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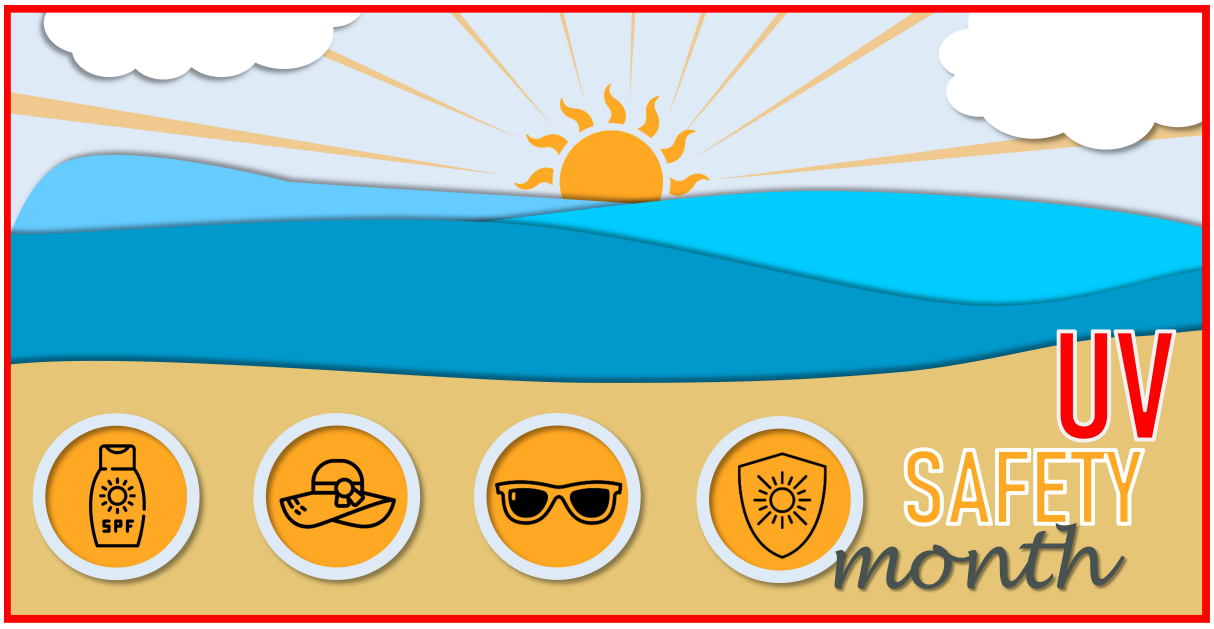

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CONTENTS / SADRŽAJ

At the initiative of the American Academy of Dermatology, 22 years ago, a campaign was launched for the first time, the goal of which was to educate the public about the dangers of excessive exposure to UV radiation. The month of July is dedicated to raising awareness about the importance of protection against the harmful effects of UV radiation. In the United States of America, in 2023, there were over 100,000 new cases recorded of skin melanoma and over 8,000 deaths. Avoid exposure to the sun during the period of the day when UV radiation is at its maximum (10 a.m.–4 p.m.).

Na inicijativu Američke akademije za dermatologiju, pre 22 godine, prvi put je pokrenuta kampanja čiji cilj je bio da se javnost edukuje o opasnostima od preteranog izlaganja UV zračenju. Jul mesec je posvećen podizanju svesti o značaju zaštite od štetnog delovanja UV zračenja. U Sjedinjenim Američkim Državama, u 2023. godini, zabeleženo je preko 100 000 novih slučajeve melanoma kože i preko 8 000 smrtnih slučajeva. Izbegavajte izlaganje suncu u periodu dana kada je UV zračenje maksimalno (10-16 časova).

ORIGINAL ARTICLES

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Comparison of effects of two analgesia methods on surgery for developmental dysplasia of the hip in children

Poređenje efekata dve metode analgezije u hirurškom lečenju razvojne displazije kuka kod dece

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[1] Both authors contributed equally to this study.

Abstract

Background/Aim. Surgery for developmental dysplasia of the hip (DDH) often needs acetabuloplasty and femoral osteotomy. This type of operation is characterized by long duration, major trauma, and severe postoperative pain. The aim of the study was to compare the analgesic effects of the caudal block (CB) and epidural block (EB) on the DDH surgery in children. **Methods.** A total of 100 children undergoing DDH surgery in our hospital from May 2018 to December 2021 were selected and randomly divided into two groups: an ultrasound-guided CB group and an EB group (each group consisting of 50 children). The dosage of ropivacaine, changes in blood pressure, heart rate, and intraoperative dosage of fentanyl were recorded. In addition, we recorded the values of several parameters after the surgery: the modified Face, Legs, Activity, Cry, and Consolability (FLACC) scale score at 2, 4, 6, 12, 24, 36, and 48 hrs after surgery; the time point of first-time request for acetaminophen and proportion of patients using pethidine; the sedation degree within 12 hrs after the operation (assessed using the Ramsay Sedation Scale – RSS); the incidence of nausea, vomiting, and urinary retention; the satisfaction degree of family members with the applied methods of postoperative analgesia in children. **Results.** The ropivacaine dosage used in the CB group exceeded that of the EB group ($p < 0.05$). The CB group had lower FLACC scores 12 and 24 hrs after the operation and a longer postoperative time until the first-time request for acetaminophen than the EB group ($p < 0.05$). No block-related complications occurred; the two groups had similar incidence rates of nausea, vomiting, and urinary retention. The RSS score within 12 hrs after operation was higher in the CB group than in the EB group, and the family members of the children in the CB group were more satisfied ($p < 0.05$). **Conclusion.** Both CB and EB can provide satisfactory intraoperative and postoperative analgesia for pediatric hip surgery. However, CB provides effective analgesia for 24 hrs after the operation and lasts longer than that accomplished by the EB.

Key words:
analgesia; anesthesia, caudal; anesthesia, epidural; child; developmental dysplasia of the hip; surgical procedures, operative.

Apstrakt

Uvod/Cilj. Za hirurško lečenje razvojne displazije kuka (RDK) često je potrebna acetabuloplastika i femoralna osteotomija. Ovu vrstu operacije karakteriše dugo trajanje, velika trauma i jak postoperativni bol. Cilj rada bio je da se uporede analgetski efekti kaudalne blokade (KB) i epiduralne blokade (EB) u hirurškom lečenju RDK kod dece. **Metode.** Ukupno je odabrano 100 dece, koja su bila podvrgnuta hirurškom lečenju RDK u našoj bolnici od maja 2018. do decembra 2021. godine, i nasumično podeljeno u dve grupe: grupu sa ultrazvučno vođenom KB i grupu sa EB (svaka grupa se sastojala od po 50 dece). Zabeležene su doze ropivakaina, promene krvnog pritiska, brzine otkucaja srca i intraoperativna doza fentanila. Osim toga, zabeležene su vrednosti još nekoliko parametara nakon operacije: procenjivan je skor modifikovane skale lica, nogu, aktivnosti, plača i utehe (*Face, Legs, Activity, Cry, and Consolability* – FLACC) 2, 4, 6, 12, 24, 36 i 48 sati nakon operacije; vreme prvog zahteva za acetaminofenom i procenat bolesnika koji su koristili petidin; stepen sedacije u toku 12 sati nakon operacije (procenjen korišćenjem Ramsay-eve skale sedacije – RSS); stopa učestalosti pojave mučnine, povraćanja i zadržavanja urina; stepen zadovoljstva članova porodice primenjenim metodama postoperativne analgezije kod

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dece. **Rezultati.** Doza ropivakaina koja je korišćena u grupi KB bila je veća od doze korišćene u grupi EB ($p < 0,05$). Grupa KB je imala niži FLACC skor 12 i 24 sata nakon operacije i duže postoperativno vreme do trenutka prvog zahteva za acetaminofenom u odnosu na grupu EB ($p < 0,05$). Nisu se pojavile nikave komplikacije povezane sa blokadama; obe grupe su imale slične stope učestalosti mučnine, povraćanja i zadržavanja urina. RSS skor tokom 12 sati nakon operacije bio je viši u grupi KB nego u grupi EB, a članovi porodice dece iz grupe KB bili su zadovoljniji ($p < 0,05$). **Zaključak.** I KB i EB mogu pružiti zadovoljavajuću intraoperativnu i postoperativnu analgeziju u pedijatrijskoj hirurgiji kuka. Međutim, KB pruža efikasnu analgeziju 24 sata nakon operacije i traje duže od one koju postiže EB.

Ključne reči:
analgezija; anestezija, kaudalna; anestezija, epiduralna; deca; kuk, razvojna displazija; hirurgija, operativne procedure.

Introduction

Surgery for developmental dysplasia of the hip (DDH) often needs acetabuloplasty and femoral osteotomy. This type of operation is characterized by long duration, major trauma, postoperative limitation of limb motion due to plaster fixation, and severe intraoperative and postoperative pain stimuli [1, 2]. Nowadays, regional block has been often applied as an auxiliary or supplementary means of general anesthesia in various operations, which can reduce the intraoperative dosage of general anesthetics, shorten postoperative recovery time, lower the incidence rate of complications caused by general anesthetics, and also offer long-term effective analgesia after operation [3, 4]. Ultrasound (US)-guided caudal block (CB) is simple to apply and renders a definite analgesic effect and few adverse reactions. Therefore, it has been applied for intraoperative and postoperative analgesia in various types of surgery [5]. However, its popularization and application are limited due to a longer learning curve. Moreover, epidural block (EB) is also a commonly used anesthesia method for pediatric lower limb and abdominal surgery [6], and it is relatively simple for anesthesiologists. The present study observed the intraoperative and postoperative analgesic and sedative effects and safety of US-guided CB and EB in DDH surgery.

Methods

General data

A total of 100 children undergoing DDH surgery in our hospital from May 2018 to December 2021 were selected, and they had proximal femoral (subtrochanteric) rotational osteotomy and acetabular (pelvic) corrective osteotomy. Ethical approval of the study protocol was obtained from the institutional Ethics Committee of the Children's Hospital of Nanjing Medical University, China (from May 06, 2014). All children's families signed the informed consent. The children were randomly divided into the US-guided CB group and the EB group, with 50 patients in each of them. Exclusion criteria were as follows: patients with infection at the puncture site or anatomic abnormalities; patients with complicated neurological diseases or coagulation dysfunction; patients allergic to ropivacaine, opioids, or acetaminophen; patients who used other drugs affecting the results within the 24 hrs before the operation; patients with a body mass index (BMI) > 30 kg/m^2.

Anesthesia preparation

The children in the two groups received no preoperative medication. The non-invasive blood pressure (BP), electrocardiogram (ECG), and pulse oximetry saturation (SpO_2) were routinely monitored in both groups, and the bispectral index (BIS) was continuously monitored using the AspectA-1000TM BIS monitor.

Anesthesia induction

All patients were intravenously administered 0.01 mg/kg atropine, 3 μg/kg fentanyl, 1.5 mg/kg propofol, and 0.7 mg/kg rocuronium. After the BIS declined below 60 and the lower jaw relaxed, a laryngeal mask was placed in the correct position. After confirming satisfactory ventilation and no leakage, an anesthesia machine was connected for mechanical ventilation, the pressure and respiratory rate were adjusted, and the partial pressure of end-tidal carbon dioxide was kept at 30–40 mmHg.

Ultrasound-guided caudal block

US-guided CB was performed after induction. The patient was placed in a lateral position, with the midpoint of bilateral sacral horns as the positioning mark. First, the sacral hiatus in dark space (also referred to as the "frog eye sign") between two hyperechoic sacral horns was positioned by the short axis, and the black region between the vertebral body and the ligaments was the sacral cavity. At the time of drug injection (0.25% ropivacaine, 1 mL/kg), the thickest part of the sacral cavity between the two sacral horns was selected by long-axis positioning and marked as the puncture point [7]. The sacral promontory and deep sacral vertebral plane in a step-like shape (step sign) were identified, and the black region and band-shaped hyperechoic area above the vertebral body were the sacral cavity gap and sacrococcygeal ligament, respectively. Then, a 5 mL syringe needle was inserted by in-plane technique and connected to an injector containing local anesthetics by injection wires through a pump. When the sacrococcygeal ligament was perforated, characteristic folds and rebounds were shown on the US image, and it was observed that the needle tip was inserted into the sacral cavity. Widening of the sacral cavity after injection and lifting of dorsal sacrococcygeal ligament during injection and its recovery after injection displayed on US images indicated the correct puncture site.

Epidural block

The patient was placed in a lateral position. The puncture point was selected between the L3 and L4 spinal vertebra, and the catheter was inserted epidurally towards the head to a depth of 2 cm. After successful catheterization, the catheter was fixed on the skin, and local anesthetics were injected through the epidural catheter. For local anesthesia, 0.2% ropivacaine was used (the initial dose of epidural anesthesia was 0.5 mL/kg, and the additional dose of intraoperative analgesia was 0.25 mL/kg, making for the total dose ≤ 1.7 mg/kg) [7]. The epidural catheter was removed after the operation.

Anesthesia maintenance

During the operation, the sevoflurane inhalation anesthesia was kept at 0.7 of Minimum Alveolar Concentration, propofol was continuously infused using a micro-pump for anesthesia maintenance, and the dosage of propofol was adjusted to keep the BIS at 40–60. Rocuronium was injected intravenously and intermittently as needed to maintain muscle relaxation. The operation was conducted 30 min after the applied block. It was observed that the fluctuations in BP and heart rate (HR) were 20% higher than the basic values during deep and shallow anesthesia, and fentanyl was added intravenously at 0.5–1 μg/kg to maintain hemodynamic stability. Sevoflurane and propofol were discontinued after postoperative plaster fixation. When the spontaneous breathing was recovered, the tidal volume was > 7 mL/kg, the respiratory rate was 16–25 breaths/min, and the inhaled air SpO_2 was > 95%. The patient was sent to the anesthesia recovery room. The laryngeal mask was removed when the patient showed a response to speech or pain stimuli. The patient could be transferred from the anesthesia recovery room to the general ward provided their vital signs were stable and the general anesthesia recovery score was ≥ 4 points.

Postoperative analgesia

No analgesic pump was connected after the operation. Tylenol® (acetaminophen) at 1.5 mg/kg was given orally if the Face, Legs, Activity, Cry, and Consolability (FLACC) scale score was ≥ 4 points. The FLACC scale score was assessed again 30 min later. If it was still ≥ 4 points, pethidine was intramuscularly injected at 1 mg/kg for analgesia. The largest dose of pethidine in a single injection was 50 mg at most, and the minimum interval of administration of Tylenol® was 6 hrs. If there was a pain outbreak during the interval, pethidine was intramuscularly injected.

Monitoring indices

The dosage of ropivacaine, changes in BP and HR before and after skin incision, and intraoperative dosage of fentanyl were recorded in the two groups. After the operation, another anesthesiologist was assigned for blind assessment of the parameters, which included: 1) FLACC score at 2, 4, 6, 12, 24, 36, and 48 hrs after operation (time points T1–T7) – the pain of patients was assessed by three grades of five aspects (facial expression, lower limb status, activity level, crying level, and consolability) with 0–10 points, and the higher the score, the more severe the pain; 2) the time point of first-time request for acetaminophen after operation and the proportion of patients using pethidine were recorded; 3) the degree of sedation within 12 hrs after the operation was assessed using the Ramsay Sedation Scale (RSS) – 1 point (not quiet and irritable), 2 points (quiet and cooperative), 3 points (lethargic and able to follow instructions), 4 points (sleepy state and able to be awakened), 5 points (slow response to call), 6 points (deep sleep state and unable to be awakened), hence, points 2–4 indicated satisfactory sedation and 5–6 excessive sedation; 4) the incidence of nausea, vomiting, and urinary retention was recorded (when the patient complained of nausea or vomiting, ondansetron was intravenously injected at 0.1 mg/kg and if the patient failed to urinate spontaneously 12 hrs after the operation, urethral catheterization was conducted once, and the urethral catheter was indwelled in case of necessity); 5) the degree of satisfaction of the children's families (parental satisfaction score) with postoperative analgesia was recorded (0 = dissatisfied, 10 = very satisfied).

