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THE SIGNIFICANCE OF IMPEDANCE AGGREGOMETRY IN CARDIAC SURGERY

Dragan Milić[1,2], Milan Lazarević[1,2], Mladjan Golubović[1,2], Velimir Perić[1,2], Aleksandar Kamenov[1,2], Vladimir Stojiljković[1,2], Marija Stošić[1,2], Saša Živić[2], Isidora Milić[1], Dimitrije Spasić[2]

The function of normal hemostasis is to prevent blood loss from an uninjured blood vessel and to stop excessive bleeding from a damaged blood vessel. Blood loss from an uninjured vessel is prevented by normal vessel structure and normal platelet function. Platelet aggregation is mediated by von Willebrand factor, a polymeric plasma glycoprotein. This protein binds to specific platelet membrane receptors and collagen. Primary aggregation of incoming platelets is facilitated by the action of thrombin. Aggregated platelets then release serotonin, thromboxane A2 and adenosine diphosphate (ADP) which stimulate vasoconstriction which is an additional stimulus for platelet aggregation and represents secondary aggregation. Many factors are related to bleeding during cardiac surgical procedures. Impedance aggregometry is a test of aggregation of platelets in whole blood, which allows us to observe the function of platelets in the presence of erythrocytes and leukocytes and prevents the artificial activation of platelets that occurs due to the separation process. Aggregometry is used to diagnose disorders of platelet function, which are rarely congenital, and most often acquired.

In our research, we proved that 31% of patients had post-operatively impaired platelet function, with postoperative bleeding after 24 hours being statistically significantly higher in patients with ADP < 300 AU/min 24 hours after surgery, as well as TRAP < 500 AU/min 24 hours after surgery (p = 0.002). Twenty-two patients (22.0%) received a platelet transfusion 3 hours after surgery - ADP test ≤ 300 AU/min, ASPI ≤ 400 AU/min, TRAP ≤ 500 AU/min. On average, 11.14 ± 4.45 doses were administered. No patient in this study needed a transfusion of platelets 24 h after the procedure. Contemporary principles such as “time is life” together with modern clinical protocols and experienced personnel are essential in the treatment of hemostatic disorders during cardiac interventions.

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Key words: *platelets, aggregometry, cardiac surgery, bleeding*

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Introduction

Blood coagulation is a complex process that takes place through a strictly regulated sequence of reactions in order to prevent blood loss from the body. The function of normal hemostasis is to prevent blood loss from an uninjured blood vessel and to stop excessive bleeding from a damaged blood vessel. Blood loss from an uninjured blood vessel is prevented by the normal structure of the blood vessel and the normal function of platelets (1). In the event of a blood vessel injury, the body fights to stop the bleeding using three main mechanisms, through three phases: vascular, platelet and blood coagulation phases. During the vascular phase, vasoconstriction of the blood vessel occurs reflexively and it lasts less than a minute, and is prolonged by serotonin from platelets and fibrinopeptide B, which is produced by the action of thrombin on fibrinogen. During the platelet phase, a platelet plug is formed. During this phase, adhesion of platelets occurs at the site of damaged blood vessels and aggregation of platelets with each other. Platelet aggregation is mediated by von Willebrandt factor, a polymeric plasma glycoprotein. This protein binds to specific platelet membrane receptors and collagen. Primary aggregation of incoming platelets is facilitated by the action of thrombin. Aggregated platelets then release serotonin, thromboxane A2 and adenosine diphosphate (ADP), which stimulate vasoconstriction, an additional stimulus for platelet aggregation, and represents secondary aggregation (2).

The coagulation phase leads to the formation of a permanent coagulum that will prevent bleeding until the injured tissue is repaired. Blood coagulum is created within the bifurcation cascade of proteolytic reactions that include nearly twenty different substances, most of which are glycoproteins synthesized in the liver (3). The coagulation system consists of proteins, lipoproteins and calcium ions. All coagulation factors (except factor III) are normally present in plasma.

Knowing the properties of coagulation factors is of particular importance. Stability to preservation *in vitro*, survival in the recipient organism and a hemostatic level of coagulation factors sufficient to prevent the patient from bleeding are essential.

Seven coagulation factors are present in the form of precursors that are proteolytically activated with the help of serine proteases in the coagulation process. Factors V and VIII are not enzyme precursors but cofactors that circulate as "precofactors". Activated forms of precursors and cofactors are marked with the lowercase letter "a". Fibrinogen (factor I) is converted to fibrin that lacks enzymatic and cofactor activities and is designated as fibrin (not factor Ia). The active form of prothrombin (factor II) is more commonly referred to as thrombin rather than factor IIa (4). Blood coagulation takes place through 4 stages:

Phase I - activation of tissue factor (thromboplastin),

Phase II - conversion of prothrombin into thrombin,

III phase - conversion of fibrinogen into fibrin, and

IV phase - coagulum retraction.

Knowledge about coagulation has changed with the progress of science, and a significant contribution was made by Rapaport when he pointed out the fact that the complex of tissue factor and factor VIIa activates factor X and IX, thus simultaneously activating the external and internal pathways of coagulation (5). Today there is a cellular model of coagulation based on the role of platelets, monocytes and endothelium in coagulation. According to this model, coagulation takes place in four stages:

- initiation phase,
- amplification phase,
- propagation phase, and
- termination phase.

At the site of blood vessel injury, tissue factor (TF) is expressed, which forms a complex with FVIIa, which under normal circumstances circulates in small amounts, but in a biologically inactive state until it forms a complex with tissue factor that leads to the activation of factors X and IX (6). Activated factor X activates factor V on the surface of cells that carry tissue factor and the created complex converts a small amount of prothrombin into thrombin, which represents the initiation phase. In the second phase, the generated thrombin leads to the activation of platelets, factors V, VIII, XI and XIII.

In the propagation phase, activated factor IXa with factor VIIIa builds a complex on the surface of platelets that strongly activates factor X. Activated factor Xa with factor Va on the surface of platelets creates a prothrombinase complex that converts significant amounts of prothrombin into thrombin (7). The generated thrombin converts fibrinogen into fibrin, which is stabilized by FXIIIa and becomes an insoluble fibrin clot.

Thrombin also activates thrombin-activated fibrinolysis inhibitor (TAFI) and thus protects the clot from lysis. At the same time, thrombin is inhibited by its potent inhibitor, antithrombin, and further binds to thrombomodulin, which activates the protein C system that neutralizes activated factors V and VIII (8). Activation of tissue pathway inhibitors stops further activation of coagulation by the tissue factor/FVIIa complex—the termination phase.

Many factors are related to bleeding during cardiac surgical procedures. It is usually related to the length of the extracorporeal circulation procedure (over 90 minutes), which involves several mechanisms that lead to bleeding.

Extended platelets contact with the plastic hoses of the extracorporeal blood flow system disturbs their function. The plastic hoses of the extracorporeal circulation system lead to the activation of platelets and the coagulase cascade, which finally manifests itself in the form of postoperative thrombocytopenia for more than 30%, and consumptive coagulopathy. Also, the pumps of the ECC system perform mechanical destruction of the same (9).

Patients undergoing cardiac intervention have overly sensitive platelets due to the fact the almost all patients referred to cardiac surgery procedures are already on dual antiplatelet therapy which impedes normal hemostatic process. Some patients are on anticoagulation therapy which leads to inhibition of factors II, VII, IX, X, preventing successful hemostasis.

The coagulation cascade is activated when the artificial surface of the intestine comes into contact with blood, and this is primarily activated by factor XII, which cascade activates factor XI, and then factor X, which ultimately leads to increased generation of thrombin. Thrombin is a very potent activator of platelets, but also of fibrinogen and the clot polymerization process, leading to their consumption. On the other hand, thrombin also activates the fibrinolysis system through plasmin, which not only breaks down fibrin threads but also affects the function of platelets by degrading their receptors on the surface of the cell membrane, without which platelets cannot fulfil their role in primary and secondary hemostasis. Increased perioperative blood loss leads to a drop in the concentration of coagulation factors and the number of platelets, but also to anemia, which in combination with the

previously mentioned disorders disrupts normal coagulation.

One of the most difficult tasks in cardiac surgery is the establishment of timely, physiological hemostasis. Hemostasis as an extremely complex process is accompanied by disorders that can be classified as acute and chronic. Chronic hemostasis disorders are most often the result of impaired liver and kidney function, impaired hematopoiesis and hereditary hemostasis disorders.

In surgical practice, acute bleeding is caused by acute traumatic bleeding, solution infusion and dilutional coagulopathy, use of heparin, antithrombotics, oral anticoagulant therapy... Cardiac surgery procedures are often related to extensive bleeding and usual tests such as INR, aPTT and PT or platelet count are insufficient and obtained not in a timely manner. In these critical conditions, point of care (POC) devices play a key role in obtaining fast and reliable hemostasis monitoring (10). A large number of powerful devices for the detection of coagulation disorders have been constructed.

Impedance aggregometry is a test of aggregation of platelets in whole blood, which allows us to observe the function of platelets in the presence of erythrocytes and leukocytes and prevents the artificial activation of platelets that occurs due to the separation process. The method of determination in whole blood detects the electrical impedance between small electrodes immersed in the blood (Multiplate® - Multiplate Platelet Function Analyzer, Roche, Germany), and the kinetics of the impedance change reflects platelet aggregation after the addition of agonists. The kinetics of impedance change reflect platelet aggregation on needles after agonist addition.

Aggregometry is used to diagnose disorders of platelet function, which are rarely congenital, and most often acquired. Acquired damage to platelets is most often caused by drugs or is a consequence of uremia. The widely used acetylsalicylic acid and many other nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the platelet enzyme cyclooxygenase, which converts arachidonic acid to thromboxane A2 (TXA2). TXA2 is a platelet agonist with a short half-life. NSAIDs can reversibly damage cyclooxygenase or behave in other damage models. There are also platelet ADP receptor blockers such as clopidogrel, ticlopidine, etc. It is also important to monitor IIb IIIa receptor blockers such as abciximab or tirofiban.

Although the monitoring of antithrombotics is certainly one of the most important roles of this device, we must not leave out the role of the MULTIPLATE analyzer in the preparation of patients for surgical intervention, and monitoring after platelet transfusion (11). As already stated, by adding different powerful platelet agonists, the response of platelets to them is monitored and their function, i.e. inhibition of function, is determined.

Regarding the role of impedance aggregometry in the preparation of patients for surgical intervention, whether they are on mono or dual antiplatelet therapy, testing is important. Based on the residual effect of drugs on platelet function, clinicians declare the possible presence of low, moderate or high risk for increased perioperative microcirculatory bleeding depending on platelet function. Not all patients respond equally to antiplatelet therapy. Several mechanisms have been identified that explain the emergence of resistance to aspirin and clopidrogel:

- Inadequate tolerability of the drug or early cessation of its introduction into the body,
- Possible drug interactions,
- Inadequate dose,
- Increased fluctuation of platelets,
- Genetic polymorphism, and
- Potential bypass mechanisms.

The curves that are detected during the analysis show the speed of aggregation of platelets and the total activity of platelets, so based on their appearance and following the reference values prescribed for each test, platelet function is separately assessed. In combination with elastometry, aggregometry can be a postoperative test and play a role in the detection of bleeding that may be a consequence of impaired platelet function.

Materials and Methods

The study included 100 patients who underwent coronary artery bypass grafting (CABG) at the Clinic of Cardiac Surgery, University Clinical Centre of Niš, during the period from June to December 2018. Twenty-two patients were females and 78 were males. All patients included in the study were preoperatively on mono or dual antiplatelet therapy (acetylsalicylic acid+-clopidogrel/ticagrelor). Antiplatelet therapy was stopped 5−7 days before surgery. CABG was performed in a standard manner. Blood samples were taken for impedance aggregometry 24 hours before operation and 3 hours and 24 hours postoperatively.

To monitor the necessary parameters, the following test were used:

- ASPI test (activation of platelets by arachidonic acid) for monitoring residual effect of acetylsalicylic acid,
- ADP test (activation of platelets by adenosine diphosphate) for measuring residual effects of clopidogrel/ticagrelor on platelet function,
- TRAP test (thrombin-activated platelet function) – for measuring the natural potential of platelets regardless of the antiplatelet therapy.

All these tests were performed on an impedance aggregometer (MULTIPLATE Roch Germany).

Blood sampling was performed in 4 ml test tubes with the anticoagulant Lithium-heparin, and

all analyses were performed within 30 minutes of sampling.

Values of monitored parameters indicating increased risk of bleeding were as follows: ADP test ≤ 310 (reference value range 570−1130) aggregation units per minute (AU/min), ASPI test ≤ 400 AU/min (reference values range 710−1490AU/min) and TRAP test ≤ 500AU/min (reference values range 923−1509 AU/min).

Statistical analysis

Arithmetic mean and standard deviation were used to present data.

The Kolomogor-Smirnov test was used to test the normality of continuous variables. In the case of normal distribution of preoperative and postoperative data, the comparison of values at 3h and 24 h was performed with the ANOVA test for repeated measurements. In the case where distribution of data was not normal, the Friedman test was used for this comparison. If distribution of data was normal, the t test was used for comparison and if data distribution was not normal, Mann-Whitney test was used for comparison.

A significance threshold of $p < 0.05$ was used to test the hypothesis. Data analysis was performed using SPSS 16.0 software.

Results

ADP values decreased in the period up to 3 h compared with preoperative values, and then the values jumped significantly between the last two measurements ($p < 0.001$). ASPI values preoperatively and 3 hours after surgery were close, then in the period up to 24 hours they increased sharply. A statistically significant difference in ASPI values between the three measurements was registered ($p < 0.001$). TRAP values were uniform comparing preoperative and postoperative measurements ($p = 0.783$) (Table 1).

Preoperatively, 13 patients had ADP < 300 AU/min (13.0%), 3 hours after surgery 31 patients had ADP < 300 AU/min (31.0%), and 24 hours after surgery, 5 patients had ADP < 300 AU/min (5.0%) (Figure 1.). TRAP < 500 AU/min was not measured preoperatively. Postoperatively, these values of the TRAP test after 3 hours were measured in 4 patients, and within 24 hours of surgery in five patients.

Preoperatively, 11 people had an ASPI < 400 AU/min (11.0%), 3 hours after surgery, 17 people had an ASPI < 400 AU/min (17.0%), and after 24 hours after surgery, 18 patients had an ASPI < 400 AU/min (18.0%) (Figure 2).

Nine patients (9.0%) had bleeding for more than one day per drain (Figure 3).

Postoperative bleeding after 24 h was statistically significantly higher in patients with ADP values < 300 AU/min 24 h after surgery ($p = 0.002$) (Table 2).

Postoperative bleeding did not differ statistically significantly in relation to ASPI values 24 hours after surgery ($p = 725$) (Table 3).

Postoperative bleeding was statistically significantly higher in patients who had TRAP < 500 AU/min 24 hours after surgery ($p = 0.002$) (Table 4).

Twenty-two patients (22.0%) received a platelet transfusion 3 hours after the operation - ADP test ≤ 300 AU/min, ASPI ≤ 400 AU/min, TRAP ≤ 500 AU/min. On average, 11.14 ± 4.45 doses were administered. No patients needed platelet transfusion 24 h after surgery.

Systemic hemostatic agents desmopressin-acetate (DDAVP) and prothrombin complex concentrate (PCC) were administered in 13 patients three hours following the surgical procedure (one ampoule of DDAVP (20 mcg)) while 7 more patients received the same agent 24 h after CABG.

Table 1. ADP, ASPI and TRAP test values before surgery and 3 h and 24 h after surgery

Parameter☐	Preoperatively	3 h postoperatively	24 h postoperatively	p-value[1]
ADP AU/min	450.19 ± 121.56	358.95 ± 145.91	519.56 ± 179.08	< 0.001
ASPI AU/min	634.81 ± 207.81	669.79 ± 326.50	794.44 ± 323.59	< 0.001
TRAP AU/min	1017.83 ± 193.12	1002.91 ± 261.34	1019.30 ± 234.50	0.783

☐ Arithmetic mean ± standard deviation, [1]Friedman's test

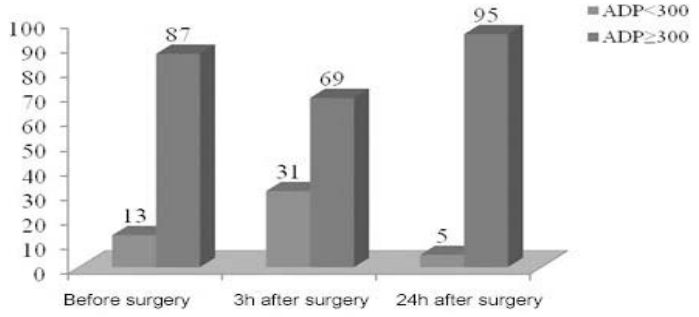

Figure 1. Distribution of patients with low ADP before surgery and 3 h and 24 h after surgery

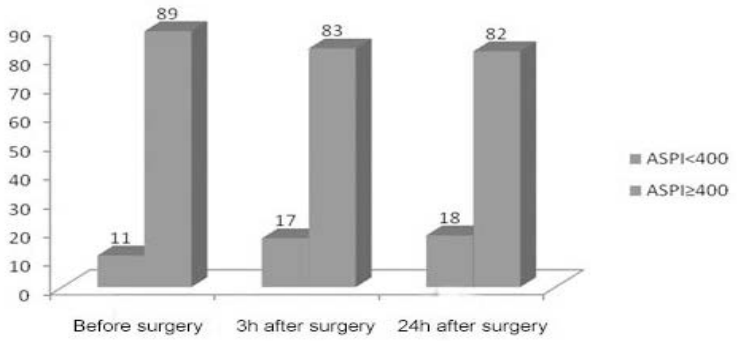

Figure 2. Distribution of patients with low ASPI before surgery and 3 h and 24 h after surgery

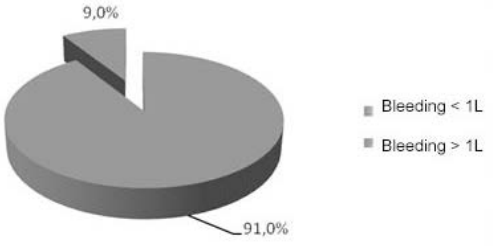

Figure 3. Frequency of drain bleeding ≥ 1 L 24 hours after surgery in the studied patients

Table 2. Postoperative bleeding in relation to low values of ADP parameters

Measurement time ☐	ADP	Postoperative bleeding	
		AS ± SD	p - value[1]
24 h after surgery	< 300	3550.00 ± 1286.47	0.002
	≥ 300	1347.89 ± 319.73	

☐ Arithmetic mean ± standard deviation, [1]Mann–Whitney test

Table 3. Postoperative bleeding in relation to low values of ASPI parameters

Measurement time ☐	ASPI	Postoperative bleeding	
		AS ± SD	p - value[1]
24 h after surgery	< 400	1816.67 ± 1282.58	0.725
	≥ 400	1379.27 ± 323.46	

☐ Arithmetic mean ± standard deviation, [1]Mann–Whitney test

Table 4. Postoperative bleeding in relation to low values of TRAP parameters

Measurement time □	TRAP	Postoperative bleeding	
		AS ± SD	p - value[1]
24 h after surgery	< 500	3550.00 ± 1286.47	0.002
	≥ 500	1347.89 ± 319.73	

☐ Arithmetic mean ± standard deviation, [1]Mann–Whitney test

Discussion

Due to hemodilution as a result of priming, patients undergoing cardiac surgery procedures frequently need a transfusion in order to correct blood loss and coagulopathy after cardiac surgery procedures (12). These patients usually have different comorbidities which may increase the risk of bleeding (13).

Our study demonstrated that decreased activity due to ADP activation was a strong predictor of increased bleeding. It was possible to determine the percentage of platelet inhibition by platelet mapping, subtracting fibrin contribution from the curve and maximizing amplitude due to platelet activators (MAADP). The study proved that either parameter was equally predictive in this dataset. ADP can predict blood loss during the cardiac surgery procedure and the need for blood transfusion. Similarly, MAADP values do have the ability to predict which patients on clopidogrel will need a platelet concentrate transfusion (14, 15, 16). It is clearly demonstrated in this study that ADP testing of patients before and early after performing cardiac surgery interventions is an independent predictor of excessive bleeding.

Using a Multiplate analyzer (ADP, ASPI, TRAP tests) 22% of monitored patients in this study received platelet concentrate. Based on the values of the ASPI test, 20 patients received hemostasis agent desmopressin acetate in order to correct platelet function.

It is very important to emphasize that acetylsalicylic acid and clopidogrel should be discontinued before the planned surgical procedure to reduce the risk of excessive bleeding and the need for blood transfusion (3, 17). No exact data are present in the medical literature describing how many days before surgical procedure antiplatelet drugs should be discontinued (18). However, published data on this issue demonstrates that stopping antiplatelet administration even only 2 days before cardiac surgery procedure significantly reduces the risk of bleeding and the need for platelet concentrate transfusion (19).

The results of our study could not demonstrate any relationship between the discontinuation of antiplatelet drugs and major prothrombotic events (cardiovascular or cerebral).

Old-fashioned surgeons still accept massive blood loss during cardiac surgery procedures as an unchangeable characteristic despite the

improvements in lowering the risk and amount of bleeding using these new techniques (20). Our study also demonstrated that only 9% of patients had an average blood loss of more than 1000 ml probably because of the timely application of hemostatic agents known in the medical literature as targeted hemostasis therapy guided by POC hemostasis testing devices.

Hemodilution is probably the most pronounced factor related to coagulopathy (thrombocytopenia or thrombocytopathy alike) emerging after cardiac surgery procedures playing a significant role in the occurrence of bleeding and excessive blood loss after cardiac surgery operations (21).

In 2011, Gorlinger et al. reviewed retrospectively more than 3000 patients after which they have concluded that implementation of POC devices significantly reduces the need for blood transfusion and thromboembolic complications (6).

In 2012, Weber et al. published the results of a prospective randomized study in which the aim was to study the effects of hemostatic therapy guided by either conventional coagulation assays or POC testing in cardiac surgical patients (22). Patients diagnosed with excessive bleeding after heparin reversal or increased blood loss during the first 24 hours after surgery were randomized into the POC group. POC testing reduced the amount of blood transfusion in comparison to standard laboratory coagulation testing. Even more, POC-guided therapy was associated with reduced use of fresh frozen plasma (FFP) and platelet transfusion leading to lower cost of treatment and better clinical outcome.

POC devices may provide fast and more reliable insight into the hemostasis disbalance creating the treatment tailored for each patient separately. Intensive variations in patient's sensitivity to clopidogrel quiet often results in grossly different tally, necessitating mandatory use of POC before, during and after performing cardiac surgery procedures. This individual approach for each patient could significantly reduce blood loss and need for blood transfusion (23).

Conclusion

Cardiac surgical procedures are complex interventions provoking dramatic hemostatic disorders in many cases. Some patients undergoing cardiac surgery procedures have preoperative hemostatic disorders due to acquired diseases or because they were treated with antiplatelet drugs.

Considering all of these features, the primary goal must be to timely detect possible hemostatic abnormalities in patients undergoing cardiac surgery procedures and to react accordingly.

Our study demonstrated that up to 31% of patients had preoperative and postoperative platelet dysfunction. As a result of these abnormalities bleeding may occur leading even to the death of the patient.

POC devices have an extremely important clinical significance in detecting coagulation disorders in a timely manner predicting excessive bleeding. In this way, the implementation of targeted hemostasis therapy can prevent or even stop the bleeding after a surgical procedure.

Using these protocols in testing platelet function with POC devices, the ease of use and the very short time needed for gaining results were key contributors to the low mortality rate of only 1% in the observed group of patients (not a consequence of hemostasis disorders).

The contemporary approach combining well-established clinical protocols, and the experience of the personnel, respecting the principle "time is life", provides excellent results in the treatment of hemostasis disorders in cardiac surgery patients.

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ZNAČAJ IMPEDANTNE AGREGOMETRIJE U KARDIOHIRURGIJI

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Hemostaza je složen biološki proces kojim se sprečava gubitak krvi iz neoštećenog krvnog suda, kao i prekomerni gubitak cirkulišuće krvi kod povređenog krvnog suda. Za normalno funkcionisanje hemostaze neophodno je da krvni sud ima histološki normalnu građu zida, kao da je i funkcija trombocita očuvana. Von Willebrandov faktor predstavlja ključni činilac agregacije trombocita. Ovaj glikoprotein plazme vezuje se za receptore trombocita i kolagen. Trombin olakšava primarnu agregaciju prisutnih trombocita, koji nakon toga oslobađaju adenozin-difosfat (ADP), tromboksan A2 i serotonin; svi oni zajedno izazivaju posledičnu vazokonstrikciju, koja dovodi do sekundarne agregacije trombocita. Krvarenje nakon aortokoronarnog bajpasa posledica je delovanja brojnih faktora. Test agregacije trombocita u celoj krvi (impedantna agregometrija) omogućava analizu funkcije trombocita u prisustvu leukocita i eritrocita. Ova metoda se koristi za dijagnostikovanje oštećene funkcije trombocita, koja je uglavnom stečenog, a izuzetno retko urođenog karaktera.

Naše istraživanje pokazalo je da je 31% bolesnika imao postoperativno poremećenu funkciju trombocita, s tim što je postoperativno krvarenje posle 24 sata bilo statistički značajno veće kod bolesnika sa vrednostima ADP < 300 AU/min 24 sata nakon operacije, kao i TRAP < 500 AU/min 24 sata posle operacije (p = 0,002). Tri sata nakon operacije, transfuziju trombocita primila su 22 bolesnika (22,0%): ADP test ≤ 300 AU/min, ASPI ≤ 400 AU/min, TRAP ≤ 500 AU/min. Prosečno je davano 11,14 ± 4,45 doza. Dvadeset četiri časa nakon intervencije nije bilo bolesnika kojima je bila potrebna transfuzija koncentrata trombocita.

Upotreba savremenih metoda, u kombinaciji sa dokazanim kliničkim protokolima i velikim kliničkim iskustvom osoblja i uz poštovanje principa „vreme je život", omogućava najbolje moguće zbrinjavanje bolesnika sa detektovanim poremećajem hemostaze u kardiohirurgiji.

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***Ključne reči**: trombociti, agregometrija, kardiohirurgija, krvarenje*

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PHARMACEUTICAL CARE IN COMMUNITY PHARMACIES DURING COVID-19 PANDEMIC: PHARMACISTS EXPERIENCE IN SERBIA

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The importance of the pharmacist's role in health care has become more pronounced during the COVID-19 pandemic as they were accessible and frontline healthcare professionals, who significantly contributed to ensuring uninterrupted access to essential healthcare services. This research aimed to examine the experiences of community pharmacists regarding the quality and extent of pharmaceutical care and patient counseling during the COVID-19 pandemic in relation to the pre-pandemic period. Also, this research aimed to investigate the informative role of community pharmacists considering the COVID-19 pandemic (possibility of infection, symptoms, protection, treatment, supplementation, etc.). In addition, the study examined whether the need for counseling and pharmacy services was greater during the period of high infection rates or not. A cross-sectional, quantitative study was conducted among community pharmacists, who accessed and filled out the online survey anonymously and voluntarily through a link on the Google platform. The obtained results showed that pharmacists who worked near COVID-19 clinics or hospitals more likely perceived that pharmaceutical care was significantly or completely compromised during the pandemic compared to other community pharmacists (38.78% *vs*. 21.88%, p = 0.005), while 40% of all respondents said that adequate information was provided to most patients compared to usual practice, including additional information they requested. Of all, 86% of pharmacists reported that many patients required additional information about COVID-19. The findings suggested that pharmacists who worked near COVID-19 clinics or hospitals perceived a lower increase in the requirement for counseling and pharmacy services during high infection rates periods compared to other pharmacists (53% *vs*. 69%, p < 0.001).

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Key words: *pharmaceutical care, patient counseling, community pharmacy, COVID-19*

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Introduction

The discovery of a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), in China at the end of 2019 led to the emergence of Coronavirus Disease 2019 (COVID-19). The declaration of the COVID-19 pandemic has brought major changes in the lives of people worldwide. Also, the pandemic has had a profound impact on healthcare systems across the globe, which had to be reorganized to address all the tasks and challenges posed by the pandemic (1, 2). Considering these circumstances, the importance of the pharmacist's role has become more pronounced, as they were accessible and frontline healthcare professionals, who significantly contributed to ensuring uninterrupted access to essential healthcare services (3). They have played a vital role in educating and supporting patients in various aspects of COVID-19, including prevention measures, disease control, symptoms, prescribed therapy, particularly antimicrobial treatment, supplementation, and vaccination process as well as provided them with reliable and timely information to counter the large number of contradictory and unverified sources (4–6). The uncertainties in the treatment of COVID-19 increased the use of over-the-counter (OTC) drugs and made patients look for alternative treatment options and natural health products. Therefore, pharmacists secured adequate supplies of drugs and other products but also guided patients on self-care, self-medication, and medication

adherence during these challenging times (7, 8). Furthermore, community pharmacists have contributed widely by providing pharmaceutical care and counseling to individuals with chronic diseases who were unable to visit their primary care physicians or general practitioners, especially during the early period of the pandemic. The concept of pharmaceutical care involves services and functions of community pharmacists towards promoting and supporting rational drug use and patient counseling as an imperative (9). In addition, the work of physicians was reorganized during the pandemic. Many doctors have been designated in the „red zone“, while certain clinics and hospital wards were transformed into specialized COVID clinics or hospitals. This situation made it very difficult for many patients to access preferred doctors and necessary healthcare, particularly the elderly population (4, 10). The International Pharmaceutical Federation (FIP) underlined the significance of community pharmacists during the pandemic and published guidelines to clarify pharmacists' roles in the healthcare system (5, 11, 12). This research aimed to examine the experiences of community pharmacists regarding the quality and extent of pharmaceutical care and patient counseling during the COVID-19 pandemic in relation to the pre-pandemic period. Also, this research aimed to investigate the informative role of community pharmacists considering the COVID-19 pandemic (possibility of infection, symptoms, protection, treatment, supplementation, etc.). In addition, the study examined whether the need for counseling and pharmacy services was greater during the period of high infection rates or not.

Material and Methods

Study design. A quantitative, non-experimental, cross-sectional study was conducted between February and June 2023 among community pharmacists in Serbia. The questions within the survey were in Serbian, closed-ended, mostly with a choice of one answer, while some were open-ended and contingency questions. Community pharmacists were able to access and fill out the online survey questionnaire anonymously and voluntarily through a link on the Google platform. The study protocols were approved by the Ethics Committee of the Faculty of Medicine, University of Niš (No 12-1258-2/2 from 2 February 2023).

Respondents. The respondents were pharmacists working in the community pharmacies in the Republic of Serbia territory. The inclusion criteria were employment in a community pharmacy before and during the COVID-19 pandemic as a licensed pharmacist. The exclusion criterion was two or more completed questionnaires by one respondent. This was ensured by response matching analysis. The respondents were contacted directly, through their employer service, or through professional organizations. The respondents did not receive any incentive for participating in the study. Of all approached pharmacists, 232 in total were enrolled based on the inclusion criteria and their willingness to answer questions from the survey. The respondents were not obligated to answer each question from the survey. Personal data of enrolled respondents (name and surname, city, e-mail and IP address, racial, religious and ethnic affiliation, and sexual orientation) were not collected. Demographic data and other respondents' answers were presented as a group, without respondents' personal data. An electronic database of respondents was formed. Access to the data in the electronic database was only granted to the principal investigator or a research team member designated by the principal investigator due to statistical analysis.

Survey development. The first phase of the survey development included question selection based on the previous research and personal experience of the research team. The first version of the survey had 21 questions. The second phase of the survey development was a focus group interview aimed to further define the survey. The focus group was composed of 4 members: a principal investigator (Ph.D. Pharmacotherapy Specialist) and three experts in the field of pharmacology, pharmacotherapy, phytotherapy, clinical pharmacy, and pharmaceutical practice. Their task was to determine the content of the survey. Some questions were reformulated during the expert panel discussion, and some were added. The pre-final version of the survey included 23 questions grouped in 5 domains. The third phase was meant to test the validity and adequacy of the questions. In order to make the terms used in the survey clear to the target population, an additional interview was conducted with 10 pharmacists from the practice. The respondents were asked to comment on the precision and clarity of all included questions within the survey. Hence, the final version of the survey was created.

Structure and content of the survey. A description and aim of the study were presented at the beginning of the survey. The participant's agreement to fill out the online survey was considered as giving consent to participate in the research. The survey consisted of 5 domains. Domain 1 included questions regarding the demographic characteristics of the participants, such as gender, age, professional experience, the proximity of the pharmacy in relation to the COVID clinic/hospital, etc. Domain 2 gathered data on the pharmacists' perception regarding the quality of pharmaceutical care and patient counseling, including chronic diseases during the pandemic. Domain 3 consisted of patient counseling regarding the pandemic itself and the immunization process against SARS-CoV-2. Domain 4 referred to the use of dietary supplements and OTC preparations during the COVID-19 pandemic from the perspective of pharmacists. Domain 5 consisted of questions

related to the desire of patients to obtain antibiotics without a prescription, the difference in patient counseling during pandemic waves and between waves, as well as the perception of pharmacists regarding how their role was recognized during pandemics. Questions from Domains 4 and some of the questions from Domains 3 and 5 were not the subject of the analysis in this paper. The survey instrument is presented in Appendix A.

Statistical analysis. Data is presented as absolute and relative numbers (percentages) for categorical variables and as mean ± standard deviation and median (interquartile range) for continuous variables. The $\chi 2$ (Chi-square) independence test was performed to compare categorical variables between defined groups of pharmacists. The significance level was set at $p < 0.05$ for all analyses. Statistical analysis was done using the IBM SPSS Statistics ver. 22.

Results

The characteristics of the study population are given in Table 1.

Of all enrolled community pharmacists, 89% were women, whereas 79% were master or graduated pharmacists (five-year integrated studies) and 21% had academic/professional specialization or doctorate. The median age of the respondents was 34 years with a median professional experience of 8 years. In addition, 64% of the pharmacists worked in the city of more than 100,000 inhabitants, while 2 out of 5 pharmacists worked in the community pharmacy near a COVID-19 clinic or hospital. In further analysis, patients were divided into two groups based on the vicinity of the COVID-19 clinics or hospitals, pharmacists who worked near the COVID-19 clinic/hospital and those who did not.

For the purpose of this study, community pharmacists were asked to share their experiences and compare the range of their activities before and during the COVID-19 pandemic period. Table 2 provides the pharmacists' experience regarding the quality of pharmaceutical care during the COVID-19 pandemic in relation to the pre-pandemic period.

