 55% of them could accurately state the names of the drugs they use and the exact regimen, indicating that a certain number of patients overestimate their discipline (5). Glaucoma belongs to a group of diseases that often progress asymptomatically until late stages, significantly influencing patient non-adherence, as confirmed in numerous studies (4). Due to the lack of symptoms, patients often do not grasp the importance of adherence and regular medication intake. From a medical standpoint, treatment effectiveness is evaluated based on the reduction of intraocular pressure, visual acuity, and structural and functional changes. On the other hand, patients must be aware that it is necessary to undergo treatment, even if they do not feel immediate relief. The assessment of treatment effectiveness, from the patient's perspective, is better in symptomatic diseases (7).

Glaucoma is a disease of older age, with prevalence increasing with age. The prevalence of glaucoma in the population over 50 years is 3%, and in those over 70 years, it rises to 5%, with the highest number of cases occurring between 65 and 75 years of age (7). The average age of our participants was 62.7 years. We demonstrated that the average age of patients who irregularly take medication is significantly higher (71.4 years) compared to the average age of patients who consistently use medication (57.4 years). Older individuals face more difficulties in using medication, including challenges in understanding how and for how long they should use the therapy, memory issues, and, particularly, the use of eye drops (6). Good coordination, dexterity, and good vision are necessary for independently applying eye drops, and these factors are often diminished in older individuals, requiring assistance in administering the therapy (8). Studies by Winfield et al. and Schwartz et al. have shown that around half of older patients experience technical difficulties when applying eye drops, such as aiming, squeezing the bottle, or blinking. The support patients receive from their families has a significant impact on consistency and persistence in therapy application (9). It is crucial for doctors to understand the importance of involving the patient's family in the treatment process, especially for asymptomatic diseases like glaucoma.

The challenge of establishing good adherence becomes more complex when considering other medications patients take for

associated systemic diseases. Different medications with varying application methods make it challenging for patients to use therapy correctly and regularly (4).

A significant number of our patients, especially the elderly, take medications for diabetes, arterial hypertension, asthma, and other diseases regularly. We demonstrated in our study that the presence of concomitant diseases, whether ophthalmological or systemic, significantly reduces patient adherence. The adherence of patients without other diseases except glaucoma was 87.5%, while the adherence of patients with associated diseases was only 35.14%. There is a clear statistically significant correlation between the presence of associated diseases and reduced patient adherence.

To achieve better adherence, the doctor needs to consider the patient's daily habits and obligations when creating a daily medication schedule. It is also desirable to associate the medication intake time with some daily activity to avoid forgetfulness as a significant reason for non-adherence (10). Studies have shown that monotherapy and once-daily dosing are associated with greater consistency and persistence in medication intake. If combined therapy is necessary, it is better to choose a fixed combination, as it simplifies the dosing regimen, leading to better adherence and satisfactory treatment effectiveness (11). Adding another medication to the treatment regimen significantly decreases patient adherence, as shown in the study by Robin et al. (12). Similar results were obtained in our study, where we demonstrated that the use of a higher number of medications leads to reduced patient adherence. The percentage of non-adherent patients among those using three drugs was 57.14%, and among those using four drugs, it was as high as 69.23%, significantly higher compared to the percentage of non-adherent patients among those using only one drug, which was 18.19%.

We demonstrated in our study that the level of education also influences the degree of adherence. Individuals with lower educational levels are often of lower socioeconomic status, limiting them to using medications covered by the National Health Insurance Fund. These patients frequently cannot afford medications without preservatives (e.g., Benzalkonium Chloride—BAK) due to financial constraints, and they have a significantly lower incidence of side effects. The most common side effects occur as a result of anterior surface eye diseases (chronic inflammation of the eyelid margins, dry eyes, chronic hyperemia, allergic reactions to preservatives in the medication) (13). These side effects were the main reason for patient non-adherence in our study, where all patients (100%) listed discomfort, itching, stinging, and redness as reasons for non-adherence. Many studies conducted worldwide have not found a connection between patient adherence and medication side effects (14, 15). The disparity between our results and theirs likely lies in the different socioeconomic profiles of patients. It is necessary in clinical practice to distinguish between side effects that genuinely require discontinuation of therapy and symptoms of a highly subjective nature not accompanied by objective symptomatology. In any case, patients must be fully informed about the nature of their disease and potential outcomes if they discontinue treatment spontaneously.

Konstans et al. showed in their study that one of the key factors in achieving good adherence is the patient's good understanding of the nature of the disease. Consequently, establishing a good relationship and trust between the doctor and patient, as well as regular check-ups, are essential factors in glaucoma treatment and preventing disease progression (10). In the GAPS study, Friedman et al. demonstrated that regular check-ups are significantly associated with better patient adherence (16). Similar results were obtained in our study, where we showed a statistically significant correlation between regular attendance of follow-up examinations and regular medication intake.

Conclusion

There is a relatively high, but not entirely satisfactory, level of adherence among outpatients with open-angle glaucoma. Optimal therapeutic regimens and medications with fewer side effects, patient education about the nature of the disease and its potential consequences, such as possible blindness, as well as emphasizing the importance of regular medication intake, could lead to an improvement in adherence levels.

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PROCENA ADHERENCIJE BOLESNIKA SA GLAUKOMOM OTVORENOG UGLA LEČENIH U AMBULANTI

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Glaukom je hronična, progresivna neuropatija optičkog živca, koja dovodi do trajnog oštećenja u vidnom polju. Iako jednom dijagnostikovan glaukom nije moguće izlečiti, pravilnim korišćenjem terapije mogu se sprečiti dalja progresija bolesti i gubitak vida. Uspostavljanje dobre adherencije prema farmakoterapijskim preporukama predstavlja jednu od ključnih komponenata u lečenju glaukoma.

Cilj ovog rada bio je da ispita stepen adherencije kod vanbolničkih pacijenata sa glaukomom otvorenog ugla.

Istraživanje je sprovedeno na Odeljenju za glaukom Klinike za očne bolesti Kliničkog centra u Nišu februara 2019. godine uz pomoć anonimne ankete na dobrovoljnoj bazi. Upitnik je popunilo 77 ljudi. Upitnik se sastojao od 11 pitanja u vezi sa demografskim i socio-ekonomskim podacima o ispitanicima, dužinom trajanja bolesti, adherencijom prema preporučenoj terapiji, te razlozima moguće neadherencije.

Od ukupnog broja ispitanika, njih 62,34% navelo je da redovno uzima terapiju; 37,66% bolesnika nije redovno uzimalo propisane lekove. Među onima koji nisu redovno uzimali terapiju više je bilo starijih osoba ($p = 0,00001$; $p < 0,05$). Nije uočena statistički značajna razlika kada je reč o polu. Pacijenti koji su u terapiji koristili više lekova imali su manju adherenciju od onih koji su koristili samo jedan lek ($p = 0,00034$; $p < 0,05$). Bolja adherencija zapažena je kod pacijenata koji nisu imali pridružene bolesti (87,5%) nego kod onih koji su imali neku pridruženu bolest (35,14%); između ovih parametara postojala je statistički značajna korelacija ($p = 0,000002$; $p < 0,005$). Kao razloge neadherencije bolesnici su najčešće navodili neželjene efekte i nelagodnost (100%), dugo korišćenje terapije (89,66%) i zaboravnost (62,01%).

Postoji relativno visok, ali ne i potpuno zadovoljavajući stepen adherencije među vanbolničkim pacijentima sa glaukomom otvorenog ugla. Izbor optimalnog terapijskog režima i lekova sa manje neželjenih efekata, edukacija bolesnika o prirodi same bolesti i njenim posledicama, tj. o mogućem slepilu, kao i naglašavanje značaja redovnog korišćenja terapije mogli bi dovesti do poboljšanja stepena adherencije.

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***Ključne reči**: adherencija, glaukom otvorenog ugla, vanbolnički pacijenti*

Original article

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TELEDENTISTRY EXAMINATION AFTER SURGICAL EXTRACTION OF THIRD MOLARS

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One of the most commonly performed surgical interventions in dentistry is the third molar extraction. This procedure may be performed routinely, but it may also be associated with complications. Numerous variations in the postoperative course have been described. Our investigation aimed to examine the reliability of postoperative follow-up of surgical third molar extraction using the method of teledentistry via patients' smartphone devices.

We performed a randomized experimental study. The follow-up examination undertaken a day after the surgical procedure consisted of two parts: a virtual one and an in-person one. Our digital examination involved photographs taken by the patients themselves and an electronic survey. The oral surgeon first evaluated the digital follow-up results before conducting the patient in-person examination. The results were processed and compared using Cohen's kappa coefficient, Z test and McNemar's χ^2 test for the statistical significance cut-off value of $p = 0.05$.

In total, 40 follow-up examinations (100%) were performed. In 39 (98%) examinations, the results obtained with in-person and virtual approaches were identical. In 7 cases (25%), the indications for a change in therapy were presented by both methods. The actual number of therapy changes recommended was 10 (100%) for the in-person approach and 9 (100%) for the teledentistry method. The following agreement values were obtained: sensitivity: 0.9750; specificity: 0.9750; efficiency: 0.9750; and Cohen's Kappa: 0.9500. These values suggested an almost perfect agreement.

The diagnostic differences between patient recovery follow-up using the virtual and in-person approaches after third molar surgical extraction were not statistically significant. In that regard, postoperative course follow-up may rely on contemporary digital communication technologies with a high degree of confidence.

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Key words: *teledentistry, third molar, follow-up examination, pericoronitis, edema*

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Introduction

Extraction of the third molars is one of the most common surgical interventions in dentistry. The reasons for third molar extraction may be different (1). They range from dental crowding, then pericoronitis, less and more serious infections caused by these teeth, all the way to complex pathological changes associated with them (2–4). The course of this surgical intervention may be routine or is accompanied by complications; moreover, numerous complications in the postoperative period have been described as well (3–6). Nevertheless, most of these interventions have a normal postoperative period and in most cases, there is no need to change the planned postoperative therapy (7–9). In order to monitor the course of recovery and, if required, to change timely the postoperative therapy, the patients are examined 24 hours after the surgery (10–12).

On the other hand, the ever-increasing presence of digital computerized and telecommunication technologies among the

population has made possible the expansion of telemedicine capacities in various areas of medicine. In some of them, it has already become the standard, and most of them are currently witnessing expansion in that regard (13, 14). Teledentistry, i.e. telemedicine applied in dentistry, offers numerous advantages reflected above all in the availability of distant dentistry consultations, better patient management and significant savings of both time and resources (15, 16).

Aim

The aim of our study was to examine the reliability of postoperative follow-up of surgical extraction of third molars using the teledentistry method based on patients' smartphones.

Material and Methods

Our investigation was a randomized experimental study. The study was approved by the Ethics Committee of the Dental Medicine Clinic in Niš and the Ethics Committee of the Faculty of Medicine in Priština—Kosovska Mitrovica. The study took place at the Dental Medicine Clinic in Niš and the Dentistry Clinic of the Faculty of Medicine in Priština—Kosovska Mitrovica. The study enrolled 37 adult patients of both genders. There were 43 (100%) surgically extracted third molars, 24 (56%) in men and 19 (44%) in women. Out of the total number, 15 teeth (35%) were upper jaw third molars, and 28 teeth (65%) were lower jaw third molars. Altogether, there were 40 (93%) postoperative follow-ups, 22 (55%) performed in men and 18 (45%) in women. Three (7%) follow-ups could not be performed since the patients did not turn up (Tables 1 and 2).

The follow-up examination consisted of two parts. In the first part, the patient was photographed by any present person, usually a patient's escort. The photographs were taken according to the procedure guidelines, but without any prior training of the person who took the photograph. The guidelines involved three extraoral patient photographs: two profiles and one en face, in order to visualize well the extraoral changes (swelling, above all). Then, a couple of photographs were taken of the inside of the mouth, in order to visualize the postoperative area and intraoral tissue in general.

The patients then were asked to fill out the digital survey (Figure 1). Together with the photographs taken, it was uploaded via a local network to the local computer server. The server started an especially created application in support of this study (Figure 2). The server fulfilled all the necessary standards and criteria, including the encryption, authorization and authentication features. In such a way performed digital control examination was then sent to a reviewer (Figure 3). The reviewer made the decision as to the local finding assessment, postoperative recovery of the patient and further therapy (Table 3). The second part of the examination involved a conventional direct, in-person examination of the patient.

Table 1. Number of extractions and follow-ups

	Number of extractions	%	Number of follow-ups	%	Number of missed follow-ups	%
	43	100%	40	93%	3	7%
Men	24	56%	22	55%	2	67%
Women	19	44%	18	45%	1	33%

Table 2. Third molar distribution according to their anatomical sites

	Left		Right		Total	
Upper jaw	7	47%	8	53%	15	35%
Lower jaw	16	57%	12	43%	28	65%
TOTAL	23	54%	20	46%	43	100%

Table 3. Agreement between two methods (in-person and teledentistry) concerning prescribed postoperative therapy after follow-up examination

Parameters	In-person n/N (%)	Teledentistry n/N (%)
Analyzed cases	40/43 (93)	40/43 (93)
identical findings	39/40 (98)	39/40 (98)
different findings	1/40 (2)	1/40 (2)
Additional treatments suggested (cases)	7/40 (17)	6/40 (15)
total number of suggestions	10 (100)	9 (100)
removal of one or more sutures	1/10 (10)	1/9 (11)
drain placement or removal	3/10 (30)	3/9 (33)
correction of antibiotic therapy	2/10 (20)	2/9 (22)
correction of antioedematous therapy	4/10 (40)	3/9 (33)
n—number of cases; N—total number		

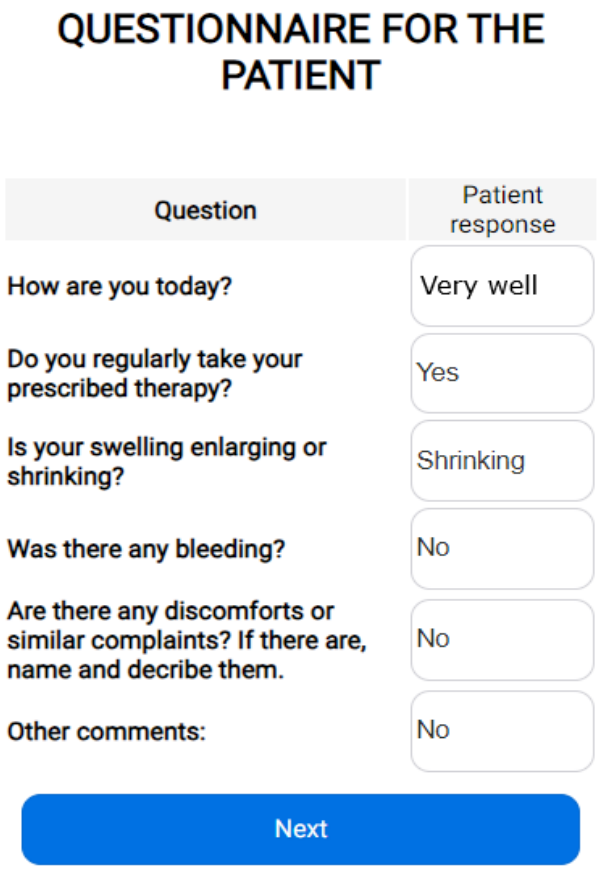

Figure 1. Digital questionnaire on patients' smartphones

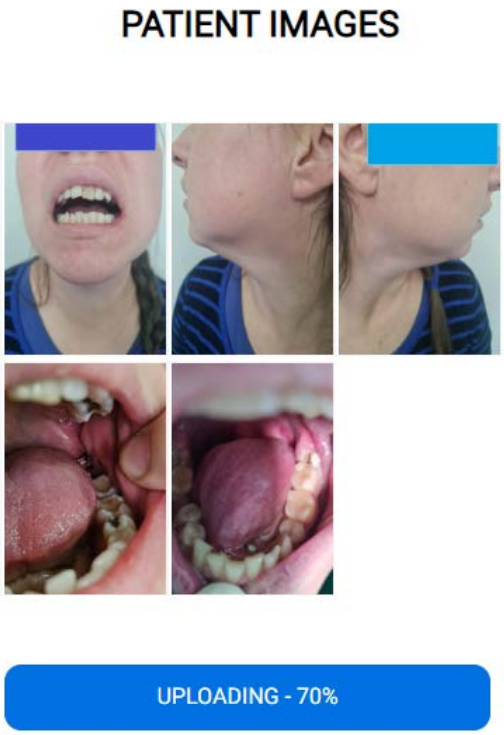

Figure 2. The upload of photos from smartphones to the local computer server

Figure 3. Digital control examination on a desktop PC of the reviewer

The degree of diagnostic accuracy was determined by the following scale:

●correct—if the teledentistry postoperative diagnosis was identical to the primary one, or if it was made as an acceptable differential diagnosis;

●incorrect—if the teledentistry postoperative diagnosis was completely different from the primary one, or the diagnosis was not made at all.

Statistical data processing was performed using the MedCalc software ver 18.6 for Windows. The degree of agreement between the examinations was determined, as well as sensitivity (SE), specificity (SP) and efficacy (EFF). Cohen's kappa coefficient was calculated, Z test comparison was done, as well as the testing with

McNemar's χ^2 test for the statistical significance cut-off of p = 0.05.

Results

In total, 40 (100%) control examinations were performed. In 39 (98%) examinations the results obtained with digital teledentistry method were identical to in-person patient examination results. In 1 examination (2%) , the results were different. A change or supplementation of therapy at the first control examination was made in 7 cases (25%) with both methods. It should be mentioned that the total number of therapy changes with the in-person method was 10 (100%), while with the teledentistry method, it was 9 (100%). With the in-person method the following indications were made: in 1 case (10%), suture removal; in 3 cases (30%), drain placement or removal; in 2 cases (20%), change of antibiotic therapy; and in 4 cases (40%), change of antiedema therapy. With the teledentistry method, the following indications were made: in 1 case (11%), suture removal; in 3 cases (33%), drain placement or removal; in 2 cases (22%), change of antibiotic therapy; and in 3 cases (33%), change of antiedema therapy.

Out of 40 follow-up examinations (100%), an agreement between the in-person method and teledentistry was detected in 39 cases (98%). The following statistical parameters should be reported as well: Sensitivity (SE): 0.9750 (95% CI: 0.8684−0.9994), specificity (SP): 0.9750 (95% CI: 0.8684−0.9994), efficiency: (Correct classification rate) = 0.9750 (95% CI: 0.9126−0.9970). Cohen's Kappa: 0.9500 (95% CI: 0.8816−1.0184). Test of Ho: Kappa = 0: z = 8.50, p = 0.0000 t.t.t. Observed agreement: 0.9750 (95% CI: 0.9126−0.9970), chance agreement: 0.5000 (95% CI: 0.0000−0.0000), positive agreement: 0.9750 (95% CI: 0.9404−1.0096), negative agreement: 0.9750 (95% CI: 0.9404−1.0096). The obtained agreement values suggested an almost perfect agreement. The diagnostic differences were not statistically significant in our study.

Discussion

The idea that teledentistry can be used in follow-up examinations in patients who have undergone surgical third molar extraction parallels the advances made in digital and telecommunication technologies. In its essence, it is comfortable for the patients in the sense that visits to their dentistry clinics are avoided, together with everything associated with the visits: traveling, waiting, expenses, additional exposure to the risk of contracting COVID-19 and other diseases (17). This makes great sense for the patients living at a distance from the place where oral surgery interventions are performed, but also for those who have to travel immediately after the intervention (18, 19). If we take into account the absence of health professionals from work to perform in-person control examinations, the savings and other benefits are significantly greater (20).

The control examination a day after the surgical third molar extraction is necessary for a normal postoperative course (without adverse events) (21, 22). In general, examinations using the methods of telemedicine are on the increase, especially after the COVID-19 epidemic (23−25). In dentoalveolar surgery, follow-up examinations using the method of teledentistry can be successfully implemented in the follow-up of patient recovery after a dental root resection. Our results agree with the results obtained by Miladinović et al. (26). They established that in-person follow-up examinations a day after the root tip resection can be successfully replaced by distant *store and forward* telemedicine examinations. Using an Android application, Krishna et al. (27) were able to monitor patient recovery after routine dental extraction, with an additional ability to provide distant instructions. They found a significant decrease in complication rates following dental extractions.

Gangwani et al. (28) reported successful use of teledentistry consultations in oral and maxillofacial surgery (OMS) procedures, especially in dentoalveolar surgery, in the domains of preoperative patient preparation and postoperative dental care. Kummerow et al. (29) followed the postoperative recovery of patients in general surgery, finding that 68% of doctors and patients thought that it was as good as a visit to a clinic. Further, 24% of them preferred clinical examination, while 8% preferred online examination. Crummey et al. (30) performed a study investigating video-assisted consultations in oral surgery patients. They found that the patients were satisfied with such examinations, but that further standardization of the examinations was required. Jiang et al. (31) established that the telemedicine method in patients undergoing total knee arthroplasty was superior to the classical face-to-face rehabilitation method. In contrast to the above-mentioned authors whose results agree with our results, Walker et al. (32) obtained rather different results in their study. In children with surgically treated clefts, they found that postoperative control examinations could not be successfully performed via electronic ways. As the reason for this, they reported essentially technical problems.

Heimes et al. (33) reported that teledentistry examinations after minor dental surgery interventions were preferred by 83.3% of patients, while 16.7% of patients preferred to adhere to conventional dental aftercare. They also found that there was no statistically significant difference regarding the frequency of symptoms or complication rate. Qari et al. (34) compared the experience of patients at follow-up examinations during the treatment of diseases affecting the temporomandibular joint. They were unable to

identify any significant differences in patient experience with virtual and conventional approach

and thus concluded that control examinations could be performed virtually with a high degree of quality. Difficulties in that regard could be encountered only with older patients, without adequate knowledge in working with virtual platforms.

All these results obtained by reputable authors are in line with our results, except for the study by Walker et al. (32). It is conspicuous that the number of studies dealing with direct comparisons is rather low, which can be explained by the still insufficiently developed presence of teledentistry in the practice of dentistry.

However, the benefits of digital communication technologies in the everyday practice of dentistry are constantly becoming clearly visible. The COVID-19 pandemic perhaps gave the process a special propulsive force.

Conclusion

The perspectives of teledentistry in the follow-up of dental patients are bright. In particular, in the monitoring of the postoperative course after surgical extraction of third molars, the method of teledentistry can be used with a high degree of reliability, i.e., there are no statistically significant differences between the virtual follow-up approach and conventional in-person patient examination.

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TELESTOMATOLOŠKI KONTROLNI PREGLED POSLE HIRURŠKOG VAĐENJA UMNJAKA

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Ekstrakcija umnjaka je jedna od najzastupljenijih stomatoloških hirurških intervencija. Može biti rutinska i komplikovana. Opisane su brojne varijacije u postoperativnom toku. Cilj našeg istraživanja bio je da ispita pouzdanost postoperativne kontrole hirurške ekstrakcije umnjaka metodom telestomatologije; u tu svrhu korišćeni su pametni telefoni ispitanika.

Sprovedena je eksperimentalna randomizovana studija. Kontrolni pregled urađen dan nakon operativnog zahvata, imao je dva dela: virtuelni i pregled pacijenata u ordinaciji. Digitalni kontrolni pregled obuhvatio je fotografije ispitanika i elektronski upitnik. Oralni hirurg je najpre ocenjivao digitalni kontrolni pregled, a potom je neposredno pregledao ispitanike. Rezultati su obrađeni i upoređeni korišćenjem Cohenovog kapa koeficijenta, Z-testa i McNemmarovog χ^2 testa; prag značajnosti bio je $p = 0,05$.

Urađeno je 40 (100%) kontrolnih pregleda. Prilikom 39 (98%) pregleda dobijeni su identični rezultati neposrednim i virtuelnim pregledom. I u jednoj i u drugoj metodi je u sedam (25%) slučajeva zapaženo da je neophodno promeniti terapiju. Broj konkretnih izmena terapije u metodi neposrednog pregleda iznosio je deset (100%), a u metodi telestomatologije devet (100%). Poređenje rezultata dobijenih prilikom kontrolnih pregleda ukazalo je na to da među njima postoji usaglašenost, čije su vrednosti bile: senzitivnost: 0,9750; specifičnost: 0,9750; efikasnost: 0,9750; Cohenov kapa koeficijent: 0,9500. Ove vrednosti ukazuju na skoro savršenu usaglašenost rezultata.

Dijagnostičke razlike između praćenja oporavka ispitanika sa hirurškom ekstrakcijom umnjaka virtuelnim putem i onog koje podrazumeva konvencionalnu metodu neposrednog pregleda nisu bile statistički značajne. Praćenje postoperativnog toka može se osloniti na moderne digitalne komunikacione tehnologije, budući da se ispostavilo da je njihova upotreba u te svrhe veoma pouzdana.

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***Ključne reči**: telestomatologija, umnjak, kontrolni pregled, perikoronitis, otok*

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IN VITRO FIBROBLASTS' RESPONSE TO THE TWO COLLAGEN MEMBRANES OF DIFFERENT ORIGIN

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Collagen-based biomaterials are largely used in tissue engineering and regenerative medicine. The sources of collagen for the design of those biomaterials are numerous. Although most collagens are highly biocompatible, the origin can influence the physicochemical and biological properties and guide the final outcome after implantation *in vivo*. A large number of collagen membranes are used in oral and maxillofacial surgery as barrier membranes to cover tissue defects in order to prevent connective tissue infiltration, and that is why it is crucial to examine their interaction with fibroblasts. In this study, we examined the fibroblasts' response to the two commercially available collagen membranes of different origins: porcine vs. equine, in cell culture *in vitro*. The effect of collagen membranes on the proliferation of L929 fibroblasts was examined in a direct cell culture system. Cells were seeded on the collagen membranes and incubated for seven days. The proliferation rate was assessed by the MTT test. There was a significant decrease in cell proliferation rate in the presence of both membranes with a pronounced anti-proliferative effect of the tested porcine membrane. This result speaks in favor of the application of both examined membranes as barrier membranes. Differences in examined collagen membranes may be due to the different origins of collagen although different manufacturing processes may significantly influence cell behavior *in vitro* as well. Further studies with more collagen membranes of various origins should be conducted in order to make final conclusions about the effect of collagen origin on cell behavior *in vitro*.

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Key words: *collagen, collagen membranes, fibroblasts, L929, in vitro, cell proliferation*

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Introduction

To restore cells, organs, or tissues that have been lost or damaged due to illness or trauma, regenerative medicine and regenerative dentistry aim to develop methods for creating new ones. Regenerative medicine and dentistry include techniques like tissue engineering, the construction of prosthetic organs, and the application of therapeutic stem cells (1). In scaffold-based approaches, cells, signals, biodegradable, and mechanically stable polymeric scaffolds are used to meet specific therapeutic needs and attain excellent cell survival and retention rates (2). In the field of regenerative dentistry, both soft and hard tissues can be restored and regenerated using tissue engineering methods (3, 4). It is an interdisciplinary field that integrates engineering and medical science ideas to produce biological replacements that maintain, repair, or improve tissue function. To treat a variety of tissue defects, tissue engineering combines cells, scaffolds and bioactive substances. Scaffold-based and scaffold-free treatment techniques have dramatically advanced thanks to the design of novel functionalized dental biomaterials and regenerative engineering techniques (5).

Collagen is the most important polymer in bone and soft tissue engineering (6). It is the most prevalent protein of the extracellular matrix (ECM) in the mammalian body and it makes up one-third of all proteins found in different tissues. Collagen is biocompatible, biodegradable and is

neither cytotoxic nor immunogenic (4, 6). Those properties make collagen a gold standard for use in regenerative medicine and tissue engineering. There are different forms of collagen found in mammals, but the most abundant is collagen type I. Collagen can be utilized as a scaffold, membrane, gel or hydrogel, in liposomes, etc (2, 6, 7). The literature describes many resorbable collagen membrane types (2). In tissue engineering, collagen-based membranes are primarily categorized by species: porcine, bovine, equine; and tissue origin: dermis, peritoneum, pericardium, etc. (8, 9). The clinician chooses the most appropriate membrane depending on their characteristics and desired outcome. In addition to supporting wound healing for soft tissue augmentation, collagen-based membranes can serve as a physical barrier to stop connective and epithelial tissue ingrowth into the defect site so that defects can heal properly without forming scarring tissue (10). The foundation of guided tissue regeneration (GTR) is the idea that placing physical barriers inhibits the flap's epithelium and connective tissue cell ingrowth and creates an isolated area for the inward migration of periodontal ligament cells and to impart resistance to bacterial contamination (11).

Collagen-based membranes can differ by added additives and manufacturing procedures in addition to variances in indication and origin. Collagen, as part of the ECM, is naturally degraded by the group of endopeptidases, specifically matrix metalloproteinases (12). Various pathogens, especially periodontal bacteria such as *Porphyromonas gingivalis* and *Treponema denticola* also produce collagenases and may affect the degradation time of collagen membranes when implanted in the oral region (13, 14). That is important in periodontal, oral and maxillofacial surgery because pathogens can jeopardise the treatment by premature degradation of the membrane. Many cross-linking methods are used to improve the physicochemical properties of collagen and to achieve control of collagen biodegradability time. Chemical cross-linking with agents such as aldehydes improves the mechanical strength and prolongs the time of degradation while physical cross-linking treatment with irradiation or biological using biological agents (transglutaminase and horseradish peroxidase) are nonchemical manufacturing techniques that lead to the control of biodegradability (2, 10, 15). However, it has been shown that modification of collagen by cross-linking techniques can lead to partial cytotoxicity (16–18). Additionally, the origin of the collagen membrane was reported to influence the physicochemical behaviour of the collagen membrane (19).

The aim of this study was to analyse and compare the *in vitro* biocompatibility and fibroblasts' response to the two collagen membranes of different species origin, porcine and equine.

Material and Methods

Collagen membranes

In this study, two commercially available collagen membranes of different species origin, porcine and equine, were analyzed:

- 4BONE RCM (MIS Implants Technologies Ltd., Israel) (membrane labeled as PM in the study) is a resorbable collagen membrane made from porcine skin collagen types I and III. According to the manufacturer, this membrane has a prolonged time of resorption achieved by a chemical cross-linking technique using formaldehyde and can be used in GTR as an effective barrier for a period of 4–6 months.
- PARASORB RESODONT® (RESORBA Medical GmbH, Germany) (membrane labeled as EM in the study) is a collagen membrane of equine origin, which contains 2.8 mg of collagen fibrils per 1 cm^2. According to the manufacturer, the production procedure involves a cross-linking technique without chemical additives. The membrane is completely absorbable with no need for secondary intervention for removal.

Cell culture

L929 mouse fibroblasts were used in this study. The cells were cultured in complete DMEM (low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM stable glutamine and 1% antibiotic-antimycotic solution, all purchased from Capricorn, Germany), in standard cell culture conditions, in a humidified atmosphere at 37 °C with 5% CO_2.

Proliferation assay

Prior to cell seeding, membranes were trimmed under sterile conditions to a square with dimensions 1 x 1 cm and one square membrane was placed per each well of the 24-well culture plate (Greiner Bio-One, Germany). Confluent culture of L929 cells was harvested using Trypsin-EDTA solution (Capricorn, Germany), centrifuged, washed in buffer solution and the number of cells was determined by Trypan blue dye exclusion method. Cells were plated out at density 10^4/well/mL and were directly seeded on examined collagen membranes in 24 well plates in complete DMEM. The cells were incubated on the membranes in standard cell culture conditions for seven days. Cells seeded in wells without membranes, in complete DMEM, incubated for seven days under the same conditions, served as a control culture. Each membrane, as well as the control culture, was examined in triplicates. Cells were microscopically analyzed under phase contrast and images were acquired on an inverted light microscope Axio Observer.Z1 equipped with the Axio Cam HRc camera and ZEN software, blue edition (Carl Zeiss, Germany). Cell proliferation

was assessed by the MTT test. Cell medium was removed, cells were washed with phosphate-buffered saline and 500 µL of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)) in concentration 1 mg/mL was added per each well. The cells were incubated with MTT solution for the next three hours. MTT is reduced by the mitochondrial dehydrogenases of the living cells and as a product purple formazan is formed. The amount of formed formazan is in direct correlation with the number of viable cells. Formazan was dissolved with 100% 2-propanol, and absorbance of the resulting solution was measured at 540 and 650 nm wavelengths on a multichannel spectrophotometer (Multiskan Ascent plate reader, ThermoLab Systems, Helsinski, Finland). The mean absorbance values were calculated for each tested membrane, as well as for the control. The cell proliferation rate was calculated according to the following formula: % cell proliferation = (absorbance value of cells incubated with membrane/absorbance value of control cell culture) × 100.

Statistical analysis

The results of the MTT test were statistically processed and the mean percentage values were calculated according to the above-mentioned formula and presented with relative standard deviations. To determine the statistically significant differences between membranes and control culture, one-way analysis of variance (ANOVA) test was performed. As statistically significant values were considered those for which $p < 0.05$.

Results

Fibroblasts' proliferation on both examined membranes, assessed by the MTT test, is shown in Figure 1. There was a noticeable difference in the cell proliferation rate among equine-derived (EM) and porcine-derived (PM) collagen membranes as well as compared to the control cell culture.

The interaction of cells with collagen membranes and proliferation pattern were monitored microscopically and images under the phase contrast were made at the end of the incubation period, prior to the MTT test, which is shown in Figure 2.

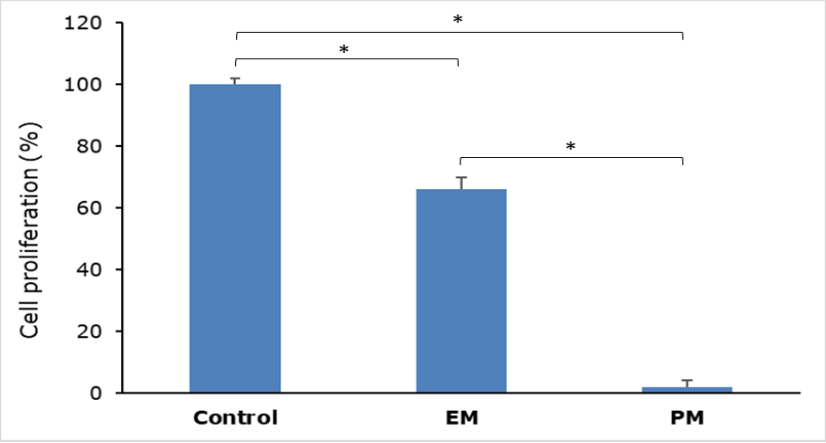

Figure 1. Proliferation of L929 cells in control cell culture, on EM and PM membrane; (*) $p < 0.001$

Figure 2. Morphological appearance of L929 cells in control cell culture (A), L929 cells cultured on EM collagen membrane of equine origin (B) and L929 cells cultured on PM collagen membrane of porcine origin (C); phase contrast, objective magnification 10x

Both examined membranes showed an anti-proliferative effect and significantly influenced cell proliferation compared to the control cell culture. In addition, the PM membrane showed a higher anti-proliferative effect than the EM membrane. Rare, elongated cells close to, onto and below the PM membrane were noticed, while a significantly higher number of cells, without significant morphological changes, were noticed in close proximity to, onto and below the EM membrane.

Discussion

There are reports in the literature that the origin of collagen may influence the physicochemical properties of collagen membranes and cell behaviour *in vitro*, but most of these studies were focused on comparing porcine and bovine membranes that are largely used in clinical practice. To the best of our knowledge, there are only a few studies with a comparative analysis of collagen membranes of porcine and equine origins, with a focus on defining the influence of the origin on their effects on cell culture *in vitro* (14, 19, 20).

Regarding the species origin of collagen, there are some concerns and questions about the risk of collagen use and expected tissue response. For instance, the implantation of collagen-based biomaterials of bovine origin carries a risk of transmission of zoonoses such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), or virus-associated diseases, while porcine originated collagen membranes can be rejected due to religious constraints (21, 22). On the contrary, the collagen of equine origin is not associated with virus disease transmission and there is no possibility of rejection due to ethical reasons (23). Furthermore, collagen originating from various species and tissue sources can differ in amino-acid sequence and consequently in its biostability (17, 24). Thus, in this study, we examined two types of collagen membranes of different species origin: porcine vs. equine. We conducted *in vitro* cytocompatibility testing on L929 cell line. *In vitro* testing of biomaterials is the first step when developing new biomaterials. It gives necessary guidance for the *in vivo* testing that comes afterwards. It is a less expensive method, experimentally controllable, repeatable and it does not raise any legal or ethical questions. The biggest disadvantage is that it cannot test chronic effects or pharmacokinetics (8, 10). In our study, the proliferation of L929 fibroblasts was tested after 7 day-cultivation period on two collagen membranes of different species origin. EM membrane demonstrated significantly higher proliferation potential than the PM membrane. Kasaj et al. (20) tested three collagen membranes and EM also showed the highest proliferation potential among tested collagen membranes. Compared to the positive control, the proliferation of cells on the tested membranes in our study was significantly lower compared to the control, which is also in accordance with the results obtained in the above-mentioned study. Data from the literature, also, indicate that the membrane of equine origin can be more suitable for cell proliferation compared to other applicable membranes for the concept of GTR. Kasaj et al. (20) demonstrated the highest rate of human gingival fibroblast (HGF) proliferation on the TutoDent® membrane of bovine origin followed by the EM membrane examined in our study as well, in an observation period of one hour and 48 hours, compared to the resorbable membrane of porcine origin and three other non-resorbable membranes. In that study, the resorbable collagen membranes, including EM, induced a significantly higher number of cells in all examined periods compared to the non-resorbable membranes in the periodontal ligament fibroblasts (PDLF) cell line (20). Authors suggested that different patterns of cell proliferation can be caused by a difference in surface topography and characteristics as well as in pore sizes (20). The above-mentioned findings about the influence of surface topography were confirmed in the study of Willershausen et al. (25) as well, where it was shown that the proliferation rate of HGF was higher on the compact layer of two examined native biomaterials of porcine origin, followed by different growth pattern, compared to spongy layer, during observation time of 48 hours. In the study of Toledano et al. (14), a difference in the biodegradation process analysed *in vitro* between membranes of different origin (porcine vs. equine) was shown, but also different results in degradation tests were obtained between two membranes of equine origin. Through the three different degradation tests, the equine collagen membrane covered with equine bone particles was more susceptible to the degradation process in comparison with other membranes, derived from the porcine dermis and equine pericardium tissue (14). The authors assumed that different biodegradation findings in this study can be related not only to different species and tissue origin but also, to the manufacturing process, in this case, the lyophilizing treatment which influenced the 3D architecture of collagen (14). Furthermore, the scaffold based on native equine collagen (PARASORB Sombrero, RESORBA), the same collagen materials and producer as the EM membrane examined in our study, was evaluated as more suitable for Human-Periosteal Cells (hPCs) proliferation than inorganic scaffolds based on PLGA alone or in combination with Hydroxyapatite (HA) (26).

Based on a comparison of data from our studies and other mentioned findings, we can assume that equine-based collagen material may be a good basis and environment for cell growth but it depends on the type of cells which is going to be seeded, as well as its 3D architecture. Thus, Raimondi et al. (27) showed that native, non-cross-linked collagen type I from equine Achile tendon (commercially available sponge Antema®) is not suitable to support human chondrocyte survival *in vitro* during the observation period of

two weeks, even newly synthesized collagen was detected (27). On the other hand, Masci et al. (28) reported that a collagen scaffold of the same origin, is a convenient scaffold for the proliferation, migration, and adhesion of murine fibroblasts (NIH 3T3), through extended filopodia and macrovesicles shedding (28). There was no literature data about previous PM membrane testing. In our study, the cell proliferation rate on the PM membrane was significantly lower than that on the EM membrane. Previous studies of porcine collagen membranes (25) showed that they caused decreased cellular proliferation and higher cytotoxic effects compared to the collagen membranes of other origins. Also, the porcine membrane was shown to lead to increased production of proinflammatory mediators by mononuclear cells at 4 and 12 h of incubation and decreased cell viability compared to the bovine membrane (29). Behring et al. (17) suggested that not only the origin of membranes is important, but also the manufacturing process. There is data in the literature about connections between the prolonged period of biodegradation caused by cross-linking modification, with a reduction in biocompatibility (30, 31). Chemical cross-linkers that are frequently used in the production of natural polymer-based biomaterials are shown to significantly influence the biocompatibility of biomaterials, making the biomaterials cytotoxic for cells (32–34). Naturally derived chemical cross-linkers are a much better solution for the cross-linking process in polymer-based biomaterials production which was shown in the case where EDC-NHS was compared with genipin for cross-linking of wound dressing material based on alginate and chitosan (35). In our study, the production of EM membrane involves a cross-linking technique without chemical additives (information provided by the manufacturer), while in the production process of PM membrane, a chemical cross-linking method was used (information provided by the manufacturer), which could cause pronounced anti-proliferative effect of PM membrane compared to EM membrane. A study by Schorn et al. (36) showed that not only origin, collagen type and modification process such as cross-linking can affect the proliferation rate, attachment, and cytotoxicity rates, but also adding other substrates on the membrane. Results from that study showed higher cell proliferation and cell viability of osteogenic cell lines on Bio-Gide® membrane of porcine origin and RESODONT® membrane of equine origin than the other membranes tested. On the other hand, the GENTA-FOIL resorb® membrane of equine origin, with added gentamycin, showed the highest cytotoxicity rate (36). Authors of the same study assumed that the rough surfaces of the RESODONT® and Bio-Gide® membranes might be one of the reasons for their high rates of cell attachment (36).

We must mention the limitations of our study. It cannot provide us with precise information regarding the tissue response to these membranes because it was only carried out on one cell line under controlled *in vitro* cell culture conditions. It merely provides us with the appropriate direction regarding what ought to be anticipated while conducting an *in vivo* study, which is the following stage in the research of biomaterials intended for regenerative medicine and tissue engineering.

Conclusion

Our results show that there is a significant difference in the proliferation rates between cells cultured on examined membranes, in examined conditions. The proliferation of fibroblasts was significantly reduced in the presence of the PM membrane (membrane of porcine origin), while slightly reduced on the EM membrane (membrane of equine origin). This suggests that both membranes, particularly PM, may be used as good barrier membranes to prevent connective tissue ingrowth into the bone defect site. The difference in the proliferation of fibroblasts on examined membranes could be due to the different origins of collagen membranes but also observed differences and anti-proliferative effect could be due to the differences in the manufacturing process that may significantly affect the cell growth *in vitro*.

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IN VITRO ODGOVOR FIBROBLASTA NA DVE KOLAGENSKE MEMBRANE RAZLIČITOG POREKLA

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Biomaterijali na bazi kolagena koriste se u velikoj meri u tkivnom inženjerstvu i u regenerativnoj medicini. Brojni su izvori kolagena za proizvodnju ovih biomaterijala. Iako je većina kolagena izuzetno biokompatibilna, poreklo kolagena može uticati na fizičko-hemijska i biološka svojstva biomaterijala i usmeriti konačni ishod nakon implantacije *in vivo*. Budući da se znatan broj kolagenskih membrana koristi u oralnoj i maksilofacijalnoj hirurgiji u svojstvu barijerne membrane za pokrivanje defekta koštanih tkiva da bi se sprečila infiltracija vezivnog tkiva, interakcija ovih membrana sa fibroblastima predstavlja ključan faktor. U ovoj studiji ispitivan je odgovor fibroblasta na dve komercijalno dostupne kolagenske membrane različitog porekla – svinjskog i konjskog – u ćelijskoj kulturi *in vitro*. Uticaj kolagenskih membrana na proliferaciju L929 fibroblasta ispitivan je u sistemu direktne ćelijske kulture. Ćelije su zasađene na kolagenske membrane i inkubirane sa njima sedam dana. Proliferacija ćelija procenjivala se MTT testom. Došlo je do značajnog smanjenja proliferacije ćelija u prisustvu obeju membrana, s tim što je uočen izraženiji antiproliferativni efekat membrane svinjskog porekla. Ovaj rezultat govori u prilogu tome da obe ispitivane membrane mogu biti primenjene kao barijerne membrane. Razlike u ispitivanim kolagenskim membranama mogu biti posledica različitog porekla kolagena, mada treba istaći i da različiti primenjeni proizvodni procesi mogu značajno uticati na ponašanje ćelija *in vitro*. Treba sprovesti dalja istraživanja sa više kolagenskih membrana različitog porekla kako bi se doneli konačni zaključci o uticaju porekla kolagena na ponašanje ćelija u prisustvu ovih biomaterijala *in vitro*.

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***Ključne reči**: kolagen, kolagenske membrane, fibroblasti, L929, in vitro, proliferacija ćelija*

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THE KI-67 CELL PROLIFERATION MARKER IN HUMAN METANEPHROGENESIS

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The kidney plays several essential roles, including the excretion of metabolic wastes, maintenance of key homeostatic parameters of the blood plasma, participation in blood pressure and hormone levels regulation. These diverse functions are enabled by the developmental process that provides the presence of specific cells for performing all diverse functions. Organogenesis of the kidney is an intricate mechanism involving cell proliferation as a fundamentally necessary process. The aim of this study was to determine proliferative activity during the metanephros stage of renal development, based on the spatial and temporal expression pattern of the cell proliferation marker Ki-67. Kidney tissue specimens of 30 human fetuses with gestational ages ranging from 11 to 36 weeks were analyzed. The specimens were divided into three groups based on gestational age, each corresponding to the earlier, mid or late gestation period. Routine histological processing yielded tissue sections. The proliferative activity of the cells (expression of the Ki-67 protein) was examined by an immunohistochemical assessment of Ki-67, according to the manufacturer's protocol. The presence of Ki-67-positive cells characterized all metanephric structures but with different intensity. The most prominent expression was revealed in the nephrogenic zone in the earlier weeks of development, indicating the role of cell proliferation in nephron formation. The intensity of Ki-67 antigen expression gradually decreased in all cortical structures until the end of the trial period. In the metanephric medulla, the proliferation was less pronounced only after week 20, and the only Ki-67 positive cells were single cells of collecting duct epithelia, narrow parts of Henle's loops and the interstitium. Cell proliferation was continuously present during metanephrogenesis. It was characterized by different intensity, more pronounced in the nephrogenic zone and renal cortex due to the dominant presence of cells in their structural components. However, the obvious developmental remodeling of the kidney tissues inevitably indicates the need to correlate proliferation with other developmental processes, apoptosis above all.

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Introduction

The kidneys are the central organs of the urinary system which perform numerous functions important for normal postnatal life. The basic functions of the kidneys are the excretion and removal of harmful products of organic decomposition of molecules from the blood plasma. The kidneys excrete approximately 200 liters of blood per day by filtration. Equally significant is the homeostatic regulation of blood plasma parameters, which includes the regulation of its ionic concentration (the balance between water and salt, acidic and basic molecules) and preserves useful molecules, which also regulates plasma volume and pressure and stabilizes blood pH. These renal functions are inextricably related to their endocrine function. By secreting renin, the kidneys become part of the regulatory RAAS system (Renin-Angiotensin-Aldosterone System), a hormonal system that by secreting signaling molecules regulates blood pressure as well as systemic vascular resistance (1).

These different functions are enabled by the phylogenetic and ontogenetic development of the kidneys, which results in the presence of cells as a tool for performing all complex functions. This process is conventionally called “nephrogenesis”, but even though millions of nephrons are the basis of its function, they are only one part of its

structure. A large part of the parenchyma is occupied by a branching system of collecting channels, a function of the organ-specific connective tissue of the interstitium and cortex, and especially the medulla as well as a single vascular bed (1).

The formation of all organs during the embryonic and fetal phases of intrauterine development, including the kidneys, depends on the spatially and temporally dependent expression of numerous signaling polypeptide factors that regulate cell mitotic activity and coordinated cell death (2-4). In the same way, nephrogenesis includes a highly controlled series of morphogenetic events within the area of the determined cell mass of the intermediate mesoderm on the dorsal side of the embryo (5). Starting from the fourth week of development, three pairs of developmental "kidneys" form consecutively in the embryo: pronephros, mesonephros and metanephros. Although they do not last for a long time and they degenerate in the earliest phases of organogenesis of the urinary systems, the pronephros and mesonephros are developmental stages that have a significant inductive effect on the formation of metanephros from which the final kidney structures will develop (6, 7).

The metanephros, a precursor of the final kidney, is built at the very beginning of development from two basic types of defined cells: epithelial cells of the ureteric bud whose origin is the urethra of the mesonephros and mesenchyme in its environment, whose cells condense to form a metanephric blastema.

Both cell types, ureteral (ductogenic) and mesenchymal (nephrogenic) are subjected to a repeated series of inductive signals that serve to organize the complex architecture of the renal parenchyma (8-10). A series of mutually inductive interactions between these tissues causes the urethral bud to sequentially branch and form the ureter, renal pelvis, calyces, and collecting tubules, while the mesenchyme undergoes a complex process of mesenchymal transition into highly specialized populations of different types of nephron epithelial cells from the renal corpuscle to the end of the distal tubules. The third type of cells, interstitial cells, also differ from the metanephrogenic mesenchyme (4, 11, 12).