Statistical analysis

SPSS 22.0 software was used for statistical analysis. Age, height, BMI, operation duration, changes in BP and HR before and after skin incision, intraoperative dosage of fentanyl, and time of first request for analgesics after the operation were expressed as mean ± standard error, and the postoperative FLACC score, RSS, and degree of satisfaction of the children's families were expressed as median (range). The general parameters of the patients were compared between the two groups using the Student's *t*-test or Fisher's exact probability test. The normal distribution of data was tested by the Kolmogorov-Smirnov test. The normal distribution of the repeated measures data (such as BP, HR, and FLACC score) was assayed by repeated measures of analysis of variance (ANOVA). Normally distributed data were compared by paired *t*-test at different time points, while data that were not distributed in a statistically normal pattern were compared by the Mann-Whitney *U* test between the two groups at each time point. The Mann-Whitney *U* test was also used for the comparison of RSS and the degree of satisfaction of the children's families. Intergroup comparison of numerical data (e.g., incidence of nausea, vomiting, and urinary retention) was conducted by Fisher's exact probability test. The time point of first-time requests for analgesics after the operation was compared using the log-rank test. A statistically significant difference was defined as $p < 0.05$.

Results

General conditions of patients and operation-related conditions

There were no significant differences in general and clinical characteristics [age, height, BMI, American Society of Anesthesiologists (ASA) grade, and operation time] between the two groups ($p > 0.05$) (Table 1). The anatomical structure of the sacral canal could be clearly seen under the US in all patients in the CB group. The block was smooth in the two groups, and no block-related complications (hemorrhage, hematoma, local anesthetic toxicity, dural puncture, cerebrospinal fluid leakage, and total spinal anesthesia) occurred. All patients received postoperative pain management as designed and were observed successfully.

Intraoperative and postoperative analgesia

The dosage of ropivacaine in the CB group was significantly larger than that in the EB group [(18.4 ± 3.4) mL vs. (17.0 ± 3.2) mL, respectively, $p = 0.036$]. No obvious changes were found in BP and HR before and after skin incision in the two groups, and the differences were not significant ($p > 0.05$) (Table 2). There was no significant difference between the two groups regarding the intraoperative dosage of fentanyl ($p > 0.05$). In the CB group, 45 patients requested acetaminophen at 18.3 ± 3.2 hrs after the operation, significantly later than in the EB group. In the EB group, 46 patients requested acetaminophen at 10.9 ± 2.3 hrs after the operation ($p < 0.05$). The FLACC score in the CB group was lower at 12 and 24 hrs after the operation, compared to that in the EB group, with a significant difference ($p < 0.05$), while there was no significant difference at 2, 4, 6, 36, and 48 hrs between the two groups. The FLACC score in the CB group was higher at 24, 36, and 48 hrs after the operation than at 2 hrs after the operation. The FLACC score in the EB group rose 12 hrs after the operation (Table 3).

Incidence of anesthesia-related adverse reactions and parents' satisfaction

The families of children in the CB group were more satisfied with postoperative analgesia than the parents of the

Table 1

Demographic and clinical characteristics of patients

Parameters	CB group	EB group	*t* or *U*	*p*-value
Gender (male/female)	14/36	13/37	0.051	0.822
Age (months)	48.6 ± 6.4	48.5 ± 6.1	0.080	0.936
Height (cm)	102.8 ± 10.3	103.2 ± 9.8	0.199	0.843
BMI (kg/m^2)	17.1 ± 3.2	18.2 ± 3.4	1.666	0.099
ASA grade (I/II)	42/8	41/9	0.071	0.790
Surgery duration (min)	229.7 ± 23.4	231.7 ± 24.1	0.421	0.675

CB – caudal block; EB – epidural block; BMI – body mass index; ASA – American Society of Anesthesiologists.
Results are shown as mean ± standard error or number.

Table 2

Blood pressure, heart rate, and perioperative dosage of analgesics

Parameters	CB group	EB group	*t*	*p*-value
Blood pressure, mmHg				
before skin incision	76.2 ± 5.4	76.5 ± 5.1	0.286	0.776
after skin incision	75.4 ± 4.2	75.5 ± 4.3	0.118	0.907
Heart rate, beats/min				
before skin incision	81.5 ± 5.3	79.8 ± 4.8	1.681	0.096
after skin incision	80.8 ± 5.1	79.7 ± 4.2	1.851	0.067
Dosage of ropivacaine (mL)	17.0 ± 3.2	18.4 ± 3.4	2.120	0.036
Intraoperative dosage of fentanyl (μg)	64.0 ± 4.6	63.9 ± 4.3	0.112	0.911
Acetaminophen after operation TPFTR (hrs)	18.3 ± 3.2	10.9 ± 2.3	13.278	< 0.001

TPFTR – time point of first-time request. For other abbreviations, see Table 1.
Results are shown as mean ± standard error.

Table 3

Face, Legs, Activity, Cry, and Consolability scale scores at different time points

Time points (T1-T7) after surgery, hrs	CB group	EB group	*t*	*p*-value
2 (T1)	1.4 ± 0.3	1.4 ± 0.2	0.000	1.000
4 (T2)	1.4 ± 0.3	1.3 ± 0.3	1.667	0.099
6 (T3)	1.5 ± 0.4	1.4 ± 0.2	1.581	0.117
12 (T4)	1.5 ± 0.3	4.4 ± 0.6	30.569	< 0.001
24 (T5)	3.5 ± 0.5	4.3 ± 0.6	7.243	< 0.001
36 (T6)	3.3 ± 0.4	3.4 ± 0.6	0.981	0.329
48 (T7)	2.1 ± 0.5	2.2 ± 0.4	1.104	0.272

For abbreviations, see Table 1. Results are shown as mean ± standard error.

Table 4

Postoperative prevalence of adverse reactions and postoperative scores

Parameters	CB group	EB group	*t* or *U*	*p*-value
Nausea	6 (12.0)	8 (16.0)	0.332	0.564
Vomiting	3 (6.0)	4 (8.0)	0.154	0.695
Urinary retention	5 (10.0)	4 (8.0)	0.1222	0.727
RSS	2 (2–3)	2 (2–2)	3.242	4.523
Parental satisfaction score	9 (9–9)	8 (7–9)	< 0.001	< 0.001

RSS – Ramsay Sedation Scale. For other abbreviations, see Table 1.
All results are shown as numbers (percentages), except RSS and parental satisfaction score which are shown as median (range).

children in the EB group, and the difference was significant ($p < 0.001$). There were no significant differences in the incidence rate of nausea and vomiting between the CB group and the EB group ($p > 0.05$), and the incidence rate of urinary retention was similar in both groups ($p > 0.05$) (Table 4). The RSS at 12 hrs after surgery was significantly higher in the CB group compared to the EB group. The parents' satisfaction score within 12 hrs after the operation was higher in the CB group than in the EB group, showing a significant difference ($p < 0.001$).

Discussion

Congenital dislocation of the hip is commonly seen in children, with an incidence rate of 1.1–3.8‰. The surgery involves the adductor muscle, proximal femur, acetabulum, and pelvis, and as a consequence, severe postoperative pain occurs. Good postoperative pain control is the basis of modern perioperative management and rapid postoperative recovery, which should not only enable the motion recovery of patients faster and reduce organ dysfunction but also make the patients take food as early as possible and, finally, be discharged [7, 8]. During the "enhanced recovery", various pain control modes are not applied individually, so selecting the optimal anesthesia and analgesia method combination is particularly important for anesthesiologists [9].

CB, a commonly used anesthesia and analgesia method, has definite intraoperative and postoperative analgesic effects, can reduce the dosage of general anesthetics and opioid analgesics, and enable the vital signs of children to be more stable [10]. However, it also has potential risk factors, such as puncture positioning error, drug toxicity, abnormally extensive block, and total spinal anesthesia, while urinary retention is a clinically common postoperative complication. According to recent studies, local anesthetics may cause poisoning in about 25% of patients undergoing CB. Therefore, accurate positioning should be paid attention to during CB injection in children in order to prevent systemic toxicity caused by intravascular injection and total spinal anesthesia due to injection into the subarachnoid space. The US assessment is characterized by accurate positioning and clear observation of drug diffusion in the correct area. That is why it has been applied in CB in children [11]. Besides accurate positioning, the volume and concentration of local anesthetics should be also controlled to minimize the incidence of adverse reactions. Therefore, the dosage should be determined according to the level of anesthesia, generally 0.5 mL/kg to the sacral spine, 1 mL/kg to the lumbar spine, and 1.25 mL/kg to the lower thoracic spine. Some scholars suggest using 0.2% ropivacaine or 0.25% levobupivacaine/bupivacaine in a single CB injection in children, and the concentration should not exceed the aforementioned level. Low-concentration ropivacaine has minimized the risk of urinary retention [12]. To prevent the occurrence of toxic reactions, a test dose of local anesthetics is recommended, and adrenaline can be added to local anesthetics to prevent the local anesthetics from being mistakenly injected into the blood vessels in children. However, regional anesthesia is mostly performed under general anesthesia or deep sedation of children. The increase in resting HR in children indicates the controversy over the effectiveness and accuracy of the test dose. Therefore, it remains inconclusive whether to use the test dose as stated in the guidelines. However, local anesthetics should be injected slowly, in small doses (0.1–0.2 mL/kg), and intermittently withdrawn. ECG monitoring should also be paid attention to. Imaging methods, such as US and fluoroscopy, may be helpful for preventing the puncture needle from mistakenly entering the blood vessel during peripheral nerve block, but there is a lack of data supporting the application value of these techniques in regional block [7]. In the present study, all children in the CB group successfully underwent US-guided CB, and no complications occurred. The intraoperative and postoperative analgesic effects were satisfactory, and the postoperative analgesic effect could last for up to 24 hrs, proving that CB is safe and effective in DDH surgery. Moreover, it was found that the duration of analgesia after CB was obviously longer than that after EB (later time of first request for acetaminophen after operation in the CB group compared to the EB group), and the FLACC score at 12 and 24 hrs after the operation was significantly lower in the CB group than that in the EB group. The reasons are as follows: 1) the concentration and volume of local anesthetics used in the study were in strict accordance with the recommendations in the guidelines (0.25% ropivacaine for CB and 0.2% ropivacaine for EB), so the effect of ropivacaine lasted for a longer time in the CB group; 2) the volume of local anesthetics used in CB was larger than that in EB, so that the effective time of the former may be longer; 3) the pharmacokinetic characteristics of ropivacaine may vary from site to site, so the postoperative analgesic effect of CB

was better than that of EB based on the concentration and volume used in this experiment.

EB, a block technique commonly used by anesthesiologists, can also be successfully applied to abdominal and lower limb surgeries. Due to poor cooperation of children, however, it often needs to be performed under general anesthesia or sedation [13]. Nowadays, multiple large-sample prospective observational studies worldwide have proven that EB is safe for children under general anesthesia and deep sedation, which can be conducted as a standard procedure. Nevertheless, severe complications may still occur, so it is necessary to remain highly skeptical of nerve injury and promptly take appropriate diagnosis and treatment measures if suspected. The results of this study showed that even though EB could also provide effective intraoperative and postoperative analgesia for DDH surgery, the duration of its postoperative analgesia was relatively shorter than that of CB. In addition, the patients might suffer from postoperative lumbago, back pain, and nerve injury. Hence, it may have no advantage after all.

In this study, there was no excessive sedation in either of the groups, but the RSS within 12 hrs after operation was higher in the CB group than in the EB group. The possible reason is that the analgesic effect was better in the CB group, so the patients were quiet and cooperative. No significant difference was found in the incidence of postoperative nausea, vomiting, and urinary retention between the two groups. Due to the longer duration of analgesia, the children's families in the CB group were more satisfied with postoperative analgesia than those in the EB group. Therefore, CB is more suitable for DDH surgery than EB.

Conclusion

Both the CB and EB can reduce the dosage of general anesthetics and opioids and offer satisfactory postoperative analgesia in pediatric hip surgery, with the intraoperative vital signs kept stable. However, the CB has a long duration of postoperative analgesia and is less likely to cause nerve injury, which makes it more suitable for DDH surgery.

Conflict of interest

The authors declare no conflict of interest.

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Clinical value of magnetic resonance spectroscopy in assessment of early curing impact of concurrent chemoradiotherapy after high-grade glioma surgery

Klinička vrednost spektroskopije magnetnom rezonancom u proceni uticaja konkurentne hemioradioterapije na rano lečenje posle operacije glioma visokog stepena

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Abstract

Background/Aim. High-grade glioma (HGG) is an interstitial cell-derived primary tumor of the nervous system. The current guidelines for the diagnosis and treatment of glioma recommend the maximum safe range of tumor resection for treatment methods. Adjuvant concurrent chemoradiotherapy is recommended after surgery, followed by six cycles of single-drug chemotherapy, temozolomide. Evaluation of the early efficacy of concurrent chemoradiotherapy after HGG surgery, especially for patients with a high risk of recurrence, is a crucial step in enhancing the treatment efficiency for patients diagnosed with HGG. In this study, we investigated the clinical utility of magnetic resonance (MR) spectroscopy (MRS) in assessing the early curing impact of concurrent chemoradiotherapy following HGG surgery. **Methods.** A total of 50 patients with incomplete resection or suspected residual postoperative HGG, treated in the radiotherapy department of our hospital between January 2016 and June 2021, were selected for routine concurrent chemoradiotherapy. Conventional MR imaging and MRS were performed one week prior to treatment and one month after treatment to assess changes in specific brain metabolites. All 50 patients were followed up for 6 to 12 months. Based on the follow-up results, the patients were divided into two groups: the tumor recurrence group and the tumor suppression group. One month after the end of the treatment, the differences in levels of brain metabolites between the two groups were analyzed using MRS. **Results.** The levels of N-acetylaspartate (NAA) and creatine (Cr) increased after radiotherapy, while choline (Cho) peak value, and Cho/Cr, NAA/Cr, and Cho/NAA ratios decreased compared to pre-treatment levels. There were statistically significant differences in the NAA peak value, and Cho/Cr, and Cho/NAA ratios in the tumor enhancement area before and after treatment ($p < 0.05$). There were also statistically significant differences in Cho/Cr ratio in the peritumoral edema area before and after treatment ($p < 0.05$). **Conclusion.** After concurrent chemoradiotherapy, MRS can be used to detect early metabolic changes in the tumor enhancement and peritumoral edema areas of HGG.

Key words:
biomarkers; chemoradiotherapy; chemotherapy, adjuvant; glioma; magnetic resonance spectroscopy; prognosis; surgery.

Apstrakt

Uvod/Cilj. Gliom visokog stepena (GVS) je primarni tumor nervnog sistema koji potiče od intersticijalnih ćelija. Aktuelne smernice za dijagnozu i lečenje glioma preporučuju maksimalan bezbedan opseg resekcije tumora kao metodu lečenja. Nakon operacije se preporučuje adjuvantna konkurentna hemioradioterapija praćena primenom šest ciklusa hemioterapije jednim lekom, temozolomidom. Procena rane efikasnosti konkurentne hemioradioterapije posle operacije GVS, posebno kod bolesnika sa visokim rizikom od recidiva, je ključni korak u poboljšanju efikasnosti lečenja bolesnika sa dijagnozom GVS. U ovoj studiji smo istražili kliničku vrednost spektroskopije magnetnom rezonancom (MR) – (SMR) u proceni ranog uticaja konkurentne hemioradioterapije na proces izlečenja, nakon operacije GVS. **Metode.** Ukupno 50 bolesnika sa nekompletnom resekcijom ili sumnjom na rezidualni postoperativni GVS, koji su lečeni na Odeljenju za radioterapiju naše bolnice u periodu od januara 2016. do juna

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2021. godine, odabrano je za rutinsku konkurentnu hemioradioterapiju. Konvencionalna MR i SMR urađene su nedelju dana pre i mesec dana nakon lečenja da bi se procenile promene u izmerenim nivoima specifičnih metabolita mozga pre i posle lečenja. Svih 50 bolesnika praćeno je tokom 6 do 12 meseci. Na osnovu rezultata praćenja, bolesnici su bili podeljeni u dve grupe: grupu kod koje je došlo do relapsa tumora i grupu kod koje je došlo do supresije tumora. Mesec dana nakon završetka lečenja, primenom SMR analizirane su razlike u nivoima metabolita mozga između dve grupe. **Rezultati.** Nivoi N-acetilaspartata (NAA) i kreatina (Kr) povećali su se nakon radioterapije, dok su najveća izmerena vrednost holina (H), i odnosi H/Kr, NAA/Kr i H/NAA bili smanjeni u poređenju sa nivoima pre lečenja. Postojale su statistički značajne razlike u najvećoj izmerenoj vrednosti NAA, i odnosima H/Kr i H/NAA u području povećanja tumora, pre i posle lečenja ($p < 0,05$). Postojale su i statistički značajne razlike u vrednostima odnosa H/Kr u području peritumorskog edema pre i posle lečenja ($p < 0,05$). **Zaključak.** Nakon konkurentne hemioradioterapije GVS, MRS se može koristiti za otkrivanje ranih metaboličkih promena u područjima povećanja tumora i peritumorskog edema.