Table 1. Characteristics of the study population

Characteristic	Value
Sample size	232 (100%)
Sex (female/male)	206/26 (88.8%/11.2%)
Age (years)	35.52 ± 8.05 34 (28-38)
Work experience (years)	9.81 ± 7.38 8 (3-13)
Education (licensed pharmacist/pharmacist with specialization or doctorate)	184/48 (79.3%/20.7%)
The community pharmacy is located in: a town/municipality with up to 10,000 inhabitants a city/municipality between 10,000 and 50,000 inhabitants a city between 50,000 and 100,000 inhabitants a city with over 100,000 inhabitants	 20 (8.6%) 30 (12.9%) 34 (14.7%) 148 (63.8%)
The pharmacy is located near a COVID-19 clinic or hospital: Yes No	 98 (42.2%) 134 (57.8%)

Results are presented as number (frequency) or mean±standard deviation and median (interquartile range)

Table 2. Comparison of pharmaceutical care in community pharmacies before and during the pandemic from the pharmacists' perspective

How do you evaluate the impact of the COVID-19 pandemic on the quality of pharmaceutical care services in a public pharmacy?	Entire study group (n = 226)*	Pharmacist-near COVID-19 clinic or hospital (98)	Pharmacist-not near COVID-19 clinic or hospital (128)	Test and sig.
Improved	58 (25.66%)	22 (22.45%)	36 (28.12%)	Pearson Chi-square = 12.827; p = 0.005
Slightly compromised	72 (31.86%)	32 (32.65%)	40 (31.25%)	
Significantly or completely compromised	66 (29.20%)	38 (38.78%)	28 (21.88%)	
It did not have an impact	30 (13.28%)	6 (6.12%)	24 (18.75%)	

Results are presented as a number and percentage of pharmacists (*6 out of 232 pharmacists did not answer this question).

The obtained results showed that pharmacists who worked near COVID-19 clinics or hospitals rather significantly perceived that pharmaceutical care was significantly or completely compromised during the pandemic compared to other community pharmacists (38.78% vs. 21.88%, p = 0.005). Also, community pharmacists were asked about the quality (scope) of patient counseling in the community pharmacy before and during the pandemic. Pharmacists should have considered quality in terms of duration, quantity of gathered/provided information, and/or patients' information needs (Table 3.)

Although there was an evident difference in the perception of patient counseling quality (scope) between pharmacists who worked near COVID-19 clinics or hospitals and those who did not (statements 2 and 5, a difference of 8.6% and 10.4%, respectively), a statistically significant difference was not reached.

Considering the number of non-COVID patients seeking pharmacist advice, the obtained results showed that the entire study population perceived an increased number of non-COVID patients requiring patient counseling/medication review during the pandemic compared to the pre-pandemic period. Still, pharmacists did not differ in relation to pharmacy proximity to COVID-19 clinic: N = 230 respondents 2 missing answers; Pearson Chi-square = 0.790; p = 0.674) (Figure 1).

Furthermore, community pharmacists were asked to rate whether or not there was a change in frequency regarding patient counseling on chronic diseases (Table 4).

Analyzing the obtained results, 40−50% of surveyed pharmacists perceived that the frequency of counseling was slightly or significantly increased compared to pre-pandemic in cases of cardiovascular, respiratory (even 75% of enrolled pharmacists), diabetes, neurological, psychiatric, and musculoskeletal disease. Considering kidney diseases, more than 82% of pharmacists thought there was no change or the number of patients who required advice decreased.

Table 3. Quality (scope) of patient counseling process before and during the pandemic form the community pharmacists' perspective

Do you believe that the quality (scope) of patient counseling processes in the pharmacy has changed during the COVID-19 pandemic compared to the pre-pandemic period?	Entire study group (n = 232)	Pharmacist-near COVID-19 clinic or hospital (98)	Pharmacist-not near COVID-19 clinic or hospital (134)	Test and sig.
Patient counseling was mostly comparable to the pre-pandemic period.	16 (6.90%)	4 (4.08%)	12 (8.96%)	Pearson Chi-square = 6.624; p = 0.157
Adequate information was provided to most patients compared to usual practice, including additional information they requested.	92 (39.66%)	34 (34.69%)	58 (43.28%)	
Adequate information was provided to most patients compared to usual practice, but their increased information demands were not adequately addressed.	26 (11.21%)	14 (14.29%)	12 (8.86%)	
Most patients received less information compared to usual practice.	22 (9.48%)	8 (8.16%)	14 (10.45%)	
Depending on the individual situation, some patients received less information compared to usual practice, while others received additional information if they requested it.	76 (32.76%)	38 (38.78%)	38 (28.36%)	

Results are presented as a number and percentage of pharmacists

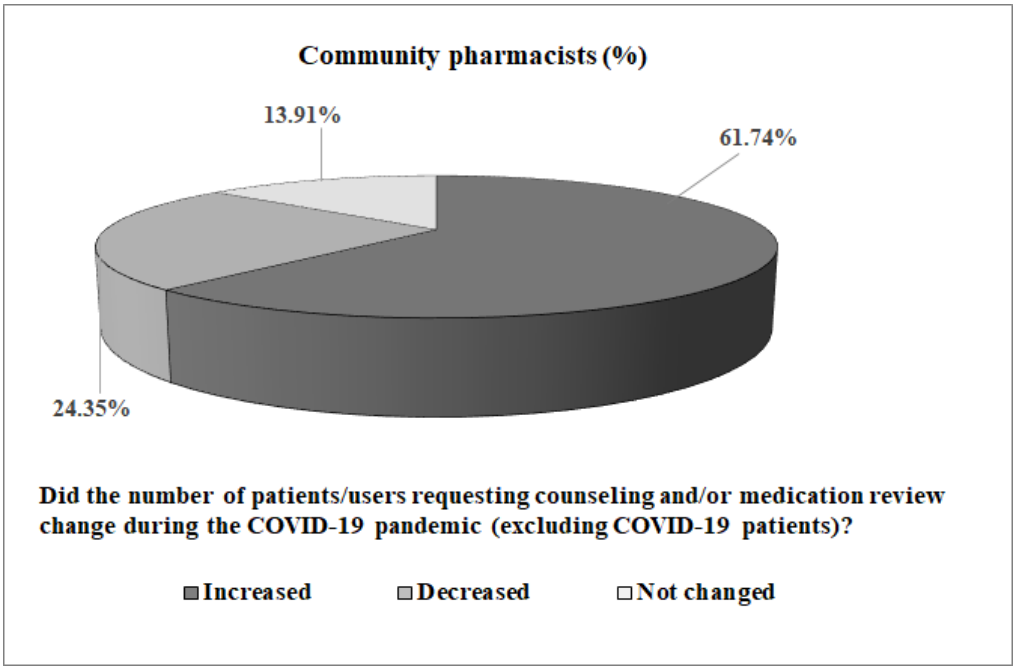

Figure 1. Comparison of the number of non-COVID patients requiring patient counseling/medication review before and during the pandemic from the pharmacists' perspective

Table 4. Community pharmacists' perception of change in the frequency of counseling for patients with chronic diseases

Did the frequency of counseling for patients with chronic diseases change during the COVID-19 pandemic?	Significantly decreased	slightly decreased	no change	slightly increased	significantly increased
Cardiovascular diseases	5.22	21.74	24.35	25.22	23.48
Respiratory diseases	0.88	13.16	10.53	26.32	49.12
Diabetes and other endocrine disorders	4.39	18.42	29.82	25.44	21.93
Anemia and other endocrine disorders	8.77	20.18	38.60	23.68	8.77
Neurological diseases	6.09	20.87	32.17	26.09	14.78
Psychiatric diseases	11.40	14.91	29.82	21.93	21.93
Musculoskeletal system disorders	6.96	17.39	33.04	26.09	16.52
Kidney diseases	10.53	27.19	44.74	12.28	5.26

Results are presented as percentage of pharmacists who chose one out of five given statement

In addition, pharmacists were asked about their perception of whether or not patients were seeking information regarding the COVID-19 pandemic (possibility of infection, symptoms, protection, treatment, supplementation, etc.) (Figure 2).

Considering COVID-19, 86% of pharmacists had an impression that a large number of patients required additional information in the pharmacy during the pandemic. The obtained results did not show a difference in relation to the proximity of the community pharmacy to the COVID-19 clinic/hospital (Pearson Chi-square = 2.692; $p = 0.260$). Furthermore, pharmacists were asked to assess whether the need for counseling and pharmacy services was greater during the period of high infection rates ("pandemic wave") or between the pandemic "waves"?

The results of the study showed that pharmacists who worked near COVID-19 clinics or hospitals perceived a lower increase in the requirement for counseling and pharmacy services during periods characterized by high infection rates compared to other pharmacists (53% vs. 69%, $p < 0.001$).

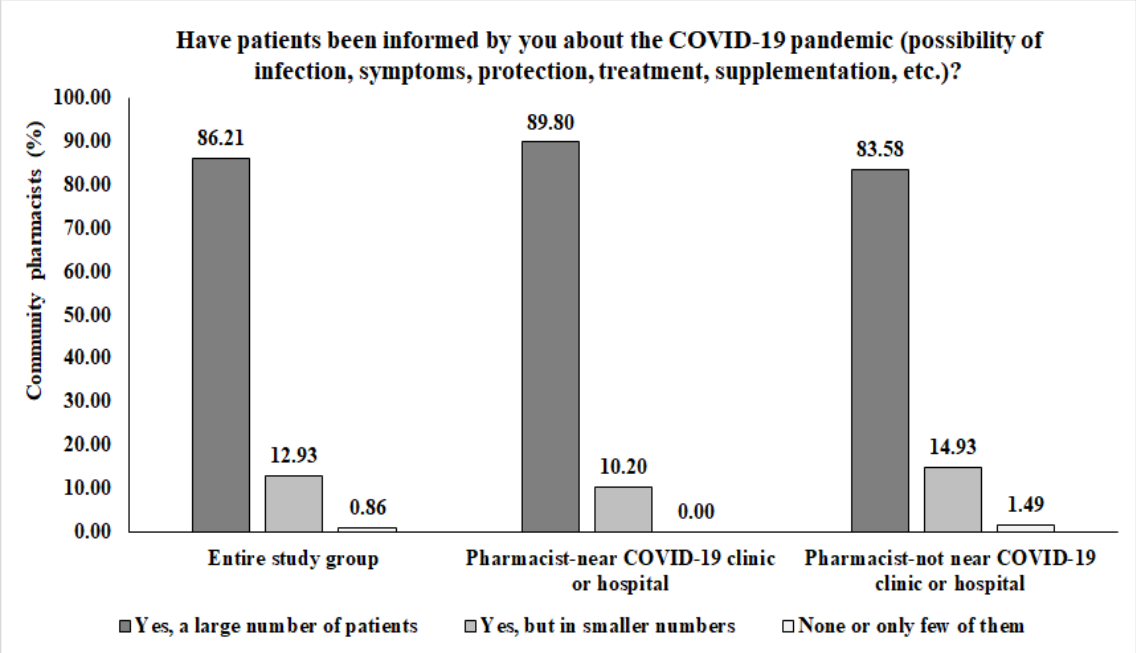

Figure 2. Pharmacists' perception regarding the number of patients seeking advice about the COVID-19 pandemic

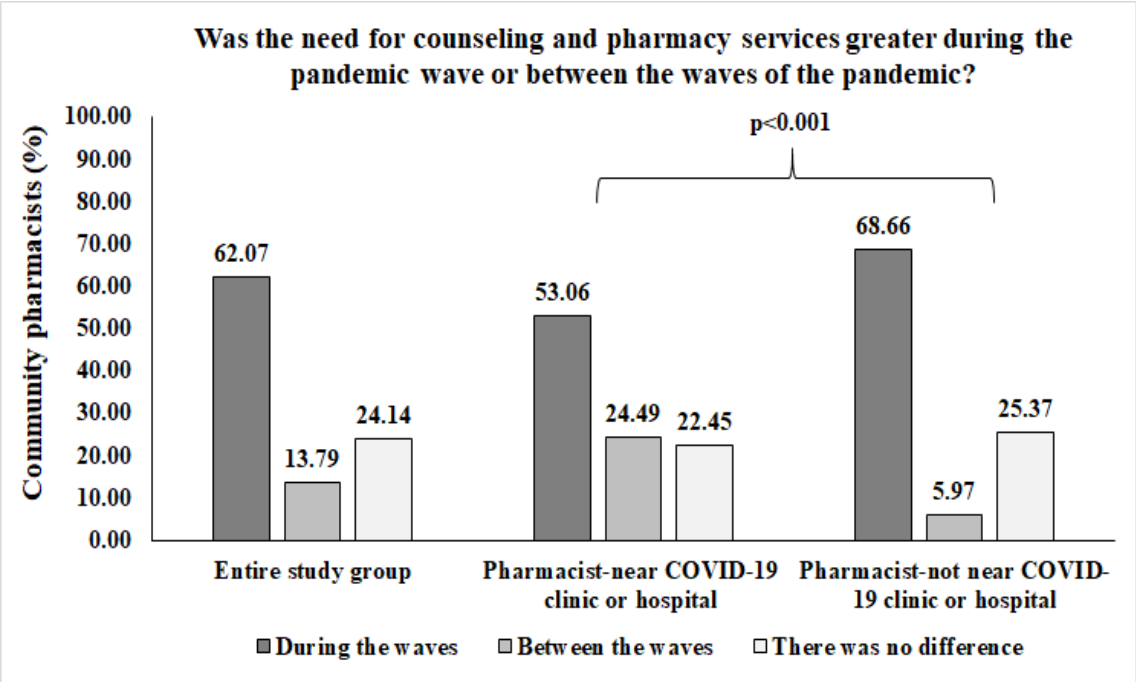

Figure 3. Pharmacists' perception of the requirement for counseling and pharmacy services during periods characterized by high infection rates

Discussion

During the COVID-19 pandemic, healthcare services in Europe, including Serbia, faced significant challenges. Visacri et al. highlighted several key responsibilities of pharmacists during the pandemic, such as prevention and infection control, provision, storage, and supply of personal protective equipment (PPE) and drugs, patient care, and support for other healthcare professionals (4). Community pharmacists played a pivotal role in directly addressing the challenges

posed by COVID-19. They ensured the dissemination of information and education regarding personal and environmental hygiene, supplied and dispensed drugs, and implemented anti-COVID-19 measures within their pharmacies to create a safe environment for pharmacists, pharmacy technicians, and patients (10). In the initial phase of the pandemic and during lockdown in Serbia, routine check-ups and medication reviews provided by chosen physicians were postponed, while prescription validity and duration were automatically extended for three months (13). Moreover, many primary health centers and hospitals were transformed into COVID-19 clinics, posing challenges for patients to access proper care, particularly for the elderly. Consequently, community pharmacists often became the primary source of reliable information and advice on medications for both COVID-19 and non-COVID-19 patients (9). According to the findings of this study, approximately 30% of the enrolled community pharmacists reported a significant or complete decline in the quality of pharmaceutical care during the COVID-19 pandemic compared to the pre-pandemic period (Table 2). The percentage was significantly higher among pharmacists working near COVID-19 clinics or hospitals in comparison to others. This can be attributed to the overwhelming number of COVID-19 patients they had to deal with on a daily basis, which likely resulted in a decline in the quality of other pharmacy activities, such as counseling for non-COVID patients. However, it is important to emphasize that pharmaceutical care remains crucial, particularly for patients with chronic diseases, and its relevance was further amplified during the COVID-19 pandemic due to drug-related concerns and problems, which were observed in many patients (9). In a photovoice study conducted by Watson et al., the roles and experiences of 21 community pharmacists during the COVID-19 pandemic were examined (14). The participants highlighted the information role of community pharmacists, emphasizing their accessibility to the public. However, it was observed that the demand for pharmacists' advice increased exponentially throughout the pandemic. As a result, the participants felt that their information role took precedence over their other duties and activities, such as proper patient-centered medication reviews and care planning for their patients (14). It appears that providing information and patient counseling were deemed crucial and time-consuming activities for community pharmacists during the pandemic. Furthermore, the present study revealed some differences, although not statistically significant, between pharmacists working near COVID-19 clinics or hospitals and those who did not (Table 3). In addition, the present study found that only 35% of pharmacists who worked near COVID-19 clinics or hospitals believed that adequate information was provided to most patients, including additional information requested by patients. Comparatively, 43% of pharmacists who did not work near COVID-19 clinics or hospitals shared the same opinion. This finding is in accordance with the earlier observation regarding the quality of pharmaceutical care.

Previous studies indicated that COVID-19 tests performed by pharmacists were also time-consuming, which could have potentially affected regular pharmaceutical activities (15). Another study by Georgina Silva-Suárez et al., which involved 302 community pharmacists in Puerto Rico, revealed that most participants considered patient education as their primary role during the pandemic and viewed themselves as a reliable source of accurate information (16). In addition, Kanaani et al. suggested that the increased workload, shortage of supplies, and frequent updates on COVID-19 management put significant pressure on community pharmacists' duties in Australia (17). Consistent with these findings, our study indicated that nearly 62% of all participating pharmacists perceived an increased number of non-COVID patients requiring patient counseling and medication review.

Furthermore, our survey showed that 40% or more of the pharmacists reported a slight or significant increase in the frequency of counseling for patients with specific chronic conditions, including cardiovascular, respiratory, endocrine, neurological, psychiatric, and musculoskeletal diseases. However, over 82% of pharmacists reported no change or a decrease in the number of patients requiring advice related to kidney disease. It is important to note that patients with chronic diseases are at a higher risk during the pandemic, as COVID-19 is best combatted by a strong immune system (13).

Studies have demonstrated the positive impact of pharmacist-led interventions during the COVID-19 pandemic. For instance, Li et al. showed that pharmacist-led telemedicine medication management for hypertension resulted in better blood pressure control and improved medication adherence compared to usual care, leading to a reduction in adverse cardiovascular events (18). Similarly, a study conducted in Italy found that pharmacists working in high COVID-19 incidence regions provided more information on symptomatic medications for respiratory tract infections, such as antitussives and antipyretics, compared to other regions. These pharmacists also received a higher number of requests for information related to blood oxygen meters (19). Ahmed et al. demonstrated that pharmacist-led telemedicine activities could lead to optimal outcomes in diabetic patients during the COVID-19 pandemic (20). Still, during the pandemic pharmacists offered accessibility, weighing patient outcomes against what they can do, as well as building trust and relationships with patients and feeling pressured to provide new services (21). In accordance with our study, whereas 86% of pharmacists perceived patients required additional information about COVID-19, Pantasri reported that 96.4% of surveyed pharmacists educated their patients about COVID-19 prevention and treatment (22). Similarly, in the paper of Merks, et al. the corresponding number was 64.5% (23).

Over 80% of community pharmacists questioned in the recent research reported an increasing number of consultations regarding COVID-19 management (24). The results of our study showed that counseling and pharmacy services were significantly higher during pandemic waves, particularly in the pharmacies that were not near COVID-19 clinics or hospitals. The single study we found available for comparison demonstrated that seeking COVID-related information was mainly increased during the first two periods of the pandemic (from May 2020 to September 2020) (25). The observed disparity between pharmacies in the vicinity of COVID-19 clinics or hospitals could have been potentially attributed to the consistently high workload experienced by pharmacies near such clinics, as the pandemic persisted, and those clinics or hospitals remained operational.

The study has a few limitations that should be acknowledged. The small number of participants may limit the generalizability of the results. However, this is an ongoing study, and it is expected that more participants will be enrolled in the future. In addition, the study primarily captures pharmacists' subjective experiences, which can introduce some degree of subjectivity and potential bias. However, the data analysis and presentation are done at the group level, focusing on overall trends and patterns rather than individual experiences. Another limiting factor was information bias due to recollection.

Conclusion

In conclusion, the role of community pharmacists emerged as crucial in the frontline response to the COVID-19 pandemic. Furthermore, community pharmacists experienced an increased workload due to COVID-19 and the additional care for non-COVID patients. Of all, 60% of the pharmacists reported a slight or significant decline in the quality of pharmaceutical care during COVID-19, while 40% of all respondents said that adequate information was provided to most patients compared to usual practice, including additional information they requested. The conducted study demonstrated that 86% of pharmacists perceived that patients required additional information about COVID-19. The findings suggested that pharmacists who worked near COVID-19 clinics or hospitals perceived a lower increase in the requirement for counseling and pharmacy services during periods of high infection rates compared to other pharmacists.

Appendix A. Online survey: „Pharmaceutical care during the COVID-19 pandemic“

Dear colleagues,

In front of you is a survey with questions related to pharmaceutical care during the COVID-19 pandemic. The aim of this research is to assess the role of pharmacists in public pharmacies, their activities, and the scope of those activities compared to the period before the pandemic. This research is part of the scientific research work of the professors and associates from the Integrated Academic Studies of Pharmacy at the Faculty of Medicine, University of Niš.

We appreciate your time in completing the survey.

Questions

1. Gender:

a. Male

b. Female

2. Age (enter):

3. Years of professional experience (enter):

4. Education:

a. Graduated Pharmacist/Master of Pharmacy

b. Graduated Pharmacist/Master of Pharmacy with specialization (academic/health) or doctorate

5. I work in a pharmacy located in:

a. a town/municipality with up to 10,000 inhabitants

b. a city/municipality with up to 50,000 inhabitants

c. a city with up to 100,000 inhabitants

d. a city with over 100,000 inhabitants

6. The pharmacy is located near a COVID clinic/hospital:

a. Yes

b. No

7. How do you evaluate the impact of the COVID-19 pandemic on the quality of pharmaceutical care services in a public pharmacy?

a. It did not have an impact on the quality of pharmaceutical healthcare services.

b. The quality of pharmaceutical care services was slightly compromised.

c. The quality of pharmaceutical care services was significantly compromised.

d. The quality of pharmaceutical care services was completely compromised/changed.

e. The quality of pharmaceutical care services improved.

8. Do you believe that the quality (scope) of patient counseling processes in the pharmacy has changed during the COVID-19 pandemic compared to the pre-pandemic period? NOTE: You can consider the quality in terms of duration, quantity of gathered/provided information, and/or patients' information needs. Please circle one answer.

a. Patient counseling was mostly comparable to the pre-pandemic period.

b. Adequate information was provided to most patients compared to usual practice, but their increased information demands were not adequately addressed.

c. Most patients received less information compared to usual practice.

d. Adequate information was provided to most patients compared to usual practice, including additional information they requested.

e. Depending on the individual situation, some patients received less information compared to usual practice, while others received additional information if they requested it.

9. Did the number of patients/users requesting counseling and/or medication review change during the COVID-19 pandemic (excluding COVID-19 patients)?

a. There was no change in the number of non-COVID patients.

b. There was an increase in the number of non-COVID patients requesting counseling/medication review.

c. There was a decrease in the number of non-COVID patients requesting counseling/medication review.

10. During the COVID-19 pandemic, were there any changes in the frequency of counseling for patients with chronic illnesses? Rate the frequency on a scale of 1-5, where 1 - significantly decreased, 2 - slightly decreased, 3 - no change, 4 - slightly increased, 5 - significantly increased:

11. Did the frequency of counseling for patients with chronic illnesses change during the COVID-19 pandemic? Rate the frequency on a scale of 1-5:

Cardiovascular diseases

Respiratory diseases

Diabetes and other endocrine disorders

Anemia and other endocrine disorders

Neurological diseases

Psychiatric diseases

Musculoskeletal system disorders

Kidney diseases

12. Have patients been informed by you about the COVID-19 pandemic (possibility of infection, symptoms, protection, treatment, supplementation, etc.)?

a. Yes, a large number of patients.

b. Yes, but in smaller numbers.

c. None or only few of them.

13. Have patients been informed by you about the importance of immunization against SARS-CoV-2?

a. Yes, a large number of patients.

b. Yes, but in smaller numbers.

c. None or only few of them.

14. Have patients been informed by you about the choice of SARS-CoV-2 vaccine?

a. Yes, a large number of patients.

b. Yes, but in smaller numbers.

c. None or only few of them.

Have patients been informed by you about the side effects of the SARS-CoV-2 vaccine?

a. Yes, a large number of patients.

b. Yes, but in smaller numbers.

c. None or only few of them.

15. Do you believe that patients have been using over-the-counter (OTC) products and/or dietary supplements to a greater extent during the COVID-19 pandemic compared to the period before the pandemic?

a. Yes, to a significant extent.

b. Yes, but not to the extent it may seem.

c. No.

If the answer to the previous question was a or b, please proceed and answer the next question.

16. What, in your opinion, has had the most significant impact on the increased use of OTC medications/dietary supplements during the COVID-19 pandemic?

a. Influence of media, family, friends, etc.

b. Recommendation of doctors (after examination in a COVID clinic)

c. Recommendation of pharmacists at the pharmacy

d. Fear for one's own health and the health of loved ones

17. What do you think has had the most significant impact on the choice of a specific OTC medication/dietary supplement during the COVID-19 pandemic?

a. Influence of media

b. Influence of family, friends, colleagues, etc.

c. Recommendation of doctors (after examination in a COVID clinic)

d. Recommendation of pharmacists at the pharmacy

18. According to you, has the influence of media on the choice of OTC medication/dietary supplement changed compared to the period before the COVID-19 pandemic?

a. It has increased.

b. It has decreased.

c. There is no significant change or it doesn't exist.

19. Have patients sought your advice on the proper use and potential interactions of OTC medications/dietary supplements during the COVID-19 pandemic?

a. More frequently than before the pandemic

b. Slightly more frequently than before the pandemic

c. No difference in the period before or during the pandemic

d. Less frequently than before the pandemic

20. Which OTC medications/dietary supplements were most commonly used during the COVID-19 pandemic? Select up to three answers.

a. Vitamins and minerals

b. Antihistamines

c. Non-opioid analgesics, antipyretics

d. Topical and systemic nasal decongestants

e. Expectorants and mucolytics

f. Non-opioid antitussives

g. Dental and oral care products

h. Antacids

i. Digestive aids

j. Laxatives

k. Antidiarrheals

l. Topical antimicrobials

m. Medications for local treatment of venous diseases

n. Medications for vaginal therapy

o. Ophthalmic vasoconstrictors

p. Herbal preparations, herbal teas

21 To what extent have you encountered patients intending to purchase antibiotics at the pharmacy without a doctor's prescription/medical report?

a. More frequently than before the pandemic

b. Slightly more frequently than before the pandemic

c. No difference compared to the period before or during the pandemic

22. In your opinion, was the need for counseling and pharmacy services greater during the period of high infection rates (during the pandemic wave) or between the waves of the pandemic?

a. There was no difference

b. During the waves

c. Between the waves

23. Rate the following statement on a scale of 1-5, where 1 - strongly disagree, 2 - mostly disagree, 3 - neither agree nor disagree, 4 - mostly agree, 5 - strongly agree:

The role and importance of pharmacists during the pandemic should have been more recognized by our healthcare system.

1 2 3 4 5

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FARMACEUTSKA ZDRAVSTVENA ZAŠTITA U JAVNIM APOTEKAMA TOKOM PANDEMIJE COVID-19: ISKUSTVO FARMACEUTA U SRBIJI

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Važnost uloge farmaceuta u zdravstvenoj zaštiti postala je dodatno izražena tokom pandemije COVID-19; kao profesionalci dostupni na prvoj liniji zdravstvene zaštite, značajno su doprineli da se nesmetani pristup osnovnim zdravstvenim uslugama održi. Ovo istraživanje imalo je za cilj da ispita iskustva farmaceuta zaposlenih u javnim apotekama u vezi sa kvalitetom i obimom farmaceutske zdravstvene zaštite (FZZ) i savetovanjem bolesnika tokom pandemije COVID-19, u odnosu na period pre pandemije. U ovom istraživanju takođe se ispitivala informativna uloga koju su farmaceuti imali u toku pandemije COVID-19 (u vezi sa mogućnošću pojave infekcije, simptomima, preventivnim merama, lečenjem, suplementacijom itd.). Još jedan cilj istraživanja bio je da se utvrdi da li je potreba za savetovanjem i za farmaceutskim uslugama bila veća tokom perioda visoke incidencije zaražavanja. Sprovedena je kvantitativna studija preseka među farmaceutima zaposlenim u javnim apotekama, koji su anonimno i dobrovoljno popunjavali onlajn anketu putem linka na *Google* platformi. Rezultati su pokazali da su farmaceuti koji su radili u apotekama blizu COVID-19 ambulanti ili bolnica smatrali da je kvalitet FZZ-a bio znatno ili potpuno narušen tokom pandemije; to su mislili i farmaceuti koji nisu radili u blizini COVID-19 ambulanti (38,78% prema 21,88%; p = 0,005). O tome da su adekvatne informacije pružene većini bolesnika izjasnilo se 40% svih ispitanika uključujući dodatno tražene informacije. Od ukupnog broja ispitanih farmaceuta, 86% njih istaklo je da su bolesnici zahtevali dodatne informacije o virusu COVID-19. Rezultati su ukazali na to da su farmaceuti koji su radili u apotekama blizu COVID-19 ambulanti ili bolnica zabeležili manji porast potrebe za savetovanjem i farmaceutskim uslugama tokom perioda visoke incidencije zaražavanja od ostalih farmaceuta (53% prema 69%; p < 0,001).

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***Ključne reči**: farmaceutska zdravstvena zaštita, savetovanje bolesnika, javna apoteka, COVID-19*

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MELD SCORE AND HEPATIC ENCEPHALOPATHY AS PREDICTORS OF MORTALITY IN PATIENTS WITH DECOMPENSATED ALCOHOLIC LIVER CIRRHOSIS

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Cirrhosis is the final stage of numerous chronic liver diseases. The disease most often occurs as a result of chronic alcohol consumption and infection with hepatitis C and B viruses. Over 3 million people die globally as a result of alcohol consumption. Prognostic scores have been developed to estimate survival rates. The MELD score (model for end-stage liver disease) is used today in the prognosis of short-term survival of patients with decompensated liver cirrhosis.

This prospective/retrospective study was conducted on a sample of 56 patients (52 male and 4 female) with an average age of 55.23 ± 10.82. MELD score values were calculated at the end of hospitalization and correlated with defined treatment outcomes. Based on the conducted receiver operating characteristic (ROC) analysis, cut-off values of MELD score over 23.5 were statistically significant in the prognosis of mortality. Of the total examined population, 72% of patients with a score higher than the obtained cut-off value died. The total number of patients with hepatic encephalopathy was 34 (61%), of which 23 died. ROC analysis in the group of patients with hepatic encephalopathy revealed a cut-off value of the MELD score of 30.5, which is statistically not significantly different from the cut-off value of the MELD score for survival. The male-to-female ratio in the subsamples of deceased and survived patients in this study was approximately equal.

The cut-off value of the MELD score proved to be statistically significant in predicting short-term survival. The increase in the cut-off value of the MELD score in patients with hepatic encephalopathy was not statistically significant. In the group of patients with hepatic encephalopathy, 92% of patients died, although no statistically significant increase in the cut-off value of the MELD score was found.

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Key words: *alcoholic liver cirrhosis, model for end stage liver disease score, hepatic encephalopathy*

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Introduction

Cirrhosis (from the Greek word kirrhos—yellow) represents the most common final stage of numerous chronic liver diseases (1, 2). This condition is characterized by irreversible damage to the liver parenchyma and permanent loss of its normal architecture. The end result of this process is the development of fibrosis and numerous regenerative nodes (2).

Cirrhosis of the liver can be developed due to chronic infection with hepatitis C and B viruses. Consumption of large amounts of alcohol is also closely associated with the development of hepatitis, cirrhosis and fibrosis (3). Non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) are both predispositions for the development of liver cirrhosis (4). A much rarer cause of liver cirrhosis can be an autoimmune or the presence of chronic cholestasis syndrome, Wilson's disease, hemochromatosis and primary sclerosing cholangitis (5, 6). The precise incidence of liver cirrhosis is difficult to determine due to its multifactorial etiology. Liver cirr

hosis is estimated to be the fourth most common cause of death in Europe and fourteenth worldwide (7). Hepatitis B virus is the main cause of disease development in Asia, and hepatitis C virus, NAFLD and chronic alcohol consumption are the primary causes in Western countries (8, 9). Frequent alcohol intake is associated with 48% to

50% of deaths from liver cirrhosis in America (10). In addition to the primary effect on liver damage, alcohol intake accelerates the progression of chronic hepatitis C to liver cirrhosis (11).

MELD score

The effect of ethanol on the liver parenchyma is complex. The main enzyme systems affected by alcohol are alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase. These enzymes are responsible for the oxidation of ethanol into acetaldehyde and the production of hydroxyethyl, superoxide and hydroxyl radicals that directly damage hepatocytes (12). In the early stages, the disease is compensated, liver function is partially preserved, and patients are often without major symptoms. With the progression of liver damage, the disease becomes decompensated and patients contact a doctor because of complications of the disease (13). Due to the decompensation of liver cirrhosis, the risk of mortality increases about 9.7 times, and the average survival of these patients is from 2 to 3 years (14, 15). Numerous scores have been developed with the aim of estimating the survival of patients with terminal disease. The MELD score (model for end-stage liver disease) was first developed in 1999 at the Mayo Clinic to estimate the survival of patients with transjugular intrahepatic portosystemic shunt (TIPS) (16, 17). This score was adopted on February 27, 2002 by the UNOS organization (The United Network for Organ Sharing) for patients on the liver transplant list. Higher score values mean higher priority for liver transplantation (18). MELD score values above 15, which are maintained during follow-up, indicate that the patient is a candidate for liver transplantation (19). The use of this score has been shown as a precise survival predictor over the next 3 months in patients with chronic, decompensated liver disease (20). This short-term patient survival is calculated using three parameters: total bilirubin value, serum creatinine value, and international normalized ratio for prothrombin time (INR) (18). An online calculator (available at the web address: http://www.unos.org/resources) allows calculating the numerical value of the MELD score using the complex formula: 9.57 × logecreatinine (mg/dL) + 3.78 × logebilirubin (mg/dL) + 11.20 × logeINR + 6.43 (19). The higher the score values, the shorter the three-month survival, so patients with a score less than 9 have the best prognosis (1.9–3.7% chance of death in the next 3 months), and patients with a score over 40 (71%–100% chance of death in the next 3 months). Alternative forms of the MELD score are often used today. Those forms are corrected with values of serum sodium and serum creatinine (sodium MELD, corrected creatinine MELD) (21). The Δ-MELD (delta-MELD) score, which represents the difference between the MELD scores at the beginning and end of treatment, can also be calculated in order to evaluate the short-term survival of patients with decompensated liver cirrhosis (22).