Renal organogenesis is based on a balanced course of events on a cellular level, such as proliferation, programmed death—apoptosis, differentiation, and morphogenesis (13-15). Despite the fact that the research is now being conducted on the level of molecular biology, these events have not yet been definitively explained. In several peer-reviewed studies (2, 3, 11-14, 16), the authors presented their most recent findings on the complex dynamics of renal development (spatially and temporally coordinated gene expression activity and the consequent presence of numerous protein factors that regulate mitotic cell activity and programmed cell death) but also warned of the fact that the results were obtained from research on experimental animals because such research is not feasible on human material due to ethical limitations (15-17).

The kidney of an adult develops from less than a thousand cells at the beginning of the development process up to several million at the end of the process, and it is self-evident that organogenesis requires extensive and accelerated cell proliferation. After induction, proliferation is an event that begins in the most vulnerable period of intrauterine development; it continues for weeks under different influences and thus carries the greatest risks for the occurrence of anomalies (18-20). Impaired proliferation and cell death have been shown to be associated with renal abnormalities involving agenesis, dysplasia, hypoplasia, obstructive uropathy, and vesicoureteral reflux, which can lead to chronic renal failure in children (21).

Understandably, proliferation as a subject of interest is still a very current topic. Standard research and diagnostics use typical representatives, which include immuno-histochemical protein markers of proliferation and apoptosis: Ki-67 and representatives of the Bcl-2 family of proteins.

Ki-67 is a non-histone nuclear protein whose presence is differently expressed during the phases of the cell cycle. In the interphase, it can be detected only inside the nucleus, while in mitosis the protein is located on the surface of condensed chromosomes. The fact that the Ki-67 protein is present in all active phases of the cell cycle (G1, S, G2, and mitosis) and that it is absent in quiet (early G1 and G0) cycles, as well as that its presence increases during cell preparation for division, allows immunohistochemical techniques to use Ki-67 as an excellent marker of cell proliferation; the more immunopositive cells, the more cell divisions. Thus, over time, this protein has become a proliferative marker, an important and reliable indicator and indirect measure of the growth fraction of the examined cell population, as well as a prognostic marker in the diagnosis of various phenomena and conditions (22).

Aim

This study aimed to examine the morphological aspects of the metanephric development in kidney samples of human fetuses of different gestational ages, and based on the presence and distribution of immunopositive cells of Ki-67 proliferation markers to determine the spatially and temporally different proliferative activity of cell-building types during the early and late weeks of the metanephros stage of kidney development.

Material and Methods

Material

The material consisted of the kidneys of 30 human fetuses of both sexes, aged 11 to 36 weeks of development, who died suddenly in utero or were autopsied within 24 hours after birth. The material was obtained from the Clinic of Pathology, Clinical Center of Niš. Fetal tissues were treated as autopsy material with the permission of the Ethics Committee of the Faculty of Medicine in Niš (No. 12-6329/4).

The fetuses were examined macroscopically; their weight and crown-rump length were measured; their gestational age was expressed in weeks of intrauterine development.

The study included only those fetuses that did not show any signs of maceration. The material was divided into three groups where Group 1 included fetal kidneys of the gestational age of 11 to 15 weeks, Group 2 of the gestational age of 15 to 28 weeks, and Group 3 of the gestational age of 28 to 36 weeks.

Methods

The kidney samples were fixed in 10% buffered formalin. Routine histological processing provided paraffin blocks that were cut into 5-micrometer thick tissue sections.

For the purposes of histological analysis and assessment of the morphological properties of metanephros of different ages, tissue sections were stained by the standard hematotoxylin-eosin (HE) method.

Proliferative cell activity (detection of the Ki-67 protein) by monoclonal antibody against Ki67 was performed with the use of a monoclonal antibody Ki-67, Clone MIB-1, (Code M7240, Dako, Denmark; dilution 1:75), according to the manufacturer's protocol (23).

Results

In the first group (the gestational age 11 to 15 weeks), the Ki-67 antigen was expressed by cells of the nephrogenic zone, where the reaction was the most intense in the structures of the most superficial part of the cortex, and the immunopositivity of the cells of the inner cortex and medulla was lower (Figure 1a). The strongest Ki-67 immunopositivity was observed in the outer part of the cortex, in the cells of the developmental forms of future nephrons (vesicles and S-forms). There were individual Ki-67 positive cells in the branches of the urethral bud, ampoules and tubular parts (Figure 1b). In the inner part of the cortex, in the formed renal corpuscles, individual cells of Bowman's capsule (the parietal and visceral leaf) were Ki-67 immunopositive (Figure 1b). In the interstitium, a smaller number of mesenchymal cells between the tubular structures showed a positive reaction to the Ki-67 antigen. The cells of the epithelium of the renal pelvis and renal calyces did not express the Ki-67 antigen (Figure 1a).

In the second group (the gestational age 15 to 28 weeks), up to 21 weeks of kidney development the Ki-67 immunopositive reaction remained in the very superficial part of the nephrogenic zone and it was the most intense form just below the capsule (Figure 2).

a) b)

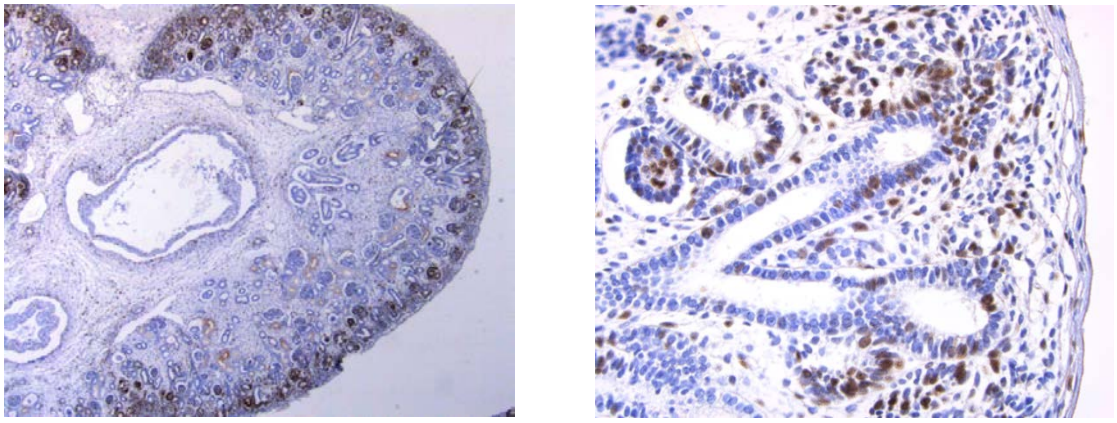


Figure 1. Ki-67 antigen expression in metanephric tissue at 13 weeks of development: a) The strongest expression of the Ki-67 antigen is observed in the nephrogenic zone, completely on the surface of the cortex; epithelial cells of the pelvic urothelium (arrow) do not express the Ki-67 antigen (x40); b) Detail from the previous image: most metanephric blastema cells and vesicles are Ki-67 immunopositive, and only rare single cells in ampoules (asterisks) of the ureteral bud express the Ki-67 antigen (x400)

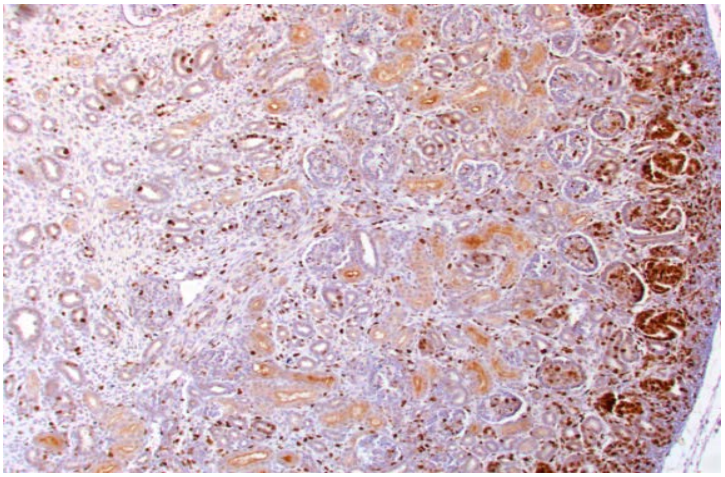


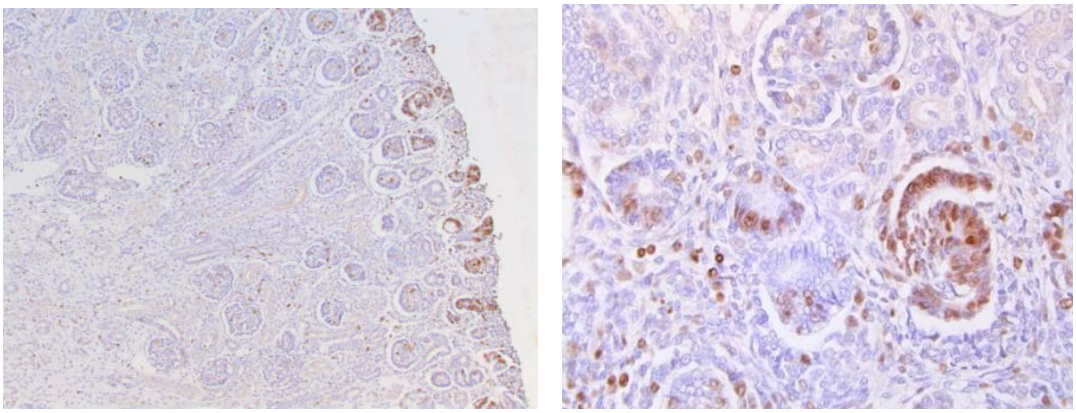
Figure 2. Ki-67 antigen expression in metanephric tissue at 15 weeks of development. The strongest expression of the Ki-67 antigen is observed in the surface layer of the nephrogenic zone. Deeper in the cortex, the immunopositive reaction is seen as a continuous line in the apical region of the epithelial cells of proximal nephron tubules (arrows) and in individual cells within renal corpuscles (x100)

From weeks 21 to 28 of development, the intensity of Ki-67 immunopositivity in metanephric tissue decreased. Only a small number of mesenchymal cells on the surface of the nephrogenic zone showed a weaker expression of the Ki-67 antigen. Somewhat more immunopositive cells were located within the immature renal corpuscles (Figures 3a and 3b). A weak positive reaction was observed in some cells of the proximal tubules (asterisks), while other tubular structures of the Ki-67 were immunonegative (Figure 3b). In the formed renal corpuscles of the internal cortex, the Ki-67 antigen was expressed by individual glomerular cells and their localization coincided with the position of endothelial cells in the capillaries of the glomeruli (Figure 3c). Individual immunopositive cells of collecting ducts and Henle's loops were present in the medulla, as well as rare mesenchymal cells of the interstitium, which sporadically expressed the Ki-67 antigen (Figure 3d).

In about 28 weeks, the strongest Ki-67 immunopositive reaction developed in the thin peripheral layer of the nephrogenic zone (Figures 4a and 4b), and in the medulla, individual cells of the collecting ducts and narrow parts of the Henle's loop (Figure 4c) showed immunopositivity.

In the third group (the gestational age 28 to 36 weeks, Figure 5), the ampoules and the nephrogenic zone disappeared between weeks 32 and 36 of metanephros development, so that the part of the renal cortex that showed the strongest expression of the Ki-67 antigen was not present. Immunopositivity is observed only in individual cells in all kidney structures.

a) b)



c) d)

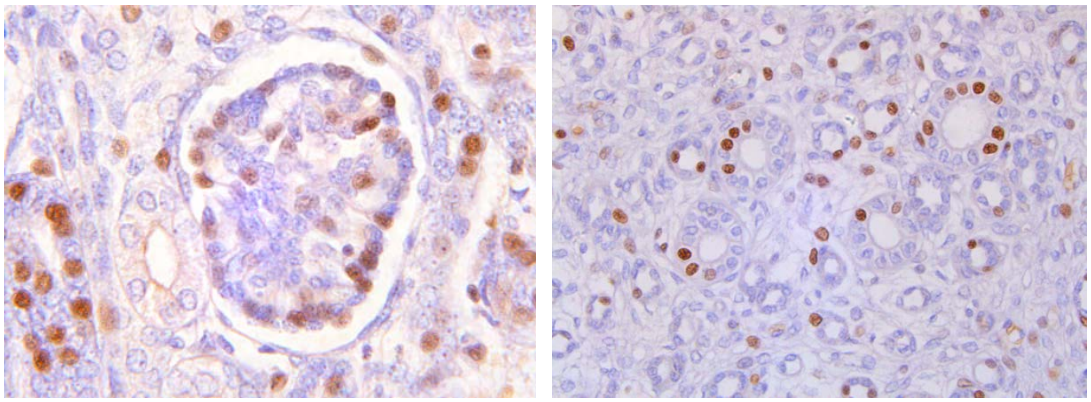
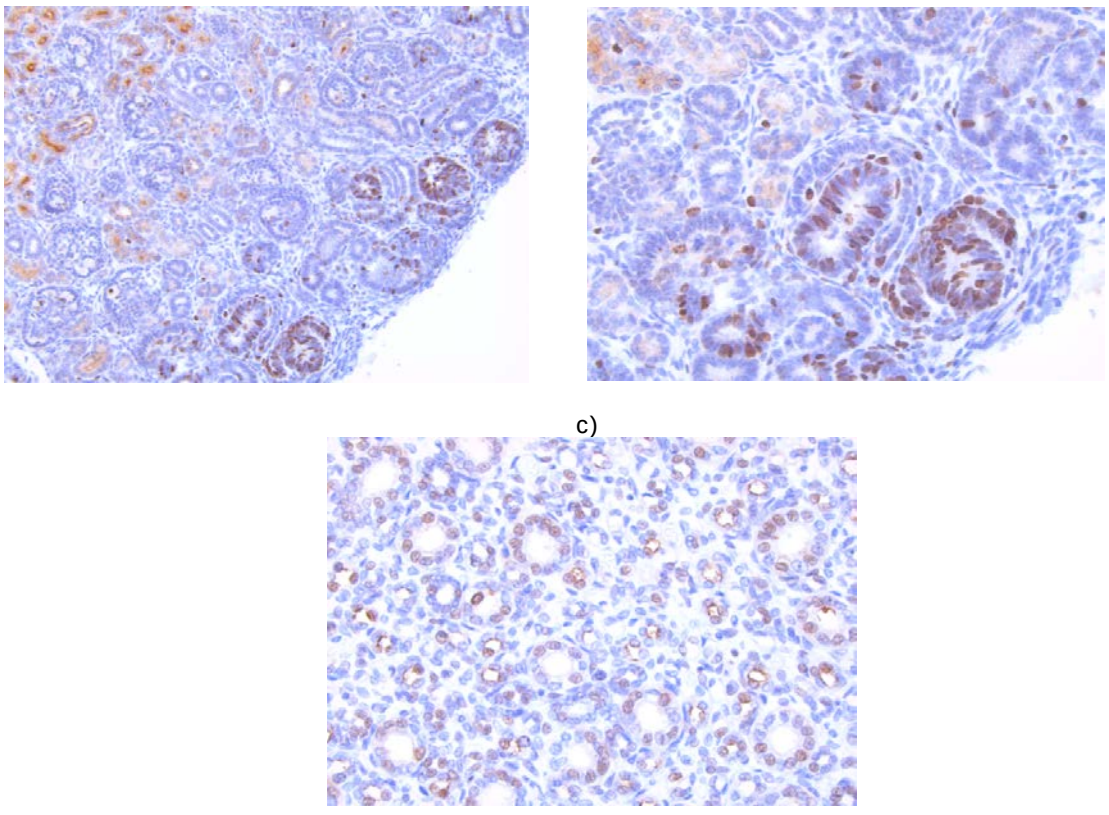


Figure 3. Ki-67 antigen expression in metanephric tissue between weeks 22 and 23 of development. a) A strong immunopositive reaction is observed in the most superficial part of the nephrogenic zone and a weaker immunopositivity is present in the cortex and medulla (x100); b) Part of the nephrogenic zone shows the expression of the Ki-67 antigen in cells of S-shaped the nephron and the forming renal corpuscle; all cells of the future visceral leaf of the Bowman's capsule are highly immunopositive while a smaller number of immunopositive cells are found in the parietal leaf of the Bowman's capsule (x400); c) The formed renal corpuscle contains Ki-67 immunopositive cells of the visceral leaf, while the cells in the parietal leaf of the Bowman's capsule are Ki-67 —immunonegative. x800); d) In the cross-section of the medulla, individual epithelial cells of collecting ducts strongly express the Ki-67 antigen, and in the epithelial cells of the narrow and wider parts of Henle's loop, a sporadic and weaker immunopositive reaction is observed. (x400)

a) b)



c)

Figure 4. Ki-67 antigen expression in metanephric tissue at 28 weeks of development: a) and b) The immunopositive reaction is strongest in immature forms of the nephron in the reduced nephrogenic zone a) x200; b) x400; c) In the cross-section of the medulla, the immunopositive cells belong to the collecting ducts and the narrow parts of Henle's loop (x400)

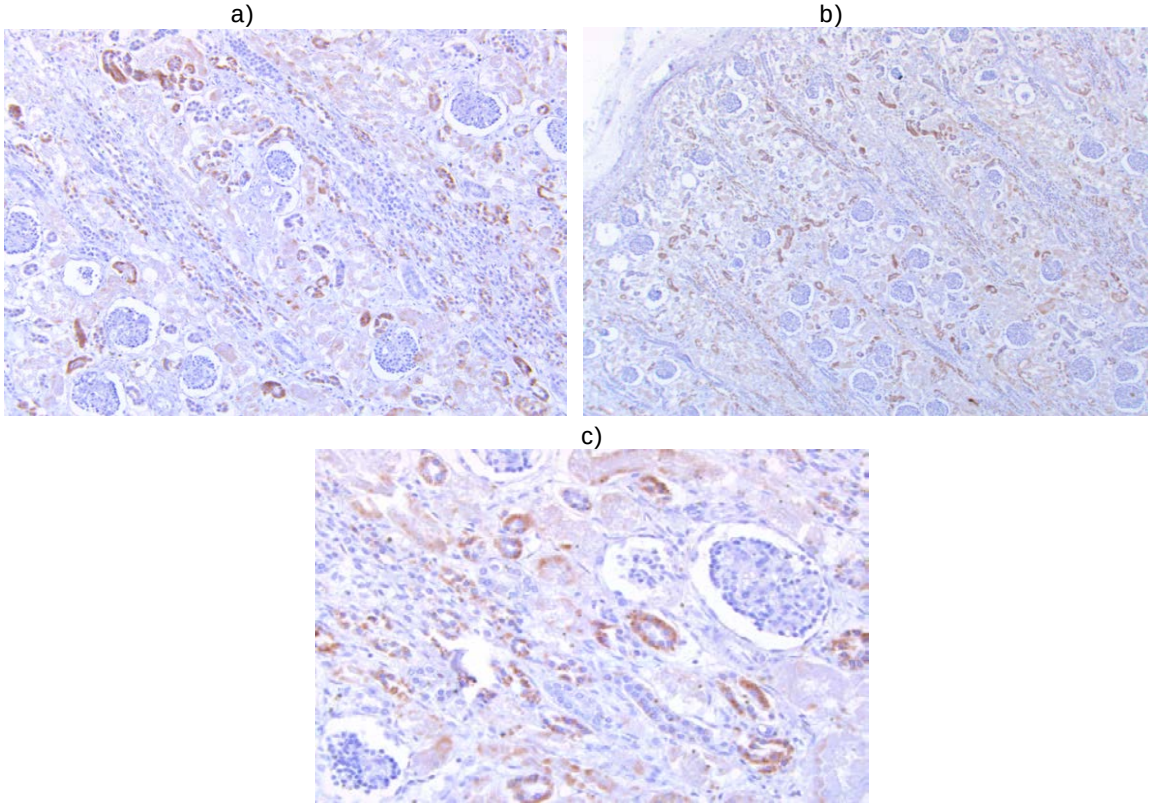
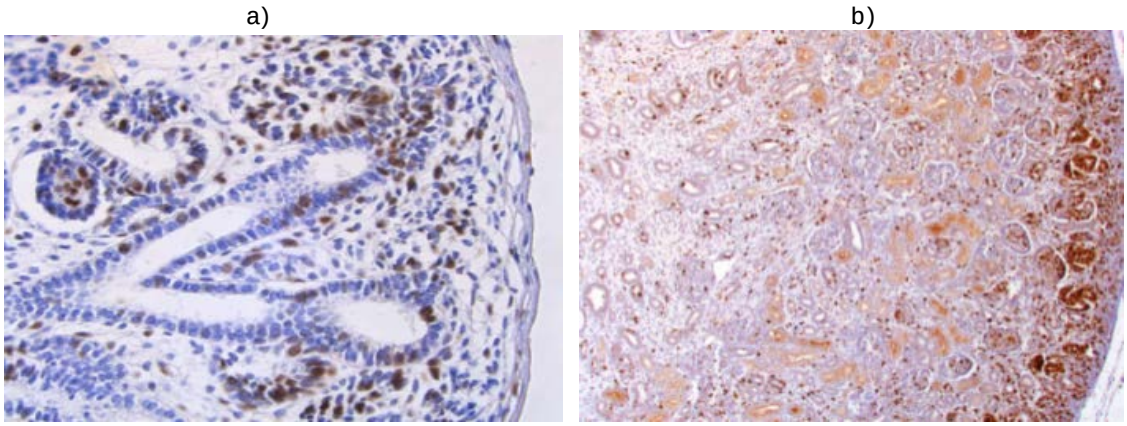


Figure 5. Ki-67 antigen expression in metanephric tissue at 36 weeks of development. A weak fine-granulated cytoplasmic Ki-67 immunopositive reaction is observed in the epithelial cells of the distal tubules of the nephron and the collecting ducts that form Ferrein's pyramids; there is no expression of the Ki-67 antigen in the renal corpuscles, a) x100; b) x200; c) x400

Taking into account the expression of the Ki-67 antigen (Figure 6), it was observed that in the nephrogenic zone, at the beginning of metanephros development (12 to 15 weeks), a strong expression of the Ki-67 (Figures 6a and 6b) was shown by metanephric blastema cells and immature forms of renal corpuscles (vesicles and S-forms). From weeks 19 to 22 of development, the intensity of Ki-67 antigen expression decreased in the renal corpuscles (Figure 6c).

The development of the metanephric medulla began later than that of the cortex, and after week 20, there was a characteristic expression of the nuclear protein Ki-67 in the few epithelial cells of the collecting channels and narrow parts of Henle's loop (Figure 6d); in the same period, the epithelial cells of the collecting ducts were immunonegative. In week 36 of development, the metanephric cortex was formed with all the histological components (renal corpuscles, proximal and distal nephron channels, medullary rays) in which only individual cells of the renal tubular system expressed Ki-67 (Figure 6e).



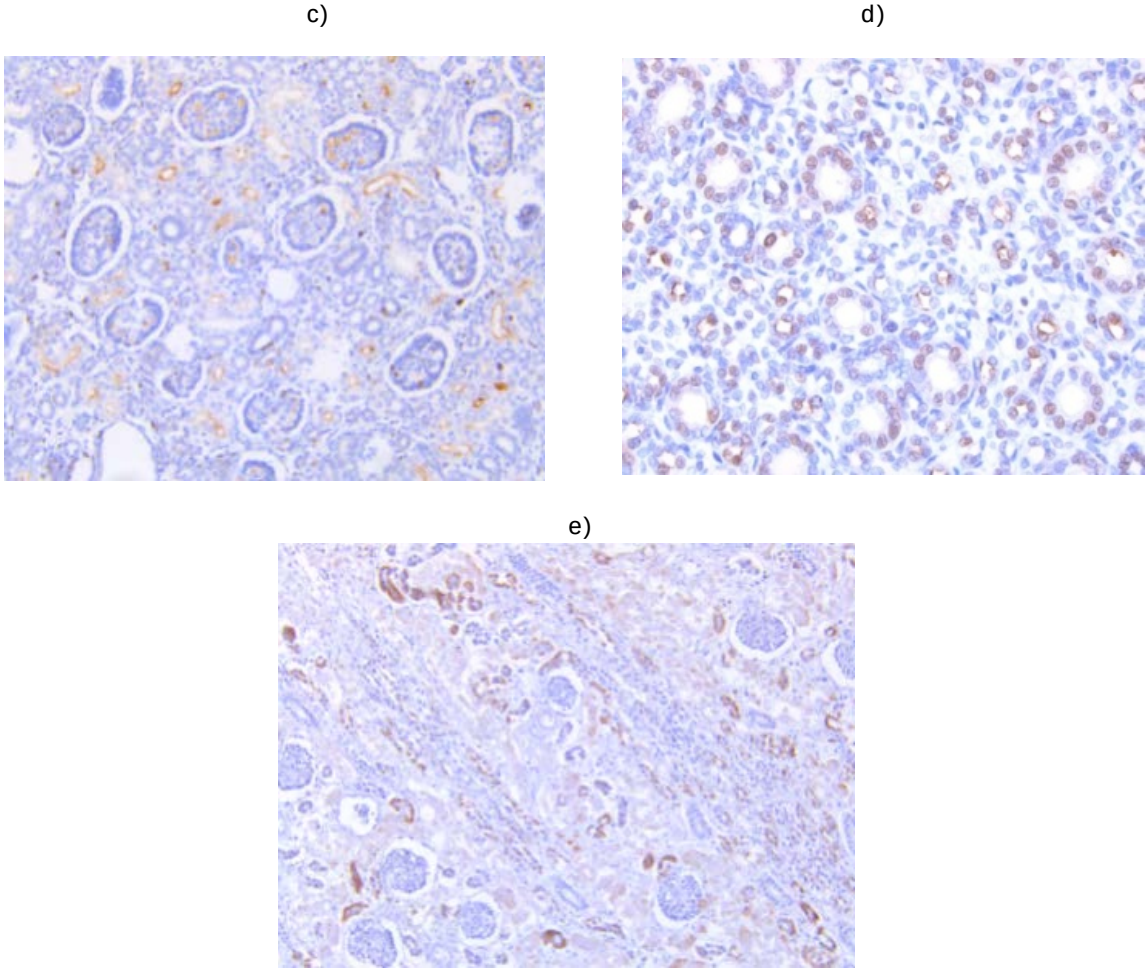


Figure 6. Ki-67 antigen expression in the metanephros in weeks 12–13 (a), 14–15 (b), 19–22 (c), 28 (d) and 28–36 (e) of development: a) The Ki-67 antigen is expressed by individual cells of the metanephric blastema, vesicles and ampoules of the ureteral bud (x400); b) The Ki-67 antigen is most strongly expressed by cells in the surface layer of the nephrogenic zone, and a weak immunopositive reaction is observed in the apical region of the epithelial cells of the proximal tubules of the nephron and individual cells within the renal corpuscles, (x100); c) The Ki-67 antigen is expressed by individual cells within the glomeruli of immature renal corpuscles and, sporadically, cells in the tubules of the forming cortex (x200); d) On the cross-section of the medulla, a small number of Ki-67 immunopositive cells can be seen in the collecting ducts and narrow parts of Henle's loop (x400); e) A Ki-67 immunopositive reaction is observed in the epithelial cells of some distal nephron tubules and medullary rays (x200)

Discussion

Almost one-third of all developmental anomalies in young children are structural or functional abnormalities of the urinary tract. Their morphological description has been well documented for decades, but the mechanisms of their origin have not been fully explained. The reason for this is that much data on normal and abnormal nephrogenesis are derived from studies of animal models, most commonly mice. There are numerous studies done with the aim of explaining key developmental processes, primarily cell proliferation and apoptosis, their genetic basis, and control mechanisms. These studies are diverse in type, methodology, and aims, while studies on human material, embryos, and fetuses are relatively few due to ethical constraints (5, 17, 19, 20). During the last 20 years, by applying gene expression manipulation techniques, as well as experimental models with cell cultures, great progress has been made in identifying cellular and molecular mechanisms that direct normal renal morphogenesis but also provide insight into the possible pathogenesis of anomalies (21).

However, caution is needed in interpreting the results of experimental studies because it has not been established with certainty whether the deciphered developmental principles and active executory molecules are the same in the case of human kidney development. In the broadest biological sense they are, but the influence of a

spectrum of genetic and epigenetic factors (race, climate, age and maternal malnutrition, drug consumption) has been proven to impact developmental processes. Therefore, very little is still known about the cell biology of most kidney malformations (11, 14).

Kidney development is a continuous series of complex processes (induction, proliferation, differentiation, apoptosis, morphogenesis) which simultaneously, but with different intensities and in different cell types, take place in the metanephros, mostly in the nephrogenic zone, the tissue of the renal cortex. Consequently, the morphology (histological picture) of the fetal kidney changes rapidly from week to week, becoming more and more complex for analysis. It is necessary to identify different cell types involved in nephrogenesis, with the help of immunohistochemical detection of their proteins expressed at the cytoplasmic or nuclear level (22).

In this study, the expression of the immunohistochemical cell proliferation marker Ki-67 was examined concerning cell type and the developmental week of metanephrogenesis to gain a better insight into the morphological aspect, as well as the dynamics of cell proliferation as a fundamental developmental process.

During the monitored developmental period, metanephros went through all the characteristic and described developmental stages, from invasion of the ureter bud into the metanephric mesenchymal blast, through all the phases of glomerulogenesis and the gradual differentiation of nephron segments, to the thinning of the nephrogenic zone into a narrow band of tissue. In all the examined tissue samples, the proliferation marker Ki-67 was expressed in different structures of the metanephros, but with different intensities and distribution.

This study also showed that nephrogenesis occurs from the deep to the external cortex (24, 25). In the examined period, the pronounced proliferation was limited almost exclusively to the external nephrogenic cortex, the subcapsular belt of tissue which, during nephrogenesis, is where new nephrons are formed under the inductive influence of growing branches of the urethral bud. Ki-67 positive cells were observed at the tips of the branches of the urethral buds indicating the elongation and branching of the collecting duct system. These cells were also present in the condensed mesenchyme of this zone, along with the developmental forms of the renal corpuscles such as vesicles and S-forms. All together, these observations reflect the activity of the formation of new nephrons. Over time, the expression of Ki-67 decreased rapidly as the nephrons matured. This pattern of expression correlates with those of previous studies (25) of human fetal kidneys, which described that in later stages of renal development, decreased Ki-67 expression occurs with progressive glomerular maturation, while in terminal differentiated glomeruli Ki-67 expression is absent (26).

The phenomena described in this study occur in individual cells but are a necessary part of morphogenesis, a developmental process by which groups of cells acquire complex three-dimensional forms, which could be tracked by analyzing the results of this study. Examples include the serial dividing of ureteral bud branches, the formation of glomeruli and winding nephron tubules from blastemic mesenchymal cells, capillary formation by angiogenesis and vasculogenesis, and the relationship between the cortical and medullary zones. It was noticed that in the earlier stages of development, the defined cortical zone is very narrow with a wide nephrogenic zone, while the medulla is wide and the corticomedullary border is unclear. The proliferative activity in the renal medulla was visible only as the presence of individual Ki-67 positive cells in the epithelium of the collecting ducts, Henle's loops, and the interstitium. Unlike the parenchyma of the cortex, which is constantly building and expanding as a result of the formation of a generation of corpuscles arranged in pillars around Ferrein's pyramids, the medulla is relatively narrowed and necessarily and constantly remodeled so that the rapidly growing number of nephrons can drain when they become functional. This interpretation is consistent with the morphometric study (16, 27) which showed that while the number of glomeruli increased 50-fold from 15 to 40 weeks, the average volume of the cortical segment of the nephron (tubuli contorti) initially decreased and then increased. Simultaneously, there was a constant increase in the average volume of the medullary segment of the nephron (Henle's loop) so that the overall fractional volume of the renal cortex decreased.

Conclusion

The results of this study showed that cell proliferation was continuously present during metanephrogenesis. It took place with different dynamics, was more pronounced in the nephrogenic zone and renal cortex due to the dominance of cells in their structural components, but the evident developmental remodeling of these tissues indicated the need to correlate proliferation with other developmental processes, apoptosis above all.

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KI-67 MARKER ĆELIJSKE PROLIFERACIJE U HUMANOJ METANEFROGENEZI

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Bubreg obavlja više neophodnih funkcija, poput izlučivanja metaboličkog otpada, održavanja ključnih parametara homeostaze krvne plazme, učešća u regulaciji krvnog pritiska i nivoa hormona. Ove raznovrsne funkcije omogućava proces razvoja, koji je osigurao prisustvo specifičnih ćelija za obavljanje svih složenih zadataka. Organogeneza bubrega je kompleksan proces koji uključuje proliferaciju ćelija kao osnovni nužni proces. Cilj ove studije bio je da se utvrdi proliferativna aktivnost u toku faze razvoja metanefrosa na osnovu profila/izgleda prostorne i vremenske ekspresije markera ćelijske proliferacije Ki-67. Analizirani su uzorci bubrežnog tkiva 30 ljudskih fetusa gestacijske starosti od 11 do 36 nedelja. Uzorci su podeljeni u tri grupe na osnovu perioda razvoja, koji su odgovarali ranijem, srednjem ili kasnijem periodu gestacije. Rutinskom histološkom obradom dobijeni su isečci tkiva na kojima je proliferativna aktivnost ćelija (ekspresija proteina Ki-67) ispitivana imunohistohemijskom metodom, monoklonskim antitelom Ki67, i to prema protokolu proizvođača.

Ćelije pozitivne na Ki-67 karakterisale su sa različitim intenzitetom sve strukture metanefrosa. Najizraženije je bilo njihovo prisustvo u nefrogenoj zoni u ranijim nedeljama razvoja, što ukazuje na ulogu proliferacije ćelija u formiranju nefrona. Intenzitet ekspresije Ki-67 antigena postepeno je opadao u svim kortikalnim strukturama do kraja ispitivanog perioda. U meduli metanefrosa proliferacija je bila slabije izražena samo nakon 20. nedelje; bile su pozitivne na Ki-67 pojedinačne epitelne ćelije sabirnih kanala, uskih delova Henleovih petlji i intersticijuma.

Proliferacija ćelija bila je kontinuirano prisutna tokom metanefrogeneze; odvijala se različitom dinamikom, a bila je izraženija u nefrogenoj zoni i bubrežnom korteksu zbog dominacije ćelija u njihovim strukturnim komponentama. Evidentno prisutno razvojno remodelovanje tkiva bubrega ukazalo je na potrebu korelacije proliferacije sa drugim razvojnim procesima, pre svega apoptozom.

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***Ključne reči**: razvoj bubrega, metanefrogeneza, proliferacija ćelija, Ki-67*

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IN SILICO STUDY OF PHYSICOCHEMICAL, PHARMACOKINETIC AND TOXICOLOGICAL PROPERTIES OF 5-LIPOXYGENASE INHIBITORS

Ana Marković

5-Lipoxygenase (5-LO) is an important enzyme involved in the production of leukotrienes, arachidonic acid metabolites which directly affect the development of the inflammatory reaction associated with numerous pathophysiological conditions. As a result, the discovery and development of selective 5-LO inhibitors for therapeutic use became a subject of active research. The study aimed to conduct first a literature review of the most potent synthetic 5-LO inhibitors (with IC_{50} values below 1 μM), focusing on their chemical structure, and then an *in silico* study of their basic physicochemical, pharmacokinetic and toxicological properties. The results showed that the investigated 5-LO inhibitors differed significantly in their physicochemical, pharmacokinetic and toxicological profiles. About half of the investigated 5-LO inhibitors fulfilled Lipinski's rule of five and Veber's rule, i.e., their good oral bioavailability was predicted, and were also predicted as compounds with no risk of mutagenic, tumorigenic, reproductive and/or irritant effects. The ability to permeate through Caco-2 cells, the possibility of intestinal absorption and the possibility of passing through the blood-brain barrier were predicted for a small number of tested compounds. Taken together, favorable physicochemical and toxicological properties were predicted for 32 out of a total of 99 tested compounds, while the most favorable pharmacokinetic profile was shown by the benzylidene derivative **22**.

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Key words: *5-lipoxygenase, in silico study, physicochemical properties, pharmacokinetic properties, toxicological properties*

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Introduction

Arachidonic acid is an essential polyunsaturated fatty acid that enters the body through food. The tetraene structure is responsible for its physiological activity, and by introducing appropriate '-yne' bonds instead of that structure, the physiological role of arachidonic acid is inhibited. 5-Lipoxygenase (5-LO) is an important enzyme involved in the production of leukotrienes, metabolites of arachidonic acid that have very significant physiological and pathophysiological functions. The 5-LO activity requires the presence of 5-LO activating protein, which is involved in the process of arachidonic acid activation. In a radical-type reaction, an unstable hydroperoxide is first formed, which is transformed into the corresponding leukotriene A4, which is further metabolized to leukotriene B4 or C4 (1). Under the direct influence of leukotrienes, the development of an inflammatory reaction occurs, which is associated with numerous pathophysiological conditions, such as asthma, allergies, cardiovascular and autoimmune disorders. Due to the significant pathophysiological role of leukotrienes, work is being done to develop pharmacological concepts that will either block their action or inhibit their biosynthesis. Therefore, 5-LO is considered a potential target in the fight against inflammation, and the discovery and development of selective 5-LO inhibitors for therapeutic use is the subject of active research (2–5). According to the mechanism of action, 5-LO inhibitors can be classified into four basic groups: redox, non-redox (competitive), iron chelators and allosteric inhibitors (2). Based on these four mechanisms of action, numerous groups of compounds were synthesized and further tested as 5-LO inhibitors. This paper provides a review of synthetic organic compounds tested for 5-LO inhibition, highlighting the most potent representatives (IC_{50} < 1 μM) and their chemical structure (Table 1, Figures 1–6).

Table 1. Groups of synthetic organic compounds tested for 5-LO inhibition, including the most potent representatives and their IC_{50} values

Comp.	IC_{50} (μM)	Ref.	Comp.	IC_{50} (μM)	Ref.	Comp.	IC_{50} (μM)	Ref.
Benzimidazole derivatives			Caffeic acid derivatives			Naphthalene derivatives		
1	0.438	(6)	**33**	0.13	(23)	**67**	0.042	(41)
2	0.12	(7)	**34**	0.24	(10)	**68**	0.063	(42)
3	0.16	(7)	**35**	0.18	(24)	**69**	0.079	(42)
Benzodithiazole derivatives			**36**	0.36	(24)	Is(oxazole) derivatives		
4	0.15	(8)	**37**	0.49	(25)	**70**	0.12	(43)
5	0.18	(8)	**38**	0.53	(25)	**71**	0.24	(44)
Benzo(*b*)furan derivatives			Coumarin derivatives			**72**	0.24	(44)
6	0.04	(9)	**39**	0.07	(26)	Phloroglucinol derivatives		
7	0.04	(9)	**40**	0.052	(26)	**73**	0.46	(45)
Benzoic acid derivatives			**41**	0.088	(26)	Propionic/propenoic acid deriv.		
8	0.18	(10)	**42**	0.0021	(27)	**74**	0.25	(46)
9	0.21	(10)	**43**	0.0028	(27)	**75**	0.28	(47)
10	0.004	(11)	Diphenylpropinones			**76**	0.5	(48)
11	0.006	(11)	**44**	0.1	(28)	Pyrazole derivatives		
12	0.1	(12)	**45**	0.3	(28)	**77**	0.003	(49)
13	0.11	(13)	**46**	0.3	(28)	**78**	0.35	(50)
Benzoquinone derivatives			Furanone derivatives			**79**	0.39	(51)
14	0.28	(14)	**47**	0.28	(29)	**80**	0.47	(51)
15	0.78	(14)	**48**	0.30	(29)	Pyrimidine derivatives		
16	0.58	(14)	Imidazole derivatives			**81**	0.6	(52)
17	0.029	(15)	**49**	0.07	(30)	**82**	0.2	(53)
Benzothiophene derivatives			**50**	0.23	(31)	**83**	0.2	(53)
18	0.021	(16)	**51**	0.32	(32)	**84**	0.2	(53)
Benzoxazole derivatives			Imidazo(1.2-a)pyridines			**85**	0.2	(53)
19	0.12	(17)	**52**	0.08	(33)	Pyrrolizine derivatives		
20	0.95	(17)	**53**	0.1	(33)	**86**	0.18	(54)
Benzylidene derivatives			**54**	0.06	(33)	**87**	0.18	(54)
21	0.16	(18)	**55**	0.030	(34)	Thiazole derivatives		
22	0.17	(18)	**56**	0.032	(34)	**88**	0.127	(55)
Carbamate derivatives			Indole derivatives			**89**	0.3	(56)
23	0.048	(19)	**57**	0.3	(35)	**90**	0.6	(56)
24	0.057	(19)	**58**	0.74	(36)	**91**	0.05	(57)
25	0.068	(19)	**59**	0.85	(36)	**92**	0.05	(57)
Chalcone derivatives			**60**	0.6	(37)	**93**	0.9	(58)
26	0.0024	(20)	**61**	0.0086	(38)	Thiazolinone derivatives		
27	0.0038	(20)	**62**	0.0097	(38)	**94**	0.09	(59)
28	0.42	(21)	**63**	0.086	(39)	**95**	0.12	(59)
29	0.45	(21)	**64**	0.17	(40)	**96**	0.08	(60)
30	0.45	(21)	**65**	0.22	(40)	Triazole derivatives		
Chromane derivatives			**66**	0.20	(40)	**97**	0.9	(61)
31	0.050	(22)				**98**	0.2	(62)
32	0.173	(22)				Triblock conjugates		
						99	0.0015	(63)

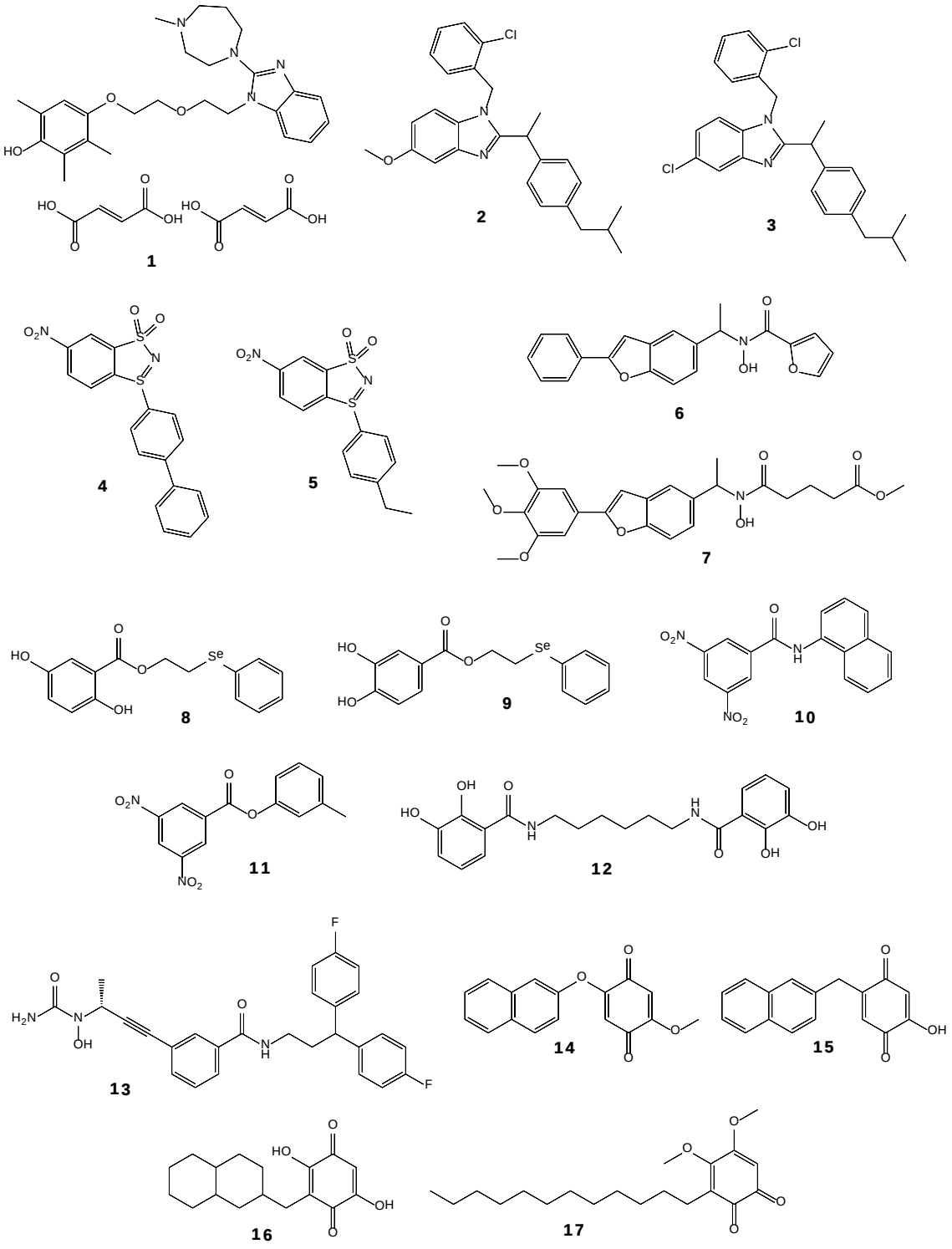

Figure 1. Chemical structures of 5-LO inhibitors: benzimidazole (**1–3**), benzodithiazole (**4**, **5**), benzo(*b*)furan (**6**, **7**), benzoic acid (**8–13**) and benzoquinone (**14–17**) derivatives

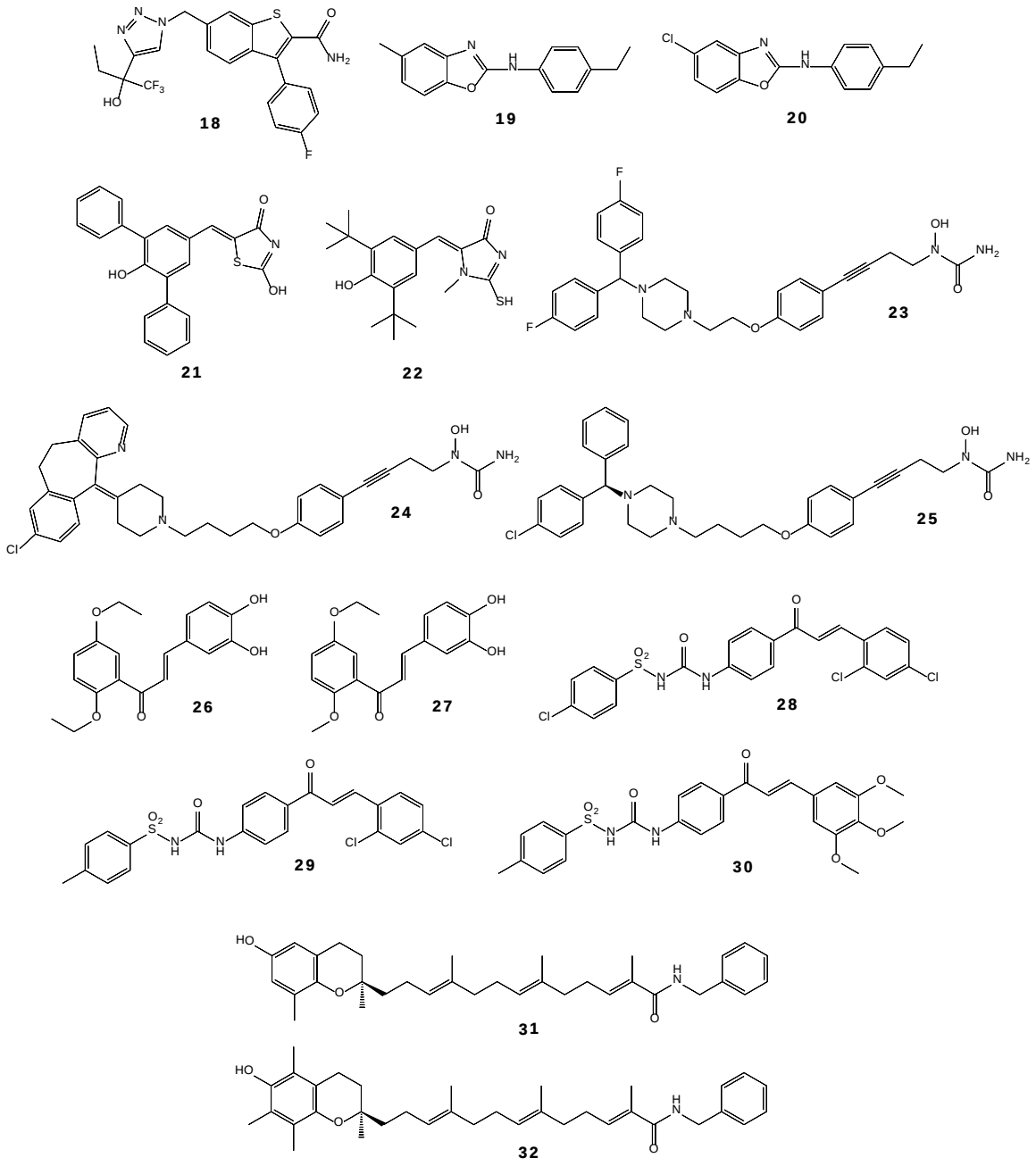

Figure 2. Chemical structures of 5-LO inhibitors: benzo(*b*)thiophene (**18**), benzoxazole (**19**, **20**), benzylidene (**21**, **22**), carbamate (**23–25**), chalcone (**26–30**) and chromane (**31**, **32**) derivatives