Ključne reči:
biomarkeri; radiohemioterapija; lečenje lekovima, adjuvantno; gliom; magnetska rezonanca, spektroskopija; prognoza; hirurgija.

Introduction

High-grade glioma (HGG) is an interstitial cell-derived primary tumor of the nervous system. It is the most prevalent type of central nervous system tumor, accounting for approximately 60% of all cases [1]. The 2018 edition of the "Guidelines for the Diagnosis and Treatment of Glioma" recommends the maximum safe range of tumor resection for treatment methods. This includes intracranial tumor resection for tumors occupying obvious space and requiring pathological biopsy due to invasive diffuse growth in the dominant hemisphere and invasion of both hemispheres. Adjuvant concurrent chemoradiotherapy is recommended after surgery, followed by six cycles of temozolomide single-drug chemotherapy. Evaluation of the early efficacy of concurrent chemoradiotherapy after HGG surgery, especially for patients with a high risk of recurrence, is a crucial step in enhancing the treatment efficiency for patients diagnosed with HGG. Currently, magnetic resonance (MR) imaging (MRI) with or without contrast is primarily used to determine whether the tumor is under control or has progressed after radiotherapy and chemotherapy for HGG. This is determined by evaluating the size of the tumor and the changes in the degree of enhancement. If the tumor metabolism can be monitored using advanced imaging technology and the efficacy of simultaneous chemoradiotherapy can be predicted prior to any change in the tumor morphology, this would be the optimal outcome for clinicians. Amin et al. [2] proposed that the change in metabolite concentration occurs always before the morphological change in HGG. Therefore, the purpose of this study was to examine the correlation between the changes in HGG metabolites before and after radiotherapy in the residual lesions and peritumoral edema areas using MR spectroscopy (MRS) and their correlation with prognosis, as well as to investigate the clinical utility of MRS in determining the early curing efficacy of concurrent chemoradiotherapy following HGG surgery.

Methods

General data

A total of 50 patients with HGG who were treated in the Radiotherapy Department of our Hospital between January 2016 and June 2021 were selected for the study. This study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University, China (No. WZ 2023062, from September 1, 2019).

The study included patients with incomplete or complete resection of the tumor but with significant enhancement of the operative cavity margin, as shown by MRI. Patients who, due to the anatomic location of the tumor, were unable to undergo surgical resection and only underwent pathological biopsy were also included.

Imaging examination

The Siemens 3.0T Skyra MR imager was used for imaging one week before concurrent chemoradiotherapy and one month after treatment for all 50 patients. Routine MRI scans with and without contrast, as well as multivoxel MRS, were performed on all patients one week before concurrent chemoradiotherapy and were re-examined one month after concurrent chemoradiotherapy. MRS is based on the principle of chemical shift, which states that different chemical substances containing the same nucleus exhibit characteristic chemical shifts at resonance frequency, allowing the chemical composition of elements to be identified. This method enables the assessment of the chemical environment in tumor cells and brain tissues from the perspective of cell metabolism. Routine MRI examination included: Ax-Magnetization Prepared Rapid Acquisition Gradient Echo (MPRAGE): echo time (TE) 2.98 ms, repetition time (TR) 5,000 ms; T_2 fast spin echo (T_2FSE): TE 117 ms, TR 5,500 ms; T_2 – weighted fluid-attenuated inversion recovery (T_2FLAIR): TE 81 ms, TR 6,000 ms; axial scanning with a layer thickness of 5.5 mm, spacing of 1.1 mm; field of view (FOV) of 23 cm, and matrix size of 320 × 224. The MRS examination was conducted using the automatic MRS technique (PROBEx/S1 proton brain exam/multiple voxels), Point RESolved Spectroscopy (PRESS) sequence, with a TR of 1,700 ms, TE of 135 ms, multi-voxel phase matrix scanning, and a layer thickness of 15 mm. Voxel dimensions were 10 mm × 10 mm × 15 mm, and the FOV dimensions were 160 mm × 160 mm. The cross-sectional T_1 image after MRI enhancement was used to identify areas of interest. This included both the

enhanced area at the edge of the operative cavity and the enhanced area of the residual tumor. If the residual tumor revealed obvious enhancement, the area of interest was positioned between the enhancement area. If the residual tumor did not demonstrate contrast enhancement, the area of interest was placed within the body of the residual tumor. Due to low-grade gliomas usually not revealing contrast enhancement on T_1, an area of interest was also placed in the edema area surrounding the lesion. Metabolic maps, spectral maps, and chemical shift maps were obtained, and peak spectral values of N-acetylaspartate (NAA), choline (Cho), and creatine (Cr) were calculated. Quantification was performed using LCModel, with metabolite concentrations typically reported in institutional units, such as institutional units per unit volume or institutional units per Cr.

Concurrent chemoradiotherapy

Using a 6MV X-ray and a 3D treatment planning system, all 50 patients underwent radiation therapy. The head was secured using a plastic surface mold. Laser light was positioned, and a continuous computed tomography (CT) scan with contrast was performed with a 2.5 mm scanning layer thickness. The CT image data were then transferred to the planning system. After fusion, the treatment target area was outlined on the MRI image. For low-grade glioma, abnormal T_2 FLAIR MRI signals and all surgical cavities were delineated by the gross tumor volume (GTV), which was then extended 0.3–0.5 cm outside to form the planning GTV relative to T_1. The clinical target volume (CTV) was created by including an additional 1.0–1.5 cm outside the tumor, and it was necessary for the CTV to encompass the edema region surrounding the tumor. The planning target volume (PTV) was formed by adding a margin of 0.3 to 0.5 cm to the CTV. For HGG, the MRI abnormal T_1 and T_2 FLAIR signals, as well as surgical cavities, were delineated by the GTV, which was then extended 0.3–0.5 cm outside to form the planning GTV. The CTV1 was formed by including an additional 1.5–2.0 cm outside the tumor, while the CTV2 was formed by including an additional 2.0–2.5 cm outside the tumor. It was necessary for the CTV1 to encompass the edema area surrounding the tumor. Based on CTV1 and CTV2, a 0.3 cm margin was added to determine the PTV1 and PTV2 dimensions. The irradiation dose was 2 Gy/day, administered five times a week, with a total dose of 60 Gy for low-grade gliomas and 64 Gy for HGG. All 50 patients with HGG were treated with intensity-modulated radiation therapy and concurrent oral temozolomide at a dose of 75 mg/m^2 for sensitization chemotherapy.

Follow-up

Six cycles of adjuvant temozolomide chemotherapy (150 mg/m^2, one cycle from Day 1 to Day 5, every 28 days) were administered to all the patients. The patients were followed up for 6 to 12 months, and routine MRI scans with and without contrast were performed. The MRI images were compared to the MRI obtained one month after concurrent chemoradiotherapy. Brain positron emission tomography (PET)-CT was used to further confirm suspected cases of recurrence. Based on the follow-up results, the patients were divided into two groups: the tumor recurrence (TR) group and the tumor suppression (TS) group.

Statistical methods

The statistical analysis of all experimental data was done using the SPSS 23.0 statistical software. The measurement data are expressed as mean ± standard deviation. The differences in metabolite concentration and ratio before and after concurrent chemoradiotherapy were analyzed using a paired sample *t*-test following a normality test. One month after concurrent chemoradiotherapy, a *t*-test was used to analyze the differences in metabolite concentration and ratio between the TR group and the TS group. The differences were considered statistically significant when $p < 0.05$.

Results

Table 1 demonstrates the baseline characteristics of the patients. The age range was between 19 to 70 years, with an average age of 46.62 ± 12.14 years.

NAA and Cr levels increased after radiotherapy, while the Cho peak value, and Cho/Cr, NAA/Cr, and Cho/NAA ratios decreased compared to pre-treatment levels.

In the intensive area, the levels of NAA and Cr were higher after concurrent chemoradiotherapy than before treatment, while the peaks of Cho were lower. There were changes in the NAA peak level before and after treatment, and the difference was statistically significant ($p < 0.05$). After concurrent chemoradiotherapy, the Cho/Cr, NAA/Cr, and Cho/NAA ratios were lower than before treatment. There were statistically significant changes in Cho/Cr and Cho/NAA ratios before and after treatment ($p < 0.05$) (Table 2).

In the edema region, the levels of NAA and Cr peaks were higher after concurrent chemoradiotherapy than before treatment, while the peaks of Cho were lower. However, there was no statistically significant difference between the changes in the three observation indicators before and after treatment ($p > 0.05$). After concurrent chemoradiotherapy, the Cho/Cr, NAA/Cr, and Cho/NAA ratios were all lower than before treatment, and there was a statistically significant difference in the Cho/Cr ratio before and after treatment ($p < 0.05$) (Table 3).

The peak values of Cho and Cho/Cr, NAA/Cr, and Cho/NAA ratios in the TR group were higher, while the peak values of NAA and Cr were lower in the TR group compared to the TS group. Seventeen out of the 50 patients who underwent HGG surgery experienced tumor recurrence after 6 to 12 months of follow-up. Among these cases, 7 patients had newly enhanced lesions in the brain tissue, while 10 patients had residual lesions that were larger than before. These larger lesions had an occupying impact resulting in a worsening of clinical symptoms. Additional brain PET-CT examinations confirmed the recurrence of the tumor in 16 of the 17 cases mentioned above. The re-

maining case was confirmed as a recurrence of the tumor through a second surgery and pathology. All 17 cases belonged to the TR group. In the remaining 33 patients, the residual tumor lesions continued to decrease or remained stable during follow-up, and the occupying impact and clinical symptoms were alleviated. These 33 patients were included in the TS group. One month after concurrent chemoradiotherapy, the spectral peak value and ratio of brain metabolites related to the enhancement and edema regions on MRS were compared between the two groups.

In the enhancement region, the peak value of Cho in the TR group was higher than that in the TS group. In addition, the peak values of NAA and Cr were lower in the TR group compared to the TS group. Furthermore, there was a significant difference in the peak value of Cho between the two groups ($p < 0.05$). The Cho/Cr, NAA/Cr, and Cho/NAA ratios were higher in the TR group compared to the TS group. The Cho/Cr and Cho/NAA ratios exhibited statistically significant differences between the two groups ($p < 0.05$) (Table 4).

Table 1

Participant demographic and baseline characteristics

Characteristics	n (%)
Gender	
male	30 (60)
female	20 (40)
KPS score	
60	4 (8)
70	10 (20)
80	28 (56)
90	8 (16)
Pathological diagnosis and grading	
astrocytoma (WHO Grade II)	7 (14)
oligodendroglioma (WHO Grade II)	6 (12)
ependymoma (WHO Grade II)	2 (4)
anaplastic astrocytoma (WHO Grade III)	21 (42)
glioblastoma (WHO Grade IV)	14 (28)
Type of surgery	
partial excision	13 (26)
pathological biopsy	6 (12)
complete resection with active lumen margin	31 (62)

WHO – World Health Organization; KPS – Karnofsky Performance Scale.

Table 2

Peak value and ratio of metabolites in enhancement area before and after concurrent chemoradiotherapy (CC)

Phase	NAA	Cho	Cr	Cho/Cr	NAA/Cr	Cho/NAA
Before CC	3.362 ± 1.202	5.809 ± 2.129	3.362 ± 1.331	1.874 ± 0.738	1.120 ± 0.477	1.801 ± 0.575
After CC	3.773 ± 1.174	5.319 ± 1.922	3.570 ± 0.956	1.581 ± 0.683	1.109 ± 0.399	1.474 ± 0.535
t-value	-2.349	1.863	-1.079	2.320	0.141	3.220
p-value	0.023	0.068	0.286	0.025	0.889	0.002

NAA – N-acetylaspartate; Cho – choline; Cr – creatine; Cho/Cr – choline/creatine ratio; NAA/Cr – N-acetylaspartate/creatine ratio; Cho/NAA – choline/N-acetylaspartate ratio. Results are shown as mean ± standard deviation.

Table 3

Peak value and ratio of metabolites in edema area before and after concurrent chemoradiotherapy (CC)

Phase	NAA	Cho	Cr	Cho/Cr	NAA/Cr	Cho/NAA
Before CC	5.788 ± 1.364	8.447 ± 2.902	5.410 ± 1.681	1.614 ± 0.454	1.150 ± 0.345	1.502 ± 0.556
After CC	5.824 ± 1.498	7.794 ± 2.615	5.821 ± 1.616	1.380 ± 0.448	1.041 ± 0.273	1.369 ± 0.393
t-value	-0.172	1.931	-1.546	3.042	1.999	1.847
p-value	0.864	0.059	0.124	0.004	0.051	0.071

For abbreviations, see Table 2. Results are shown as mean ± standard deviation.

Table 4

Peak value and ratio of related metabolites in the tumor recurrence (TR) and tumor suppression (TS) groups in the enhancement area

Group	NAA	Cho	Cr	Cho/Cr	NAA/Cr	Cho/NAA
TR	3.736 ± 0.926	6.433 ± 1.800	3.505 ± 1.133	1.985 ± 0.754	1.168 ± 0.477	1.791 ± 0.506
TS	3.792 ± 1.296	4.745 ± 1.743	3.603 ± 0.868	1.373 ± 0.547	1.079 ± 0.357	1.312 ± 0.478
t-value	-0.160	3.209	-0.341	3.286	0.739	3.289
p-value	0.874	0.002	0.735	0.002	0.463	0.002

For abbreviations see Table 2. Results are shown as mean ± standard deviation.

Table 5

Peak value and ratio of related metabolites in the tumor recurrence (TR) and tumor suppression (TS) groups in the edema region

Group	NAA	Cho	Cr	Cho/Cr	NAA/Cr	Cho/NAA
TR	5.555 ± 1.379	8.629 ± 2.570	5.461 ± 1.418	1.649 ± 0.465	1.057 ± 0.265	1.565 ± 0.322
TS	5.963 ± 1.558	7.364 ± 2.570	6.006 ± 1.700	1.254 ± 0.415	1.032 ± 0.280	1.268 ± 0.392
t-value	-0.911	1.648	-1.134	3.062	0.307	2.690
p-value	0.367	0.106	0.262	0.004	0.760	0.010

For abbreviations, see Table 2. Results are shown as mean ± standard deviation.

In the edema area, the peak value of Cho was greater in the TR group than in the TS group, whereas the peak values of NAA and Cr were lower than that in the TS group. The three observation indices did not differ significantly between the two groups ($p > 0.05$). The Cho/Cr, NAA/Cr, and Cho/NAA ratios were higher in the TR group than in the TS group. In addition, there were statistically significant differences between the two groups in the Cho/Cr and Cho/NAA ratios ($p < 0.05$) (Table 5).

Discussion

HGG is a primary tumor of the central nervous system with a relatively high level of malignancy. The epidemiological characteristics of the disease include a high incidence, high disability rate, and a high mortality rate, and pose a serious threat to human health. Surgical resection within the maximum safe range remains the preferred initial treatment option for HGG. In the early stages of the disease, however, the highly invasive growth of HGG cells causes them to spread to the surrounding normal brain tissue and develop into highly irregular lesions. This makes complete surgical resection extremely difficult [3]. Postoperative concurrent chemoradiotherapy is, therefore, an important adjunctive treatment for HGG, and accurate monitoring of the early response to concurrent chemoradiotherapy is essential for determining prognosis. In the early stages of treatment, MRS can be used to detect the microscopic infiltration of HGG cells and determine their aggressiveness [4] and is effective in evaluating the early efficacy of concurrent chemoradiotherapy. In this study, the spectral peaks of NAA and Cr were found to be higher in both the enhancement and edema regions after treatment, than before treatment, and there was a statistically significant difference between the spectral peaks of NAA in the tumor-enhanced area before and after concurrent chemoradiotherapy ($p < 0.05$), which is consistent with most reports. The increase in NAA suggests the recovery of neuronal function in the brain tissue, while the increase in Cr indicates that the level of energy metabolism in brain tissue has been restored, indicating that the treatment is effective. In addition, the results of this study revealed that the Cho spectral peaks in the enhancement area and edema area were lower after treatment compared to before treatment with no statistically significant difference between the changes in the two areas before and after treatment ($p > 0.05$). The decrease in the Cho spectral peak indicates that the proliferation of tumor cells is being inhibited, and the disease may be effectively controlled. Lotumolo et al. [5] suggested that tumorigenesis and progression are characterized by elevated levels of Cho and decreased levels of NAA. The molar concentration of Cho in the brain tissue of patients who responded positively to the treatment was reduced.