Objective

The aim of this research was to determine the prognostic validity of the MELD score in relation to survival only in the population of patients with decompensated alcoholic liver cirrhosis and developed hepatic encephalopathy examined in the period from admission to hospital treatment to defined treatment outcomes.

Material and Methods

The prospective/retrospective study was conducted at the Clinic of Gastroenterology and Hepatology of the University Clinical Center in Niš. The research included 56 patients with a diagnosis of decompensated alcoholic liver cirrhosis treated between January and December 2022. For each admitted patient, the MELD score was calculated as standard at the end of hospital treatment using an online calculator. In order to calculate the MELD score, a blood sample was taken from the patients for analysis of the blood count and biochemical parameters. Patients were also examined for the presence of hepatic encephalopathy and asked about the accompanying diseases and alcohol consumption. After the insight into the obtained anamnestic data, the statistical processing of the data was performed.

The state of decompensation of alcoholic cirrhosis of the liver meant aggravation of the terminal phase of the disease due to the development of one or more complications (hepatic encephalopathy, ascites, spontaneous bacterial peritonitis, bleeding from esophageal varices, hepato-renal syndrome). Hepatic encephalopathy was diagnosed based on the clinical condition of the patient, lack of orientation, specific shaking movement of the limbs (asterixis) and exclusion of other potential etiologies of encephalopathy such as intracranial lesions, stroke of hemorrhage, seizure activity etc.

The defined treatment outcomes in this study were:

1. survival of the patient in the episode of the decompensated phase of the disease, and
2. fatal outcome of the patient in the episode of the decompensated phase of the disease.

Differences in gender structure between patients who died and those who survived were tested with the chi-square test. The difference in mean age between patients who died and those who survived was tested by t-test for two independent samples. The normality of the data was confirmed by the Kolmogorov–Smirnov test. Finally, the z/proportion test was used to test the difference in the number of patients who died and patients who survived.

Results

Based on the obtained results, it can be concluded that the ratio of males and females in the subsamples of deceased and survived patients was approximately equal. The average age of survived and deceased patients did not show a statistically significant difference. There was also no significant difference between the number of deceased and the number of survived patients in this sample.

The difference in the number of patients with and without hepatic encephalopathy was tested using the *z*/proportion test.

No significant difference between the number of patients with and without hepatic encephalopathy was found in this sample.

The cut-off value of the MELD score was calculated using ROC analysis.

Based on the ROC analysis, it was found that the value of the MELT score at discharge from hospital treatment could be used as a diagnostic predictor for survival. Based on the ratio of sensitivity and specificity, a cut-off value of 23.5 was determined. Patients with a measured value of less than 23.5 were more likely to survive. The predictive power was statistically significant.

The chi-square test confirmed the good diagnostic power of the MELD score.

The chi-square test was used to check whether there was a relationship between survival rate and the presence of hepatic encephalopathy in patients.

Table 1. Demographic characteristics of the studied population

Population characteristics		Survived/Deceased		Total	Chi2/t/z	sig
		survived	deceased			
gender	Male	28 (90.32%)	24 (96%)	52 (92.86%)	.673	0.412
	Female	3 (9.68%)	1 (4%)	4 (7.14%)		
age		54.03 ± 11	56.72 ± 10.62	55.23 ± 10.82	-0.923	0.360
	Total	31 (55%)	25 (45%)	56 (100%)	0.668	0.504

Table 2. Demographic characteristics in relation to hepatic encephalopathy

	Hepatic encephalopathy		Total	z	sig
	no	yes			
Total	22 (39%)	34 (61%)	56 (100%)	-1.472	0.141

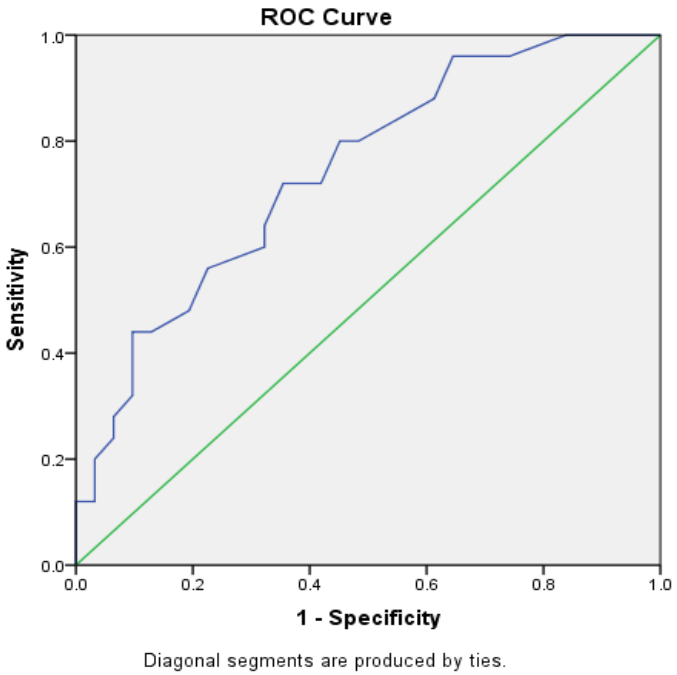

Figure 1. ROC analysis of the cut-off value of the MELD score

Table 3. Cut-off value of the MELD score of survival

Area Under the Curve					Cut off value
Test Result Variable(s): MELD					
Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		
			Lower Bound	Upper Bound	
.743	.065	**.002**	.615	.871	**23.5**

Based on the results of the chi-square test, it can be concluded that there was a statistically significant connection between these two characteristics. The percentage of deceased patients was significantly higher in the subsample of patients with hepatic encephalopathy.

The cut-off value of the MELD score in patients with hepatic encephalopathy was calculated using ROC analysis.

ROC analysis only determined the cut-off value of the MELD score at discharge from hospital treatment in patients with hepatic encephalopathy. The obtained result was not statistically significant, although the obtained value was close to the threshold of significance. This would mean that the MELD score should not be used as a prognostic survival score in patients with hepatic encephalopathy. In addition, based on the ratio of sensitivity and specificity, the established cut-off value was 30.5. This obtained cut-off value of the MELD score in the group of patients with hepatic encephalopathy who died was higher than the initial value of 23.5, previously shown to be statistically significant for survival. In the group of patients with hepatic encephalopathy, 92% of patients died, although no statistically significant increase in the cut-off value of the MELD score was found.

Table 4. Survival rate in relation to the cut-off value of the MELD score

		Survived/Deceased		Total	Chi2	sig
		survived	deceased			
MELD score positive (>23.5)	no	20 (64.52%)	7 (28%)	27 (48.21%)	7.391	.007
	yes	11 (35.48%)	18 (72%)	29 (51.79%)		

Table 5. Survival rate in relation to the presence of hepatic encephalopathy

		Survived/Deceased		Total	Chi2	sig
		survived	deceased			
Hepatic encephalopathy	no	20 (64.52%)	2 (8%)	22 (39.29%)	18.532	**0.000**
	yes	11 (35.48%)	23 (92%)	34 (60.71%)		

Table 6. Cut-off value of the MELD score in patients with hepatic encephalopathy

Area Under the Curve					
Test Result Variable(s): MELD					
Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		Cut off value
			Lower Bound	Upper Bound	
.680	.094	.094	.495	.864	30.5

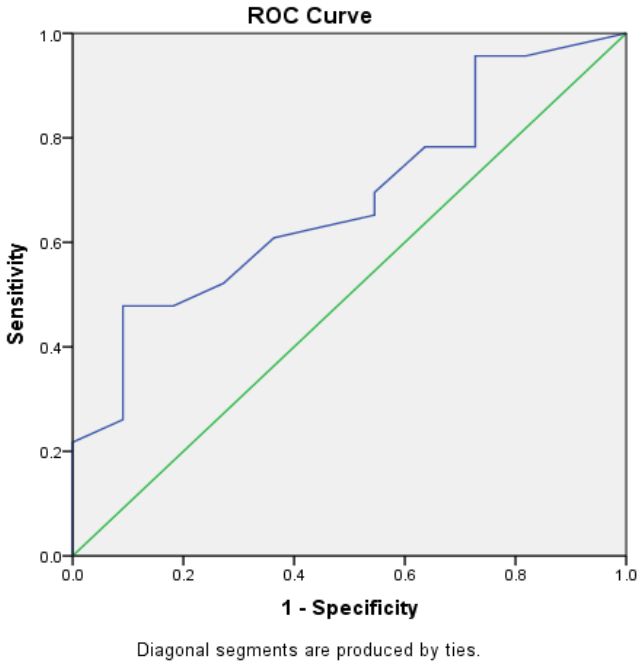

Figure 2. ROC analysis of the cut-off value of the MELD score only in patients with hepatic encephalopathy

Discussion

In this research, we examined the significance of the MELD score as a prognostic parameter of survival in a selected population of patients with decompensated alcoholic cirrhosis of the liver observed in the period from admission to hospital treatment to defined treatment outcomes.

According to the report of the World Health Organization (WHO), about 3.3 million people around the world die as a result of long-term alcohol consumption (23). The WHO records that the average alcohol consumption is 13.5 g of alcohol per day, or about 6.2 liters of alcohol per year. (24). It is estimated that 1 in 12 adults consumes alcohol daily, men more than 3 and women more than 2 drinks per day. The National Institute for Alcoholism also defines alcohol abuse in the form of acute intake of over 5 drinks in men and over 4 drinks in women in a period of 2 hours (25). The largest number of alcoholic cirrhosis of the liver is recorded in individuals between 45 and 54 years of age, but this limit has been moving for a whole decade towards a younger age (26). Men are more likely to develop liver cirrhosis than women, due to more frequent and higher intake of alcohol. However, women are sensitive to the direct toxic effects of alcohol, so the risk of developing alcoholic cirrhosis in women who drink alcohol is up to 2 times higher than in the male population (27, 28). Patients with alcoholic cirrhosis of the liver usually contact a doctor due to the development of jaundice, elevated body temperature and weight loss. In the decompensated phase of the disease, there are ascites, hepatic encephalopathy, bleeding from esophageal varices and kidney failure (29). In patients who are already treated for alcoholic cirrhosis of the liver, further intake of alcohol increases mortality in the next 5 years, so it is estimated that the 5-year survival rate in this case is about 35% (30). In addition to well-known diagnostic methods, the MELD score is often used as a predictor of three-month survival in patients with decompensated alcoholic cirrhosis of the liver (18).

The MELD score was implemented to assess the risk of mortality in patients undergoing transjugular intrahepatic portosystemic shunt (TIPS) (18). The score has also been used in patients who are candidates for liver transplantation, and it is widely used as a simple score to predict the survival of patients with decompensated liver cirrhosis (31, 32). Numerous studies have addressed the importance of the MELD score. A large meta-analysis including 16 studies and a sample of 2, 337 patients was conducted by Wu Shi-Lan et al. This analysis established the reliability of the MELD score in clinical application, and above all in predicting mortality in the next 6 to 12 months (33). Wu Shi-Lan points to the importance of the MELD score because of the reliable values of the laboratory parameters used to calculate the score. The results obtained in this way are more objective and most often correlate with the clinical condition of the patients (34). Our conducted research shows similar results. Measured MELD score values that were higher than the obtained cut-off value (23.5) are associated with a shorter survival rate. In practice, the clinical condition of the patient includes other parameters such as the

presence of hepatic encephalopathy, ascites, spontaneous bacterial peritonitis and acute variceal bleeding. These independent mortality factors are not included in the MELD score, although they are very important in the treatment outcome of patients (35). Stewart CA et al. conducted a study on a sample of 271 patients with decompensated liver cirrhosis and developed hepatic encephalopathy. The study determined the statistical significance of the MELD score in surviving patients, however, hepatic encephalopathy was shown to be an independent predictor of mortality (36). Patients who were treated in our hospital due to decompensated liver cirrhosis with hepatic encephalopathy had a shorter survival rate than patients without this complication. This difference was statistically significant. The established cut-off value of the MELD score for the patient with hepatic encephalopathy (30.5) was slightly higher compared to the cut-off value for survival (24.5). Although this increase in the cut-off value was not statistically significant, the largest number of deceased patients had hepatic encephalopathy. This complication develops relatively late in the decompensated phase of the disease as a consequence of the appearance of portosystemic shunts. Toxic substances bypass the liver via shunts, their detoxification is absent and they reach the cerebral circulation directly. Because of this, the presence of hepatic encephalopathy could directly affect the biochemical parameters, and therefore the values of the MELD score (36). The research conducted by Kamath concluded that the MELD score is highly reliable in the assessment of the three-month mortality rate in patients with decompensated liver cirrhosis, regardless of its etiology. The results of this study were that albumin values, the presence of hepatic encephalopathy, ascites and spontaneous bacterial peritonitis did not significantly increase the accuracy of the MELD score (18). The results of our research have shown similarities to this research. Hepatic encephalopathy was an independent predictor of mortality. Although this complication had a partial effect on the change in the cut-off value of the MELD score in patients who died, this effect was not statistically significant. This topic of association between MELD score and hepatic encephalopathy was addressed by Yoo Iwan and Edwin David. By examining a smaller population of 66 patients, they found that the subclinical and clinical presence of hepatic encephalopathy correlates weakly with the change in the MELD score. Therefore, they point out that the MELD score may not be the most reliable score for assessing the survival of patients with advanced encephalopathy (37). The group of subjects in the present study counted only 10 patients less, but the results we obtained were similar to theirs. The presence of this complication primarily influenced the increase in patient mortality, but not a significant change in the MELD score. The impact of hepatic encephalopathy on the survival of patients with decompensated cirrhosis was also studied by Bjerring Peter and Gluud Lise. In their 2017 study, hepatic encephalopathy again stood out as an independent prognostic factor from the MELD score. The conclusion of this study suggests that the accuracy of the MELD score increases if it is combined with hepatic encephalopathy (38). Similar results on a sample of 1,560 patients with cirrhosis were obtained by Bajaj who claims that hepatic encephalopathy must be considered when calculating the MELD score (39). The evaluation of the effectiveness of the MELD score was also done by Elzouki Aiello et al. On a sample of 109 patients diagnosed with decompensated liver cirrhosis with hepatic encephalopathy, the MELD score was a reliable predictor of mortality in patients over the age of 60 (40). The average age of deceased patients in our present study was 56.72 ± 10.62. The largest number of these patients had MELD score values higher than the calculated cut-off value for survival. These obtained results are very similar to the results of the previous research. Florencia et al. point out the imperfection of the MELD score, which is why it is not the most reliable prognostic marker of mortality. The reason for this is the lack of precision of the creatinine value as one of the parameters of the MELD score. These values may vary individually in relation to age, sex, protein intake and liver and kidney function (41). Due to the difference in standardized creatinine values between the sexes, the score values are often higher in women. This would indicate worse survival in women, which is not always the case when dealing with decompensated liver cirrhosis (42). Numerous substances such as pyruvate and various drugs can also affect serum creatinine values, so such measured creatinine values can be unreliable (43). In our country, the level of serum creatinine is expressed in millimoles per liter (mmol/L). The exact creatinine values used to calculate the MELD score were obtained by dividing the value in mmol/L by the coefficient 88.4, which gives the value in milligrams per deciliter (mg/dL). Different to the study by Florencia et al., the cut-off value of the MELD score in our study was statistically significant for survival, and therefore the value of serum creatinine as part of this score. A large cohort study of 830 subjects by Peeraphatdit Thoetchai points to certain shortcomings of the MELD score. In the decompensated phase of the disease, bilirubin values are often unchanged on a daily basis, considering the chronic state of the disease. Also, INR values may vary due to the administration of anticoagulant therapy, fresh frozen plasma or vitamin K. These are the reasons why the MELD score at the end of treatment does not have to be precise, and often does not correlate with the more severe clinical condition of the patient. According to this study, the MELD score at discharge from treatment does not precisely correlate with the patient survival rate (22). Porte RJ et al. dealt with similar challenges. Their study indicates a large variability of INR values because of the laboratories where this analysis was performed. The MELD score calculated in this way could differ by 3 to 5 MELD

points, which significantly reduces the accuracy of this prognostic marker. For this reason, it is considered that INR is not a reliable parameter for any prognosis in patients with decompensated liver cirrhosis (44). Our MELD score calculations used the INR obtained at the end of hospitalization, but we cannot say with certainty how reliable this parameter was. Given that a higher MELD score meant shorter survival, INR values would probably be reliable. Although Kamath et al. have previously spoken about the reliability of the MELD score, they are also pointing out its flaws. Similar to previous studies, they claim that the measurement of renal function by determining the clearance of some other substances, besides creatinine, could increase the reliability of this prognostic marker if they are combined with the MELD score (18). Since the score is based on parameters that are likely to change, there was a need to increase the accuracy of this score. Shortly after the creation of the MELD score, in 2003, Ruf et al. suggested adding the value of serum sodium as an additional parameter of the MELD score. It has been noted that patients with hyponatremia have ascites, which is one of the independent prognostic parameters of mortality (45). Accordingly, the modified MELD-Natrium (MELD-Na) score more accurately predicts the mortality of these patients (45, 46). A meta-analysis by Wu Shi-Lan shows a higher prognostic accuracy of the MELD-Na score compared to the MELD score. According to this meta-analysis, the MELD-Na score is superior in estimating mortality in the next 12 months (33). Chen Si-Hai in his examination of markers for the prognosis of liver cirrhosis, suggests the combination of the MELD score with other parameters of inflammation, such as C-reactive protein (CRP) and procalcitonin (PCT). The MELD-CRP and MELD-PCT scores improve the prediction of mortality in the next 30 days. These results are in agreement with results on the same topic of research conducted at the Mayo Clinic (47). The previously mentioned problem of accuracy of the MELD score due to the variability of INR as one of the score parameters could be overcome by simply eliminating INR from this score. The MELD score calculated without the INR value (the so-called MELD-XI) would be a suitable prognostic marker of mortality only in patients whose renal function is significantly impaired due to the primary disease. Although a large number of decompensated liver cirrhosis is followed by hepatorenal syndrome with renal failure, this may not always be the case. MELD-XI could be prognostically quite accurate in decompensated alcoholic liver cirrhosis, but not in liver cirrhosis of other etiologies (48). It is also suggested to replace the INR with coagulation factor V or coagulation factor VII. MELD scores modified in this way have not yet found wider application in clinical practice (49). Our experience with modified MELD scores is very poor. Further research on this topic, adding the sodium value to the score and correlating it with other complications of decompensated liver disease could increase the accuracy of the MELD score. Possible combination of this score with the values of serum CRP and procalcitonin, or some other laboratory parameter, would probably give a better short-term prognosis of mortality in patients suffering from decompensated alcoholic liver cirrhosis.

Conclusion

This study of a selected patient population aimed to determine the prognostic validity of the MELD score in relation to the survival rate of patients with decompensated alcoholic cirrhosis of the liver with developed hepatic encephalopathy. The MELD score is proved to be a statistically significant predictor of mortality, but hepatic encephalopathy did not significantly affect changes in this score. Statistically, there was a significant difference in the group of deceased patients who developed hepatic encephalopathy compared to the group without this complication. The cut-off value of the MELD score in the group of deceased patients who developed hepatic encephalopathy was not statistically significantly different from the cut-off value of the MELD score of survival. To determine if hepatic encephalopathy is an independent mortality predictor, it is necessary to perform a study on a larger patient population.

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MELD SKOR KAO PREDIKTOR MORTALITETA KOD BOLESNIKA SA DEKOMPENZOVANOM ALKOHOLNOM CIROZOM JETRE I RAZVIJENOM HEPATIČNOM ENCEFALOPATIJOM

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Ciroza je završni stadijum brojnih hroničnih bolesti jetre. Bolest najčešće nastaje usled hroničnog konzumiranja alkohola i infekcije virusima hepatitisa C i B. U svetu od posledica unosa alkohola godišnje umre više od tri miliona ljudi. Prognostički skorovi razvijeni su sa ciljem procene preživljavanja. Prilikom prognoze kratkoročnog preživljavanja bolesnika sa dekompenzovanom cirozom jetre danas se koristi MELD (engl. *Model for End-stage Liver Disease*) skor.

Ova prospektivno/retrospektivna studija sprovedena je na uzorku od 56 bolesnika (52 muškarca i četiri žene), čija je prosečna starost bila 55,23 ± 10,82 godine. Vrednosti MELD skora računate su na kraju hospitalizacije i bile su u korelaciji sa definisanim ishodima lečenja. Sprovedena ROC (engl. *Receiver operating characteristic*) analiza pokazala je da su *cut-off* vrednosti MELD skora preko 23,5 bile statistički značajne u prognozi mortaliteta. U ukupno ispitivanoj populaciji, preminulo je 72% bolesnika sa skorom većim od dobijene *cut-off* vrednosti. Ukupan broj bolesnika sa razvijenom hepatičnom encefalopatijom bio je 34 (61%); od toga, preminulo je njih 23. ROC analizom u grupi bolesnika sa hepatičnom encefalopatijom utvrđena je *cut-off* vrednost MELD skora 30,5, što se statistički signifikantno ne razlikuje od *cut-off* vrednosti MELD skora za preživljavanje. Odnos muškog i ženskog pola u poduzorcima preminulih i preživelih bolesnika u ovom istraživanju bio je skoro ujednačen.

Cut-off vrednost MELD skora pokazala se kao statistički značajna u prognozi kratkoročnog preživljavanja. Zabeležen porast *cut-off* vrednosti MELD skora kod bolesnika sa hepatičnom encefalopatijom nije bio statistički značajan. U grupi bolesnika sa hepatičnom encefalopatijom zabeležen je smrtni ishod kod 92% bolesnika, premda nije utvrđeno statistički signifikantno povećanje *cut-off* vrednosti MELD skora.

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***Ključne reči**: alkoholna ciroza jetre, model for end stage liver disease skor, hepatična encefalopatija*

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LIPID PEROXIDATION INHIBITION STUDY OF FLOWER EXTRACT AND TWO COUMARINS ISOLATED FROM *DAPHNE MEZEREUM* L.

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The medicinal importance of the genus *Daphne* L. is related to the richness in the expansive range of different classes of natural products and bioactive phytochemicals, such as coumarins, flavonoids, lignans and different classes of terpenes. The current study reports on the lipid peroxidation effect of diethyl-ether macerate of *Daphne mezereum* L. flowers and of two coumarins we have isolated from the aqueous subfraction of the crude diethyl-ether extract. All three tested samples, *D. mezereum* flowers extract (IC_{50} = 25.1 ± 2.9 mM) and isolated coumarins: umbelliferone (IC_{50} = 7.1 ± 2.6 mM) and herniarin (IC_{50} = 19.0 ± 1.3 mM), exhibited notable antioxidative potential in lipid peroxidation assay. None of the samples, however, had an inhibitory effect as pronounced as standardly applied antioxidants Trolox (IC_{50} = 22 ± 6 μM), caffeic acid (IC_{50} = 15 ± 3 μM) and quercetin (IC_{50} = 23 ± 6 μM). Taken altogether, the results of our studies bring forward new data regarding the antioxidant activities of *D. mezereum* species.

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Key words: *Daphne mezereum, coumarin isolation, umbelliferone, herniarin, lipid peroxidation*

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Introduction

Named after the water nymph in Greek myth that was turned into a laurel tree, encompassing 95 species of flowering shrubs, *Daphne* L. is the most diverse genus of the Thymelaeaceae family. The genus is native to certain regions of sub-tropical Asia but is also distributed in Europe and North Africa. To this day, 17 species of this genus have been evidenced in Europe's Flora (1). *Daphne mezereum* L. is among plants that grow mostly in Europe and Asia and one of seven Daphne species native to Serbia (2). The decorative, strongly scented flowers are produced in early spring on the bare stems before the leaves appear. The fruit is a bright red berry, poisonous to humans, but despite striking toxicity, because of its desirable horticultural characteristics, *D. mezereum* has become one of the most popular perennial flowering shrubs (3).

As was confirmed in earlier studies, *Daphne* plants are a rich source of pharmacologically important molecules, indicating broad potential use in medicine (4–12). The genus has a long history in traditional medicine as a remedy for the treatment of rheumatism, ulcers, and treatments for aches, inflammation, and abortificient (13, 14). Previous phytochemical studies of the genus report a large number of classes of bioactive secondary metabolites, dominated by coumarins, flavonoids, lignans, diterpenes and steroids (5, 6, 9, 11, 12).

Daphne mezereum was already subjected to phytochemical research (4, 15–22). The extracts and essential oil of *D. mezereum* demonstrated several biological properties; the plant is suspected to have an immune-stimulating effect, the water-alcohol extract has antileukemic activity on P-388 lymphocytic cells in mice (4), while pure compound mezerein, isolated from *D. mezereum*, shows an inhibitory effect against P-388 cells and L-1210 type of leukemia in mice in the 50 μg dosage (23). Interestingly, only one paper analyzed *D. mezereum* flowers in an analysis related to the floral fragrance chemistry (20).

Despite numerous data on the compositional analysis of *Daphne* species from Serbia (*Daphne*

alpina subsp. *alpina* (24), *Daphne cneorum* L. (25), *Daphne blagayana* L. Freyer (26, 27) and *Daphne malyana* Blečić (28)), with antioxidant capacity determined and correlated to coumarins, phenolic acids and flavonoids, a similar investigation was never published for *D. mezereum* (except the congress announcement we have reported in 2022 (29)). Therefore, we decided to provide information on the antioxidant effects of extract obtained from flowers by maceration with diethyl-ether. As part of our investigation, we isolated two simple coumarins from *D. mezereum* and tested their antioxidant properties in the lipid peroxidation assay.

Material and Methods

Chemicals

All inorganic and organic reagents were of analytical grade, obtained from commercial sources (unless specified otherwise, all chemicals were purchased from Merck (Darmstadt, Germany)). Phospholipids (Phospholipon® 90 – PL90) were obtained by courtesy of Phospholipid GMBH, Cologne, Germany. Analytical thin-layer chromatography was carried out on precoated TLC sheets ALUGRAM® Xtra SIL G/UV_{254} (Macherey-Nagel). Preparative column chromatography was carried out on Silica Gel 60 (70–230 mesh).

Spectrophotometric measurements were performed using Thermo Scientific Evolution 60 Spectrophotometer (Fisher Scientific, UK). HPLC analysis was performed using the Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and a diode array (DAD) detector and NMR spectra were recorded on a Bruker AVANCE DPX300 spectrometer.

Plant material and extraction procedure

Aerial parts of *D. mezereum* were collected in the flowering phase at Devojački grob, Suva Planina in June 2007. Taxonomic identification was performed by Professor B. Zlatković (Faculty of Science and Mathematics, University of Niš). Fresh samples were subjected to exhaustive macerations (800 mL, 3 × 48 h) with diethyl-ether (DE) as a solvent. The resultant solution was evaporated to dryness under reduced pressure below 40 °C, to give 3.1 g of DE extract. Extraction yield, expressed in % of used plant material, was 0.5%. The extract was purged with nitrogen and kept at -20 °C, under nitrogen atmosphere, until the final use.

Isolation of simple coumarins

Dry DE extract was subfractioned according to the modified procedures given by Komissarenko et al., 1994 (30) and Ness et al., 1996 (31) with hot water, filtrated and re-extracted with chloroform. Upon drying, chloroform extract was evaporated to give 1.2 g, or 0.2% if expressed in % of used plant material. Subfractionated extract, i.e., water fraction was fractionated further with column chromatography first on a silica gel column (1.2 x 20 cm) and further chromatographed on Sephadex LH-20 column (2.5 cm × 150 cm) and eluted successively with deionized water (50 mL), aqueous ethanol (20%, 40%, 70% and 90% ethanol, 50 mL for each) and aqueous acetone (50% and 90% acetone, 50 mL for each) at room temperature (32). Coumarins umbelliferone and herniarin were eluted in different fractions, yielding 124.2 mg (extraction yield 0.02%) and 54.8 mg (extraction yield 0.009%), respectively. The composition of the extract and isolated coumarins were analyzed with HPLC-DAD and confirmed by recording ^{1}H and ^{13}C NMR. For this purpose, samples were either diluted in methanol HPLC grade, filtered through 0.45 μm PTFE filters and subjected to HPLC analyses or dissolved in 1 mL DMSO-d^6, and 0.7 ml of the solution was transferred into a 5-mm Wilmad, 528-TR-7 NMR tube.

NMR analysis

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE DPX300 spectrometer at 300 MHz and 75 MHz, respectively. All NMR spectra were recorded at 298 K in DMSO-d6 (isotopic enrichment 99.95%) solution. Chemical shifts (δ) were given as parts per million (ppm), relative to tetramethylsilane (TMS) as an internal standard with multiplicity reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; coupling constants (*J*) are shown in hertz (Hz); number and assignment of protons. The experimental error in the measured ^{1}H-^{1}H coupling constants was ± 0.5 Hz.

HPLC analysis

HPLC analysis was performed using the Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump and a diode array detector. The test sample solutions were prepared in methanol diluted up to 100 ppm. The analyses were carried out on a reverse phase Purospher STAR RP-18e column (125 mm x 3 mm, 3.0 μm, Merck, KGaA, 64271 Darmstadt, Germany) by maintaining column temperature at 30 °C. Mobile phase A was a trifluoroacetic acid (0.1%) solution which was prepared by dissolving 1.0 mL of trifluoroacetic acid in 1000 mL of water, and methanol was used as mobile phase B. The injection volume was 10 μL and the flow rate was 0.5 mL/min. The wavelength was fixed at 254 nm. For crude DE extract (***A***) the data were acquired by using a gradient elution system: 0−10min, 50% A, increasing the ratio of phase B to 90% and decreasing phase A to 10%; 10–12 min, holding 10% A and 90% B phase; 12−13 min decreasing of B phase to 50% and increasing A phase to

50%; 13–15 min, holding 50% A and 50% B phase. For subfractionated extract (***B***) and coumarins (***C*** and ***D***) isolated therefrom, data were acquired by using gradient elution system: 0–18 min, 80% A, increasing the ratio of phase B to 90% and decreasing phase A to 10%; 18–20 min, holding 10% A and 90% B phase; 20–21 min decreasing of B phase to 20% and increasing A phase to 80%; 21–22 min, holding 20% A and 80% B phase.

Lipid peroxidation inhibition by thiobarbituric acid-malondialdehyde assay

Lipid peroxidation (LP) is a free radical-mediated chain reaction that once initiated results in the oxidative degradation of polyunsaturated lipids. The final product of lipid peroxidation is malondialdehyde (MDA), a short-chain aldehyde, which is a biochemical marker of cell membrane oxidative damage (33).

Lipid peroxidation and LP inhibition in the presence of the tested compounds were measured by thiobarbituric acid-malondialdehyde (TBA-MDA) assay according to the procedure given by Lazarević *et al*., 2020 (34).

The absorbance of MDA-$(TBA)^2$ adduct in the supernatant, read at 530 nm, was used to calculate the inhibition percentage of LP using the following equation:

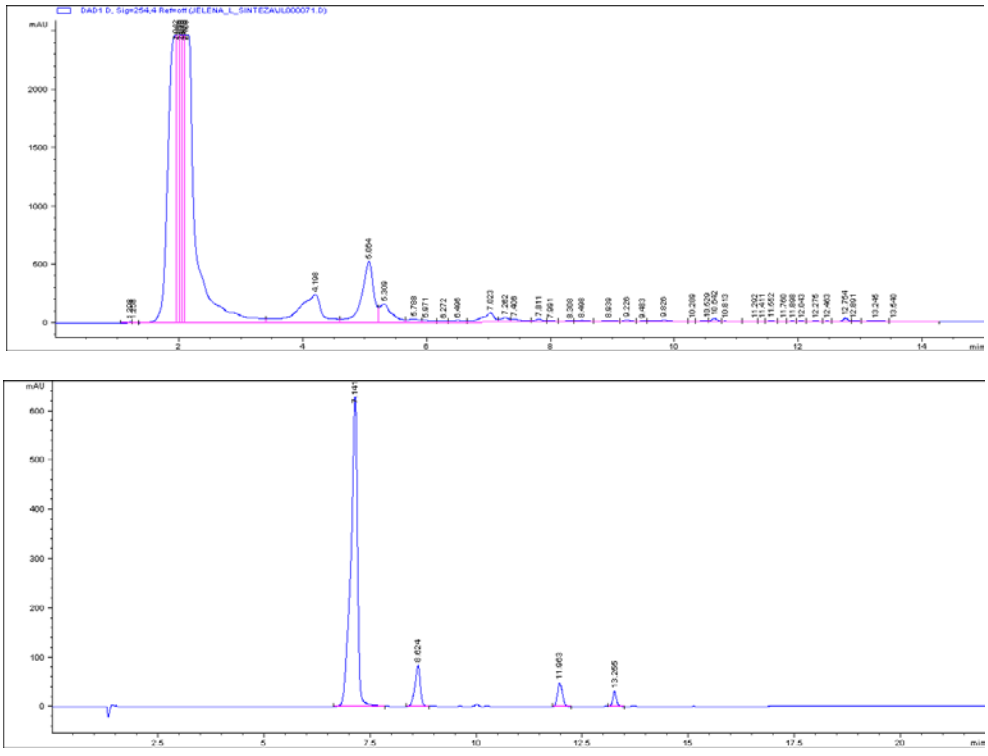
LP inhibition (%) = 100 x (Ac-As)/(Ac-Ab)

Ac - the absorbance of control (PL90 in methanol treated with the AAPH and TBA solution), As - the absorbance of samples (tested extract/compounds dissolved in PL90 solution, afterwards treated with the AAPH and TBA solution) and Ab - the absorbance of blank (PL90 in methanol, not treated with AAPH, but with TBA solution).

Samples were evaluated for LP-inhibitory activity, and only those showing inhibition greater than 50% at 500 μM were investigated further in a broader concentration range to allow calculation of IC_{50} values. The same type of experiment was done by using frequently used antioxidants as standards: caffeic acid, Trolox or quercetin. The standards were evaluated for LP-inhibitory activity at concentrations of 50 μM (caffeic acid) and 80 μM (quercetin and Trolox) in the final reaction mixture. All experiments were performed in triplicate.

Results and discussion

HPLC chromatograms of crude DE extract (Figure 1.A.), subfractionated water extract (1.B.) and of coumarins isolated from *D. mezereum*: umbelliferone (1.C.) and herniarin (1.D.), at wavelength detection of 254 nm, are presented in Figure 1. Unfortunately, a mixture of several co-eluting compounds, representing the most abundant compound from the chromatogram, was not detected. On the other hand, the presence of two simple coumarins umbelliferone and herniarin was confirmed by the comparison of the retention time and UV spectrum with the standards. Coumarins are among the important constituents of *Daphne* species and have been previously reported on many occasions (5, 10, 13, 18, 22, 25–27).