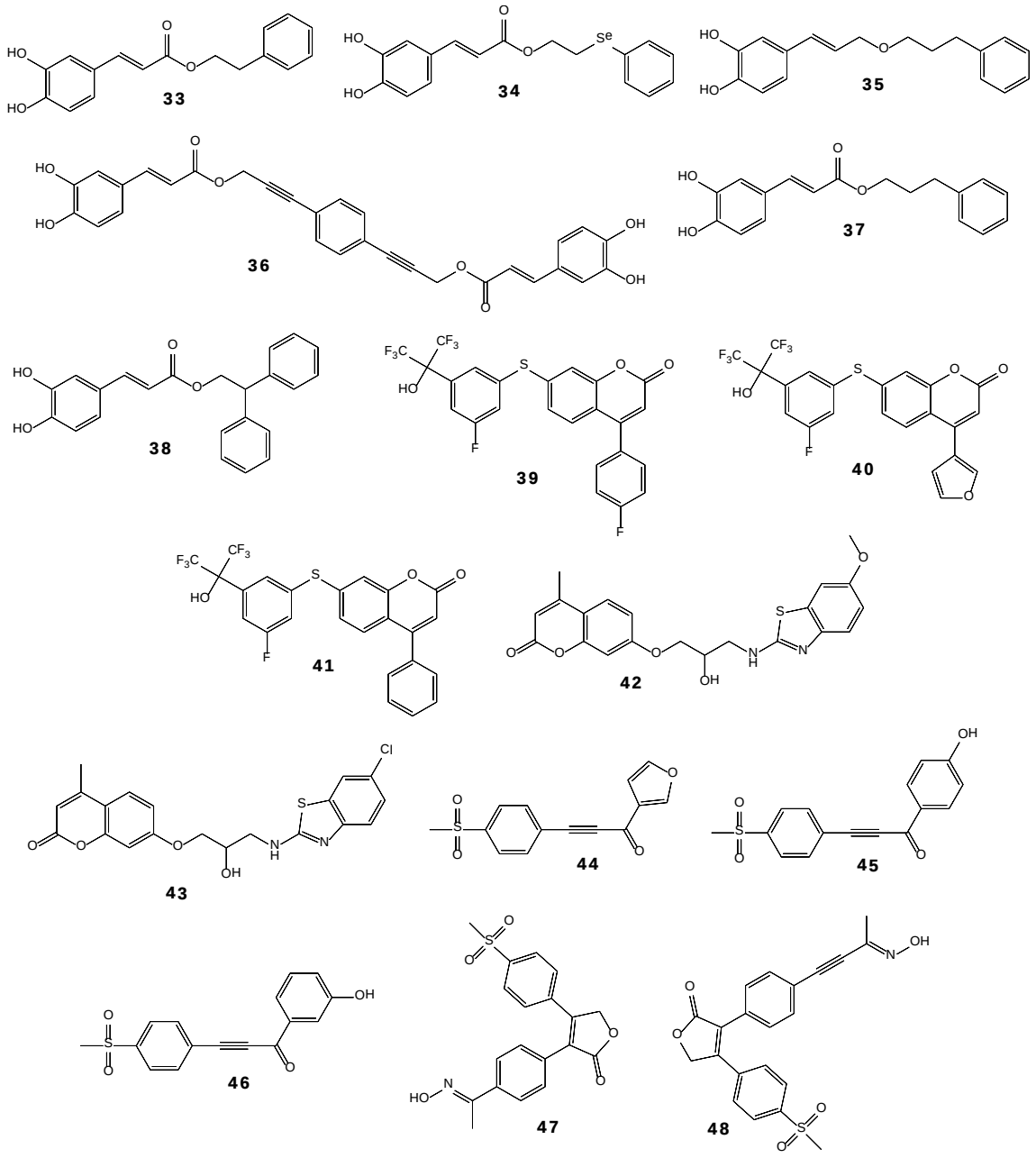

Figure 3. Chemical structures of 5-LO inhibitors: caffeic acid (**33–38**), coumarin (**39–43**), diphenylpropinone (**44–46**) and furanone (**47**, **48**) derivatives

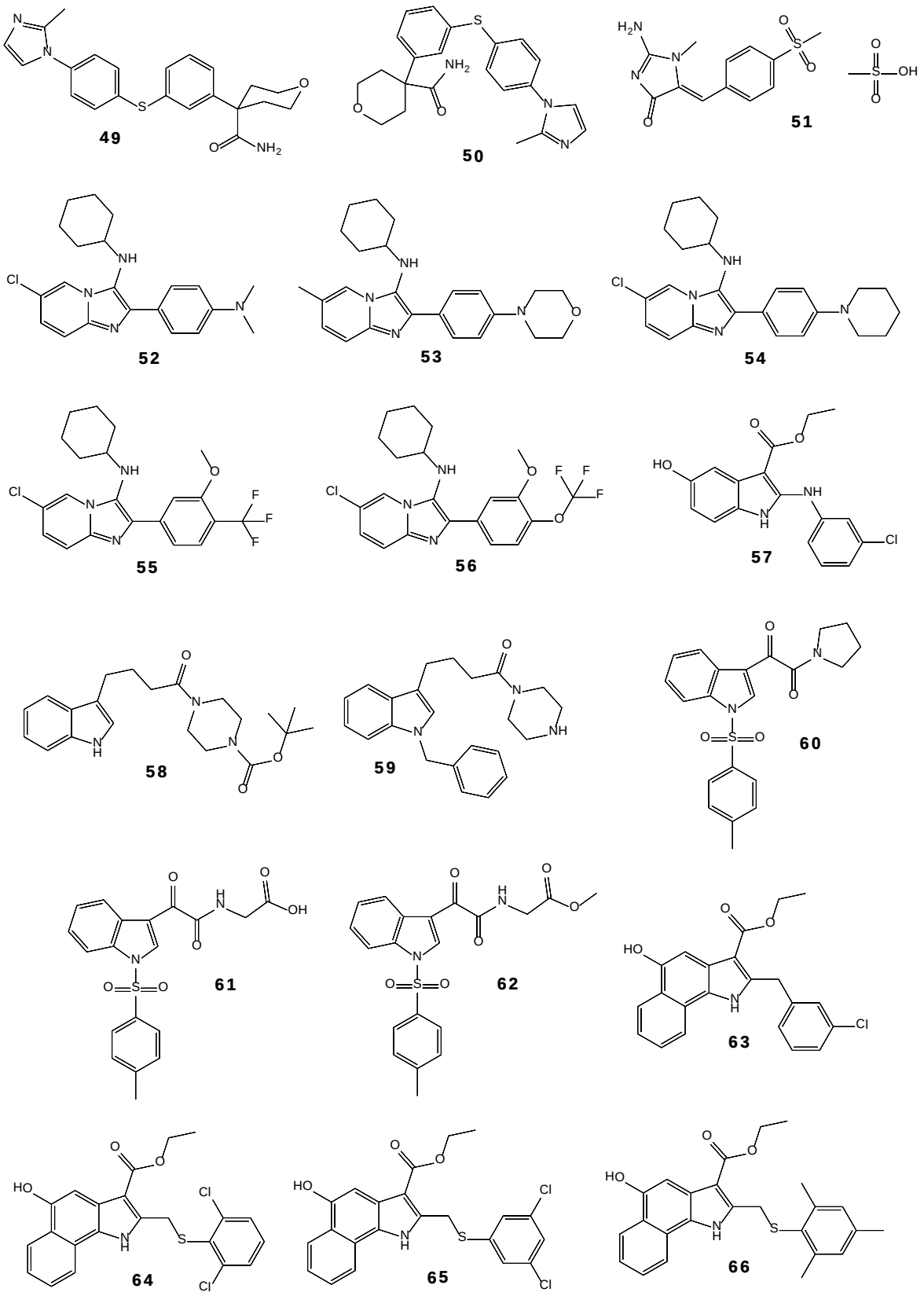

Figure 4. Chemical structures of 5-LO inhibitors: imidazole (**49–51**), imidazo(1,2-*a*)pyridine (**52–56**) and indole (**57–66**) derivatives

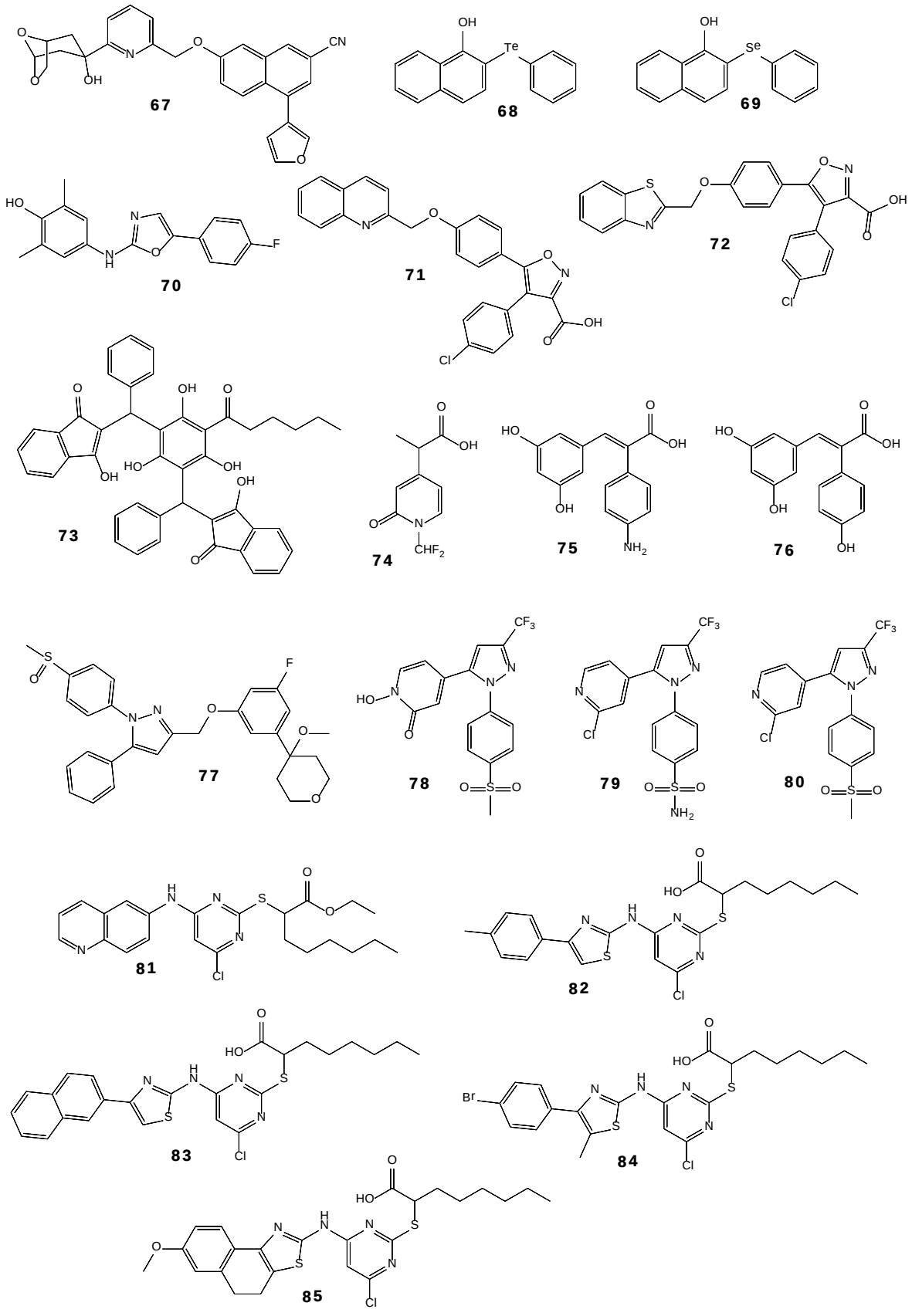

Figure 5. Chemical structures of 5-LO inhibitors: naphthalene (**67–69**), (is)oxazole (**70–72**), phloroglucinol (**73**), propionic and propenoic acid (**74–76**), pyrazole (**77–80**) and pyrimidine (**81–85**) derivatives

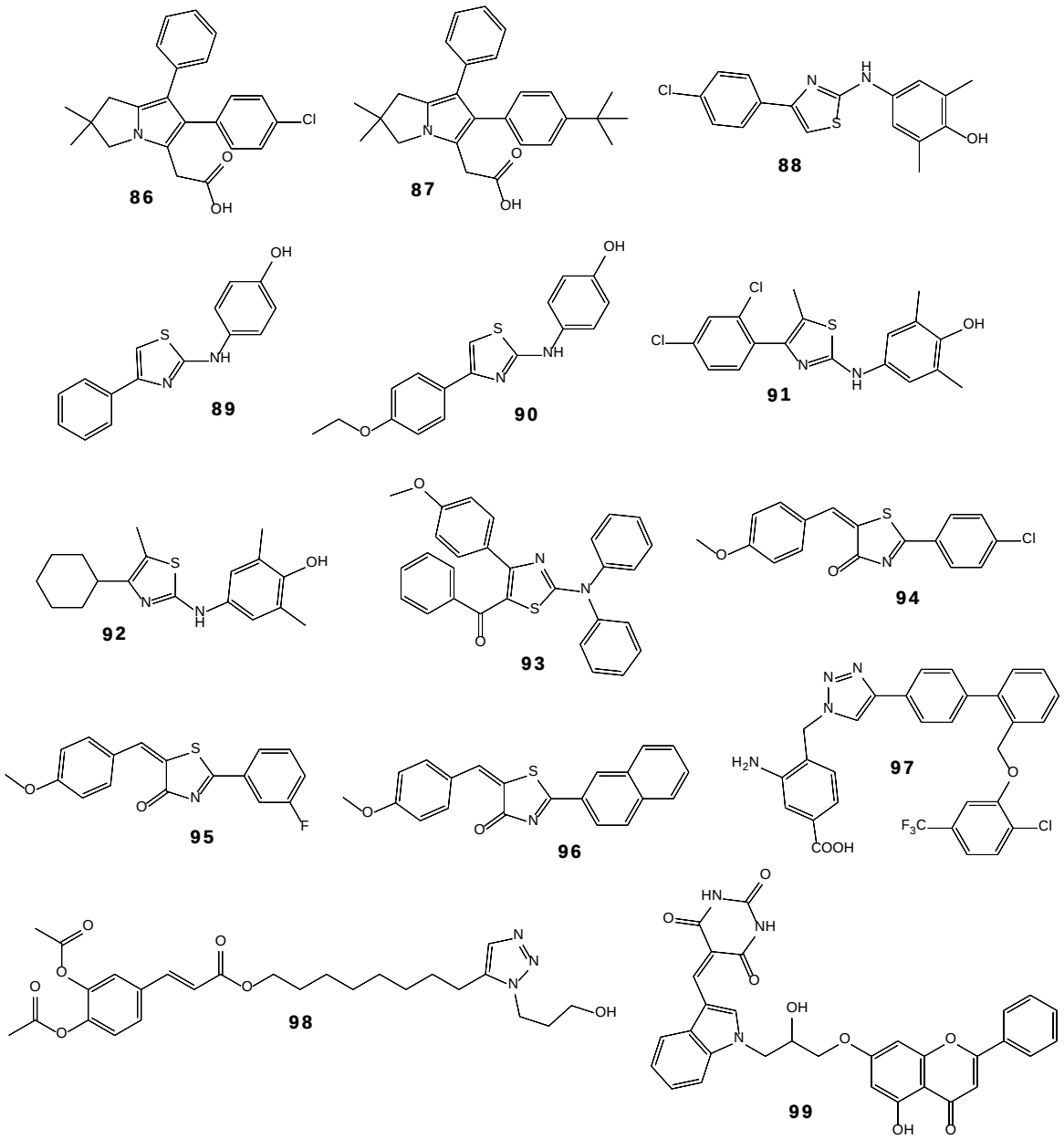

Figure 6. Chemical structures of 5-LO inhibitors: pyrrolizine (**86**, **87**), thiazole (**88**–**93**), thiazolinone (**94**–**96**), triazole (**97**, **98**) derivatives and triblock conjugates (**99**)

Aim

The aim of this study was an *in silico* study of the physicochemical, pharmacokinetic and toxicological characteristics of selected 5-LO inhibitors (**1**–**99**).

Material and Methods

Physicochemical properties of 5-LO inhibitors (**1**–**99**) were predicted using the *Molinspiration* tool (64), pharmacokinetic properties were predicted using *ADMETlab* 2.0. software (65), and the toxicological properties of the examined 5-LO inhibitors were predicted using *DataWarrior* (66) and *Toxtree* (67) softwares.

Results and Discussion

Physicochemical properties of 5-LO inhibitors

Based on the predicted physicochemical properties, the biological availability of the tested compounds in *in vitro* and/or *in vivo* conditions can be assumed. Octanol/water partition coefficient (m_iLogP) values indicate the

lipophilicity of the investigated compounds. Compounds with m_iLogP values less than 5 are considered hydrophilic, i.e., they are predicted to show sufficiently good oral bioavailability. Compounds with m_iLogP values above 5 are predicted to be lipophilic, that is, to show poor oral bioavailability (68). Oral bioavailability can also be predicted on the basis of the polar surface area of the molecule. Thus, sufficiently good oral bioavailability is predicted for compounds with topological polar surface values below 140 Å^2 (69–71). The capacity to form hydrogen bonds can be represented by the number of hydrogen bond donors (n_{OHNH}) and the number of hydrogen bond acceptors (n_{ON}). The number of rotatable bonds (n_{rot}) indicates conformational flexibility, which is another important factor for optimal bioavailability. For compounds with less than 10 hydrogen bond acceptors, less than 5 hydrogen bond donors, and less than 10 rotatable bonds, good oral bioavailability is predicted (69).

Summarizing the *in silico* predicted physicochemical parameters (Table 2), 50 of the 99 compounds (**1**, **4–6**, **8–11**, **13–16**, **18**, **22**, **26**, **27**, **33–35**, **37**, **38**, **42–51**, **57–62**, **67**, **68**, **70**, **74–76**, **78–80**, **89**, **90**, **94**, **95**) fulfilled Lipinski's rule of five and Veber's rule (m_iLogP ≤ 5, TPSA ≤ 140 Å^2, n_{ON} ≤ 10, n_{OHNH} ≤ 5, n_{rot} ≤ 10, M_r ≤ 500), which predicts their good oral bioavailability.

Pharmacokinetic properties of 5-LO inhibitors

The results obtained from an *in silico* pharmacokinetic study (Table 3) indicate that a small number (24 compounds) of the tested 5-LO inhibitors (**1–99**) were predicted with the ability to permeate through Caco-2 cells, epithelial human colon cancer cells used as a model for assessment of human intestinal permeability (72, 73). The ability to pass through the blood-brain barrier and penetrate the central nervous system was also predicted for a minority of the investigated 5-LO inhibitors (20 out of 99 compounds). The possibility of intestinal absorption was predicted for only four compounds (**12**, **22**, **36** and **99**). Slightly more than half of the tested 5-LO inhibitors (55 out of 99 compounds) were potentially not inhibitors, and the vast majority (86 out of 99 compounds) were potentially not substrates of P-glycoprotein, an efflux membrane transporter that mediates the transfer of structurally diverse substrates through the cell membrane. This transmembrane protein is important for the absorption of orally administered drugs, as well as for penetration through the blood-brain barrier (74–77).

Table 2. Physicochemical properties of 5-LO inhibitors predicted by *Molinspiration* (64)

Physicochemical properties	Compounds
m_iLogP[a] > 5	2, 3, 17, 19–21, 24, 25, 28, 29, 31, 32, 39–41, 52–56, 63–66, 69, 71–73, 81–88, 91–93, 96, 97
m_iLogP ≤ 5	1, 4–16, 18, 22, 23, 26, 27, 30, 33–38, 42–51, 57–62, 67, 68, 70, 74–80, 89, 90, 94, 95, 98, 99
TPSA[b] > 140 Å^2	73, 99
TPSA ≤ 140 Å^2	1–72, 74–98
M_r[c] > 500	23–25, 28, 30–32, 36, 39–41, 73, 77, 83–85, 97–99
M_r ≤ 500	1–22, 26, 27, 29, 33–35, 37, 38, 42–72, 74–76, 78–82, 86–96
n_{ON}[d] > 10	99
n_{ON} ≤ 10	1–98
n_{OHNH}[e] > 5	12
n_{OHNH} ≤ 5	1–11, 13–99
n_{rot}[f] > 10	7, 17, 25, 31, 32, 73, 81–85, 98
n_{rot} ≤ 10	1–6, 8–16, 18–24, 26–30, 33–72, 74–80, 86–97, 99

[a]octanol/water partition coefficient calculated using the methodology developed by *Molinspiration*; [b]topological polar surface of the molecule (Å^2); [c]molecular mass; [d]number of hydrogen bond acceptors (O and N atoms); [e]number of hydrogen bond donors (OH and NH groups); [f]number of rotatable bonds

Table 3. Absorption properties of 5-LO inhibitors predicted by *ADMETlab 2.0* (65)

Absorption properties	Compounds
Caco-2 cells	12, 13, 22, 24, 28–30, 36, 42, 43, 47, 48, 51, 59, 63–66, 73, 75–78, 99
Intestinal absorption	12, 22, 36, 99
Blood-brain barrier	1, 3, 22–24, 49–52, 54, 58–60, 74, 77–81, 92
P-glycoprotein inhibitor	1–3, 6, 7, 13, 14, 19, 21, 22, 24, 30, 32, 35, 36, 38–41, 43, 51–56, 58–60, 63–66, 71–73, 77, 86–88, 93, 95, 98, 99
P-glycoprotein substrate	6, 12, 13, 24, 42, 43, 52, 55, 56, 58, 59, 70, 98

The obtained results showed that the examined 5-LO inhibitors (**1–99**) differed from each other in their metabolic properties, depending on whether they were potential substrates and/or inhibitors of certain CYP450 isoenzymes (Table 4).

Toxicological properties of 5-LO inhibitors

The results obtained from an *in silico* toxicological study (Table 5) indicate that 52 compounds (**2–6**, **8**, **9**, **11–13**, **16**, **17**, **21–27**, **31**, **32**, **34–38**, **40**, **44–46**, **49–51**, **57–59**, **70**, **74**, **77–80**, **86–89**, **91**, **92**, **94–96** and **98**) were predicted with no risk of mutagenic, tumorigenic, reproductive and/or irritating effects, and 10 more compounds (**14**, **15**, **19**, **20**, **28**, **33**, **68**, **69**, **75** and **97**) were predicted with low risk of these effects.

The identification of compounds with the possibility of covalent binding to proteins and/or DNA is of great importance in the assessment of toxicity. The formation of a covalent adduct with a biological macromolecule represents the initial event, that is, the first step in a series that can result in toxic effects (78, 79). Using the *Toxtree* tool (67), structural predispositions of selected 5-LO inhibitors for covalent binding to DNA and/or proteins were evaluated, such as the possibility of acylation, Michael addition, formation of Schiff bases, aromatic and aliphatic nucleophilic substitution. The results indicated that all 99 investigated compounds have at least one structural predisposition for binding to DNA and/or proteins (Tables 6 and 7). Given predispositions refer to the chemical mechanism by which a given compound can covalently interact with a biological macromolecule, but do not necessarily indicate that the given compound is toxic (78, 79).

Table 4. Metabolic properties of 5-LO inhibitors predicted by *ADMETlab 2.0* (65)

Metabolic properties	Compounds
CYP450 inhibitor	
1A2	1, 6, 8–11, 14–22, 26, 27, 33–38, 42–48, 51–57, 59, 63–72, 78–86, 88–96, 98
2C19	1–4, 6–11, 13–15, 17–22, 24–29, 31, 33–50, 52–60, 62–70, 72, 73, 77, 80–99
2C9	1–16, 18, 21–23, 25–46, 48–50, 52, 55–58, 60, 62–70, 73, 77–86, 88–99
2D6	6, 8, 9, 12–17, 19, 20, 23–27, 31–35, 37, 38, 40, 42, 43, 47, 52, 54–57, 59, 63–66, 70, 81, 88–92
3A4	2, 3, 6, 7, 10, 11, 13–15, 18, 22–25, 27, 31–33, 35, 37, 40, 42, 43, 46, 55, 57–60, 62–64, 67, 75, 76, 81, 85, 91, 99
CYP450 substrate	
1A2	2, 7, 13, 14, 16–20, 22–25, 27, 30, 32, 39–43, 49–51, 55, 56, 60, 66, 67, 70, 73, 74, 80, 85, 88, 91, 92, 95
2C19	5, 7, 22, 25, 30, 32, 49–51, 58, 59
2C9	1–11, 13–17, 21–23, 25–37, 39, 41, 42, 45–48, 55–58, 60–66, 69, 73, 75–87, 89, 90, 93–96, 98, 99
2D6	2, 8, 9, 13, 16, 17, 19, 22–27, 31–35, 37–40, 42, 43, 52–59, 63, 64, 66, 68, 69, 80, 85–91, 94–96
3A4	2–7, 13, 18–20, 22–25, 28–30, 39–43, 49, 50, 58–60, 62, 67, 77–80, 88, 90–93

Table 5. Toxicological properties of 5-LO inhibitors predicted by *DataWarrior* (66)

Toxicological properties	No risk compounds	Low-risk compounds	High-risk compounds
Mutagenic effects	1–9, 11–13, 16–18, 21–41, 43–59, 67, 70–74, 76–80, 82–96, 98, 99	14, 15, 19, 20, 68, 69, 97	10, 42, 60–66, 75, 81
Tumorigenic effects	1–9, 11–27, 29–32, 34–51, 53–62, 70–74, 76–80, 86–96, 98, 99	33, 69, 75	10, 28, 52, 63–68, 81–85, 97
Reproductive effects	1–6, 8–17, 19–27, 31–38, 40, 44–46, 49–51, 57–66, 68–70, 73, 74, 77–84, 86–89, 91, 92, 94–98	28	7, 18, 29, 30, 39, 41–43, 47, 48, 52–56, 67, 71, 72, 75, 76, 85, 90, 93, 99
Irritant effects	2–32, 34–59, 61–72, 74, 76–96, 98, 99	33	1, 60, 73, 75, 97

Table 6. DNA binding ability of 5-LO inhibitors predicted by *Toxtree* (67)

Toxicological properties	Compounds
Possibility of S_N1 aliphatic nucleophilic substitution	1, 4, 5, 10, 11, 15, 19, 20, 22–25, 28–30, 42, 43, 51–60, 70, 74, 75, 81–85, 88–92, 97
Possibility of forming Schiff bases	60–62, 98
Possibility of Michael's addition	1–99
Possibility of acylation	28–30
Possibility of S_N2 aliphatic nucleophilic substitution	/

Table 7. Protein binding ability of 5-LO inhibitors predicted by *Toxtree* (67)

Toxicological properties	Compounds
Possibility of aromatic nucleophilic substitution	10, 11, 24, 52–56, 67, 71, 79–81
Possibility of forming Schiff bases	98
Possibility of Michael's addition	1–99
Possibility of acylation	10, 11, 39–43, 74, 78, 98
Possibility of S_N2 aliphatic nucleophilic substitution	2, 3, 5–7, 13, 15, 18–20, 22–25, 31–33, 35–38, 47, 48, 58, 59, 63–67, 71–74, 77, 85–87, 92, 97, 98

Conclusion

The results obtained from the *in silico* study showed that the tested 5-LO inhibitors differ significantly from each other in their physicochemical, pharmacokinetic and toxicological properties. For 32 compounds (**4–6**, **8**, **9**, **11**, **13**, **16**, **22**, **26**, **27**, **34**, **35**, **37**, **38**, **44–46**, **49–51**, **57–59**, **70**, **74**, **78–80**, **89**, **94** and **95**), out of a total of 99 examined, favorable physicochemical and toxicological characteristics were predicted. Namely, it was

predicted that the listed compounds fulfil Lipinski's rule of five and Veber's rule, as well as that they are without the risk of mutagenic, tumorigenic, reproductive and/or irritating effects. The benzylidene derivative, compound **22**, stood out with a favorable pharmacokinetic profile, for which the possibility of intestinal absorption and permeation through Caco-2 cells, as well as the possibility of passing through the blood-brain barrier and penetration into the central nervous system was predicted. Generally, the results of this study provide a good basis for further *in vivo* research, as well as for the design of novel therapeutically significant 5-LO inhibitors with favorable physicochemical, pharmacokinetic and toxicological profiles.

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IN SILICO FIZIČKO-HEMIJSKA, FARMAKOKINETIČKA I TOKSIKOLOŠKA ISPITIVANJA INHIBITORA 5-LIPOKSIGENAZE

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Enzim 5-lipoksigenaza (5-LO) predstavlja važan enzim koji učestvuje u proizvodnji leukotriena, metabolita arahidonske kiseline pod čijim direktnim uticajem dolazi do razvoja reakcije inflamacije koja je povezana sa brojnim patofiziološkim stanjima. Stoga, otkrivanje i razvoj selektivnih 5-LO inhibitora za primenu u terapiji predstavljaju predmet istraživanja koja se trenutno sprovode. Cilj ove studije bio je da se najpre da pregled literature u vezi sa najaktivnijim sintetskim 5-LO inhibitorima (sa IC_{50} vrednostima manjim od 1 μM), usmeren prvenstveno na njihovu hemijsku strukturu, a potom predstave rezultati *in silico* studije njihovih osnovnih fizičko-hemijskih, farmakokinetičkih i toksikoloških karakteristika. Rezultati su pokazali da se fizičko-hemijski, farmakokinetički i toksikološki profili ispitivanih 5-LO inhibitora značajno razlikuju. Oko polovine ispitivanih 5-LO inhibitora ispunilo je „pravilo pet Lipinskog" i „pravilo Vebera", što znači da je predviđena njihova dobra oralna bioraspoloživost. Takođe, predviđeno je da su posredi jedinjenja koja ne izazivaju mutagene, tumoralne, reproduktivne i/ili iritacione efekte. Sposobnost penetracije kroz Caco-2 ćelije, mogućnost intestinalne apsorpcije i mogućnost prolaska kroz krvno-moždanu barijeru predviđene su za mali broj ispitivanih jedinjenja. U suštini, povoljna fizičko-hemijska i toksikološka svojstva predviđena su za 32 od ukupno 99 testiranih jedinjenja. Sa najpovoljnijim farmakokinetičkim profilom izdvojio se derivat benzilidena **22**.

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***Ključne reči**: 5-lipoksigenaza, in silico studija, fizičko-hemijske osobine, farmakokinetičke osobine, toksikološke osobine*

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PREVALENCE AND SPECIES DISTRIBUTION PATTERN OF SUPERFICIAL FUNGAL INFECTIONS IN THE NIŠAVA DISTRICT, SERBIA

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An assessment of distribution patterns of infectious disease causative agents is crucial in cases of infections managed empirically, without laboratory-based evidence.

The study aimed to explore the prevalence of superficial fungal infections (SFI) and identify the most common causative agents.

This study included a mycological examination of skin and adnexa samples of patients with clinical symptoms and signs of SFI. Standard mycological methods, microscopy and cultivation, were used for the detection and identification of causative agents. Dermatophytes were determined based on macroscopic and microscopic morphological characteristics to genus or species level, while the identification of yeast species was done by using commercial Integral System YEASTS plus (Liofilchem®, Italy) tests. The results were elaborated with the statistical method of descriptive and quantitative analysis (SPSS 14.0 for Windows 2003).

Statistical analysis revealed a high prevalence of SFI (30.2%), with a significant difference observed concerning patients' age ($p < 0.001$), while no significant difference was noted regarding patients' gender ($p = 0.504$). SFI did not exhibit a seasonal pattern ($p = 0.783$). Superficial fungal infection was confirmed by isolating and identifying fungi in 188 patients (15.1%). *Candida* spp. were isolated from 113 patients (60.1%), with *Candida albicans* identified as the causative agent of superficial candidosis in 46 patients (40.7%), while non-*albicans Candida* (NAC) species were detected in significantly more patients (59.3%). Dermatophytoses were diagnosed in 75 patients (39.9%), with *Microsporum canis* being the predominant species (38.7%).

The increasing incidence of superficial yeast infections caused by previously classified NAC species underscores the necessity for mycological analyses to determine the etiology of SFI and evaluate the *in vitro* effectiveness of antimycotics. The notable prevalence of zoophilic dermatophyte species highlights the imperative for epidemic and epizootic preventive measures.

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Key words: *superficial fungal infections, prevalence, dermatophytes, Candida* spp.

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Introduction

Superficial fungal infections (SFI) are among the most common human infections in the world with incidence rates from 20–25%, and present a significant public health problem (1). The high incidence and prevalence of dermatological fungal diseases can be attributed to various socio-economic factors, lifestyle choices, continuous migrations, and climate changes, particularly global warming.

Dermatophytes are the predominant causative agents of SFI. According to the latest suggested classification, based on molecular analyses, this group includes numerous genera, namely *Trichophyton* spp., *Epidermophyton* spp., *Nannizzia* spp., *Microsporum* spp., *Lophophyton* spp., *Arthroderma* spp., *Ctenomyces* spp., *Guarromyces* spp. and *Paraphyton* spp. The most medically significant species belong to the *Trichophyton, Epidermophyton*, and *Microsporum* genera (2). In addition to the aforementioned classification, it is essential to differentiate these species based on their natural habitat, into

antropophiles, zoophiles, and geophiles (1). Superficial fungal infections are frequently caused by yeasts of the genera *Malassezia* (3) and *Candida*. Based on the new taxonomy, which led to the exclusion of many medically significant *Candida* species from this genus, it can be highlighted that yeasts *Nakaseomyces* spp., *Pichia* spp., *Meyerozyma* spp. and *Kluyveromyces* spp. (4–6) are also causative agents of this infection. Even though species, previously classified within the genus *Candida* have been regrouped, in this article we will continue to use the old terms because this taxonomic revision could be overlooked or misinterpreted among clinicians. Furthermore, *Cryptococcus* spp. (7) and *Trichosporon* spp. can cause this form of fungal infection, primarily in immunocompromised patients. In recent years, there has been increasing data on non-dermatophyte molds as causative agents of SFI. These include molds of the genus *Aspergillus*, fungi of the Mucorales order, and members of hyaline and dermateaceae molds, which can cause infections not only in immunocompromised but also in immunocompetent individuals (4).

Despite the widespread occurrence of SFI, there are still no rapid diagnostic tests with adequate sensitivity and specificity. Consequently, routine mycological analyses for determining causative agents still need to be improved (8). The prevalence of certain species causing SFI and the localization of these infections vary depending on geographic, socio-economic, and ecological characteristics. Continuous monitoring of specific species' incidence in a particular region can help identify changes in the spectrum of the SFI causative agents. These findings can significantly impact the selection of the most effective therapy regimen, often based on empirical approaches. This retrospective study aimed to determine the prevalence of SFI, as well as the distribution of genera and species which had caused those infections from 2019 to 2022 in patients from the Nišava district in Southeast Serbia.

Material and Methods

The study entailed an analysis of data derived from mycological examination of skin and adnexa samples collected from 1243 patients suspected of having superficial fungal infections between the beginning of 2019 and the end of 2022. Only patients from the Nišava district were included with the aim of determining the incidence of SFI. The research was conducted at the laboratories of the Department of Parasitology and Mycology at the Institute of Public Health in Niš. The research was approved by the Ethic Committee of the Faculty of Medicine of the University of Niš (Decision No. 12-6316-2/1-2014) and the Ethic Committee of the Institute of Public Health in Niš (Decision No. 07-4665/2014).

The standard mycological analysis included microscopic examination using 10% potassium hydroxide and chlorine lactophenol to identify fungal elements in active reproduction (conidia, germination, pseudohyphae, and hyphae) in patients' material, along with cultivation to obtain fungal growth. Positive findings of one of those two methods, along with characteristic clinical findings, were considered as confirmation of SFI. Cultivation of fungi was done on selective media (Liofichem®, Italy) for dermatophyte and yeast isolation: Sabouraud dextrose agar (SDA); SDA with the addition of chloramphenicol and cyclohexamide; Dermatophyte test medium agar (DTM). Genera and species of dermatophytes were identified based on macroscopic and microscopic morphological characteristics (9), while the yeast species were identified using CHROMagar Candida and commercial Integral System YEASTS Plus (Liofichem®, Italy) test.

Statistical analysis

The interpretation of obtained, systematized, and encrypted data was done using a statistics calculator within the Epi Info program (Version 6.04) and statistical package SPSS (16.0 for Windows). The data were presented as arithmetic mean, standard deviation, and absolute and relative numbers. A T-test was used for the comparison of values between two groups of examinees. Fisher's test of exact probability, as well as the $\chi 2$ test with or without Yates' correction, were used for the comparison of different frequency distributions. The hypothesis was tested with a level of significance of $p < 0.05$.

Results

The research included 1243 patients: I) 456 men and 787 women; II) with an average age of 39.72 ± 23.09 years (min 1 year, max 86 years). Among them, 256 patients (20.9%) were referred with a diagnosis of tinea (t.) capitis; 117 (9.4%) with t. faciei; 171 (13.8%) with t. pedis; 111 (8.9%) with t. manuum; 19 (1.5%) with t. inguinalis; and 569 (45.7%) patients were referred due to onychomycosis, with 264 (21.2%) having changes in fingernails and 305 (24.5%) with changes in toenails.

Using the standard procedure, positive mycological findings were recorded in 375 patients, and among them, fungal isolation and identification confirmed the findings in 188 patients (15.1%). During the examined time frame, a high prevalence of fungal infections was noted (30.2%), exhibiting statistically significant variations across the years (25.6% in 2019; 43.5% in 2020; 33.3% in 2021; 24.1% in 2022; $p < 0.001$; Figure 1).

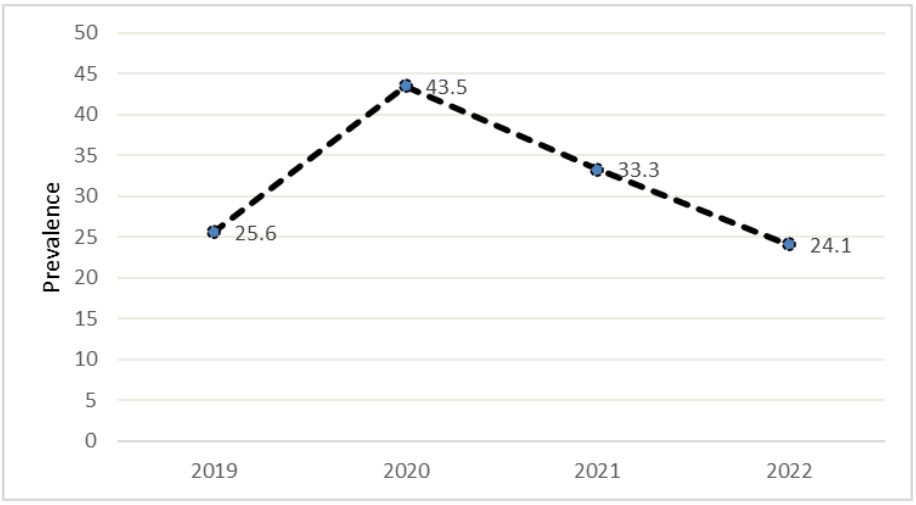

Figure 1. Trend of prevalence of superficial fungal infections from 2019 to 2022

Furthermore, a high cumulative incidence (26/105 inhabitants) was determined and calculated based on the assessment of inhabitants of the Nišava district in 2022 (343,950 inhabitants). Statistical analysis showed no statistically significant difference regarding gender ($p = 0.504$) and season ($p = 0.783$). Patients with fungal infections were statistically significantly younger than those without infection (35.66 ± 24.07 vs. 41.38 ± 22.53, $p < 0.001$; Table 1).

Table 1. Demographic characteristics of patients and season of superficial fungal infections occurrence

	Patients without SFI		Patients with SFI		p[1]
Age (years)	41.38 ± 22.53		35.66 ± 24.07		< 0.001[2]
Age categories	Examinee number	Percentage (%)	Examinee number	Percentage (%)	
< 18	189	22.0	130	34.9	< 0.001
18–65	507	58.9	192	51.5	
65 or older	165	19.2	51	13.7	
Sex					
Male	311	36.0	143	38.1	0.504
Female	554	64.0	232	61.9	
Season					
Winter	200	23.2	91	24.5	0.783
Spring	181	21.0	73	19.7	
Summer	244	28.3	98	26.4	
Autumn	237	27.5	109	29.4	

SFI—superficial fungal infections

Candida spp. were isolated from the materials of 113 patients (60.1%), among which, as a causative agent of superficial candidosis, *Candida* (*C.*) *albicans* species was confirmed in 46 patients (40.7%), while non-*albicans Candida* (NAC) species were found in a significantly more patients (59.3%). Among the NAC species, 15 isolates were not identified at the species level. In the identified NAC species group, *C. parapsilosis* was isolated as the dominant species (32; 47.8%), followed by *C. guilliermondi* (11; 16.4%). Other NAC species were found in significantly fewer patients (*C. glabrata* in 4; *C. tropicalis* in 3; *C. kefyr* in 1; *C. krusei* in 1) (Figure 2).

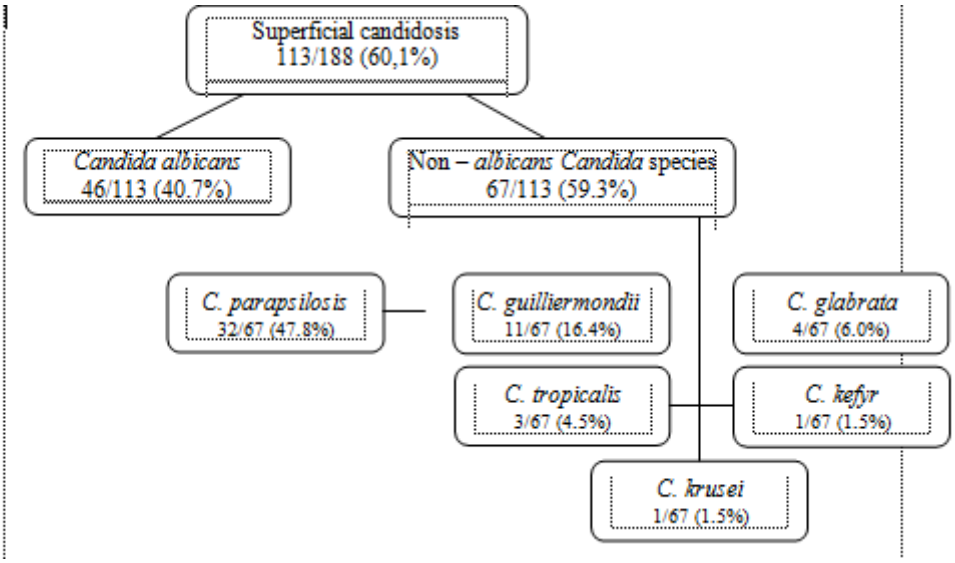

Figure 2. Yeast species isolated from materials of patients with SFI

Dermatophytoses were confirmed in a smaller number of patients (75; 39.9%). In this group, causative agents were *Trichophyton* species in 58.7% of patients, while species from the genus *Microsporum* were isolated from the materials of 41.3% of patients with dermatophytosis. The most prevalent dermatophyte species identified was *Microsporum (M.) canis* (38.7%), followed by the most prevalent species from the genus *Trichophyton*, *Trichophyton* (*T.*) *mentagrophytes* (12.0%). Additionally, species *T. interdigitale* and *T. tonsurans* each accounted for 8.0% of cases. Other species in this genus, *T. rubrum*, and *T. verrucosum*, comprised a smaller percentage of isolates (Figure 3).

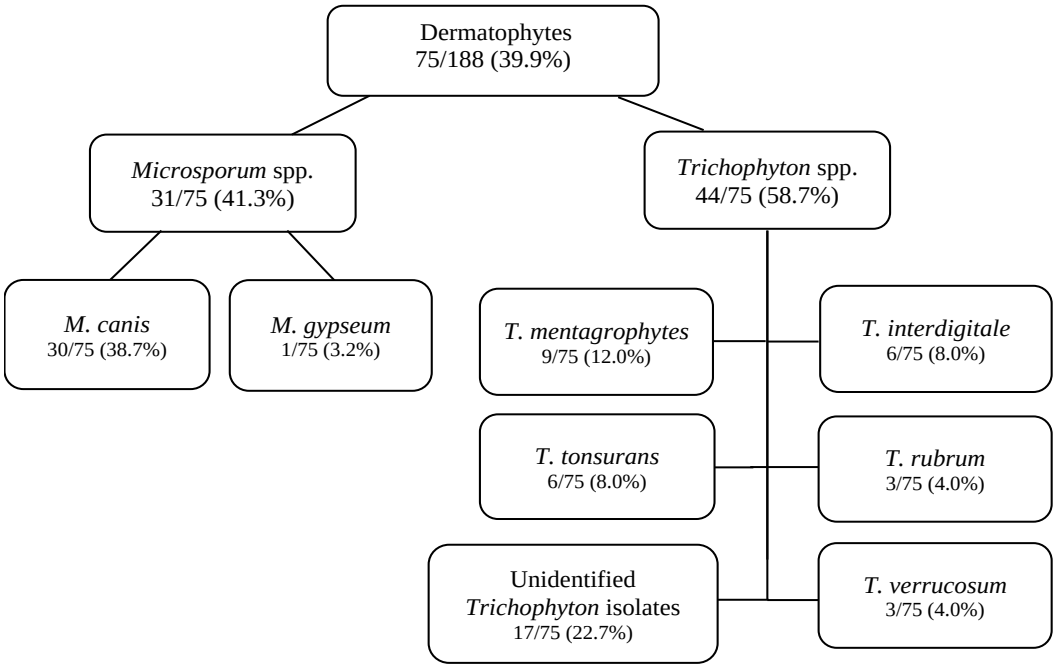

Figure 3. Dermatophyte species isolated from materials of patients with SFI

Discussion

The prevalence of fungal species causing SFI, as well as the localization of fungal infections, varies depending on the geographic, socio-economic, ecological, and climatic characteristics of a particular region, alongside the habits and traditions of its inhabitants. Given the lack of rapid fungal diagnostic tests, diagnoses are primarily based on clinical findings, while therapy is empirical, and chosen based on recommended treatments (8–12). Monitoring the incidence and prevalence of the most common causes of SFI could assist dermatologists in selecting the most effective, primarily empirically-based therapy, as well as in implementing appropriate preventive measures to halt the spread of infectious diseases, including SFI.

The results of this study show a high cumulative incidence of SFI over the examined period, consistent with the incidence levels reported in earlier studies (13, 14). The prevalence of SFI is also notably high, with variations observed depending on the year of research, particularly evident in 2020 and 2021. This is understandable, as these two years coincided with the COVID-19 pandemic, during which only patients with more intense, severe symptoms and indicative clinical findings were referred for mycological examination. Statistical analysis did not reveal a significant difference regarding gender, nor did the infections demonstrate seasonal patterns. However, a statistically significant age difference was observed, with a higher frequency of infections recorded in younger individuals. This can be attributed to the high number of children diagnosed with t. capitis among the study participants (15, 16).

Additionally, this study showed that SFI caused by yeasts were present in significantly more patients compared to dermatophytoses. This reflects a consistent upward trend in yeast SFI prevalence, previously recorded only as sporadic infections, increasing to 30%, then to 50%, and now up to 60% (13, 14). Dermatophytes, formerly the dominant causative agents accounting for over 90% of cases, were identified as causes in approximately 40% of our study participants. Based on the results of the recent studies, it can be emphasized that the most common causes of SFI in European countries are species *T. rubrum, M. canis, T. mentagrophytes*, and *T. verrucosum* (1, 14). *Species M. audouinii, T. soudanense*, and *T. violaceum*, known as causes of so-called immigrational SFI, are rarely detected in this region, as their endemic zones are in Africa and some parts of Asia (16). However, it can be assumed that the incidence of mycoses caused by these species could increase due to ever-increasing migration and tourism.

The dynamic nature of the spectrum of dermatophyte fungi within a specific area is evidenced by the long-term monitoring of dermatophytoses in the Nišava district, which commenced in the 1950s. Over this extended period, the incidence of species causing dermatophytoses has changed. It evolved from epidemic occurrences of SFI caused by anthropophilic species (17) to a plethora of species including *T. verrucosum, T. violaceum, T. tonsurans, T. quinceaneum, T. rubrum, M. gypseum* and *M. ferrugineum* (18, 19), then to a significant increase in the prevalence of species *M. Canis*, which emerged as the predominant species in the 1990s, persisting until today. Until 2011, this species was responsible for causing SFI in more than half of the infected patients (13).