In this study, the NAA/Cr ratio in the enhancement area and the edema area was found to be lower after treatment compared to that before the treatment with no statistically significant difference before and after treatment ($p > 0.05$). Wen et al. [6] studied MRS performance in patients diagnosed with HGG after chemotherapy and discovered that the NAA/Cr ratio decreased in patients who responded to the treatment. In addition, our study revealed that the Cho/Cr and Cho/NAA ratios in the enhancement area and the edema area were lower following treatment than they were prior to treatment with no statistically significant difference between the changes in the Cho/NAA ratio before and after concurrent chemoradiotherapy in the edema area ($p > 0.05$). Zhang et al. [7] discovered a strong positive correlation between the invasion ability of glioma cells and the Cho/Cr and Cho/NAA ratios. Based on the significant changes in the ratios between the tumor enhancement area and the edema area observed in this study before and after concurrent chemoradiotherapy, it can be concluded that the aggressiveness of the tumor cells was inhibited in patients diagnosed with HGG who received concurrent chemoradiotherapy, indicating the effectiveness of the treatment. Lotumolo et al. [5] suggested that patients diagnosed with HGG who received effective treatment exhibited decreased Cho/Cr and NAA/Cr ratios post-treatment, which is consistent with our experimental findings.

After concurrent chemoradiotherapy, the peak value of the Cr spectrum increased in both groups compared to that before treatment. However, the peak value of the Cr spectrum was lower in the TR group than in the TS group. There was no statistically significant difference in the peak value of the Cr spectrum between the two groups ($p > 0.05$). Sauwen et al. [8] believe that Cr exists as a raw material for energy supply in brain HGG with active energy metabolism. They argue that the more vigorous the glioma cell metabolism, the more Cr is consumed, leading to a decrease in its content. Therefore, there is a negative correlation between the Cr level in brain tissue and the proliferation and activity of glioma cells. It is not difficult to conclude based on our experimental data, that the glioma cells in the TS group continued to proliferate and metabolize vigorously in the early stage after synchronous chemoradiotherapy, compared to the glioma cells in the TR group. This ultimately results in different tumor outcomes.

In this experimental study, we discovered that the Cho/Cr, NAA/Cr, and Cho/NAA ratios in the enhancement and edema areas of patients diagnosed with HGG were significantly lower after concurrent chemoradiotherapy than before treatment. However, the Cho/Cr, NAA/Cr, and Cho/NAA ratios after concurrent chemoradiotherapy at the above two sites were significantly higher in the TR group than in the TS group. There were statistically significant differences in the Cho/Cr and Cho/NAA ratios between the two groups ($p < 0.05$). As mentioned previously, patients who responded positively to chemotherapy had a lower NAA/Cr ratio, while patients in the TR group displayed a higher NAA/Cr ratio than the TS group. However, there was no statistically significant difference in the NAA/Cr ratio between the two groups ($p > 0.05$). Furthermore, it was observed that patients in the TR group exhibited relatively low sensitivity to concurrent chemoradiotherapy. Zhang et al. [7] observed the ratios of Cho/NAA > 2, Cho/Cr > 2, lactic acid (Lac)/Cr >1, and lipid (Lip)/Cr > 1 in metabolically active tumor voxels. Although no spectral peaks of Lac and Lip were observed in our study, the other indicators observed were similar to those found in the study by Zhang et al. [7].

In light of the significant differences in the spectral peaks and ratios of related metabolites between the TR and the TS group, the detection of higher Cho peak and Cho/Cr and Cho/NAA ratios using MRS in the early period after concurrent chemoradiotherapy for patients diagnosed with HGG following surgery, strongly suggests that the tumor cells are still metabolically active and pose a high risk of recurrence. This finding serves as a reminder for clinicians to enhance patient follow-up, consider increasing adjuvant drugs, and intensify late temozolomide chemotherapy, if necessary, to prevent tumor recurrence in patients.

It is difficult for conventional MRI to detect the information on the invasion of tumor cells in patients diagnosed with HGG in the early stage after the completion of concurrent radiotherapy and chemotherapy following surgery. In addition, most HGG tumor recurrences are detected during long-term follow-up after treatment. By detecting changes in the peak values and ratios of brain tissue-related metabolites, MRS can be used to accurately evaluate the efficacy of concurrent radiotherapy and chemotherapy in patients with HGG after earlier surgery than traditional MRI. This is especially advantageous for patients with a high risk of recurrence and provides clinicians with valuable reference information, enabling them to develop comprehensive follow-up and treatment strategies for patients diagnosed with HGG with varying treatment outcomes, to minimize tumor recurrence following combined radiotherapy and chemotherapy administered after surgery. This strategy aims to enhance the quality of life of patients and extend their lives.

MRS is also effective in distinguishing between HGG recurrence and radiation brain necrosis. The specificity of this technique lies in its capacity to distinguish between tumor recurrence and brain radionecrosis. This is accomplished by evaluating the extent of HGG invasion and identifying the metabolic information of the brain tissue at the tumor margin [9]. Lotumolo et al. [5] suggested that tumor recurrence following HGG treatment is linked to an increase in Cho levels. Radioactive brain necrosis on the other hand is linked to an increase in Lac or Lip concentration, whereas other brain metabolites demonstrate a decreased state in cases of radioactive brain necrosis. After HGG treatment, Chuang et al. [10] discovered significant differences in the Cho/NAA and Cho/Cr ratios between tumor recurrence and radiation-induced brain necrosis. Crain et al. [11] proposed five indicators for diagnosing HGG recurrence following treatment in their study: Cho/Cr > 1.54 (sensitivity: 66%, specificity: 79%), Cr/Cho ≤ 0.63 (sensitivity: 65%, specificity: 79%), Lac/Cho ≤ 2.67 (sensitivity: 85%, specificity: 58%), Lac/Lip ≤ 1.64 (sensitivity: 54%, specificity: 95%), and Lip/Lac > 0.58 (sensitivity: 56%, specificity: 95%). Thust et al. [12] confirmed that the Cho/NAA and Cho/Cr ratios distinguished tumor recurrence from radiation-induced brain necrosis with an accuracy of 80% to 97%. MRS has important clinical application value in differentiating long-term tumor recurrence from radiation brain necrosis after concurrent chemoradiotherapy for HGG surgery, as shown by the aforementioned data.

Conclusion

In light of the previously reported clinical trial data and the results of this experimental study, it is evident that MRS has significant clinical value for the early assessment of treatment effectiveness in patients diagnosed with HGG undergoing concurrent chemoradiotherapy after surgery, thus it is an essential imaging technique in clinical practice.

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Influence of gender on the diagnostic and prognostic significance of the dobutamine stress echocardiography for ischemia

Uticaj pola na dijagnostički i prognostički značaj dobutaminskog stres-ehokardiografskog testa na ishemiju

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Abstract

Background/Aim. Cardiovascular diseases are the most common cause of mortality and morbidity worldwide, with the highest incidence in low-middle-income countries. Dobutamine stress echocardiography (DSE) plays a significant role in diagnosing coronary artery disease. The aim of the study was to examine the influence of gender on the accuracy of DSE and on the prognostic significance of the left ventricular wall motion score index (WMSI) as a parameter of DSE for adverse cardiovascular events (ACEs). **Methods.** The prospective, observational study conducted at the Clinic for Cardiovascular Diseases of the University Clinical Center of the Republic of Srpska, Bosnia and Herzegovina, included 143 patients who, due to suspicion of coronary disease, underwent a DSE from January 1, 2021, until February 1, 2022. ACEs that we observed one year after DSE were: unstable angina, acute myocardial infarction, percutaneous coronary intervention, in-stent restenosis, aortocoronary bypass, heart failure, and death. **Results.** A positive DSE was more common in men (45%) compared to women (25%), which was statistically significant ($p < 0.05$). The sensitivity of the test in men was 96.0%, and the specificity was 93.9%. In women, the sensitivity was 86.7%, and specificity was also 86.7%. ACEs were more frequent in men (43%) than in women (19%), which was statistically significant ($p < 0.01$). In men, WMSI had a sensitivity of 96.0% and a specificity of 82.4%. In women, WMSI had a sensitivity of 93.3% and a specificity of 90.6% in predicting ACEs. **Conclusion.** Our results confirmed the influence of gender on the accuracy of DSE in diagnosing coronary artery disease. WMSI as a parameter of DSE was identified as a significant prognostic factor for ACEs in both sexes, with the sensitivity being higher in men and specificity in women.

Key words:
coronary disease; diagnosis; echocardiography, stress; sensitivity and specificity; sex factors.

Apstrakt

Uvod/Cilj. Kardiovaskularne bolesti su najčešći uzrok mortaliteta i morbiditeta u celom svetu, sa najvećom incidencijom u zemljama sa niskim i srednjim dohotkom. Dobutaminski stres-ehokardiografski (DSE) test ima značajnu ulogu u dijagnostici koronarne arterijske bolesti. Cilj rada bio je da se ispita uticaj pola na tačnost DSE, kao i na prognostički značaj skora indeksa pokretljivosti zidova (*wall motion score index* – WMSI) leve komore, kao parametra DSE za neželjene kardiovaskularne događaje (NKD). **Metode.** Prospektivnom, opservacionom studijom koja je rađena na Klinici za kardiovaskularne bolesti Univerzitetskog kliničkog centra Republike Srpske, Bosna i Hercegovina, obuhvaćena su 143 bolesnika koji su zbog sumnje na koronarnu bolest bili podvrgnuti DSE u periodu od 1. januara 2021. do 1. februara 2022. godine. Godinu dana od urađenog DSE testa, uočeni su sledeći NKD: nestabilna angina pektoris, akutni infarkt miokarda, perkutana koronarna intervencija, restenoza u stentu (*in-stent restenosis*), aortokoronarni *bypass*, srčana insuficijencija i smrt. **Rezultati.** Pozitivan DSE test bio je češći kod muškaraca (45%) u odnosu na žene (25%), što je bilo statistički značajno ($p < 0,05$). Senzitivnost testa kod muškaraca iznosila je 96,0%, a specifičnost 93,9%. Kod žena su i senzitivnost i specifičnost iznosile 86,7%. NKD su bili češći kod muškaraca (43%) nego kod žena (19%) i ta razlika je bila statistički značajna ($p < 0,01$). Indeks WMSI je kod muškaraca imao senzitivnost 96,0%, a

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specifičnost 82,4%. Kod žena je WMSI za predviđanje NKD imao senzitivnost 93,3% i specifičnost 90,6%. **Zaključak**. Naši rezultati su potvrdili uticaj pola na tačnost DSE testa u dijagnostici koronarne arterijske bolesti. Indeks WMSI, kao parametar DSE, identifikovan je kao značajan prognostički faktor za NKD kod oba pola, s tim što je senzitivnost bila viša kod muškaraca, a specifičnost kod žena.

Ključne reči:
koronarna bolest; dijagnoza; ehokardiografija, stres; senzitivnost i specifičnost; pol, faktor.

Introduction

Cardiovascular diseases are the most common cause of mortality and morbidity worldwide, with the highest incidence in low and middle-income countries [1, 2]. Ischemic heart disease accounts for about 38% of all cardiovascular causes of death in women and 44% in men. The decline in mortality from cardiovascular diseases in men was dramatic. The observed decrease in mortality is significantly less for women than for men [3, 4]. Compared with men, women consistently receive less intensive care, including fewer anti-anginal drugs, less frequent coronary angiography or revascularization, and fewer treatments that modify lifestyle or risk factors [5–8].

Stress echocardiography plays a significant role in diagnosing coronary artery disease (CAD). The two most popular pharmacological stress echocardiography tests used are dobutamine and dipyridamole. Dobutamine is the prototype of pharmacological adrenergic or inotropic stress. The high accuracy of the dobutamine stress echocardiography (DSE) in detecting angiographically assessed CAD, with sensitivity and specificity of 81% and 84%, respectively, is reported in a meta-analysis of 102 studies with more than 7,900 patients [9].

Several studies examined the role of DSE as a predictor of patient outcomes [10–15]. Controversy exists regarding the presence of a sex-based difference in the accuracy of DSE in detecting CAD [16, 17]. Compared to men, the noninvasive assessment of CAD in women is more challenging for several reasons: a lower incidence of single-vessel disease and less predictability and atypical symptoms for CAD. In the study by Elhendy et al. [16], sensitivity, specificity, and accuracy of DSE for ischemia in women were 76% [confidence interval (CI) 67–84], 94% (CI 89–99), and 82% (CI 75–90), respectively, and in men, 73% (CI 67–79), 77% (CI 71–83), 74% (CI 68–80), respectively. It was observed that the majority of false-positive DSE occur in women [18, 19].

The aim of the study was to examine differences in the diagnostic and prognostic significance of the ischemia test according to the gender of the patient. Based on that, we assessed the significance of the left ventricle (LV) wall motion score index (WMSI).

Methods

The prospective, observational study was conducted at the Cardiovascular Clinic of the University Clinical Center of the Republic of Srpska (UCCRS), Bosnia and Herzegovina. The research was conducted according to the permission of the Ethics Committee of UCCRS (No. 01-19-239-2/23, from June 28, 2023). All patients undergoing the test signed informed consent to protect privacy in the publication process.

The study included 143 patients who, due to suspicion of coronary disease, underwent a DSE from January 1, 2021, to February 1, 2022. The follow-up period was one year. The study inclusion criteria were the following: patients with suspected coronary heart disease, patients with typical anginal pain, patients with atypical anginal pain, patients with risk factors for cardiovascular diseases, patients with left bundle branch block and chest pain, patients with a previous coronary event and recurrent chest pain, and patients with inconclusive ergometric test. Exclusion criteria for the study were valvular or congenital heart disease, significant comorbidities with an expected survival of less than one year, and a poor acoustic window. Before the test, beta blockers and anti-aging drugs were excluded. The test was performed according to the protocol in which the dose of dobutamine was increased every three minutes, starting from 5 mcg/kg/min up to 40 mcg/kg/min. Study endpoints included electrocardiograms, echocardiographic evidence of ischemia, significant symptoms or arrhythmia, target heart rate of 85% of submaximal frequency predicted for age ($220 - \text{age} \times 0.85$), symptomatic hypotension, or protocol completion. If the patient failed to achieve the submaximal frequency of age-predicted heart rate, atropine was added up to a maximum dose of 2 mg intravenously (i.v.). Metoprolol was used (1 to 5 mg i.v.) to reverse the effects of the dobutamine or dobutamine-atropine combination if these did not revert spontaneously. Subjects who underwent DSE had a high percentage of angiotensin-converting enzyme inhibitors (78%), beta-blockers (90%), aspirin (97%), clopidogrel (60%), and statins (68%) in their therapy. Calcium channel blockers (48%) and nitrates (43%) were used in a smaller percentage. Examinations were performed using the Vivid 7 echocardiography device (General Electrics). LV was recorded in apical four-chamber, two-chamber, and three-chamber sections as well as the parasternal two-chamber circular section at rest at the end of the fifth minute in the peak (submaximal age-predicted heart rate) and in the recovery period. The test was considered positive if contractility worsened in two or more segments in the form of hypokinesia and akinesia, new regional wall motion abnormality, and biphasic responses. We used a 17-segment model of LV to calculate the WMSI. WMSI was calculated by dividing the sum of all individual segmental values by the number of myocardial segments. A normal value of the WMSI is 1, greater than 2.5, indicating a poor prognosis. Visual evaluation of the contractility of each LV segment included a four-step scale: 1-normokinetic, 2-

hypokinetic, 4-akinetic, and 5-dyskinetic. The test was negative if there was no abnormality of segmental contractility of LV. The data from the UCCRS information system regarding four monthly control cardiology examinations and echocardiography were recorded. Observed cardiovascular events were: unstable angina (UA), acute myocardial infarction (MI), in-stent restenosis (ISR), percutaneous coronary intervention (PCI), aortocoronary bypass (ACB), heart failure (HF), and death. Patients who had UA, acute MI, and suspected ISR underwent coronary angiography and treatment according to the recommendations of the European Association of Cardiologists (EAC). All patients who had a positive test underwent coronary angiography. Patients with more severe forms of HF were also hospitalized and treated according to the recommendations of EAC.

Statistical analysis

We used Chi-Square tests to determine the difference between the sexes, the probability of CAD, and adverse cardiovascular events (ACEs). We used the independent samples test (*t*-test) to determine differences between the sexes in echocardiographic parameters during DSE. The values of $p < 0.05$ were considered statistically significant.

The optimal cut-off value for WMSI to predict poor outcomes was determined by receiver operating characteristics (ROC) curve analysis. The optimal cut-off value was defined as the one that provides maximum accuracy in distinguishing between patients with and without CAD and ACEs. The value of $p = 0.05$ was considered significant.

Statistical data analysis was performed using IBM SPSS Statistics 22 (SPSS Inc. Chicago, IL, USA).