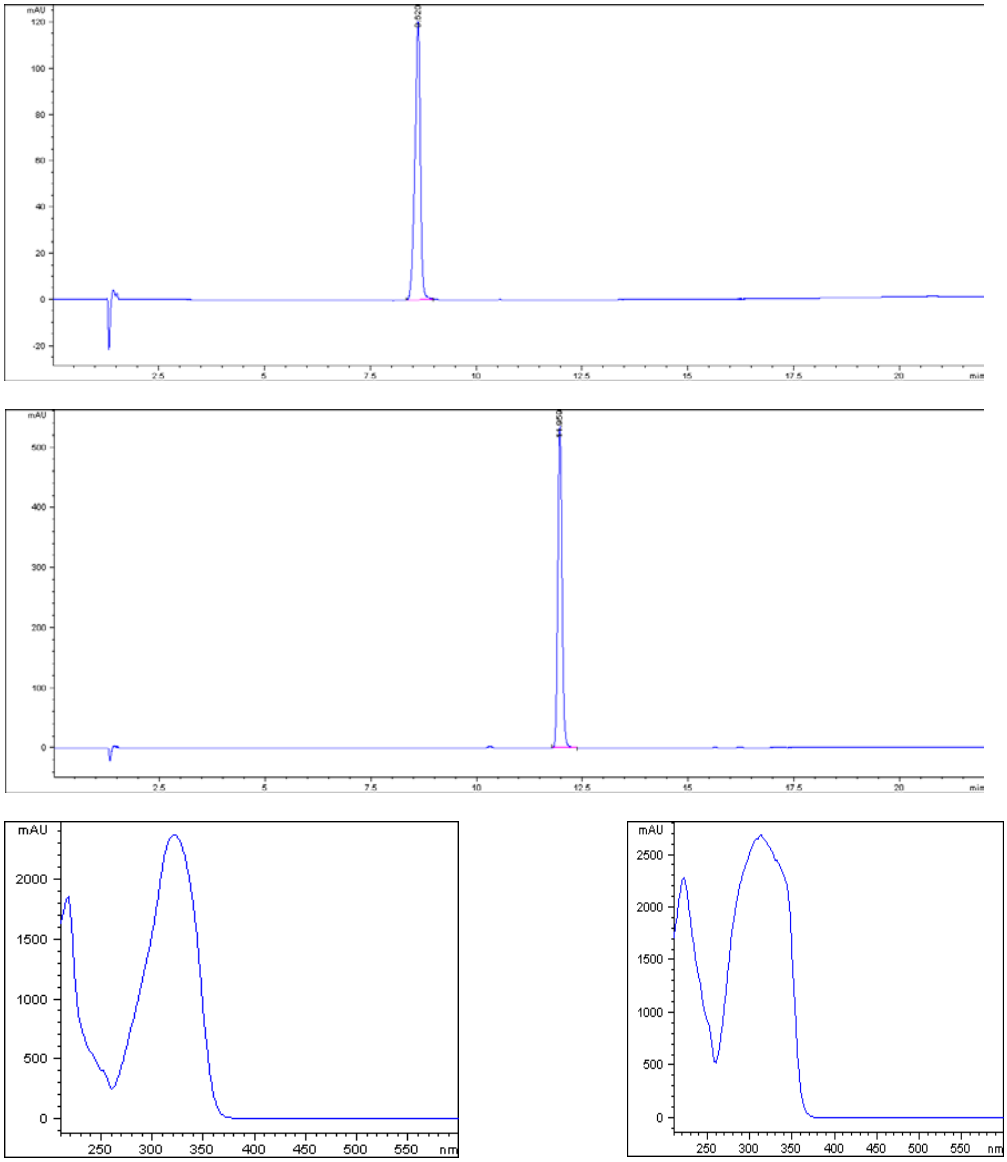


Figure 1. HPLC chromatograms of crude DE extract (***A***), subfractionated water extract (***B***) and of coumarins isolated from *D. mezereum*: umbelliferone (***C***) and herniarin (***D***) and UV and UV spectra of umbelliferone (t_R = 8.62 min) and herniarin (t_R = 11.96 min)

The composition of the extract was analyzed and confirmed with HPLC-DAD, by the comparison of the retention time and UV spectrum with the coumarin standards, and by recording ^{1}H and ^{13}C NMR for the isolated compounds. The obtained spectral data have confirmed the identity of the isolated coumarins. These data coincide well with the previous reports (35, 36) and fully assigned ^{1}H and ^{13}C spectra are presented in Figure 2.

Figure 2. ^{1}H and ^{13}C NMR spectra of umbelliferone (A) and herniarin (B)

Analytical properties of both isolated coumarins are given as follows:

Umbelliferone (7-Hydroxy-2*H*-chromen-2-one): White amorphous solid, $C_9H_6O_3$ (M = 162.14), HPLC purity ≥ 99%, ^{1}H NMR (DMSO-d^6, 300.13 MHz, δ, ppm): 10.59 (s, 1H, O-H), 7.95 (d, *J* = 9.16 Hz, 1H, C-H), 7.54 (d, *J* = 8.53 Hz, 1H, Ar-H), 6.81 (dd, *J* = 8.47, 2.32 Hz 1H, Ar-H), 6.73 (d, *J* = 2.38 Hz, 1H, Ar-H), 6.22 (d, *J* = 9.41 Hz, 1H, C-H). ^{13}C NMR (DMSO-d^6, 75 MHz, δ, ppm): 161.2, 160.4, 155.5, 144.5, 129.7, 113.1, 111.4, 111.2, 102.0.

Herniarin (7-Methoxy-2*H*-1-benzopyran-2-one): White amorphous solid, $C_{10}H_8O_3$ (M = 176.17), HPLC purity ≥ 99%, ^{1}H NMR (DMSO-d^6, 300.13 MHz, δ, ppm): 7.99 (d, *J* = 9.54 Hz, 1H, C-H), 7.63 (d, *J* = 8.66 Hz, 1H, Ar-H), 7.0-6.93 (m, 2H, Ar-H), 6.3 (d, *J* = 9.54 Hz, 1H, =C-H), 3.87 (s, 3H, =C-H). ^{13}C NMR (DMSO-d^6, 75 MHz, δ, ppm): 162.4, 160.3, 155.4, 144.3, 129.4, 112.4, 112.3, 112.3, 100.6, 55.9.

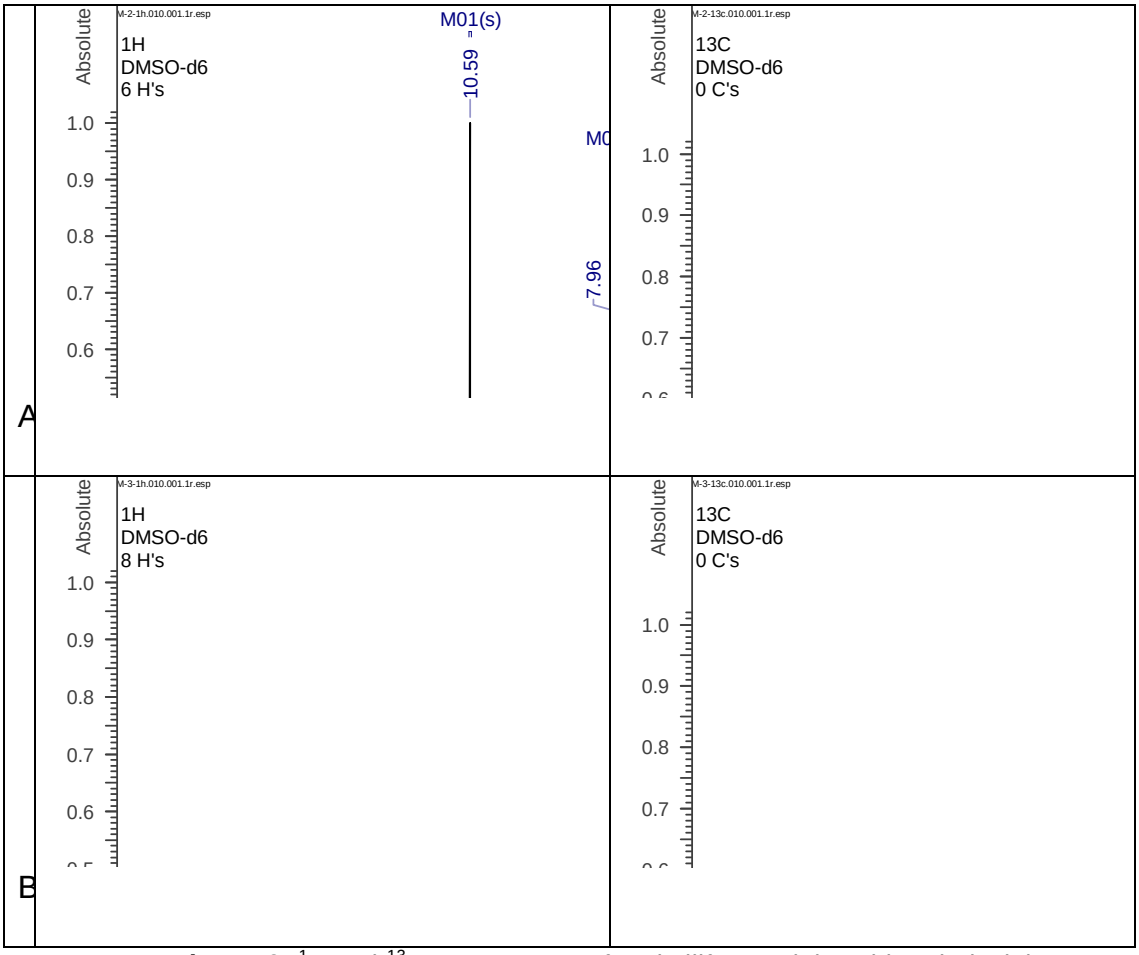


Figure 2. ^{1}H and ^{13}C NMR spectra of umbelliferone (A) and herniarin (B)

The lipid peroxidation inhibition effect of the tested samples: diethyl-ether macerate of *D. mezereum* flowers and isolated coumarins: umbelliferone and herniarin, measured using the method based on MDA-TBA assay, was notable. After performing experiments, the obtained results were plotted, IC_{50} values were calculated and reported in Table 1. The obtained results indicated that all samples [*D. mezereum* flowers DE (IC_{50} = 25.1 ± 2.9 mM) and isolated umbelliferone (IC_{50} = 7.1 ± 2.6 mM) and herniarin (IC_{50} = 19.0 ± 1.3 mM)] exhibited significant potential in LP assay. However, none of the tested samples were as effective as standardly applied antioxidants Trolox (IC_{50} = 22 ± 6 μM), caffeic acid (IC_{50} = 15 ± 3 μM) and quercetin (IC_{50} = 23 ± 6 μM). Preliminary results have shown that *D. mezereum* DE extract, by inhibiting the LP process, has antioxidant properties and that this effect can be partially attributed to the presence of simple coumarins umbelliferone and herniarin, whose antioxidant effects have been investigated and reported in a number of studies (37, 38).

Table 1. Lipid peroxidation inhibition effects of the three tested samples (IC_{50} values given in mM) and the selected antioxidants (IC_{50} values given in μM)

Compound	**LP inhibition IC_{50} (mM) ± SD**	**Compound**	**LP inhibition IC_{50} (μM) ± SD**
Daphne mezereum flowers' DE	25.1 ± 2.9	Trolox	22 ± 6
umbelliferone	7.1 ± 2.6	caffeic acid	15 ± 3
herniarin	19.0 ± 1.3	quercetin	23 ± 6

Conclusion

Epidemiological studies link dietary intake of coumarin-based compounds with beneficial health effects, mainly due to their antioxidant activity. Exhibiting antioxidant activity by the inhibition of lipid peroxidation, studied coumarins represent such compounds. Additional phytochemical and pharmacological evaluations are needed before shedding further light on the potential application of *D. mezereum*.

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ISPITIVANJE LIPIDNE PEROKSIDACIJE EKSTRAKTA CVETA I DVA KUMARINA IZOLOVANA IZ BILJKE *DAPHNE MEZEREUM* L.

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Fitohemijskim ispitivanjem vrsta *Daphne* L. potvrđeno je prisustvo aktivnih sastojaka koji po svojoj strukturi pripadaju kumarinima, flavonoidima, lignanima i terpenima. U ovom radu ispitan je efekat inhibicije lipidne peroksidacije etarskog ekstrakta cveta *Daphne mezereum* L. i kumarina koji su postupkom frakcionisanja izolovani iz ekstrakta. Sva tri testirana uzorka – sirov etarski ekstrakt cvetova *D. mezereum* (IC_{50} = 25,1 mM ± 2,9 mM) i izolovani jednostavni kumarini, umbeliferon (IC_{50} = 7,1 mM ± 2,6 mM) i hernijarin (IC_{50} = 19,0 mM ± 1,3 mM) – pokazala su dobar antioksidativni potencijal. Efikasnost ekstrakta i ispitivanih jedinjenja upoređivana je sa standardno primenjivanim antioksidansima: troloksom (IC_{50} = 22 mM ± 6 mM), kafenom kiselinom (IC_{50} = 15 mM ± 3 mM) i kvercetinom (IC_{50} = 23 mM ± 6 mM). Rezultati ove studije doprinose razumevanju fitohemijske karakterizacije vrste *D. mezereum*, ukazujući na to da se ekstrakt pomenute biljke može koristiti kao izvor prirodnih antioksidanasa.

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***Ključne reči**: Daphne mezereum, izolovanje kumarina, umbeliferon, hernijarin, lipidna peroksidacija*

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IMPACT OF COVID-19 PANDEMIC ON THE STRUCTURE OF PATIENTS UNDERGOING SURGICAL TREATMENT FOR URO-ONCOLOGICAL INDICATIONS

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The aim of this retrospective study was to examine the impact of the COVID-19 pandemic on the structure of patients who had undergone operative treatment for oncological indications at the Urology Clinic of the University Clinical Center Niš in the period from March 2018 to June 2022. The following operations were included: nephrectomy for kidney cancer, nephroureterectomy for upper urothelial cancer, prostatectomy for prostate cancer, cystectomy for bladder cancer, orchiectomy for testicular cancer and penectomy for penile cancer. Data were taken on the number of operations, patients' age and gender and postoperative histopathological findings. Depending on the time the surgery was performed, patients were divided into two groups: the preCOVID group - procedures carried out before the start of the pandemic (March 1, 2018 – March 1, 2020) and the COVID group - procedures carried out after the start of the pandemic (June 2020 – June 2022). A total of 569 investigated operations were performed, 320 before and 249 after the beginning of the pandemic. Nephrectomies were the most frequently performed procedures in both study groups. During the pandemic, a significant decrease in number of prostatectomies and nephrectomies was registered. The proportion of prostatectomies was significantly lower in the COVID group, while the proportion of cystectomies significantly increased. The frequency of stage T4 bladder cancer was significantly higher during the pandemic, while the frequency of stage T3a was significantly lower. When we consider the results of tumor stages after all operative procedures in our study, significantly more patients with T4 stage were registered during the pandemic, the majority with bladder cancer.

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Key words: *COVID-19 pandemic, urologic surgical procedures, urologic cancers*

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Introduction

The rapid spread of the Coronavirus disease (COVID-19) caused by the new Beta Coronavirus SARS-CoV-2, has significantly threatened the functioning of the entire health system (1). The great and urgent need for hospital and human capacities in the treatment of COVID-19 patients has led to a rapid overload of health institutions all over the world. In this way, diagnostic and therapeutic procedures for patients who needed medical treatment due to other diseases and conditions were postponed in most cases, especially at the beginning of the pandemic (2). Fear of viral transmission, the implementation of epidemiological measures, as well as the reduced availability of medical practitioners at all levels of health care were the most common reasons why non-COVID patients did not regularly attend medical examinations (3, 4). This was especially the case with the elderly population, which was more susceptible to serious illness from COVID-19 (5). Considering that a significant part of uro-oncology patients consists of this geriatric population, the pandemic has also affected their treatment (6).

In the Republic of Serbia, the first case of COVID-19 was officially registered on March 6, and the epidemic of this disease was declared on March 19, 2020 (7). Since then, the statistical curve of patients who suffered or died from COVID-19 has been changing, with several registered waves, and the last one recorded in March 2022 (8). Since the beginning of the epidemic, a large number of COVID patients have been hospitalized at the University Clinical Center

Niš. The first, sudden hospital patient admission was recorded in March and April 2020, when the capacities of the University Clinical Center Niš, including the Clinic of Urology, were designated for the treatment of COVID patients. During that period, only emergency procedures were carried out at our institution. After that, we started the strategy of conducting also elective, oncological procedures.

Aim

This study aimed to examine the impact of the COVID-19 pandemic on the structure of patients who had undergone surgical treatment for oncological indications at the Clinic of Urology of the University Clinical Center Niš. In this sense, we compared the number of performed uro-oncological surgical procedures, as well as post-operative, histopathological findings between the period before and the period during the pandemic.

Material and Methods

The retrospective study included data on oncological surgical procedures performed at the Clinic of Urology, University Clinical Center Niš between March 2018 and June 2022. The following procedures were included in the study: nephrectomy for renal cancer, nephroureterectomy for upper urothelial cancer, prostatectomy for prostate cancer, cystectomy for bladder cancer, orchiectomy for testicular cancer and penectomy for penile cancer. Data on the number of procedures, patients' age and gender, as well as postoperative, histopathological tumor stage, grade and type were taken into account. Depending on the time the surgery was performed, patients were divided into two groups: the pre-COVID group - procedures carried out in the period before the pandemic (March 1, 2018 – March 1, 2020) and the COVID group - procedures carried out during the period of the COVID-19 pandemic (June 1, 2020 – June 1, 2022). March and April 2020 were not included in the study, because only emergency procedures were performed during that period.

Data analysis was performed using the MedCalc program (version 22). The Chi-square test was used to test the statistical significance of absolute frequency differences between samples. The comparison of arithmetic means of two samples was performed by Student's t-test. A p-value < 0.05 was considered statistically significant.

Results

A total of 569 uro-oncological surgical procedures were performed at the Clinic of Urology, University Clinical Center Niš during the study period, 320 in the period before and 249 during the COVID-19 pandemic. No statistically significant difference was observed in terms of age and gender distribution between patients of both groups who underwent the same surgical procedures. The total number of operations, as well as their number by group, is shown in Figure 1. The most frequently performed surgical procedures in both groups were nephrectomies. In relation to the pre-pandemic period, during the pandemic the largest and statistically significant decrease was registered in the number of prostatectomies (by 47.7%, $p = 0.0003$), followed by nephrectomies (by 37.3%, $p = 0.0015$). A decrease was also registered in the number of nephroureterectomies (by 24.1%). Operations with an increase in number during the pandemic were cystectomy (by 38%) and orchiectomy (by 7.8%). The number of operations for penile cancer was identical before and during the pandemic (Figure 1). Figure 2 shows a comparative view of the structure of surgical procedures between the examined groups. The proportion of prostatectomies in relation to the total number of operations was significantly lower in the COVID group compared to the pre-COVID group ($p = 0.0134$). On the other hand, the percentage of cystectomies increased significantly during the COVID-19 pandemic ($p = 0.0004$). The proportion of other procedures by group did not differ significantly.

In our research, stage T1a renal cancer was more prevalent before the pandemic compared to the pandemic period, but this difference was not statistically significant. The frequency of stage T1b and T2 was similar in both studied groups. On the other hand, a higher percentage of stage > T2 was registered during the pandemic compared to the pre-pandemic period (35.1% vs. 27.9%), but without a significant difference. Also, no significant difference was observed between the examined groups in terms of the histopathology type, as well as kidney cancer grade (Table 1). When it comes to prostate cancer, the prevalence of tumor stage and Gleason score did not significantly differ between the examined groups. The incidence of locally advanced prostate cancer (T3–4) was similar between the pre-COVID and COVID groups (41.9% vs. 40%) (Table 2). The distribution of tumor stage in testicular cancer was similar in both studied groups. The ratio of seminoma to non-seminomatous testicular cancer was 1 : 1 before the pandemic, while a slight increase in the number of non-seminomatous tumors was recorded afterwards (Table 3). No significant difference was observed regarding the stage and grade of penile cancer (Table 4).

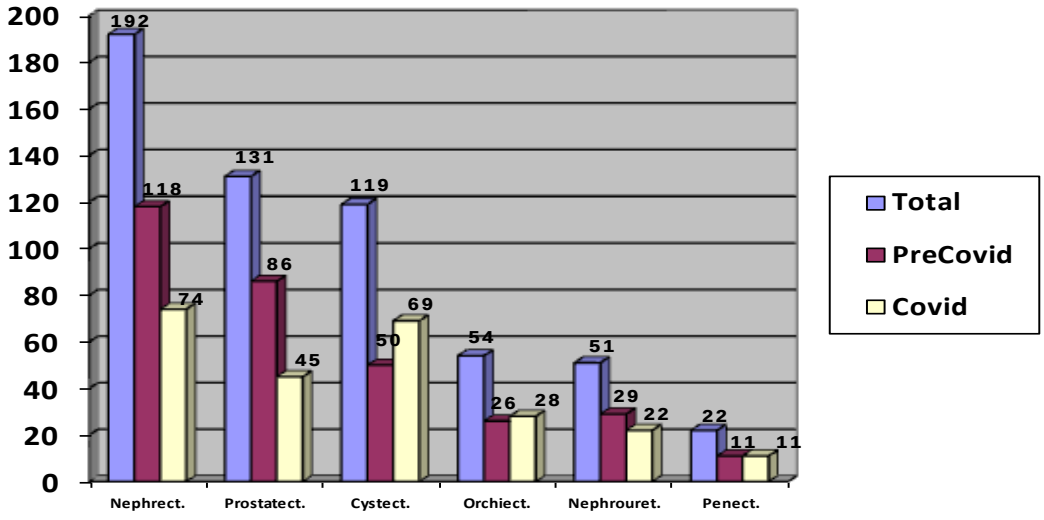

Figure 1. Number of operative procedures in total and by groups

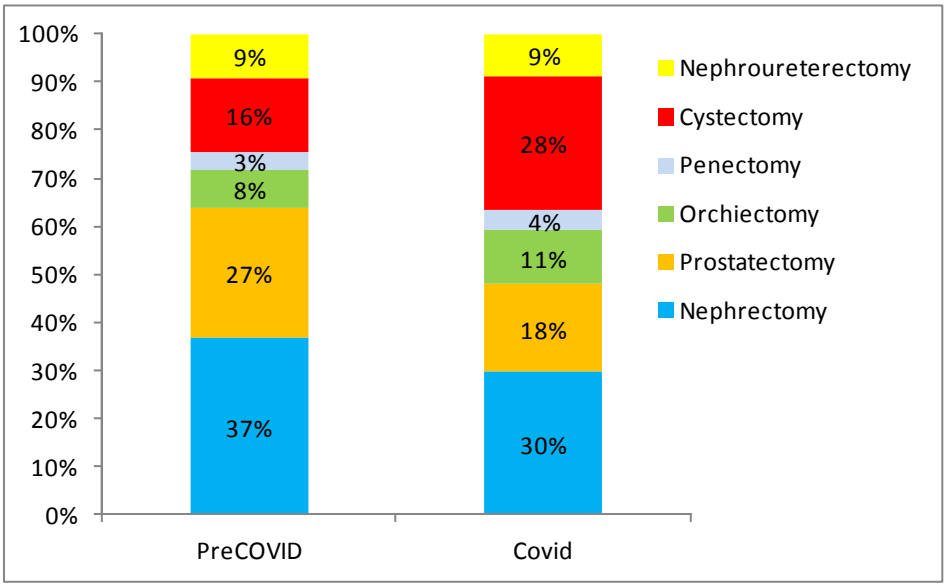

Figure 2. Comparative view of the percentage of surgical procedures between the examined groups

Table 1. Characteristics of patients who underwent nephrectomy

	PreCOVID group	COVID group	p-value
Number of patients	118	74	0.0015
Age (years)	61.4 ± 12.5	59.7 ± 13	0.3676
Gender			
Male	67 (56.8%)	41 (55.4%)	0.8522
Female	51 (43.2%)	33 (44.6%)	
Tumor stage			
T1a	37 (31.4%)	18 (24.3%)	0.2955
T1b	29 (24.6%)	19 (25.7%)	0.8644
T2a	19 (16.1%)	5 (6.8%)	0.0574
T2b	3 (2.5%)	6 (8.1%)	0.0765
T3a	29 (24.6%)	25 (33.8%)	0.1684
T3b	/	/	
T3c	/	/	
T4	1 (0.8%)	1 (1.3%)	0.7385
Tumor grade			
G1	20 (16.9%)	12 (16.2%)	0.8948
G2	61 (51.8%)	33 (44.6%)	0.3394
G3	24 (20.3%)	14 (18.9%)	0.8105
G4	13 (11%)	15 (20.3%)	0.0778
Tumor type			
ccRCC	103 (87.3%)	61 (82.4%)	0.3548
chRCC	1 (0.8%)	2 (2.7%)	0.3143
pRCC	13 (11.1%)	10 (13.5%)	0.6051
other	1 (0.8%)	1 (1.4%)	0.7385

Table 2. Characteristics of patients who underwent prostatectomy

	PreCOVID group	COVID group	p-value
Number of patients	86	45	0.0003
Age (years)	61.3 ± 4.7	60.9 ± 5.3	0.6588
Tumor stage			
T2a	10 (11.6%)	4 (8.9%)	0.6312
T2b	10 (11.6%)	5 (11.1%)	0.9300
T2c	30 (34.9%)	18 (40%)	0.5653
T3a	16 (18.6%)	7 (15.6%)	0.6643
T3b	20 (23.3%)	10 (22.2%)	0.8940
T4	/	1 (2.2%)	
Gleason score (GS)			
GS 6	27 (31.4%)	20 (44.4%)	0.1407
GS 7	48 (55.8%)	19 (42.2%)	0.1410
GS 8	10 (11.6%)	4 (8.9%)	0.6312
GS 9	1 (1.2%)	2 (4.5%)	0.2349

Table 3. Characteristics of patients who underwent orchiectomy

	PreCOVID group	COVID group	p-value
Number of patients	26	28	0.7877
Age (years)	34.5 ± 14.7	33.9 ± 13.1	0.8746
Tumor stage			
T1	10 (38.5%)	11 (39.3%)	0.9510
T2	14 (53.8%)	16 (57.1%)	0.8093
T3	2 (7.7%)	1 (3.6%)	0.5128
T4	/	/	
Tumor type			
Seminoma	13 (50%)	13 (46.4%)	0.7949
Non-seminoma	13 (50%)	15 (53.6%)	

Table 4. Characteristics of patients who underwent penectomy

	PreCOVID group	COVID group	p-value
Number of patients	11	11	0.8890
Age (years)	66.8 ± 9.6	67.4 ± 10.3	
Tumor stage			0.4028
T1	6 (54.6%)	4 (36.4%)	0.6770
T2	5 (45.4%)	6 (54.6%)	
T3	/	1 (9.1%)	
T4	/	/	
Tumor grade			0.3496
G1	4 (45.5%)	2 (18.2%)	0.1797
G2	6 (54.5%)	9 (81.8%)	
G3	1 (9.1%)	/	
G4	/	/	0.8890

The prevalence of stage ≤ T2 bladder urothelial cancers between the pre-COVID and COVID groups was not significantly different (36% vs. 28.9%, p = 0.4198). However, the frequency of T4 stage was significantly higher in the COVID group (p = 0.0342), whereby 1/3 of the patients in this group had this stage of the disease. Also, when we consider the proportion of patients with stage ≥ T3b, there is an even greater statistical significance between the COVID and pre-COVID group (59.5% vs. 34%, p = 0.0064). In our study, two cases of stage T4b after cystectomy were registered, both in the COVID group. On the other hand, in the period before the pandemic, there were significantly more patients with stage T3a (p = 0.0124). The incidence of high-grade bladder cancer was higher during the pandemic, but with no statistical significance (Table 5). There was no significant difference in tumor stage or grade for upper urothelial cancer. However, the only case of the T4 stage was recorded during the pandemic (Table 6).

When we consider the results of tumor stages after all operative procedures in our study, the COVID group had significantly more patients with T4 stage compared to the pre-COVID group (p = 0.0002). The distribution of other tumor stages was similar between the examined groups (Figure 3). The largest number of stage T4 tumors was verified after cystectomy (23 cases), while one case each was recorded after nephrectomy, nephroureterectomy and prostatectomy.

Table 5. Characteristics of patients who underwent cystectomy

	PreCOVID group	COVID group	p-value
Number of patients	50	69	0.0824
Age (years)	66.7 ± 10.4	65.4 ± 9.6	0.4828
Gender			
Male	39 (78%)	57 (82.6%)	0.5315
Female	11 (22%)	12 (17.4%)	
Tumor stage			
T1	5 (10%)	9 (13%)	0.6125
T2a	4 (8%)	3 (4.3%)	0.4053
T2b	9 (18%)	8 (11.6%)	0.3263
T3a	15 (30%)	8 (11.6%)	0.0124
T3b	9 (18%)	18 (26.1%)	0.3005
T4	8 (16%)	23 (33.4%)	0.0342
Tumor grade			
Low grade	11 (22%)	8 (11.6%)	0.1277
High grade	39 (78%)	61 (88.4%)	

Table 6. Characteristics of patients who underwent nephroureterectomy

	PreCOVID group	COVID group	p-value
Number of patients	29	22	0.3317
Age (years)	63.4 ± 6.2	62.8 ± 5.8	0.7265
Gender			
Male	18 (62.1%)	14 (63.6%)	0.9096
Female	11 (37.9%)	8 (36.4%)	
Tumor stage			
Ta	9 (31%)	6 (27.3%)	0.7725
T1	3 (10.3%)	1 (4.55%)	0.4500
T2	5 (17.3%)	3 (13.6%)	0.7285
T3	12 (41.4%)	11 (50%)	0.5440
T4	/	1 (4.55%)	
Tumor grade			
Low grade	15 (51.7%)	8 (36.4%)	0.2797
High grade	14 (48.3%)	14 (63.6%)	

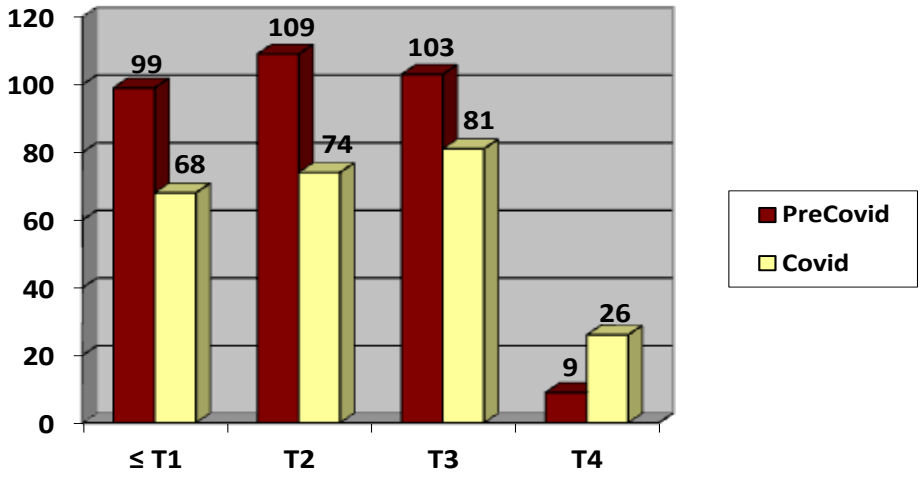

Figure 3. Distribution of tumor stages after all operative procedures

Discussion

There was a 22.2% decrease in the number of uro-oncological surgical procedures during the pandemic, compared to the pre-pandemic period. A number of studies by other authors have also registered a decrease in the number of uro-oncological procedures during the pandemic. A large retrospective study in the United Kingdom, which included more than 110 thousand uro-oncological procedures, registered a decrease of 7.6% (9). In our study, nephrectomies due to renal cancer were the most frequently performed procedures in both investigated time periods. However, almost 40% more nephrectomies were performed before the pandemic. Renal cancer is often asymptomatic, especially in the lower stages of the disease, and is often discovered as an incidental finding during radiological, systematic examinations (10). The reduced availability of radiological examinations, as well as the reduced number of systematic examinations during the pandemic, could be one of the reasons why a smaller number of kidney tumors, especially those of smaller diameter, were diagnosed in this period. In our study, the percentage of stage T1a tumors was higher in the prepandemic compared to the pandemic period, but this difference is not statistically significant. At the beginning of the pandemic, the European Association of Urologists (EAU) recommended that nephrectomy for renal cancer stage < T2 can be safely delayed for 3 months. On the other hand, treatment of advanced renal cancer, especially those with

associated venous thrombosis, should not be delayed (11). In our study, a higher percentage of stage > T2 was registered during the pandemic compared to the pre-pandemic period, but the difference was not significant. Similar results were also shown in a study by Turkish authors. They recorded a decrease in the number of nephrectomies by almost 50% during the pandemic, with a similar ratio of tumor stage and grade as in our study (12). On the other hand, some studies did not record a significant decrease in the number of nephrectomies during the pandemic period (9, 13).

Patients with prostate cancer are also often asymptomatic, and timely diagnosis largely depends on regular screening. Studies have shown that fewer men reported for prostate cancer screening during the pandemic (14, 15). At the beginning of the COVID-19 pandemic, EAU guidelines recommended that radical prostatectomy in patients with low-, intermediate-, and even in some cases high-risk cancers can be postponed, without a clearly defined time distance (11). The fact is that an alternative modality of treatment (hormonal and radiation therapy), which requires less direct contact with medical personnel and does not require hospitalization of the patient, was a safer treatment option when it comes to the risk of viral transmission. This was certainly one of the important reasons why patients chose this treatment modality instead of radical prostatectomy (16). In our study, the largest and statistically significant decrease in the number of procedures during the pandemic was recorded in radical prostatectomies. The aforementioned study in the United Kingdom has also recorded the greatest decrease in the number of radical prostatectomies among all uro-oncological procedures (9). Other studies also registered a decrease in the number of prostatectomies of up to 50%, with no difference in the stage and grade of tumors before and during the pandemic (12, 17).