This trend continued in the later years (14), with zoophilic species *M. canis* and *T. mentagrophytes* being the most common causes of dermatophytoses in patients residing in the Nišava district, accounting for 60.1% of cases. *M. canis* has been recognized as the most common cause of dermatophytoses for quite some time, not only in this region but also in the former Yugoslavia countries, southeast European countries, and rural areas of economically developed European countries (10, 20, 21). The results of this study indicate that the prevalence of these species remains relatively stable, with both species still dominating as causative agents, accounting for as much as 50.7% of identified dermatophyte isolates. However, unlike the previous study covering the period from 2012 to 2017, species *T. tonsurans* and *T. verrucosum* were isolated among dermatophytes in this region, which had not occurred since the 1990s. Although the number of patients infected with these species is currently low, an increase in prevalence can be anticipated in the future, particularly among those infected by *T. tonsurans*, given its anthropophilic nature and the possibility of interhuman transmission (22, 23).

In addition to dermatophytes, SFI can be caused by yeasts. Previous studies involving patients from our region have indicated a decreasing prevalence of dermatophytoses relative to superficial candidosis, with nearly equivalent prevalence of SFI. However, contrary to previous findings, this study reveals a lower prevalence of *C. albicans* alongside an increasing prevalence of other yeasts. In previous research, *C. albicans* was identified as the causative agent in 55.6% of patients, whereas in the current study, this number has declined to 40.7%. Besides epidemiological significance, this information can be important for devising appropriate therapy regimens. While previous research on yeasts causing SFI did not demonstrate resistance of NAC species and newly classified yeasts to antifungal medicines (4), the prevailing opinion is that NAC species exibit a more frequent occurrence of resistance, lower sensitivity, as well as dose-dependent sensitivity to antifungal medicines. The widely accepted division of the *Candida* genus into *C. albicans* and NAC species may soon be discarded as recent extensive phylogenetic studies on its members have prompted reclassification. As a result, the mentioned NAC species no longer

belong to this genus, which explains their lower sensitivity to antifungal agents and discrepancies in sensitivity/resistance. Species *C. albicans*, *C. parapsilosis,* and *C. tropicalis* remain classified in the *Candida* genus (6), while others, due to their characteristics, have been assigned to different or newly created genera. For example, the widespread species C. glabrata was added to the *Nakaseomyces (N.)* genus, and its name is now changed to *N. glabrata*. Similarly, one of the most prevalent species for human pathology, *C. krusei*, was reclassified into the *Pichia (P.*) genus, so now it is referred to as *P. kudriavzevii*. Species *C. guilliermondii* and *C. kefyr* have also been reassigned and are now members of the genera *Meyerozyma (M. guilliermondii*) and *Kluyveromyces (K. marxianus*) (5). However, the newly determined classification of yeasts poses a new challenge in medical mycology. Adopting and defining isolates according to the new taxonomy could confuse clinicians when interpreting mycological analyses. Many authorities argue against changing the old names of yeasts, now classified into separate genera. Alternatively, at this point, retaining the old nomenclature while writing the new names in parentheses is suggested. Additionally, the clinically recognizable entity of superficial candidosis, recognized for many years, will also change, whether using "superficial fungal infection" or, more precisely, "superficial yeast infection".

Considering that conventional diagnosis of SFI often requires a long time, there is an effort to introduce and develop new, rapid molecular methods, such as multiplex PCR, to detect fungi directly in patient samples (24, 25). Also, for more accurate identification of the causative agent, in recent years, the MALDI TOF (matrix-assisted laser desorption/ionization-time of flight) method has become very useful in diagnostics since it identifies yeasts and molds at the species level based on the detection of specific fungal proteins (26). All these methods aim to contribute to the rapid diagnosis of SGI as well as the adequate treatment of these infections.

Conclusion

Results of this retrospective study revealed a significantly high prevalence (30.2%) and cumulative incidence (26/105 inhabitants) of SFI. The high prevalence of zoophilic species, *M. canis* and *T. mentagrophytes*, emphasize the need for epidemic and epizootic preventive measures in order to stop the spread of dermatophytoses through diagnostics and treatment of not only people but infected animals as well. The emergence of anthropophilic species, not documented in the past 30 years, calls for constant monitoring of these SFI due to their potential to cause infections with epidemic characteristics. Ever-higher numbers of patients with superficial candidosis caused by NAC species demand mycological analyses in order to determine the cause and examine the *in vitro* effectiveness of antimycotics.

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PREVALENCIJA I DISTRIBUCIJA VRSTA UZROČNIKA SUPERFICIJALNIH GLJIVIČNIH INFEKCIJA U NIŠAVSKOM OKRUGU U SRBIJI

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Procena distribucije vrsta uzročnika infektivnih bolesti ključna je u slučajevima infekcija čije se lečenje sprovodi empirijski, bez laboratorijskih dokaza.

Cilj ovog rada bio je da utvrdi prevalenciju superficijalnih gljivičnih infekcija (SGI) i da identifikuje njihove najčešće uzročnike.

Za potrebe ove studije sproveden je mikološki pregled uzoraka kože i adneksa bolesnika sa kliničkim simptomima i znacima SGI-ja. Za detekciju i identifikaciju uzročnika korišćene su standardne mikološke metode – mikroskopija i kultivacija. Dermatofiti su identifikovani na osnovu makroskopskih i mikroskopskih morfoloških karakteristika do nivoa roda ili vrste, dok je identifikacija vrsta kvasnica obavljena komercijalnim testom *Integral System Yeasts plus* (Liofilchem®, Italija). Pri statističkoj obradi podataka korišćene su metode deskriptivne i kvantitativne analize (*SPSS* 14.0 za *Windows* 2003).

Statističkom analizom utvrđena je visoka prevalencija SGI-ja (30,2%); pritom, uočena je značajna razlika u pogledu starosti bolesnika ($p < 0,001$), ali nije zabeležena značajna razlika kada je reč o polu bolesnika ($p = 0,504$). Osim toga, SGI nije pokazao sezonski obrazac ($p = 0,783$). Superficijalne gljivične infekcije potvrđene su izolacijom i identifikacijom kvasnica kod 188 bolesnika (15,1%). *Candida* spp. izolovana je kod 113 bolesnika (60,1%); *Candida albicans* identifikovana je kao uzročnik superficijalne kandidijaze kod 46 bolesnika (40,7%), a ne-*albicans Candida* (NAC) vrste otkrivene su kod značajno većeg broja bolesnika (59,3%). Dermatofitoze su dijagnostikovane kod 75 bolesnika (39,9%), a dominantna vrsta bila je *Microsporum canis* (38,7%).

Viša incidencija SGI-ja čiji su uzrok NAC vrste ukazuje na to da su mikološke analize potrebne da bi se utvrdila etiologija SGI-ja i procenila *in vitro* efikasnost antigljivičnih lekova. Značajna zastupljenost zoofilnih vrsta dermatofita ukazuje na potrebu za osmišljavanjem strategije sprovođenja epidemioloških i epizootioloških preventivnih mera.

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***Ključne reči**: superficijalne gljivične infekcije, prevalencija, dermatofiti, Candida* spp.

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LONG-TERM USE OF BENZODIAZEPINES IN PATIENTS WITH AND WITHOUT PERSONALITY DISORDERS

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Personality disorders (PD) are prevalent co-morbid conditions among addicted individuals. Our study aimed to determine whether long-term, continuous use of benzodiazepines (over a year) leads to the development of symptoms of dependence considering the presence or absence of personality disorders. The group consisted of 78 benzodiazepine users who used benzodiazepine as a monotherapy for at least 1 year before a screening. Patients completed a group of questionnaires: a semi-structured questionnaire for sociodemographic data as well as for basic data on the use of benzodiazepines, Wisconsin Personality Inventory, and Benzodiazepine Dependence Self-Report Questionnaire. The group was divided into two subgroups: the group of subjects with personality disorders (60.26%) and those without personality disorders (39.74%). These two groups were mutually compared concerning: (a) correlates of benzodiazepine dependence (problematic use of benzodiazepines, preoccupation with benzodiazepines, lack of compliance, and withdrawal syndrome) and (b) intensity of benzodiazepine dependence. In the whole group, approximately 70% of subjects had positive indicators for physical dependence (lack of compliance due to a rise of tolerance in 73.08% and withdrawal in 70.51% of subjects). The psychological dependence indicator (preoccupation with benzodiazepines) was positive in 94.87% of subjects, as well as for social aspects of dependence (problematic use of BDZs) in 93.59%. The total score, or intensity of benzodiazepine dependence, was statistically higher in the group with personality disorder. Patients with a personality disorder had more frequent and more intensive preoccupation with benzodiazepine and lack of compliance. Co-occurrence of two or more personality disorders increases the intensity of preoccupation with a benzodiazepine.

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Key words: *benzodiazepine, dependence, personality, withdrawal*

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Introduction

Benzodiazepines (BDZ) are a group of drugs with a wide range of uses, of which particularly frequent one is for the managementing of anxiety and insomnia. They have sedative, anxiolytic, hypnotic, anticonvulsant and muscle relaxant properties. The short-term use of benzodiazepines is safe and has many benefits. In contrast, long-term use leads to dependence (1). The shortest period for the development of dependence due to continuous use of benzodiazepines varies in different studies. It ranges from one month (2) to, in most studies, 3 to 6 months (3, 4). Those most at risk are people who take benzodiazepines for more than four months, people who take high doses of benzodiazepines, older people, and people with a current or previous history of another type of dependence, as well as people who take high-potency benzodiazepines with a short half-life (2, 5).

Previous research has shown that certain personality traits are important for the development, maintenance, and progression of benzodiazepine dependence and the intensity of withdrawal symptoms. The temperament and character profile of benzodiazepine users differed from that of other drug users in the association between higher harm avoidance, self-transcendence, and lower self-centeredness (6). Neuroticism and introversion, a high degree of

passivity and dependence as specific personality traits, have been associated with a more pronounced abstinence syndrome in benzodiazepine addicts and with a more complicated process of reduction and withdrawal from the benzodiazepine drug (7, 8).

Unfortunately, the approach has rarely been applied to personality pathology, i.e., the relationship between personality disorders (PD) and benzodiazepine dependence. According to various systematic reviews, personality pathology is present in 5–91% of individuals with substance use disorders. However, little is known about the presence of personality pathology in patients with benzodiazepine dependence. Personality pathology has a significant and independent impact on the process of discontinuing benzodiazepines. It exacerbates the subjective severity of withdrawal symptoms. It also increases the likelihood of premature withdrawal failure (7). Adults diagnosed with personality disorders and addictions such as benzodiazepine dependence represent a significantly impaired subpopulation. They are a challenge for the treatment and rehabilitation of people with a substance use disorder (9).

Aims

Our study aimed to determine whether long-term continuous use of benzodiazepines (over one year) leads to the development of dependence symptoms, taking into account the presence or absence of personality disorders. We also wanted to investigate any differences in the clinical manifestation of benzodiazepine dependence between individuals with and without personality disorder.

Material and Methods

This cross-sectional study was conducted for 12 months at the Centre for Mental Health, Clinical Center Niš.

Subjects

One hundred and forty-nine patients were referred to a psychiatrist by a general practitioner for long-term use of benzodiazepines as monotherapy. At baseline, we conducted an in-depth psychiatric interview, assessed current psychiatric status and the presence of psychiatric illness, and examined medical records from previous medical/psychiatric history. We obtained data on the history of benzodiazepine use and the reasons for initiating these medications.

The following criteria should be fulfilled for inclusion of patients in the study: (1) long-term use of benzodiazepines before screening (daily use for more than one year); (2) no other psychiatric disorders during screening; (3) age of 18–65 years.

After the clinical examination, the inclusion criteria were met in 83 patients. They were informed about the study and had to sign a consent form for inclusion in the study. All participants completed a set of questionnaires and then returned them in a sealed envelope. Five patients did not complete the questionnaires correctly (omission of items), so their data were excluded from further statistical analysis. The final group consisted of 78 subjects who had been taking benzodiazepines as monotherapy for at least one year at the time of screening (diazepam, bromazepam, lorazepam, alprazolam and clonazepam).

Instruments

Patients completed a questionnaire consisting of the following:

1. The semi-structured questionnaire with which we collected data on the general socio-demographic characteristics of the patients (age, place of residence, marital status, educational level) and the basic data on benzodiazepine use (duration of BDZ use, type of BDZ, frequency of use).

2. **Wisconsin Personality Inventory**

The participants completed the (WISPI) in paper and pencil form (10). This self-report questionnaire provides both categorical diagnoses and dimensional scores for 11 categories of personality disorders. The questionnaire consists of 214 items rated on a 10-point scale. We obtained two scores produced by the WISPI scoring program: (a) mean scores (the average of the ratings for the items on each scale) and (b) z-scores (calculated using the standard sample data from Klein et al., 1993). In the second method, a specific personality disorder was diagnosed if a patient had a z-score of 1.96 or more on a PD scale (i.e., their score was significantly higher than that of the norm sample at $p < 0.05$) (11). The WISPI measures the following personality dimensions: paranoid (PAR), schizoid (SCH), schizotypal (STP), histrionic (HIS), narcissistic (NAR), antisocial (AS), borderline (BL), avoidant (AVO), dependent (DEP), obsessive-compulsive (OC) and passive-aggressive (PA).

3. **Benzodiazepine Dependence Self-Report Questionnaire (Bendep-SRQ)**

The (BDEPQ) is the first scale to comprehensively assess dependence on BZDs, as all existing scales focus solely on withdrawal symptoms (12). This questionnaire consisted of 20 items and was used to measure the severity of benzodiazepine dependence. Each item of the Bendep-SRQ had five response options ranging from 1 – 'Not at all true for me' to 5 –'Completely true for me'. After processing the data, we obtained the results in four homogeneous subscales (13). Each subscale represents a correlate or indicator of benzodiazepine dependence:

a. Problematic use of benzodiazepines subscale measures the degree of awareness of one's problematic benzodiazepine use.

b. Preoccupation with benzodiazepines measures the level of concern, preoccupation, or obsession with the availability of benzodiazepines.

c. Lack of compliance measures the degree of compliance with a prescription or therapeutic benzodiazepine regimen.

d. Withdrawal syndrome measures the degree of benzodiazepine withdrawal experienced.

Based on the responses, it was possible to calculate dimensional (providing a continuous measure of severity) and/or categorical (classifying severity into distinct groups) scores for the indicators of benzodiazepine dependence and the overall severity of benzodiazepine dependence.

The intensity for each subscale was calculated by summing the items in each scale (five items with response options from 1 to 5, meaning that each subscale can have a value from 5 to 25) (dimensional approach) (14).

We calculated the intensity and estimated the presence of indicators (categorical approach) based on dichotomous scores of Rasch modeling. In this process, each item's response was assigned a score of either 0 or 1, depending on its severity. If the response to the item was 1 or 2, the dichotomous score was zero; if the response was 3, 4, or 5, the dichotomous score was one. Since each indicator consists of 5 items, each indicator has a score/intensity ranging from zero to five. We consider an indicator positive/present/clinically significant if the score is between one and five (13).

This questionnaire is highly valid in the outpatient setting and has been used in many previous research studies to measure BZD dependence (14).

The group was then divided into two subgroups based on the Wisconsin Personality Inventory (WISPY) (10):

a) Group of subjects with personality disorders (N = 47; 60.26%)

b) Group of subjects without personality disorders (N = 31; 39.74%).

These two groups were compared concerning the correlates of benzodiazepine dependence (problematic use of benzodiazepines, preoccupation with benzodiazepines, lack of compliance, and withdrawal syndrome) and the intensity of benzodiazepine dependence.

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp, 2012). Student's t-tests and chi-square tests determined statistically significant differences between groups. A P-value of < 0.05 was considered statistically significant.

Results

A. Characteristics of the Sample

The group had a significantly higher percentage of female subjects (88.46%). The average age of participants was 39.58 years. The highest percentage of patients were from urban areas (76.92%), in a relationship or married (82.05%), and had at least 12 years of education (79.49%) (Table 1).

The average duration of using benzodiazepines was 87.33 months (8–360). The frequency of positive benzodiazepine dependence indicators among respondents with chronic benzodiazepine use was very high. Preoccupation with benzodiazepines was present in 94.87% of the subjects, problematic use in 93.59%, while lower compliance was observed in 73.08% of the subjects. The symptoms of physical dependence or withdrawal symptoms were present in 70.51% of the subjects (Table 1).

Regarding personality disorders, the most frequent ones were obsessive-compulsive personality disorder (30.77%) and paranoid personality disorder (25.64%). These two disorders had the highest average scores (4.47 and 4.93, respectively) (Table 1).

The average intensity of benzodiazepine dependence was 54.85 ± 16.89, however, in the group with personality disorder, the intensity was significantly higher than in the group without it (59.15 ± 15.22 vs. 49.04 ± 17.50; p = 0.007).

Table 1. Sociodemographic and clinical characteristics of the group (N = 78)

	N	%	Mean	SD
Age			39. 58	11.56
Female gender	69	88.46		
Place of residence				
City	60	76.92		
In a relationship/married	64	82.05		

Education				
8 grades	2	2.56		
12 grades	62	79.49		
15 grades	7	8.97		
≥ 16 grades	7	8.97		
Number of PD				
None	31	39.74		
One	17	21.79		
Two or more	30	38.47		
Personality disorders				
Obsessive-compulsive	24	30.77	4.93	1.29
Paranoid	20	25.64	4.47	1.47
Schizoid	15	19.23	3.62	1.28
Borderline	11	14.10	3.28	1.39
Narcissistic	11	14.10	3.81	1.31
Dependent	11	14.10	3.26	1.55
Passive-aggressive	11	14.10	3.67	1.12
Histrionic	9	11.54	3.77	1.00
Avoidant	7	8.97	4.14	1.86
Schizotypal	5	6.41	2.58	0.94
Antisocial	2	2.56	1.43	0.43
Mean duration of using BDZ (months)			87.33	76.41
Correlates of BDZ dependence frequency and mean intensity scores				
Problematic use of BDZ	73	93.59	3.31	1.58
Preoccupation with BDZ	74	94.87	3.19	1.41
Lack of compliance	57	73.08	1.87	1.56
Withdrawal	55	70.51	2.18	1.81

PD = personality disorder; BDZ = benzodiazepines; SD = standard deviation

B. Indicators of benzodiazepine dependence concerning the presence of personality disorders

1. Differences in frequency of benzodiazepine dependence indicators between the groups with and without personality disorder

Comparing the groups of subjects with and without personality disorders, a significantly higher frequency of two correlates was found in the group of subjects with personality disorders - preoccupation with benzodiazepines and low compliance (Table 2).

2. *Intensity of the benzodiazepine dependence indicators in the groups with and without personality disorders*

A similar result was obtained when these two groups were compared in terms of the intensity of benzodiazepine indicators. A statistically significant difference was found concerning preoccupation with benzodiazepines and low compliance (Table 3).

The co-occurrence of two or more personality disorders was of significance concerning the intensity of preoccupation with benzodiazepines. The highest rates of preoccupation with benzodiazepines were found in patients with co-occurrence of 1–3 personality disorders ($p < 0.003$).

Table 2. Frequency of benzodiazepine dependency indicators in the group with and without personality disorders

	Problematic use of BDZ		Preoccupation with BDZ		Lack of compliance		Withdrawal syndrome	
	N	%	N	%	N	%	N	%
With personality disorder	45	95.7%	**47**	**100.0%**	**39**	**83.0%**	34	72.3%
Without personality disorder	22	90.3%	**27**	**87.1%**	**18**	**58.1%**	21	67.7%
Pearson Chi-square	0.915		**6.392**		**5.893**		0.19	
df	1		**1**		**1**		1	
p-value	0.339		**0.011***		**0.015***		0.663	

BDZ = benzodiazepines; CI = Confidence interval; *p < 0.05

Table 3. Intensity of the benzodiazepine dependency indicators in the groups with and without personality disorder

		N	Mean	Std. Deviation	Std. Error Mean	T-test	df	P value
Problematic use	With PD	47	3.53	1.472	0.215	1.556	76	0.124
	Without PD	31	2.97	1.703	0.306			
Preoccupation with benzodiazepines	With PD	47	**3.64**	1.206	0.176	**3.700**	**76**	**0.000**
	Without PD	31	**2.52**	1.458	0.262			**
Lack of compliance	With PD	47	**2.19**	1.527	0.223	**2.293**	**76**	**0.025**
	Without PD	31	**1.39**	1.498	0.269			*
Withdrawal syndrome	With PD	47	2.23	1.879	0.274	0.326	76	0.745
	Without PD	31	2.1	1.72	0.309			

PD = personality disorder; *p < 0.05; **p < 0.001

Discussion

Benzodiazepine dependence is the condition resulting from the repeated and continuous use of benzodiazepine drugs. Since 1981, the WHO has propagated a psycho-physiological-social model for dependence on all psychoactive substances, the so-called drug dependence syndrome. The four scales of the Bendep-SRQ reflect the psychological, physiological and social aspects of BZD dependence (13, 14). If we consider the content of the Bendep-SRQ items for the assessment of benzodiazepine indicators (13), we can conclude that 'problematic use' seems to reflect a social aspect of benzodiazepine dependence, 'preoccupation' the psychological aspect, 'lack of compliance' reflects an increase in tolerance or the physiological aspect, and 'withdrawal' highlights the physical dependence associated with this substance (14).

In our study, approximately 70% of subjects had positive indicators for physical dependence (lack of compliance in 73.08% and withdrawal in 70.51% of subjects). The psychological dependence indicator was highly frequent (preoccupation with BDZs in 94.87% of subjects), as well as for social aspects (problematic use of BDZs in 93.59%). A significantly higher number of subjects was of the female gender, which is consistent with previous research that has shown that the female gender is more frequent in benzodiazepine users (15). Gender is a well-known factor associated with the development of benzodiazepine dependence since several studies suggested that women are more vulnerable to this condition (2).

The result of our study was in concordance with previous studies and confirms that the frequency of personality disorders is high among people with dependence on psychoactive substances. Current data indicate that the frequency ranges between 65% and 90% of subjects treated for substance abuse or dependence (16). Research that dealt with personality disorders in benzodiazepine addicts found that predominantly borderline personality disorder, but also antisocial personality disorder, are associated with misuse of benzodiazepines and a more severe form of abstinence syndrome (17). Borderline personality disorder, avoidant personality disorder, and dependent personality disorder are the most common disorders among benzodiazepine addicts. Patients with cluster B personality disorders have the worst prognosis regarding discontinuing BZD (18). Some authors indicate that histrionic, dependent, or anankastic personality disorders are personality factors that predispose to dependence (19).

In our study, a large percentage of subjects with benzodiazepine dependence had at least one personality disorder (60.26%). Among them, the most frequent were obsessive-compulsive personality disorder (30.77%) and paranoid personality disorder (25.64%).

After the division into subgroups, it was established that there were differences in the expression of benzodiazepine dependence in the groups with and without personality disorders. First, the total score (e.g. intensity) of benzodiazepine dependence was statistically higher in the group with personality disorder. That means greater symptom severity and more suffering for a person. When comorbidity with personality disorders was present, there was a greater likelihood that certain indicators of benzodiazepine dependence would be present as well.

Our results indicate that individuals with benzodiazepine dependence who also have a personality disorder are more likely to present frequent and intensive behavior associated with poor compliance, which is usually the result of an increase in tolerance. Our patients with a comorbid personality disorder had a higher chance of expressing more frequent and more intensive preoccupation with benzodiazepine or craving and safety behavior related to the constant need for availability of the drug.

The co-occurrence of two or more personality disorders in the same person was associated with a more frequent occurrence of preoccupation with benzodiazepine, too. They spend a great deal of time thinking about medication and its availability, get nervous if medication is out of reach, feel safe only if medication is with them, expect scheduled time to take a medication, etc.

The limiting factor of this research was a relatively small number of subjects. Also, the study involved a significantly higher number of women, but this relationship was probably the result of a greater use of benzodiazepines among females, as well as of the fact that men are less likely to seek treatment (20).

The importance of the data obtained was predominantly clinical. Information about which patients are at a high risk of developing benzodiazepine dependence can help clinicians take measures to modify the risk. Those may involve selecting therapeutic methods and techniques, pre-preparation of specific treatment, close monitoring during treatment, or adding adjunctive interventions. The conclusions based on our results are that more caution is needed when administering benzodiazepines to people who have a comorbid personality disorder. In our opinion, the presence of personality disorders should be considered in the duration of the treatment planning. A personality assessment should guide clinical decisions on tapering BDZ in addition to considerations such as dosage, residual psychopathology, duration, etc. (7).

Conclusion

Our results suggested that benzodiazepine dependence was commonly associated with personality disorders. This association could

contribute to the increase in the intensity of benzodiazepine dependence, as well as in the increased intensity and frequency of preoccupation with benzodiazepine and lack of compliance during treatment.

Cumulative effects of the co-occurrence of two or more personality disorders may additionally increase preoccupation with a benzodiazepine.

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DUGOTRAJNA UPOTREBA BENZODIAZEPINA KOD PACIJENATA SA POREMEĆAJEM LIČNOSTI I KOD PACIJENATA BEZ POREMEĆAJA LIČNOSTI

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Poremećaji ličnosti predstavljaju veoma česta komorbidna stanja kod zavisnika. Cilj ove studije bio je da, uzimajući u obzir prisustvo ili odsustvo poremećaja ličnosti, utvrdi da li dugotrajna, kontinuirana upotreba benzodiazepina (duža od jedne godine) dovodi do razvoja simptoma zavisnosti. U ispitanoj grupi bilo je 78 osoba koje su koristile benzodiazepin kao monoterapiju najmanje jednu godinu pre skrininga. Pacijenti su popunjavali grupu upitnika: polustrukturisani upitnik za sociodemografske podatke i za osnovne podatke o upotrebi benzodiazepina, Viskonsin upitnik za procenu ličnosti i samoupitnik o zavisnosti od benzodiazepina. Grupa je potom podeljena u dve podgrupe: grupu ispitanika sa poremećajima ličnosti (60,26%) i grupu ispitanika bez poremećaja ličnosti (39,74%). Pri poređenju ovih dveju grupa u obzir su uzeti korelat zavisnosti od benzodiazepina (problematična upotreba benzodiazepina, preokupacija benzodiazepinima, nedostatak komplijanse i apstinencijalni sindrom) i intenzitet zavisnosti od benzodiazepina. Kod oko 70% svih ispitanika zapaženi su pozitivni pokazatelji fizičke zavisnosti (nedostatak komplijanse zbog porasta tolerancije kod 73,08% ispitanika i apstinencijalni sindrom kod 70,51% ispitanika). Pokazatelj psihološke zavisnosti (preokupacija benzodiazepinima) bio je pozitivan kod 94,87% ispitanika, a pokazatelj socijalnih aspekata zavisnosti (problematična upotreba benzodiazepina) kod 93,59% ispitanika. Ukupan skor, tj. intenzitet zavisnosti od benzodiazepina, bio je na statistički značajnom nivou veći u grupi ispitanika sa poremećajem ličnosti. Kod pacijenata sa poremećajem ličnosti zabeleženi su češća i intenzivnija preokupacija benzodiazepinima i nedostatak komplijanse. Istovremena pojava dvaju ili više poremećaja ličnosti povećava intenzitet preokupacije benzodiazepinom.

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***Ključne reči**: benzodiazepini, zavisnost, ličnost, apstinencijalni sindrom*

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ANEMIA AS A COMMON COMORBIDITY AND PROGNOSTIC MARKER IN HEART FAILURE

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Heart failure (HF) is a clinical syndrome characterized by the inability of the heart to pump the amount of blood necessary to meet the body's needs for oxygen and energy substances in proportion to physical activity, provided that the venous blood flow to the heart is preserved.

The frequency of HF and left ventricular dysfunction increases with the age of patients. It is considered that the diagnosis of heart failure is the most common discharge diagnosis at the age of 65. Comorbidities are very important in HF patients for several reasons. Chronic anemia is very often associated with HF (up to 55% of patients). The aim of the work was to assess the presence of anemia in patients with HF and its impact on the prognosis of these patients.

The total number of subjects was 201. Anemia was more common in women than in men and was equally prevalent in systolic and diastolic HF, which is also consistent with previous reports. However, anemia was not more common in elderly HF patients. Patients with New York Heart Association (NYHA) class IV HF were significantly more likely to have anemia than those with NYHA class I or II, which is consistent with previous reports. In patients with HF, there is a significant frequency of anemia as non-cardiac comorbidity. The presence of anemia significantly impacts the hospital and post-hospital course. Therapy should be started even with subclinical anemia or with reduced iron depots even though the hemoglobin is still within the reference values because this improves the prognosis of our patients.

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Key words: *heart failure, comorbidities, anemia, prognosis*

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Introduction

Heart failure (HF) is a clinical syndrome characterized by the heart's inability to pump the amount of blood necessary to meet the body's needs for oxygen and energy in proportion to physical activity, provided that the venous blood flow to the heart is preserved (1).

The frequency of HF and left ventricular dysfunction increases with the age of patients. It is considered that the diagnosis of HF is the most common discharge diagnosis at the age of 65. It is estimated that 23 million people worldwide suffer from HF. New, modern methods of treatment in cardiology, primarily in coronary disease, reduce patient mortality and a large number of patients with chronic HF are recruited from the group of survivors (1). The mortality of HF patients is high and ranges from 15% to 60% per year. Patients with New York Heart Association (NYHA) class III/IV have a mortality of 50% compared to patients with NYHA class II/III where the two-year mortality is 25%. The prognosis is much more serious in older patients and men (2, 3).

In recent years, HF has become one of the biggest health and social problems in the field of cardiovascular pathology and one of the most common causes of hospitalization in the world. A special problem is the socioeconomic importance of HF, due to frequent hospitalizations and a greater number of medications that are used for a long time (4).

Comorbidities are very important in HF patients for several reasons. They can directly affect the therapy of patients with HF in the sense

of preventing the use of certain medications, medications used in the therapy of comorbidities can worsen HF, and drugs used in HF and comorbidities can show an interaction (beta-blockers in HF and beta-agonists in chronic obstructive pulmonary disease) (5).

Many comorbidities are associated with poor clinical status, which further aggravates HF, i.e., they are associated with a worse prognosis (diabetes mellitus) (5). Due to the possible improvement of the clinical status of HF, sometimes the treatment should be focused primarily on the treatment of comorbidities (anemia).

We should not ignore the cost of treating these patients, which is higher due to associated diseases, more frequent and longer hospitalizations, and the greater number of medications used (6). Comorbidities can be cardiac and extracardiac. Cardiac comorbidities can be causes of HF, but they can be present and associated with other primary causes. The most common are arterial hypertension (HTA), coronary disease, and valvular insufficiency (5). The most common noncardiac comorbidities in HF are anemia, diabetes mellitus, renal failure, obstructive lung disease, depression and infection, and cognitive dysfunction (7). Chronic anemia is very often associated with HF (up to 55% of patients). Anemia is defined as a state of decreased hemoglobin concentration < 12g/dl in women or < 13g/dl in men. It is more common in hospitalized HF patients, women, the elderly, and in patients with renal impairment. Anemia is associated with many symptoms, poor functional status and represents a high risk for hospitalized HF patients because it increases mortality (8). Anemia in these patients is associated with increased left ventricular mass. There is an increase in markers of inflammation and biohumoral parameters N terminal pro-BNP and C reactive protein. A systematic follow-up of more than 150,000 patients with HF and anemia showed the presence of an increased risk of death. Forty-eight percent of anemic patients died within 6 months of HF diagnosis compared to 29.5% of non-anemic patients (7). The etiology of anemia in HF is multifactorial. Additional factors that worsen anemia are the already mentioned elderly population and renal failure, but also hemodilution, increased circulation of pro-inflammatory cytokines (IL6, TNF-α), reduced bone marrow function, and therapy with aspirin and ACE inhibitors (2). Repeated hospitalizations due to decompensation and death are directly correlated with hemoglobin concentration. Probable mechanisms of deterioration are expansion of intravascular volume, increase in neurohumoral activity and worsening of myocardial ischemia. Absolute or relative deficiency of iron and/or erythropoietin is the leading factor in the pathophysiology of anemia in these patients, especially when a certain degree of renal insufficiency is present. Nanas et al. found that 73% of patients with HF and anemia had decreased iron levels on bone marrow aspiration (9).

Anemia therapy should be directed towards the causative factor, but even in the case of anemia of unknown etiology, intravenous iron and erythropoietin are used (10). Anemia improvement correlates with a reduction in HF symptoms, an increase in exercise tolerance, and an improvement in heart muscle condition.

Aim

The work aimed to assess the presence of anemia in patients with heart failure and its impact on the prognosis of these patients.

Patients and Methods

The study included patients who were treated at the Intensive Care Unit of the Clinic for Cardiovascular Diseases (CVD), University Clinical Center (UKC) Niš. They were hospitalized for signs of HF. The total number of patients was 201. During the processing of the patients, detailed anamnestic data were taken. In case of impossibility of cooperation with the patient, data were obtained heteroanamnestically from the closest family members. The examination included: the most important complaints, the time sequence of the occurrence of certain complaints; duration of the underlying disease that led to HF; way of treating HF; the presence of risk factors for heart diseases (smoking, arterial hypertension, family burden); frequency of hospitalizations, present comorbidities; previous illnesses; and medications used so far.

The clinical examination included the observation of HF signs: tachycardia, arrhythmias, crackles over the lungs, weakened breathing over the lung bases; the presence of peripheral edema, hepatomegaly; the presence of III or IV tones over the heart; swollen neck veins; clinical signs of anemia, hyperthyroidism or myxedema. The classification of heart failure used was NYHA classification. The ECG was performed on a twelve-channel ECG (Scheler). Heart rate, possible ischemic changes, and occurrence of acute myocardial infarction were monitored along with the presence of rhythm disorders such as atrial fibrillation, extrasystoles, and conduction disorders. Upon admission to the Clinic for CVD of UKC Niš, blood was taken for laboratory analysis at the Central Biochemical Laboratory and the Hematology Clinic of UKC Niš. The following values were determined: general laboratory analyses including glucose, urea, creatinine, total cholesterol, HDL and LDL fractions, triglycerides, sodium, potassium, Acidum uricum, transaminases, total proteins as well as cardiac markers of myocardial damage troponin I and CKMB. In the case of a negative finding and suspected acute coronary syndrome, the analyses were repeated after 6 hours; Markers of risk

factors: markers of inflammation (C-reactive protein, albumins), markers of coagulation (fibrinogen), markers of thrombosis (D dimer), markers of ischemia and remodeling of the left ventricle (BNP), thyroid hormones (T3, T4, TSH); Complete blood count: the total number of leukocytes, erythrocytes, hemoglobin, hematocrit and platelet count were determined. The blood test was done at the Hematology Clinic of UKC Niš. Each patient underwent a transthoracic echocardiographic examination on a General Electric Vivid 4 ultrasound machine. The techniques of one-dimensional examination, two-dimensional examination and Doppler technique from standard sections were used. The size of the left and right heart cavities and their volumes were assessed. The systolic and diastolic function of the left ventricle was evaluated as well as the function of the valvular apparatus. Disorders of regional contractility were monitored, and pericardial effusion was observed. Further, the possible presence of additional echoes in the cavities (dense spontaneous echoes, thrombi) or on the valvular apparatus (vegetation) was observed. A radiological examination of the lungs was performed in patients for possible evidence of an inflammatory process on the lung parenchyma (in patients with clinical signs of lung infection) and for the evidence and evolution of the presence of fluid in the pleural space. In addition, 24 Holter ECGs were performed in patients to monitor the presence of significant arrhythmias (paroxysmal atrial fibrillation, multifocal VES and ventricular tachycardia). We followed changes in the ST segment in terms of subendocardial ischemia, especially asymptomatic ones. An ultrasound examination of the abdomen was performed in patients to possibly prove the presence of free fluid in the abdomen (ascites) and to monitor the state of the parenchymatous organs (liver, gall bladder, kidneys). For a more precise diagnosis of comorbidities, examinations by doctors of other specialties were included: pulmonologists, neurologists and psychiatrists, hematologists, gastroenterologists and surgeons.

Statistical Analysis

Data were processed using standard descriptive statistical methods including mean value, standard deviation, percentage representation, and median. Pearson's linear correlation coefficient was used to determine the relationship. Statistical processing was done with Excel 7.0 and SPSS version 17 in the Windows XP environment, and the results are presented tabularly and graphically.

Results

A total of 201 patients with acute decompensation of chronic heart failure were included in the research. Among the patients, 60.7% were male, and 39.3% were female. The average age of patients was 71.55 ± 10.354 years. The average duration of heart failure and the average length of hospital treatment are given in Table 1.

On admission, 55 patients (27.4%) had NYHA class II, NYHA class III was the most frequent, i.e., it was present in 108 patients (53.7%), while 38 patients (18.9%) had NYHA class IV. There were no patients with NYHA class I, i.e., they were not included in the research (Figure 1).

There were 70 patients (34.8%) without peripheral edema on admission, 83 (41.3%) with peripheral edema, and 48 (23.9%) who had effusions along with edema, which is presented in Table 2.

Table 1. Baseline patients' characteristics at hospital admission

	X min	X max	$\bar{x}$	SD
Age (years)	40	90	71.55	10.354
Duration of heart failure (years)	0	3	1.69	1.129
Duration of hospitalization (years)	0	9	4.98	2.233

Table 2. Clinical presentation on hospital admission

	No.	%
0	70	34.8
1	83	41.3
2	48	23.9
Total	201	100.0

0—without edema and effusion, 1—peripheral edema, 2—pleural/pericardial effusion

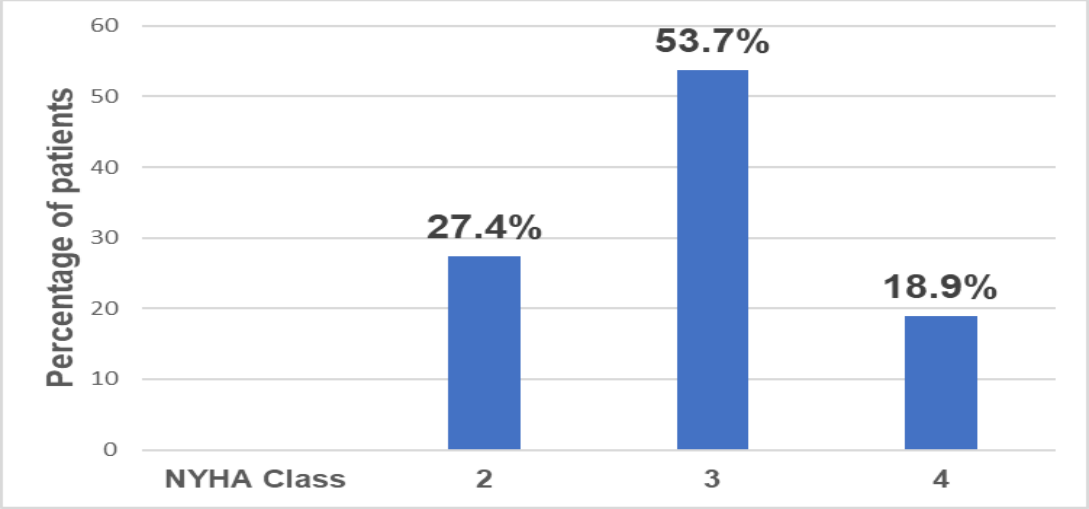

Figure 1. NYHA class on admission

Among the examined patients, 67 (33.3%) had HF with a preserved ejection fraction (Table 3).

Laboratory parameters on admission to the hospital are given in Table 4 (min, max, mean value and standard deviation). Table 5 shows the basic echocardiographic parameters examined on the patient's admission.

Of the cardiac comorbidities, arterial hypertension and atrial fibrillation were present in a high percentage. Among the non-cardiological comorbidities, diabetes, chronic renal failure, acute infections, anemia, chronic obstructive pulmonary disease (COPD), depression, cerebrovascular insult (CVI), hypothyroidism, hyperthyroidism, alcoholism and hematological diseases were monitored. The so-called "other comorbidities" were represented in a small percentage (up to 0.5%) and included the following associated diseases: oncological diseases of various localizations, sleep apnea, systemic connective tissue diseases, duodenal ulcer, psoriasis, osteoarthrosis and prostate adenoma (Table 6).

Of the examined patients, who were followed up for 1 year, 42.8% did not survive. 20 (10%) patients died during hospitalization, 26 (12.9%) patients died within 6 months of hospital discharge, and 40 (19.9%) patients died within 6–12 months after hospital discharge.

The largest number of patients who died (total, intra-hospital and in the period from 6 to 12 months) had three comorbidities, while the largest number of those patients who died within the period up to six months after discharge from the hospital had 2 comorbidities.

Table 3. Type of heart failure

HF with preserved LVEF	No.	%
No	134	66.7
Yes	67	33.3
Total	201	100.0

HF heart failure, LVEF—left ventricle ejection fraction

Table 4. Laboratory parameters on hospital admission

	X min	X max	$\bar{x}$	± SD
BNP	149.00	7714.30	1554.1426	1243.39835
TnI	0	12.2	12.5	10.0.827
Er	2	7	4.16	.867
Hb	65	197	122.77	23.189
Hct	17	55	36.58	7.742
Le	0	24	12.34	5.184
Tr	84	488	277.88	102.297
Blood glucose	2	28	8.77	4.924
Creatinine	53	665	152.18	87.602
Urea	3	617	15.72	45.508
Uric acid	195	921	471.07	137.794
Sodium	123	148	137.41	3.886
Potassium	3	7	4.46	.701
HOL	2	9	4.64	1.494
HDL	1	2	1.10	.306
LDL	1	6	2.93	1.091
TG	0	7	1.57	.929
AST	12	798	71.54	86.975
ALT	5	568	58.89	71.751
Albumin	22	49	33.75	5.096
D-dimer	95	5200	461.61	567.162
CRP	1	435	40.72	59.759
Fib	1	13	6.25	2.707
Hs CRP	11	83	53.95	37.781

BNP—brain or B-type natriuretic peptide, TnI—troponin I, Er—red blood cells, Hb—hemoglobin, Hct—hematocrit, Le—leukocyets, Tr—platelets, HOL—total cholesterol, HDL high density lipoprotein cholesterol, LDL—low density lipoprotein cholesterol, TG—triglycerides, AST—aspartate aminotransferase, ALT—Alanine aminotransferase, CRP—C reactive protein, Fib—fibrinogen

Table 5. Baseline echo parameters

	X min	X max	$\bar{x}$	± SD
EF (%)	11	79	37.40	13.743
ESD (mm)	37	88	61.37	9.007
EDD (mm)	20	74	48.11	10.019
SPDK (mmHg)	19	78	46.83	13.750

EF—left ventricular ejection fraction, ESD end systolic diameter of left ventricle, EDD end-systolic diameter of left ventricle, SPDK systolic pressure in right ventricle

Table 6. Comorbidities

	No.	%
Arterial hypertension	162	80.6
Diabetes mellitus	110	54.7
Atrial fibrillation	95	47.3
Chronic renal failure	87	43.3
Acute infection	66	32.8
Anemia	53	26.4
COPD	48	23.9
Depression	20	10
Hypothyreosis	16	8
Stroke	15	7.5
Hyperthyreosis	15	7.5.
Alcoholism	11	5.5
Other comorbidities	4	2
Hematological comorbidities	3	1.5

Discussion

A total of 201 patients with acute decompensation of chronic heart failure were included in our observational study. The majority of respondents were men (60.7%), with an average age of 71.55 years. The highest percentage of patients had Killip class II (patients with the acute coronary syndrome) on admission and peripheral edema (lower leg edema), and belonged to NYHA functional class III. Our results are consistent with those obtained by Rudiger et al. In a multicenter study on the clinical presentation of patients with acute heart failure as well as their one-year prognosis and factors affecting it, the average age of the patients was 73 years and 56% of the study sample was male. A slightly higher percentage of patients with

pulmonary edema (29.4%) in our study compared to 13% (from the aforementioned work by Rudiger et al.) can be explained by the fact that our research was conducted in the intensive care unit where patients with more severe forms of heart failure receive treatment and did not include all patients who were hospitalized with this diagnosis (11). Three percent of the patients were in cardiogenic shock, i.e. with seriously compromised hemodynamics, which is again in line with previous reports on the clinical presentation of patients with acute cardiac decompensation, which ranges from less than 1% in Euro-HF I to 7.7% in the Italian Survey study, while one of the largest registries of patients with acute heart failure, ADHERE, also reported rate of 3% (12–14). The average length of treatment lasted 2.43 days, which is in line with the length of treatment in intensive care units in Europe and America, which ranges from 2.6 days in the ADHERE registry including 105,388 patients to 7.6 days in the EFICA study performed on 599 subjects (14–16). Our subjects suffered from heart failure for an average of 1.69 years and were hospitalized due to worsening of the underlying disease. As the presumed root cause of heart failure, the following were found in almost the same percentage: coronary disease, dilated cardiomyopathy, hypertension and, with a slightly lower frequency, valvular disease. In a meta-analysis of 31 studies that included patients with heart failure, Tavazzi found that in 15–17% of patients with acute HF, arterial hypertension is an etiological factor, similarly, in our country this percentage is 18.4% (13). In a meta-analysis, Bui et al. conducted in subjects in North America, about 50% of patients had coronary disease as the main etiological factor (17). Tavazzi et al. state that coronary disease as an etiological factor of HF is mentioned with a significant difference in different studies ranging from 29% to 52%, while in our patients it was found in 29.9% of respondents (18). The same authors in their meta-analysis state that dilated cardiomyopathy is the most common cause of HF after coronary heart disease, which aligns with the results obtained in our study, where it was present in 27.9% of cases. In addition, the frequency of valvular disease decreases significantly in developed countries, whereas in our research, it was the cause of HF in 18.4% of patients (13, 17). This is in line with the results of the recently conducted EFICA study, where dilated cardiomyopathy and hypertension were reported in 15% of subjects, and valvular disease in 21% as the main cause of heart failure (15). One-third of our patients (33.3%) had heart failure with preserved ejection fraction. Large prospective European national registries indicate a high frequency of heart failure with preserved ejection fraction in hospitalized patients ranging from 46% to 51%. This type of heart failure is more common in elderly women who have been suffering from heart failure for a long time (18–22). In most Western European countries, the frequency of heart failure with a preserved ejection fraction is increasing, while in our country a greater number of patients have the type of heart failure with a reduced ejection fraction (23). It is assumed that this is a consequence of the high percentage of patients with coronary disease due to exposure to specific socioeconomic risk factors, but also due to less engagement in primary prevention (18, 21, 24).

The average value of arterial blood pressure on admission to the hospital was 134/80 mmHg, which corresponds to reports in previous studies in patients with acute HF (25–27). Patients were mildly tachycardic with an average heart rate of 97/min which is consistent with reports from other studies where heart rates ranged from 75/min in the Euro-HF I study (including 11,327 subjects) to 97/min in the Italian Survey study conducted on 2,807 patients (12, 13).

Of the laboratory parameters on admission, our subjects had significantly elevated BNP and D dimer values, which is consistent with biohumoral disorders in patients with acute HF. Natriuretic peptides type A and type B (BNP) play a significant role in the pathogenesis of heart failure with effects on the kidneys, heart and blood vessels. Today, the measurement of natriuretic peptides is of great importance for diagnosis and prognosis in patients with acute HF (28, 29). The largest percentage of our respondents have three and two comorbidities, respectively, which is in line with the majority of studies investigating this area (5, 6). The most common comorbidities are arterial hypertension, atrial fibrillation and diabetes mellitus. A significantly smaller percentage of respondents (1.5%) had no comorbidities, that is, they had as many as 6 associated comorbidities (30). During the one-year follow-up, 42.8% of patients did not survive, 10% of patients died during hospitalization, 12.9% of patients died within 6 months of discharge from the hospital, while 19.9% of patients died in the period from 6 to 12 months after discharge from the hospital. The obtained results are consistent with the results from the studies in which in-hospital mortality was monitored, among which the largest are the MAGGIC and EFICA studies (11, 15, 20). In the EFICA study, one-year mortality in patients treated in the Intensive Care Unit was slightly higher than in the non-implantation study (49%). The reason is probably the higher NYHA class in their patients (NYHA III/IV) (15).

We found a statistically significant association between the duration of hospital treatment and, therefore, the cost of treatment in patients with a greater number of comorbidities. This indicates the importance of comorbidity on the prognosis and mortality of patients with heart failure, but also the significant financial costs to society as a whole (31).