Results

The study included 143 patients with suspected CAD who underwent a DSE. The study comprised 43% of men and 57% of women. The average age of men was 66.02 years, and of women, 65.99 years, which was not statistically significant ($p = 0.985$).

The baseline characteristics of examined patients are presented in Table 1. Male patients had a higher percentage of risk factors for CAD, such as diabetes mellitus ($p < 0.05$) and elevated blood lipid levels ($p < 0.01$). The family history of MI ($p < 0.01$), as well as revascularization in the form of PCI ($p < 0.01$) and ACB ($p = 0.018$), was more common in men (Table 1).

Parameters of DSE according to gender are presented in Table 2. In the observed echocardiographic characteristics using the *t*-test, a statistically significant difference was found in the LV ejection fraction (LVEF) between men and women ($p < 0.01$). During the test, women had a higher value of the LVEF. Men had a higher value of WMSI at the submaximal frequency at the peak of the test ($p < 0.05$).

The differences in ACEs between men and women are presented in Table 3. A positive DSE test was more common in men (45%) than in women (25%); it was statistically significant. The Chi-Square test showed that ACEs were more frequent in men (43%) than in women (19%). The difference was statistically significant ($p < 0.01$). UA ($p < 0.05$) and PCI ($p < 0.01$) were significantly more common in men than in women.

The sensitivity of the DSE in men was 96.0%, and the specificity was 93.9%. In women, the sensitivity was 86.7%, and specificity was also 86.7%.

Log-Rank tests indicated no statistically significant difference in survival between male and female patients, while

Table 1

Characteristics of patients

Parameter	Men	Women	*p*-value
Age, years	66.02 ± 9.70	65.99 ± 8.97	0.985
Arterial hypertension	51 (86)	71 (90)	0.533
Diabetes mellitus	22 (38)	15 (18)	< 0.05
Hyperlipoproteinemia	41 (71)	34 (43)	< 0.01
Positive family history	19 (32)	19 (24)	0.289
Previous myocardial infarction	25 (42)	12 (15)	< 0.01
Previous PCI	25 (42)	12 (15)	< 0.01
Previous bypass	3 (5)	1 (1)	0.018

PCI – percutaneous coronary intervention.
All values are given as numbers (percentages) or mean ± standard deviation.

Table 2

Parameters of dobutamine stress echocardiography according to gender

Parameter	Men	Women	*p*-value
WMSI before test	1.07 ± 0.19	1.03 ± 0.12	0.141[a]
WMSI in test peak	1.24 ± 0.31	1.12 ± 0.25	< 0.05[a]
Heart frequency	130.78 ± 9.52	132.03 ± 8.21	0.412
Ejection fraction	55.05 ± 6.35	58.50 ± 5.47	< 0.01[a]

WMSI – wall motion score index. All values are given as mean ± standard deviation. [a]Independent samples test (*t*-test).

older patients, as expected, had a lower survival rate in the observed time (Figure 1).

During the follow-up, three patients died – two men and a woman.

By analyzing the ROC curve, we determined that the sensitivity of WMSI in the peak of the DSE in men for the discrimination of ACEs was 96.0% and specificity was 82.4% for the value of WMSI > 1. The area under the curve (AUC) was 90.6% (95% CI 80.2–96.7) ($p < 0.001$). The value of $p \leq 0.001$ confirms the statistical significance of these results (Figure 2).

The sensitivity of the WMSI in women by peak DSE test for the discrimination of ACEs was 93.3%, and the specificity was 90.6%. The optimal WMSI threshold value was >

Table 3

Frequency of adverse cardiovascular events (ACEs) according to gender

Test results	Men	Women	*p*-value
Positive DSE	27 (45)	20 (25)	< 0.05
Unstable angina	14 (24)	8 (10)	< 0.05
Acute myocardial infarction	3 (5)	1 (1)	0.186
PCI	26 (44)	15 (19)	< 0.01
In-stent restenosis	1 (2)	1 (1)	0.842
CABG	5 (8)	3 (4)	0.246
Heart failure	4 (7)	2 (3)	0.226
Death	3 (5)	2 (2)	0.415
ACE	25 (43)	15(19)	0.01

DSE – dobutamine stress echocardiography; PCI – percutaneous coronary intervention; CABG – coronary artery bypass grafting.
All values are given as numbers (percentages).

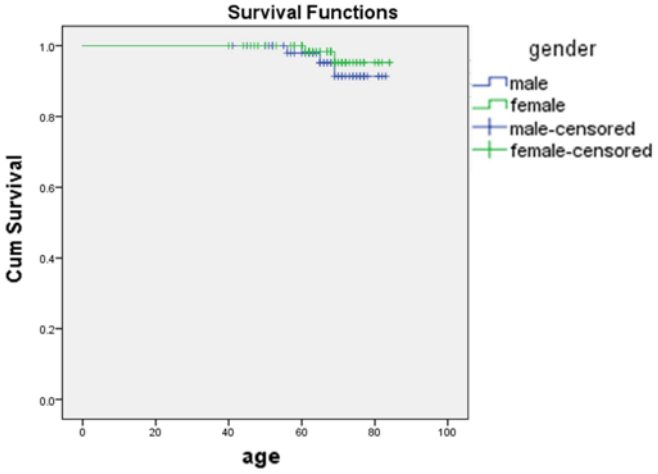

Fig. 1 – Gender differences in survival: Kaplan-Meier curve.
cum – cumulative.

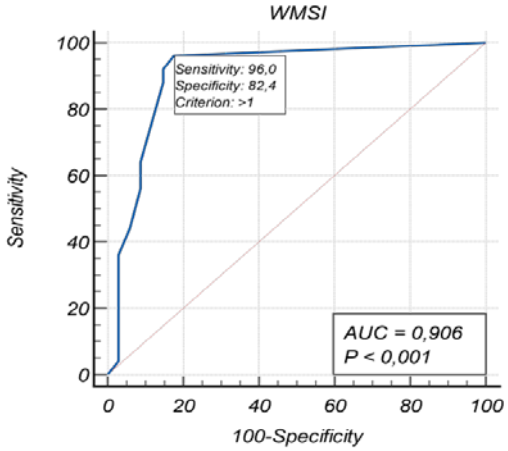

Fig. 2 – Prognostic value of wall motion score index in men: sensitivity 96.0%, specificity 82.4%, optimal threshold value > 1. Area under the curve (AUC) is 90.6% [95% confidence interval (CI) 80.2–96.7] (p < 0.001).

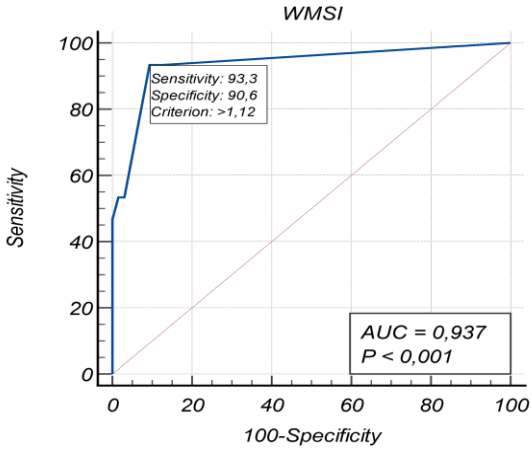

Fig. 3 – Prognostic value of wall motion score index (WMSI) in women: sensitivity 93.3%, specificity 90.6%; optimal threshold value is > 1.12. AUC is 93.7% (95% CI 85.9–97.9) (p < 0.001). For abbreviations, see Figure 2.

Table 4

Prognostic impact of wall motion score index on adverse cardiovascular events (ACEs)

ACEs	B	SE	Wald	df	*p*-value
Unstable angina	4.02	1.05	14.68	1	0.000
AMI	6.09	2.07	8.68	1	0.003
PCI	9.49	1.61	34.89	1	0.000
In-stent restenosis	2.18	2.64	0.67	1	0.410
CABG	5.22	1.48	12.46	1	0.000
Heart failure	2.84	1.54	3.38	1	0.066
Death	8.57	2.52	11.57	1	0.001

B – coefficient for the constant; SE – standard error; df – degrees of freedom; AMI – acute myocardial infarction; PCI – percutaneous coronary intervention; CABG – coronary artery bypass grafting.

1.12. AUC was 93.7% (95% CI 85.9–97.9) ($p < 0.001$). These results indicate a high degree of accuracy of WMSI in identifying ACEs in women (Figure 3).

We examined the difference in WMSI values before the test and at the peak of the test and proved the prognostic significance of this variable. Using binary logistic regression, we proved that an increase in the WMSI value for each unit increased the possibility of the occurrence of ACEs except in ISR and HF (Table 4).

Discussion

The results of our study showed the great importance and usefulness of the application of the DSE in everyday work in diagnosing CAD. In our environment, the DSE is one of the most accessible and economical diagnostic methods.

A significantly higher number of positive tests were in men with clinical suspicion of coronary disease (46% compared to 25% in women). This finding is in line with expectations, considering that men have a significantly higher presence of risk factors for coronary disease. Clinical assessment of CAD has traditionally been more challenging in women compared to men due to lower disease prevalence, higher incidence of referral bias, and intrinsic performance of different stress testing modalities. DSE has been reported as an effective tool in the diagnosis and prognosis of CAD in women [20, 21]. In our work, false positive tests were registered in a very small number of female patients, as shown by Bach et al. [19]. After a positive DSE, coronary angiography findings in these patients were normal. In our study, men had a more frequent occurrence of ACEs compared to women in the observed period. They had UA, MI, ISR, ACB, death, and heart failure more often.

Our results indicate a higher sensitivity and specificity of DSE in men than in women. The sensitivity of DSE in men was 96%, the specificity was 93.4%, while in women, the sensitivity of DSE was 86.7%, and the specificity was 88.9%. The accuracy was 94% and 88,9% in men and women, respectively. The Echocardiography: Value and Accuracy at Rest and Stress (EVAREST) study demonstrated a sensitivity and specificity of the DSE for predicting cardiac outcomes of 95.4% and 96.0%, respectively, with an accuracy of 95.9%, which is consistent with our results. This study provides insight into the current use and accuracy of stress echocardiography in real-world practice in 31 UK-based hospitals. The results of this recent multicenter study provide confidence that stress echocardiography can be safely used

as a first-line test in the treatment of patients with stable chest pain [22].

There was no difference in survival between men and women in our study. The survival rate decreases with age. Sedlak et al. [23] proved a worse short-term prognosis for women with non-obstructive disease compared to men. The intermediate and long-term prognosis in subjects with obstructive coronary disease did not differ between genders.

In addition to the analysis of the diagnostic and prognostic significance of DSE, we analyzed and evaluated the prognostic value of WMSI in relation to gender. WMSI in DSE in our research showed high specificity and sensitivity in predicting ACEs. The sensitivity of WMSI in men was 96% and 93.3% in women, while the specificity was lower in men (82.4%) compared to women (90.6%). This analysis indicates that WMSI is effective in predicting unfavorable cardiovascular events in men. Savage et al. [24] proved that WMSI is a superior predictor of 12-month mortality over LVEF in ST-elevation MI patients treated with primary PCI. The discriminatory ability of WMSI (AUC: 0.77; 95% CI: 0.68–0.84) was significantly better than LVEF (AUC: 0.71; 95% CI: 0.61–0.79; $p = 0.034$).

Some more recent studies have investigated WMSI compared to other echocardiographic measurements, including global longitudinal strain (GLS), and have shown a good correlation with LVEF, with both WMSI and GLS scores showing superiority over LVEF in predicting ACEs and patient mortality with acute MI [25]. Wierzbowska-Drabik et al. [26] showed that in patients with coronary disease at the peak of DSE, WMSI was a better predictor of coronary SYNTAX and Gensini scores and severity of coronary disease than GLS or LVEF. However, in patients after MI, the correlation of GLS with the coronary score improved and approached the visual assessment.

Our results show that an increase in WMSI for each unit increases the possibility of unwanted cardiovascular events, which correlates with other studies. Playford et al. [27] confirmed the prognostic significance of the increase in WMSI values. In men, there was an increase in all-cause mortality from 38.9% to 49.8%, with an increase in WMSI values from > 1 to > 3. According to calculated WMSI, all-cause mortality rose in women from 38.5%, with a score > 1.0, to 49.5%, with a score ≥ 3.0. The follow-up period in this study was five years. Chuah et al. [28] pointed to the predictive significance of WMSI, abnormal LV end-systolic volume response, and percentage fixed wall motion abnormalities. In this examination difference between WMSI at rest and peak DSE was 0.14 ± 0.31 in patients with ACEs. In patients without ACEs, the difference was 0.05 ± 0.23.

Conclusion

The positive DSE was more common in men. The sensitivity of the DSE in men was 96%, and the specificity was 93.9%. In women, the sensitivity was 86.7%, and the specificity was 86.7%. ACEs were statistically significantly more frequent in men than women. Our study identified WMSI as a predictor of ACEs. In men, WMSI had a higher sensitivity in predicting ACEs, while in women, it showed a higher specificity.

Conflict of interest

The authors declare no conflict of interest associated with the publication of this article.

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Interplay between dental pulp tissue osteoprotegerin and TNF-α levels with micromorphological changes in the teeth of patients with chronic pulpitis

Interakcija između nivoa osteoprotegerina i TNF-α u dentalnoj pulpi sa mikromorfološkim promenama zuba kod pacijenata sa hroničnim pulpitisom

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Abstract

Background/Aim. Chronic pulpitis (CP) is an inflammatory dental pulp disorder associated with various pathophysiological mechanisms in its origin. The aim of the study was to evaluate the changes in the pulp tissue of osteoprotegerin (OPG) and tumor necrosis factor (TNF)-α and establish their relationship with the histological changes of pulp tissue, as well as with the micromorphological changes, occurring in the mineralized tissue. **Methods.** The study examined the dental pulp of 41 patients with CP and 12 healthy individuals. The group of the diseased subjects was subdivided based on the presence of communication of the pulp with the oral cavity, i.e., open (n = 22) or closed (*clausa*) (n = 19) CP. **Results.** The levels of TNF-α were statistically significantly increased, while OPG levels were decreased in the pulp of patients with CP, compared to the control group. TNF-α levels were almost the same in the pulp of patients with closed CP and the control group, while values were significantly increased in those with open CP compared to control. Histopathological analysis showed a significant increase in the number of mononuclear inflammatory cells in the diseased pulp. Scanning electron microscopy showed distinct changes, which correlate with internal resorption. **Conclusion.** The results indicate a much greater and intensified process of root resorption in patients with closed CP, which is unassociated with dental pulp OPG and TNF-α level changes.

Key words:
cytokines; dental pulp; histological techniques; microscopy, electron, scanning; osteoprotegerin; tumor necrosis factor-alpha.

Apstrakt

Uvod/Cilj. Hronični pulpitis (HP) je inflamacijski poremećaj zubne pulpe, u čijoj osnovi se nalaze različiti patofiziološki mehanizmi. Cilj rada bio je da se procene promene osteoprotegerina (OPG) i faktora nekroze tumora (*tumor necrosis factor*-TNF)-α u tkivu pulpe i ustanovi njihova povezanost sa histološkim promenama tkiva pulpe, kao i sa mikromorfološkim promenama koje se javljaju u mineralizovanom tkivu. **Metode.** Studijom je ispitana zubna pulpa 41 pacijenta sa HP i zubna pulpa 12 zdravih osoba. Grupa obolelih osoba podeljena je na osnovu prisustva komunikacije pulpe sa usnom dupljom, odnosno otvorenog (n = 22) ili zatvorenog (*clausa*) (n = 19) HP. **Rezultati.** Nivoi TNF-α bili su statistički značajno povišeni, dok je nivo OPG bio snižen u pulpi pacijenata sa HP, u poređenju sa kontrolnom grupom. Nivoi TNF-α bili su skoro jednaki u pulpi pacijenata sa zatvorenim HP i kontrolnoj grupi, a značajno povišeni kod pacijenata sa otvorenim HP u odnosu na kontrolnu grupu. Histopatološkom analizom uočeno je značajno povećanje broja mononuklearnih inflamacijskih ćelija u bolesnoj pulpi. Skenirajućom elektronskom mikroskopijom pokazane su jasne promene, koje su bile u korelaciji sa unutrašnjom resorpcijom. **Zaključak.** Rezultati ukazuju na znatno veći i intenzivirani proces resorpcije korena kod pacijenata sa zatvorenim HP, koji nije povezan sa promenama nivoa OPG i TNF-α u dentalnoj pulpi.

Ključne reči:
citokini; zub, pulpa; histološke tehnike; mikroskopija, elektronska, skenirajuća; osteoprotegerin; faktor nekroze tumora alfa.