One of the main results of our study concerns the data on performed cystectomies for bladder cancer. The proportion of cystectomies, unlike other operations, increased significantly during the pandemic period. Significantly more stage ≥ T3b bladder cancers were registered in the COVID group, which were also registered in more than half of the cystectomy cases in this group. In about 1/3 of the cases in COVID group, cystectomy was performed in patients with bladder cancer stage T4. Total, painless hematuria is generally the main clinical sign in patients with bladder cancer and its presentation is often intermittent. The author's experience shows that patients who were later diagnosed with bladder cancer, often ignored the initial appearance of painless hematuria and came for an examination only after the recurrent or massive hematuria, which delayed diagnosis and treatment even for several months. The pandemic also affected patients with non-muscle-invasive bladder cancer, who delayed cystoscopic examinations during the follow-up, which influenced their further treatment (18, 19). At the beginning of the COVID pandemic, most of the relevant uro-oncology organizations considered that the necessary diagnostics in patients with total, painless hematuria should not be postponed, but there were also those who recommended that it could be postponed for 1−2 months (11). It is known that delaying a cystectomy for ≥ 12 weeks increases the chance of tumor stage progression and decreases survival in patients with muscle-invasive carcinoma (20). The results of other studies on the pandemic impact on the treatment of bladder cancer patients are various. In the study by Romanian authors, significantly more cystectomies, as well as stage T3 and T4 bladder cancers, were registered during the pandemic compared to the pre-pandemic period (21). Other studies also registered an increase in the number of cystectomies, but without a significant difference in tumor stage (12, 17). In the study by Brument M. et al., a decrease in the number of cystectomies by 2.4% was recorded (9). When it comes to upper urothelial cancer, our study results are different compared to cancer of the lower urothelium. The percentage of nephroureterectomies was identical in both investigated time periods in our study. Although the only T4 stage was registered in the COVID group, there was no statistically significant difference comparing the other stages between the pre-COVID and COVID groups. The percentage of high-grade tumors was higher during the pandemic but without statistical significance. A study by Japanese authors also showed no significant difference in tumor stage after nephroureterectomy before and during the pandemic (22). In our study, there was no difference in terms of tumor stage and grade between the examined groups when it comes to testicular and penile cancers. The results of other studies are similar to our results (12, 23).

Conclusion

During the pandemic, a decrease in the total number of uro-oncological surgical procedures was recorded, of which a statistically significant decrease was registered in the number of prostatectomies and nephrectomies. Unlike other procedures, the percentage of cystectomies increased significantly during the pandemic period. In this period, significantly more bladder cancers of stage ≥ T3b and significantly fewer cancers of stage T3a were registered. When we consider the results of tumor stages after all procedures in our study, significantly more patients with T4 stage were registered during the pandemic, most of them with bladder cancer.

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UTICAJ COVID-19 PANDEMIJE NA STRUKTURU BOLESNIKA PODVRGNUTIH OPERATIVNOM LEČENJU ZBOG UROONKOLOŠKIH INDIKACIJA

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Cilj ove retrospektivne studije bio je da ispita uticaj pandemije COVID-19 na strukturu bolesnika koji su zbog onkoloških indikacija bili podvrgnuti operativnom lečenju na Klinici za urologiju Univerzitetskog kliničkog centra u Nišu u periodu od marta 2018. godine do juna 2022. godine. Istraživanjem su bili obuhvaćeni sledeći operativni zahvati: nefrektomija zbog karcinoma bubrega, nefroureterektomija zbog karcinoma gornjeg urotela, prostatektomija zbog karcinoma prostate, cistektomija zbog karcinoma mokraćne bešike, orhiektomija zbog tumora testisa i amputacija penisa zbog karcinoma penisa. Uzeti su podaci o broju operacija, godinama starosti i polu bolesnika, kao i postoperativni histopatološki nalazi. Operativne procedure podeljene su u dve grupe: pre COVID grupa uključila je procedure sprovedene pre početka pandemije (1. mart 2018. godine – 1. mart 2020. godine), a COVID grupa one sprovedene nakon početka pandemije COVID-19 (jun 2020. godine – jun 2022. godine). Urađeno je ukupno 569 uroonkoloških operacija – 320 pre početka pandemije i 249 nakon početka pandemije. U obema ispitivanim grupama najčešće sprovedene procedure bile su nefrektomije. Statistički značajan pad broja prostatektomija i nefrektomija registrovan je u toku pandemije. Udeo prostatektomija bio je statistički značajno manji u COVID grupi, dok je udeo cistektomija u njoj statistički značajno porastao. Učestalost T4 stadijuma karcinoma mokraćne bešike bila je statistički značajno veća tokom pandemije, a učestalost T3a stadijuma značajno manja. Rezultati u vezi sa tumorskim stadijumima, dobijeni nakon svih operativnih procedura ispitanih u ovoj studiji, pokazali su da je značajno više bolesnika sa T4 stadijumom registrovano u toku pandemije; pritom, u većini slučajeva radilo se o karcinomu mokraćne bešike.

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***Ključne reči**: COVID-19 pandemija, urološke operacije, karcinomi u urologiji*

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ASSOCIATION OF THE GENETIC POLYMORPHISM RS11640851 *MT1A* 80 C/A AND TYPE 2 DIABETES MELLITUS IN THE CENTRAL BALKAN POPULATION

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Type 2 diabetes mellitus (T2DM) is the most common type of diabetes and is becoming an increasingly prevalent global health issue. Polymorphisms in genes coding metallothioneins, a group of small zinc-binding proteins that participate in antioxidative protection, are believed to be involved in T2DM pathogenesis. This study aimed to investigate the potential association of the single nucleotide polymorphism (SNP) rs11640851 MT1A 80 C/A and the T2DM risk and to determine the impact of the genotype and allelic distribution on the diabetes-related biochemical parameters. The study included 298 subjects, 112 with T2DM and 186 healthy, non-diabetic controls. The participants' fasting glycemia and HbA1c levels were measured, while the SNP in the MT1A gene was determined using the PCR-RFLP method. There were no significant differences in the genetic distribution and allele frequency between control subjects and diabetic patients ($p > 0.05$). There was likewise no association between the SNP and diabetes-associated laboratory parameters, fasting serum glucose and HbA1c levels. However, 79.6% of allele C carriers had fasting glucose levels above 7 mmol/L, versus 53.3% of subjects homozygous for allele A ($p = 0.005$). Although our study did not find a direct association between the MT1A genetic variants and the occurrence of T2DM, we observed an effect of the allele C on glycemic control in the patients. Further research in a larger population is needed to expand these findings and to improve the understanding of metallothionein genes and their impact on the development of T2DM.

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Key words: *type 2 diabetes, metallothionein, zinc, single nucleotide polymorphism*

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Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia caused by a relative insulin deficiency (1). It is the most common type of diabetes by far, accounting for roughly 95% of all cases, affecting over 400 million people worldwide (2). Overall incidence of DM has doubled between 1990 and 2017, with the vast majority of T2DM cases (3). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs and systems, especially cardiovascular and peripheral nervous systems, as well as the eyes and kidneys (1).

Alterations in the metabolism of essential trace elements, including zinc, have been consistently observed in patients with T2DM (4). Reduced levels of serum zinc in diabetic patients have been reported in multiple studies (5), and some linked hypozincemia to the severity of diabetes and associated complications, such as diabetic retinopathy, diabetic nephropathy and diabetic peripheral neuropathy (6 – 9). Earlier studies have also found that Zn ions are involved in insulin synthesis, storage, secretion, and as insulin signaling, leading some authors to propose the inclusion of impaired zinc metabolism to the list of metabolic disorders in diabetes (10).

Moreover, studies have demonstrated that a family of small, cysteine-rich proteins called metallothioneins (MTs), may have an important role in Zn ion buffering and signaling, as well as in nitric oxide (NO) signaling and oxidative stress defense. Previous studies have suggested that MTs have important roles in essential metals homeostasis, defense against heavy metal poison-

ing and as anti-oxidative protection (11) due to their ability to scavenge reactive oxygen and nitrogen species (ROS/RNS) (12 – 14).

There are four main MTs isoforms in humans (15, 16), two of which, MT1 and MT2, are expressed in the majority of tissues, most abundantly in the liver, pancreas, intestine and kidney (17).

Interest in MTs has grown over the years, as they were found to be implicated in a variety of pathological processes, from accumulation and toxicity of heavy metals (Cd, Hg, Pb), to association with multiple types of cancer, cardiovascular diseases and diabetes mellitus, and psychiatric disorders such as autism (18).

The expression levels and function of MTs are highly variable and dependent on polymorphisms present in the MT coding genes, which may contribute to the occurrence and development of various pathologies. To date, only a handful of studies have investigated the association between MT1 and MT2 gene single nucleotide polymorphisms (SNPs) and their contribution to the development of T2DM (18 – 20).

One of the SNPs of particular interest in T2DM is rs11640851 MT1A 80 C/A, also known as MT1A Thr27Asn or MT1A +647 A/C, a polymorphism located in the coding region of MT1A gene that leads to an amino acid substitution. Association of this polymorphism and T2DM was suspected (21), but it was never reported before in the Central Balkan population.

This study aimed to investigate the potential association of SNP rs11640851 MT1A 80 C/A and the T2DM risk, and to determine the impact of the genotype and allelic distribution on the diabetes-related biochemical parameters, specifically patients' glycemia and glycated hemoglobin levels.

Materials and Methods

Patients

In the study, a total of 112 patients with T2DM were recruited from the Endocrinology, Diabetes and Metabolic Diseases Clinic, University Clinical Center Niš. The patients were recruited while attending the clinic for their routine check-ups. Additionally, 186 healthy subjects were recruited as non-diabetic controls.

Biochemical analyses

Fasting serum glucose and glycated hemoglobin (HbA1c) levels were measured in all of the study participants using standard methods on an automated clinical chemistry analyzer Beckman-Coulter AU680 at the Medical and Clinical Biochemistry Center, University Clinical Center Niš. Genotyping was performed in the Laboratory for Functional Genomics and Proteomics of the Scientific Research Center for Biomedicine of the Medical Faculty in Niš.

MT1A 80 C/A rs11640851 genotyping

The subject's DNA was extracted from 200 µL of whole blood, which was sampled in 3 mL EDTA tubes. A commercial DNA purification kit (Genomic DNA Purification Kit, Thermo Scientific, Lithuania) was used for DNA extraction according to the manufacturer's instructions. Genotyping of the SNP in MT1A gene 80 C/A rs11640851 was performed using the PCR-RFLP method adapted from Cipriano et al. 2006 (22). The primers used were forward, 5'-CACTCAGCTGGCAGCATTTG-3' and reverse 5'-ACTTGGCTCAGCCCCAGATT-3'. The reaction mixture consisted of 0.1 µL HotStart DNA polymerase (FIREPol DNA polymerase, Solis BioDyne, Tartu, Estonia), 2 µL FIREPol Buffer B 10x, 1.2 µL $MgCl_2$, 0.2 µL dNTP mix (20 mM of each), 0.4 µM of each primer, 1 ng/µL of DNA template, and PCR grade water was added up to a total volume of 20 µL. Amplification was performed using the following program: initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 61 °C for 45 s, elongation at 72 °C for 60 s, and final elongation at 72 °C for 7 minutes. The PCR product (187 bp) was digested using the MnlI restriction enzyme (NEB, Ipswich, MA, USA) at 37 °C for 50 minutes, and the resulting fragments were resolved using vertical electrophoresis on an 8% polyacrylamide gel. The gel was then stained in ethidium bromide solution and observed under UV light. The presence of the allele A was identified by the undigested 187 bp band, while the allele C was identified by two bands of 140 and 47 bp. Heterozygous samples displayed all three bands.

Statistical analysis

The distribution of the genotypes for the polymorphism was assessed for deviation from the Hardy-Weinberg equilibrium (HWE). The characteristics of the study group were expressed as the median and interquartile range, or the mean and standard deviation or frequency (with or without percentages). Student's t-test (for normally distributed data) or the Mann-Whitney U test (non-normally distributed data) was employed for the comparison of two independent samples. ANOVA (for normally distributed data) with Tukey as Post Hoc Test and Kruskal Wallis (non-normally distributed data) with Mann-Whitney U test as Post Hoc were used to compare more than two defined groups. Chi-square (χ^2) test was used to compare data between groups when data were defined as categorical. All analyses were performed using IBM SPSS Statistics for Windows, v.24.0 (IBM Corp, Armonk, NY, USA) with the significance level set at $p < 0.05$.

Results

General characteristics and biochemical parameters of the subjects are displayed in Table 1. Subjects with diabetes were on average of older

Table 1. Characteristics of the study population

	Non-Diabetics (n = 186)	Diabetics (n = 112)	Test (t/Z, χ^2) and significance
Sex (male/female)	30.65%/69.35%	50.00%/50.00%	χ^2 = 11.124; p = 0.001
Age (years)	60.31 ± 9.70 60 (13)	65.12 ± 9.19 65 (12)	T = -4.229; p < 0.001
Weight (kg)	80.52 ± 15.33 78 (20)	88.63 ± 15.40 88 (22)	Z = -4.393; p < 0.001
Body mass index (kg/m^2)	28.09 ± 4.11 27.92 (5.68)	30.63 ± 4.44 31.00 (5.83)	T = -4.972; p < 0.001
Serum glucose level (mmol/L)	5.57 ± 0.53 5.50 (0.70)	8.34 ± 2.65 8.00 (2.70)	Z = -11.820; p < 0.001
HBA1c (%)	5.43 ± 0.40 5.40 (0.50)	7.60 ± 1.71 7.00 (2.40)	Z = -13.257; p < 0.001

t- Data are presented as mean ± standard deviation and media (interquartile range) or frequency (%). Student – t Test; Z-Mann Whitney U Test; Chi-Square (χ^2) Test

Table 2. Genotypes and alleles of MT1A in relation to diabetes presence

	MT1A AA	*MT1A* AC	*MT1A* CC	Allele A frequency	Allele C frequency
Non-diabetics (n = 180)	75	78	27	0.63	0.37
Diabetics (n = 99)	45	41	13	0.66	0.34
Test and significance	χ^2 = 0.424; p = 0.809			χ^2 = 0.4453; p = 0.505	

Table 3. Genetic dominant model of MT1A genotypes in relation to diabetes presence

	MT1A AA genotype	*MT1A* C allele (AC+CC genotype)	Test and significance
Non-diabetics (n = 180)	75	105	χ^2 = 0.374;
Diabetics (n = 99)	45	54	p = 0.541

age, had greater overall body weight, and had increased BMI (p < 0.001). Diabetic patients had significantly increased fasting glucose levels compared to the control subjects (p < 0.001), as well as significantly increased levels of HbA1c (p < 0.001).

Of the total number of 298 subjects enrolled in the study, 279 were genotyped for MT1A 80 C/A polymorphism. Of these, 180 were control subjects and 99 had diabetes.

The distribution of genotypes of MT1A 80 C/A in subjects is summarized in Table 2, and was in Hardy-Weinberg equilibrium in both the control and diabetic groups (p > 0.05). No significant differences in genotypes were found between the diabetes patients and controls. Minor allele frequency (allele C) was 0.37 in the control group and 0.34 in the diabetes group, which was not a significant difference (p> 0.05).

To assess the potential impact of the C allele on diabetes risk, a genetic dominant model that classified AA genotypes differently from AC and CC genotypes was used (Table 3). However, no significant difference in C allele frequency was found between the control and diabetes groups.

The association of MT1A 80 C/A and the laboratory parameters of diabetes, fasting glucose and HbA1c levels, was also explored in the diabetes group (Table 4). Average glycemia in the allele C carriers was 8.68 ± 2.82 mmol/L vs. 8.15 ± 2.60 mmol/L in the genotype AA patients, which was not a significant difference (p > 0.05). Also, HbA1c levels were similar between allele C carriers and non-carriers (7.67 ± 1.89% vs. 7.59 ± 1.67%, p > 0.05).

As the subjects in the diabetes group were all receiving anti-diabetic medication, a proportion of them did not show serum glucose and HbA1c values characteristic of diabetes, which were ≥ 7.0 mmol/L for glycemia and ≥ 6.5% for HbA1c, i.e. medication was masking the true diabetes phenotype. For this reason patients were stratified based on the aforementioned cutoff values for glycemia and HbA1c, both individually and together (Table 5). After the exclusion of subjects with regulated biochemical parameters, it was revealed that 79.6% of allele C carriers had fasting glucose levels above 7 mmol/L, versus 53.3% of subjects homozygous for allele A (p = 0.005). On the other hand, patients with values of

Table 4. Serum glucose level and HbA1c with respect to MT1A genotype

	MT1A AA genotype	*MT1A* C allele (AC+CC genotype)	Test and significance
Fasting glucose (mmol/L)	8.15 ± 2.60 7.20 (3.80)	8.68 ± 2.82 8.30 (2.20)	Z = -1.409; p = 0.159
HBA1c (%)	7.67 ± 1.89 6.80 (2.90)	7.59 ± 1.67 7.45 (2.50)	Z = -0.197; p = 0.844

Table 5. Genotype frequencies of MT1A with stratification by laboratory parameters

	MT1A AA genotype (n = 45)	*MT1A* AC+CC genotype (n = 54)	Test and significance
Fasting glucose (> 7 mmol/L)	24 (53.3%)	43 (79.6%)	X^2 = 7.759; p = 0.005
HBA1c (> 6.5 %)	32 (71.1%)	39 (72.2%)	X^2 = 0.015; p = 0.903
Fasting glucose (> 7 mmol/L) and HBA1c (> 6.5 %)	20 (44.4%)	36 (66.7%)	X^2 = 4.934; p = 0.026

HbA1c ≥ 6.5% revealed no differences regarding the presence or absence of allele C (72.2% vs. 71.1%, respectively, p > 0.05). In addition to that, among patients that displayed both glycemia and HbA1c levels above the cutoff values, 66.7% were allele C carriers, versus 44.4% allele A homozygotes (p = 0.026).

Discussion

Type 2 diabetes mellitus is a metabolic disorder characterized by hyperglycemia dominantly caused by insulin resistance. It manifests with a complex pathology involving dyslipidemia and hyperlipidemia, an increase in weight due to accumulation of adipose tissue, as well as increased oxidative stress, proinflammatory state and endothelial dysfunction (1, 23, 24). Elevated glucose causes non-enzymatic glycation of proteins and lipids, leading to the accumulation of advanced glycation end products (AGEs), which further exacerbate oxidative stress, LDL oxidation and activation of immune cells (25, 26).

Oxidative stress is a crucial mechanism of pathogenesis in diabetes. Some of the typical findings in both diabetic patient and experimental animal models are impaired functionality of antioxidative protection enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, catalase and paraoxonase (PON), as well as increased production of ROS/RNS and levels of oxidative stress markers, such as malondialdehyde (27). Excessive production of ROS/RNS worsens insulin resistance and accelerates the development of the microvascular and macrovascular complications of diabetes (28). Some authors even suggested that diabetes mellitus itself should be considered an oxidative stress disease (29).

Metallothioneins provide a substantial contribution to antioxidative defenses. Thanks to their high thiol (-SH) group content, metallothioneins react directly with ROS/RNS, neutralizing them similar to glutathione (GSH). In the case of •OH radicals, MTs neutralize them at a rate of approximately 340 times higher than that of GSH (13). Metallothioneins also sequester dangerous Fenton metal ions, such as Cu+ and Fe^{2+} (30). Additionally, MTs provide a stable availability of Zn ions, which are crucial cofactors of numerous enzymes, including SOD (31). Given the involvement of MTs in antioxidative defenses, a number of researchers have explored the possible associations between MT alterations and various pathological conditions (32).

In this study, we investigated the potential association of the genetic polymorphism MT1A 80 C/A rs11640851, which causes amino acid substitution asparagine (allele A) to threonine (allele C) at position 27 of the MT1A polypeptide chain, where the Asn variant is the wild type. We found that there were no significant differences in the genotype distribution and allelic frequency of MT1A 80 C/A SNP between control subjects and patients with T2DM. Previous studies conducted in the central Italian population found a significant association of rs11640851 allele C with longevity in elderly women, as well as an increased T2DM occurrence with a higher risk of diabetic cardiovascular complications in the general population (21, 22). With this in mind, a genetic dominant model was used to assess whether allele C was a potential risk allele in our study, but no significant differences were found between the control group and the group of patients with diabetes.

There was likewise no association between MT1A 80 C/A SNP and diabetes-related laboratory parameters, fasting serum glucose and HbA1c levels. Interestingly, the Italian group found an

association between the allele C and elevated fasting glucose and HbA1c levels in the group of diabetic patients with cardiovascular complications (21). Another, surprising, finding in the same study was that peripheral blood mononuclear cells of allele C carriers exhibited increased intracellular MT content, as well as reduced intracellular release of Zn ions upon NO stimulation. A more recent study in India did not find any association between MT1A 80 C/A and laboratory parameters of diabetes, but it did find an increased frequency of allele C in the group of diabetic patients (33).

Since the subjects in the diabetes group were receiving various types and doses of antidiabetic medication, there was a possibility that this variation in medication, along with its varying effectiveness, could have influenced the laboratory measurements of fasting serum glucose and glycated hemoglobin A1c levels. To address this issue, we have stratified the patient group based on their glycemia and HbA1c levels by implementing cutoff values of 7 mmol/L for glycemia, and 6.5% for HbA1c. When stratified by HbA1c values alone, no difference was observed in the genotype and allelic representation. However, there was a clear difference in serum glucose levels between the AA genotype patients and the patients carrying allele C, where 79.6% of allele C carriers had glycemia above 7 mmol/L, compared to 53.3% of the AA homozygotes. When cutoff values for both glycemia and HbA1c were implemented, the significance remained. This finding suggests that MT1A 80 C/A does have an impact on the level of glycemia control in diabetic patients.

The group of researchers led by Mocchegiani provided an explanation for how the presence of the MT1A 80 C allele influences the risk of diabetes including glycemic control in patients with type 2 diabetes. Under conditions of increased oxidative stress and pro-inflammatory signaling the expression of MT1 and MT2 genes is continuously upregulated, as these genes are induced by IL-6 signaling and the presence of ROS/RNS. However, under these altered conditions, increased total Zn binding capacity, without the corresponding increase in zinc pool leads to Zn ion sequestration. Reduction in inducible Zn ion availability inside cells not only impairs the function of antioxidative protection enzymes that require Zn as a cofactor but also disrupts zinc signaling and causes NF-κB over activation (34 – 37).

However, the majority of studies have found that MTs exhibit both antioxidant and anti-apoptotic properties, improving cell survival and functionality (14). Overexpression of MT protects cardiomyocytes from oxidative damage, loss of contractility and apoptosis induced by high-fat diet, while the absence of MT exacerbates diabetic cardiomyopathy in a rodent model (38 – 42). Other studies have found that MTs have neuroprotective properties in the retinal and brain tissues against oxidative stress damage (31, 43 – 46). Additionally, MTs have been found to help in the prevention of diabetes-induced tissue ischemia, by activation of angiogenesis through induction of HIF-1/SDF-1/VEGF pathway (47, 48). It should be noted that experimental animal models are unable to accurately predict physiological and pathological processes in humans reliably, indicating that the mechanisms related to metallothioneins and their function in metabolism are still not fully understood.

Conclusion

This study did not find a direct association between genetic polymorphism in MT1A 80 C/A and the occurrence of type 2 diabetes mellitus. However, there was evidence of poorer glycemic control in diabetic patients with respect to the MT1A alleles they are carrying. A possible implication of the study is in the implementation of personalized medicine, by the inclusion of genetic testing in the diagnostics, for better optimization of treatment. Further research in a larger population is needed to expand these findings and to improve the understanding of metallothionein gene polymorphisms and their impact on the occurrence and progression of diabetes.

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POVEZANOST GENSKOG POLIMORFIZMA RS11640851 MT1A 80 C/A SA DIJABETESOM MELITUSOM TIPA 2 U POPULACIJI CENTRALNOG BALKANA

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Tip 2 dijabetesa melitusa (T2DM) najčešći je tip dijabetesa, koji postaje sve zastupljeniji globalni zdravstveni problem. Veruje se da polimorfizmi u genima koji kodiraju metalotioneine, grupu malih proteina koji vezuju cink i uključeni su u antioksidativnu zaštitu, učestvuju u patogenezi T2DM-a. Cilj ovog istraživanja bilo je ispitivanje potencijalne povezanosti pojedinačnog polimorfizma nukleotida rs11640851 MT1A 80 C/A sa rizikom od T2DM-a, kao i utvrđivanje uticaja genotipa i raspodele alela na biohemijske parametre povezane sa dijabetesom. U istraživanju je učestvovalo 298 ispitanika – njih 112 imalo je T2DM, a preostalih 186 zdravih ispitanika činilo je kontrolnu grupu. Ispitanicima su mereni glikemija natašte i nivo HbA1c. Polimorfizam u MT1A genu utvrđivan je pomoću PCR-RFLP metode. Nisu primećene značajne razlike u distribuciji genotipova i frekvenciji alela između kontrolne grupe i bolesnika sa dijabetesom ($p > 0,05$). Takođe, nije bilo povezanosti između polimorfizma i laboratorijskih parametara povezanih sa dijabetesom, glikemije natašte i nivoa HbA1c. Ipak, nivo glukoze iznad 7 mmol/L zabeležen je kod 79,6% nosilaca alela C, odnosno kod 53,3% ispitanika koji su homozigoti za alel A ($p = 0,005$). Iako u našem istraživanju nije pronađena direktna povezanost između genetskih varijanti MT1A gena i pojave T2DM-a, uočen je uticaj alela C na kontrolu glikemije kod bolesnika. Kako bi se proširila navedena saznanja i unapredilo razumevanje uticaja gena za metalotioneine na razvoj T2DM-a, neophodna su dalja istraživanja sa većim brojem ispitanika.

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***Ključne reči**: tip 2 dijabetesa, metalotionein, cink, pojedinačni polimorfizam nukleotida*

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EFFECT OF SEVERITY OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE ON THE RIGHT VENTRICULAR SYSTOLIC FUNCTION

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According to the World Health Organization, the most common complication of chronic obstructive pulmonary disease (COPD) is chronic pulmonary heart disease (*cor pulmonale chronicum*). It represents myocardial hypertrophy of the right ventricle, dilatation and insufficiency of the right ventricle occurring as a result of changes in lung function/structure in the absence of left heart disease. The gold standard in detecting changes in right heart function in patients with COPD is an echocardiographic examination. The primary goal of this research was to determine the influence of the severity of COPD on the values of the right ventricular systolic function parameter, and the secondary goal of this research was to determine the frequency of tricuspid regurgitation in relation to the degree of COPD. For a detailed assessment of the systolic function of the right ventricle, which is important for the objectives of the study, the following parameters were performed: The fractional area change (FAC) of the right ventricle and tricuspid annular plane systolic excursion (TAPSE) in 44 patients with COPD which were divided into four groups according to the global initiative for obstructive lung disease (GOLD) criteria. There was no statistically significant difference between the groups for anthropometric indicators and FEV1 values (%) ($p > 0.05$).

The Kruskal–Wallis test shows that the TAPSE index and FAC values are significantly higher in patients with severe and very severe COPD ($p < 0.05$). The results of our research show that echocardiographic parameters of the right ventricle such as TAPSE and FAC are very important for assessing its systolic function and that these values decrease proportionally with the progression of the COPD disease.

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Key words: *chronic obstructive pulmonary disease, tricuspid annular plane systolic excursion index, fractional area change*

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by airflow limitation that is progressive and is the result of airway inflammation and remodelling associated with parenchymal destruction and the development of emphysema (1). The leading risk factor for the development of COPD is the consumption of cigarettes, and in areas of air pollution, the disease has an endemic character. It occurs mainly in people over 40 years of age. Patients have symptoms more often in the morning (productive cough, dyspnea) (2).

It is estimated that 210 million people in the world suffer from COPD and that about 3 million people die from this disease every year. It is the 3rd leading cause of death in the world, and over 500,000 patients have been registered in the Republic of Serbia (2). The diagnosis of COPD is determined by a clinical examination based on symptoms, auscultatory findings and diagnostic procedures—spirometry and a post-bronchodilator test that indicates the relationship FEV1/FVC < 0.70%. Based on the The Global Initiative for Obstructive Lung Disease (GOLD) classification, it is divided into mild, moderate, severe and very severe COPD (2).

COPD is associated with significant extrapulmonary (systemic) effects, among which cardiac complications are the most common. Diseases of CVD and COPD are linked by: a large number of common risk factors (e.g. tobacco smoke in coronary disease and COPD), heart dysfunction as the last change in lung functions (primary pulmonary hypertension, increase in intrathoracic pressure), high level of inflammatory proteins in the blood (CRP) (3), disturbance of pulmonary ventilation (FEV1 and FVC), disturbance of blood gases indicating the development of hypoxia and respiratory acidosis (4), endogenous metabolic disorders and failure of neural compensatory mechanisms (5).

By increasing the resistance in the pulmonary blood vessels, changes occur in the pulmonary blood vessels and ventricles, which can lead to the development of pulmonary hypertension and chronic pulmonary heart disease, right systolic dysfunction and left ventricular diastolic dysfunction (6). According to the World Health Organization, the most common complication of COPD is chronic pulmonary heart disease (*cor pulmonale chronicum*). It represents myocardial hypertrophy of the right ventricle, dilatation and insufficiency of the right ventricle occurring as the result of changes in lung function/structure in the absence of left heart disease (7).

The gold standard in detecting changes in right heart function in patients with COPD is an echocardiographic examination. Echocardiographic characteristics of chronic pulmonary heart disease: enlarged right atrium and right ventricle, right ventricular hypertrophy, and paradoxical septum movements that occur as a result of loading the right ventricle with increased pressure in the pulmonary circulation (8, 9). To assess the function of the circumferential myocardial fibres of the right ventricle, FAC is determined, a parameter that indicates the percentage change in the area of the right ventricle in systole compared to diastole and directly correlates with the ejection fraction of the right ventricle obtained by magnetic resonance imaging.

Left ventricular dysfunction parameter values in COPD patients are often the subject of studies by pulmonologists and cardiologists around the world, and pulmonary hypertension is often investigated. However, parameters of right ventricular dysfunction are rarely examined.

Aim

The primary goal of this research was to determine the influence of the severity of COPD on the values of the right ventricular systolic function parameter, and the secondary goal of this research was to determine the frequency of tricuspid regurgitation in relation to the degree of COPD.

Material and Methods

This prospective study included 44 patients with chronic obstructive pulmonary disease. The research was conducted at the Pulmonary Clinic of the University Clinical Center in Niš in the period from January 2021 to January 2022. Patients were included in the research during regular examinations at the Pulmonary Diseases Clinic of the University Hospital Niš. During the examination, before hospitalization or previous outpatient examinations, patients were proven to suffer from COPD according to the standardized GOLD criteria (Figure 1.). Data on the clinical picture of each patient were obtained through anamnesis and clinical examination. Among the pulmonological diagnostic methods, spirometry, bronchodilator test and blood gas analysis were performed. Of the cardiological diagnostic methods, blood pressure was measured with a sphingometer, a standard 12-channel electro-cardiogram (ECG) and an echocardiographic examination were performed. All patients had their body weight and body height measured.

The criteria for inclusion in the study were: chronic obstructive pulmonary disease proven by GOLD criteria, stable phase of chronic obstructive pulmonary disease and preserved left ventricular function (LVEF > 50%).

STADIUM	DISTURBANCE OF VENTILATION	FEV1 (%)
GOLD 1	mild	≥ 80
GOLD 2	moderate	50–79
GOLD 3	severe	30–49
GOLD 4	very severe	< 30

Figure 1. Spirometric classification of COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria for the year 2022.

Criteria for excluding patients from the study: other lung diseases (interstitial lung diseases, pulmonary thromboembolism, lung cancer), coronary disease and recent acute myocardial infarction, hemodynamically significant heart defects, previous cardiac surgery, diabetes mellitus type I and type II, chronic renal insufficiency, reduced left ventricular function (EF < 55%), signs of left ventricular hypertrophy on ECG findings.

At the end of the regular checkup, the patients received information about the purpose and protocol of the research and signed their consent to participate in the research. Only doctors/researchers have access to data from the medical history and the results obtained from diagnostic procedures, and no information other than gender and age will be published or used for illegal purposes.

Pulmonological diagnostic methods and blood gas analysis

Arterial blood gas analyses were performed using the micromethod, by taking blood samples from the radial artery and measuring them using an AVL99 gas analyzer from the company AVL Gratz, Austria. Values of PaO2 and PaCO2 (10) were evaluated. The bronchodilator test was performed according to the recommendations of the European Respiratory Society ERS/ATS (11). Spirometric tests were performed with a pneumotachograph of the company Erich Jaeger, Würzburg, model Masterlab. The results are presented in absolute measurement units and relative measurement units (%). The following spirometric measurements were used in the diagnosis of COPD: FVC (forced vital capacity): the maximum volume of air during forced expiration, FEV1 (forced expiratory volume in the first second): the volume of air that is blown out in the first second of maximum expiration after a full inhalation. It is a measure of lung emptying rate, FEV1/FVC: FEV1, expressed as a percentage of FVC, provides a clinically useful index of airflow limitation.

In patients with a post-bronchodilator FEV1/FVC value < 70%, numbers from 1 to 4 according to the GOLD criteria provide information on the degree of reduction in the percentage value of FEV1 and limited airflow in the airways. According to the GOLD criteria, COPD is classified as a disease with mild, severe, severe and very severe ventilation disorder (12).

Cardiological diagnostic methods—Echocardiography

Standard two-dimensional echocardiography (performed on a GE Vivid 4 machine using a 2.5 MHz probe) includes a longitudinal parasternal (PLAX) (PSAX), parasternal transverse, apical, and subcostal echocardio-graphic window. Two-dimensional (2D), M-mode, continuous (CW), pulsed (PW) color Doppler and tissue Doppler were used.

For a detailed assessment of the systolic function of the right ventricle, which is important for the study objectives, the following parameters were performed: The fractional area change of the right ventricle and tricuspid annular plane systolic excursion.

Assessments of all parameters were determined by an echocardiographer according to echocardiographic guidelines (13, 14, 15). TAPSE characterizes the function of longitudinal right ventricular myocardial fibers and is measured in a four-chamber apical section by placing an M-mode cursor through the lateral tricuspid annulus, (normal values > 15 mm). FAC values are obtained by measuring the area of the right ventricle in diastole and systole using the formula Ad-As/Ad (Ad—area of the right ventricle in diastole, As—area of the right ventricle in systole) expressed as a percentage, (normal value > 35%).

Statistical analyses

In all tests, the obtained level of statistical significance was expressed, and a value of $p < 0.05$ or $p < 0.01$ was considered statistically significant, depending on the obtained results of the corresponding test. The collected data were entered into a specially created database on a personal computer, and statistical processing was done with the help of the Statistical Package for the Social Sciences (SPSS) for Windows program. The results are presented in tables and graphs with a textual commentary.