Iron deficiency is the main cause of anemia. Despite this known cause, there are a number of questions regarding the best choice of therapy. Commonly used drugs are ferrous sulfate, ferrous gluconate, or ferrous fumarate. These forms of iron (dionic) are more soluble than the ferric form,

with twice the absorption capacity (32, 33). Approximately 30–40% of patients with chronic HF had anemia (34). If iron deficiency in chronic HF is defined as a serum ferritin level < 100 mg/L, together with a transferrin saturation level < 20%, approximately 24% of all patients with chronic HF (e.g. about 40% of non-anemic patients) have iron deficiency (35). There are several possible reasons for iron deficiency in patients with chronic HF. Some patients with chronic HF are anemic not because their RBC mass is low, but because their plasma volume is high, which is described as hemodilution (36). The proteinuria often encountered in chronic HF can cause urinary loss of erythropoietin, as well as transferrin loss, which can lead to iron deficiency anemia (37). Anemia may be part of a chronic inflammatory process. Some studies suggest that about 60% of patients with chronic HF may have anemia of the aforementioned type, characterized by low iron levels and iron-binding capacity, but elevated ferritin levels (38). The disadvantage of ferritin is that it is an acute phase reactant and its level can be elevated during inflammation. The precise limit for defining anemia in CHF was mostly arbitrary in previous research. According to the World Health Organization (WHO), anemia is defined as hemoglobin concentrations < 13 g/dL for men or < 12 g/dL for women, but some authors use more conservative definitions. The prevalence and severity of anemia increase during the progression of chronic HF (37). Some studies have shown the importance of correcting anemia in patients with chronic HF. Treatment strategies include administration of erythropoietin, iron supplementation, or both (39). Most of these studies were conducted using intravenous formulations, but few observations were made with oral formulations of iron. Anemia is an independent prognostic factor for morbidity and mortality in patients with chronic HF. Previous studies have shown a beneficial effect of anemia therapy in patients with chronic HF. The prevalence of anemia in our patients is in accordance with previously published data (40). Anemia was more common in women than in men and was equally prevalent in systolic and diastolic HF, which is also consistent with previous reports (41). However, anemia was not more common in elderly HF patients. Patients with NYHA class IV were significantly more likely to have anemia than those with NYHA class I or II, which is consistent with previous reports (39). Anemia is a comorbidity that should be treated in HF patients. Potential beneficial effects of this treatment are improved oxygen delivery to tissues and inhibition of cardiomyocyte apoptosis due to ischemia, slowing of harmful left ventricular remodeling, improved exercise tolerance, and improved quality of life (42). It should be noted that the use of oral iron therapy is often associated with gastrointestinal side effects (20–30%), and a long duration of therapy is necessary to replenish iron stores. These side effects lead to poor therapeutic adherence. This is the main reason for switching to parenteral therapy. Regardless of whether iron deficiency is absolute or relative in HF, it appears to be a comorbidity per se (39). In a recent study of 459 anemic and iron-deficient patients, all prognostic markers were improved by supplementation (43). This finding also indicates that iron deficiency is a significant comorbidity in SI, even without anemia. The prognostic markers examined in these patients were variations in maximum oxygen consumption assessed by ergospirometry, NYHA class, BNP levels, quality of life questionnaires (Kansas City and EQ5D), LVEF, rehospitalization and HF mortality (43).

Conclusion

In patients with heart failure, there is a significant frequency of anemia as non-cardiac comorbidity. The presence of anemia has a significant impact on the hospital and post-hospital course. Therapy should be started even with subclinical anemia or with reduced iron depots even though the hemoglobin is still within the reference values because this improves the prognosis of HF patients.

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ANEMIJA KAO ČEST KOMORBIDITET I PROGNOSTIČKI MARKER U SRČANOJ SLABOSTI

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Srčana insuficijencija (SI) je klinički sindrom koji karakteriše nesposobnost srca da ispumpa količinu krvi neophodnu za zadovoljenje potreba organizma za kiseonikom i energetskim materijama srazmerno fizičkoj aktivnosti, pod uslovom da je dotok krvi kroz vene na srcu očuvan.

Učestalost srčane insuficijencije i disfunkcije leve komore raste sa starošću bolesnika. Smatra se da je dijagnoza srčane insuficijencije najčešća otpusna dijagnoza nakon 65. godine. Komorbiditeti su veoma važni kod bolesnika sa srčanom insuficijencijom iz nekoliko razloga. Hronična anemija veoma je često udružena sa srčanom insuficijencijom (javlja se kod čak 55% bolesnika). Cilj ovog rada bilo je sagledavanje prisustva anemije kod bolesnika sa srčanom insuficijencijom i uticaja koji anemija ima na prognozu pomenute bolesti.

U studiju je bio uključen 201 ispitanik. Anemija je bila češća kod žena nego kod muškaraca i bila je podjednako zastupljena u sistolnom i dijastolnom SI-ju; to je u skladu sa prethodno zabeleženim rezultatima. Međutim, anemija nije bila češća kod starijih bolesnika sa SI-jem. Bolesnici sa klasom IV Njujorške asocijacije za srce (engl. *New York Heart Association* – NYHA) imali su anemiju značajno češće od onih sa NYHA klasom I ili II, što odgovara stanju zabeleženom u ranijim saopštenjima. Kod bolesnika sa srčanom insuficijencijom uočena je značajna učestalost anemije kao nekardijalnog komorbiditeta. Prisustvo anemije ima važan uticaj na hospitalni i posthospitalni tok lečenja. Terapiju treba započeti čak i ako je posredi supklinička anemija, tj. ako su depoi gvožda sniženi iako je hemoglobin i dalje u okviru referentnih vrednosti, budući da to poboljšava prognozu bolesti kod osoba sa srčanom insuficijencijom.

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***Ključne reči**: srčana slabost, komorbiditeti, anemija, prognoza*

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CANNABINOIDS IN THE TREATMENT OF EPILEPSY: A REVIEW OF CURRENT EVIDENCE OF EFFICACY AND SAFETY

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Despite the use of appropriate pharmacotherapy, a significant proportion of epilepsy patients still struggle with inadequate seizure control. Consequently, there has been a surge in research exploring alternative therapeutic options. Over the past two decades, there has been a growing focus on investigating the potential of cannabinoids as a treatment for epilepsy. While various cannabis-based preparations are available, their compositions and quality vary widely, posing diverse risks.

Among these cannabinoids, cannabidiol (CBD) stands out as the only one with scientifically supported benefits, balancing both efficacy and safety after a comprehensive assessment of risks. Notably, CBD distinguishes itself by consistently demonstrating efficacy without inducing psychoactive effects. The highly purified form of CBD has obtained approval from both US and EU regulatory agencies for addressing pharmacoresistant seizures linked to rare and severe childhood-onset epileptic syndromes.

Short-term side effects associated with CBD are generally mild to moderate and tend to ameliorate with dose adjustments. However, to gain a deeper understanding of the therapeutic mechanisms, expand the assessment of CBD's effectiveness across various epilepsy types, compare its efficacy with other antiseizure medications, and ensure long-term safety, additional research studies are imperative.

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Key words: *cannabidiol, epilepsy, safety, efficacy*

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Introduction

Epilepsy is a neurological disorder marked by a lasting tendency to experience epileptic seizures, leading to various neurobiological, cognitive, psychological, and social repercussions (1). With a prevalence of approximately 1% across the general population, epilepsy stands as one of the most common chronic neurological conditions affecting individuals of all ages. Despite adequate pharmacotherapy, approximately 30% of epilepsy patients struggle to attain effective seizure control (2). Although newer-generation drugs exhibit improved tolerability and interaction profiles compared to their predecessors, the incidence of pharmacoresistant epilepsy has not shown significant changes over time (3, 4). Consequently, the exploration of novel therapeutic alternatives remains a significant challenge for both healthcare professionals and patients alike.

While cannabis products have been utilized as herbal remedies for managing epileptic seizures since ancient times (5), their potential therapeutic value in this context has garnered significant media attention over the past two decades. Specifically, following the discovery of the endogenous cannabinoid system—an intricate cellular signaling system influenced by cannabis (6)—renewed interest in the clinical application of cannabidiol (CBD)-rich cannabis preparations emerged, driven by the allure of a 'natural' alternative treatment (7). Media coverage highlighting notable cases of efficacy, particularly in children with severe pharmacoresistant epilepsy (8), along with changes in local regulations on cannabis use, such as in Colorado, USA (9), further fueled this interest. Consequently, numerous clinical studies were initiated in the USA to assess the efficacy and safety of a purified form of CBD (Epidiolex; > 99% CBD) in treating pharmacoresistant epilepsy. The outcomes of

these studies prompted approval from the US Food and Drug Administration (FDA) for this purified CBD formulation in the treatment of pharmacoresistant seizures associated with Dravet syndrome (DS), Lennox–Gastaut syndrome (LGS), and tuberous sclerosis complex (TSC) in patients aged two or older. Subsequent approvals were granted by the European Medicines Agency (EMA). Thus, CBD achieved the distinction of being the first non-synthetic preparation derived from the cannabis plant to receive official regulatory approval.

The objective of this paper is to comprehensively review the current scientific evidence regarding the efficacy and safety of CBD in the treatment of epilepsy.

Efficacy

When assessing the efficacy of certain preparations, various types of evidence can be employed. The most robust scientific evidence is derived from well-conducted randomized controlled studies of high quality, involving a thorough comparison between the test substance and an appropriate comparator. Regulatory agencies and professional organizations demand evidence from such research (10, 11), as it alone facilitates a comprehensive evaluation of the balance between benefits and risks, thereby safeguarding the public interest. On the opposite end of the spectrum, we find case reports or anecdotal evidence, which may capture media attention but do not constitute a suitable mechanism for safeguarding public welfare (12).

Diverse forms of cannabinoids are available in the market, exhibiting significant differences in the evidence supporting their usage. Figure 1 presents a general categorization of cannabis and cannabinoid preparations utilized for medicinal purposes. These preparations can be classified into four primary categories based on the available evidence concerning their efficacy and safety, as detailed in Table 1:

1. Regulatory approved cannabis-based medications
2. Non-regulatory approved cannabis-based medications
3. CBD contenting consumer or food products
4. Recreational cannabis

For preparations lacking registration by drug regulatory agencies, there exists considerable variability in quality and safety. Moreover, precise labeling of content is often non-binding, and substance concentrations may not align with stated claims. The commercial oil preparations display vast differences, underscoring the necessity for stringent regulatory oversight and development.

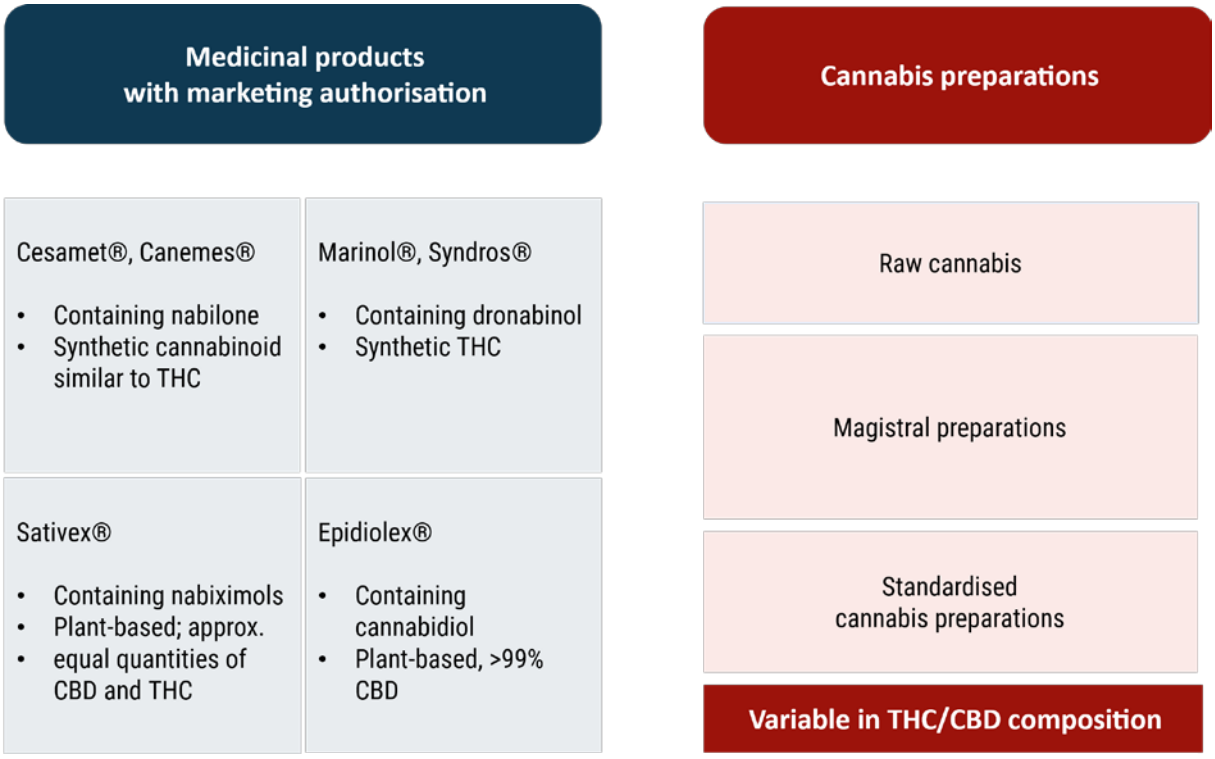

Figure 1. General topology of cannabis preparations and cannabinoids used for medicinal purposes

THC Tetrahydrocannabinol; CBD cannabidiol
(modified and adapted from reference 13)

Table 1. Classification of cannabis-based products based on available scientific evidence of efficacy

	Product class			
	Regulatory approved cannabis-based medications	Non-regulatory approved cannabis-based medications	Consumer or food products containing CBD	Recreational cannabis
Description	Medicines approved by regulatory agencies (e.g. EMA) after assessment of evidence	Medical cannabis available via prescription without the approval of regulatory agencies	Commercially available products containing CBD	Cannabis obtained from non-medical sources, usually by those seeking to achieve feelings of euphoria and relaxation
Evidence to support use	Evidence from RKS proves that the benefits outweigh the potential harms of side effects (13)	Evidence supporting safety and efficacy is lacking. Medication monitoring varies from country to country (13, 14, 15)	Evidence supporting their safety, efficacy, and quality is lacking (16)	Evidence supporting its medicinal use is lacking, but evidence of side effects is increasing (17)

In the 1980s and 1990s, several case reports and studies involving small patient cohorts explored the use of cannabis extracts for epilepsy treatment, yielding conflicting results (18, 19). Subsequent reports remained inconclusive (20), marked by notable methodological flaws or insufficient statistical power. Consequently, systematic reviews conducted by the Cochrane Group and the American Academy of Neurology in 2014 concluded that there was insufficient scientific evidence supporting the use of cannabis for epilepsy treatment (21, 22).

However, even during that period, cannabidiol (CBD) emerged as a potential candidate for epilepsy treatment, driven by its observed effects in experimental seizure and epilepsy models, as well as early pilot trials in epilepsy patients (18, 23). In comparison to delta-9-THC, CBD exhibited a more consistent anticonvulsant profile in animal models (24) and did not induce the adverse psychoactive effects associated with delta-9-THC (25, 26).

The anticonvulsant properties of CBD have been extensively documented across various experimental models (27, 28). However, the specific molecular mechanisms driving these effects remain unclear. This ambiguity partly stems from the intricate interactions of cannabinoids with numerous receptors and biological systems, many of which influence neuronal excitability (29). Unlike delta-9 THC, CBD exhibits minimal affinity for CB1 and CB2 receptors (30). Recent studies propose the involvement of three key mechanisms: 1) antagonism of G protein-coupled receptor 55 (GPR55), 2) desensitization of transient receptor potential vanilloid type 1 (TRPV1) channels, and 3) enhancement of adenosine-mediated signaling by inhibiting equilibrate nucleoside transporter 1 (ENT-1) (31). These mechanisms are believed to align plausibly with the concentrations of CBD at which the anticonvulsant effects have been demonstrated.

The effectiveness of adjunctive CBD treatment has been demonstrated in five placebo-controlled clinical studies. Two trials focused on patients with DS (32, 33), two on those with LGS (34, 35), and one on epilepsy linked to TSC (36). Table 2 showcases the primary methodological features of these studies.

Across these trials, CBD treatment led to a notable decrease in the frequency of convulsive seizures associated with DS, atonic seizures linked to LGS, and focal or generalized seizures related to TSC (Figure 2). These effects were consistent across all tested daily doses: 10 and 20 mg/kg (in DS and LGS), as well as 25 and 50 mg/kg in TSC. However, the benefit-risk assessment did not favor the utilization of the 50 mg/kg dose. An emerging concern following the publication of these findings was that most CBD-treated patients were also taking clobazam. This has led to investigations to determine whether the observed enhancement in seizure control is directly attributed to CBD or if it results from a

pharmacological interaction with clobazam, potentially resulting in elevated plasma levels of N-desmethylclobazam (37−39).

The impact of an interaction effect with clobazam was explored in three recent studies assessing CBD efficacy in patients with and without clobazam co-medication (28, 40, 41). These studies presented evidence supporting the independent anticonvulsant effects of CBD, albeit with more pronounced effects observed in patients using clobazam. However, methodological limitations such as the absence of randomization for clobazam co-medication, small sample sizes, and the inclusion of patients with different epilepsy syndromes (40, 42) pose challenges in interpreting the data on CBD efficacy, potentially influencing study outcomes. Nevertheless, despite these challenges, the US Food and Drug Administration (FDA) has granted approval for CBD use irrespective of the presence or absence of co-medication (43). In contrast, the European Medicines Agency (EMA) has restricted approval only to the use of CBD in patients concurrently using clobazam (44).

It is crucial to highlight that existing evidence pertains solely to the effectiveness of CBD as an adjunctive therapy in these syndromes when compared to a placebo. To date, there have been no studies directly comparing the efficacy of CBD with other antiseizure medications (ASMs). While preclinical models indicate a potentially broad spectrum of CBD effects, it is imperative to assess its effectiveness under appropriate clinical conditions. For instance, recent pilot studies suggest that CBD may not exhibit efficacy in typical absence seizures (45). Therefore, further research is essential to evaluate the effectiveness of CBD in various types of epilepsy and to directly compare its efficacy with other ASMs.

Table 2. Key methodological characteristics of studies that supported the efficacy of cannabidiol as adjunctive therapy in rare epileptic syndromes

Syndrome	**Dravet syndrome**		**Lenox−Gastaut syndrome**		**Tuberous−sclerotic complex**
Age (years)	2−18		2−55		1−65
Seizure type and number of attacks during the last 4 weeks	Convulsive ≥ 4/week		Drop attacks ≥ 2/week		Focal and generalized ≥ 8
Number of ASM	≥ 1		≥ 1		≥ 1
Name of the study	GWPCARE1 (DS 1332b)	GWPCARE2 (DS 1424)	GWPCARE3 (LGS 1414)	GWPCARE4 (LGS 1423)	GWPCARE6
Sample size	n = 120	n = 199	n = 225	n = 171	n = 225
CBD dose (mg/kg/d)	20	10 & 20	10 & 20	20	25/50
Reference	(32)	(33)	(34)	(35)	(36)

Safety

Cannabidiol

The short-term adverse effects of cannabidiol have been thoroughly identified and documented in the clinical studies that facilitated its registration. Generally, CBD is well tolerated, manifesting as transient, dose-dependent, mild to moderate effects like drowsiness, decreased appetite, or diarrhea. Nevertheless, it is important to note that severe, life-threatening side effects can occur, particularly in association with toxic combinations involving other drugs commonly used in this patient group, such as valproate or clobazam. Comprehensive studies are imperative to evaluate long-term outcomes and ensure the continued assessment of safety.

In randomized controlled trials conducted in patients with DS and LGS, adverse events were more frequently reported in CBD-treated patients, with an absolute difference in incidence of more than 5% compared to placebo-treated patients. These adverse events included somnolence, decreased appetite, increased transaminases, infections, rash, diarrhea, fatigue, sleep disturbances, irritability/agitation, and lethargy (23). Notably, in these trials, 8.9% of patients receiving CBD discontinued treatment due to side effects, in contrast to 1.8% of those receiving placebo (28).

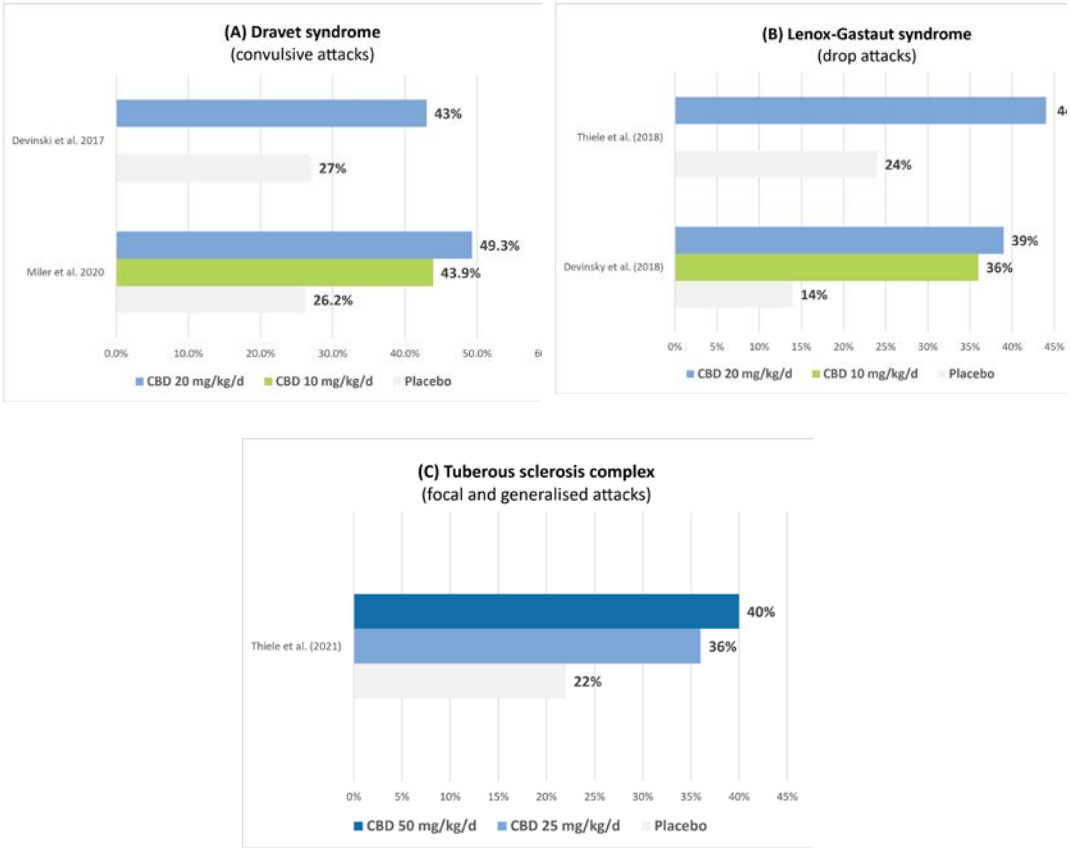

Figure 2. Proportions of patients with a ≥ 50% reduction in seizure frequency compared to baseline in 5 randomized, placebo-controlled trials of cannabidiol (CBD) as adjunctive therapy in (A) convulsive seizures associated with DS (32, 33), (B) drop attacks associated with LGS (34, 35) and (C) generalized and focal attacks associated with TSC (36)

Similarly, in the group receiving 25 mg/kg CBD in patients with TSC, the most reported adverse events were increased transaminases, pyrexia, vomiting, decreased appetite, weight loss, nausea, diarrhea, and anemia. Significantly, 13% of patients receiving CBD discontinued the study due to adverse events, compared to 3% in the placebo group (36).

Serious side effects were observed in approximately 15% of patients undergoing CBD treatment, with the most significant being a clinically substantial elevation (three times the upper limits of reference values) in alanine transaminase (ALT) and aspartate transaminase (AST) levels. This elevation in enzyme levels was more frequent with higher CBD doses and concurrent valproate therapy (46). Rash incidents were infrequent but were typically associated with pyrexia and often led to the discontinuation of treatment (34).

Instances of increased liver enzymes were more prevalent in patients also using valproate, while somnolence, heightened secretion, and pneumonia occurred more frequently in the group concurrently using clobazam (23, 47). Generally, these side effects can be managed by reducing the CBD or clobazam dose.

In a recent study, the side effects of CBD were assessed based on the findings from double-blind randomized placebo-controlled studies across various health conditions (48). A meta-analysis was conducted on data from 12 trials involving 803 participants. The results indicated that compared to the placebo, CBD was more likely to be discontinued due to side effects. This trend was observed for both serious adverse events (such as abnormal liver function tests and pneumonia) and milder adverse events (including reduced appetite, diarrhea, drowsiness, and sedation).

Associations with abnormal liver function tests, somnolence, sedation, and pneumonia were specifically identified in studies involving pediatric patients. However, upon excluding these studies, the only adverse event consistently associated with CBD was diarrhea. The authors suggested that interactions with other drugs, particularly valproate and clobazam, contribute to apparent differences in treatment outcomes between patients with epilepsy and those with other conditions.

Non-regulatory approved cannabis-based medications

In certain countries, these preparations are accessible with a doctor's prescription. The primary challenge lies in the absence of evidence substantiating their safety and efficacy. Medication monitoring practices differ from one country to another (14–16).

Consumer or food products containing CBD

Commercially available products such as CBD oils are commonly accessible, and there exists a misconception that their use is risk-free. However, several crucial considerations should be noted:

a) They are marketed without substantiated proof of efficacy and safety.

b) Quality is not consistently assured, potentially impacting their safety (16, 49–52).

c) The actual CBD content may differ from what is stated on the packaging (16, 49–51).

d) There is a risk of contamination with pesticides, heavy metals, or other phytocannabinoids, including THC (48, 51, 53–55).

Recreational cannabis

There are notable risks linked to the usage of this kind of preparation. In Europe, there has been an observed increase in THC levels in recreational cannabis preparations in recent years (56). The utilization of such products is associated with significant acute and long-term side effects. Acute effects encompass anxiety and memory impairment (17, 57), while long-term side effects may involve mental disorders, cardiovascular issues, and respiratory diseases (17,58). Additionally, recreational cannabis carries the risk of addiction (59). Despite the growing understanding of the side effects associated with this type of preparation, routine monitoring of these effects, like other drugs, is not commonly implemented.

Conclusion

Various cannabis-based preparations exhibit considerable differences in their composition and quality, leading to varying associated risks.

Among these preparations, the sole one currently supported by scientific evidence, following a comprehensive assessment of its benefits and risks, is the purified form of cannabidiol (CBD). This form has shown benefits as an adjunct therapy for patients with pharmacoresistant seizures associated with Dravet syndrome (DS), Lennox–Gastaut syndrome (LGS), and tuberous sclerosis (TS).

CBD is generally well tolerated, with most short-term side effects being mild to moderate and often improving with dosage adjustments.

However, further studies are imperative to assess CBD's efficacy through direct comparisons with other antiseizure medications (ASMs) and to ensure a thorough evaluation of its long-term safety.

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PRIMENA KANABINOIDA U LEČENJU EPILEPSIJE: AKTUELNI DOKAZI O EFIKASNOSTI I BEZBEDNOSTI

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Činjenica da kod jedne trećine osoba sa epilepsijom ne postoji adekvatna kontrola napada uprkos primeni odgovarajuće farmakoterapije motivisala je istraživanja novih terapijskih opcija. U poslednje dve decenije zabeleženo je povećanje interesovanja za proučavanje terapijskih potencijala kanabinoida za lečenje epilepsije. Na tržištu su dostupni razni preparati bazirani na kanabisu; oni znatno variraju po sastavu i kvalitetu, a njihova primena udružena je sa različitim rizicima. Jedini kanabinoid o čijim prednostima upotrebe trenutno postoje naučni dokazi, dobijeni nakon procene benefita i rizika, jeste kanabidiol (engl. *Cannabidiol* – CBD). Kanabidiol se razlikuje od drugih kanabinoida po tome što postoje dokazi o njegovoj konstantnoj efikasnosti i o odsustvu psihoaktivnih efekata. Visoko prečišćeni oblik CBD-a prva je supstanca dobijena od biljke kanabisa koja je dobila odobrenje regulatornih agencija Sjedinjenih Američkih Država i Evropske unije za lečenje napada rezistentnih na farmakoterapiju koji su udruženi sa retkim i teškim epileptičkim sindromima koji su se pojavili u detinjstvu. Kratkoročni neželjeni efekti blagog su i umerenog stepena i popravljaju se nakon prilagođavanja doze. Da bi se otkrili precizni mehanizmi terapijskih efekata, procenila efikasnost CBD-a i u drugim vrstama epilepsije, izvršilo direktno poređenje sa drugim antiepileptičkim lekovima i procenila dugotrajna bezbednost njegove upotrebe, neophodno je sprovesti dalja istraživanja.

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***Ključne reči**: kanabidiol, epilepsija, efikasnost, bezbednost*

Review article

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THE ROLE OF NEUROPSYCHOLOGY IN DIAGNOSTICS AND TREATMENT OF PATIENTS WITH EPILEPSY

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Epilepsy represents a neurological disease with a prevalence of approximately 1% of the world population. Besides unprovoked seizures which are the main characteristic of this disease, there can be a decline in cognitive functioning, including memory and concentration dysfunctions, executive dysfunction, and visuoconstructional and visuospatial dysfunctions. However, behavioral changes can also be seen throughout the disease duration and/or during postoperative treatment of drug-resistant epilepsy. This article aims to emphasize that neuropsychological diagnostics and neuropsychology as a science are making a valuable contribution to the diagnostic process and can be used as a tool for examining the localization and/or lateralization of brain damage, determining the severity of cognitive deficits, monitoring disease and treatment, which improves quality and safety of treatment, as well as further detection of neuropsychological comorbidities and their rehabilitation.

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Key words: *neuropsychological evaluation, epilepsy surgery, cognitive dysfunction*

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Epilepsy and cognitive functioning

Epilepsy is a common disease characterized by two types of conditions: 1. At least two unprovoked (or reflex) seizures occurring greater than 24 hours apart; 2. One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; 3. Diagnosis of epilepsy syndrome (1) occurs with neuropsychological and psychiatric comorbidity (2).

Cognitive functions represent the brain's ability to analyze and use information from the environment (3) through functional systems (combination of mental abilities) in the brain, specifically attention, memory, language, praxis, and executive functions (4).

The issue of mental impairment and cognitive decline in epilepsy dates back to the late 19th century (5–7) when Gowers named it "epileptic dementia" and described it as a slow cognitive decline resulting from chronicity and the accumulation of negative effects of seizures, treatment and other factors associated with epilepsy. Cognitive impairment in epilepsy can have different etiologies (8): 1. It may be the repercussion of epilepsy (9, 10), 2. Impaired cognitive domains can be comorbidity resulting from the same underlying pathology (11), 3. Seizures themselves can lead to cognitive impairment (12, 13), 4. Antiepileptic drug therapy may cause cognitive decline (14).

Over time, neuropsychology has demonstrated its great clinical role in characterizing the impact of epilepsy on the degree of cognitive impairment. The number of factors is reduced to the following ones: age of onset (15), as children with the early-onset disease are at higher risk of losing scores on intelligence tests; disease duration, a factor often identified with the age of onset (16–18); seizure type and frequency (19) (status epilepticus on already vulnerable brain changes cognitive status by reducing cognitive reserve capacity); epileptic encephalopathy, several studies in adult patients with encephalopathy have shown an important role of inflammation related to cognitive impairment as an essential feature (20, 21); comorbidities—patients with seizures after stroke or traumatic head injury show a more pronounced cognitive decline compared to the patients without seizures, hence seizures can be understood as exacerbating factor on an already vulnerable brain (22); and antiepileptic medications which can induce cognitive decline as a reaction to treatment (20, 23).

Causes of epilepsy can be congenital or acquired. Young people are more often affected by genetic form, congenital or developmental factors, while acquired factors, such as brain tumors or stroke, are more common among older people (24). With a prevalence from 0.8% to 1.2%, epilepsy is one of the most common chronic neurological disorders (25, 26); 30% to 40% of patients in this group suffer from a drug-resistant form of epilepsy (epilepsy that cannot be adequately controlled by antiepileptic drugs) (27, 28).

The place where Neuropsychological assessment meets epilepsy

One of the first cognition studies in epilepsy was conducted in England when a "healthy group" was compared with two groups of patients with epilepsy. The results showed that there were differences between groups in recognition, registration, and reactions. However, in areas such as sensory discrimination, voluntary movement rapidity, rhythmic movement, and maximal voluntary contractions—relatively simple processes—the differences between groups were insignificant (29).

In the US in 1912, Wallin (30) used the first Binet–Simon IQ scale in two groups: "brightest epileptic school children" and "bright, average and backward pupils in public school" where he found that there was less overall intellectual damage in patients with epilepsy compared to “feeble-minded” and at the same time he pointed out the importance of a clinical psychologist in research and practical work. As early as the 1980s, tests used to examine the cognitive decline in epilepsy were mainly WAIS and Halstead neuro-psychological test battery (31–33).

In the 20th century, psychological and neuropsychological tests were primarily used as diagnostic and prognostic measures. Modern practices in the field of epilepsy evaluation and management include neuropsychology as an important component of research and patient treatment, hence epilepsy and neuropsychology enjoy a special and synergistic relationship (34, 35).

Neuropsychological assessment of epilepsy can provide information on the prediction of cognitive and psychiatric outcomes, the degree of cognitive and behavioral functioning after surgery, lateralization and/or localization of present brain damage (36), it can provide a baseline evaluation of cognitive functions, antiepileptic effects and identification and formulation of a treatment plan for patients with psychogenic non-epileptic seizures as well (2), which is why certain patterns can be identified in certain epileptic syndromes (4). Additionally, neuropsychological assessment aims to define the impact of epileptogenic foci in the interictal period (3), the relationship between localization and lateralization of epileptogenic foci, and the dysfunction of certain cortical and subcortical regions in the interictal period.

Partial or focal seizures may be the product of focal temporal, frontal, or occipital lobes, with or without secondary generalization (37, 38). Focal epilepsy or localization-related epilepsy is usually characterized in terms of their lobar site of origin, and neuropsychology has historically focused on the relationship between structure and function such as executive function in frontal lobe epilepsy (FLE) and memory in temporal lobe epilepsy (TLE) (34).

Cognitive characteristics have been mostly studied in temporal lobe epilepsy, as it is the largest group of epilepsies eligible for surgical treatment and has the most favorable surgical outcomes (39), especially in patients with hippocampal sclerosis or those undergoing anterior temporal lobe resection (40). Studies have shown an association between right temporal lobe epilepsy and visual learning (41, 42) or decreased visual memory (43), although recent neuropsychological and neuroimaging studies have progressively challenged this model (44), while left temporal lobe epilepsy has been associated with language impairment (45) and verbal memory (46). One of the goals of a neuropsychological evaluation is to determine the risk of memory and speech impairment postoperatively, after temporal lobe resection. The first step in this direction is to determine whether the epileptogenic zone is in the dominant or non-dominant hemisphere with several methods and techniques: Edinburgh handedness inventory (47, 48) seizure semiology analysis (49), postictal neuropsychological test (50), neuropsychological assessment (51), functional magnetic resonance imaging (52), Wada test (53, 54).

Other cognitive functions are expected to remain relatively intact because both seizure onset and focal epileptiform abnormalities are limited to temporal lobe structures encoding new contents (34). However, it is concluded that greater attention needs to be directed to the domains of executive functions and speed (55). Neuropsychological impairments, for this reason, are more prevalent than expected in temporal lobe epilepsy and more associated with distributed brain anomalies (56). It can be seen that the difference between focal epileptic syndromes (e.g. temporal versus frontal) is less pronounced in children with epilepsy due to the impact of epilepsy on the neurodevelopmental process (57).

It has been anticipated that epilepsy will continue to be a major clinical area that reflects the importance and benefits of neuropsychological testing which will continue to play an important role in establishing phenotypes (and perhaps endophenotypes) of epilepsy (35). Neuro-psychological evaluation remains, in addition to MRI and EEG, the most important method for indicating cognitive deficits in epilepsy and determining the epileptic focus (41).

We cannot continue without mentioning the impact of the psychological share in the neuropsychological testing, important for detecting the psychological comorbidities of epilepsy (most often anxiety and depression), which should certainly be included in the comprehensive treatment of patients with epilepsy (58). Neuropsychologists in epilepsy centers are generally preoccupied with conducting a comprehensive evaluation of patients with epilepsy (59) and for this reason probably do not treat depression or anxiety more often, despite the ideal position for that kind of treatment (34). While the importance of mental health work is recognized by the epileptologist community, more work should be invested and mental health care should be implemented to provide adequate care to patients with epilepsy (60).

The role of neuropsychology in epilepsy surgery

Historically, epilepsy surgery provided one of the most important pieces of evidence for the relevance of certain brain structures in cognitive functions through one of the most significant cases in the history of neuropsychology, the surgery of Henry Gustav Molaison, better known as HM, in 1953, who developed profound anterograde amnesia after surgical removal of mesial structures of both temporal lobes 8 cm long from the temporal pole (61) including hippocampus bilaterally (4, 62, 63). More studies are analyzing the long-term outcomes of epilepsy surgery and have found stable or even improved cognitive status (28, 64), which is why epilepsy surgery has become an evidence-based treatment for patients with drug-resistant epilepsy (65). Successful epilepsy surgery leads to seizure freedom and stabilization of cognitive functioning in two-thirds of patients (66).

It should be noted that there are always risks for the bad outcome of postoperative recovery of cognitive functions. Risk factors for postoperative verbal memory deficits may be the following (67): 1. Left temporal resection (dominant hemisphere surgery) (64); 2. Adequate function of the ipsilateral hippocampus (68); 3. Insufficient functional capacity of the contralateral hippocampus (69); 4. Size of the resection (70); 5. Surgery in older age (71); 6. Later epilepsy onset (72) and 7. Absence of postoperative remission (73).

Surgery also has potential neuropsychological risks within visual memory after right temporal lobe surgery (74). Spatial memory impairment is 6 times more pronounced in patients who underwent right anterior temporal lobe resection than left anterior temporal lobe resection (75). Some studies have shown improvement in executive functions after surgery (64, 76).

Another progress in this domain is the evaluation of the super-selective surgical procedure for mesial temporal lobe epilepsy (12). With improvements in neuroimaging, neuropathology, and genetics, the number of epilepsies with unknown etiology has significantly decreased, encouraging changes in the classification of seizures and epilepsies, and also putting cognitive dysfunction in better perspective (77). The neuropsychological literature in this field has accumulated a lot of knowledge, which raises the question of why neuropsychology is still not fully integrated into the routine treatment of patients with epilepsy (12). A little over 30 years after Michael Trimble's work (78) and more than 80 years after the beginning of epilepsy surgery, neuropsychological knowledge is still not included in the required treatments of patients. Although early neuropsychological evaluation is often implied in children with early-onset epilepsy, it has not yet, although it should become a routine in the treatment of adult patients with epilepsy (12).

Cognitive rehabilitation among epilepsy patients

Cognitive rehabilitation has a positive effect on the outcomes of memory therapy before and after surgery, but its use is still being considered among groups of patients at risk (79). Modalities and outcomes of rehabilitation are important issues for clinical treatment and research. A holistic approach to rehabilitation seems to be more useful than selective interventions (80). Predicting postoperative cognitive changes enables the design and implementation of pre-rehabilitation programs as a part of preoperative treatment, to prepare the patient for postoperative changes (81).

A study conducted by Geraldi et al. (82) supports the role of memory rehabilitation after left temporal lobe surgery, and fMRI results suggest concomitant changes in the brain networks that underlie the results achieved after rehabilitation. Such findings are encouraging and suggest that rehabilitation should be considered as part of mandatory surgical evaluation and preparation. Also, research shows the importance of the application of cognitive rehabilitation in attention-deficit (80).

A period of intensive pre-surgical training can be useful for cognitive functions that have previously been shown to be impaired after surgery, especially for high-risk patients (e.g. verbal memory in left temporal lobe resections) by giving them some "reserve skills" to use after surgery. The post-surgical rehabilitation period (after a 6-month recovery period) should be adjusted individually to target the specifically identified weaknesses in each patient (83).

Conclusion

Epilepsy is a common neurological disease that can lead to cognitive impairment worsened by antiepileptic therapy. Successful epilepsy surgery

can lead to seizure freedom and stabilization or even improvement of cognitive status. Neuropsychological diagnostics play an important role in predicting postoperative cognitive and behavioral functioning, formulating treatment strategies, and examining the effect of antiepileptic drugs and the impact of the epileptogenic focus. Neuropsychological deficits are a leading comorbidity of epilepsy and can be exacerbated by treatment, making regular neuropsychological evaluation crucial. Cognitive rehabilitation tailored to individual patient needs is proposed as part of neuropsychological treatment and has shown significant results, with studies encouraging its use before surgical treatment in certain patient groups. Overall, neuropsychology in epilepsy has been trusted for decades and has contributed positively to the diagnosis and treatment of epilepsy.

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ULOGA NEUROPSIHOLOGIJE U DIJAGNOSTICI I LEČENJU OSOBA SA EPILEPSIJOM

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Epilepsija predstavlja neurološko oboljenje koje se javlja kod otprilike 1% svetske populacije. Osim neprovociranih epileptičnih napada, koji su glavna odlika, kod obolelih se javljaju smetnje u kognitivnom funkcionisanju; one koje se tiču pamćenja, koncentracije, egzekutivnih funkcija, vizuokonstruktivne i vizuospacijalne smetnje. Takođe, mogu se pojaviti bihevioralne promene u toku trajanja bolesti i/ili nakon operativnog tretmana tipova epilepsije rezistentnih na farmakoterapiju. Cilj ovog rada bio je da ukaže na to da neuropsihološka dijagnostika i neuropsihologija kao nauka daju veoma vredan doprinos dijagnostičkom procesu i da mogu poslužiti kao alat za ispitivanje lokalizacije i/ili lateralizacije oštećenja mozga, za određivanje težine kognitivnog deficita, kao i za praćenje bolesti i lečenja. Na taj način poboljšava se kvalitet i bezbednost tretmana obolelih od epilepsije, a doprinosi se i daljem otkrivanju neuropsiholoških komorbiditeta i njihovoj rehabilitaciji.

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***Ključne reči**: neuropsihološka procena, hirurgija epilepsije, kognitivna disfunkcija*

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POSTOPERATIVE THREE-DIMENSIONAL CONFORMAL RADIOTHERAPY IN THE PATIENT WITH A SIGNIFICANT CARDIOVASCULAR RISK: A CASE REPORT

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Radiotherapy of breast cancer represents an essential component in the overall multidisciplinary breast cancer treatment. Considering the satisfactory results of the application of the multimodal treatment as well as its role in the decrease in the mortality rate of breast cancer patients, the focus has shifted towards monitoring acute as well as chronic complications occurring as a consequence of oncological treatment, intending to preserve the patient's quality of life. Complications are numerous and vary from the local ones (dermatitis) to more serious forms including dysfunctions of the cardiovascular system. The application of 3D radiotherapy on the patient in this case report as the most used method in our centre points to its low acute toxic effect, while the observed negative effects in the high-risk patient were removed, which resulted in satisfactory therapeutic effects despite the limited technical equipment of the centre.

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Key words: *breast cancer, cardiotoxicity, radiotherapy*

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Introduction

Breast cancer represents a huge global challenge for public health considering the fact it is one of the most common neoplasms in humans, accounting for one-quarter of all cancers in women all over the world and 27% of cancers in developed countries. Breast cancer can also appear in men, but it is 100 times more frequent in women (1, 2). Persons who are at a greater risk of becoming ill are those with genetic predisposition, primarily relatives, then those being on hormonal therapy for a number of years, obese people having unhealthy lifestyles. There are several types of cancer, and in clinical practice ductal is the most frequent one, then lobular, while inflammatory cancer is the rarest (3, 4).

Upon receiving pathohistological verification, complete biochemical and diagnostic processing of the patient, the stage of illness and the treatment plan are defined. The Medical Consilium brings the initial plan of treatment of these patients, basing its decision not only on scientifically proven facts but also taking care of maximal individualistic approach to each patient, taking into consideration his/her general health and existing comorbidities. Methods of breast cancer treatment include operative treatment, radiotherapy, chemotherapy, hormonal therapy, target therapy and immunotherapy. Depending on the type and stage of cancer, these methods of treatment can be combined, and surgical removal of cancer is most frequently followed by radiotherapy or system therapy.

Radiotherapy of breast cancer represents an essential component in the overall treatment of early-stage and locally advanced breast cancer. It is based on the application of local and locoregional treatment of ionising radiation where local refers to the radiation treatment of the rest of the breast or the scar region after radical surgery. Also, the corresponding lymphatic drainage regions can be treated by radiotherapy. The role of radiotherapy is reflected in "the sterilisation'' of the treated region to prevent ipsilateral recurrences, locoregional recurrences and potential dissemination of tumour cells (5).

Since the number of patients who survive breast cancer is on the increase, the focus has shifted towards monitoring and assessment of

toxicity, with the emphasis put not only on acute complications of usually reversible character but also on later consequences of radiotherapy. Complications are numerous and vary from the local ones (dermatitis) to more serious forms including dysfunctions of the cardiovascular system (6, 7).

Case report

At the beginning of 2022, patient D.Č. aged 54 discovered a lump the size of a walnut at the intersection of the lateral quadrants of the right breast. As part of diagnostic processing, mammography detected two stellate shadows, one next to the other, 15 mm and 14 mm in diameter, in the region of the right breast, at the intersection of the lateral quadrants, as well as one more stellate shadow 11 x 10 mm in the upper lateral quadrant, which retracted the surrounding parenchyma. No abnormality was detected in the axillae and contralateral left breast. The mammography result of the right breast was classified as Bi Rads 5, while the left breast was classified as Bi Rads 1. Taking into account Bi Rads classification, the Oncological Consilium suggested ultrasound "core" biopsy and it was pathohistologically determined that it was invasive breast cancer, with histological characteristics ER 8, PR 8, HER2 1+, Ki 67 20%.

By looking at the anamnesis and available medical documentation, it was observed that in 2017 the patient underwent surgical revascularisation of the myocard with one vein graft on RCA coronary artery, replacement of the mitral valve by the artificial mechanical valve and the plastic repair of the tricuspid valve with ring implantation. Since then she has been on the prescribed internistic therapy – application of oral anticoagulant therapy, regularly controlled hemodynamic status and regular echo sonography of the heart.

Considering a significant cardiovascular disease burden as well as the multicentricity of the malignant change verified by pathohistological biopsy, the Consilium decided that a specific oncological treatment should begin with surgery; therefore, the patient underwent surgery in May 2022 when radical mastectomy with the dissection of the axilla was performed.

Clinical, laboratory and diagnostic processing as well as heart and lungs X-ray, MSCT of the abdomen and the lesser pelvis were done prior to the surgery in order to exclude the dissemination of the malignant process. The patient was thoroughly examined by the cardiologist, when the echo sonography of the heart was done and anticoagulant therapy was prescribed during and after the surgery to keep the INR in the therapeutic range between 2.5 and 3.5.

A definite pathohistological result indicated that it was micropapillary grade 2 invasive breast cancer, dimensions were 15 mm and 9 mm, where in 6 out of 15 extirpated lymph nodes the presence of malignant cells was detected. The disease was determined to be at stage T1cN2Mx. Biological characteristics of the tumour indicated that it was a hormone-dependent breast tumour with highly positive estrogen and progesterone receptors ER 8, PR 7, Her 2 negative gene expression.

Taking this into account, in the continuation of treatment, the patient was prescribed hormonal therapy with aromatase inhibitors, 1 mg daily, with regular monitoring. The treatment was afterwards presented to the Consilium that indicated the continuation of the prescribed hormonal therapy with the application of locoregional radiotherapy.

During the first visit to the radiotherapy ambulance, the patient submitted control MSCT results of the thorax and abdomen, a cardiologist report with the consent to perform radiotherapy, after which she was clinically examined. The results corresponded to the performed procedures, no abnormality was detected regarding the scar tissue, and also no subjective symptoms were reported. She was processed on an MSCT simulator in order to perform postoperative radiotherapy of the right hemithorax and regional lymphatics. The recommendations of the national radiotherapeutic protocol for the treatment of malignant illnesses by the Ministry of the Republic of Serbia of 2022 were followed for the evaluation of the series of CT slices, which showed that a careful delineation of target volumes and organs from risk helped define the contours encompassing the scar region with the surrounding skin, where the upper and lower boundaries were at the level of tissue projection of the collateral breast. The contours of regional lymphatics included all three levels of the same side axilla and supra and infraclavicular regions (Figure 1).

Technical equipment of the radiotherapy centre in Niš referred to the application of 3D conformal radiotherapy, using the techniques of beforehand directed planning, when by combining two lateral fields with two added segment fields with a view to optimization, a satisfactory distribution of the dose was achieved. The patient was processed and was to receive TD 50 Gy in 25 fractions. By arranging the fields and using the experience of the medical physicist, it was attempted to maximally exclude the heart volume from the radiation volume, therefore, based on the Quantum constraints determined at V 10% < 10 Gy, the heart in our case received only 2.88 Gy by 10% of the volume.