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Introduction

Although teeth are located in a small area of the human body, the oral cavity, their unique structure and function, as well as their interaction with the rest of the body, make them a very complex structure to study. Teeth are comprised of outer mineralized tissues (dentin, enamel, cementum) that surround and protect the soft tissue within (dental pulp) [1]. The structure of the dental pulp, apart from the connective tissue, includes numerous cells, among which dental pulp stem cells have a vital role in dentine regeneration, multilineage differentiation, etc. [2]. The secretion of cytokines and chemokines by the cells in this milieu, as well as the influence of extra pulpal stimuli, are responsible for the dynamics of the processes occurring within the pulp. The cytokine level disbalance, especially during inflammatory stimulation, is responsible for the disturbances in both mineralized and soft tissue, accompanied by subjective symptoms. Dental pulp disorders can be caused by numerous factors influencing exposed pulpo-dentinal tissue and most frequently involve a progression of dental caries caused by different bacterial species [3].

Inflammatory and immune responses within the dental pulp are elicited in reaction to microorganisms, encompassing both anaerobic and aerobic bacterial species, along with their products that permeate dentinal tubules [1, 2]. The pathological process of internal root resorption leads to dentine loss and the potential invasion of the cementum due to activities originating within pulp [1]. Pulpitis, an inflammatory condition affecting dental pulp, is characterized by accumulating inflammatory cells and different mediators to specific regions [3]. Depending on the type of pulp changes and whether it occurs in an open or a closed pulp cavity, a distinction can be made between ulcerative and hyperplastic pulpitis. Chronic open pulpitis is characterized by vasodilation, infiltration of mononuclear inflammatory cells, and the occurrence of exudation, as well as cellular infiltration by neutrophil leukocytes [4]. Microscopic analysis of the tissue affected by pulpitis reveals granulation tissue comprised of new and immature capillary networks rich in inflammatory cells [5]. The infiltration of the pulp that occurs at the beginning of this process is comprised mainly of mononuclear cells (MNCs) such as lymphocytes and monocytes [1, 6]. We can make a difference between the two forms of chronic closed pulpitis – pulpitis *clausa* alternative *seu* parenchymatosa and granulomatosa interna. Closed pulpitis is identified by the infiltration of small round cells and concurrent varying degenerative changes. The pathohistological analysis of internal granuloma reveals the existence of granulation tissue surrounded with odontoclasts [5]. The inflamed area encompasses almost all types of leukocytes but mainly includes neutrophils and various forms of MNCs [7]. Cells in the infiltrate produce locally large amounts of inflammatory mediators, as well as other cytokines and chemokines [3]. The extent of inflammation can represent a diagnostic issue, which further influences the therapeutic option.

Although numerous studies reveal the mechanism underlying pulpitis, the exact nature of these biological processes is still unclear [3, 5, 7]. Modern-day laboratories, molecular biology and genetic-based, are equipped with sophisticated equipment adequate for quantitative and qualitative determination of cytokines in body fluids or tissue cell cultures obtained from different sources. Cytokines or biological response modulators include various proteins that affect inflammation, immunity, and hematopoiesis. Altered levels of cytokines have been found in inflamed pulp tissue (interleukins (IL), such as IL-8 and IL-2); however, their exact function is not completely understood [5]. The assessment of biomarkers can help improve the prediction or treatment of inflammatory pulp disease, and their investigation might thus prove to be of great value.

Osteoprotegerin (OPG) and receptor activator of nuclear factor κβ (RANK) ligand (RANKL), together with the associated RANK receptor, are proteins that share a great homology with tumor necrosis factor (TNF) receptor superfamily and are involved in the bone metabolism and osteoclastic cell function [5]. OPG is expressed by odontoblasts, ameloblasts, and dental pulp cells [8] and is involved in the maintenance of bone mineral matrix homeostasis [9]. Namely, cells expressing RANKL, such as osteoblasts, previously believed to be the only ones responsible for osteoclastic activity, and periodontal ligament fibroblasts could modulate and/or initiate root resorption [7]. During the process of root resorption induced by orthodontic forces, there is a significant shift in OPG and RANKL levels, as well as in the level of different cytokines in the pulp [5, 10]. The OPG/RANKL/RANK system could be referred to as a crucial connection in the interaction between bone, vascular, and immune cells, which is not fully understood [5]. These data suggest an association between cytokines and mineralized tissue destruction, potentially originating from the cells present in the dental pulp.

The balance that exists between pro- and anti-inflammatory cytokines predetermines the response of the body toward antigen stimulation in both acute and chronic inflammatory states [11]. The stimulation of inflammatory response causes the production of various cytokines, among which TNF-α plays an important role [12]. This cytokine induces an increase in RANKL levels in the cell, which further engages in the process of mineralized tissue destruction [5]. Moreover, TNF-α is known to be associated with the development of periodontal inflammation and the destruction of periodontal tissue [13]. These facts suggest that numerous stimuli are capable of inducing the destruction of mineralized tissue; however, their exact process remains unclear. The extent of pulp inflammation could be correlated with the progression of mineralized tissue destruction, and its estimation could help determine the course of treatment. However, up to now, there are no objective, quantitative, or clinically practical methods to assess process [4]. Likewise, there are no studies correlating changes in TNF-α levels with the inflammatory cells present in the pulp tissue or with changes in mineralized tissue ultrastructure.

The aim of the study was to determine dental pulp OPG and TNF-α level changes occurring in patients with chronic pulpitis (CP) (opened and closed) and compare the results with the ones obtained from healthy subjects. Furthermore, histopathological analysis (HPA) of the soft pulp tissue

obtained from the same patients would be performed in order to corroborate the biochemical findings. Additionally, this study encompassed the examination of the microstructural changes in the mineral tissue of teeth during the asymptomatic internal root resorption.

Methods

Study population

The study included a total of 53 patients from the Department of Dentistry. Before the commencement of the study, the study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Priština/Kosovska Mitrovica, Serbia (decision No. 05-83, from March 26, 2013). The study was conducted according to the standards given in the Declaration of Helsinki (revised in 2008). Each subject signed an informed consent form given by the lead researcher and was informed about the study details. Patients with CP (n = 41) were initially diagnosed based on the anamnestic data and auxiliary diagnostic methods (radiological findings). They were further subdivided into two groups based on the presence of communication of the pulp and the oral cavity, i.e., groups with open CP (n = 22) or closed (*clausa*) CP (n = 19). The pulp in the control group (n = 12) was obtained from the subjects with healthy teeth extirpated for prosthetic reasons.

Tissue homogenization and sample preparation

Dental pulp extraction was done under locoregional anesthesia with 2% lidocaine (lidocaine-chloride ampules, obtained from Hemofarm, Belgrade, Serbia) after the application of appropriate procedures to the teeth, gingiva, and mucous membrane in order to create an aseptic working environment (Elastic dental dam, Dental World, Italy). Pulp chamber trepanation was performed using a round dental burr while the cavity was prepared, and pulp extirpation was done with an instrument of appropriate size [3, 4]. Immediately after the extraction, healthy teeth were disinfected with 70% ethanol, and the enamel surface was crack-opened with a hammer. The pulpal tissue was removed from the exposed pulpal cavity using straight forceps. The extirpated content was randomly placed in a petri dish, and afterward, one-half of the mixed sample was placed in a sterile tube and snap-frozen (kept at -80 °C) prior to homogenization. The other randomly selected part of the tissue was immersed in a fixation medium for light microscopy. The process of homogenization [10% weight by volume (w/v)] was done using a Teflon® pestle in an ice-cold phosphate buffer (pH 7.4). The clear supernatant, used for determining OPG and TNF-α, was obtained by homogenate centrifugation at 4,000 × g for 15 min at 4 °C.

Determination of OPG and TNF-α concentrations

The obtained tissue homogenate supernatants were used for determining OPG and TNF-α concentrations in the pulp. The TNF-α enzyme-linked immunosorbent assay (ELISA) kit used in this study was obtained from R&D systems (Inc, Minneapolis, USA) with a standard curve range from 62.5 to 4,000 pg/mL. The OPG ELISA kit was obtained from R&D systems (Inc, Minneapolis, USA) with a standard curve range from 0.5 to 5.5 pg/mL. The obtained data are expressed as pg/mL.

Light microscopy tissue analysis

Extracted pulp tissue was fixated for 24 hr in a fixation medium [Zamboni fixative – phosphate buffered picric acid-formaldehyde fixative], after which the samples were washed with Millonig's buffer and then prefixed with 2% osmium-tetroxide. Dehydration was performed by a series of alcohol solutions of ascending concentrations (starting from 50% up to 100%), followed by propylene oxide. Prepared samples were left overnight in an epon resin mixture (Epon 820, DDSA, MNA, DMP30 as an accelerator) and were then molded and cut into semithin sections. Staining was performed using a basic fuchsin dissolved in ethanol and methylene blue dissolved in sodium tetraborate. Stained samples were examined under a polarization microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) equipped with a camera. Furthermore, the appearance of inflammatory cells and changes was examined on 5–10 randomly selected high-power fields (x40) [14], based solely on the shape of the nucleus, which enabled us to distinguish between polymorphonuclear cells and MNCs [15].

Scanning electron microscope analysis of the extracted teeth

In the case where internal root resorption of the teeth was in an advanced stage (n = 6), the teeth were extracted, washed, and left in sterile saline at 4 °C until the analysis. Furthermore, the teeth from the control group extracted due to prosthetic reasons were all analyzed by scanning electron microscopy (SEM). All samples were analyzed by a single examiner. Occlusal surfaces (2-3 mm thick) of the crown were cut circularly with the thinnest diamond fissure bur. The roots were cut with a separation disc longitudinally, thus enabling the separation of the root into the oral and vestibular half. Any superficial debris was removed by a subsequent wash in distilled water and dried with compressed air. Using separation pliers, the occlusal surfaces were separated and then separated longitudinally following the already prepared cuts. Each half was placed in a carrier and covered with gold under a vacuum. The examination on SEM was done using the JEOL-JSM-5300 microscope (JEOL, Tokyo, Japan).

Statistical analysis

The obtained data are given as mean values ± standard deviation, obtained from several different measurements. Initially, the differences in OPG and TNF-α concentrations between the two groups (control and experimental) were estimated using Student's *t*-test for two independent samples, while in the second case, when the experimental group was subdivided into two groups (open and closed CP), the differ-

ences between the three groups were compared using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test (GraphPad Prism, 8.0, San Diego, CA, USA). Probability values (p) equal to or less than 0.05 were taken to be statistically significant.

Results

The results of the present study revealed that OPG levels in healthy pulp tissue were statistically significantly higher ($p < 0.001$) than in the pulp of patients with CP. In the control group, pulpal tissue OPG values were around 50 pg/mL, while in the group of CP, values of OPG were around 10 pg/mL, i.e., some 5-fold decrease (Figure 1A). In the subsequent analysis of pulp OPG levels of the control subjects and those with closed CP and open CP, it was revealed that OPG was most significantly decreased in open CP patients, compared to both control and closed CP patients (Figure 1B).

TNF-α concentrations in the dental pulp tissue of the subjects with CP were statistically significantly ($p < 0.001$) higher than in the tissues obtained from the control group subjects. In the control group, pulpal tissue TNF-α values were below 5 pg/mL, while in the group of CP values of TNF-α were above 50 pg/mL, i.e., some 10-fold increase (Figure 2A). Analysis of the differences between TNF-α

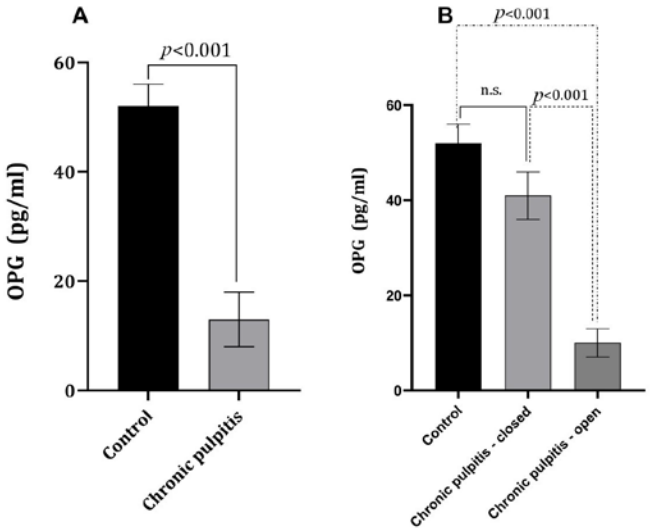

Fig. 1 – A) Comparison between the concentration of osteoprotegerin (OPG) in the dental pulp of subjects with chronic pulpitis (CP) and from the control group; B) OPG in the dental pulp of subjects with open CP, closed CP, and those from the control group. n.s. – no statistical difference; $p \leq 0.05$ was considered statistically significant [Student's *t*-test (A); ANOVA and Tukey's tests (B)].

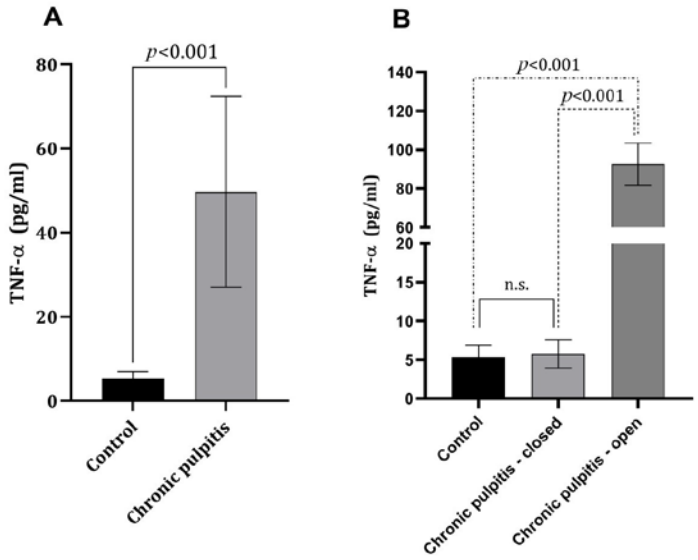

Fig. 2 – A) Comparison between the concentration of tumor necrosis factor (TNF)-α in the dental pulp of subjects with chronic pulpitis (CP) and from the control group; B) TNF-α in the dental pulp of subjects with open CP, closed CP, and those from the control group. n.s. – no statistical difference; $p \leq 0.05$ was considered statistically significant [Student's *t*-test (A); ANOVA and Tukey's tests (B)].

concentrations in the dental pulp of the control subjects and those with closed CP and open CP revealed a significant difference among the three groups ($p < 0.001$). Further analysis revealed that the levels of TNF-α in the dental pulp of the subjects with open CP were statistically significantly higher than the levels of TNF-α in the subjects belonging to either the control group or the group with closed CP. When the levels of TNF-α in the dental pulp of the subjects from the control group and the group with closed CP were compared, no statistically significant differences were found ($p > 0.05$). This means that the levels of TNF-α in the control group or the group with closed CP were around 5 pg/mL, while in the open CP, the levels were around 100 pg/mL (Figure 2B).

Analysis of the dental pulp obtained from the control group of subjects revealed a normal histoarchitecture with blood vessels, nerve fibers, and pulp cells (Figure 3A). Examination of the dental pulp with granulomatous formations that occurred during CP revealed the presence of massive necrotic fields, inflamed pulp tissue with scattered inflammatory cells, poor collagen fiber deposition, and blood vessels (sinusoids) filled with red blood cells, as well as a mass of bacteria and occasional multinuclear cell, odontoclast (Figures 3B and 3C). The number of inflammatory cells in the control group and the group of patients with closed CP was almost identical. On the other hand, the number of cells in the group of subjects with opened CP was significantly higher than in the other two groups (Figure 3D).

SEM of the teeth obtained from the subjects with healthy teeth extirpated due to prosthetic reasons showed an undamaged enamel surface, while the pulpo-dentine wall appeared regular with evenly distributed dentine canals (Figures 4A and 4B). The teeth obtained from the subjects with internal resorption revealed cracks in the enamel on the occlusal surface. The examined pulpo-dentine wall is characterized by both normal and irregular dentine structures (Figure 4C). Irregular canal distribution (Figure 4D), Howship's lacunae, and activated odontoclasts, which possess filamentous parts (filopodia) spread to the periphery (Figures 4E and 4F), sometimes up to the edge of the cracks and firmly attached to the dentine surface are seen during the SEM analysis.