Results

The research included 44 examinees suffering from COPD (28 males and 16 females). The examinees were divided into four groups according to the severity of the disease previously determined by the GOLD criteria. There were 11 patients in each group.

The patients were from 50 to 80 years old. The average age of patients who made up the first group of subjects with a mild form of COPD was 58.70 ± 7.9, in the group of patients with decreased COPD 57.80 ± 7.5, in the group with severe COPD 61.54 ± 6.7, while in the group of patients with very severe COPD, the average age was 60.56 ± 4.4. There was no statistically significant difference in age distribution between the groups.

The anthropometric indicators of the patients are presented in Table 1 and Table 2. Table 1 shows the average values for the body mass and body height of the patients by group, while the calculated values for the body surface area and BMI are presented in Table 2. There was no significant difference for any of the values of anthropometric indicators among the groups of examinees ($p > 0.05$).

FEV1 values (%) obtained by spirometry are shown in Table 3. The Kruskal–Wallis test proved that there was a significant difference for FEV1 among all groups (p = 0.05), except for FEV1 values between the third and fourth groups.

Out of 44 patients, 7 (15.8%) had TAPSE ≤ 15 mm. In the group of patients with mild or moderate COPD, there were no patients with TAPSE ≤ 15 mm, the prevalence in severe disease was 26.67% (3 patients) and in very severe 36.67% (4 patients). There were statistically significant differences in prevalence between groups (p < 0.01). With the progression of the disease, the systolic function of the myocardium of the right ventricle decreases significantly. In terminal illness, the prevalence of decreased TAPSE, which indicates decreased systolic function of the right ventricle, is the highest.

Table 4 lists the values of the TAPSE index by group. By comparing the values of the TAPSE index using the Kruskal–Wallis test, a statistically significant difference could be observed between the second and third groups of patients, the first and fourth, as well as the second and fourth (p < 0.05).

The values for FAC are shown in Table 5. The Kruskal–Wallis test proved that FAC values were statistically significantly lower in patients with severe and very severe COPD, compared to values in patients with mild or moderate form of COPD (p < 0.05).

Table 1. Anthropometric indicators: values of body mass (kg) and body height (cm)

		TM			TV		
STADIUM		X±SD	Minimum	Maximum	X ± SD	Minimum	Maximum
I		78.73 ± 17.86	54	114	175.4 ± 9.3	158	186
II		78 .43 ± 18.4	52	119	174.05 ± 6.6	158	185
III		69.8 ± 12.8	42	96	173.2 ± 5.8	160	189
IV		68.3 ± 13.4	50	109	172.55 ± 7.4	159	186

Table 2. Anthropometric indicators: body surface area (m^2) and BMI (kg/m^2)

		TP			BMI		
STADIUM	N	X±SD	Minimum	Maximum	X±SD	Minimum	Maximum
I	11	1.99 ± 0.28	1.61	2.80	25.71 ± 4.3	19.36	33.24
II	11	1.95 ± 0.20	1.61	2.25	26.33 ± 6.5	17.70	41.52
III		1.85 ± 0.16	1.45	2.10	23.55 ± 4.1	14.52	33.06
IV		1.82 ± 0.15	1.50	2.30	23.3 ± 4.8	15.82	36.32

Table 3. FEV1 values (%)

STADIUM	N	X ± SD	Minimum	Maximum
I	11	92.87 ± 10.0	80.00	113.00
II	11	61.8 ± 7.6	50.00	78.2
III	11	32.32 ± 7.2	21.49	47.8
IV	11	27.13 ± 5.8	17.70	41.0

Table 4. TAPSE index values

STADIUM	N	X ± SD
I	11	2.05 ± 0.30
II	11	2.09 ± 0.29
III	11	1.81 ± 0.34
IV	11	1.72 ± 0.32

Table 5. FAC values (mm)

STADIUM	N	X ± SD
I	11	40.05 ± 0.30
II	11	40.09 ± 0.29
III	11	35.01 ± 0.34
IV	11	34.02 ± 0.32

Discussion

The function of the right ventricle represents an important component of the entire cardiac function. It is of prognostic and predictive significance for the origin, development and outcome of various cardiovascular diseases, non-ischemic cardiomyopathies and pulmonary hypertension (16). Changes in the pulmonary circulation often lead to diseases of the cardiovascular system, especially to the development of secondary pulmonary hypertension due to increased resistance in the pulmonary blood vessels. As a result of remodelling of the right heart, insufficiency and dilatation of the right ventricle occur over time, which in the absence of left heart disease is called cor pulmonale chronicum and is the most common complication of COPD (6, 7).

By echocardiographic examination in patients with COPD, we can determine numerous parameters of the right heart cavities used to evaluate the function of the right ventricle. In our research, the following parameters were used for a detailed assessment of the systolic function of the right ventricle: tricuspid annular plane systolic excursion, the fractional area change of the right ventricle and tricuspid regurgitation.

Kaul et al. first confirmed the use of TAPSE as a parameter of right ventricular systolic function in 1984, when a strong correlation of this parameter with right ventricular ejection fraction was established by radionucleotide angiography. TAPSE is known as tricuspid ring motion and represents the longitudinal function of the right ventricular myocardium (17). A decrease in TAPSE values in patients with COPD has been described in various studies, but the mechanism of this connection is complex and not fully elucidated (18).

According to the results of our study, the TAPSE value significantly decreases with the progression of COPD. In severe COPD, the prevalence of reduced TAPSE, which indicates reduced systolic function, is the highest ($p < 0.01$). Similar to our results, Vizza et al. described in 1998 that the drop in the ejection fraction of the right ventricle is most pronounced in patients in the terminal phase of COPD (19). In other studies, a reduced TAPSE was described in patients with COPD, only if these patients had also developed pulmonary hypertension (20). Reduced right ventricular ejection fraction assessed by TAPSE is a predictor of mortality in all patients with heart failure, regardless of the presence of COPD in the patient (21).

In 2020 and 2021, TAPSE was used as an indicator of mortality in patients with COVID-19 in several studies, the results of which were included in a systematic review of researchers from Indonesia. According to the results, for every 1 mm of TAPSE decrease, the mortality rate of patients with COVID-19 increases by approximately 20% (22). TAPSE, as a significant parameter of right ventricular systolic dysfunction, can be used to differentiate sub-massive from non-massive pulmonary embolism (23).

Measurement of right ventricular ejection fraction is an important indicator of morbidity and mortality in patients with COPD (24, 16). In addition to TAPSE, another parameter strongly correlates with EFRV: the percentage fraction of change in the right ventricular area. FAC values are obtained by measuring the area of the right ventricle in diastole and systole using the formula Ad−As/Ad (Ad—area of the right ventricle in diastole, As—area of the right ventricle in systole) expressed as a percentage. FAC less than 35% indicates right ventricular systolic dysfunction. The percentage of FAC correlates with right ventricular

ejection fraction (RVEF) measured by magnetic resonance imaging (MRI) (25).

Numerous studies have shown that a decrease in TAPSE is accompanied by a decrease in FAC (14, 26, 27, 28). In line with those results, ours show the same observations. FAC values decrease with the progression of COPD, and the lowest percentage values are found in patients with stage IV COPD ($p < 0.01$). Gosh et al. proved that the reduction of FAC according to the stages of COPD is highly proportional (29). In a study by Chen et al., it was shown that FAC and TAPSE were significantly lower in patients with COPD compared to healthy individuals. Also, it has been proven that in addition to these parameters TR, tricuspid E/A, E/e, RVMPI, RVTD and TRPG can be used to assess the change of right ventricular function in early stages (30). According to the results of the study by Ghio et al., FAC is a less reproducible measure of reduced right ventricular systolic function than TAPSE (24). These observations are probably due to the fact that the FAC measure is much more variable because it is mathematically determined on the basis of echocardiographic measures of the systolic and diastolic area of the right ventricle.

Conclusion

COPD as a disease is accompanied by a large number of extrapulmonary manifestations, among which the most frequent are cardiac manifestations. The results of our research show that echocardiographic parameters of the right ventricle such as TAPSE, FAC and TR are very important for the assessment of its systolic function and that these values decrease proportionally with the progression of the COPD disease. Based on the above results and by comparing the results of other studies, it can be concluded that an echocardiographic examination is necessary for monitoring the cardiac function of these patients and that it is necessary to introduce it routinely for COPD patients in order to reduce the load on the heart, especially the right one, keep it under control and improve the quality of life with appropriate therapy.

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UTICAJ TEŽINE HRONIČNE OPSTRUKTIVNE BOLESTI PLUĆA NA SISTOLNU FUNKCIJU DESNE KOMORE

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Prema navodima Svetske zdravstvene organizacije, najčešća komplikacija hronične opstruktivne bolesti pluća (HOBP) jeste hronično plućno srce (*cor pulmonale chronicum*); ono podrazumeva hipertrofiju miokarda desne komore, dilataciju i insuficijenciju desne komore, koja nastaje kao posledica promena funkcije/strukture pluća, u odsustvu bolesti leve strane srca. Ehokardiografski pregled je zlatni standard u otkrivanju promena funkcije desne strane srca kod obolelih od HOBP-a. Glavni cilj ovog istraživanja bio je da se utvrdi uticaj stepena težine HOBP-a na vrednosti parametara sistolne funkcije desne komore. Sporedni cilj istraživanja bio je da se odredi učestalost trikuspidne regrugitacije u odnosu na stepen HOBP-a. Radi detaljne procene sistolne funkcije desne komore, značajne za ciljeve ove studije, ispitani su sledeći parametri: procentualna frakcija promene površine desne komore (engl. *fractional area change* – FAC), trikuspidna regurgitacija (TR), amplituda sistolne pokretljivosti trikuspidnog prstena (engl. *tricuspid annular plane systolic excursion* – TAPSE) (mm). U ispitivanju su učestvovala 44 bolesnika sa HOBP-om, podeljena u četiri grupe prema GOLD (engl. *global initiative for obstructive lung disease*) kriterijumima. Nije bilo statistički značajne razlike među grupama u pogledu antropometrijskih pokazatelja i vrednosti FEV1(%) ($p > 0,05$). Na osnovu Kruskal–Wallisovog testa uočeno je da su vrednosti TAPSE indeksa i FAC-a bile značajno veće kod osoba sa teškim i veoma teškim HOBP-om ($p < 0,05$). Prilikom poređenja učestalosti TR-a u blagom HOBP-u sa onom u umerenom i u teškom HOBP-u, zapažena je statistički značajna razlika; kada je reč o ovom parametru, statistički značajna razlika postojala je i između umerenog i teškog HOBP-a ($p < 0,05$). Rezultati našeg istraživanja pokazali su da su ehokardiografski parametri desne komore, kakvi su TAPSE, FAC i TR, veoma važni za procenu njene sistolne funkcije, kao i da je opadanje ovih vrednosti srazmerno sa progresijom HOBP-a.

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***Ključne reči**: hronična opstruktivna bolest pluća, indeks amplitude sistolne pokretljivosti trikuspidnog prstena, frakcija promene površine desne komore, trikuspidna regurgitacija*

Review article

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CD4$^+$ T CELL PROFILES IN AUTOIMMUNE HEMOLYTIC ANEMIA

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Autoimmune hemolytic anemia (AIHA) is an immune-mediated disorder characterized by the reduced lifespan of red blood cells (RBCs) due to enhanced intravascular and extravascular destruction. Traditionally, the immunopathogenesis of AIHA has been considered in the context of the immunological tolerance breakdown of B cells, since the autoantibodies are the main disease mediators. However, more recent data suggest that the production of anti-RBC antibodies by B cells is only an epiphenomenon and that the tolerance breakdown in the CD4$^+$ T cell compartment is a key point in early AIHA development. In AIHA, there are numerical and functional alterations of the essential CD4$^+$ T cell subpopulations, including Th1, Th2, Th17, regulatory T cells and follicular helper T cells. In this review, the main characteristics of the cellular immune response during the development of AIHA, as well as the potential mechanisms by which CD4$^+$ T cells promote the initiation and maintenance of the autoimmune process, are summarized. Identification of these characteristics and mechanisms would be of practical importance in the therapeutic sense because it opens up the possibility of designing more specific immunotherapy that is still not available for AIHA patients.

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Key words: *autoimmune hemolytic anemia, red blood cells, Th1 cells, Th2 cells, Th17 cells, Treg cells, Tfh cells*

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AIHA pathophysiology

Autoimmune hemolytic anemia (AIHA) is an immune-mediated disorder characterized by the reduced lifespan of red blood cells (RBCs) due to enhanced intravascular and extravascular destruction. The main pathological feature of AIHA is the production of autoantibodies directed against different RBC antigens, which are only the reflection of much profound dysregulation of immune mechanisms involving multiple cell types.

Based on the clinical features and laboratory findings, there are three basic types of AIHA: worm AIHA, cold AIHA, and mixed-type AIHA (1). These different types of AIHA are usually distinguished by the use of direct antiglobulin test (DAT), also known as direct Coombs test, which detects autoantibodies and/or components of the complement system (C3d most commonly) on the surface of the RBCs.

Worm AIHA (wAIHA) is the most common type, comprising 70%–80% of all adult cases and 50% of childhood cases (2). It is named after the autoantibodies that optimally react with RBCs at 37 ºC, worm agglutinins. These antibodies are mainly of IgG class and polyclonal by nature—directed against different RBC membrane proteins, such as the Rh antigen system but also the antigens of Kell, Kidd, Duffy, and Diego blood group systems (3). When attached, these antibodies facilitate and accelerate RBC phagocytosis by spleen macrophages that express multiple receptors for the Fc fragment of IgG antibodies (FcγR), thus leading to extravascular hemolysis. However, more often, only the parts of the RBC membrane are phagocytized but not their cytoplasm, which disrupts their typical, discoid biconcave appearance, i.e., RBCs become smaller in size and assume a more spherical shape, which is designated as spherocytosis. Spherocytes are subjected to further degradation during their re-passage through the spleen. Antibody-dependent cellular cytotoxicity (ADCC) involving NK cells and CD8$^+$ T cells is also proposed as a possible mechanism of extravascular RBC destruction in the spleen and other secondary lymphoid organs. IgG antibodies are able to activate the complement cascade by classical pathway; however, to a much lesser extent compared to IgM antibodies. During this process, the C3b

component of the complement is formed and deposed at the RBC surface, rendering them as a target for phagocytosis by complement receptor (CR) expressing Kupffer cells in the liver. Besides extravascular hemolysis, which is the dominant form of hemolysis in wAIHA, intravascular hemolysis can also occur due to the formation of membrane attack complex, the end product of the complement system activation, which perforates cell membrane of circulating RBCs (4). Based on etiology, wAIHA can be classified as primary, idiopathic, when it is presented as an isolated clinical syndrome (no underlying disorder can be identified) or as secondary when it is associated with various other disorders including lymphoid malignancies (chronic lymphocytic leukemia, non-Hodgkin's lymphoma), autoimmune disorders (systemic lupus erythematosus and other systemic autoimmune disorders of connective tissue), immunodeficiencies, viral infections, drugs etc. Considering these pathological features of wAIHA, DAT is positive for IgG only or IgG and C3d complement component (1, 4, 5).

Cold AIHA (cAIHA) encompasses three basic clinical entities: cold agglutinin disease (CAD), secondary cold agglutinin syndrome (CAS), and paroxysmal cold hemoglobinuria (PCH). Generally, these entities are characterized by the presence of autoantibodies that react with RBCs at lower temperatures, which is why they are designated as cold agglutinins (4).

CAD is the second most common form of AIHA (20%–25%), characterized by the production of autoantibodies that optimally react with RBCs at 4 ºC; however, these cold agglutinins can be also functional at higher temperatures, exceeding 28 ºC to 30 ºC, what actually makes them pathogenic. They are mainly of IgM class, in the form of pentameric or hexameric macromolecules, and monospecific, directed against the I/i antigen system of the RBCs (4). The monoclonality of these antibodies suggests underlying clonal B-cell lymphoproliferative disorder, which was recognized by the World Health Organization (WHO) as a distinct lymphoid neoplasm and a special type of monoclonal gammopathy in 2022 (6). In the distal, acral areas of the body, such as fingers, toes, nose, and ears, the temperature of the blood is lower than in the central parts, thus allowing IgM molecules to attach to the RBC surface and cause their agglutination. Being the most potent complement activator, IgM leads to the excessive formation of the C3b component which also deposes on the RBC surface. When the blood temperature rises again, in the central parts of the body, IgM detaches from the RBCs allowing them to separate from each other, but C3b molecules remain bound to their surface. A portion of C3b-coated RBCs is recognized by the cells of the mononuclear phagocytic system, predominantly by the Kupffer cells of the liver, and removed from the circulation. In this case, the entire RBCs are phagocytized rather than the parts of their membrane, which is why spherocytosis is rarely seen in CAD. On the other hand, C3b can be enzymatically cleaved to C3d, which allows RBCs to survive. In the circulation, complement activation at the RBC surface can proceed to the final phase, resulting in the formation of membrane attack complex and intravascular hemolysis. In CAD, DAT is IgG negative but C3d positive and typically, a spontaneous RBC agglutination occurs at room temperature (4, 7).

In contrast to CAD, CAS develops in the context of various other diseases. It is also characterized by the presence of cold reacting autoantibodies mainly of IgM class, however, these antibodies can be both monoclonal if CAS accompanies lymphoid malignancy (non-Hodgkin lymphoma, Waldenström macroglobulinemia) and polyclonal if it is associated with infections (M. pneumoniae, Epstein-Barr virus) or autoimmune disorders (systemic lupus erythematosus, rheumatoid arthritis) (4, 7).

PCH is a rare form of cAIHA mainly affecting children, which almost always arises as a postinfectious complication (8). It is mediated by Donath-Landsteiner hemolysins which are cold reacting, polyclonal IgG antibodies directed against the P antigen of RBCs. These antibodies are biphasic by nature, meaning that they attach to the RBC surface at lower temperatures and fix the components of the complement system; however, at high temperatures (37 ºC) IgG detaches, but complement components then become active, causing massive intravascular hemolysis. In contrast to CAD and CAS, in PCH there is no RBC agglutination and the hemolysis is completely complement-dependent (4).

Mixed type AIHA (mAIHA) is defined by the presence of both warm and cold agglutinins. In this case, DAT is positive for IgG and C3d; however, cold IgM agglutinins with high thermal amplitude are also present in high titers. They cause spontaneous agglutination at 20 °C which diagnostically differentiates this condition from IgG^+C3d^+ wAIHA (9). Due to the difficulties in the diagnosis, particularly, clinically irrelevant cold agglutinins with a low thermal amplitude that can also be present in patients with wAIHA as well as in healthy persons; mAIHA is probably much less common than it was previously believed (10).

In some patients with wAIHA, autoantibodies of the IgA class can be found in addition to IgG and IgM molecules; however, there are also very rare cases of IgA exclusively mediated wAIHA. They are characterized by severe clinical presentation, refractoriness to glucocorticosteroid therapy and interestingly, very little free autoantibodies detectable in the serum, probably due to their high RBC binding affinity (11, 12). The precise mechanism of hemolysis in this type of wAIHA is still not clear; however, massive hemagglutination and subsequent RBC sequestration in spleen is often seen. It appears that complement activation, FcαRI mediated phagocytosis, ADCC, and RBC apoptosis due to

membrane alterations do not play substantial role in RBC destruction (12).

CD4+ T cells in AIHA pathogenesis

Traditionally, the immunopathogenesis of AIHA has been considered in the context of the immunological tolerance breakdown of B cells, since the autoantibodies are the main disease mediators. However, the production of RBC-specific antibodies by B cells is only an epiphenomenon, caused by more profound immune dysregulation involving multiple other cell types. From the immunological point of view, AIHA can be classified as either IgG or IgM mediated, taking into account the different immune mechanisms that mediate the production of these classes of antibodies as well as the mechanisms of RBC destruction. Namely, B cells need to cooperate with antigen-specific $CD4^+$ T cells to undergo the process of immunoglobulin class switching and produce antibodies of the IgG class. IgG antibodies mainly induce hemolysis by FcγR-mediated phagocytosis or cytotoxicity. On the other hand, although IgM antibody production is a T cell-independent process, $CD4^+$ T cells also have the ability to facilitate B cells production of this antibody class (13). In this case, hemolysis is mainly complement dependent. Therefore, RBC-specific $CD4^+$ T cells are of particular importance during AIHA pathogenesis, especially wAIHA which is the most common disease type. Accordingly, it has been demonstrated on a murine model, that RBC-specific B cells are constantly present in healthy animals and are able to respond to $CD4^+$ T cell stimulation by autoantibody production. On the contrary, RBC-specific $CD4^+$ T cells, although also present in healthy animals, following antigen stimulation become functionally non-responsive, and anergic. Taken together, these findings suggest that the breakdown of immunological tolerance in the T, but not the B cell compartment is actually a key point in early AIHA development (14). Also, splenic T cells isolated from New Zealand Black (NZB) mice which spontaneously develop AIHA, underwent an extensive clonal expansion *in vitro* after RBC antigen challenging, predominantly in the $CD4^+$ T cell compartment (15). In the same model, anti-CD4 monoclonal antibody treatment was able to prevent and abrogate the production of RBC-specific antibodies, but not the development of anemia, indicating that $CD4^+$ T cells are crucial for the autoantibody production but also normal erythropoiesis (16–18). Similar findings were reported in humans. In contrast to healthy donors, T cells of AIHA patients were able to mount recall response to Rh protein epitopes *in vitro*. This effect was abrogated by anti-HLA-DR antibodies, specifying that $CD4^+$ T cells were the main responding cell population, since their process of antigen recognition is HLA class II-restricted (19). Recently, the expansion of a special subset of $CD4^+$ T cells ($CD3^+CD4^+CD28^-$) was documented in patients with idiopathic wAIHA (20). This T cell subset is highly pro-inflammatory profiled and interestingly has cytotoxic capacities due to the secretion of cytolytic enzymes, perforin, and granzyme A and B; therefore, besides the supportive role, $CD4^+$ T cells could be also directly involved in RBC destruction (20, 21).

Phenotypically, $CD4^+$ T cells are a very heterogeneous cell population and depending on the microenvironment conditions, they can differentiate into diverse polarization profiles, classified as Th1, Th2, Th17, Th9, Th22, regulatory T cells and follicular helper T cells. Each of these $CD4^+$ T cell subsets has unique characteristics including the master transcription factor, cytokine profile and specific functions.

While the importance of $CD4^+$ T cells in the pathogenesis of AIHA is currently being intensively studied, there are still many unknowns related to the precise phenotypic characteristics that determine the pathogenicity of these cells during the disease development. Identification of these characteristics as well as the mechanisms by which $CD4^+$ T cells promote the disease onset and maintenance would be of practical importance in the therapeutic sense because it opens up the possibility of designing more specific immunotherapy that is still not available for AIHA patients.

Th1 and Th2 cells in AIHA

Historically, the pathogenesis of most autoimmune disorders has been discussed in the context of a disturbed balance between Th1 and Th2 cellular immune responses, bearing in mind that these are the first two identified subtypes of $CD4^+$ T cells. Th1 differentiation program is initiated by IL-12 and/or IFN-γ, and it involves the activation of T-bet as the master transcription factor. Fully differentiated Th1 cells produce large quantities of IFN-γ, but also IL-2, TNF-α and lymphotoxin (TNF-β), thus promoting cell-mediated immunity primarily by inducing phenotype switch of phagocytes towards pro-inflammatory M1 phenotype. On the other hand, Th2 cells require IL-4 and the expression of GATA-3 as the master transcription factor for complete differentiation and produce cytokines IL-4, IL-5, IL-9, IL-10, and IL-13. Generally, this T cell lineage promotes antibody-mediated immunity by stimulating B cell antibody production but also promotes the development of M2 phenotype of macrophages that is associated with tissue regeneration and fibrosis (22, 23).

Pioneering studies investigating Th profiles in the pathogenesis of AIHA supported the notion that AIHA is a predominantly Th2-mediated disease. Namely, in basal conditions, peripheral blood mononuclear cells (PBMCs) isolated from AIHA patients produced a significantly higher amount of IL-4, a signature cytokine of Th2 cells, and less IFN-γ, a signature cytokine of Th1 cells, compared with healthy controls. When artificially

stimulated, AIHA PBMC cultures also behaved differently in the sense of higher IL-10 production and lower production of IL-12, which further favored Th2 polarization and the inhibition of IFN-γ production (24). Similar results were obtained on the whole blood cultures of AIHA patients, which following mitogen stimulation showed an increase in the production of Th2 profiled cytokines (IL-4, IL-10 and IL-13) and reduced IFN-γ production, that was more pronounced in AIHA patients with active hemolysis. Interestingly, the addition of Th2 cytokines into the cell cultures increased both autoantibody production and their binding to autologous RBCs (25). In order to minimize individual variation in cytokine production, *Kruizinga et al*. (2018) calculated the Th1/Th2 cytokine ratio in the serum of hematopoietic stem cell transplanted patients with autoimmune cytopenias, including AIHA, and also found a more pronounced Th2 cytokine profile compared with control subjects without such complications (26). Moreover, some therapeutic strategies that proved efficient, especially in wAIHA treatment, such as low doses of rituximab together with a short course of corticosteroids, were able to partially restore Th1/Th2 balance by enhancing the levels of IFN-γ, IL-12, and TNF-α and transitory reduction of IL-4 production (27).

The very origin of the Th2 immunity dominance in the pathogenesis of AIHA is still unclear, however, recently increased levels of the cytokine IL-33 have been reported in patients with wAIHA (28). Initially, this cytokine was identified as a strong inducer of Th2 cell differentiation; although more recent studies have shown that IL-33 also has stimulatory effects on a number of other cells, including Treg and Th1 cells (29). The levels of IL-33 were positively correlated with disease activity (measured by the number of reticulocytes and the levels of hemoglobin and lactate dehydrogenase (LDH)) but also with the production of anti-RBC antibodies. In PBMC cultures isolated from the patients with active AIHA, IL-33 promoted anti-RBC antibody production in a dose-dependent manner, mainly by increasing the production of the Th2 cytokines IL-4, IL-6, and IL-13 (28). RBCs contain a substantial amount of IL-33, and due to hemolysis, they can be a significant source of this cytokine during AIHA development (30).

Considering the physiological functions of Th2 cells in promoting humoral immunity, data supporting the importance of Th2 polarization during AIHA pathogenesis appear to be expected and logical. However, certain facts do not fit into the concept of AIHA as a classical Th2-mediated disease.

Firstly, the signature cytokine of Th2 cells, IL-4 as well as IL-13 preferably stimulate the production of antibodies of the IgE and IgG4 class, which do not fix the complement (31), whereas the complement is an important factor predominantly during cAIHA, but also at a lesser extent wAIHA pathogenesis (4, 32). In fact, in wAIHA, anti-RBC antibodies are most often of the IgG1 and IgG3 subclass, the latter being the most effective in RBC destruction (33, 34). In humans, such antibody isotype switching pattern is primarily associated with biological functions of IL-10 (35), which levels were indeed found to be elevated in patients with AIHA (24, 36, 37). Based on the mice studies, IL-10 was originally included in the Th2 cytokine palette; however, in humans, this cytokine can be produced by different types of immune cells including monocytes, macrophages, DCs, NK cells, B cells, T regulatory (Treg) cells, even Th1 and Th17 cells (38). Consistent with this, the higher basal production of IL-10 was observed in the monocyte cultures obtained from AIHA patients (39). Additionally, immune complexes (including IgG-coated RBCs) have the ability to polarize macrophages towards a specific M2b phenotype, which differs from the M2a phenotype typically induced by Th2 cells (40). M2b macrophages are characterized by extensive IL-10 production and the absence of IL-12 secretion, and their pathogenicity is well documented in the settings of systemic lupus erythematosus (SLE) (40, 41). Therefore, the importance of IL-10 in the pathogenesis of AIHA, as suggested by some authors (24, 25, 42), cannot be strictly attributed to Th2 cell dominance.

Secondly, FcγR-mediated phagocytosis is the most important mechanism of RBC destruction in wAIHA (43). In monocytes, IL-4 down-regulated the expression of stimulatory FcγRs (FcγRI, FcγRIIa, and FcγRIIIa), while stimulating the expression of inhibitory FcγRIIb and thus actually compromised their ability to internalize IgG coated particles (44, 45). In contrast, peripheral blood monocytes of AIHA patients showed higher expression levels of FcγRI compared with healthy controls (46). On the other hand, in cAIHA, the dominant mechanism of hemolysis was CR-mediated phagocytosis of C3b-coated RBCs (4). Th2 cytokines, IL-4 and IL-13 were also found to down-regulate the expression of immunoglobulin superfamily complement receptor (CRIg) and decrease the ability of macrophages to phagocytize complement opsonized particles (47). Considering these data, one could speculate that Th2 cells actually have a protective function during the development of AIHA as they reduce the degree of RBC hemolysis.

Third, AIHA does not show the typical features of classical Th2-mediated inflammation, such as atopic reactions and parasite infestation, in which, in addition to an increased titer of IgE antibodies, there is also an increased infiltration and activation of eosinophils. By producing IL-5, Th2 cells support eosinophil hematopoiesis in the bone marrow, while IL-4 and IL-13 induce the expression of eotaxin I (CCL11) in stromal cells and thus enhance eosinophilic infiltration. However, the cooperation between Th2 cells and eosinophils appears to be much more complex in nature and of particular importance in initiating and maintaining the Th2 immune response (48,

49). To our current knowledge, there are only a few reported cases of the coexistence of AIHA and eosinophilia in the literature and their interconnection was not investigated (50–52).

Although most studies point to Th2 cell polarization, it is clear that the pathogenesis of AIHA cannot be explained by a simplistic model of Th1/Th2 imbalance. In concordance to that, new subtypes of $CD4^+$ T cells were described as well as many different intermediate phenotypes, which have also been shown to play an important role during the AIHA onset and development.

Th17 and Treg cells in AIHA

The established Th1/Th2 paradigm was questioned by the discovery of Th17 cells, which were shown to mediate protection against fungi and extracellular bacteria, but at the same time have an important role in the development of various autoimmune diseases. This subpopulation of $CD4^+$ T cells is characterized by the production of cytokines IL-17, IL-22, IL-21, and IL-26, expression of RORγt as a master transcription factor, while their differentiation program, although still enigmatic, most likely includes IL-6 and TGF-β for initial Th17 development, and IL-21, IL-1β, and IL-23 for phenotype stabilization (53).

The first evidence to suggest the pathogenicity of Th17 cells in AIHA was based on higher levels of their signature cytokine, IL-17 in the patient's serum, which closely correlated with disease activity (54, 55). Moreover, PBMCs of AIHA patients when co-cultured with autologous RBCs as well as Rh peptides produced significantly higher amount of IL-17 compared with healthy controls (55). These results were reconfirmed by the work of Xu et al. (2012), who also detected elevated frequency of Th17 cells in the patient's blood. In this study, both Th17 cell frequency and serum IL-17 levels were closely correlated with the levels of anti-RBC antibodies, hemoglobin, the C3 component, and the activity of LDH (56). The important role of Th17 cells during disease development was additionally emphasized in the murine models of AIHA. Adoptive transfer of Th17 cells purified from AIHA mice was able to induce a higher incidence and more severe clinical presentation of AIHA in healthy animals compared with the adoptive transfer of Th0 cells. IL-17 was proposed to be the main mediator of Th17 cell pathogenicity, considering a significantly lower incidence of AIHA induction in the animals pretreated with IL-17 neutralizing antibodies or in *IL-17*$^{-/-}$ animals (56).

Treg cells represent a special subset of $CD4^+$ T cells that mediates the suppression of the inflammatory response and the establishment of immunological tolerance. In addition to CD4, these cells express CD25 and FoxP3 as the master transcription factor, and these molecules are the most commonly used identification markers of so-called conventional Treg cells. However, there is a whole spectrum of phenotypically different cells, some of which do not express FoxP3 but perform immunosuppressive functions, and they are designated as non-conventional Treg cells. IL-2 is required for TGF-β to induce FoxP3 expression and the differentiation program of conventional Treg cells (57). When activated, these cells achieve immunosuppression by different mechanisms including the secretion of immunomodulatory cytokines IL-10, IL-35 and TGF-β, the expression of inhibitory molecules PD-1L and CTLA-4, IL-2 deprivation and the generation of cAMP and adenosine (58).

A significant role of Treg cells during AIHA pathogenesis was suggested in the Marshall-Clarke and Playfair model of murine AIHA. Specifically, mice pretreated with anti CD25 antibody, that depletes Treg cells, had a much higher incidence of AIHA following rat RBC immunization. Additionally, the adopted transfer of splenic $CD4^+CD25^+$ cells from AIHA mice was able to prevent the production of anti-RBC antibodies in healthy mice following immunization with rat RBCs. This effect was not observed when the population of splenic $CD4^+CD25^-$ cells was adoptively transferred (59). In IL-2αR deficient mice which develop systemic autoimmune disease and the lethal form of AIHA, decreased number and impaired function of $CD8^+$ Treg cells was found to be a decisive factor for the early appearance of a more severe form of the disease (60). In humans, reduced percentage of conventional Treg cells in the blood of AIHA patient was detected, and their number was closely associated with the parameters of hemolysis: reticulocytes and haptoglobin (36). Another indirect support of Treg cell role in AIHA control, comes from the frequent occurrence of autoimmune hemolysis after treatment with purine analogs, especially fludarabine (61). Fludarabine has excessive toxicity to T cells, among which are Treg cells as well. According to some authors, this could be beneficial in immune reconstitution during treatment (62), but the depletion of Treg cells might consequently lead to immune tolerance disruption and autoimmune hemolysis.

Although perform completely different functions, Th17 and Treg cells share a common differentiation factor, and that is TGF-β, meaning that additional microenvironment factors are crucial in deciding the final fate of the naïve $CD4^+$ T cell differentiation process. More specifically, TGF-β in combination with IL-2 promotes Treg differentiation; whereas in combination with IL-6 drives the Th17 differentiation program (53, 57). Higher serum levels of TGF-β were found in AIHA patients (63,64), as well as higher frequency of TGF-β single nucleotide polymorphisms (SNPs) associated with enhanced production of this cytokine, especially in patients with more severe clinical presentation of the disease (65). Additionally, in the mitogen-stimulated whole blood cultures, TGF-β production was significantly

increased whereas IL-2 secretion was reduced in patients with the active form of AIHA compared with non-haemolytic AIHA patients (25). This cytokine milieu generally favors the generation of Th17 cells and, thus, may be the cause of the disturbed balance between Th17 and Treg cells observed in AIHA patients.