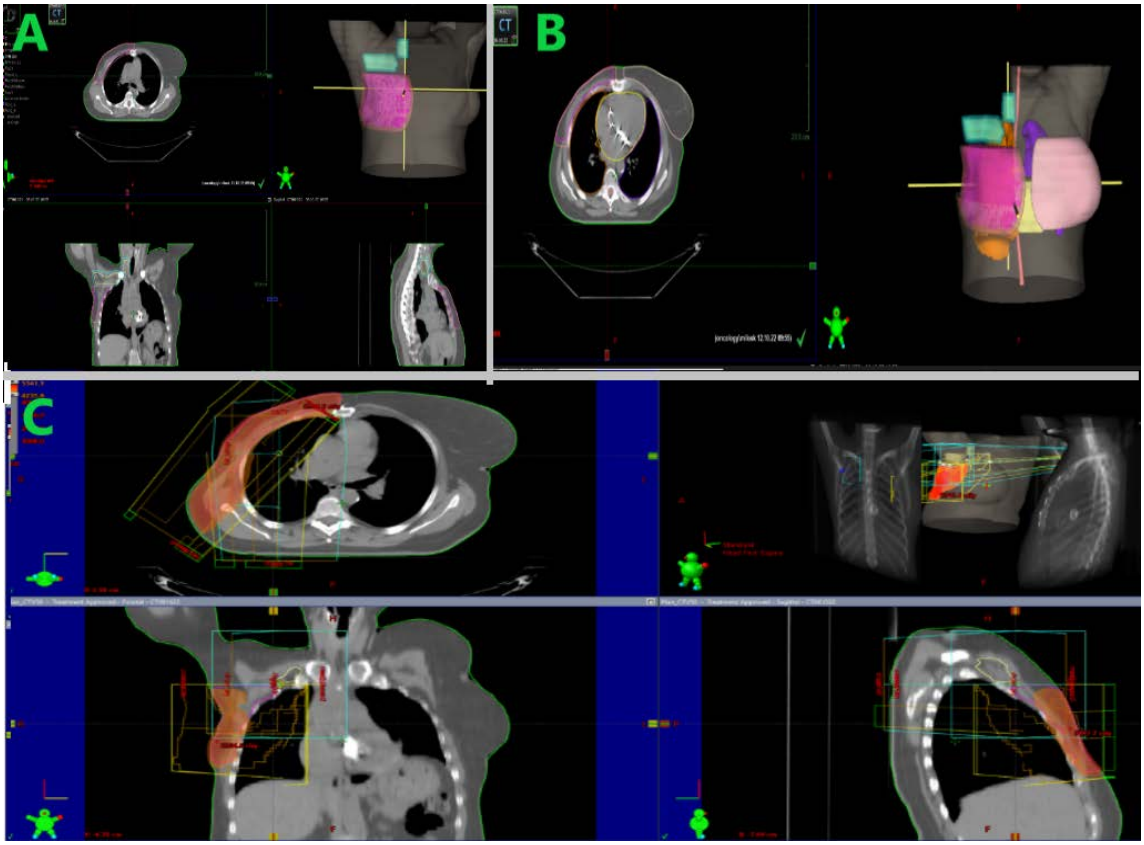

Figure 1. Delineation of the target volumes (A and B); Dose distribution to target volumes (C).

Discussion

Current options for the protection/avoidance of the heart to decrease cardiotoxicity of radiation include manoeuvers which transfer the heart from the field, such as coordination of the breathing cycles or positioning of the patient in the supine position. Technological advancement enabled therapies such as intensity modulated radiation therapy (IMRT) or proton beam therapy (PBT) and techniques treating lesser volume around the lumpectomy cavity, such as accelerated partial breast irradiation (APBI) or intraoperative radiation therapy (IORT) (7). Although these techniques have dosimetrically proven to be promising, there are limited possibilities of their application (8, 9). They are primarily related to the limited technical equipment of the centres, and on the other hand, there are insufficient data regarding later heart attacks due to difficulties related to long-term monitoring. The application of 3D conformal radiotherapy represents a standard in the radiotherapeutic treatment of patients, within which a precise delineation of target volumes is done on CT slices, not only because it is based on the recommendations but also because it is the only available treatment in our centre. Therefore, the purpose of this review was to assess how the applied technique could lead to the decrease of heart dose in the patient with a significant cardiological burden. Future studies are necessary in order to confirm the efficiency of the advanced techniques which spare the heart dose and can use surrogates for heart attacks such as biomarkers or perfusion scanning.

The most common heart problems during radiotherapy are acute pericarditis, pericardial haemorrhage and arrhythmia. Acute damage of pericardial and intimal coronary endocytosis caused by radiation results in ischemia of myocytes and fibrosis. It seems that the risk of coronary diseases increases decades after radiation therapy (10). The majority of heart diseases was noticed in patients who underwent the radiation of the left thoracic wall after the left-side mastectomy, but present-day radiotherapy techniques expose the heart to less radiation than those 30 years ago, even in those patients with tumors on the left side (11). Earlier epidemiological cohort studies noticed a greater risk of death due to heart failure among the patients having cancer on the left breast as compared to those having cancer on the right breast, with the risk increasing as the time after treatment progresses (10, 11). However, the analysis of results of epidemiological surveillance

of the patients who had radiation of the left breast as compared to those who had radiation of the right breast did not show that the passage of time made a significant difference regarding the hospitalisation due to heart diseases or heart insufficiency (12), suggesting that the occurrence of complications is independent of the side on which the tumour change is present.

In our case, the patient with a high cardiovascular risk (burden) did not develop acute serious complications related to the cardiovascular or respiratory systems after the application of 3D conformal radiotherapy. The complications that developed in the patient included skin changes such as erythema (grade I) which was locally treated with corticosteroids. The incidence of skin toxicity caused by radiation is an important clinical problem which affects a majority of patients with breast cancer who were subjected to adjuvant therapy. This problem is related to the radiation technique, dose homogeneity, PTV receiving a dose bigger than 100% of the prescribed dose and prophylactic use of the local therapy (13) and is present in almost all patients exposed to radiotherapy, going up to 70–100% (14). The fluctuation of values of INR is common among oncological patients, and it was also noticed during the radiotherapeutic treatment of the patient, thus it can be viewed as one more complication of this therapeutic protocol (15). On the other hand, this can be regarded as deterioration, i.e., change of the basic illness in the patient.

Conclusion

This case report indicates the applicability of 3D conformal radiotherapy in patients after breast cancer surgery with a high cardiovascular risk. Since the application of this technique is the most frequent at our centre, despite the implementation of new techniques, this case report confirms its safety and applicability in high-risk patients despite the implementation of new techniques. The applied radiation therapy in the patient in this case report points to its low toxic effect, while the noticed negative effects in high-risk patients were removed. The treatment was finished a few months ago and since then there have not been delayed complications, and in the future monitoring, they will be noted.

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POSTOPERATIVNA TRODIMENZIONALNA KONFORMALNA RADIOTERAPIJA KOD BOLESNICE SA ZNAČAJNIM RIZIKOM OD NASTANKA KARDIOVASKULARIH KOMPLIKACIJA: PRIKAZ SLUČAJA

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Radioterapija raka dojke predstavlja esencijalnu komponentu u ukupnom lečenju i ranog stadijuma raka dojke i lokalno uznapredovalog raka dojke. S obzirom na zadovoljavajuće rezultate i udeo u smanjenju stope mortaliteta obolelih od raka dojke, primenom multimodalnog lečenja fokus je pomeren na praćenje akutnih, ali i hroničnih komplikacija koje nastaju kao posledica onkološkog lečenja. Cilj multimodalnog lečenja jeste da očuva kvalitet života bolesnika. Komplikacije su mnogobrojne i variraju od lokalnih (dermatitis) do ozbiljnijih, koje obuhvataju i disfunkcije kardiovaskularnog sistema. Primena trodimenzionalne (3D) zračne terapije kao najzastupljenije metode u našem centru kod bolesnice prikazane u ovom radu ukazala je na njen nizak akutni toksični efekat. Budući da su primećeni neželjeni efekti kod bolesnice kod koje postoji visok rizik od pojave kardiovaskularnih komplikacija bili otklonjeni, postignut je zadovoljavajući terapijski efekat uprkos limitiranoj tehničkoj opremljenosti centra.

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***Ključne reči**: karcinom dojke, kardiotoksičnost, radioterapija*

Review Article **UDC: 612.753:547.973**
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BERRIES AS A NATURAL SOURCE OF BONE SUPPORT: THE INVOLVEMENT OF ANTHOCYANINS IN THE MOLECULAR MECHANISMS OF THE HEALING AND REGENERATION PROCESSES

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Bone tissue has a remarkable self-regenerating ability which, in case of injury, enables return to a completely functional, pre-injury state. However, ageing, disease, obesity, traumas, multiple fractures, infections and tumor removal cause large bone defects that cannot be healed spontaneously. To achieve successful bone healing and regeneration, plenty of approaches, including the application of autografts, allografts and bone tissue engineering (BTE), have been developed. One of the approaches is based on the findings that bone loss in humans and many animals during aging is partially caused by accumulation of reactive oxygen species (ROS). Due to the spectrum of biological activities, including antioxidative, essential polyphenolic components–anthocyanins (ACNs), are a part of a significant research area regarding means and methods for bone healing and regeneration. Berries are especially rich in ACNs. Based on *in vitro* and *in vivo* studies regarding molecular mechanisms involved in bone healing and regeneration supported with berries' ACNs and on observational research in human populations, it has been found that berries' ACNs enhance osteoblastogenesis, suppress osteoclastogenesis and have osteoimmunological activity. Therefore, berries' ACNs should be considered as naturally widespread therapeutics for bone support. Nevertheless, before implementation of berries as a natural source of bone support, there are some issues left to resolve: clarification of molecular mechanisms of ACNs action in bone metabolism, identification of effective doses of particular ACNs for bone regeneration therapies and performing clinical studies for determination of therapeutic efficacy of different types and concentrations of ACNs.

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Key words: *bone healing, bone regeneration, berries, anthocyanins, molecular mechanisms*

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Introduction

Bone-forming osteoblasts (OBs) and bone-resorbing osteoclasts (OCs) control normal bone metabolism (1). Remarkable bone tissue self-regenerating ability is maintained throughout adulthood to a certain degree which, in case of injury, enables the return to a completely functional, pre-injury state (2). However, a high rate of bone tissue degradation and decreased production of new bone lead to bone loss (1). Bone mass declines and bone microarchitecture weakens with ageing so that, in advanced age, the bone resorption rate surpasses the bone formation rate (3). Besides ageing, other systemic factors, such as disease and/or obesity, also have an influence on bone healing and regeneration (4). Moreover, traumas, multiple fractures, infections and tumor removal cause large defects that cannot be healed spontaneously (5).

Bone healing and bone regeneration approaches

To achieve successful bone healing and regeneration, plenty of approaches have been developed. The gold standard in the treatment of posttraumatic conditions—fractures, delayed unions and nonunions, is the application of autografts—the grafts constructed out of the

patient's own bone (6). Autografts' structure is similar to the original bone, so bone growth and regeneration are enabled (7), but taking autologous bone is usually associated with health risks to the patient and the source of the tissue is limited (8). Processed bone allografts are good alternatives because of their higher availability and the lack of donor-site complications in the recipient (6). However, the limitation of allografts is the induction of immune reactions which is followed by graft rejection, the possibility of infection and the chance of disease transmission (9). In the field of bone tissue engineering (BTE), great efforts have been made to avoid complications caused by bone grafts. The idea of BTE is to mimic the structure of a natural bone and construct an implant based on a biological triad—biomaterial as a scaffold, regulatory molecules and cells (10). In light of that, implants based on combinations of different biomaterials, various regulatory molecules and different types of cells were constructed. The promising results in BTE include the application of calcium phosphate-based biomaterials as a scaffold due to their similarity with the natural bone structure (11, 12, 13). Also, the inclusion of natural sources of growth factors, such as platelet-rich plasma (PRP) (14) and blood (15) into the implants' structure deserves special attention in BTE. Biomaterials and regulatory molecules can be combined with macrophages (13, 16) or stem cells, including adipose tissue-derived mesenchymal stem cells (ADSCs). ADSCs can be applied either freshly isolated (17), *in vitro* expanded before implantation (18) or *in vitro* differentiated into various types of cells, including OBs and endothelial cells, before implantation (11, 12, 18).

Another approach for bone healing and regeneration is based on findings that bone loss in humans and many animals during ageing is partially caused by the accumulation of reactive oxygen species (ROS) (19). Cells normally prevent the excessive presence of ROS thanks to their antioxidant defense system which includes vitamins, enzymes and other substances (20). Excessive ROS accumulation leads to DNA damage, lipid peroxidation and oxidation of amino acids with consequent changes in the cells' structures and functions (21). In bone tissue, oxidative stress leads to OBs and osteocyte apoptosis (22) and stimulates osteoclastogenesis (19). During osteoclastogenesis, the transcription factor nuclear factor-kappa B (NF-κB) signaling pathway plays a crucial role (1). The existence of such an association between oxidative stress and bone mass loss during ageing points out to the need to improve an individual's antioxidant defense. Standard pharmacological agents that improve bone mass and reduce fractures may show side effects and/or poor efficacy in bone healing and regeneration (23).

Folk medicine has various approaches to bone healing, and some of them are a source of valuable facts for creating more effective modern bone treatments. For example, water extracts of flavonoid-rich plants, such as *Labisia pumila* (Blume) Fern.-Vill. (Myrsinaceae) and *Piper sarmentosum* Roxb. (Piperaceae), are used in folk medicine by Malay women since these plants maintain the estrogen level at the post-menopausal stage and consequently increase bone formation and reduce bone resorption (24). Root barks and stem barks rich in flavonoids are traditionally used for bone fracture healing by the indigenous people of Eastern Ghat (25). Several flavonoid-rich plants, including *Pholidota articulata* Lindl. and *Coelogyne cristata* Lindl. (Orchidaceae), are part of folk tradition in India for the treatment of bone-related disorders and fractured bones (26).

In recent decades, there has been a noticeable trend in the application of a suitable diet for the additional treatment of certain bone conditions and diseases. All-natural nutritional therapies are expected to be safer therapeutic options for bone loss and restoration of normal bone metabolism. A plethora of preclinical and clinical researches indicate that fruits and vegetables-rich diets could help bone fracture healing (27). Due to the spectrum of observed biological activities, flavonoid substances, especially anthocyanins (ACNs) as bioactive components of natural origin, are part of a significant research area regarding means and methods for bone healing and regeneration.

Anthocyanins: classifications, biological activities and health effects

With around 8000 polyphenols among which nearly 500 are bioactive, plants represent an abundant source of antioxidants (28). Berries are especially rich in polyphenols. Due to the health benefits associated with polyphenols, the intake of berries and the possibility of their application in medicine have gained much interest within scientific circles (29). Phenolic compounds have a spectrum of biological activities including anticancer, antidiabetic, anti-inflammatory, and anti-platelet, and represent one of the most powerful natural antioxidants (30).

Polyphenols are categorized into four groups: phenolic acids, flavonoids, stilbenes, and lignans (31). It has been found that flavonoids can promote bone formation and stimulate osteogenic differentiation of mesenchymal stem cells (MSCs) (32). The idea for the application of flavonoids for bone healing and regeneration in clinical medicine comes from the experience of using flavonoid-rich compounds in folk medicine worldwide (33).

Flavonoids can be classified into flavones, flavonols, isoflavones, flavanones, flavanes, chalcone, isoflavanes and ACNs (34). ACNs are essential polyphenolic water-soluble plant pigments which consist of an anthocyanidin (aglycone) bound to sugar fraction (35). According to the position and number of hydroxyl and methoxy groups, more than 635 ACNs have been

identified (1). Cyanidins are the most abundant group of ACNs found in food, afterwards delphinidins, pelargonidins, peonidins, malvidins, and petunidins (36). These plant pigments are responsible for the wide range of colors (from dark red to blue) visible to the human eye. ACNs are indicators of the ripeness as well as the quality of the fruit (37, 38). The berry skin contains the highest amount of ACNs, although they are also present in the pulp (39, 40). Their production is affected by various environmental conditions, such as light, temperature, presence of minerals, climate and many other factors.

In vitro and *in vivo* studies indicate that ACNs have anti-inflammatory and antioxidative properties (41) that prevent or delay the onset of chronic diseases involving oxidative stress and inflammation (29, 42). ACNs stop pro-inflammatory mediators by blocking their production or activity, which is the foundation of their anti-inflammatory potential (43). Some types of ACNs possess ROS scavenging properties thus preventing DNA damage (44).

Among berries, blackcurrant (*Ribes nigrum* L.) stands out as a rich source of ACNs (45), with 250 mg of ACNs per 100 g of fresh fruit (46) and ACNs' concentrations that are up to four-fold higher in comparison with other similar fruits (47). Blackcurrant contains delphinidin-3-O-rutinoside (del-3-rut), cyanidin-3-O-rutinoside (cya-3-rut), delphinidin-3-O-glucoside (del-3-glc) and cyanidin-3-O-glucoside (cya-3-glc), contributing to approximately 98% of total ACNs (48). Delphinidins contribute to approximately 74% of total ACNs in blackcurrant. They are of particular importance for the prevention of bone resorption (49) due to the higher free-radical scavenging capacity in comparison with all other major ACNs (50).

A review of the literature revealed other berries valuable as a beneficial source of ACNs. Blueberries and grapes are abundant in delphinidin, cyanidin, petunidin and malvidin glycosides, making up over 90% of the total ACN content (51, 52). Malvidins account for 16% of the total blueberry ACNs (48), whereas strawberries' attractive color and health benefits are derived from pelargonidin-3-O-glucoside as the major ACN of this popular red berry (38, 53). Cranberries' dominant ACNs are peonidin glycosides (54), whilst cyanidin glycosides are most represented in red currants, blackberries and raspberries (48, 55, 56). The role of these ACNs in bone healing and regeneration will be thoroughly elucidated in this paper.

Anthocyanins in bone healing and regeneration: mechanisms of action

Discovering the possibilities for bone healing and regeneration supported with berries' ACNs opens the door to explore the potential use of ACNs in therapeutic interventions for people who suffer from bone degeneration related to inflammation, menopause or ageing (57). Potential benefits of ACNs are especially important for those populations that are more and more prone to osteoporosis (57), the most common bone disease characterized by low bone mineral density (BMD) and bone matrix fragility that predisposes patients to increased risk of fractures (28, 58). Osteoporosis incidence is influenced by various factors including ageing, insufficient estrogen levels, increased oxidative stress, chronic inflammation, and genetics (58).

Human (59) and animal (60) studies have shown that a strong positive correlation exists between excessive ROS and bone loss. ROS directly contribute to bone degradation by osteoclast (OC)-generated superoxide or can cause an increase in OCs' differentiation and function (60). At the same time, excessive ROS inhibit osteogenic differentiation through extracellular signal-regulated kinases (ERK) and ERK-dependent NF-κB signaling pathways (22). Also, an important factor in the oxidant–antioxidant balance is the capacity of OBs to produce antioxidants in response to ROS (61).

It has been reported that ACNs can enhance osteoblastogenesis, suppress osteoclastogenesis and also have osteoimmunological activity (28). The main modes of ACNs' action on osteogenesis are achieved by involving in the molecular mechanisms of bone morphogenetic protein (BMP-2), WNT-β catenin and fibroblast growth factor (FGF). Del-3-rut and cya-3-glc activate the FGF pathway thus accomplishing the influence on OBs' differentiation. Most of the ACNs that can promote osteogenesis also up-regulate the expression of genes for transcription factors Sox9, Runx2, and Osterix (Osx) and genes for type 1 collagen (Col1), osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP) (1). The effects of different ACNs on osteoclastogenesis are mainly achieved by involvement in the molecular mechanisms of some pathways such as c-Fos, NF-κB, JNK, Ca^{2+} and ROS (1). In addition, three subfamilies of mitogen-activated protein kinases (MAPKs) are important in RANK signal-mediated OC generation (62), while the nuclear factor of activated T-cells 1 (NFATc1) represents a major pathway that regulates osteoclastogenesis.

Bone healing and regeneration supported with ACNs and berries compounds: *in vitro* studies

The involvement of ACNs and berries compounds in bone healing and regeneration was examined by using various *in vitro* models. One type of applied *in vitro* models mimic the conditions in an organism that are the consequence of estrogen deficiency and/or microdamage. In one of such models, oxidative stress was induced in the human OB-like cell line SaOS-2 by an intracellular depletion of glutathione (GSH), in the period before the beginning of osteogenic differentiation and during the early

mineralization process (63). In GSH-depleted SaOS-2 cells, blueberry juice (BJ) rich in ACNs prevented inhibition of osteogenic differentiation and mineralization process caused by oxidative stress. BJ also modulated signals which up-regulate the expression and activity of osteogenic factors. Likewise, increased expression of sirtuin type 1 deacetylase, an enzyme that regulates osteogenic differentiation of tendons and MSCs and represents a positive Runx2 regulator (64), is probably related to the osteogenic action of BJ (63).

Inhibitory effects of delphinidin, cyanidin and peonidin on osteoclastogenesis were examined and compared—delphinidin suppressed *in vitro* OC formation, while cyanidin and peonidin did not showed such strong impact on osteoclastogenesis (58). On the other hand, Ostos Mendoza et al. (65) revealed that, peonidin-3-O-glucoside applied in low concentrations improved OBs' viability and reduced apoptosis in serum-starved human OBs. This treatment favored the cell growth and OBs differentiation as well as the alteration in the expression of proinflammatory interleukins and downregulation of RANKL expression, which suggests the possible use of peonidin as therapeutic in bone diseases.

In vitro osteogenic effects of delphinidin glycoside-enriched maqui berry extract (MBE) were also examined (66). Up-regulated bone-related gene expression for proteins such as BMP-2, OSX, and OCN indicated that MBE stimulated osteogenic differentiation of MC3T3-E1 cells. The research conducted on RAW264.7 cell line showed that petunidin (> 5 μg/ml) significantly suppressed OCs' differentiation and down-regulated expression of genes for c-Fos, NFATc1, matrix metalloproteinase 9 and cathepsin K (67).

The anti-inflammatory effects of ACNs contained in blueberry, blackberry and blackcurrant were compared, and the relationship between their antioxidant capacity and anti-inflammatory effect in macrophages was determined (48). These berries achieved anti-inflammatory effects in macrophages, at least partially, due to inhibition of nuclear translocation of NF-κB independent of the nuclear factor E2-related factor 2 (NRF2)-mediated pathways.

Multiple effects of cya-3-glc on OCs are well-known, but the mechanisms of its impact on OBs are not yet completely clarified. Therefore, the effects of cya-3-glc on proliferation and differentiation of the hip joint-derived OBs taken from osteoporotic patients and on mice OB cell line MC3T3-E1 were examined (68). The ability of OBs to mineralize after cya-3-glc treatment as well as the role of ERK signaling pathway in cya-3-glc regulation of OBs were also evaluated. ERK, a crucial member of MAPK cascades, positively regulates OB differentiation and bone formation (69). Cya-3-glc enhanced OBs' proliferation rate and OBs' mineralization points, up-regulated OCN gene and protein expression, and increased the level of ERK phosphorylation (68), which proves that ERK pathway is involved in cya-3-glc regulation of osteogenic differentiation and indicates that OBs can be targets for prevention and treatment of osteoporosis.

Del-3-rut protects MC3T3-E1 from oxidative damage and promotes osteogenic differentiation of this cell line via PI3K/AKT pathway (1), which means that del-3-rut could be used as dietary supplement for the prevention of OBs' dysfunction in age-related osteoporosis (70). Also, malvidins are considered to be responsible for bone formation by inducing significantly higher calcium deposits in MSCs (71).

Bone healing and regeneration supported with ACNs and berries compounds: *in vivo* studies

Numerous *in vivo* studies reveal a valuable relationship between ACNs and bone health. Bone-protective roles of phenolic and flavonoid ingredients derived from dried plum have been shown in rat osteoporosis models (72). The fact that dried plum and blueberry have several identical phenolic and flavonoid ingredients was used to perform research on an ovariectomized (OVX) rat model of postmenopausal osteoporosis (42). It was hypothesized that blueberry-derived phenolic compounds can prevent bone loss in ovarian hormone deficiency (42). OCN, Col1 and bone-specific ALP were chosen as markers of bone formation, and tartrate-resistant acid phosphatase (TRAP) as a bone resorption marker. Analyses at gene and protein expression levels indicated that treatment with 5% blueberry (w/w) prevented bone loss by suppression of ovariectomy-caused bone turnover. The OVX rat model was also applied to examine the effects of rabbiteye blueberry on osteoporosis (73). Rabbiteye blueberries effectively inhibited bone resorption, bone loss, and reduction of bone strength of OVX rats. In another study, blackcurrant extract supplementation reduced trabecular and cortical bone loss in an OVX mice model (74), which was the same effect estimated upon supplementation of OVX mice with delphinidin glycoside-enriched MBE (66). On the other hand, bilberry extract that has 15 various ACNs did not show an impact on bone metabolism in the OVX rat model (19).

Besides the OVX model, which mimics postmenopausal estrogen loss but does not specifically mimic the effects of ageing, an age-related model of bone loss can also be used to evaluate the influence of ACNs on bone loss prevention. In this model, the influence of blackcurrant extracts on the improvement of mice bone mass was evaluated (75). Young and old female C57BL/6J mice were fed with either a standard chow diet or a chow diet enriched with 1% (w/w) blackcurrant extract for four months. Since supplementation with blackcurrant extracts improved glutathione peroxidase and catalase activity and led to an increase in trabecular bone volume, OB surface, and bone mineral content in

young mice, it was concluded that consumption of blackcurrant early in life—when a substantial amount of bone mass is still present, could prevent ageing-associated bone loss.

Besides being the direct bone remodeling mechanisms, high oxidative stress and chronic inflammation can also lead to obesity and, consequently, to bone loss. Low levels of vitamins C and E, carotenoids, superoxide dismutase, glutathione peroxidase, catalase and other plasma antioxidants and antioxidant enzymes in obesity can cause augmented bone resorption (76). Therefore, the effects of blueberry, blackberry and blackcurrant on bone health were examined on a diet-induced obesity mice model. High-fat (HF) diet-induced obese C57BL mice were fed a HF diet, with or without berry supplementation, for 12 weeks. The results confirmed that there was a negative correlation between fat and bone mass, but that consumption of berries with different ACNs' composition could affect bone turnover via mechanisms that should be clarified in the future (76).

It was discovered that pelargonidin-3-O-glucoside acts as an anti-inflammatory agent by suppressing the NF-κB pathway in an experimental model of osteoarthritis. In this way, the inflammation and cartilage damage can be reduced as well as the progression of osteoarthritis (77). Petunidin prevented bone mass loss in a RANKL-induced osteopenic mice model (67). Cyanidin-chloride (CC) and cya-3-glc, can regulate bone homeostasis, but the literature data regarding their specific role in osteoclastogenesis are controversial. According to Cheng et al. (78), CC inhibits osteoclastogenesis, hydroxyapatite resorption, and RANKL-induced signal pathways *in vitro* and protects against OVX-induced bone loss *in vivo*. Other data indicate that, at high doses (> 10 μg/ml), cyanidins suppress osteoclastogenesis and OCs fusion, but at low doses (< 1 μg/ml) the effect is the opposite (79). Moreover, cya-3-glc improved OBs proliferation and up-regulated OCN gene and protein expression, mainly via the ERK1/2 pathway (68).

The application of flavonoids, including ACNs, in the field of BTE is becoming an increasingly attractive way to promote bone healing. Their role, in addition to protecting cells from oxidative stress, is also reflected in the promotion of proliferation and osteogenic differentiation of MSCs (80). Attempts to incorporate flavonoids into different types of biomaterials to promote bone defects' healing have proven to be more than successful. Their beneficial effect is reflected in the increase in osteogenic and angiogenic markers' expression, activation of the Wnt signaling pathway and reduction of inflammatory factors' levels (34).

The effects of anthocyanins and other flavonoids on bone healing and regeneration in population studies

The information regarding the effects of different flavonoid subclasses on bone health in humans are limited. An observational research conducted in the group of 3160 female twins revealed that total flavonoid intake was positively correlated with BMD of the hip and spine (81). Also, a strong positive correlation between consummation of fruits and BMD and bone mineral content (BMC) in boys and girls (11–14 years), young women (20–34 years), and postmenopausal women (50–70 years) has been reported (82). In addition, positive association between high fruit intake and high BMD in men and women aged 25–64 years was found (83). Another study was conducted only in women—women who consumed high amounts of fruit in childhood had higher BMD of the femoral neck compared to the women who had a medium or low intake of fruits during childhood (84).

Conclusion

Based on *in vitro* and *in vivo* studies regarding molecular mechanisms involved in bone healing and regeneration supported with berries' ACNs and on observational research in human populations, it is unequivocally clear that berries' ACNs could be used for the prevention and treatment of the certain bone conditions and diseases. Berries' ACNs are part of nature, so they should be considered as naturally widespread therapeutics for bone support. Furthermore, research into the possibility of bone healing and regeneration using substances from natural sources, such as ACNs, may contribute to the development of new, less invasive therapeutic methods. However, some discrepancies are noticed regarding the influence of ACNs on bone healing and regeneration which can be explained by the different experimental models that were chosen, different classes of ACNs that were used, or different concentrations of the same type of applied ACNs. Despite that, plenty of encouraging results speak in favor of the medical use of ACNs in bone healing and regeneration as a safer and cheaper solution for human health in comparison with standard medical therapies. Nevertheless, before the implementation of berries as a natural source of bone support, there are some issues left to resolve. First, further studies regarding clarification of molecular mechanisms of ACNs' action in bone metabolism are needed. Then, the identification of effective doses of particular ACNs for bone regeneration therapies needs to be determined. Finally, clinical studies for the determination of the therapeutic efficacy of different types and concentrations of ACNs must be performed. Resolving these issues will make an additional contribution to the prevention and treatment of osteoporosis, which is a global public health problem primarily in the elderly population. Also, it will contribute to the quality of life of people with bone injuries, osteoporosis or other bone diseases.

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BOBIČASTO VOĆE KAO PRIRODNO SREDSTVO POTPORE KOSTIJU: UČEŠĆE ANTOCIJANA U MOLEKULARNIM MEHANIZMIMA PROCESA ZALEČENJA I REGENERACIJE

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Koštano tkivo ima izuzetan potencijal samoregeneracije koji, u slučaju da dođe do povrede, omogućava povratak u potpuno funkcionalno stanje pre povrede. Međutim, starenje, bolesti, gojaznost, traume, višestruki prelomi, infekcije i uklanjanje tumora uzrokuju velike koštane defekte koji se ne mogu spontano zalečiti. Kako bi zaceljenje i regeneracija kostiju bili uspešni, razvijeni su različiti pristupi, među kojima su i primena autografta, alografta i tkivnog inženjerstva kosti. Jedan od pristupa zasniva se na saznanju da gubitak kostiju kod ljudi i kod mnogih životinja u toku starenja delimično izaziva akumulacija reaktivnih vrsta kiseonika. Zbog spektra bioloških aktivnosti, uključujući i onu antioksidativnu, esencijalna polifenolna jedinjenja – antocijani – deo su značajne oblasti istraživanja povezane sa sredstvima i metodama koje se koriste za zaceljenje i regeneraciju kostiju. Bobičasto voće je posebno bogato antocijanima. Na osnovu *in vitro* i *in vivo* proučavanja molekularnih mehanizama uključenih u zaceljenje i regeneraciju kostiju koji su potpomognuti antocijanima iz bobičastog voća i na osnovu opservacionih istraživanja sprovedenih među ljudima, utvrđeno je da antocijani bobičastog voća pospešuju osteoblastogenezu, suzbijaju osteoklastogenezu i imaju osteoimunološku aktivnost. Dakle, antocijane iz bobičastog voća treba smatrati potencijalnim, široko rasprostranjenim terapijskim sredstvom davanja potpore kostima. Ipak, pre primene bobičastog voća kao prirodnog sredstva potpore kostima, treba se pozabaviti pojašnjenjem molekularnih mehanizama delovanja antocijana u metabolizmu kostiju i utvrđivanjem efikasnih doza konkretnih antocijana za terapiju regeneracije kostiju. Takođe, treba sprovesti kliničke studije kako bi se utvrdila terapijska efikasnost različitih tipova i koncentracija antocijana.

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***Ključne reči**: zaceljenje kostiju, regeneracija kostiju, bobičasto voće, antocijani, molekularni mehanizmi*

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PREDICTIVE VALUE OF CLAUDIN-4 EXPRESSION IN NON-MUSCLE INVASIVE UROTHELIAL BLADDER CANCER

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Claudin-4 is an integral membrane protein of tight junctions, and its expression is frequently altered in epithelial cancers. Non-muscle-invasive urothelial bladder cancer (NMIBC) is a common neoplasm with an unpredictable clinical course that requires more precise stratification and risk assessment. The aim of this study was to investigate the association between Claudin-4 expression and clinicopathologic features of NMIBC, and to assess the predictive impact of Claudin-4 regarding to disease prognosis. The study comprised tumor tissue samples obtained from 441 patients with urothelial bladder cancer who had undergone transurethral resection. Samples were embedded in tissue microarrays and analyzed immunohistochemically for Claudin-4 expression. High expression was found in 41.6% of pTa and 47.8% of pT1 tumors. High Claudin-4 expression significantly correlated to high histologic grade ($p = 0.002$), and hematuria ($p = 0.038$). High Claudin-4 expression was more frequently observed in tumors with divergent differentiation, early invasive cancers associated with carcinoma in situ, and recurrent disease, however, these associations were not statistically significant. Kaplan—Meier survival analysis failed to indicate a significant difference in overall survival between the patients with high and low Claudin-4 expression. Conversely, recurrence-free survival was significantly associated with Claudin-4 expression ($p = 0.023$). In conclusion, overexpression of Claudin-4 is associated with high tumor grade and shorter recurrence-free survival. As an indicator of aggressive tumor behavior, Claudin-4 may serve as a potentially useful and accessible addition to the pathohistological panel for the prediction of clinical behavior of urothelial bladder cancer, as well as a promising therapeutic target.

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Key words: *urothelial bladder cancer, Claudin-4, tumor grade, recurrence, prognosis*

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Introduction

Claudin-4 is an integral membrane protein of tight junctions in epithelial cells, responsible for maintaining cell adhesion and polarity. A decrease of its expression may be associated with the loss of intercellular bonds and thus contribute to the progression of epithelial cancers, especially in the early stages of tumor invasion (1, 2). However, recent studies have indicated that Claudin-4 is not just a mere adhesion molecule that supports mechanical stability and integrity of the cell, but a much more versatile factor with many important roles in intracellular signalling with an impact on epithelial-mesenchymal transition, cell proliferation andlstemness, DNA repair and genomic instability (1, 3, 4).

Claudin-4 expression is frequently altered in epithelial cancers (5–8). Overexpression of Claudin-4 has been reported in many types of cancer, including breast, ovarian, gastric, pancreatic carcinoma, and oral squamous cell carcinoma. In most of them, Claudin-4 has been found to correlate with aggressiveness of the disease and poor prognosis. However, in some types of cancer, its decreased expression favors tumor invasiveness and progression, including mesothelioma, prostate and thyroid carcinoma (9–11).

Urothelial bladder cancer is a very common neoplasm of the genitourinary tract, frequently associated with exposure to environmental carcinogens, and has a strong association with smoking. It is a heterogeneous disease in terms of clinical behavior that reflects various genetic and epigenetic alterations that underlie the pathogenesis of urothelial carcinoma (12–14). The

majority of patients require life-long cystoscopic surveillance due to frequent recurrence of the disease (13).

Non-muscle-invasive urothelial carcinoma (NMIBC) is an early-stage urinary bladder carcinoma without invasion into the muscle layer of the bladder wall (detrusor muscle of the bladder). It comprises the majority of bladder cancer cases at the time of diagnosis, but this group is quite heterogeneous and associated with a notable risk of recurrence and progression (13, 14). NMIBC groups together the different entities: tumors staged as pTa, tumors with papillary architecture involving only urothelium with preserved basal membrane, tumors staged pT1, that have overt infiltration of lamina propria of the bladder mucosa, and carcinoma *in situ* (CIS). Patients with NMIBC tumors require careful estimation of risk progression and may be treated by several clinically diverse management protocols, from immediate chemotherapy instillation and intravesical bacillus Calmette–Guérin (BCG) immunotherapy to radical cystectomy which is considered in very high-risk patients. Therefore, NMIBC requires as precise as possible risk assessment and stratification (14, 15).

Several studies investigated the expression of Claudin-4 in urothelial bladder neoplasms (16–21). Immunohistochemical studies of Claudin-4 included a limited number of cases (under 100 tumors) and investigated and compared tumors of different pathologic stages. Heterogeneous results have been reported on the correlation between Claudin-4 expression and clinicopathologic features of urothelial cancer, while the prognostic significance varies between different types of urothelial lesions.

The aim of this study was to investigate the association between Claudin-4 expression and clinicopathologic features of NMIBC, and to assess the predictive impact of Claudin-4 in regard to disease prognosis.

Material and methods

Patients and histopathological analysis

This study comprised tumor tissue samples obtained from 441 patients with urothelial bladder cancer who had undergone transurethral resection during a 6-year period in the Clinic of Urology, University Clinical Center Niš, Serbia. All cases were diagnosed at the Center for Pathology, University Clinical Center Niš, according to the WHO classification (WHO, 2022, 5th edition) and staged according to the TNM pathological staging system (TNM classification 2016, 8th edition).

Average patients' age was 65.3 ± 9.6 years. Male patients comprised the majority of the study group, only 25% of the patients were women. Hematuria was the most common clinical symptom precluding the diagnosis, and it was detected in 86.6% of the patients. For every patient included in the study, detailed clinical data were obtained, including recurrence-free survival, as well as overall survival of the patients, and, if a patient died during the 5-year follow-up period, the cause of death was noted.

The study was approved by the Ethics Committee of the Faculty of Medicine, University of Niš, Serbia (Decision No 12-1250/8).

Immunohistochemical analysis

Following the diagnosis of NMIBC, including 173 cases of non-invasive papillary urothelial carcinoma (stage pTa), and 268 cases of superficially invasive urothelial carcinoma (stage pT1), the selected, representative areas of the tumor were incorporated in tissue microarrays, constructed using the manual tissue arrayer (Arraymold Paraffin Tissue Microarrayer, Arraymold, Utah, USA). Two core samples with a diameter of 2mm were selected from each case. Tissue microarray composite paraffin blocks were then cut in 3-micrometer thick sections and immunostained. Immunohistochemical analysis was performed using the primary antibody to Claudin-4, Rabbit polyclonal Anti-Claudin 4 antibody (ab15104, Abcam, Cambridge, UK). The slides were reviewed by two independent pathologists and staining intensity and distribution were assessed. Claudin-4 displayed a membranous staining pattern in urothelial cells, and, rarely, cytoplasmic immunoactivity. Moderate or strong immunostaining intensity in ≥50% of tumor cells was considered a high expression, according to the previously described methodology (18).

Statistical analysis

Analyses were performed using the statistical software for data processing SPSS version 20.0. The frequencies of categorical variables were tested by using the $\chi 2$ test with Yates's correction and Fisher's exact test. Overall survival and recurrence-free survival analysis about Claudin-4 expression were presented with Kaplan–Meier curves. $P \leq 0.05$ values were considered statistically significant.

Results

Immunohistohemical staining to Claudin-4 was found in the majority of investigated tumors, 80.9% of pTa and 87.3% of pT1 tumors (Figure 1). Only 33 tumors staged pTa, and 34 staged pT1 were negative, while 174 NMIBC displayed diffuse membranous staining of low intensity (faint yellowish precipitate), or focal staining in less than 50% of tumor cells with intermediate to strong intensity. These tumors were designated as low expressors. High expression was found in 41.6% of pTa and 47.8% of pT1 tumors, without statistically significant difference between the stages. Strong Claudin-4 expression significantly

correlated to high histologic grade (p = 0.002). Namely, 52.6% of high-grade tumors showed high Claudin-4 expression compared to 41.6% of low-grade tumors. High Claudin-4 expression was more frequently observed in tumors with divergent differentiation, early invasive cancers associated with carcinoma *in situ* in the immediate surroundings, and recurrent disease, however these associations were not statistically significant. High Claudin-4 was associated with hematuria (p = 0.038) (Table 1).

The median follow-up in the study group was 60 months. During that period, 41.3% of the patients had tumor recurrence, most of them had only one (105, 57.7%), while the rest had two or more recurrent tumors. Seventeen percent of the patients succumbed to the disease. The patients with cancer-specific mortality developed aggressive disease with locally advanced growth and metastatic spread. Kaplan–Meier survival analysis failed to indicate significant difference in overall survival between the patients with high and low Claudin-4 expression (Figure 2). Conversely, recurrence-free survival was significantly associated with Claudin-4 expression (p = 0.023). The analysis showed that patients with high Claudin-4 expression had shorter disease-free time and earlier occurrence of novel intravesical tumor growth.

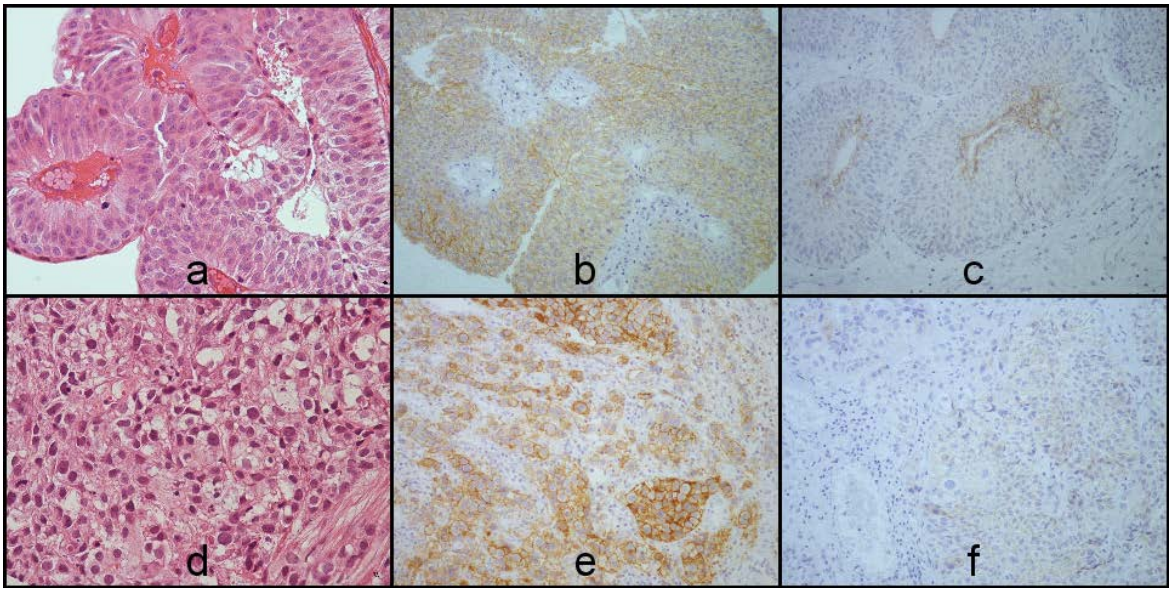

Figure 1. Representative immunohistochemical staining of Claudin-4 in non-muscle-invasive bladder cancer. The upper row shows pTa urothelial carcinoma on H&E stain (a), high Claudin-4 (b) and low Claudin-4 expression (c). The lower row displays superficially invasive pT1 bladder cancer with the infiltration of lamina propria on H&E stain (d), with high Claudin-4 (e) and low Claudin-4 immunohistochemical expression (f).

Table 1. Association of Claudin-4 expression with clinicopathologic features of non-muscle invasive bladder cancer (NMIBC)

Claudin-4 in NMIBC								
Claudin-4				**Low**		**High**		
	Total N (%)	441	(100)	241	(54.6)	200	(66.5)	**P *value***
Histologic grade								
	Low	228	(51.7)	140	(61.4)	88	(38.6)	**0.002**
	High	213	(48.3)	101	(47.4)	112	(68.1)	
Pathological stage								
	pTa	173	(39.2)	101	(58.4)	72	(41.6)	0.121
	pT1	268	(60.8)	140	(52.2)	128	(47.8)	
Carcinoma *in situ*								

	Yes	21	(4.8)	10	(4.1)	11	(5.5)	0.329
	No	420	(95.2)	206	(95.9)	394	(94.5)	
Divergent differentiation								
	Absent	400	(90.7)	222	(92.1)	178	(89.0)	0.169
	Present	41	(9.3)	19	(7.9)	22	(11.0)	
Hematuria								
	Yes	382	(86.6)	202	(52.9)	180	(47.1)	**0.038**
	No	59	(13.4)	39	(66.1)	20	(33.9)	
Recurrence								
	Yes	182	(41.3)	94	(51.6)	88	(48.4)	0.168
	No	259	(58.7)	147	(56.8)	112	(43.2)	
Cancer specific mortality								
	Yes	75	(17.0)	35	(46.7)	40	(53.3)	0.081
	No	366	(83.0)	206	(56.3)	160	(43.7)	

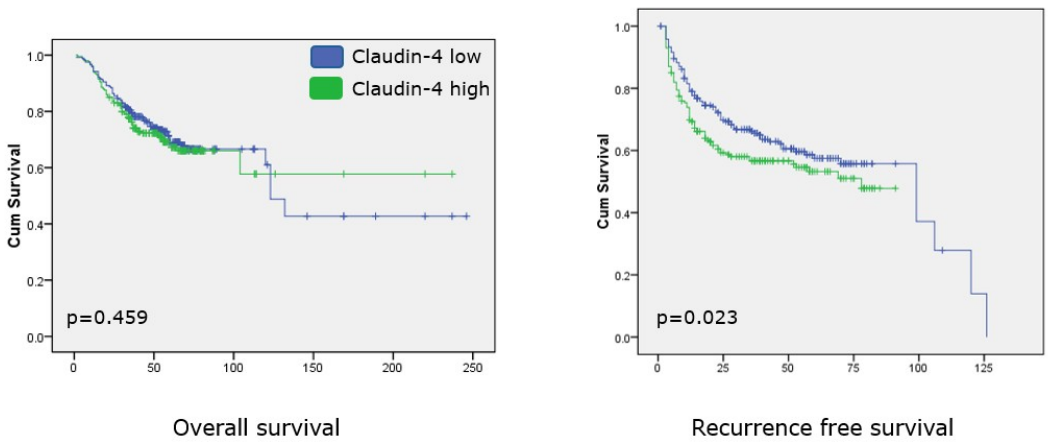

Figure 2. Kaplan–Meier survival curves showing overall survival and recurrence-free survival (the *x* axis represents the time in months) of 441 patients with non-muscle-invasive urothelial bladder cancer with low and high Claudin-4 expression.

Discussion

About 75% of urothelial bladder cancer is diagnosed at the stage of early cancer, NMIBC. The unpredictive nature of NMIBC emphasizes the need for more precise stratification of the disease in order to enhance the recognition of the patients who require more precise follow-up, cystoscopic surveillance or even early aggressive management. The 5-year recurrence rate of 31–78% for NIMBC and 10-20% progression to muscle invasive disease warrants close and expensive clinical monitoring (13–15).

The role of tight junction molecules has been recognized in the carcinogenesis of various epithelial neoplasms (3–8, 22). Claudins are major integral transmembrane proteins of tight junctions which expression profile varies between different tissues. During carcinogenesis, aberrant expression of certain claudin may contribute to invasiveness or impede tumor progression, in a tissue-specific manner. Claudin-4 is predominantly expressed in the kidneys and urinary tract, including the urothelial lining of the pelvis, ureters and bladder (2). In normal urothelium, Claudin-4 has a membrane expression pattern, with strong staining of the upper layers and progressive decrease towards the basal layers.

Accumulated evidence suggests that Claudin-4 overexpression represents an early event in carcinogenesis in many tumors (23, 24). Increased expression and abnormal distribution of Claudin-4 were found in many precancerous lesions, including premalignant changes in the

genitourinary, gastrointestinal and respiratory tract. In tumor cells, the function of tight junctions in polarity maintenance and pericellular trafficking is disturbed. Claudin-4 overexpression in those conditions may contribute to the enforcement of barrier subordinated to retain the tumor microenvironment (1, 3). In addition, Claudin-4 upregulates VEGF and Interleukin-8, thus promoting tumor angiogenesis (25). Recently, the role of Claudin-4 in the suppression of apoptosis and cell survival has been recognized (26, 27).

To our knowledge, this study is the largest investigation of immunohistochemical expression of Claudin-4 in urothelial carcinoma of the urinary bladder. This research comprised 173 pTa and 268 pT1 urothelial carcinomas, while previously published studies analyzed significantly smaller study samples. One of the pivotal studies that investigated Claudin-4 in various low-grade urothelial neoplasms, among other members of the claudin family, indicated that high claudin-4 expression in case of low-grade papillary urothelial cancer is associated with shorter recurrence-free survival (18). This is in accordance with our findings of statistically significant association of high Claudin-4 and recurrence-free survival, but not with the overall survival of the patients as well. Moreover, our results demonstrated a strong correlation between high Claudin-4 expression and high histologic grade of the tumors, indicating markedly worse clinical outcome in high expressor tumors.

On the contrary, several studies published in bladder cancer patients from the Egyptian population found a correlation between Claudin-4 expression and earlier T stage, and low-tumor grade (19, 20). The discrepancy between the conclusions of these authors and our results may be caused by the differences in the scoring of the immunohistochemical staining. The authors (19, 20) decided to enlist tumors with moderate staining scores in the group of high expressor tumors, while in the present study only the patients that they would designate as strong were considered high Claudin-4 expressors. Moreover, in these studies muscle-invasive cancers were more numerous than NMIBC, which was the subject of the present research. A recently published study of Claudin-4 expression in 50 cases of bladder cancer in the European population (21) indicated significantly higher scores of Claudin-4 immunoexpression in high-grade carcinomas. Moreover, the authors reported that Claudin-4 increases in muscle invasive tumors, suggesting the involvement of Claudin-4 in the progression of bladder cancer.