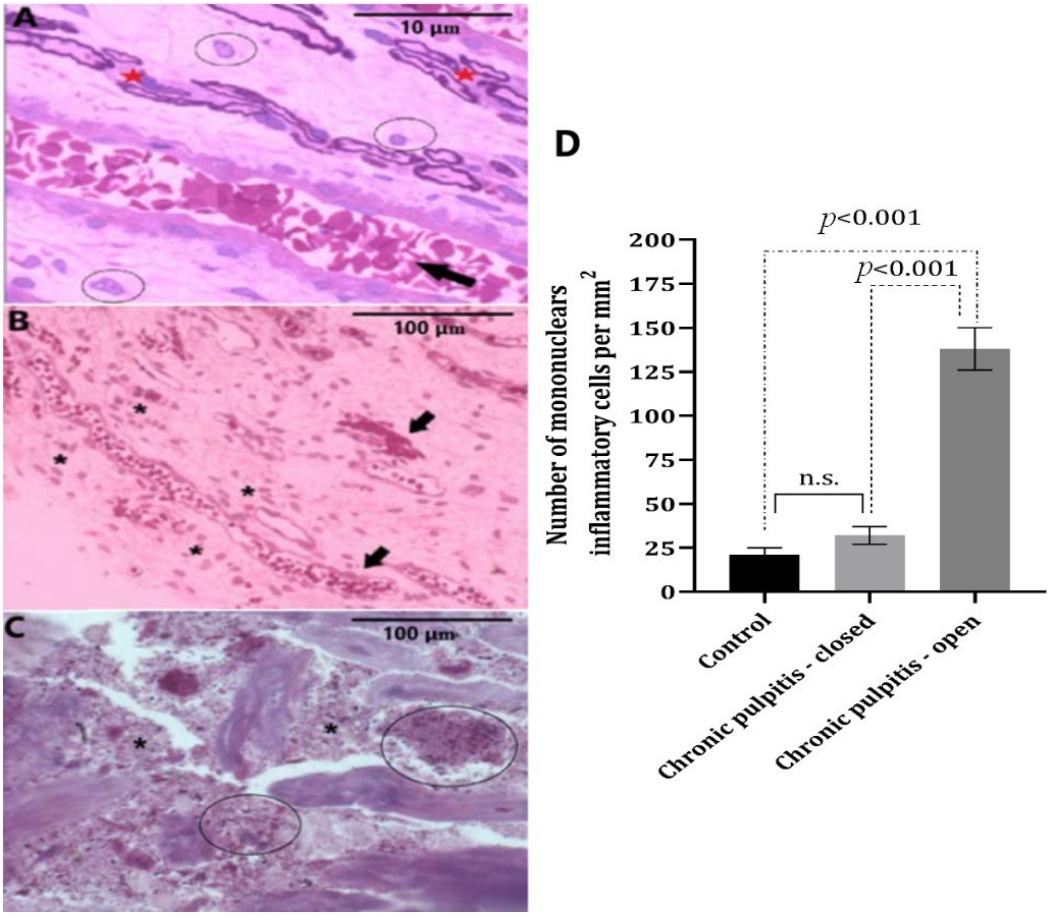

Fig. 3 – A) Histopathological appearance of the healthy dental pulp and dental pulp from patients with chronic pulpitis, normal dental pulp with red blood cells (arrow), nerve fibers (star), and dental pulp cells (circled) (×40; Fuscin staining); B) Dental pulp with hyperemia (arrow) and numerous inflammatory cells (asterisk) (×20; Fuscin staining); C) Dental pulp with necrotic field (asterisk) and bacterial infiltration (circled) (×20; Fuscin staining); D) Number of inflammatory cells in the dental pulp of subjects with chronic open pulpitis, chronic closed pulpitis, and those from the control group.
n.s. – no statistical difference; $p \leq 0.05$ was considered statistically significant (ANOVA and Tukey's tests).

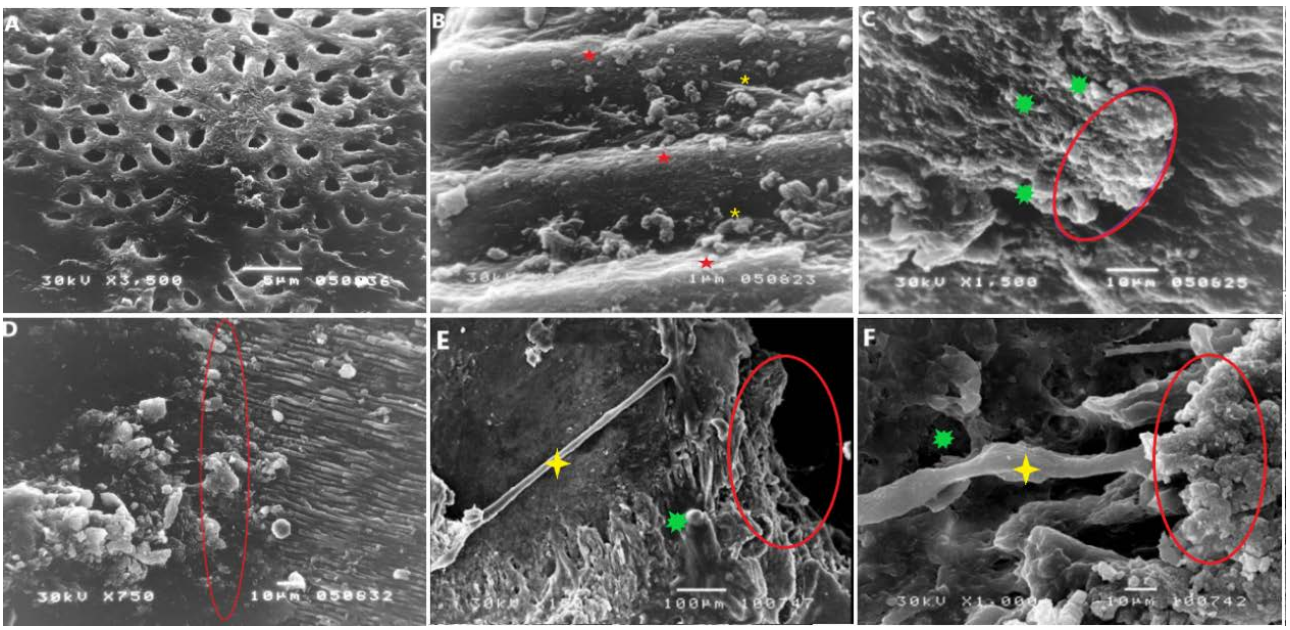

Fig. 4 – Images of healthy teeth (A and B) and teeth from patients with internal resorption (C–F) obtained by scanning electron microscopic analysis: A) regular dentine with transversely cut dental tubules and clear intertubular and peritubular dentine; B) longitudinal cut through a healthy tooth, peritubular (red star) and intratubular (yellow asterisk) dentine; C) irregular dentine structure (circled) with lacunes (multipoint star); D) the border between regular and irregular dentine (circled); E) filamentous part (four point star) of the odontoclast spreading to the surface of dentine (circled) and with formed lacunes (multipoint star); F) odontoclast filopodia (four point star) firmly attached to the damaged dentine surface (circled) with some surrounding lacunes (multipoint star).

Discussion

The destruction of the root *via* the process of internal resorption is a form of a chronic asymptomatic inflammatory disorder discovered by accident during radiography. It could be recognized as a radiolucency along the dentine surface. This inflammatory process is believed to be exclusively associated with the activity of osteoclasts. However, up-to-date studies have pointed out the role of different cells and cytokines during root resorption [4, 7]. Apart from osteoclast, fibroblasts are known to play an important role in this process, and it was proven that pulp tissue fibroblasts obtained from teeth of patients affected with root resorption in *in vitro* conditions produce significant amounts of inflammatory cytokines, e.g., TNF-α, when stimulated by the release of substance P [16]. Moreover, inflammatory cells (polymorphonuclear and mononuclear) invading dental pulp and some resident cells (fibroblasts and macrophages) could initiate and further promote internal root resorption [17]. The significant production of this cytokine is observed in patients with symptomatic pulpitis rather than in patients without symptoms [18]. This seems to be in accordance with a significantly increased number of inflammatory MNCs in the pulpal tissue of these patients (Figure 3D). Previous reports suggest that the cells comprising the inflammatory infiltrate are mainly macrophages and B cells, followed by different subsets of T cells to a much lesser extent [14]. The herein obtained data suggest that there is an increase of TNF-α in the pulp tissue of patients with CP of different origin, which could be produced by different cells observed to infiltrate the dental pulp, as well as by the resident ones. In the case of closed CP, the upper dental pulp tissue is altered and is mainly comprised of granulation tissue [19]. The removal of the dental pulp (pulpectomy), i.e., the removal of the granulation tissue and the blood supply to the process, represents one of the standard approaches in the treatment of internal root resorption [20], which was performed in patients where this process was not in an advanced stage.

The damage on the occlusal surfaces almost specifically stems from the traumatic occlusion, which further enables the invasion of the pulp and dentine with bacteria, all leading to internal resorption [3]. In some cases, during the analysis of the extracted teeth, enamel cracks on the occlusion surface were noted in the teeth analyzed by SEM. HPA of the corresponding dental pulp tissue revealed a mass of invading bacteria and inflammatory cells, mainly consisting of MNCs. These MNCs are most probably resident macrophages and/or attracted monocytes, now transformed into macrophages or belong to lymphocytes. Bacteria causing pulpitis, the ones believed to be initial invaders of the pulp, come from *Lactobacillus, Prevotella, Pseudoramibacter, Olsenella, Streptococcus,* and *Stenotrophomonas genera* [21]. Some studies suggest that without bacterial infection, the process of internal resorption would be self-limiting since the bacteria are the ones enhancing the process [22]. One of the first cells that recognize bacterial products is odontoblasts, through pattern recognition receptor toll-like receptor (TLR)-2, causing a further immune response involving cell activation, proliferation, and cytokine secretion [23]. The association between TNF-α and TLR-2 further supports the role

of TNF-α signaling in alveolar bone loss after infection with *Porphyromonas gingivalis* [24]. One of the major cell populations producing TNF-α, as well as IL-1β, is macrophages [25], the most numerous cells detected in patients with opened CP.

During the process of chronic inflammation, a cytokines could affect the dynamics of both mineral and soft tissue through the activation or inhibition of the effector cells. Namely, OPG is responsible for maintaining the symbiosis between bone resorption formation [9]. The role of OPG is the inhibition of RANK-RANKL interactions and their binding to nuclear factor (NF)-kB, thus it is essential in suppressing osteoclastogenesis and bone resorption [26]. We found that OPG levels in the healthy pulp were significantly higher than in the pulp of patients with pulpitis suggesting that the teeth bone homeostasis is maintained. This is clearly an indicator of decreased teeth mineral matrix observed both during the initial teeth examination (radiolucency) and during SEM. In some animal studies, an antibody mimicking OPG action (denosumab) was found to prevent rat root resorption when applied locally [27], further confirming the role of this molecule in root resorption.

Secreted TNF-α could also activate osteoclastic resorption [28], and, together with IL-1 lead to the activation and differentiation of osteoclasts, and induction of prostaglandin (e.g. PGE2) production from fibroblasts and osteoblasts [29]. In the present study, levels of TNF-α in the dental pulp of healthy teeth and in those with closed CP were significantly lower than the levels in the subjects with open CP, which suggests that the invasion of bacteria provoked the processes of TNF-α production. Furthermore, the produced TNF-α could originate from different inflammatory (macrophages) and residual (fibroblasts) cells, stimulated by bacterial antigens, which were both detected in the soft pulpal tissue. Apart from TNF-α, previous studies on inflamed dental pulp showed that inflammatory cells and stimulated pulpal fibroblasts strongly express and secrete IL-1β and IL-8 [30], which agrees with an increase of TNF-α and IL-1β in the same patients reported on previous occasions [4]. In addition, as mentioned, the greatest number of cells of inflammatory infiltrate is macrophages [14] and this overlaps with the findings of the present study. Interestingly, contemporary studies showed that the presence of bacterial endotoxins provokes less TNF-α production than the protein derived from dental pulp cells [31].

In this study, in the teeth analyzed using SEM and obtained mainly from the subjects with closed CP, where the extraction was indicated and performed, the resorption and evident dentine damage were always present. The active osteoclastic cells are evident in the teeth analyzed under SEM, where these cells spread with numerous filamentous parts all the way to the periphery. These findings are not fully in agreement with the increase in pulpal tissue OPG and TNF-α levels nor with the potential of these molecules to induce osteoclastic resorption as suggested elsewhere [9, 28], even in very low concentrations in the case of TNF-α [32]. Previous findings revealed an increase in pulpal tissue IL-1β [13], which should have caused, together with a local increase in TNF-α, the differentiation of osteoclasts [29]. These findings might be potentially explained by the different, non-inflammation-related, closed CP, found after certain teeth trauma or its exposure to heat [33].

The present study has a few shortcomings and weaknesses that include only descriptive SEM analysis and some morphometric analysis of the mineralized tissue ultrastructure, which might reveal additional characteristics of internal resorption that should be further performed. Moreover, further analysis of the soft tissue samples using some immunohistochemical analysis (specific molecules associated with different cell types) should reveal which cells might be the source of the cytokine production. Finally, a broader panel of cytokines, pro- and anti-inflammatory ones, could be analyzed to better understand their interactions and impact on soft and mineralized tissue function. We should also emphasize the strength of this study which lies in the multidisciplinary, biochemical, pathological, and electron microscopic approach to studying changes in the teeth under chronic inflammation.

Conclusion

The results of the present study revealed that the dental pulp tissue obtained from the subjects with open CP has significantly higher levels of TNF-α than the one obtained from the control group or the group of subjects with closed CP. Furthermore, OPG levels were found to be statistically significantly higher in the healthy dental pulp than in the one obtained from the patients with CP. The results point to the fact that OPG level changes reversely follow the changes in TNF-α levels within the dental pulp tissue. The detailed HPA of the inflamed dental pulp and the SEM, micromorphological investigation of the extracted teeth further confirmed and partially overlapped with the biochemical findings. These data suggest a much greater and intensified process of root resorption in the here studied patients with closed CP, which is based on the results unassociated with the OPG and TNF-α concentrations.

Conflict of interest

The authors declare no conflict of interest.

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Multimorbidity in the working-age population of Serbia: results from the 2019 National Health Survey

Multimorbiditet radnoaktivnog stanovništva Srbije: rezultati nacionalnog istraživanja zdravlja iz 2019. godine

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Abstract

Background/Aim. Population aging and the increase in the prevalence of chronic diseases led to a rise in the number of people who live with more than one disease. The aim of the study was to determine the prevalence and predictors of multimorbidity in the working-age population (WAP) of Serbia. **Methods.** The study is part of "The 2019 Serbian National Health Survey", a cross-sectional study conducted on a representative stratified two-stage sample. For this paper, a representative data sample for 9,473 persons of the WAP (aged 15–64 years) was used. Multimorbidity was defined as the co-occurrence of two or more of 13 chronic conditions. Data on chronic conditions were self-reported, and data on body mass and body height were measured. Multivariable logistic regression was used to assess predictors of multimorbidity. **Results.** Multimorbidity prevalence among WAP was 12.0%, and it was significantly higher among women (13.3%) than in men (10.6%). The predictors of multimorbidity were: female gender, increasing age, lower level of education, lower income, unemployment, retirement, widowhood, and divorce. Being overweight and obese were associated with higher odds of multimorbidity in both men and women. **Conclusion.** Multimorbidity is an important public health problem amongst WAP in Serbia due to its high prevalence, especially among vulnerable groups, and its inequality in frequency among different socioeconomic groups.

Key words:
multimorbidity; occupational groups; prevalence; risk factors; serbia; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Starenje stanovništva i porast prevalencije hroničnih bolesti doveli su do porasta broja ljudi koji boluju od dve ili više bolesti. Cilj rada bio je da se utvrde prevalencija i prediktori multimorbiditeta kod radnoaktivnog stanovništva (RAS) u Srbiji. **Metode.** Ovi rezultati su deo studije „Istraživanje zdravlja stanovništva Srbije 2019. godine", sprovedene kao studija preseka na reprezentativnom stratifikovanom dvostepenom uzorku. Za potrebe ovog rada korišćeni su podaci 9 473 osoba iz populacije RAS (životnog doba 15–64 godina). Multimorbiditet je definisan kao prisustvo dve ili više hroničnih bolesti od ukupno 13 ispitivanih bolesti. Podaci o hroničnim bolestima dobijeni su samoizjašnjavanjem, a podaci o telesnoj masi i telesnoj visini dobijeni su merenjem ispitanika. Za procenu prediktora multimorbiditeta korišćena je multivarijabilna logistička regresija. **Rezultati.** Prevalencija multimorbiditeta kod RAS iznosila je 12,0% i bila je značajno viša kod žena (13,3%) nego kod muškaraca (10,6%). Prediktori multimorbiditeta bili su: ženski pol, starije životno doba, niži nivoi obrazovanja, niži prihodi, nezaposlenost, penzionisanost, udovištvo i status razveden(a). Predgojaznost i gojaznost bili su povezani sa višim šansama za multimorbiditet i kod muškaraca i kod žena. **Zaključak.** Multimorbiditet je značajan javnozdravstveni problem kod RAS Srbije zbog njegove visoke prevalencije, posebno među ranjivim kategorijama stanovništva i zbog nejednake zastupljenosti među različitim socio-ekonomskim grupama.