T follicular helper cells in AIHA

T follicular helper (Tfh) cells are particularly interesting from the aspect of AIHA pathogenesis, bearing in mind that these cells provide necessary signals for B cell maturation, but also stimulate antibody production, class switching and affinity maturation. Due to the specific pattern of chemokine receptor expression ($CXCR5^+CCR7^-$), Tfh cells migrate towards B cell-rich zones; where they regulate B cell maturation and differentiation by the expression of both co-stimulatory (ICOS, CD40L) and inhibitory (PD-1) molecules and the production of IL-21. The differentiation of this unique $CD4^+$ T cell subset is dependent on IL-6 and ICOSL signaling, while B cell lymphoma 6 (Bcl-6) is identified as the master transcription factor (66). Recently, T follicular regulatory (Tfr) cells have been described which, in addition to ICOS, CD40L, PD1, and Bcl-6, express markers of Treg cells, CD25 and FoxP3, and thus are specialized in suppressing Tfh mediated B cell activation and antibody production (67, 68).

The disturbed balance between Tfh and Tfr cells was previously implicated in the pathogenesis of various autoimmune disorders including rheumatoid arthritis, SLE, Sjögren's syndrome and others (68). In the mouse model of AIHA, a higher frequency of $CD4^+CXCR5^+CD25^-$ Tfh cells was documented in autoantibody-positive mice, as well as a high ratio of Tfh/Tfr cells. Moreover, the adoptive transfer of $CD4^+CXCR5^+CD25^-$ T cells, but not $CD4^+CXCR5^-CD25^-$ T cells, was able to promote the induction of autoantibody production (69). In antibody-positive mice, serum levels of Tfh-associated cytokines IL-6 and IL-21 were also found to be elevated as well as the T cell mRNA expression of IL-21 and transcription factor Bcl-6 (69). In IL-2 deficient BALB/c mice which early develop a lethal form of AIHA, a higher number of $CD4^+$ but also specific $CD8^+$ Tfh cells was documented, considering that IL-2 is a negative regulator of the differentiation program of this T cell subset (70, 71). In humans, the expansion of the circulating Tfh cell population was reported in the peripheral blood of AIHA patients (72). Besides germinal center B cells, Tfh and Tfr cells also regulate the development and the activity of regulatory B (Breg) cells which perform immunosuppressive functions (73). Previously, it has been shown that Breg cells have a significant role during autoimmunity development (74); however, we were unable to find studies investigating the importance of Breg cells in the pathogenesis of AIHA.

Conclusion

Today, the importance of $CD4^+$ T cells in the pathogenesis of AIHA, especially wAIHA, is indisputable; however, there are many conflicting results regarding the phenotypic characteristics of these cells. The reason for this discrepancy may be the pronounced heterogeneity of the study designs and participant inclusion criteria, as well as the differences in the animal models of AIHA used to study the disease. On the other hand, AIHA per se is a very heterogeneous disease, so there may be several different mechanisms that result in the production of anti-RBC antibodies, depending on the cause of the disease itself. In general, the altered $CD4^+$ T cell compartment is in the background of pathological B cell activation and the production of autoantibodies therefore should be considered as a potential target of future therapeutic strategies. To achieve this goal, additional studies are needed that will determine the precise pathogenic profile of $CD4^+$ T cells active during the onset of AIHA.

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CD4⁺ T ĆELIJSKI PROFILI U AUTOIMUNOJ HEMOLITIČNOJ ANEMIJI

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Autoimuna hemolitična anemija (AIHA) predstavlja imunoposredovanu bolest koju karakteriše skraćenje životnog veka eritrocita usled pojačane intravaskularne i ekstravaskularne destrukcije. Tradicionalno, imunopatogeneza AIHA sagledavala se u kontekstu prekida imunske tolerancije B ćelija, budući da su autoantitela osnovni medijatori bolesti. Međutim, skorašnji podaci sugerišu da je produkcija antieritrocitnih antitela od strane B ćelija samo epifenomen i da je prekid tolerancije u odeljku CD4⁺ T ćelija zapravo centralni događaj u ranom razvoju AIHA. U AIHA postoje i numeričke i funkcionalne alteracije osnovnih subpopulacija CD4⁺ T ćelija, uključujući Th1, Th2, Th17, regulatorne T ćelije, kao i folikularne pomoćničke T ćelije. U ovom preglednom radu prikazane su osnovne karakteristike celularnog imunskog odgovora tokom razvoja AIHA, kao i potencijalni mehanizmi kojima CD4⁺ T ćelije promovišu inicijaciju i održanje autoimunog procesa. S obzirom na to da otvara mogućnost dizajniranja specifične imunoterapije, još uvek nedostupne bolesnicima sa AIHA, definisanje ovih karakteristika i mehanizama bilo bi od praktičnog značaja u terapiji.

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***Ključne reči**: autoimuna hemolitična anemija, eritrociti, Th1 ćelija, Th2 ćelija, Th17 ćelija, Treg ćelija, Tfh ćelija*

Review article

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RELAPSING POLYCHONDRITIS: FROM ETIOPATHOGENESIS TO THERAPY

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Relapsing polychondritis (RP) is a rare autoimmune systemic disease of nature, with insufficiently elucidated etiopathogenesis, characterized by a predominantly relapsing-remitting course, involving elastic, hyaline and fibrous cartilage and tissues abundant in proteoglycans. It may lead to anatomical and functional impairments, with a potentially fatal outcome despite treatment. It usually manifests in the form of auricular and nasal chondritis and polyarthritis. Involvement of the laryngotracheobronchial tree, as well as heart valves and aorta, with the onset of secondary infections of primarily lower portions of the respiratory tract, are the most common reasons for the lethal outcome. Involvement of the eye in the form of episcleritis, scleritis etc., involvement of the inner ear in the form of vestibular disorders and sensorineuronal symptoms, as well as central and peripheral nervous system involvement, comprise a probable clinical spectrum of RP. The diagnosis of the disease is usually significantly delayed; for the diagnosis, clinical presentation is essential, while laboratory findings play only a supportive role, and imaging methods (CT, PET-CT, MRI) are important in disease activity assessments. Mild forms of RP should be treated with non-steroidal anti-inflammatory agents and low doses of corticosteroids, while severe forms are treated using higher or, as needed, pulse doses of corticosteroids, and with conventional and biological disease-modifying drugs (DMARDs). More advanced forms of aortic and valvular disease require surgical treatment.

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Key words: *cartilage, relapsing polychondritis, therapy*

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Introduction

Relapsing polychondritis (RP) represents a rare, systemic immune-mediated disease, characterized by recurrent episodes of inflammation affecting the cartilage and tissues abundant in proteoglycans, with consequential anatomical and functional impairments and a potentially fatal outcome. In addition to the involvement of elastic cartilage in the ear and nose, hyaline cartilage of peripheral joints, axial fibrocartilage, and laryngeal and cartilage of the tracheobronchial tree may be affected as well (1). Since different cartilaginous structures are involved, with different resultant symptomatology, the diagnosis is very often delayed. Auricular and nasal cartilages are the ones most commonly involved, with accompanying polyarteritis (2). Since there is a possibility of involvement of the cartilage in the laryngotracheobronchal tree, and tissues rich in proteoglycans, such as the sclera, heart valves and blood vessels, there is an increased risk of permanent or life-threatening consequences. In 30% of RP patients, this disease is associated with other autoimmune diseases, most commonly with rheumatoid arthritis, but also with myelodysplasia and/or systemic inflammatory conditions (3).

The disease was originally described in 1923 and termed "polychondropathia", while the current term RP was first used by Pearson et al. in 1960, who described it as a recurrent disease in 12 patients. The first diagnostic criteria were presented by McAdam et al. in 1976, and their modification was subsequently performed by Damiani and Levine in 1979, and by Michet et al. in 1986 (4).

Epidemiology

The incidence of the disease has been 3.5 cases per one million people, according to the data for the United States (5). The data reported about the population of the affected in the United

Kingdom has indicated the incidence of 0.71 per one million a year, and the prevalence of 9 per one million, suggesting also a frequently delayed diagnosis and a mortality rate more than twice as high as that in the general population (6).

The disease usually starts between the fourth and fifth decades of life, being slightly more common in women compared to men. It occurs among all ethnicities and races, though being more common in whites. In less than 5% of the cases it may occur in pediatric populations, with similar symptomatology as in adults (7).

Etiopathogenesis

A histopathological finding of involved cartilage typically shows an inflammatory infiltrate which consists of T-lymphocytes (mostly CD4 T-cells), macrophages, plasma cells and immune deposits, limited to the perichondrium initially, and extending subsequently to the cartilage itself (1). In a later phase of the disease, chondrocyte apoptosis and focal calcifications or fibrosis can be seen (2).

The disease pathogenesis has not been fully elucidated. It is thought that the presence of HLA-DR4 antigen is important for the disease onset, and humoral and cellular immunities are implicated as well. In RP patients, cartilage-specific autoimmunity is essential, since the presence of circulating autoantibodies against collagen types II, IX and XI has been demonstrated. Autoantibodies against collagen type II are especially important since this type constitutes 95% of collagen in the cartilage and is present in the sclera as well. These antibodies have been found in one-third of the affected with an active disease form, and their titre is positively correlated with disease severity. In addition to collagen II, significant autoantigens are also matrilin-1 and cartilage oligomeric matrix proteins (COMPs). Matrilin-1 is an intercellular matrix protein of the cartilage, contained in a considerable amount by the tracheal, nasal, auricular and chondro-sternal cartilage, while COMPs are mainly contained in the extracellular matrix of the cartilage, ligaments and tendons. There have been reports indicating a positive correlation between the titre of autoantibodies against matrilin-1 and disease severity, while the results indicating a correlation between the titre of autoantibodies against COMP and disease severity have been controversial (1).

The importance of cellular immunity is reflected in the role of chemokines consistent with the Th-1 profile, such as interferon, interleukin (Il)-2 and IL-12, released during this inflammatory process. With disease progression, there occurs a high expression of proteolytic enzymes in perichondral cells and chondrocytes, with matrix metalloproteinases (MMPs) -3, -8 and -9, elastase and cathepsin K and L being the most significant (2, 8).

Finally, it has been thought that still unknown factors, such as possible infectious agents and/or mechanical and chemical aggression in genetically predisposed individuals can cause protein breakdown, with consequential release of cartilage antigens and creation of autoantibodies. Further, the production of proinflammatory cytokines, recruitment of infiltrating cells and action of proteolytic enzymes released by apoptotic chondrocytes result in cartilage destruction (1, 8). There have been descriptions of RP appearing during pregnancy, after a trauma, piercing, after using glucosamine chondroitin preparations, as well as after the use of an anti-TNF drug in patients with ankylosing spondylitis (2, 8).

It has recently been found that a subgroup of RP patients carry somatic mutations in the gene coding for ubiquitin-activating enzyme 1 (UBA1), which is considered to be the cause of the Vacuoles, E1 enzyme, X-linked, Autoinflammatory, Somatic (VEXAS) syndrome. The patients with VEXAS-RP are mostly middle-aged or older males, with frequent hematological disorders and increased mortality rates (9).

Clinical picture

The clinical spectrum of the disease may vary from occasional inflammatory episodes, leading to non-esthetic structural deformities, to serious progressive multiorgan damage, with cardiopulmonary manifestations being the most serious and life-threatening (such as the collapse of the airways, involvement of the aorta and valvular regurgitation) (1–3). In over 80% of RP patients, the disease manifests in the form of auricular chondritis and polyarthritis. The disease can have an abrupt onset, while in milder cases the onset can be rather insidious. Constitutional symptoms in the form of fever, weight loss, night sweats, exhaustion and lymphadenomegaly are often present.

Involvement of the auricular cartilage manifests in the form of pain, swelling and redness of the auricle, with possible additional involvement of the outer and middle ear. Involvement of the vestibular structures or vasculitis of the branches of the internal auditory artery may produce vestibular or sensorineural symptoms. Nasal cartilages are often involved, with the onset of nasal pain, hoarseness, throat pain, difficulty speaking, and nose deformities such as saddle nose or flat nose tip in later phases of the disease (3).

In almost 50% of patients with RP, there is laryngeal or tracheobronchial involvement, which, if remains unrecognized, may lead to strictures, mucosal edema and collapse of the cartilage, with consequential airway obstruction and lethal outcome even in early disease stages (10, 11). Pain and stiffness of the thyroid cartilage and trachea are also possible, as well as hoarseness, non-productive cough, dyspnea, stridor and

wheezing, and also subglottic inflammation, collapse of the trachea, tracheobronchomalacia or secondary pulmonary infection. Infiltrations in the lungs may be the consequence of vasculitis (2, 3). Diagnostic and therapeutic procedures in cases with involved laryngotracheobronchial tree have to be executed with extreme caution since there is a high risk of complications and possibly fatal outcomes.

A significant percentage of RP patients have polyarthralgias/polyarthritis or oligoarthritis, with the wrist joints, metacarpophalangeal and proximal interphalangeal joints in the hands being most frequently involved, as well as the knees. Arthritis is frequently episodic, asymmetric, migratory, non-deforming, and mostly non-erosive, although associations with rheumatoid arthritis have been described as well (3).

Involvement of the eye is present in 20%–60% of patients, with different described manifestations in the form of proptosis, eyelid edema, episcleritis and scleritis, conjunctivitis, iridocyclitis, as well as possible retinopathy and optic neuritis. An early involvement of the eye has been considered as a marker of the serious, multisystem form of RP (3, 10, 12).

Dermatological manifestations have been described in 14%–37% of cases, most commonly in the form of changes resembling *erythema* nodosum or urticaria, in the form of purpura on the extremities, papules, *livedo reticularis*, superficial phlebitis or oral aphthous lesions (13). Changes affecting the skin are especially common in patients in whom RP is associated with myelodysplastic syndrome (10).

Other, rarer manifestations include cardiac, neurological and renal changes.

Cardiovascular (CV) manifestations of RP are present in 24%–52% of cases, representing the second most common cause of death in these patients. Valvular heart disease is the most common CV manifestation of RP, with the aortic valve being more frequently affected (10% of patients) than the mitral valve (2%–4%) (14). On the aortic valve, a dilation in the aortic root occurs, with aortic regurgitation, although the cases with aortic cusp rupture have been described, with a normal aortic root (3). The second most common cardiovascular manifestation is an acquired aneurysm of the thoracic aorta, developing in 5%–7% of patients with RP, with the aortic root and ascending thoracic portion being most frequently affected, while it rarely extends to involve major arterial blood vessels and abdominal aorta. The pathogenetic mechanism underlying the onset of aneurysm is slowly progressing aortitis with inflammation and gradual disruption of medial layers, carrying the risk of aortic rupture (10, 15). A systemic review of the literature, including the patients with polychondritis and involved aorta, including the thoracic and abdominal aorta, aortic valve and coronary arteries, has shown that aortic involvement (in the form of an aneurysm or ectasia) predominates, being present in 82% of patients, while aortic valve involvement has been reported in 36% (12). There have been descriptions of sporadic cases of atrioventricular block, including complete heart block, mitral regurgitation, acute pericarditis, myocarditis and silent myocardial infarction (3).

Vasculitis of any blood vessel is also possible, as well as RP associated with Takayasu's arteritis, *polyarteritis nodosa*, granulomatosis with polyangiitis and Churg–Strauss syndrome. The onset of arterial and venous thrombosis is thought to be the consequence of vasculitis or the presence of antiphospholipid antibodies.

In addition to CVS involvement, the kidney and central nervous system may be affected as well, although much less often. Kidney involvement can be the consequence of primary injury to the kidney, vasculitis or some other autoimmune disease. Renal failure has been reported in 10% of patients, and urine abnormalities in 26% of RP patients.

Vasculitis is the culprit when central and peripheral nervous system involvement is concerned, with the most common manifestations being headache, epileptic seizures, hemiplegia, aseptic meningitis, meningoencephalitis and cerebral aneurysm. Mononeuritis multiplex and cranial nerve paralysis may occur as well (3).

The prevalence of gastrointestinal involvement in RP patients is relatively low. However, in recent years there have been more and more cases of RP occurring in association with inflammatory bowel disease (IBD), including ulcerous colitis and Crohn's disease. In the literature, more than 1000 cases of RP have been described, out of which over 30 cases with accompanying IBD. The onset of arthralgias, eye involvement, aphthous lesions and *erythema nodosum* in both RP and IBD patients suggests a similar or identical autoimmune etiology of these diseases (16).

Diagnosis

RP is a rare disease which is difficult to recognize early in its course, especially when the typical involvement of the auricle and nose, or joint involvement is absent (3, 13, 17). The rate of misdiagnosis in RP reaches as high as 73%, and the time from the onset of first symptoms to diagnosis ranges from 2.9 to 5 years (18). It is necessary to emphasize the association of immune-mediated diseases, such as rheumatoid arthritis, spondyloarthritis, systemic lupus erythematosus, Sjogren's syndrome, vasculitis, antiphospholipid syndrome, inflammatory bowel disease, thyroiditis, Behcet's disease and others with RP in about 30% of cases (19). In addition to this overlapping with other diseases, the reasons for limited clinical investigations of RP are a very diverse clinical spectrum and low incidence of the disease (a couple of cases per one million people a year).

The diagnosis of relapsing polychondritis is based mostly on clinical disease parameters, while laboratory data play only a supportive role. There are no characteristic laboratory analyses for RP, although the inflammatory syndrome with elevated sedimentation and C-reactive protein (CRP) values is present (not persistently, though) in over 60% of patients (3, 20). Further, we may also encounter normocytic normochromic anemia, leukocytosis, thrombocytosis and polyclonal hypergammaglobulinemia. In some of the cases, antinuclear antibodies (ANA), antineutrophil cytoplasmic (ANCA) and antiphospholipid antibodies may be positive (3). Autoantibodies against collagen structures are not sensitive, nor specific enough, and thus have not entered clinical practice. Even the value of biopsy is rather limited, since positive findings have been reported in only two-thirds of the cases (19). Imaging methods are important in the assessment of the degree of systemic involvement and disease activity, especially computed tomography (CT), positron emission tomography—CT (PET-CT), and magnetic resonance imaging (MRI) (2).

In addition to establishing the diagnosis, it is necessary to assess disease activity and involvement and damage to the organs, and to establish or exclude the presence of other autoimmune diseases or a malignancy, since RP may appear as part of the paraneoplastic syndrome. The patient should be examined by an otorhinolaryngologist, pulmonologist, cardiologist (because of aortic and valvular involvement), hematologist (because of myelodysplastic syndrome). It is also necessary to check the renal function and perform an ANCA testing (19).

Three disease phenotypes with different clinical presentations have been recently described: hematological form in 10% of the cases; respiratory form in 25%; and mild disease form with a favorable prognosis in about 65% of the cases (19, 21).

The classification criteria for RP by Michet et al. require the presence of a proven inflammation in at least two of three auricular, nasal or laryngotracheal cartilages, or a proven inflammation in one cartilage, plus two of the other signs, including eye inflammation, vestibular dysfunction, seronegative arthritis or loss of hearing (4). The classification criteria should not be identified as diagnostic criteria—the diagnosis should be made based both on the classification criteria and the clinical experience of the practising physician.

Disease course and prognosis

RP is a chronic disease, commonly with a relapsing-remitting course. Poor prognostic factors include the onset of aortitis, vasculitis, eye involvement, and male gender is associated with a poor prognosis and higher prevalence of uveitis, loss of hearing, vestibular disorders, and a greater need for pulse doses of methylprednisolone and cyclophosphamide (19).

Although the survival of these patients has been significantly prolonged, the relapse rate is still very high, which is a substantial problem for both the patients and their doctors. In a study performed by Japanese authors, it has been shown that the risk factors for relapse are tracheal involvement, high pre-treatment values of CRP and initial monotherapy with prednisolone. The onset of relapse could possibly be prevented or delayed by the administration of combined therapy with prednisolone and immunosuppressants from the onset of the disease (22).

The principal causes of a lethal outcome are airway obstruction, CVS involvement and infections (6, 12, 17).

According to the information from 1986, the five-year survival of RP cases was 74%, and ten-year survival 55%, while according to the most recent information from 2016, these percentages were 95% and 91%, respectively. The improved survival percentages of RP patients are probably the consequence of earlier diagnosis, more effective and more aggressive treatments, and the availability of new immunosuppressive drugs, including the administration of biological agents. Renal disease significantly reduces ten-year survival rates to as low as 30% (6, 22, 23).

Therapy

Milder forms of RP should be treated with non-steroidal anti-inflammatory drugs and low doses of corticosteroids; the use of colchicine and dapsone is also possible. More severe disease forms should be treated with high doses of glucocorticoids and conventional immunosuppressants or biological agents (24). Conventional disease-modifying drugs (DMARDs) (methotrexate, azathioprine, cyclosporine, leflunomide, mycophenolate mofetil, cyclophosphamide) constitute the first line of treatment; in case of their failure or for more severe manifestations biological drugs may be used. The use of conventional DMARDs is also important because of the glucocorticoid-sparing effect (25).

Therapeutic approaches in RP are generally based on reports about individual cases and case series studies since as yet there have not been any randomized studies, as it is a very rare disease. There are no standardized guidelines for the treatment of RP, which means that the selection of drugs has to be empirical. In addition to the disease phenotype, the selection of drugs is also influenced by the presence of comorbid conditions, potential side effects and cost-effectiveness.

In a recent report by Petitdemange et al., an effectivity assessment has been done of conventional immunosuppressants and biological therapies used for RP based on the literature information. Anti-TNF drugs (infliximab and adalimumab, above all), tocilizumab and methotrexate have been shown to be the most effective DMARDs. A therapy of methotrexate

combined with some of these biological drugs is also recommended for the purpose of improving the effectiveness and in order to suppress the development of antibodies against the biological agent. The percentage of adequate responses was lower when anakinra and rituximab were used, while abatacept was used in a very small number of patients (24). So far, there has been very little data about the use of JAK inhibitors. When biological agents are used, there is an associated risk of adverse effects, especially infections. Despite an aggressive treatment approach and use of biological drugs, a French study has demonstrated that disease remission, i.e. complete response in the first 6 months of treatment, is achieved in only 19% of patients (26). A recent study has shown that most RP patients demonstrate persistent disease activity despite the treatment (27).

In life-threatening situations encountered in RP patients, the use of cyclophosphamide is indicated, since this agent can produce a rapid therapeutic response (24).

A treatment of eye involvement with developed necrotizing scleritis is rather hazardous, since it may lead to eye perforation. It is therefore necessary that the patient be examined by an ophthalmologist, receiving if required pulse doses of methylprednisolone and cyclophosphamide or biological agents, while a topical treatment can suffice only in mild cases of episcleritis (19).

In 25% of cases with airway involvement, a laryngotracheal stricture will develop, and therefore signs of tracheobronchomalacia should be sought for early during the disease course. Lung fibroscopy should be strictly avoided in RP patients because of the risk of tracheal perforation.

Corticosteroids and immunosuppressants are not effective in the treatment of more advanced forms of aortic and valvular involvement—they should be treated surgically (10).

Conclusion

RP is a rare systemic disease with the incidence of several cases per one million people a year and insufficiently clarified etiopathogenesis, affecting the elastic, hyaline and fibrous cartilages and proteoglycan-abundant tissues, such as the eye, blood vessels, heart and inner ear. It is characterized by a diverse clinical picture with accompanying constitutional symptoms, and its diagnosis is usually significantly delayed. In 30% of cases, it is associated with other autoimmune and malignant diseases. Involvement of the laryngotracheal tree, cardiovascular manifestations and secondary infections are the most common causes of lethal outcomes. Despite an aggressive treatment approach, use of corticosteroids and conventional and biological DMARDs, the disease may demonstrate a persistent activity and produce complications. The treatment is nowadays empirical, and for better control of the disease in the future, randomized controlled studies on a larger number of patients are required.

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RELAPSNI POLIHONDRITIS: OD ETIOPATOGENEZE DO TERAPIJE

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Relapsni polihondritis (RP) jeste retka autoimuna sistemska bolest, nedovoljno poznate etiopatogeneze, uglavnom relapsno-remitentnog toka, a zahvata elastičnu, hijalinu i fibroznu hrskavicu i tkiva bogata proteoglikanima. Može dovesti do anatomskog i funkcionalnog oštećenja, a uprkos lečenju, i do fatalnog ishoda. Najčešće se manifestuje u vidu aurikularnog i nazalnog hondritisa i poliartritisa. Zahvatanje laringotraheobronhijalnog stabla, kao i srčanih zalistaka i aorte, uz pojavu sekundarne infekcije – pre svega donjih delova respiratornog trakta – najčešći su uzroci letalnog ishoda. U mogući klinički spektar RP-a spadaju: zahvatanje oka episkleritisom, skleritisom i dr., unutrašnjeg uha vestibularnim poremećajima i pojava senzoneuronalnih simptoma,kao i zahvatanje centralnog i perifernog nervnog sistema. Uglavnom se značajno kasni sa postavljanjem dijagnoze, za koju je najbitnija klinička prezentacija, dok su laboratorijski nalazi od suportivnog značaja; *imaging* metode (CT, PET-CT, MR) važne su pak za procenu aktivnosti bolesti. Blaže oblike RP-a treba lečiti primenom nesteroidnih antiinflamatornih lekova i malim dozama kortikosteroida, dok se teži oblici leče primenom većih i, ukoliko je to potrebno, pulsnih doza kortikosteroida, kao i konvencionalnim i biološkim lekovima za modifikaciju bolesti. Uznapredovali oblici bolesti aorte i srčanih zalistaka zahtevaju hirurško lečenje.

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***Ključne reči**: hrskavica, relapsni polihondritis, terapija*

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CORRELATION ANALYSIS OF PITUITARY LUTEINIZING AND SOMATOTROPIC CELLS IN MALE CADAVERS DURING AGING

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This manuscript focused on examining the correlation between immunoreactive luteinizing (LH) and immunoreactive somatotropic (GH) cells in men during ageing. Anti-LH and anti-GH are the antibodies used to label the mentioned pituitary cells in 14 male cadavers. The cells identified in this way were analyzed with ImageJ. The obtained results were statistically analyzed using the SPSS statistical software package. The results of the morphometric analysis showed that during ageing, the surface area of LH and GH cells increased significantly ($p < 0.05$), and that the nuclear-cytoplasmic ratio decreased, and that the obtained changes were particularly significant ($p < 0.05$) in elderly cadavers over 70 years of age. These results showed that after the mentioned period, there was a hypertrophy of the examined cells. The resulting changes were of a functional nature and showed that cadavers after the age of 70 have a significantly reduced hormonal capacity. Based on this, it can be concluded that the investigated morphometric parameters of gonadotropic LH and GH cells correlate significantly, which indicates the parallel occurrence of adaptation and compensatory mechanisms in these cells in men during ageing.

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Introduction

Aging in humans leads to physical, mental and functional changes over time, which is reflected in the dysfunction of the neuroendocrine system (1). The ageing process has always attracted the attention of many researchers, especially in recent decades when people's life expectancy has increased (2). Therefore, the study of endocrine regulation of the ageing process occupies a significant place in many scientific studies of researchers around the world (3). Late hypogonadism or gonadopause is characterized by progressive dysfunction of the hypothalamus-pituitary-testis system, reduction of maximum and average luteinizing hormone (LH) pulse amplitude, reduction of LH concentration and reduction of negative feedback mediated by testosterone (4). Literature data show that ageing is not associated with any measurable disturbance of gonadotropic secretion either active or immunologically reactive LH cells (5). The causes of such progressively dysregulated LH secretion and testosterone release are still unclear. Decreased expression of androgen receptors in the brain and pituitary gland in older men may be the reason for impaired testosterone feedback efficiency (6).

The activity of the somatotropic axis changes during life

Hull and Harvey (7) have suggested that growth hormone (GH) may affect gonadal function by increasing gonadotropin secretion in the hypothalamus and pituitary gonadotropic cells. GH secretion rises during gestation, falls during the neonatal period, remains stable during childhood, rises during puberty, and afterwards drops during adult life (8). It has been observed that the synthesis of GH decreases by 14% per decade and that over 35% of men have a deficiency of this hormone at the age of 60 (9). GH levels decline

significantly in people over 70 years of age and represent about one-third of the value compared to late puberty (10). The causes and mechanisms responsible for somatopause and late hyposomatotropism have not been sufficiently studied and described in the literature.

Based on these and other observations in the literature, it is clear that functional disorders during ageing at the level of two human anabolic axes, gonadotropic and somatotropic, are currently not adequately histomorphologically analyzed and described. Precisely because of the many unknown facts about the relationship between LH and GH cells during ageing, the focus of this work was to examine the correlation of immunohistological characteristics of gonadotropic LH and GH cells in men during ageing.

Materials and Methods

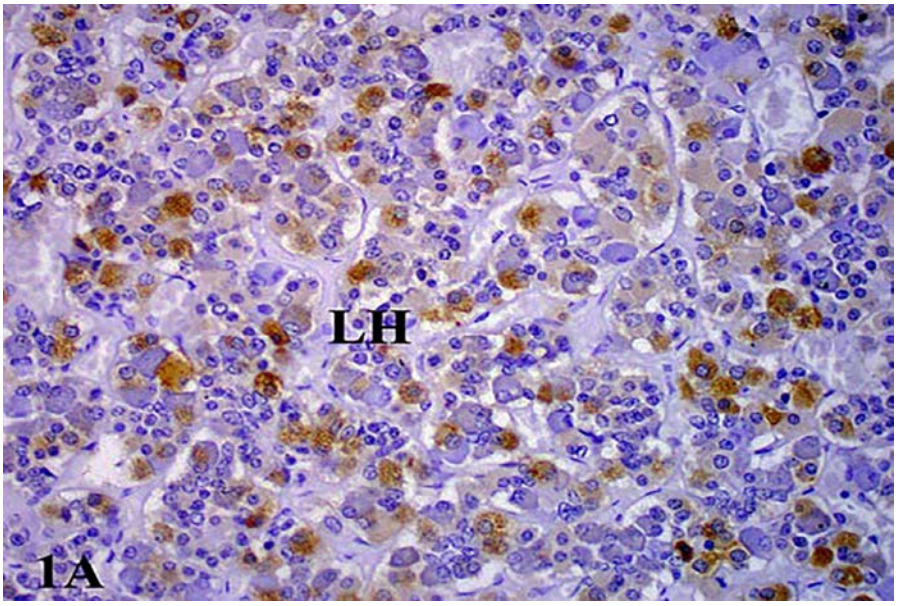
Pituitaries for this research were taken from 14 male cadavers aged 41 to 87 years. The cadaveric material was taken in accordance with the ethical norms approved by the Ethics Committee of the Faculty of Medicine of the University of Niš (Decision No. 12-2307-2/8 of March 10, 2016), which regulates the use of cadaveric material in biomedical research. Autopsy material was taken during a routine autopsy at the Center for Forensic Medicine in Niš, Serbia, within a period of no longer than 24 hours. The cadavers used in this study had not been diagnosed with neurological, endocrine or psychiatric disorders during life. No visible damage to the brain or pituitary gland was observed during the autopsy. The obtained cadaveric material was divided into groups that were described in detail in our earlier work (11). Isolated pituitary glands of male cadavers were immunohistomorphometrically processed according to a previously established procedure (12–15).

Statistical analysis

All obtained results were statistically processed using the SPSS software package (version 16) and analyzed using One Way ANOVA and Tukey-Kramer post hoc test.

Results

In the pars distalis of the pituitary gland of men aged 47 and 87 years, LH cells were oval or polygonal in shape and centrally scattered in the lateral wings of the adenohypophysis, while in the mucoid, wedge-shaped part of the gland, these cells were localized within acinar formations (Fig. 1A, B). The cytoplasm contained numerous secretory granules and was darkly stained, with a markedly granular appearance (Figures 1A and 1B). In 87-year-old men, more numerous, oval LH cells had eccentrically smaller, hyperchromatic, immunonegative nuclei compared with younger cases (Figure 1B). Immunoreactive GH cells were polygonal in shape and with an eccentric euchromatic nucleus (Figures 2A and 2B). In younger cases, GH cells were rare and scattered in the pars intermedia, while their presence in the lateral wings of the adenohypophysis was much more noticeable (Figure 2A). In older cases, these cells were larger, showing a slightly stronger immunoreactive reaction in the pars intermedia of the adenohypophysis (Figure 2B).