During the last decade, claudins have become a focus of interest for targeting therapies (1, 3, 25). Claudin-4 is currently being investigated as a possible treatment target, although no clinical trials have started yet. Targeting Claudin-4 can lead to a direct attack of cancer cells with Claudin-4 overexpression, but may also cause the disruption of tight junctions that stabilize and maintain tumor microenvironment, which supports and promotes cancer phenotype. Cancer cells expressing Claudin-4 could serve as a docker molecule for cytotoxic fusion proteins in a targeted therapy approach.

Conclusion

Non-muscle-invasive urothelial bladder cancer is a common neoplasm with an unpredictable clinical course that requires more precise stratification and risk assessment. Overexpression of Claudin-4 is associated with high tumor grade and shorter recurrence-free survival. As an indicator of aggressive tumor behavior, Claudin-4 may serve as a potentially useful and accessible addition to the pathohistological panel for the prediction of clinical behavior of urothelial bladder cancer, as well as a promising therapeutic target.

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PREDIKTIVNI ZNAČAJ EKSPRESIJE PROTEINA KLAUDIN-4 U UROTELNOM KARCINOMU MOKRAĆNE BEŠIKE BEZ ZAHVATANJA MIŠIĆNOG SLOJA

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Klaudin-4 je integralni membranski protein tesnih spojeva epitelih ćelija, čija je ekspresija često izmenjena u karcinomima. Karcinom mokraćne bešike bez zahvatanja mišićnog sloja (engl. *non-muscle-invasive urothelial bladder cancer* – NMIBC) čest je tumor sa nepredvidivim kliničkim tokom koji zahteva precizniju prognostičku stratifikaciju i procenu rizika. Cilj ove studije bio je da se ispita povezanost između ekspresije klaudina-4 i kliničkopatoloških karakteristika NMIBC-a, kao i da se proceni prediktivni značaj klaudina-4 za prognozu bolesti. Studija je obuhvatila uzorke tumorskog tkiva 441 bolesnika sa urotelnim karcinomom bešike, koji su dobijeni transuretalnom resekcijom. Uzorci su inkorporirani u tkivne mikroareje i analizirani imunohistohemijski na ekspresiju klaudina-4. Visoka ekspresija uočena je kod 41,6% pTa i 47,8% pT1 tumora. Visoka ekspresija klaudina-4 značajno korelira sa visokim histološkim gradusom ($p = 0,002$) i pojavom hematurije ($p = 0,038$). Visoka ekspresija klaudina-4 bila je češća kod tumora sa divergentnom diferencijacijom ranih invazivnih karcinoma povezanih sa karcinomom *in situ* i rekurentnom bolešću. Međutim, ove povezanosti nisu bile statistički značajne. Kaplan–Majerova analiza preživljavanja pokazala je da nije bilo značajne razlike u ukupnom preživljavanju između bolesnika sa visokom ekspresijom klaudina-4 i bolesnika sa niskom ekspresijom klaudina-4. Nasuprot tome, preživljavanje bez recidiva bolesti značajno je povezano sa ekspresijom klaudina-4 ($p = 0,023$). Može se zaključiti da je prekomerna ekspresija klaudina-4 povezana sa visokim tumorskim gradusom i kraćim preživljavanjem bez recidiva. Kao indikator agresivnog ponašanja tumora, klaudin-4 može poslužiti kao potencijalno koristan i pristupačan dodatak patohistološkom panelu za predviđanje kliničkog ponašanja karcinoma mokraćne bešike, a i kao i potencijalna meta ciljane terapije.

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***Ključne reči**: urotelni karcinom mokraćne bešike, klaudin-4, gradus tumora, recidiv, prognoza*

Case report **UDC: 616.36:616.14-005.6]:616.24-006.6**
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BUDD–CHIARI SYNDROME IN A PATIENT WITH SMALL CELL LUNG CANCER: A CASE REPORT

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Budd–Chiari syndrome (BCS) is a rare disease caused by hepatic venous outflow tract obstruction which can occur anywhere along the path from the hepatic venules to the inferior vena cava inflow into the right ventricle. Liver congestion results in hypoxic damage of hepatocytes. Etiologic factors related to BCS are hematologic and malignant disease. Budd–Chiari syndrome is a rare condition in lung cancer patients. Only a few cases have been reported during the last decades. We present a very rare case of acute BCS syndrome in a patient with primary small cell lung cancer caused by tumor thrombus of the inferior vena cava. The diagnosis was made based on ultrasound findings. Thereafter treatment with anticoagulant therapy was started. Due to the poor performance status and significant liver damage, specific oncological treatment with chemotherapy was not started. The patient was discharged from the hospital and advised to continue symptomatic therapy. Pulmonologists should be aware that BCS syndrome could be a presenting feature of an unrecognized lung cancer.

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Key words: *Budd–Chiari syndrome, lung cancer*

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Introduction

Budd–Chiari syndrome (BCS) is a rare disease caused by impaired venous outflow which can occur anywhere along the path from the hepatic venules to the inferior vena cava inflow into the right ventricle. It is quite often defined as congestive hepatopathy (1-5). British internist George Budd described in 1845 a triad of symptoms characteristic of disease, hepatomegaly, ascites and abdominal pain (6). After more than half a decade Austrian pathologist Hans Chiary described its pathohystology (7). Etiologic factors related to BCS are hematologic and malignant disease. Malignancies are an etiologic factor in 10% of patients with BCS, predominantly in the liver and kidney. Budd–Chiari syndrome is a very rare condition in lung cancer patients. The incidence of BCS differs between Western and Eastern countries. It is estimated that BSC occurs in 1/100000 of the general population worldwide (8). BSC is classified according to etiologic factors into primary (caused by primary hematological disorders or hypercoagulable conditions) and secondary (invasion or compression of inferior vena cava or hepatic veins with their thrombosis) (9). Two major hepatic veins must be blocked for clinically manifest disease. Blockage of hepatic veins increases the sinusoidal pressure and reduces sinusoidal blood flow. Asymptomatic BCS syndrome accounts for 15–20% of cases and is associated with the existence of collateral veins (4). Liver congestion results in hypoxic damage of hepatocytes. According to the course, it can be fulminant, chronic, or asymptomatic (3). The clinical picture is characterized by a triad in the form of pain in the right upper quadrant of the abdomen, ascites, and hepatomegaly. Leg swelling and jaundice are also common (10). The diagnosis is established by using non-invasive imaging techniques (ultrasound, computed tomography, magnetic resonance) (11). Doppler ultrasonography, with a sensitivity and specificity of 85%, is the imaging technique of choice for initial investigation when BCS is suspected. Laboratory analyses are also important. The recommended therapeutic approach to BCS consists of medical treatment (anticoagulation

therapy), endovascular treatment to restore vessel patency, and liver transplantation as a rescue treatment. The prognosis depends on etiology and the presence of risk factors. Survival rates range from 42% to 100% (12). We present a case of acute BCS syndrome in a patient with primary small cell lung cancer.

Case report

A male patient aged 51 years was referred to our clinic due to a non-productive irritating cough. A month before admission he had a productive bloody cough, after which only a dry irritating cough remained. Among associated diseases, he had a rapid heart rate. He had a 30 pack-year history of smoking. The performance status of the patient was 0 on the Eastern Cooperative Oncology Group scale. Lung auscultation revealed weakened right basal breath without accompanying murmurs. In routine laboratory analyses, hematocrit: 0.393 (0.410–0.560) L/L, thrombocytes: 435 (120–380) x 10^9/L, glucose: 7.2 (3.9–6.1) mmol/L, C reactive protein: 69.2 (0.0–5.0) mg/L, and other laboratory findings were within the reference values. On the chest X-ray in the middle and lower lung fields, a homogeneous soft-tissue shadow was found, masking the right hemidiaphragm and costophrenic sinus with clearly delineating cranial boundaries (Figure 1). This shadow may correspond to atelectasis or superimposed pleural effusion. On the right side, close to hilus lightening zones were described with a partially present bronchial pattern. On the same day, diagnostic and therapeutic thoracocentesis of the right pleural space was performed and 850 ml of serohemorrhagic content was evacuated. Pleural fluid was sent for biochemical, microbiological, and cytological analysis. According to the biochemical characteristics and Light's criteria, the pleural effusion corresponded to exudate (lactate dehydrogenase (LDH) punctate (p)/serum (s) ratio: 0.84, total proteins p/s ratio 0.65, and LDH in the punctate: 320). No pathogenic bacteria or fungi were isolated from the pleural punctate. The results of the cytological examination described a mixed type of pleural effusion, probably of inflammatory etiology. In addition, computed tomography (CT) of the chest and upper abdomen was performed. CT described a massive right - sided pleural effusion with a thickness of 94 mm and compressive atelectasis. Enlarged lymph nodes were described, with the largest in the lower mediastinum measuring 74 x 82 x 59 mm including the lower mediastinum and right hilus. The organs of the upper abdomen, as well as the bone structures, were free of secondary deposits (Figure 2). Furthermore, a bronchological examination was conducted. An enlarged carina for the upper right lobe of the lung was seen in the right bronchial tree. The confluence, as well as the initial part of the bronchus for the lower lobe, were stenosed and infiltrated. The mucous membrane was rough, with strongly accentuated folds of the mucosa from longitudinal striae. Endobronchial biopsies were taken for pathohistological verification. The pathohistological findings showed that it was small-cell cancer of the lung T4N2M1a stage IV A. The patient was presented to the Board for malignant lung and pleural diseases in April 2023. The board decided to start the treatment with the first line of chemotherapy according to the Etoposide/Cisplatin regimen for 4 cycles. Until the start of specific oncological treatment, the patient was discharged from the hospital in good general condition. Specific oncological treatment was not started due to complaints in the form of dizziness, pallor, nausea, urge to vomit, swelling of the lower legs, as well as pronounced weakness and malaise. Therefore, the patient was hospitalized again for symptomatic treatment. Lung auscultation revealed weak to inaudible breath sounds without accompanying murmurs on the right. He had retromalleolar and pretibial leg oedema. Blood pressure was 84/55 mmHg. In laboratory analyses, leukocytes: 19.8 (4.0-9.0) x 10^9/L, neutrophils: 13.53 (2.10-7.50) x 10^9/L, glucose: 11.6 (3.9-6.1) mmol/L, urea: 9.1 (2.5-7.5) mmol/L, creatinine: 151.8 (53.0-115.0) µmol/L, uric acid: 523 (208-428) µmol/L, direct bilirubin: 9.3 (0.0-3.4) µmol/L, CRP: 35.8 (0.0-5.0) mg/L, albumin: 28 (35-52) g/L, cholesterol: 2.05 (3.90-5.20) mmol/L, K: 5.5 (3.5-5.5) mmol/L, chlorides: 96 (98-108) mmol/L, Ca: 2.16 (2.20-2.65) mmol/L, aspartate aminotransferase (AST): 58 (10-37) U/L, alkaline phosphatase (ALP): 154 (30-120) U/L, gama glutamil transpeptidase (GGT): 339 (0.0-55) U/L, lactate dehydrogenase (LDH): 623 (220-450) U/L, prothrombin time (PT): 19 (9-15) sec, activated partial thromboplastin time (APTT): 23.9 (24-35) sec, D-Dimer: 1200 (0.0-250) ng/ml, INR: 1.69, hs Troponin I: 0.001 (0.000-0.040). In gas analyses, pH: 7.36 (7.35-7.45), pCO_2: 24 (35-45) mmHg, bicarbonate (HCO3): 13.6 (22-26) mmol/L, lactates: 9.2 (0.00-1.80) mmol/L, base excess extracellular fluid (BEecf): -11.8 (-2.3-+2.3) mmol/L, pO2: 76 (70-100) mmHg, SpO2: 94%. On the chest X-ray, a homogeneous soft-tissue shadow was seen on the right in the middle and lower radiological field, overshadowing the right hemidiaphragm and the right costophrenic sinus (Figure 3). Control laboratory analyses on the second day of hospitalization were urea: 16.0 (2.5-7.5) mmol/L, creatinine: 251.2 (53.0-115) µmol/L, sodium: 133 (135-148) mmol/L, potassium 6.0 (3.5-5.5) mmol/L, chlorides: 94 (98-108) mmol/L, calcium: 1.71 (2.20-2.65) mmol/L, AST: 13411 (10-37) U/L, ALT: 4650 (10–42) U/L, GGT: 277 (0.0–55) U/L, LDH: 33129 (220–450) U/L, PT: 45 (9-15) sec, APTT: 37.7 (24-35) sec D-Dimer: 10451 (0.0-250) ng/ml, INR: 4.05. In control gas analyses, metabolic acidosis was registered pH: 7.27 (7.35-7.45), pCO2: 21 (35-45) mmHg, Lac: 13.4 (0.00-1.80), and HCO3: 9.6 (22-26) mmol/L. BEecf: -17.3 (-

2.3-+2.3) mmol/L; pO2: 76 (70-100) mmHg; Spo2: 93%. A gastroenterologist was consulted due to the sudden extreme increase of transaminases and LDH values, as well as marked hypotension. On physical examination, the abdomen was symmetrical, distended and flatulent in the upper parts, soft on palpation, and not sensitive to superficial and deep palpation. The liver and spleen were not palpable. The kidney lodges were free. An abdominal ultrasound was performed, which described an enlarged liver with an oval hyperechoic shadow with a diameter of 3.33 cm in the basin of the hepatic veins, which extends into the vena cava and gives the impression that it obliterates it (Figure 4a). A moderate amount of ascites was found in the abdomen (Figure 4b), and a small right-sided pleural effusion was also observed (Figure 4c). Ultrasonographic findings of gallbladder, pancreas, spleen and kidneys were normal. The gastroenterologist prescribed symptomatic therapy and recommended further examination by a vascular surgeon. The vascular surgeon confirmed thrombosis of the inferior vena cava and prescribed therapeutic doses of low-molecular-weight heparin. Due to hypotension and deterioration of renal function, a nephrologist was consulted. He prescribed symptomatic therapy. On the control abdominal ultrasonography after 7 days of therapy, the previously described thrombosis of the vena cava was partly resolved. In the control blood count, a decrease in platelets Tr 52 (120–380) x 10^9/L was registered. The hematologist suggested the introduction of therapeutic doses of fondaparinux. After 7 days of therapy, laboratory results were repeated: Le 13.8 (4.0-9.0) x 10^9/L, HCT 0.403 (0.410-0.560) L/L, glucose 7.0 (3.9-6.1) mmol/L, urea: 10.5 (2.5-7.5) mmol/L. L, uric acid: 186 (208-428) µmol/L, total bilirubin 54.8 (5.0-21.0) µmol/L, direct bilirubin 23.6 (0.0-3.4) µmol/L, total proteins: 51 (62-81) g/L, CRP 8.1 (0.0-5.0) mg/L, albumin: 26 (35-51) g/L, cholesterol: 3.21 (3.90-5.20) mmol/L, Na 130 (135-148) mmol/L, chlorides: 93 (98-108) mmol/L, Ca 1.94 (2.20-2.65) mmol/L AST 52 (10-37) U/L, ALT 147 (10-42) U/L, LDH 673 (220-450) U/L After the control laboratory analyses the patient was discharged from the hospital. Symptomatic treatment was recommended.

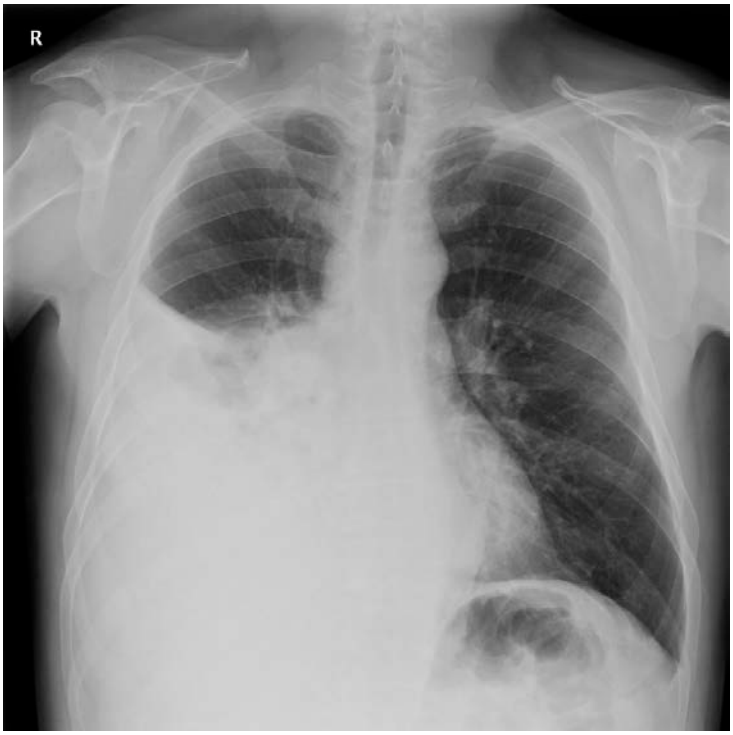

Figure 1. Initial chest radiography

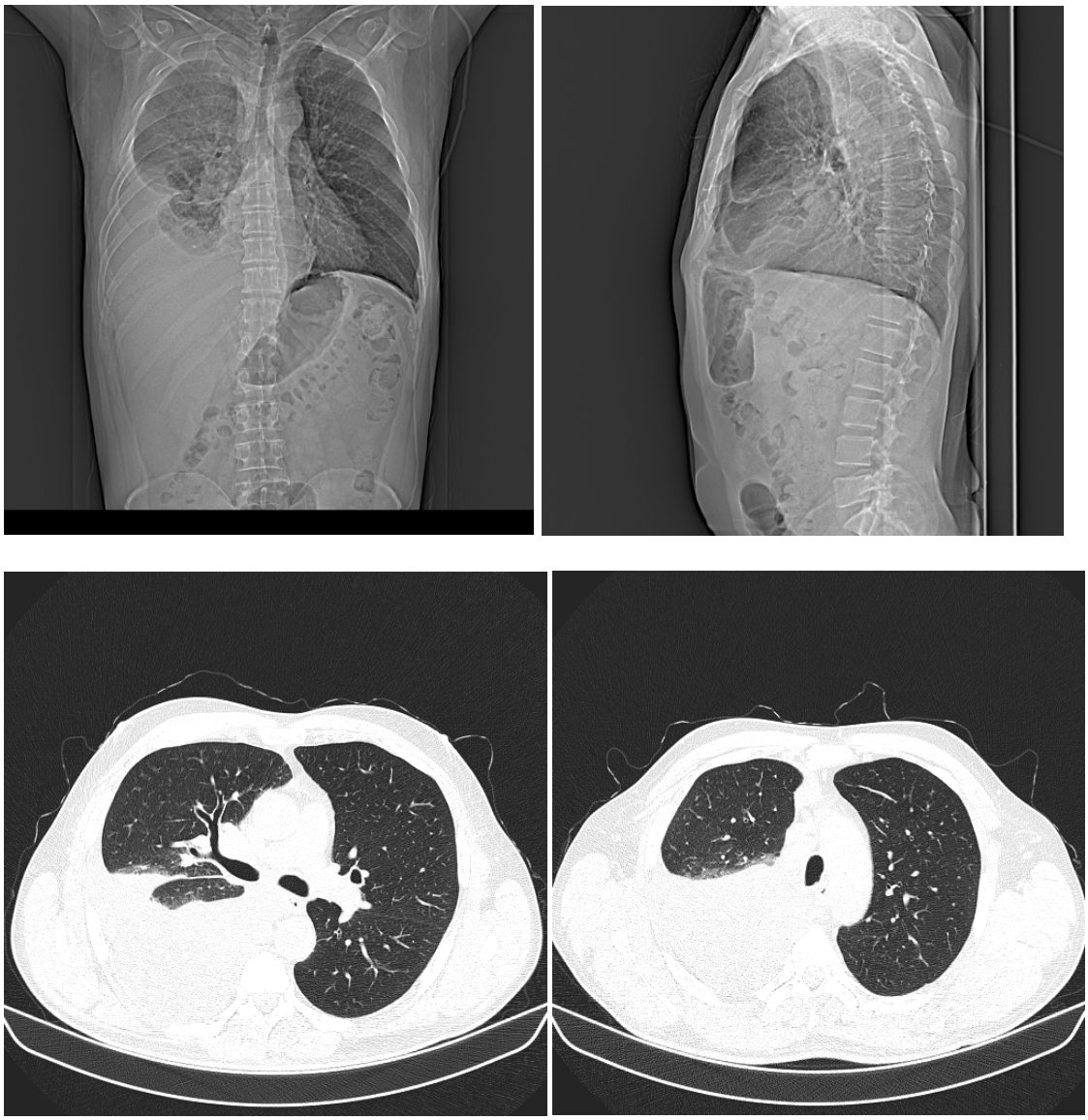

Figure 2. Computed tomography of the chest

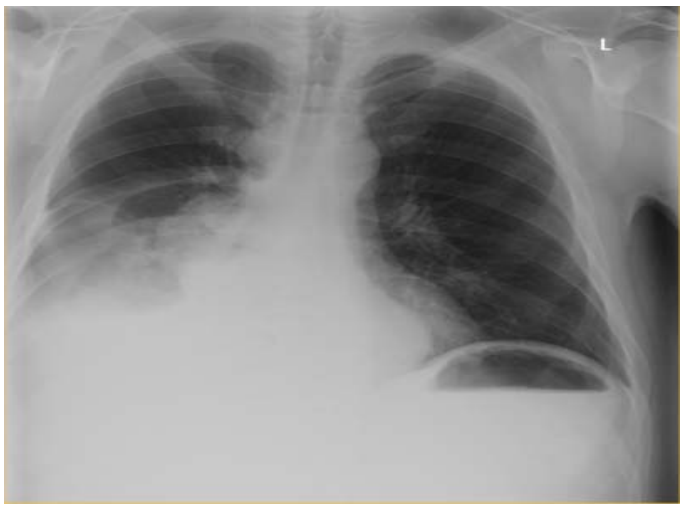

Figure 3. Control chest radiography

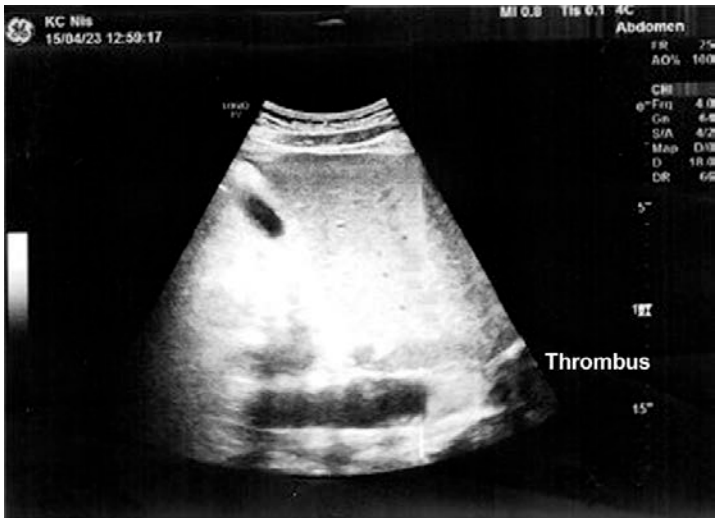

Figure 4a. Abdominal ultrasonography – visible thrombus in vena cava inferior

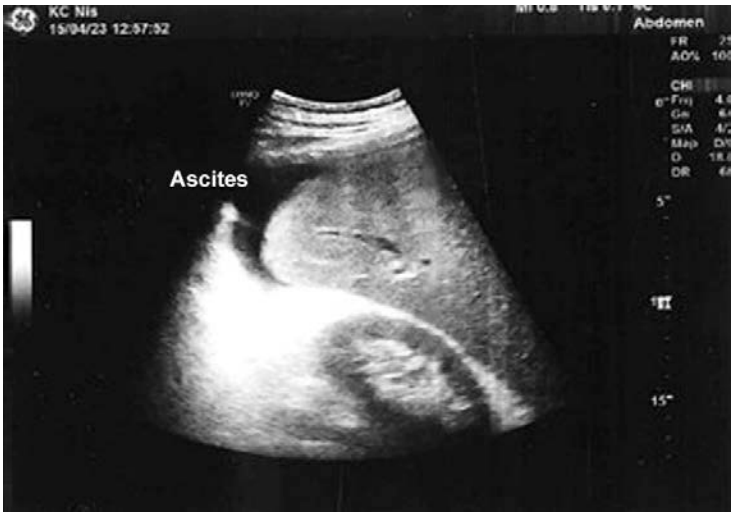

Figure 4b. Abdominal ultrasonography – ascites

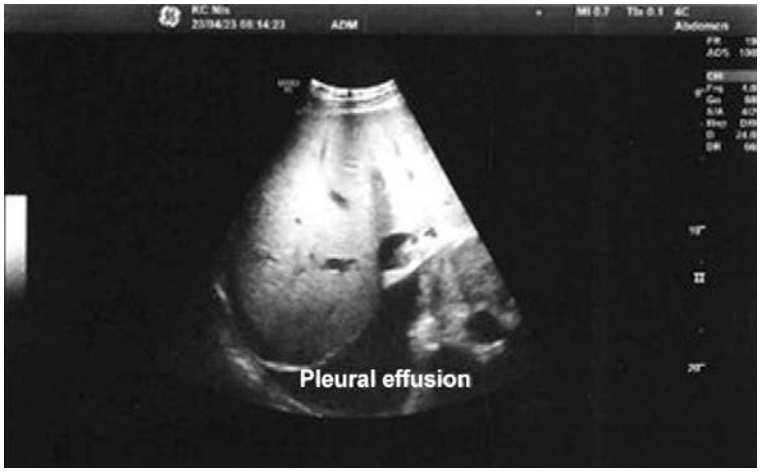

Figure 4c. Abdominal ultrasonography – pleural effusion

Discussion

We present a very rare case of BCS syndrome associated with small-cell lung cancer. According to etiology, this is the primary BCS caused by tumor thrombus of the inferior vena cava. According to the course, the described case represents a fulminant form of BCS syndrome. The symptoms are most often manifested in the form of a triad of pain in the upper part of the abdomen, ascites, and hepatomegaly. In general, patients with BCS syndrome have serious liver damage at the time of diagnosis. Doppler ultrasonography, CT or MR are non-invasive imaging techniques of choice for initial investigation when BCS is suspected (9). The prognosis depends on the degree of obstruction. Patients with complete obstruction of the inferior vena cava die within 3 years of liver failure, while in patients with incomplete obstruction, the course of the disease differs. In half of the patients, etiologic factors cannot be detected. The most common cause of BCS syndrome is diseases that cause blood hypercoagulability, such as polycythemia rubra vera and myeloproliferative diseases. Malignancies are the cause of BCS in about 10% of cases (13). The most common tumors that cause thrombus formation are liver and kidney tumors, rarely pancreas, and stomach tumors.

Although lung cancer is the leading cause of cancer death worldwide, (14) it is very rarely the cause of BCS syndrome. To date, only a few cases of BCS associated with lung cancer have been described. Patients had non-small lung cancer predominantly and rarely small cell lung cancer. (15) So, our case would be a very rare case in a patient with SCLC. Similarly, Japanese authors described a case of small cell cancer causing BCS syndrome by tumor thrombus of the inferior vena cava (16) Barbosa-Martins et al. (17) described metastatic lung cancer with multiorgan thrombosis and BCS syndrome. A rare case of BCS syndrome caused by tumor thrombus in the inferior vena cava secondary to lung cancer was reported by Dhali et al. (18). Huang et al. (19) reported a case of a patient with small cell lung cancer with an unusual initial presentation of both acute pancreatitis and acute BCS syndrome.

Clinicians rarely think about BCS syndrome. Therefore, the diagnosis is established late after severe liver damages occur. In our case, the diagnosis was made on time and treatment with low-molecular-weight heparin in therapeutic doses was started. However, significant liver damage developed early in the course of BCS syndrome in our case. Consequently, chemotherapy was not indicated.

Conclusion

We presented a very rare case of primary BCS syndrome in a patient with small cell lung cancer caused by tumor thrombus of the inferior vena cava. The diagnosis was made using Doppler ultrasound after which treatment with anticoagulant therapy was started. Due to the poor performance status and significant liver damage specific oncological treatment according to the Etoposide/Cisplatin regimen, which is the only choice of treatment in our country for patients with small cell lung cancer at this stage of disease, was not started. The patient was discharged from the hospital and advised to continue symptomatic therapy. Clinicians should be aware that acute BCS syndrome could be a feature of an undiagnosed lung cancer.

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BAD–KJARIJEV SINDROM KOD BOLESNIKA SA SITNOĆELIJSKIM KANCEROM PLUĆA: PRIKAZ SLUČAJA

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Bad–Kjarijev sindrom (*Budd–Chiari Syndrome* – BCS) predstavlja retku bolest uzrokovanu opstrukcijom hepatičnih vena koja se može javiti bilo gde na putu od hepatičnih venula do mesta gde se donja šuplja vena uliva u desnu komoru. Kongestija jetre dovodi do hipoksičnog oštećenja hepatocita. Hematološke i maligne bolesti predstavljaju etiološke faktore povezane sa Bad–Kjarijevijem sindromom. Pomenuti sindrom je retko stanje kod osoba sa kancerom pluća; poslednjih decenija opisano je samo nekoliko takvih slučajeva. U ovom radu prikazan je veoma redak slučaj akutnog Bad–Kjarijevog sindroma kod bolesnika sa primarnim sitnoćelijskim kancerom pluća izazvanim tumorskim trombom donje šuplje vene. Nakon što je dijagnoza postavljena na osnovu nalaza ultrazvuka, započeto je lečenje antikoagulantnom terapijom. Specifično onkološko lečenje nije započeto zbog lošeg opšteg stanja bolesnika i zbog značajnog oštećenja njegove jetre. Bolesnik je otpušten iz bolnice uz preporuku da nastavi simptomatsko lečenje. Valjalo bi da kliničari imaju na umu da akutni Bad–Kjarijev sindrom može biti obeležje kancera pluća koji nije prepoznat.

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***Ključne reči**: Bad–Kjarijev sindrom, kancer pluća*

Original article

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CESAREAN SCAR ECTOPIC PREGNANCY: SURGICAL TREATMENT

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Cesarean scar ectopic pregnancy is one of the rarest forms of ectopic pregnancy. It occurs in approximately 1 out of 2000 pregnancies. There are many challenges in its diagnosis and management which if not recognised can endanger the patient and provide poor outcomes, with a high rate of maternal morbidity or mortality. In cesarean scar ectopic pregnancies an embryo attaches to the scar tissue from the previous cesarean section and grows in the uterine wall that is not strong, so it may cause life-threatening hemorrhage, leading to a hysterectomy and potential devastating consequences.

This is a case report of a 35-year-old pregnant woman, with cesarean scar ectopic pregnancy, who has one cesarean section in her medical history and was treated by ultrasound-guided suction curettage.

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Key words: *ectopic pregnancy, cesarean scar, suction curettage*

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Introduction

The ectopic pregnancy is increased with the increase of the incidence of cesarean section, which also results in a higher rate of cesarean scar ectopic pregnancies. It is the rarest form of ectopic pregnancy. It occurs in approximately 1 out of 2000 pregnancies (1). The cesarean scar ectopic pregnancies can be presented in two forms: endogenous where the gestational sac enlargement does not show a tendence to devastate the uterine wall, and may reach viable gestation, and the other, exogenous, which grows through the myometrial defect toward the bladder and have a high risk for uterine rupture and intra-abdominal hemorrhage (2). In cesarean scar ectopic pregnancies an embryo attaches to the scar tissue from the previous cesarean section and grows in the uterine wall that is not strong, so it may cause life-threatening hemorrhage, leading to a hysterectomy and potential devastating consequences.

In this report, the authors present surgical treatment of cesarean scar ectopic pregnancy using ultrasound-guided suction curetage.

Case report

Our patient is a 35-year-old woman, who came to our clinic with suspected ectopic pregnancy with painless vaginal bleeding. A cesarean section was performed in a previous birth, 7 years ago. We performed a physical examination: her vital signs were stable. A pelvic examination showed slight vaginal bleeding, with a closed cervix. There was no pain in the uterusand no enlargement. Adnexal masses weren't present. The rectouterine pouch (cul-de-sac) was empty. The Prust sighn was negative. Beta human chorionic gonadotropin (beta HCG) was positive–3650 mIU/ml. Transabdominal and transvaginal ultrasound showed a gestational sac with an embryo and positive fetal cardiac activity localised in the scar of the previous cesarean section (Figure 1). CRL was 3.7 mm. There was also a yolk sac. The uterine cavity was empty, and the cervical canal was also empty. A thin myometrium was visualized but did not invade the bladder wall (Figure 2). We used diagnostic criteria as described in the Green-top Guideline of Royal College of Obstetrics and Gynecology: Diagnosis and Management of Ectopic Pregnancy GTG 21 (3). Beta HCG was repeated after two days, and it almost doubled–7120 mIU/ml. We discussed with the patient her options for treatment after the diagnosis was confirmed. She was presented with possible management including administration of Methotrexate, which can be local and muscular, or

surgical treatment using ultrasound-guided suction curetage, a hysteroscopic suction or laparoscopic resection. She gave her consent to ultrasound-guided suction curetage. She got an explanation of the potential risks of massive bleeding, possible laparotomy and even hysterectomy. After preparation, and informed written consent, under ultrasound guidance, we performed complete suction evacuation of the conceptus with Suction Cannula 10 (Figure 3). We did not have any complications during this procedure, which was confirmed using post-procedure ultrasound. There was no active bleeding in the uterine or abdominal cavity, or retained products. The uterine wall was intact.

After a full recovery, on the same day, the patient was discharged home. Beta HCG was repeated a few more times, until it was negative.

Two weeks after surgery the patient was fine, and the ultrasound examination showed an empty uterine cavity with an intact uterine wall (Figure 4). Histopathology showed the products of conception.

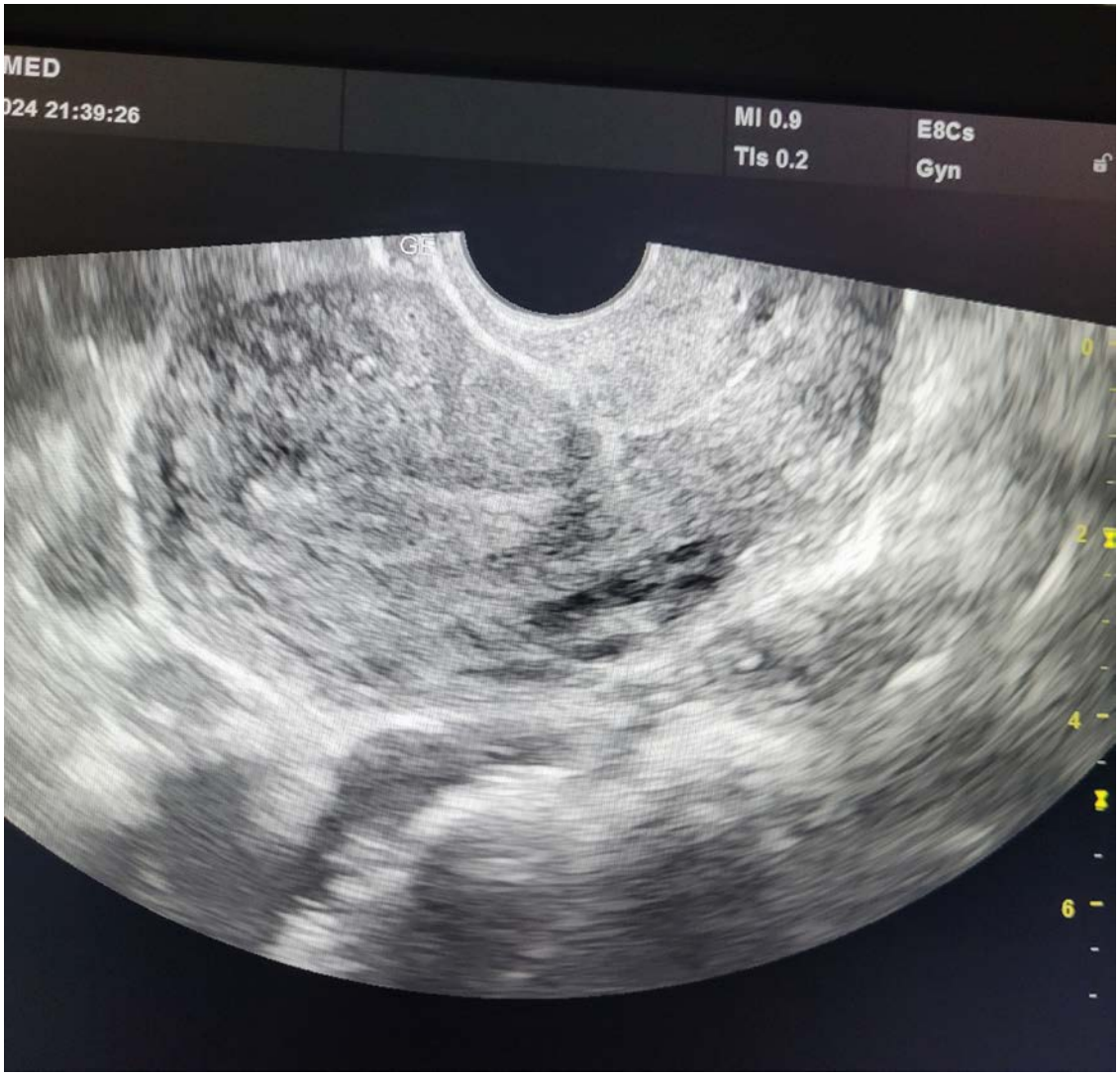

Figure 1. Uterus two weeks later

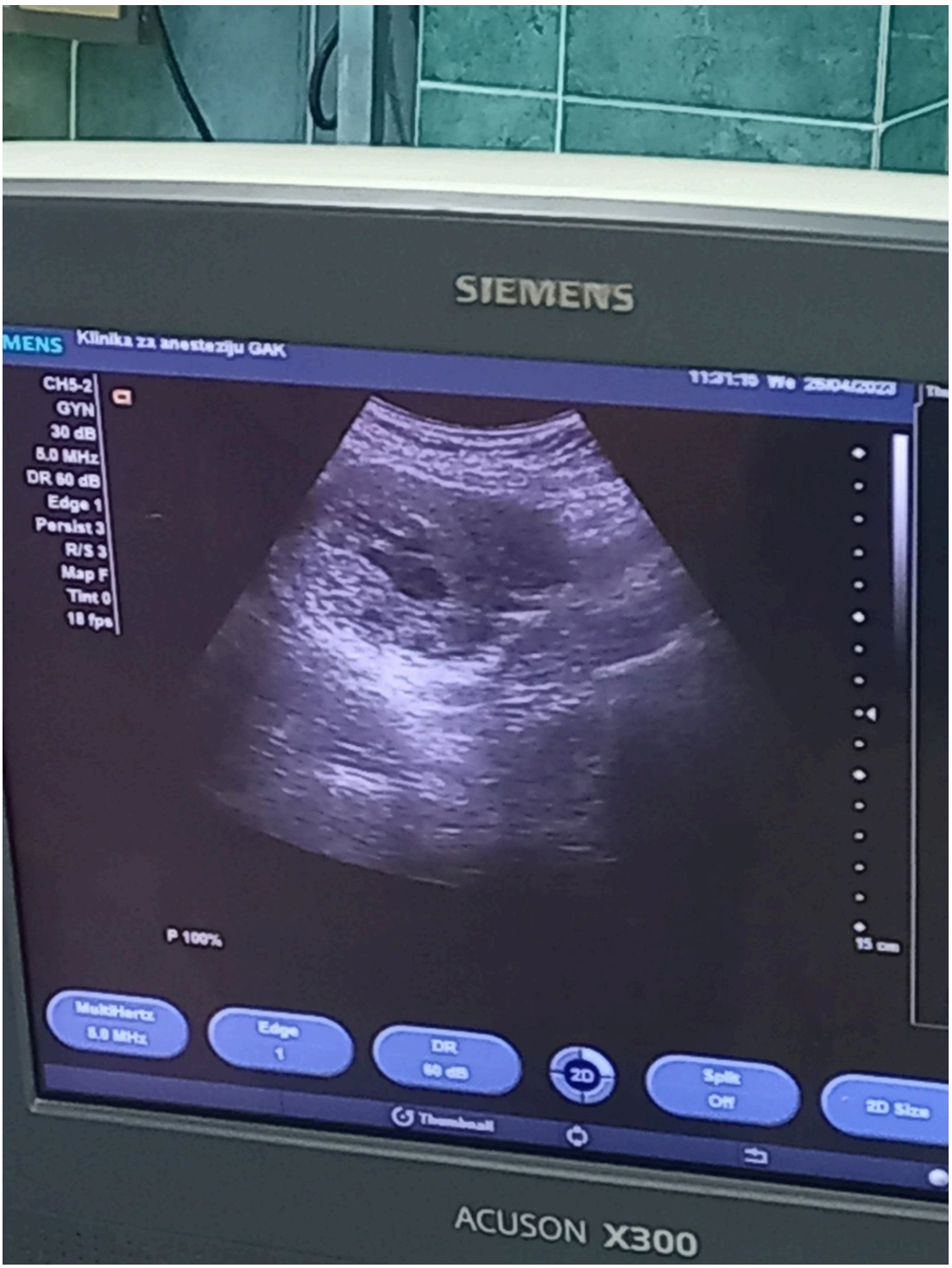

Figure 2. Collapsed gestational sac

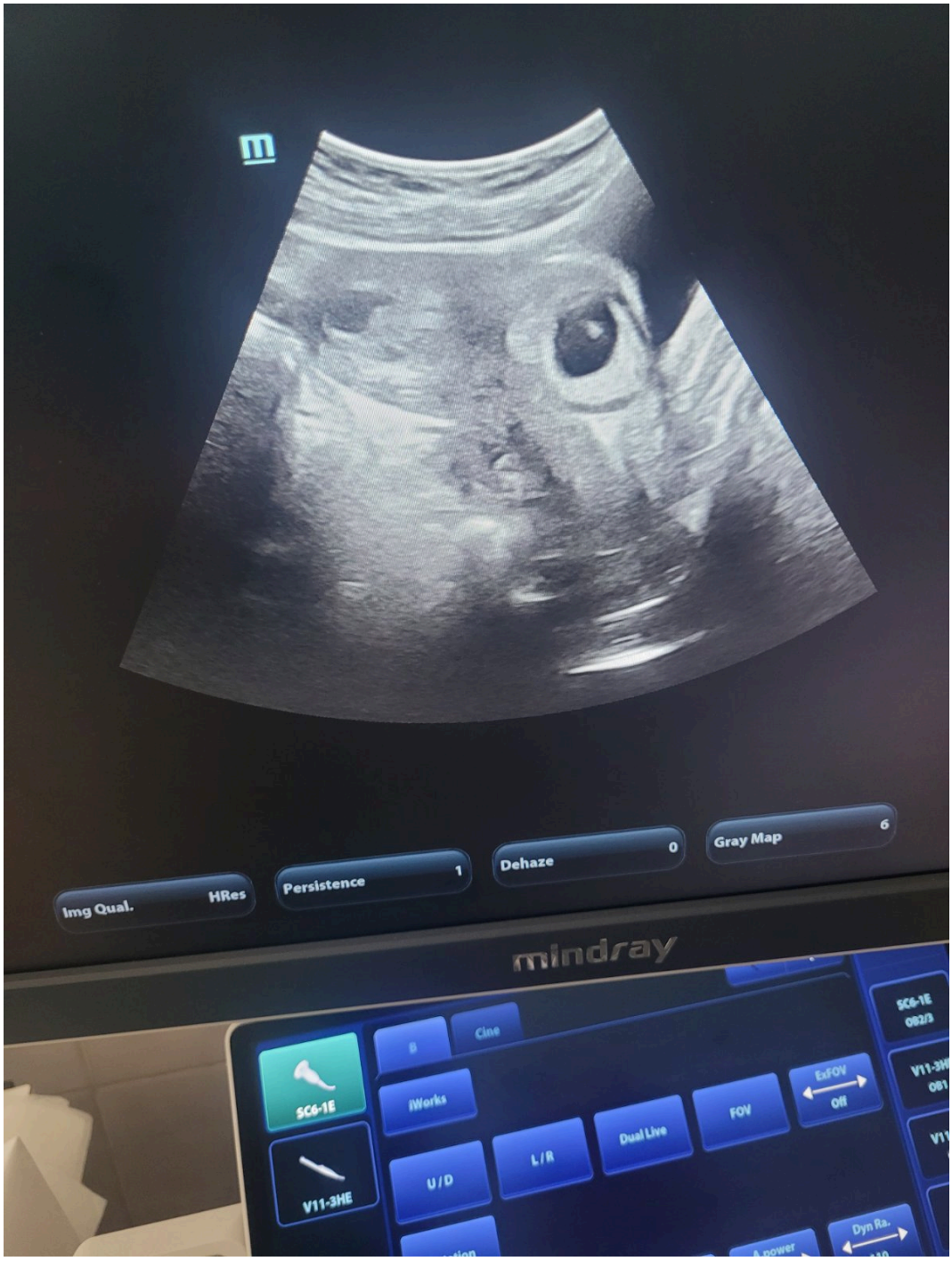

Figure 3. Vital pregnancy with intact bladder wall

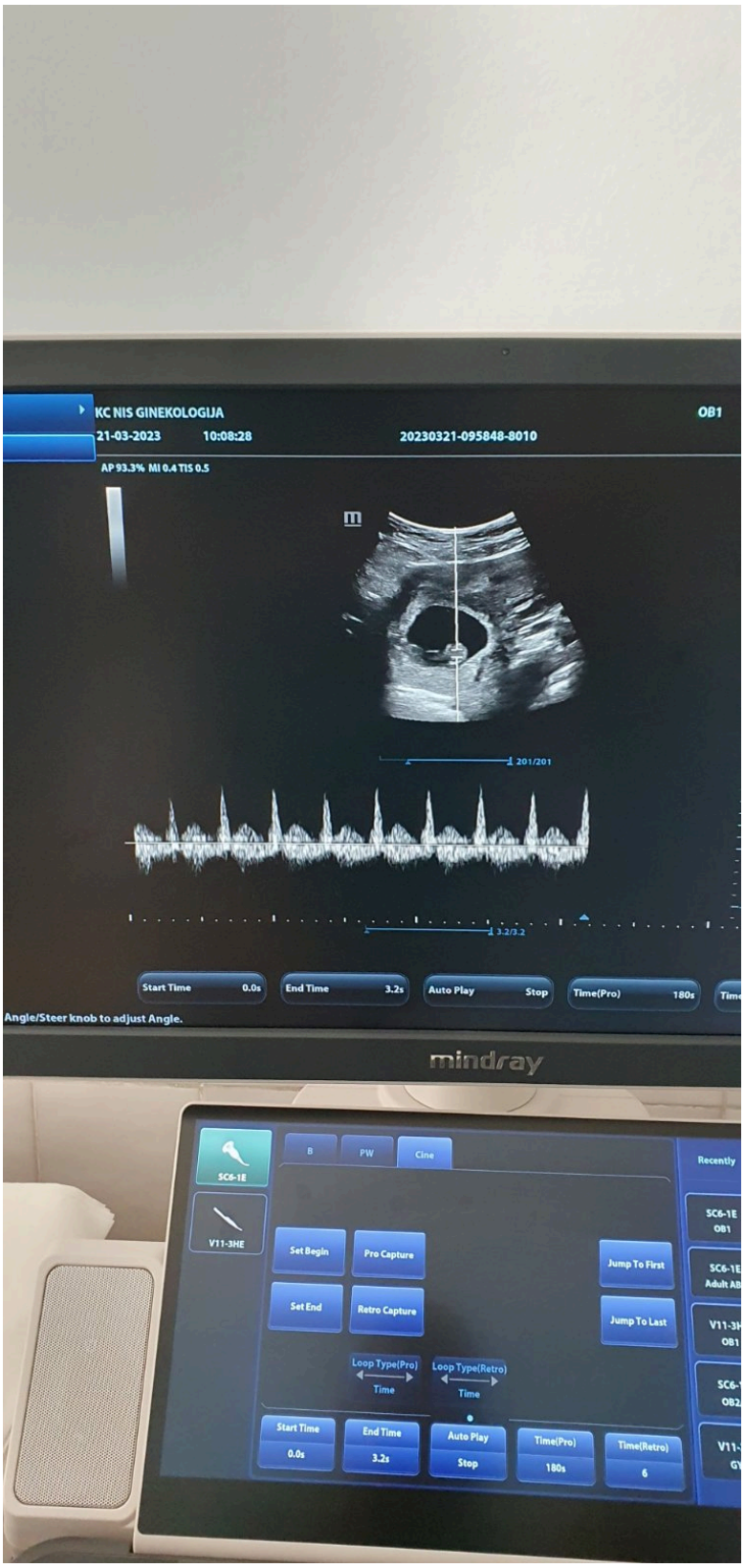

Figure 4. Vital pregnancy localised ob scar of previous SC

Discussion

Cesarean scar ectopic pregnancy is one of the types of ectopic pregnancy, where the conceptus implants itself into the scar in the uterus. It is associated with high morbidity and mortality in pregnant women, and may cause fatal outcome (4).

The conceptus may implant itself in the scar tissue in the uterus made by cesarean section, or other uterine surgery, such as myomectomy (5), manual removal of placenta in previous labour (6), or after *in vitro* fertilisation in the absence of uterine surgery history (7). However, this type of pregnancy is always abnormal, conceptus implants itself in the scar tissue, without decidualized endometrium. Symptoms are very scarce and slight vaginal bleeding, or abdominal pain may occur (8). There are no pathognomonic symptoms for cesarean scar ectopic pregnancy (9). The first step to getting right diagnosis is the use of transvaginal and transabdominal ultrasound. There are several criteria: 1. implantation settled in the scar of the previous cesarean section, 2. visualized functional throphoblastic tissue by ultrasound Color Doppler, 3. impossibility for removing the gestational sac with pressure applied by transvaginal probe (10). The best way to manage this rare condition is early termination of pregnancy, which provides a lower risk of hysterectomy and preserves the women's fertility.

There are a few ways to manage this condition: medical or surgical. Surgical approach can be transabdominal or transvaginal (transcervical) (11). We can use a transabdominal approach for treatment, which can be laparotomy or laparoscopy according to a patient's condition (12−14). The transvaginal approach can be suction curettage or hysteroscopic management (15−17). Laparoscopic managament can be performed itself or combined with transcervical. Medical treatment with Methotrexate can also be performed (18). In order to reduce bleeding during the procedures uterine artery embolisation can be used, or a double balloon catheter to tamponade the bleeding.

In this pregnant woman, the ovum was implanted in the scar of the previous cesarean section. There was positive fetal heart rate and CRL was about 6 to 7 weeks of gestation. The vascularisation was seen at the implanation site. The uterus was empty and slightly enlarged. Poush of Douglas was empty. The cervical canal was closed. The present authors have suggested ultrasound-guided suction curettage. The patient gave her consent to the suggested method to terminate the pregnancy. The post-procedure ultrasound confirmed no complications, such as uterine and abdominal bleeding, or retained products. The fertility was preserved.

Conclusion

There is a higher rate of cesarean sections worldwide. United with increased artificial fertilisation and other instrumental treatments in the uterine cavity contribute to a higher incidence of ectopic pregnancies. (19). Implantation of the conceptus placed in the cesarean section scar is a very rare form of ectopic pregnancies. Early diagnosis is crucial, combined with an adequate method of management, because of the complications that can jeopardize a patient's life, including massive bleeding and ruptured uterine wall. The early termination of pregnancy is suggested once it is diagnosed.

Our case showed that treatment of early cesarean scar ectopic pregnancy can be successfully performed by using ultrasound-guided suction curetage, which can provide complete and fast evacuation of the conceptus (20) and low risk for uterine and abdominal bleeding, transfusion of blood products with preservation of women's fertility.

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EKTOPIČNA TRUDNOĆA LOKALIZOVANA NA OŽILJKU OD PRETHODNOG CARSKOG REZA: HIRURŠKI TRETMAN

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Ektopična trudnoća lokalizovana na ožiljku od prethodnog carskog reza jedna je od najređih formi ektopičnih trudnoća. Javlja se otprilike u jednoj od dve hiljade trudnoća. Postoji mnogo izazova u njenoj dijagnostici i zbrinjavanju. Ako se ne prepozna, ona može ugroziti život pacijentkinje, dovesti do lošeg ishoda i visokog morbiditeta i mortaliteta majki. U trudnoći lokalizovanoj na ožiljku od prethodnog carskog reza embrion se implantira na ožiljnom tkivu i raste u zidu uterusa, što može izazvati krvarenje koje ugrožava život; ono pak može dovesti do histerektomije i potencijalno pogubnih posledica.

U ovom radu prikazan je slučaj ektopične trudnoće lokalizovane na ožiljku od prethodnog carskog reza kod tridesetpetogodišnje pacijentkinje. Prekid trudnoće uspešno je izvršen sukcionom kiretažom vođenom ultrazvukom.

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***Ključne reči**: ektopična trudnoća, ožiljak od carskog reza, sukciona kiretaža*

Original article

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doi: 10.5633/amm.2024.0416

ANTIOXIDANT ACTIVITY OF HYDROLATE OBTAINED FROM THE AERIAL PART OF SWEET BASIL *OCIMUM BASILICUM* L.

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Ocimum basilicum is a well-known aromatic, medicinal, and culinary plant. The essential oils obtained through steam distillation from the leaves and flowering tops of sweet basil have antiseptic, antimicrobial, antioxidant, antiviral, and anti-inflammatory properties. As a product of hydrodestination hydrolates can be obtained. The main objective of the study was the spectrophotometric quantification of the content of total phenols, tannins, nontannins and flavonoids in the hydrolate obtained from aerial part of *O. basilicum*, the evaluation of antioxidant activity and the correlation analysis of certain phenolic compounds and antioxidant activity. Quantitative analysis of the concentration of total phenolic, flavonoid, tannin and nontannin in the hydrolate determined 151.91 ± 23.491mg CE/L , 23.34 ± 3.978 mg CE/L, 119.75 ± 8.09 mg CE/L and 0.86 ± 0.07 mg Ru/L, respectively. The hydrolate showed antioxidant potential in three assays for study: scavenged 2,2-diphenyl-1 picrylhydrazyl (DPPH) radicals with IC_{50} 0.51 ± 1.07 %; total antioxidant potential, 392.15 ± 16.299 mmol of Fe^{2+}/L and prevention of lipid peroxidation in a way that depends on concentration. In addition, correlation between phenolic compounds contents and antioxidant activity in hydrolated was noted. The demonstrated antioxidant properties of *O. basilicum* hydrolate may be crucial to its future as a potential natural antioxidant.

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Key words: *antioxidant, ferrie reducing ability ofplasma, hydrosol, polyphenols, sweet basil*

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Introduction

The increasing appeal of using natural products has led to a growing interest in aromatic and medicinal plants. This is especially noticeable in the abundance of phytochemicals produced for use in various industries such as food, chemicals, pharmaceuticals, and cosmetics. A good example of this trend is the move towards the use of healthier and more organic substitutes for synthetic products, including aromatic chemicals derived from plants. Aside from the obvious benefits of using natural resources for human consumption, this approach offers a more commercially viable and environmentally sustainable method of production (1).

Hydrolates are obtained during the extraction of essential oils from aromatic plants. These are aqueous solutions that are a by-product of distillation and contain a certain amount of bioactive molecules that quantitatively and qualitatively might be different from the essential oils (2). As hydrolates are no longer classified as waste, their use is in line with the concepts of biorefinery and green extraction (3,4). Compared to essential oils, the production of hydrolate is easy and inexpensive. Furthermore, it appears to be less toxic to human health. Since hydrolates have such significant biological activity, it could be beneficial to turn them into valuable products. Furthermore, despite the published scientific articles and the various proposed practical applications, it is crucial to conduct further research on plant hydrolysis (2,5,6).

The Lamiaceae family is a world-renowned family of aromatic plants with strong antimicrobial and antioxidant properties. It is also the family of the well-known spices (7). Depending on the variety, *Ocimum* species vary greatly in form and chemical composition. These variations include phenolic profile and volatile

organic chemicals, as well as variations in leaf and flower size and color, plant height, flowering time and other characteristics. Of the 150 species of *Ocimum basilicum* L., so much is known about it, its cultivation, distribution and uses that it is referred to as sweet basil and the "*king of herbs*" as it is one of the most widely cultivated aromatic plants in the world. *O. basilicum* is also used in traditional medicine for a wide range of diseases and conditions of the respiratory and urinary tract as well as in the prevention of neurodegenerative and cardiovascular disorders (8−10). It also has antipyretic, hypoglycemic and antihypertensive effects. Additionally, it is even thought to be an anti-cancer agent. However, because of its relaxing effect on the nervous system, sweet basil essential oil is often used in aromatherapy. It can also help to improve memory, relieve migraines and combat mental fatigue and insomnia (11). Sweet basil essential oil has antiseptic, antimicrobial, antioxidant, antiviral and anti-inflammatory properties (10). In addition, both the plant itself and its essential oil have an insecticidal and fungicidal effect. When using essential oil as a biopesticide, it is important to pay attention to the dosage, as a higher concentration can be harmful to certain plants (12). Although a wide range of bioactive properties are attributed to *O. basilicum*, the antimicrobial and antioxidant properties are currently the most studied (1). These properties form the basis for many of the applications of *O. basilicum* mentioned above.

The study of chemical compositions of hydrolates obtained from fresh and dried aerial parts of sweet basil *O. basilicum* was conducted. The main compounds in hydrolates obtained from fresh and dried aerial parts of basil were methyleugenol (51.0 %, 33.4 %), eugenol (26.0 %, 5.8%) and linalool (11.3 %, 10.2 %), retrospectively (13). In aid to creating safe, eco-friendly preservatives that prevent food spoilage by fungi the investigation of antifungal activity of hydrolate obtained from leaves and flowers of *O. basilicum* against *Rhizoctonia solani*, *Fusarium oxysporum* f. sp *tulipae*, *Botrytis cinerea* and *Alternaria citri* was done (14). Furthermore, research on basil hydrolate as a bioherbicide, which is now preferred as a natural compound in organic agriculture, has been done. Under laboratory conditions, the different concentrations of hydrolate obtained from *O. basilicum* leaves significantly decreased the germination of both *O. basilicum* and *Chenopodium quinoa* seeds (15).

Therefore, even as research about hydrolate obtained from aerial part of *O. basilicum* is still limited, there is a potential for use in pharmacy.

Aim

The aim of this study was: (i) spectrophotometric quantification of total phenolic, tannin, nontannin and flavonoid content in hydrolate obtained from aerial part of *O. basilicum*, (ii) *in vitro* evaluation of the antioxidant activity of the hydrolate obtained from aerial part of *O. basilicum* employing three different assays: 2,2-diphenyl-1 picrylhydrazyl assay, *β*-carotene bleaching assay and ferrie reducing ability ofplasma assay, (iii) correlation analysis of certain phenolic compounds and antioxidant activity.

Material and methods

Plant material and chemicals

The hydrolate of the aerial part of *O. basilicum* was obtained through the industrial steam distillation process by the company "PROMONTIS production", Vilandrica, Gadžin Han After the distillation process, double microbiological filtration was performed.

All chemicals used were obtained from Sigma Aldrich (USA), or Zorka pharma (Šabac, Serbia). All solvents and chemicals were of analytical grade.

Determination of total phenolic content

The total phenolic content of hydrolate obtained from the aerial part of *O. basilicum* was determined using the Folin−Ciocalteu method (16,17). The Folin-Ciocalteu reagent, which had previously been diluted 1:1 v/v with distilled water, and a 20% Na_2CO_3 solution were added to the test tubes into which the studied hydrolate had previously been added. The tubes were then shaken vigorously and allowed to stand for 40 minutes to develop a blue color, and the absorbance was measured spectrophotometrically at 725 nm in comparison to a blank containing the extraction solvent (water) instead of the sample. The experiment was done in triplicate. The total phenolic content of the sweet basil hydrolate was calculated using a (+)-catechin calibration curve (range 1−5 μg/mL) and expressed as mg catechin equivalents (CE) per L of hydrolate.

Determination of total tannin content

The total tannin content of hydrolate obtained from the aerial part of *O. basilicum* was determined using the same Folin−Ciocalteu method (18,19). Polyvinylpolypyrrolidone was added to the test tubes containing the sample and then shaken vigorously. The supernatant contains all phenolic compounds except the tannins. The test was performed on the clear supernatant and the results were expressed in mg catechin equivalents (CE) per L of hydrolate. The experiment was done in triplicate. The content of nontannin polyphenols is also expressed as mg catechin equivalents per L of hydrolate and results from the difference

between the total phenolic content and the total tannin content.

Determination of total flavonoid content

The total flavonoid content of hydrolate obtained from the aerial part of *O. basilicum* was estimated according to Lamaison and Carnat (18). Aluminum trichloride ($AlCl_3$) in ethanol was mixed with the same volume of the hydrolate. The blank sample consisted $AlCl_3$ with ethanol without extract solution. After incubation for 1 hour, the absorbance was measured spectrophotometrically at 430 nm. The experiment was done in triplicate. The total flavonoid content of sweet basil hydrolate was calculated and expressed as mg rutin (Ru) per L of hydrolate using a rutin calibration curve (range 0.5−5 µg/mL).

Antioxidant activity examination

DPPH assay

The antioxidant activity of hydrolate obtained from the aerial part of *O. basilicum* was assessed using the DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical scavenging assay. The color changed from violet to yellow when DPPH reduced to DPPHH, and an ELISA microplate reader was used to measure it at 540 nm (13). The assay was performed according to Pavlović et al. (19) by incubating different concentrations of hydrolate (10−70% v/v) with DPPH in 96% (v/v) ethanol solution for 30 minutes at room temperature and in the dark and then absorbance was measured on ELISA microplate reader at 540 nm (20). The distilled water is present in the blank sample. As a control, 96% (v/v) ethanol containing DPPH was used. Synthetic antioxidants BHT and BHA were used as the reference compounds. The experiment was done in triplicate. The following formula was used to determine the percentage of DPPH free radical inhibition:

% DPPH = (Ac – As) /Ac x 100

where Ac is the absorbance of the control, and As is the absorbance of the sample.

β-carotene bleaching assay

The *β*-carotene bleaching method assesses the capacity of hydrolate obtained from the aerial part of *O. basilicum* to impede the process of lipid peroxidation. The microplate with freshly prepared *β*-carotene linoleic acid emulsion (200 mg of Tween-20 and 25 µl of linoleic acid were combined with 1 ml of *β*-carotene solution in chloroform 1 mg/5 ml) and different concentrations of hydrolate (1.11 – 11.1 % v/v) was read in a microplate reader immediately (t 0 min) and after 120 minutes of incubation at 55 °C (t = 120 min) on 450 nm (21). The percent inhibition of *β*-carotene bleaching by the samples was calculated according to formula (22):

% inhibition = (A_{120} / A_0) x 100

where A_{120} is the absorbance of the sample at t = 120 min and A_0 is the absorbance of the sample at t = 0 min. The experiment was done in triplicate. The reference compounds were synthetic antioxidants BHT and BHA.

FRAP assay

The total reduction potential of hydrolate obtained from the aerial part of *O. basilicum* was measured using the FRAP (ferric reducing antioxidant power) assay. The method is based on reducing the ferric tripyrydiltriazine (Fe^{3+}-TPTZ) complex to the ferrous tripyrydiltriazine (Fe^{2+}-TPTZ) form in the presence of antioxidants at a low pH (23). After incubation at 37 °C for 30 min, a solution containing FRAP working reagent (10 mmol/L TPTZ in 40 mmol/L HCl, 300 mmol/L sodium acetate buffer, pH 3.6 and 20 mmol/L $FeCl_3$ x $6H_2O$ solution, each in a ratio of 10:1:1 (v/v/v)) was mixed with hydrolate and the absorbance was measured at 593 nm. The result of the FRAP assay of sweet basil hydrolate was calculated using an Fe^{2+} sulfate calibration curve (range 100−1000 mmol/L) and expressed as FRAP value, as mmol of Fe^{2+} per L of hydrolate. The experiment was done in triplicate.

Statistical analysis

All experimental measurements were performed in triplicate and expressed as the standard deviation of the average of the three measurements. The normality of the distribution of the continuous variants was determined using the Shapiro−Wilk test. The comparison of variable values between several groups was carried out using One-Way ANOVA followed by Tuckey's post hoc test. Pearson's linear correlation coefficient was used for the correlation analysis. A significance level of $p < 0.05$ is considered statistically significant. Statistical analyses were performed using SPSS v. 20.0 software. The heatmap was obtained by using OriginLab graphing and data analysis software.

Results

Determination of total phenolic content

The results of the spectrophotometric determination of the total phenolic content of the hydrolate obtained from the aerial part of *O. basilicum* are shown in Table 1. The total phenolic content of sweet basil hydrolate calculated using a (+)-catechin calibration curve (range 1−5 µg/mL) was 151.91 ± 23.491 mg CE/L.

Determination of total tannin and nontannin content

The results of the spectrophotometric determination of the total tannin and nontannin content of the hydrolate obtained from the aerial part of *O. basilicum* are shown in Table 1. The total tannin content of sweet basil hydrolate calculated using a (+)-catechin calibration curve (range 1−5 μg/mL) was 23.34 ± 3.978 mg CE/L. The total nontannin content of hydrolate obtained from the aerial part of *O. basilicum* was 119.75 ± 8.09 mg CE/ L.

Determination of total flavonoid content

The results of the spectrophotometric determination of the total flavonoid content of the hydrolate obtained from the aerial part of *O. basilicum* using aluminum trichloride complexing agent are shown in Table 1. The total flavonoid content of sweet basil hydrolate was calculated using a rutin calibration curve (range 0.5−5 μg/mL) was 0.86 ± 0.07 mg Ru/L.

Antioxidant activity examination

DPPH assay

The results of the ability of different concentrations (10−70% v/v) of the hydrolate obtained from the aerial part of *O. basilicum* to inhibit the DPPH radical are presented in Figure 1. According to DPPH assay all tested concetracions of sweet basil hydrolate possess anti-radical activity, IC_{50} value 0.51 ± 1.07 % (v/v). A statistically significant difference ($p < 0.05$) in the percent of inhibition of DPPH radical was recorded between the second and third concentrations of the hydrolate, 20% *vs* 40%. Under the same conditions IC_{50} values for BHT and BHA, commercial synthetic antioxidants, were 22.82 ± 2.07 μg/ml and 2.44 ± 0.09 μg/ml, respectively.

β-carotene bleaching assay

The results of the determination percent of *β*-carotene bleaching as a function of concentration of the hydrolate obtained from the aerial part of *O. basilicum* expressed in percent inhibition are presented in Figure 2. In the current study sweer basil hydrolate showed mean inhibition of bleaching from 5.023 ± 0.809 to 21.37 ± 3.207, for concentracion range 1.11−11.1% (v/v), respectively. A statistically significant difference ($p < 0.05$) in the percent of *β*-carotene bleaching was recorded between the second and third concentration (2.78% *vs* 5.56%) and between the third and fourth concentration (8.3% *vs* 11.1%). Under the same conditions conditions IC_{50} values for BHT and BHA, commercial synthetic antioxidants, were 0.03 ± 0.00 μg/ml and 0.04 ± 0.01 μg/ml, respectively.

FRAP assay

The results of *in vitro* determination of the total reduction potential of the hydrolate obtained from the aerial part of *O. basilicum* using the FRAP assay are shown in Table 1. The total reduction potential of basil hydrolate was measured using the standard curve of known concentrations of ferrous sulfate solutions (range 100−1000 mmol/l) was 392.15 ± 16.299 expressed as mmol of Fe^{2+} per L of hydrolate.

Correlation between phenolic compounds contents and antioxidant activity

Pearson correlation coefficients analysis was worked out among total phenolic, flavonoid, tannin, nontannin content and antioxidant activity of hydrolate obtained from the aerial part of *O. basilicum*. The total phenolic, tannin and nontannin content showed a weak correlation with the percentage of DPPH free radical inhibition, while total flavonoids content showed a strong negative correlation (r = -0.961) (Figure 3). Furthermore, the examined phenolic compounds showed a weak negative correlation with the percent of *β*-carotene discoloration. Similar results were noted between phenolic compounds and total antioxidant potential, except for total flavonoid content where we observed a significant positive correlation (r = 0.987)(Figure 3).

Table 1. Phenolic compounds contents and *in vitro* antioxidant assay of *O. basilicum* hydrolate

Hydrolate	**Phenolic compounds content**				**Antioxidant activity**
	Total phenolic content	Total tannin content	Total nontannin content	Total flavonoid content	FRAP assay
	(mgCE/L)	(mg CE/L)	(mg CE/L)	(mg Ru/L)	(mmol Fe^{2+}/L)
Ocimum basilicum L.	151.91 ± 23.491	23.34 ± 3.978	119.75 ± 8.09	0.86 ± 0.07	392 ± 16.299

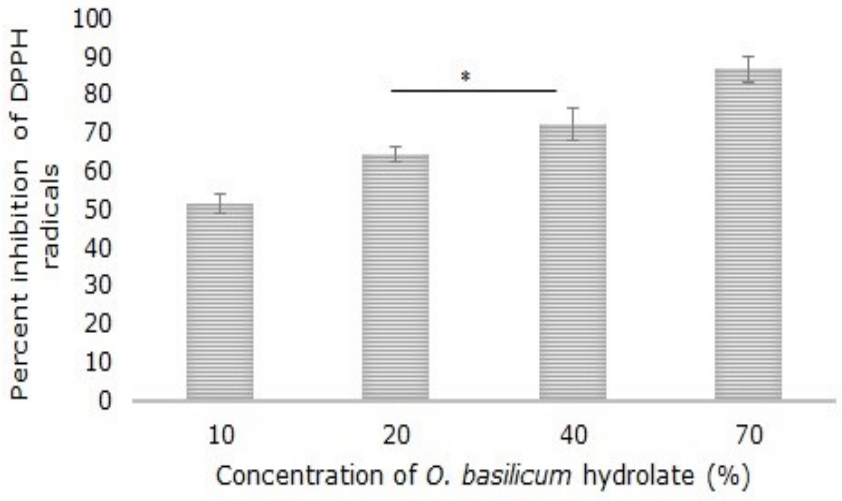

Figure 1 Results of antioxidant testing: inhibition of DPPH radicals. Data are presented as mean ± SD and further compared using One-Way ANOVA followed by Tuckey's post hoc test. * $p < 0.05$

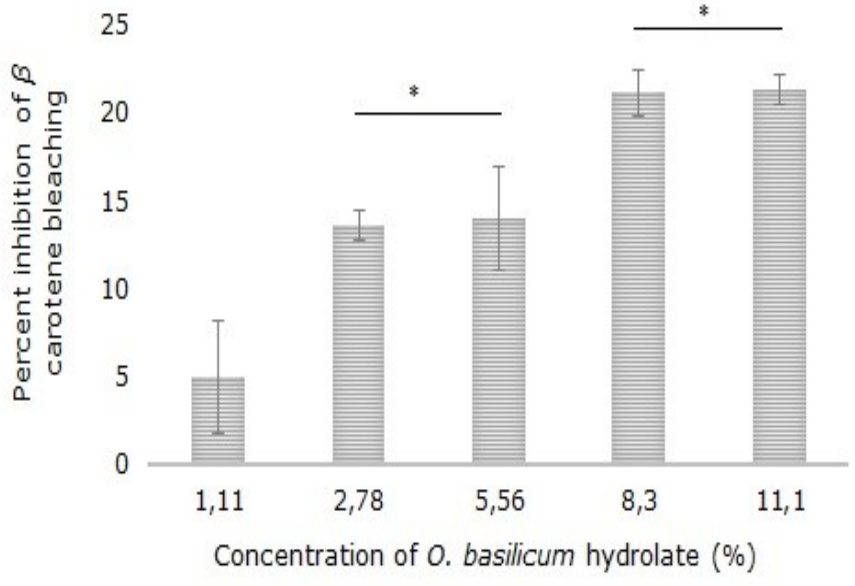

Figure 2 Results of antioxidant testing: inhibition of β-carotene bleaching. Data are presented as mean ± SD and further compared using One-Way ANOVA followed by Tuckey's post hoc test. * $p < 0.05$

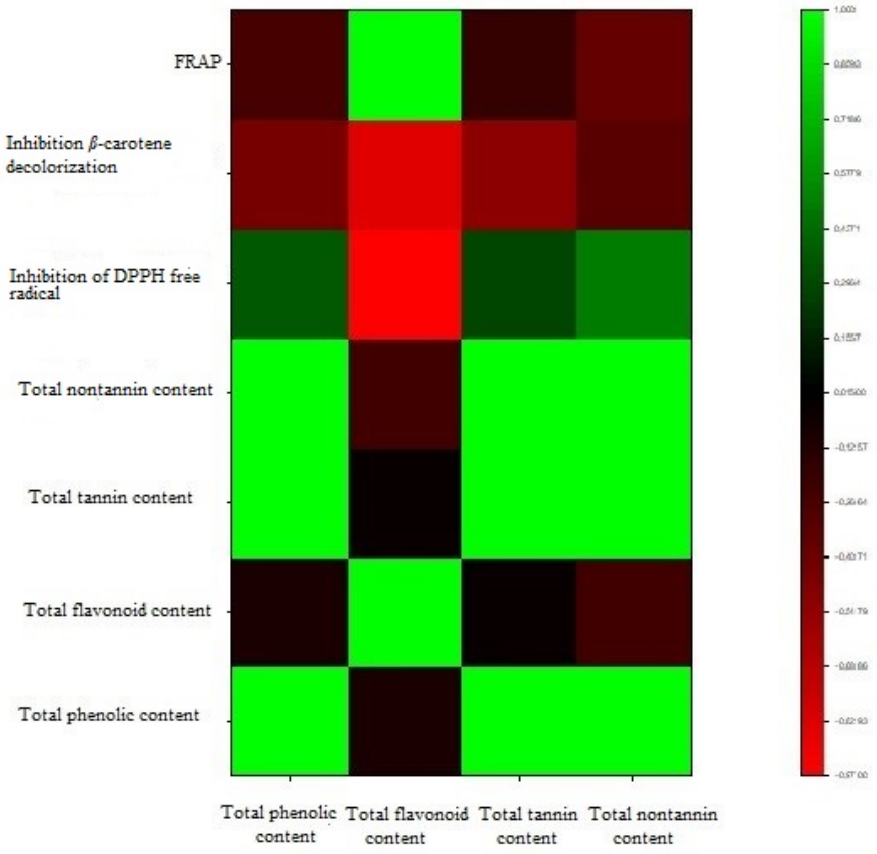

Figure 3. Heatmap of the correlation matrix generated by the Pearson r correlation coefficient for the phenolic compounds and antioxidant activity (inhibition of DPPH free radical, inhibition of *β*-carotene decolorization and FRAP) for *O. basilicum* hydrolate. The scale ranges from -0.961 (red) to +1 (green)

Discussion

Many diseases of the 21st century, including malignancies, cardiovascular disease and inflammation, are thought to be caused by oxidative stress. An imbalance between the antioxidant systems and the production of oxidant species leads to oxidative stress.

These diseases are caused by oxidative damage to vital elements such as nucleic acids, proteins, carbohydrates and lipids. Studies have shown that the consumption of antioxidants, especially those found in foods, can be beneficial in combating these effects. Plants are rich sources of antioxidants such as ascorbic acid, carotenoids, various phenolic compounds, alcohols, stilbenes, tocopherols and tocotrienols (24).

In recent decades, the food industry has focused on finding natural antioxidants from plants that are effective for human use and have minimal side effects. This search has become a major industry and research problem in the field of food technology. Despite extensive research, the potential of plant antioxidants has not yet been fully understood, and their benefits have not been fully realized yet. Therefore, there is a need for reliable *in vitro* methods to screen for antioxidant activity (25).

Hydrolates have historically been employed in food and functional drink items. For instance, in Turkey, sage hydrolate is consumed as a natural antibiotic to treat digestive issues and a variety of bacterial illnesses (26). Also, hydrolates are utilized as drinks in traditional and folk Persian medicine to cure a variety of ailments (27,28). These facts are highlighted only that hydrolates are valuable, innovative natural products that have great potential for use in the food industry as functional beverages.

Phenols are one of the main compounds with antioxidant activity, because they have an aromatic ring in their structure that allows the stabilization and relocation of unpaired electrons. Further, the aromatic ring also makes it easier for their hydroxyl groups to donate hydrogen atoms and electrons (29). The species, tissue, and developmental stage of the plant affect the

overall phenolic content. Also, environmental elements such as water stress, light conditions and temperature have an impact on phenolic content (30). As shown in Table 1. the total phenolic content of the hydrolate obtained from the aerial part of *O. basilicum* was 151.91 ± 23.491 mg CE/L determined by Folin–Ciocalteu method. Thanks to the presence of free OH groups, particularly 3-OH groups flavonoids, secondary metabolites from plants have the potential to be antioxidants (31). According to the results of our study, hydrolate obtained from the aerial part of *O. basilicum* contained 0.86 ± 0.07 mg Ru/L, as shown in Table 1. Also, phenolic compounds include tannins, which, like other phenolic compounds, among other activities, have proven antioxidant activity (31). The total tannin content was also examined using the Folin–Ciocalteu method. We found that the hydrolate obtained from the aerial part of *O. basilicum* contained 23.34 ± 3.978 CE/L as shown in Table 1. As the obtained results indicate, the studied hydrolate obtained from the aerial part of *O. basilicum* contains phenolic, tannic and flavonoid compounds. These phytochemical compounds may be responsible for many of the health benefits of this hydrolate.

Plant extracts can be assessed for their antioxidant activity using various methods. It is suggested that a minimum of two different methods be used for accurate measurement. In our study, we utilized three assays, each with a different mechanism of reaction (32). One of the assays was based on hydrogen atom transfer, while the other two were based on electron transfer.

The synthetic free radical generator DPPH is used to test the scavenging power of certain antioxidants in DPPH assay. When the H^+-donating antioxidant reacts with DPPH, it results in a reduction in DPHH to hydrazine, which causes a decrease in the absorbance of the reaction (33). Additionally, the response causes a changed color from purple to yellow. The degree of discolouration and absorbance drop is correlated with the sample's antioxidant activity, concentration, and capacity to donate H^+ (34). Proteins and nucleic acids, two important macromolecules, can be harmed by hydroxyl radicals, which are highly reactive and have a short lifespan when produced by the Haber–Weiss/Fenton process (35). Because of their high sensitivity, hydroxyl radicals seriously damage both individual cells and the components that make them up, as well as entire organisms (36). Further, by measuring the process by which antioxidants prevent lipid oxidation, DPPH free-radical scavenging is used to evaluate antioxidant activity and, consequently, free-radical scavenging capacity. As a result, a moderate DPPH scavenging action of hydrolate could be related to the existence of phenols, as shown in the current work. To assess the antioxidant capacities of the hydrolate obtained from the aerial part of *O. basilicum in vitro*, we used the FRAP assayand compared it with two other assays. The molecular mechanisam of FRAP assay is performed on electron-transfer processes. The reduction of iron(III)-2,4,6-tripyridyl-S-triazine at low pH to an intense blue colored iron (II) tripyridyltriazine complex is the reaction mechanism (37). According to the result of the assay sweet basil hydrolate might be consider possessing moderate antioxidant capacities. In the *β*-carotene bleaching assay, the yellow color of *β*-carotene is lost due to its reactivity with radicals formed in an emulsion when linoleic acid oxidizes. Antioxidants are able to slow down the bleaching of *β*-carotene (24). In the current study, all tested concentracions of sweer basil hydrolate showed mean inhibition of *β*-carotene bleaching. The obtained results might be considered significant due to the inhibition of lipid peroxidation using the *in vitro β*-carotene-linoleic acid system mimics *in vivo* oxidation/protection of valuable biological fatty acids present in cell membranes.

This is the first study to determine the phenolic compound content and antioxidant activity of the hydrolate from the aerial part of *O. basilicum*. The results show that the sweet basil hydrolate contains certain phenolic, flavonoid and tannic compounds. The overall phenolic, tannic and flavonoid properties of basil hydrolate are associated with its antioxidant activity. However, a comprehensive phytochemical analysis is required before using hydrolates to treat diseases associated with oxidative stress.

Conclusion

Spectrophotometric quantification of hydrolate obtained from the aerial part of *O. basilicum* a certain concentration of total tannin, flavonoid, tannin and nontannin was observed 151.91 ± 23.491mg CE/L , 23.34 ± 3.978 mg CE/L, 119.75 ± 8.09 mg CE/L and 0.86 ± 0.07 mg Ru/L, respectively. The tested concentration of hydrolate affected the neutralization of DPPH radicals from 51.57 ± 2.592% to 86.87 ± 3.304% and *β*-caroten bleaching from 5.023 ± 0.809% to 21.37 ± 3.207%. The total reducing power in the FRAP assay was 392.15 ± 16.299 mmol Fe^{2+}/L hydrolate. Additionally, the correlation between certain phenolic compounds and antioxidant activity has been noted.

Acknowledgments

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ANTIOKSIDATIVNA AKTIVNOST HIDROLATA DOBIJENOG IZ NADZEMNOG DELA BOSILJKA *OCMIUM BASILICUM* L.

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Ocimum basilicum je poznata aromatična, lekovita i kulinarska biljka. Etarsko ulje dobijeno parnom destilacijom iz listova i vršnih cvetova bosiljka ima antiseptička, antimikrobna, antioksidativna, antivirusna i antiinflamatorna svojstva. Hidrolati se mogu dobiti kao proizvodi hidrodestilacije aromatičih biljaka. Za potrebe ove studije izvršene su spektrofotometrijska kvantifikacija ukupnog sadržaja fenola, tanina, netanina i flavonoida u hidrolatu dobijenom iz nadzemnog dela biljke *O. basilicum*, procena antioksidativne aktivnosti i korelaciona analiza fenolnih jedinjenja i antioksidativne aktivnosti. Kvantitativnom analizom ukupnih fenola, flavonoida, tanina i netanina u hidrolatu utvrđene su sledeće koncentracije: 151,91 mg CE/L ± 23,491 mg CE/L, 23,34 mg CE/L ± 3,978 mg CE/L, 119,75 mg CE/L ± 8,09 mg CE/L i 0,86 mg CE/L ± 0,07 mg Ru/L, redom. Hidrolat je pokazao antioksidativni potencijal u trima testovima: sposobnost uklanjanja slobodnih 2,2-diphenyl-1 picrylhydrazyl (DPPH) radikala, IC_{50} 0,51% ± 1,07%; ukupni antioksidativni potencijal 392,15 mmol Fe^{2+}/L ± 16,299 mmol Fe^{2+}/L i prevencija lipidne peroksidacije na način koji zavisi od koncentracije. Osim toga, zabeležena je korelacija sadržaja fenolnih jedinjenja i antioksidativne aktivnosti u hidrolatu. Pokazana antioksidativna aktivnost hidrolata *O. basilicum* može biti važna za njegovu buduću upotrebu u svojstvu potencijalnog prirodnog antioksidansa.

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***Ključne reči**: antioksidans, antioksidativna moć redukcijom gvožđa, hidrosol, polifenoli, bosiljak*

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CHARACTERISTICS OF IMMUNE RESPONSE DURING HERPES SIMPLEX VIRUS INFECTION IN CHILDHOOD

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The infection with the herpes simplex virus (HSV) basically implies the chronic activation of the immune system. Interferon gamma (IFN-γ) and Interleukin 4 (IL-4) are part of the mechanisms that control the immune system's response to recurrent herpes simplex virus.

The research included 40 children (2−15 years old) with clinical herpes simplex virus infection. Routine laboratory tests were performed on the patients: leukocyte count, creatinine-kinase-CPK, oxidative stress (nitro-blue tetrazolium), NBT test, lactate-LDH dehydrogenation, IFN-γ, IL-4 levels in serum were measured by ELISA test. The serological test for HSV type I virus was positive in all patients.

A high level of LDH, CPK was detected as well as a low ability to reduce NBT. An increased level of IFN-γ, IL-4 was observed compared to the control group of patients (who did not have clinical manifestations of herpes virus infection). Patients with a high concentration of IFN-γ are associated with a low concentration of NBT-test.

During infection of virus herpes simplex, an immune response is activated (lymphocyte Th1and Th2 type are stimulated). Different clinical manifestations are based on a certain type of immune response. Our results presented the dominance of the Th1 type of response over the Th2 type. The production of IFN gamma was higher compared to IL4. Oxidative stress parameters were also associated with the dominant Th1 type of immune response. This is all important for prognosis, prevention and therapy.

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Key words: *herpes simplex virus infection, interferon gamma, interleukin 4, immune system, children*

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Introduction

Herpes simplex virus type 1 (HSV-1) is a DNA virus, a member of the Herpes viridian's family (25 family members) associated with manifestations like: dermatologic, immunologic, or neurologic disorders (1,2,3). Infection of HSV-1 can manifest as primary, latent and recurrent (3). Xu et al report that 36% of children younger than 14 years of age have serological evidence of HSV-1 infection in the United States (4).

In children and young adults, primary infection (gingival stomatitis) usually occurs with HSV-1 (5). Studies show that primary HSV-1 infection manifests itself in two groups of children: the first group of children, between 6 months and 5 years of age, and the second in adolescence (6,7).

Often the primary infection is asymptomatic. Primary infection is manifested with damaged oral mucosa and gingiva accompanied by pyrexia, lethargy, loss of appetite (8,9). Also, patients can have irritability headaches and bilateral cervical lymphadenopathy (10−13).

Shaffer et al. point out that HSV can cause lifelong infection, through the spectrum of diseases depending on host factors such as immune competence, age and site of infection (5) Theil et al. report that latency infection HSV-1 is a part of the inflammatory process where virus factors are a crucial role for of latency (14,15).

The interactions between the host immune system and HSV-1 are very complex.

Many investigators tried to explain the mechanisms of the pathogenesis of infection HSV-1. Previous studies demonstrated that monocytes, natural killer (NK) cells and different T lymphocytes populations all have important functions in the control of HSV infections (16−19).

Usually during virus infection, the immune system is activated with the intent to destroy the virus. Many studies described that CD4+ and CD8+ T lymphocytes and their soluble products (cytokines) have a function in the control of disorders during HSV-1 infection (20).

Mogensen et al demonstrate that the HSV down-modulates the production of proinflammatory cytokines. Also, Mogensen et al described the role of macrophages during HSV infection (21). In this study, the author and colleagues demonstrate that HSV-1 suppresses the production of cytokines (22).

Cytokines control cell survival. Antiviral functions are triggered by cytokines and lead to the activation of inflammatory factors. Therefore, it is not surprising that one of the strategies of viruses is to target cytokines to avoid removal by the immune system (23). Many studies try to explain the mechanisms of latent infection of HSV. Herpes simplex virus type 1 (HSV-1) after a primary infection of the skin or mucous membrane, attacks sensory neurons and is transmitted by in sensory ganglia. Therefore, remittent illness is not the result of exogenous reinfection, but the awakening of a latent virus (24). Also, investigators point out the main role of cytokines during infection of HSV (25).

The purpose of this study was to explain the physiology of immunoregulation (parameters of oxidative metabolism, clinical manifestations, haematological and biochemical parameters, cell injury enzymes) and their effect on the clinical manifestation of disease in HSV-1 seropositive children.

Matherial and Methods

Fourty children were involved in the study(from 2 to 15 years old) with signals of herpes virus (HSV) herpes simplex virus infection and medical history of frequent inflammatory reactions such arelapse of labial herpes, urticaria, remittent respiratory tract infections. Inclusive measures were positive ELISA test and age for viruses HSV. None of the children were under any treatment before blood collection for analysis.

Blood was taken after their verbal agreement from patients and controls. There are 20 healthy patients in the control group.

Quantification of NBT test/Tetrazolium reduction test

The Nitro-blue-tetrasolium (NBT) test was performed according to the method to Park et.al., 1968.

Serologic survey using ELISA assay

Level of IgM and IgG antibody for HSV were determined by ELISA test.

Statistical analysis

Data are reported as mean x, ± SD and as a percentage of certain parameters. The statistical significance of differences was estimated by using Student's t-test. Microsoft program SSPS version 7,5 was used for statistical calculation of data.

Results

Control group had healthy children (20) and 40 children.

There are very heterogeneous clinical manifestations which comprised different organs and systems (Table 1).

52% of the patients were positive for anti-HSV whereas only 4% of the patients were positive for IgM against HSV (Table 2).

Means levels of IFN-γ and IL-4 were high values in relation to control group (Table 3). Results showed significantly low values of mean level of IFN-γ ($p < 0,01$) − Student t-test. There was no normal level of IFN-γ. Values of IL-4 were significantly high and low ($p < 0,01$). A higher value of INF-γ was present in about 75% of patients and high value of IL4 was present in about 46% of patients. High values of IFN-γ followed change in haematologic parametars as low values of leukopenia (74%), Hb (62%), monocytosis (82%).

A high concentration of IFN-γ followed leukopenia (74%). We used low levels of haemoglobin about 80-90 g/lit, in relation to the age of the children, in our study. Monocytosis (35%) and low values of Hb (55%) were associated with high values of IL-4 (Table 4).

There was no significant deviation at low values of IFN-γ. High values of IL-4 are associated with high values of GOT (49%), but low level of IL-4 are associated with low values of CPK (25%) (Table 5).

Domination of low levels of NBT in patients with high levels of IFN-γ (Table6).

Table 1: Clinically manifestations

Clinical manifestations	n	(%)
Labial HSV infection	7	17,5
Urticariarecidivans	6	15
BHR	5	12,5
Atopic dermatitis	5	12,5
Stomatitsaftosa	5	12,5
Laryngitis	4	10
Erythema anulare	4	10
Pneumonia	3	7,5
Encephalitis	1	2.5

Table 2: Positive ELISA test for HSV in patients

HSV	
IgM +	IgG +
4%	52%

Table 3: Mean values of IFN-γ, IL-4

	IFN-γ (IU/l)	IL-4 (pgr/ml)
Study group(n=40)	0.59 ± 0.21	1.42 ± 0.42
Control group (n=40)	0.25 ± 0.41	1.20 ± 0.21

Results show as mean values ± SD; n-number of patients

Table 4: Comparision between haematological parametars and IFN-γ, IL-4. Interleukins, and low levels of Hb, leukopenia, monocytosis

	Low Hb	Leukopenia	Monocytosis
high IFN-γ	62%	74%	82%
high IL-4	55%		35%

Table 5: Comparision between enzymes, IFN-γ and IL-4. Interleukins (IFN-γ, IL-4) and enzymes (LDH, CPK, GOT, GPT)

	High LDH	High CPK	High GOT	High GPT
High IFN-γ	72%	80%	62%	61%
High IL-4	Normal	Normal	49%	Normal
Low IL-4	Normal	25%	Normal	Normal

Table 6: Comparision between NBT test, IFN-γ, and IL4. Interleukine IFN-γ, IL4 and NBT-test

	Low spont. NBT test	Low. stim. NBT-test
High IFN-γ	35%	72%
High IL4	17%	47%

Discussion

Children are infected primarily with orolabial HSV by 5 years of age, with infection rates of 33% in populations that are of lower socioeconomic status and 20% in those who have improved socioeconomic status. By adulthood, HSV affects 70% to 80% people of the lower socioeconomic population and 40% to 60% of the higher socioeconomic population (26).

This study showed that herpes virus infection leads to activation of the immune system and phagocytes.

Patients with other evidence of the disease in this study, (labial HSV:17,5%, urticaria:15%, BHR:12,5%, atopic dermatitis:12,5%, stomatitis aphtosa:12,5%, laryngitis:10%,) are indicated in Table 1.

All mothers of children included in the study had herpes labialis.

HSV infection in persons with eczema or other skin diseases causes eczema herpetic (27).

Exaggerated Th2 responses to common allergens are characterized for atopic eczema and such answers may be showed in the physiology of eczema herpetic (28).

In our study we did not have aggressive form of HSV infection on the skin. In 52% of patients ELISA test was positive (IgG antibody on HSV). Positive IgM antibody on HSV was 4% in patients.

Our results show high concentrations of IL-4 were connected to a low percent of Hb (55%) and high concentration of monocytes (35%). Otherwise, high concentrations of IFN-γ were connected to a high percent of monocytosis (82%) and low percent of Hb (62%), leukopenia (74%).

During HSV-1 infection many investigations analysed the roles of subpopulations (CD4+ and CD8+ T lymphocytes) (21). CD4+ T cells are sufficient to clear the infection from both peripheral and neuronal sites in the absence of CD8+ T cells, (21). CD4+ T cells have been suggested to be responsible for the inflammatory response in HSV keratitis proved by investigation (29).

The cytokines IL-4, IL-5, IL-10 and IL-13 express Th2 cell lineage and are involved in the activation of B cells. In HSV-1 infection IL-4 and IL-10 have been detected, but some of these data are controversial, probably due to different mouse models and virus strains used (25). Increased IL-4 levels correlated with increased HSV-1 replication in the eye indicate that IL-4 might function by down-regulating IL-2 in the HSV-1 infection. When IL-2 is down producing, HSV-1 titers increase (30).

In our investigation values of IFN-γ, IL-4, were compared with values of LDH, CPK, GOT, GPT (Table 5). Patients with high levels of IFN-γ had high level of CPK (80%), LDH (72%), GOT (62%) and GPT (61%). The presence of risen levels of this immune modulation cytokine in insistent infection proposes that when the immune system is unable to mediate viral clearance it may contribute to injury of hepatocyte (31). Patients with low levels of IL-4 had high levels of CPK (25%), while patients with high levels of IL-4 had high levels of GOT (49%). The presence of risen values of CPK may contribute to cell injury during herpes virus reactivation.

IL-4 mRNA and protein were detected at days 7 through 14 after HSV-1 infection; In a study by Heilinhenhaus et al. compared to IL-2 and IFNgama, IL-4 staining intensities were lower (32).

In resistance to HSV infection IFN-γ may be critical. HSV-specific IFN-γ production by cultured peripheral blood mononuclear cells (PBMC) is lacking in some patients who have frequent episodes of herpes labialis (33). A recent study (29) compared titers of antibody to HSV and cytokine production by cultured PBMC for seropositive patients with or without a history of herpes labialis.

Research has shown that activation of lymphocytes causes HSV-1 keratitis, then type 1 cells release IL-2 and IFN-γ. Type 2 cytokines play a role in better disease prognosis (34).

Lekstrom-Himes et al. suggest that IFN-γ plays two roles in HSV-1 infection. First, it prevents acute disease and limits the quantity of virus amenable to ganglionic latency; second, it limits the spread of the virus once reactivated.

Viruses trigger an oxidative metabolism and function of phagocytes. In our investigation, most patients with high concentraions of IFN-γ,had low NBT test (35).

Conclusion

During HSV infection, an immune response is activated that goes in the direction of Th1 and

Th2 type of response. Different clinical manifestations are based on a certain type of immune response. Our results determined the dominance of the Th1 type of response over the Th2 type. The production of IFN gamma was higher compared to IL4. Oxidative stress parameters were also associated with the dominant Th1 type of immune response. It is important to recognize the mechanisms and the activity of the immune answer in a viral infection. This is all important for prognosis, prevention and therapy.

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KARAKTERISTIKE IMUNSKOG ODGOVORA U INFEKCIJI HERPES SIMPLEKS VIRUSOM KOD DECE

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U osnovi infekcije herpes simpleks virusom (HSV) jeste hronična aktivacija imunosistema. Interferon gama (IFN-γ) i interleukin 4 (IL-4) čine deo regulatornih mehanizama reakcije imunosistema na rekurentne infekcije herpes simpleks virusom.

Analizirano je četrdesetoro dece (starosti 2–5) sa infekcijom herpes simpleks virusom tipa 1. Urađena je laboratorijska analiza bolesnika kojom su određeni broj leukocita, nivo laktat dehidrogenize (LDH), kreatinin kinaze (CPK) i oksidativni metabolizam fagocita (sposobnost fagocita da redukuje boju *nitro-blue-tetrazolium* – NBT). Nivo IL-4 i IFN-γ određivan je pomoću ELISA testa. Serološka analiza za HSV tipa 1 bila je pozitivna kod svih bolesnika.

Naši rezultati su pokazali visok nivo LDH, CPK i niske vrednosti NBT testa. Bile su povećane vrednosti IFN-γ i IL-4. Kod bolesnika sa visokim vrednostima IFN-γ bile su povišene vrednosti LDH, CPK, GOT, GPT, a zabeleženi su i nizak nivo hemoglobina (Hb), leukopenija i monocitoza.

Povišene vrednosti IFN-γ udružene su sa nižim vrednostima NBT testa.

U toku infekcije herpes simpleks virusom aktivira se imunski odgovor (stimulisani su limfociti Th1 i Th2 tipa). Različite kliničke manifestacije zasnivaju se na određenoj vrsti imunskog odgovora. Naši rezultati pokazali su dominaciju Th1 tipa odgovora nad Th2 tipom odgovora. Proizvodnja IFN-γ bila je veća nego proizvodnja IL-4. Parametri oksidativnog stresa takođe su bili povezani sa dominantnim Th1 tipom imunskog odgovora. Važno je prepoznati mehanizme patogeneze i aktivnost imunskog odgovora kod virusne infekcije. Sve ovo je važno za prognozu, prevenciju i terapiju.

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***Ključne reči**: infekcija virusom herpes simpleks, interferon gama, interleukin 4, imunosistem, deca*

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ERRATUM: CLINICAL FEATURES OF 22q11.2 DELETION SYNDROME: A LITERATURE REVIEW AND CASE SERIES REPORTS (Vol. 63, p. 127, 2024)

Editorial Board of the journal Acta Medica Medianae

In the article *Clinical Features Of 22q11.2 Deletion Syndrome: A Literature Review And Case Series Reports* authored by: Tatjana Stanković, Katarina Harfman Mihajlović, Dragana Lazarević, Karin Vasić, Hristina Stamenković published in Volume 63, issue 3, pages 127–132., in Katarina Harfman Mihiajlović's afiliations, afiliation number 2 should be removed and afiliation number 3 should remain, so Katarina Harfman Mihajlović's afilitaion should be [3]Univeristy of Niš, Faculty of Medicine, doctoral studies, Niš, Serbia.

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