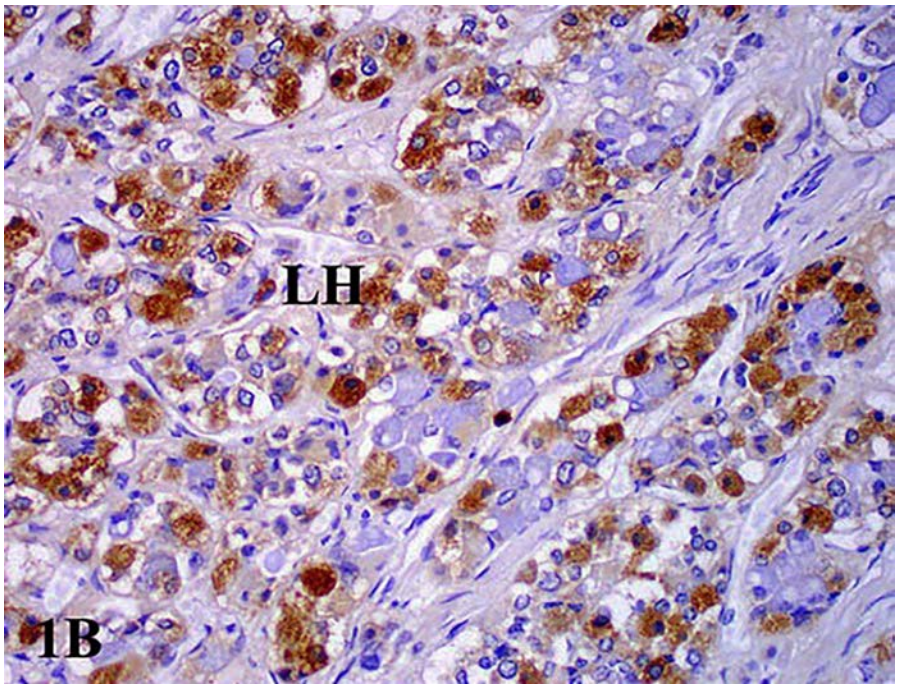
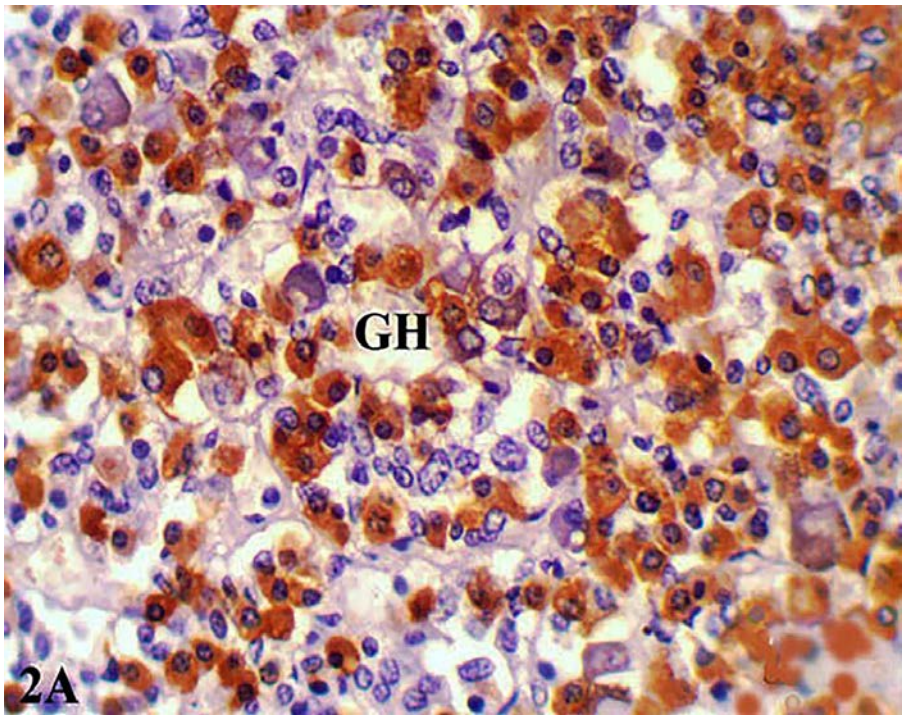


Figure 1. Representative micrographs of immunopositive LH cells in a 41 years old man, with eccentric or central euchromatic nuclei (marked with arrows) (A); large immunopositive LH cells with small eccentric hyperchromatic nuclei in a 87 years old man (B), PAP, objective lens magnification 10x



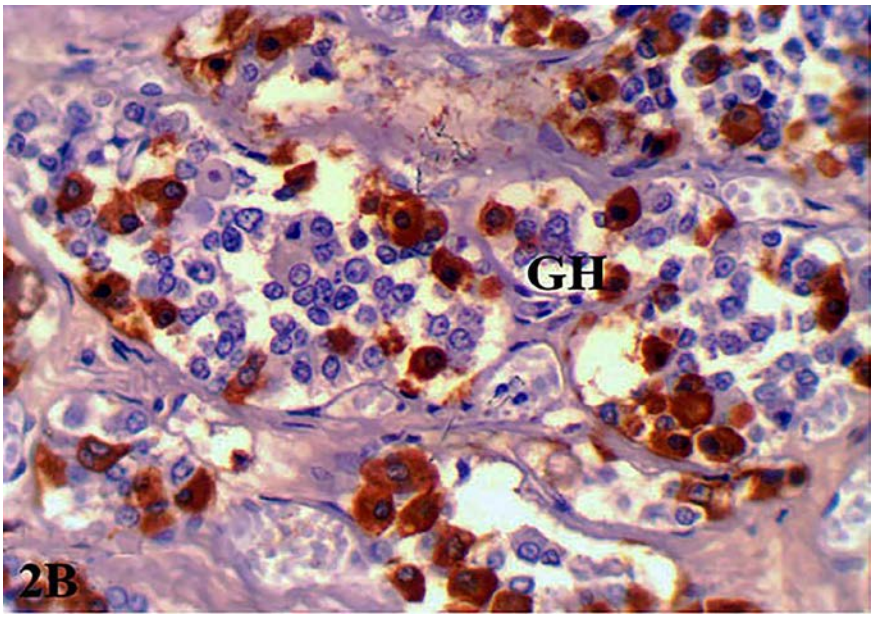


Figure 2. Representative micrographs of immunopositive GH cells in a younger man: these are predominantly polygonal, with eccentric euchromatic nuclei (A); large GH cells with immunopositive cytoplasm, with eccentric hyperchromatic immunonegative nuclei in an older man (B), PAP 40x

Table 1 shows the correlation of the same parameters of GH cells. Other morphometric parameters of LH and GH pituitary immunoreactive cells in the analyzed cases. The area and nuclear-cytoplasmic ratio of LH cells statistically significantly ($p < 0.05$) correlated with morphometric parameters did not show a statistically significant correlation between these two groups of cells.

Table 1. Correlation between morphometric parameters of gonadotropic LH and somatotropic cells of the adenohypophysis in the analyzed cases

Parameter		A_{GH}	A_{NGH}	$(N/C)_{GH}$	V_{VGH}
A_{LH}	R	0.69	-0.1	-0.65	-0.02
	p	0.006	0.74	0.012	0.94
	N	14	14	14	14
A_{NLH}	R	0.36	0.34	-0.01	-0.24
	p	0.2	0.24	0.96	0.41
	N	14	14	14	14
$(N/C)_{LH}$	R	-0.47	0.31	0.65	-0.14
	p	0.09	0.29	0.012	0.64
	N	14	14	14	14
V_{VLH}	R	0.38	0.06	-0.31	0.21
	p	0.18	0.83	0.28	0.47
	N	14	14	14	14

A_{GH} – area of GH cells; A_{NGH} – area of GH nuclei; $(N/C)_{GH}$ – nuclear–cytoplasmic ratio of GH cells; VV_{GH} – volume density of GH cells; A_{LH} – area of LH cells; A_{NLH} – area of LH nuclei; $(N/C)_{LH}$ – nuclear–cytoplasmic ratio of LH cells; VV_{LH} – volume density of LH cells

Discussion

During ageing, somatopause occurs, which is characterized by a decrease in the level of GH in the blood (16). The correlation between reproductive status and GH secretion, indicates that GH may have a modulating role during this process (7). Osamura and Watanabe (17) showed that in the normal adult anterior pituitary, about 10% of GH cells contained beta LH and alpha and beta follicle-stimulating hormone (FSH) subunits, and had an appearance that suggested the coexistence of GH with gonadotropic LH and FSH.

Changes in the adenohypophysis are a link in the chain of changes that include the hypothalamic-pituitary axis, hypothalamic-somatotropic axis and hypothalamic-pituitary-adrenal axis (16, 18–19).

The analysis of immunohistochemically labeled LH and GH cells of the pituitary gland of an 87-year-old man showed that they were larger, more often oval and with an eccentric, smaller, hyperchromatic nucleus compared to younger cases. The obtained results of the analysis of gonadotropic LH cells were in accordance with our previous work (14). In younger cases, GH cells showed a slightly stronger immunopositive response in the pars intermedia of the adenohypophysis. Results similar to ours were obtained by Antić et al. (20), who examined immunoreactive GH cells in 27 cadavers of both sexes, aged from 30 to 90 years. In contrast to our results, Sun et al. (21) showed that human pituitary GH-immunopositive cells decrease during ageing. Published results of the study by Sano et al. (22), showed that in 88% of elderly people, mostly men, interstitial perivascular fibrosis was present, which progressed over time and affected the parenchyma of the anterior pituitary gland.

The obtained results showed that the surface area and nuclear-cytoplasmic ratio of immuno-reactive LH cells are significantly correlated with the surface area of immunoreactive GH cells. Previous studies have shown that in elderly men, the correlation between morphometric parameters of gonadotropic LH and somatotropic cells is the result of anabolic synergy of GH and androgens under normal physiological conditions (23).

Conclusion

A significant correlation between some of the examined morphometric parameters of gonadotropic LH and GH cells indicates the parallel occurrence of adaptation, i.e. compensatory mechanisms, in these cells in men during ageing, or their potential mutual interaction during this process.

Acknowlegments

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KORELACIONA ANALIZA LUTEINIZIRAJUĆIH I SOMATOTROPNIH ĆELIJA HIPOFIZE KOD MUŠKIH KADAVERA TOKOM STARENJA

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U fokusu ovog rada bilo je ispitivanje korelacije imunoreaktivnih luteinizirajućih (LH) i imunoreaktivnih somatotropnih (GH) ćelija kod muških kadavera tokom starenja. Anti-LH i anti-GH antitela obeležila su gorepomenute ćelije hipofize kod 14 muških kadavera. Ćelije identifikovane na ovaj način analizirane su sistemom *ImageJ*. Dobijeni rezultati statistički su analizirani pomoću statističkog softverskog paketa SPSS. Rezultati morfometrijske analize pokazali su da se tokom starenja površina LH i GH ćelija značajno povećala ($p < 0,05$), da se nuklearno-citoplazmatski odnos smanjio, kao i da su dobijene promene bile od posebnog značaja ($p < 0,05$) kod kadavera muškaraca starijih od 70 godina. Ovakvi rezultati ukazali su na to da je nakon 70. godine starosti došlo do hipertrofije ispitivanih ćelija. Nastale promene bile su funkcionalne prirode i pokazale su da je hormonski kapacitet bio značajno smanjen kod kadavera muškaraca koji su imali više od 70 godina. Na osnovu navedenog, može se zaključiti da ispitivani morfometrijski parametri gonadotropnih LH i GH ćelija značajno koreliraju, što može upućivati na paralelnu pojavu adaptacionih i kompenzacionih mehanizama u pomenutim ćelijama kod muškaraca u procesu starenja.

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***Ključne reči**: starenje, muškarci, imunoreaktivne luteinizirajuće ćelije, imunoreaktivne somatotropne ćelije, imunohistomorfometrija*

Case report

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ALLEVIATED ANAPHYLACTIC REACTION TO MUSCLE RELAXANTS DUE TO PREOPERATIVE ADMINISTRATION OF CORTICOSTEROIDS

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Anaphylactic reaction during general anaesthesia represents a rare event in clinical practice with a possible fatal outcome. We present a case of a female patient with no prior history of allergic reactions to rocuronium. Shortly after rocuronium administration, she developed the following signs: tachycardia, low blood pressure and bronchospasm. Skin rash and peripheral signs were absent, probably due to previous premedication. Events like this could be potentially fatal if not recognized and treated in time.

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Key words: *anaphylaxis, drug hypersensitivity, hypersensitivity, neuromuscular agents, period, perioperative, rocuronium bromide*

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Introduction

Anaphylaxis during anaesthesia is a rare event in clinical practice, and it can result in complications and death in 9% of cases. Considering that an anaphylactic reaction during the perioperative period may remain unrecognized, data on its frequency varies from 1:3,500 to 1:445,000 of cases, depending on the country (1, 2). According to research, the most common cause of anaphylaxis during anaesthesia (in as many as 60%–70% of cases) are muscle relaxants, predominantly suxamethonium and rocuronium (1, 3). Cross-reactivity to other muscle relaxants, most often suxamethonium, is present in as many as 65% of patients allergic to rocuronium (3). Timely recognition of an anaphylactic reaction, its treatment and informing the patient about the event are extremely important.

Allergic reactions to rocuronium are mainly mediated by immunoglobulin E. Given the fact that 75% of allergic reactions to rocuronium occur during the first contact with the agent, there is a suspicion of possible cross-reactivity with apparently unrelated agents as well as with certain foods, cosmetic products and industrial materials (2, 4). Genetic studies show the existence of bypassing of IgE antibodies in situations when a mutation in Mas-related G protein-coupled receptor-X2 is indicated as the cause (5).

Signs of anaphylactic reaction during general anaesthesia do not differ from the symptoms of anaphylaxis in a conscious state, however, considering that a large number of agents are administered in the perioperative period, the signs can be altered and/or masked by hypovolemia, the depth of anaesthesia or the regional blockade (6). Also, there are a large number of other clinical conditions with high incidence during general anaesthesia which can give a similar clinical picture to anaphylaxis.

On this occasion, we present a case report of an anaphylactic reaction most likely caused by rocuronium, which was alleviated by previous premedication and preoperative administration of corticosteroids.

Case report

A 49-year-old female patient was admitted to the hospital for the procedure of septoplasty,

with a diagnosis of deviated nasal septum. During the preoperative anaesthesiology examination, the patient's general health was assessed, and a clinical examination was performed. The patient was on regular antihypertensive (ramipril, hydrochlorothiazide 2 x 5 mg + 25 mg and bisoprolol 5 mg 1 x 1/2) and endocrinological (levothyroxine-sodium 75 mg) therapy. When it comes to previous surgeries, the patient mentioned having had an appendectomy 10 years ago and a tonsillectomy in childhood. Preoperative laboratory, including thyroid hormones were normal. There were no deviations in the chest X-ray, the electrocardiogram (ECG), and the preoperative clinical examination. The patient denied allergies to food and medical agents, and only mentioned an allergy to "feathers, animal hair and house dust". Anamnesis revealed the reactions exclusively in the form of urticaria. During the patient's previous hospitalization at the Clinic of Otorhinolaryngology, University Clinical Center of Niš, after the induction of anaesthesia and placement of the tracheal tube, there was a sudden drop in oxygen saturation. This event was interpreted by the attending anaesthesiologist as a consequence of the tracheal tube malpositioning while positioning the patient on the operating table. After the repositioning of the tube, saturation stabilization did not occur, and sugammadex was administered. After the clinical parameters stabilized, the patient was awakened and extubated.

After analysing these data from previous intubation and postintubation period, a detailed examination of the patient was undertaken. Spirometry was performed, which showed no deviations, together with an examination by a pulmonologist. The anaesthesiologic examination revealed that the patient was obese with a BMI of 34.72, increased girth and reduced neck height. Neck mobility was preserved, Mallampati score II–III, with normal thyromental distance. The day before the surgery, preoperative bronchodilator therapy (aminophylline NoI/12 h and methylprednisolone 80 mg/12 h) was prescribed to the patient.

Thirty minutes before entering the operating room, premedication was given in the form of an intramuscular injection of 5 mg of midazolam and 0.5 mg of atropine. The response to premedication was satisfactory. Upon entering the operating room, non-invasive monitoring was provided: ECG, pulse oximeter, manometer for blood pressure (BP) measurement. Vital parameters after placing the patient on the operating table were BP 131/82 mmHg, heart rate (HR) 77/min and SpO2 97%. After administration of oxygen therapy, saturation improved to SpO2 99%. Introduction to general anaesthesia was started with 2 mg of midazolam, 100 mcg of fentanyl and 170 mg of propofol. When loss of consciousness and respiratory suppression were established, manual ventilation with the help of a face mask was started. One minute after the administration of 50 mg of rocuronium, the HR increased to 125–135/min with occasional ventricular extrasystoles. Blood pressure was measured, and it was as low as 65–75/35–45 mmHg. Clinical examination at that moment showed no visible skin changes or changes in the patient's oxygenation. The patient's peripheral pulse was stable, skin was not flushed nor pale. Two minutes after the administration of the muscle relaxant, the patient was intubated without any difficulties, with a Cormack-Lehane score of 1. Immediately after intubation, resistance during manual ventilation was observed. After placing the patient on the mechanical ventilator, transpulmonary pressure ranged from 25 to 27 mmHg, therefore, manual ventilation was continued. Despite attempts to maintain adequate ventilation, SpO2 dropped to 88%. Considering dropping in SpO2 and low BP, 100% oxygen was administered with a flow rate of 6 L/min together with rapid administration of crystalloid fluids. A dose of 50 mcg of phenylephrine was administered on two occasions with close monitoring of BR and HR. This was followed by the rise of BP to 80/40 mmHg, the HR maintained 115–120/min. Saturation rose to 93%. After the systolic BP reached a value above 100 mmHg and HR dropped to 100–105/min, aminophylline NoI was administered in slow bolus.

Surgery was not started until the patient's condition stabilized. After the stabilization of vital parameters, the surgical intervention started, and general anaesthesia was maintained with 1.5–2 Vol% sevoflurane together with a combination of 60% oxygen and 40% air with a flow rate of 3.6 L/min. During the further course of anaesthesia, vital parameters ranged from BP 120–135/65–80 mmHg, HR 85–95/min, SpO2 95–97%. The total duration of general anaesthesia was 85 minutes. After the end of the surgical intervention, the surgical sheets were removed when a skin rash was observed in the lower part of the abdomen with extension towards the back. The patient's general condition and vital parameters were stable, without the need for administration of antishock, bronchodilator and antioedematous therapy. The patient was awakened and extubated. After a short postoperative follow-up, the patient was transferred to the ward. Postoperative anamnesis shows that the patient had no subjective complaints except the feeling of "heaviness in the head" the day after surgery. When the patient checked in for the control surgical examination, she was informed about the well-founded suspicion of the allergy to rocuronium with the advice to conduct a more detailed examination in the next period.

Discussion

Most of the allergic reactions to intravenous anaesthetics develop in the first minutes after the induction of anaesthesia (6). Common symptoms of an anaphylactic reaction are: urticaria,

erythema or oedema, symptoms of the respiratory tract, gastrointestinal tract, cardiovascular system and central nervous system.

The most commonly reported initial symptoms are the absence of peripheral pulse, difficulty in patient ventilation and desaturation with reduced End-Tidal CO2 (etCO2) (7). Clinicians often describe skin changes as the first sign of an intraoperative anaphylactic reaction, however, according to research, the skin reaction to allergen may be absent in the perioperative period, which makes prompt diagnosis difficult (2, 8). The existence of skin signs is very often overlooked due to the covering of visible skin surfaces with surgical sheets (6, 9). Cardiovascular symptoms include hypotension and tachycardia, however, if adequate treatment is not promptly provided, they can soon progress to arrhythmia and cardiovascular collapse (6). The advantage of the occurrence of anaphylactic shock in the operating room is easy and prompt recognition of changes in vital parameters through present monitoring (8, 10).

Bronchospasm occurs less frequently but may be present in patients with asthma or in patients of atopic constitution (6). During the perioperative period, the patient is sedated or under general anaesthesia and is unable to report the presence of signs such as pruritis, hoarseness, dizziness, dysphagia and/or blurred vision (8). The very introduction to anaesthesia leads to the blockade of sympathetic nerves (9), and simultaneously, the administration of medical agents together with previously administered premedication can change the clinical picture of anaphylactic shock and/or lead to diagnosis delay (7). It is important to rule out other clinical conditions that may have similar or the same signs as anaphylaxis. This is extremely important when the patient is under general anaesthesia and when the clinical picture of anaphylaxis is altered or incomplete (7). All this affects the identification and prompt treatment of perioperative anaphylaxis, which contributes to complications and mortality in clinical practice (9).

Meng et al. indicate that they encountered patients with a negative history of the existence of anaphylactic reactions to rocuronium during or after previous surgical interventions (11). This is explained by the fact that the patient was sensitized to the agent during previous general anaesthesia. Our patient previously had two surgical interventions, however, no data were available on the previously used agents. An allergic reaction in the form of bronchoconstriction and a rapid drop in saturation most likely developed during the first hospitalization at the Clinic for otorhinolaryngology. The lack of recognition of an allergy to rocuronium can be explained by the fact that sugammadex was administered as a reversal of the muscle relaxant before more severe signs of anaphylaxis occurred. Several previous case reports have reported stabilization of vital parameters and reversal of anaphylactic signs after administration of sugammadex. It is believed that sugammadex encapsulates the neuromuscular blocking agent's molecule and thus stops the allergic reaction. However, laboratory and clinical studies did not support this. The conclusion of such discrepancies in science and practice requires additional studies in the near future (8).

When it comes to the second introduction to anaesthesia after the observation of the first sign, e.g., extreme tachycardia, malignant hyperthermia was first suspected. This was immediately ruled out considering the agents used during induction. In the absence of filiform peripheral pulse, skin reactions and cold and moist periphery, hypotension and tachycardia were understood as a complication of hypothyroidism in terms of hypersensitivity to the cardio depressant effects of anaesthetics, although preoperative thyroid hormone values were normal. Cardio-depressive effects in hypothyroidism are due to reduced intravascular volume, reduced preload, reduced baroreceptor response, and reduced cardiac output (12). Also, there is clinical evidence as well as research confirming that prescribing levothyroxine in patients with subclinical hypothyroidism reduces blood pressure values by reducing TSH levels (13, 14).

Symptoms in the form of tachycardia, hypotension and increased resistance in the respiratory tract that occurred in our patient were also reported in other case reports, but they were mostly accompanied by skin changes (5, 15–17). Considering that it is very difficult to recognize an anaphylactic reaction in the absence of skin changes, clinical data indicate that it is necessary to suspect the presence of an anaphylactic reaction if hypotension persists despite the administration of inotropes and vasopressors (10). When it comes to our case, the appearance of skin changes as the first sign was expected given the personal history, and its absence is explained by preoperative preparation and the administration of corticosteroids.

An intraoperative anaphylactic reaction to rocuronium can be life-threatening, and there are a large number of case reports in which cardiopulmonary resuscitation had to be performed (18, 19). Considering the atopic constitution of our patient with a history of developing only mild allergic symptoms, and considering that she had received corticosteroids preoperatively, we believe that the intraoperative anaphylactic reaction we witnessed was mild. There was a relatively quick stabilization of the patient's general condition, and this was maintained until the very end of the intervention. Considering that, the intervention was safely continued.

In the case of suspicion of perioperative anaphylactic reaction, it is necessary to inform the patient and write a report about it. The second step is to refer the patient for histamine/tryptase tests, which are ideally performed within 15

minutes of the reaction onset. In our country, this kind of practice is impossible in smaller medical centers. It is extremely important to verify the occurrence of anaphylaxis, due to the administration of anaesthesia in the future. It is important to point out that cisatracurium has the lowest degree of cross-reaction in patients who have previously experienced an anaphylactic reaction to rocuronium and vecuronium, even if there exists only a reasonable doubt. Therefore, cisatracurium is an alternative muscle relaxant for future surgical interventions (2).

Conclusion

Perioperative anaphylaxis caused by muscle relaxants is a relatively rare and potentially fatal complication of anaesthesia. For this reason, constant education of anaesthesiologists about possible symptoms and triggers is extremely important. Also, it is necessary to develop official recommendations on further steps if an anaphylactic reaction is recognized intraoperatively. Lack of diagnosis and failure to inform the patient about the existence of suspicion as well as referral for further examination can lead to a fatal outcome after the administration of the agent.

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UBLAŽENA ANAFILAKTIČKA REAKCIJA NA MIŠIĆNE RELAKSANTE USLED PREOPERATIVNE PRIPREME KORTIKOSTEROIDIMA

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Anafilaksa tokom anestezije predstavlja redak događaj u kliničkoj praksi, koji može dovesti i do smrtnog ishoda. Prikazan je slučaj bolesnice bez prethodne istorije alergijskih reakcija izazvanih rokuronijumom. Ubrzo nakon primene rokuronijuma, došlo je do pojave tahikardije, niskog krvnog pritiska i bronhospazma. Urtikarija i periferni simptomi su izostali, najverovatnije usled prethodne premedikacije kortikosteroidima. U praksi, situacije poput ove mogu biti fatalne ukoliko se ne prepoznaju i ne tretiraju pravovremeno.

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***Ključne reči**: anafilaksa, preosetljivost na lekove, hipersenzitivnost, neuromišićni agensi, perioperativni period, rokuronijum-bromid*

Case report

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A CASE REPORT OF A PATIENT WITH FOUR METACHRONOUS CANCERS

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Multiple primary malignant neoplasms (MPMNs) are two or more histopathologically distinct malignancies in one or more organs such that one tumor is not a recurrence or metastasis of the other. Although there are well-known genes associated with hereditary cancers, sometimes it is not possible to find a genetic link between neoplasms. Our patient had four metachronous primary malignancies: breast cancer (BC), rectal cancer (RC), parotid cancer, lung cancer (LC), and highly suspected contralateral BC, over a 28-year period. We presented the challenges in diagnosis and therapeutic approach and highlighted the importance of genetic counseling and testing in these patients. To achieve better treatment, we need to find out which patients are at risk for MPMNs, and which tumors are more likely to occur synchronously or metachronously and enroll these patients in clinical trials.

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Key words: *neoplasms, multiple primary, neoplastic syndromes, hereditary, genetic testing*

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Introduction

Multiple primary malignant neoplasms (MPMNs) are two or more histopathologically (HP) distinct malignancies in one or more organs, such that one tumor is not a recurrence or metastasis of the other (1). They may be synchronous or metachronous, depending on the time of occurrence. Risk factors include genetic predisposition, exposure factors (tobacco, alcohol, hormones, immunodeficiency, infections), and carcinogenic effects of cancer treatment. The incidence (2.4%–8%) is low, tending to increase with longer survival and early detection. It depends on the primary cancer and prior treatment. In patients with breast cancer (BC), the incidence of MPMNs is approximately 4.1% to 16.4% (2). MPMNs occur in hereditary syndromes. Although there are well-known genes associated with hereditary cancers (3), sometimes it is not possible to find a genetic link between neoplasms.

This article presents the case of a patient with four metachronous primary malignancies (BC, rectal cancer, parotid cancer, lung cancer, and highly suspected contralateral BC) over a 28-year period, explains the challenges in diagnosis and therapeutic approach, and highlights the importance of genetic counseling and testing in these patients.

Case report

The female patient had a verified ductal invasive right BC as her first malignancy at the age of 33. Immunohistochemical examination of receptor status was not performed in the 1990s, and she was treated with preoperative radiotherapy (RT) followed by radical mastectomy in October 1994 at the Institute of Oncology and Radiology of Serbia. Thereafter, she underwent regular follow-up. In May 2004, after a colonoscopy with biopsy, she underwent anterior and inferior rectal resection with total mesorectal excision and coloanal anastomosis. Histo-pathologically, it was a villous adenocarcinoma of the rectum, Dukes B, Astler-Coller B2, without lymph node invasion, classified as T1N0Mx. Accordingly, in stage I, the patient underwent follow-up. In December 2018, after palpating a nodule in her right parotid gland, she underwent a subtotal parotidectomy with preservation of the right facial nerve and was diagnosed with parotid gland cancer. Local recurrence occurred after 2 years and was confirmed after extirpation. Due to frequent epi attacks, severe right-sided weakness, and severe motor dysphasia, a brain magnetic resonance imaging (MRI) was performed, which showed an expansive change in the left

temporoparietal lobe. Complete tumor extirpation was performed in September 2019 at the Neurosurgery Clinic of the University Clinical Center Niš. Based on the immune profile, it was a metastasis of lung adenocarcinoma with papillary growth. She underwent palliative RT to the brain with 30 Gy TD in 10 sessions. There was no evidence of recurrence at the last endocranial MRI in December 2021. She had no respiratory symptoms until November 2021, and this was the first time she saw a pulmonologist. She presented with a cough with yellowish sputum, dyspnea, and fatigue without fever. Multi-slice computed tomography (MSCT) of the chest showed a 60 mm pleural effusion on the right side, an irregular lung tumor lesion of 53 x 43 x 64 mm, a reduction of the tracheal lumen ipsilateral to the main bronchi, and secondary deposits of both adrenal glands of 53 x 21 mm and 40 x 36 mm. A bronchological examination was indicated to clarify the etiology of the lung changes, but it was not performed because the patient was not motivated. Treatment for the lung cancer was limited to symptomatic therapy due to the patient's poor Eastern Cooperative Oncology Group performance status (ECOG PS) 3. In February 2022, she presented to the breast multidisciplinary (MTD) with a highly suspicious 60 x 40 mm left BC and marked lymphedema on the right hand. According to the Breast Imaging Reporting and Database System, the change was graded as BI-RADS 5. The MDT recommended a CORE biopsy for possible hormone therapy in case of hormone receptor-positive disease.

The patient is a non-smoker and denies hereditary diseases.

Discussion

This is a rare case of a patient with a metachronous cancer quartet that includes the breast, rectum, parotid gland, and lung. In such a case, it is important to determine whether the tumor is hereditary. Our patient met some of the criteria (4): first cancer at age 33, MPMNs, but negative family history, and searching the literature we could not find a specific syndrome for this quarter. An Asian study showed that patients with MPMNs were more likely to be carriers of pathogenetic variants, most frequently in BRCA 1 and BRCA 2 and mismatch repair genes and less frequently in APC, ATM, MUTIH, PALB2, RAD50, and TP53 (5).

While a Turkish study described BC with gynecologic carcinoma as the most common pair of primary cancers in women (6), Halamkova J. et al. showed that in 32.7% of cases and with a mean time of 8.9 years, the second was colorectal carcinoma (CRC), consistent with our case. This association may be sporadic or germinal. Older patients, with early-stage disease and without recurrence have a higher risk of secondary cancer (7, 8). We can establish a link between LC and preoperative BC RT (2) or the fact that BC is the most common cancer in women, LC the second most common primary cancer, and that patients' lives have been prolonged thanks to better screening and advanced therapies (8). The risk of contralateral BC cancer is five times higher in patients who have had this cancer before (9), which supports our suspicion. There is an association with lobular cancer and family history.

The treatment decision is multidisciplinary and based on the characteristics of the cancer, the patient and the drugs. These patients are usually excluded from clinical trials, so there are no specific guidelines for their treatment. Because of the marked neurological symptoms and poor PS, our patient underwent tumor extirpation in the brain, followed by palliative RT. She was not a candidate for specific oncological treatment for LC, so she received only symptomatic therapy. In the case of BC, she could be a candidate for hormonal therapy in hormone receptor-positive disease.

Genetic counseling and testing are very important in these patients because of therapeutic options (e.g., BRCA, PALB2 mutation) and investigation of predisposition to other cancers in the carrier of a particular mutation or first-degree relatives. Previously, genetic testing was expensive and time-consuming, but now next-generation sequencing (NGS) allows the investigation of a whole range of genes at a lower cost and with faster testing speed (10).

Conclusion

This quartet of cancers may occur sporadically, due to immunodeficiency or previous therapies, or in the context of hereditary syndromes. Therefore, it is necessary to perform genetic testing in patients with MPMNs, for themselves but also for their family members. The incidence of MPMNs will tend to increase in the future. To achieve better treatment, we need to find out which patients are at risk for MPMNs, which tumors are more likely to occur synchronously or metachronously and enroll these patients in clinical trials.

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PRIKAZ SLUČAJA BOLESNICE SA ČETIRI METAHRONA PRIMARNA KARCINOMA

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Multiple primarne maligne neoplazme (MPMN) predstavljaju pojavu dvaju ili više histopatološki različitih maligniteta u jednom organu ili u više organa, i to tako da jedan tumor nije recidiv ili metastaza drugog. Iako su dobro poznati geni udruženi sa hereditarnim karcinomima, ponekad nije moguće pronaći genetsku vezu između neoplazmi. Prikazan je slučaj bolesnice koja je u periodu od 28 godina imala četiri metahrona primarna maligniteta: karcinom dojke, karcinom rektuma, karcinom parotida, karcinom pluća i vrlo sumnjiv kontralateralni karcinom dojke. U radu su predstavljeni izazovi na koje smo naišli u dijagnostici i terapijskom pristupu. Takođe, istaknut je značaj genetskog savetovanja i testiranja ovakvih bolesnika. Da bi se postigao bolji tretman, mora se saznati koji su bolesnici u riziku od pojave MPMN-a, kao i kod kojih tumora postoji veća verovatnoća da će se pojaviti sinhrono ili metahrono. Osim toga, potrebno je uključiti ove bolesnike u klinička ispitivanja.

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***Ključne reči**: neoplazme, višestruki primarni, neoplastični sindromi, nasledni, genetsko testiranje*

PROPOZICIJE ZA PISANJE RADOVA U ACTA MEDICA MEDIANAE

Acta Medica Medianae (AMM) je tematski časopis iz oblasti medicinskih nauka. Časopis objavljuje originalne radove koji nisu prethodno publikovani.

U AMM se objavljuju: uvodnici, naučni i stručni članci, prethodna ili kratka saopštenja, revijski radovi tipa opšteg pregleda, aktuelne teme, meta-analize, prikazi slučajeva, prikazi knjiga i drugi prilozi. Radovi se štampaju na srpskom ili engleskom jeziku sa apstrak-tom na srpskom i engleskom jeziku. Radovi na engles-kom jeziku se prezentuju u elektronskom formatu na sajtu Medicinskog fakulteta u Nišu, kao i na među-narodnim sajtovima iz oblasti medicinskih nauka. Acta Medica Medianae izlazi četiri puta godišnje, od 1962 godine.

Svi radovi koji se objavljuju u AMM podležu anonimnoj recenziji, a Uređivački odbor određuje redosled njihovog štampanja. Primedbe i sugestije urednika i recenzenata dostavljaju se autoru radi konačnog oblikovanja. Radovi se predaju u pisanom ili elektronskom obliku na srpskom i engleskom jeziku. Rukopisi radova prihvaćenih za štampu ne vraćaju se autoru.

Rukopis treba predati sa jednostrukim proredom, formata A4, sa levom marginom od 3 cm.

Prva strana rada treba da sadrži: a) naslov rada b) puna imena i prezimena autora c) puni nazivi ustanova i organizacijskih jedinica u kojima je rad realizovan i mesta u kojima se ustanove nalaze, d) znacima *, **, ***, #, ##, ###,…označavaju se redom autori i njihove institucije e) puna adresa, broj telefona i e-mail osobe zadužene za korespondenciju u vezi predatog rukopisa.

Druga strana treba da sadrži samo naslov rada, rezime i ključne reči, bez imena autora i institucija. Veličina rezimea za naučne i stručne članke, revijske radove tipa opšteg pregleda i meta–analize može da bude do 250 reči. Ispod rezimea sa podnaslovom "Klučne reči" navesti 3-5 ključnih reči ili izraza. Poželjno je da autori za ključne reči koriste odgovarajuće deskriptore, tj. definisane termine iz *Medical Subject Heading* (MeSH) liste *Index Medicus-a*. Prva i druga strana se predaju na srpskom i engleskom jeziku i ne obeležavaju se brojevima.

Tekst članka: Naučni i stručni članci, kao i opšti pregledi i meta-analize ne smeju prelaziti 11 stranica sa prilozima; aktuelne teme– 6 stranica; kazuistika 6–stranica; prethodna saopštenja– 5 stranica, a izveštaji sa skupova i prikazi knjiga 2 stranice. Naučni i stručni članci obavezno treba da sadrže poglavlja: uvod, cilj, materijal i metode, rezultati, diskusija i zaključak. Zahvalnost ili komentar povodom sponzorstva rada dati na kraju teksta članka iza poglavlja "zaključak".

U tekstu naznačiti mesta priloga i obeležiti ih onako kako su obeleženi u prilogu.

Literatura se daje u posebnom poglavlju, pri čemu se navodi onim redosledom kojim se citati pojavljuju u tekstu. Broj literaturne reference se u tekstu označava arapskim brojem u zagradi. Za navođenje literature koristiti pravila Vankuverske konvencije. Strane se numerišu arapskim brojevima u donjem desnom uglu

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