Ključne reči:
bolesti, interakcije; radnici; prevalenca; faktori rizika; srbija; ankete i upitnici.

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Introduction

Multimorbidity can be defined as "the co-existence of two or more chronic conditions (CCs) in the same individual" [1]. Population aging and the increase in the prevalence of chronic diseases (CDs) led to a rise in the number of people with more than one disease. The prevalence of multimorbidity varies from study to study, depending mainly on the study population and the number of CDs considered. One of the recent systematic reviews that included 49 countries showed that the overall prevalence of multimorbidity at a global level is 33.1% [2]. The prevalence of multimorbidity does increase with age, but it does not exclusively affect older populations, and many studies report high rates of multimorbidity among working-age populations (WAPs) [3, 4]. CDs are prevalent in Republic of Serbia (RS) and pose an important public health problem. In 2019, almost every second person in RS aged 15 and above stated that they had at least one of the 17 CDs/conditions. The most frequent CDs/conditions were arterial hypertension (AH) (29.6%), chronic low back disorder (17.2%), chronic neck disorder (12.1%), hyperlipidemias (10.8%), coronary heart disease (CHD)/angina pectoris (8.9%), and diabetes mellitus (DM) (7.8%) [5]. Results from the previous 2013 National Health Survey in RS revealed that the prevalence of multimorbidity among adults was 26.9% [6]. The Serbian population, with an average age of 43.5 years and almost a fifth of the population (21.3%) aged 65 and above, is one of the oldest populations in Europe, which makes age-related health issues more challenging [5]. Due to population aging and the increase in the prevalence of CDs (DM, chronic heart disease, stroke, and malignant diseases) [7, 8], it is expected that the public health importance of multimorbidity will continue to increase.

Even though the recent coronavirus disease 2019 (COVID-19) pandemic brought acute diseases back to the spotlight, it did not diminish the public health importance of multimorbidity. Since people with CDs have a higher risk of infection, they are thus more prone to suffering from some consequences of a disease like a more severe clinical picture and worse outcomes of COVID-19 [9, 10].

It is well known that CDs reduce the quality of life (QoL); hence, an increasing number of diseases significantly reduces the QoL. Physical health is affected more than mental health, and younger populations and females are burdened more [11].

Multimorbidity is a major challenge not only for patients but also for healthcare workers and the healthcare systems. People affected by multiple CDs have more complex needs; they are the most challenging patients to manage and also have a higher probability of poor outcomes. All this leads to using healthcare more frequently and is associated with higher healthcare costs [12, 13].

Not only is multimorbidity more prevalent in persons of lower socioeconomic (SE) status, but also the associated costs of long-term care result in greater health expenditure for these patients and their households, pushing them deeper into poverty and increasing health inequalities [12, 14–16].

Multimorbidity increases sickness absence, primarily due to musculoskeletal, cardiovascular, and mental illnesses [17, 18]. CDs decrease work productivity by limiting both the physical and psychosocial ability to perform specific work demands. With the increase in the number of CCs, the odds of having a work limitation increase significantly [18].

One of the facts that highlights the public health importance of multimorbidity is that it is associated with higher mortality. Jani et al. [19] found that all-cause mortality risk for individuals with four or more long-term conditions was nearly three times higher than those with no long-term condition.

The prevalence of multimorbidity increases with age, and most studies on multimorbidity have predominantly focused on older populations. However, in absolute numbers, the majority of patients with multimorbidity are younger than 65 [3]. A better understanding of the epidemiology of multimorbidity in WAP is necessary for the development of interventions to reduce the burden of multimorbidity, especially in the context of an aging population, such as the population of RS. The aim of the study was to estimate the prevalence and predictors of multimorbidity in WAP of RS.

Methods

"The 2019 Serbian National Health Survey" was conducted in line with the ethical standards of the Declaration of Helsinki and the legislation of the RS. Participants were given a written document with the necessary study information and they signed informed consent before participating in the study. In order to keep the anonymity of the participants in the study, data that could identify the participant were not collected (the necessary identification that was replaced by code) [5]. The ethical aspects of the study were approved by the Ethics Committee of the Institute of Public Health of Vojvodina, RS (approval No. 01-969/1).

"The 2019 Serbian National Health Survey" is a cross-sectional study conducted by the Ministry of Health of RS, the Statistical Office of RS, and the Institute of Public Health of Serbia "Dr. Milan Jovanovic Batut". It was conducted in 2019 on a representative stratified two-stage sample and included 5,114 households with a response rate of 80.7%. Sample stratification was done according to the area type (urban, other) and region (Vojvodina, Belgrade, Southern and Eastern Serbia, and Šumadija and Western Serbia). The survey included people living in private households. The survey did not include population groups in collective households (student dormitories, dormitories for children and young people with disabilities, homes for socially endangered children, retirement homes, homes for the elderly and infirm, homes for adults with disabilities, convents, monasteries, etc.) [5]. For this paper, a representative sample of WAP (aged 15–64) of the RS was used. The analysis included 9,473 persons aged 15–64 years.

The instruments were questionnaires designed in line with the European Health Interview Survey questionnaire. The interview was conducted by teams comprised of two

members; one of the two members was a health professional. For this paper, data from two questionnaires were used. The first questionnaire was a household info panel used to collect information about all members of the household, i.e., SE characteristics of the household itself. This questionnaire had 18 questions, and we used the question about household income for this manuscript. The second questionnaire had 118 questions about background variables on demography and SE status (gender, age, education, employment status), health status (self-perceived health), CCs, limitation in everyday activities, disease-specific morbidity, physical and sensory functional limitations, healthcare use, unmet healthcare needs, use of medicines, preventive actions, health determinants, such as height and weight, fruit and vegetable consumption, etc. Data were collected by "face-to-face" interviews. The following data were used from this questionnaire for analysis: gender, age, level of education, employment status, marital status, region of residence, and data on CDs.

The outcome variable was multimorbidity, and it was defined as a co-occurrence of two or more CCs. Information on CCs was ascertained based on responses to the question: "Have you had any of the following diseases or conditions in the previous 12 months?". A total of 13 CCs were selected for analysis (chronic bronchitis/chronic obstructive pulmonary diseases/emphysema, bronchial asthma, AH, myocardial infarction (MI)/consequences of infarction, stroke/consequences of stroke, coronary disease/angina pectoris, DM, arthrosis/degenerative joint disease (excluding arthritis), liver cirrhosis, allergies, depression, kidney problems, malignant disease). The presence of CDs was dichotomized into having or not having CDs, and variables were coded as dummy variables (yes/no).

Information on independent variables used in the analyses was obtained using the questionnaire, except for nutritional status (NS). NS was assessed using the body mass index (BMI), which was calculated based on the measured weight and height and was categorized as follows: obesity (BMI $\geq$ 30 kg/m^2), overweight (BMI 25.0–29.9 kg/m^2), normal weight (18.5–24.9 kg/m^2) and underweight (BMI < 18.5 kg/m^2). BMI data was available for 80.7% of participants.

The following independent variables were analyzed: Age was categorized into five 10-year age groups (15–24, 25–34, 35–44, 45–54, 55–64). Marital status was categorized as married (or in a relationship), never-married, widowed, and divorced. SE status was measured using the highest education attainment, employment status, and household income. The level of education was categorized into three educational groups: high (postsecondary diploma or university), intermediate (secondary school graduation), and low education (elementary school or lower). Employment status was categorized into four groups: employed, unemployed, in retirement/unable to work, and other inactive (students, housewives, other answer). Data on income were collected at the household level and disseminated by quintile of the income distribution (first, second, third, fourth, and fifth quintile groups). The first and second quintile groups were merged into a low-income group (most disadvantaged), and the fourth and fifth quintile groups were merged into a high-income group (most advantaged). All independent variables were analyzed as categorical variables.

Descriptive statistics were used to describe sample characteristics [means, standard deviations (SD), and proportions]. For testing the differences in variables between population groups with and without multimorbidity, independent *t*-tests and Chi-square tests were performed depending on the type of variables. Multivariable logistic regression was used to model the odds of multimorbidity (enter method). Separate logistic regression analyses were done for men and women. The dependent variable was the co-occurrence of two or more diseases, and the comparison group for analysis was one or no CDs. All logistic regression analyses were adjusted for the following independent variables: age, marital status, level of education, income, region, and NS. The fully adjusted logistic regression model for the whole sample was additionally adjusted for gender. All independent variables were significantly associated with the outcome variable (multimorbidity). That is why they were included in the final model. The odds of multimorbidity were presented as an adjusted odds ratio (OR) with a corresponding 95% confidence interval (CI). All the analyses used weighting to be representative of the population of RS. Statistical Package for the Social Sciences (SPSS) version 23 was used for statistical analyses.

Results

The study population included 9,473 individuals with a mean age of 40.81 years (± SD 14.06), and 50.0% were females. More than half of the participants had an intermediate level of education (59.8%). Every eighth person of working age had multimorbidity (12.0%). Multimorbidity was more prevalent in women than men (13.3% vs. 10.6%). More people had overweight or obesity (53.9%) than normal weight (43.5%). Other demographic and SE characteristics are presented in Table 1. The average age of women was higher than that of men ($p < 0.001$). Among women, there were more individuals with a high level of education than among men ($p < 0.001$). Men had a higher proportion of employed individuals than women ($p < 0.001$). Overweight and obesity were more prevalent among men than women ($p < 0.001$).

Table 2 shows the prevalence of multimorbidity according to gender. The most prevalent single condition amongst males and females was AH. Almost every fifth person of working age had AH, and it was more prevalent among women (19.5%) than among men (17.9%). After AH, the most prevalent conditions in men and women were allergies, DM, and CHD/angina pectoris. The prevalence of CDs was higher in women than in men, except DM, MI/consequences of MI, stroke/consequences of stroke, and liver cirrhosis, which were more prevalent amongst men.

Table 1

Sample characteristics by gender, Serbia 2019

Parameter	All	Men	Women	*p*-value*
Age (years)				
15–24	1,519 (16.1)	793 (16.6)	726 (15.7)	< 0.001
25–34	1,629 (19.5)	866 (20.0)	763 (19.1)	
35–44	1,949 (21.9)	991 (22.2)	958 (21.6)	
45–54	1,989 (20.7)	991 (20.5)	998 (20.9)	
55–64	2,387 (21.7)	1,126 (20.7)	1,261 (22.8)	
Level of education				
high	1,888 (22.3)	860 (20.2)	1,028 (24.3)	< 0.001
intermediate	5,776 (59.8)	3,065 (63.2)	2,711 (56.4)	
low	1,803 (17.9)	836 (16.6)	967 (19.3)	
Employment status				
employed	4,758 (52.6)	2,675 (57.9)	2,083 (47.2)	< 0.001
unemployed	2,274 (23.5)	1,180 (24.3)	1,094 (22.8)	
retired	826 (7.8)	327 (5.9)	499 (9.6)	
other inactive	1,590 (16.1)	575 (11.9)	1,051 (20.4)	
Marital status				
married	5,714 (59.5)	2,693 (56.8)	3,021 (62.3)	< 0.001
never married	2,886 (30.9)	1,768 (37.0)	1,118 (24.9)	
widowed	325 (3.6)	58 (1.2)	267 (5.9)	
divorced	527 (6.0)	238 (5.0)	289 (6.9)	
Income				
high	3,756 (42.1)	1,880 (41.7)	1,885 (42.4)	< 0.001
intermediate	1,839 (18.9)	914 (18.7)	925 (19.2)	
low	3,869 (39.0)	1,973 (39.6)	1,896 (38.4)	
Region of Serbia				
Belgrade	2,207 (24.5)	1,059 (23.6)	1,148 (25.4)	< 0.001
Vojvodina	2,104 (27.0)	1,060 (27.3)	1,044 (26.8)	
Šumadija and Western	3,127 (27.2)	1,617 (27.5)	1,510 (27.0)	
Southern and Eastern	2,034 (21.2)	1,031 (21.7)	1,004 (20.8)	
Nutritional status				
normal weight	3,255 (43.5)	1,297 (34.1)	1,958 (52.6)	< 0.001
underweight	203 (2.6)	52 (1.3)	151 (3.8)	
overweight	2,711 (35.4)	1,619 (43.5)	1,092 (27.6)	
obesity	1,478 (18.5)	817 (21.1)	661 (15.9)	
Number of chronic diseases				
none or one	8,222 (88.0)	4,210 (89.4)	4,012 (86.7)	< 0.001
two or more	1,223 (12.0)	542 (10.6)	681 (13.3)	
total	9,473 (100.0)	4,767 (50.0)	4,706 (50.0)	

All values are expressed as unweighted numbers of participants (weighted percentages).
*** Chi-square for testing differences between men and women.**

The prevalence of multimorbidity increased substantially with age in both men and women. According to age and gender, the prevalence of multimorbidity reached the highest value (32.1%) in women aged 55–64 years. Multimorbidity was significantly more prevalent in the population with a lower educational level. Gender differences in multimorbidity prevalence were especially pronounced between men (14.5%) and women (22.4%) with low levels of education. There were also significant differences in prevalences of multimorbidity in the four regions in RS. Every third obese woman (34.1%) and every fifth obese man (19.5%) had two or more CDs. Other values for the prevalence of multimorbidity for the examined population in 2019 are shown in Table 3.

Table 4 presents ORs for multimorbidity from the multivariable logistic regression. The logistic regression models used as the outcome variable two or more CCs vs. no or one CC. In a fully adjusted logistic regression model being female was associated with 14% higher odds of multimorbidity (OR = 1.14; 95% CI: 1.13–1.15). The association between age and multimorbidity was positive in both men and women. The age gradient in multimorbidity was more pronounced in women than in men. Women aged 55–64 years had almost 19 times higher odds of multimorbidity compared to women aged 15–24 years (OR = 18.88; 95% CI: 18.12–19.68). The population with a lower level of education had higher odds of multimorbidity. Never-married men and women had lesser odds of multimorbidity compared to married men and women. Divorced and widowed persons had higher odds of multimorbidity compared to married ones. Men with low income had 5% higher odds of multimorbidity (OR = 1.05; 95% CI:

Table 2

Prevalence and 95% confidence interval (CI) of chronic diseases in the working-age population according to gender

Chronic disease	All		Men		Women		*p*-value*
	n**	% (CI)***	n**	% (CI)***	n**	% (CI)***	
Arterial hypertension	1,915	18.7 (18.67–18.74)	911	17.9 (17.84–17.94)	1,004	19.5 (19.46–19.57)	< 0.001
Allergies	626	6.9 (6.89–6.94)	252	5.7 (5.67–5.73)	374	8.2 (8.10–8.17)	< 0.001
Diabetes mellitus	447	4.4 (4.34–4.38)	229	4.6 (4.53–4.58)	218	4.2 (4.14–4.19)	< 0.001
CHD/angina pectoris	408	3.8 (3.82–3.85)	181	3.1 (3.13–3.17)	227	4.5 (4.49–4.54)	< 0.001
Arthrosis/degenerative joint disease	369	3.4 (3.31–3.34)	118	2.2 (2.21–2.25)	251	4.5 (4.39–4.44)	< 0.001
Depression	338	3.2 (3.19–3.22)	129	2.5 (2.51–2.55)	209	3.9 (3.86–3.91)	< 0.001
Bronchial asthma	265	2.8 (2.75–2.78)	121	2.5 (2.52–2.57)	144	3.0 (2.96–3.00)	< 0.001
Kidney disease	257	2.6 (2.55–2.58)	94	1.8 (1.77–1.81)	163	3.3 (3.31–3.36)	< 0.001
Chronic bronchitis/ COPD/emphysema	230	2.4 (2.33–2.36)	100	2.0 (1.97–2.00)	130	2.7 (2.68–2.72)	< 0.001
Malignant disease	117	1.2 (1.15–1.17)	35	0.8 (0.78–0.80)	82	1.5 (1.50–1.54)	< 0.001
MI/consequences of MI	90	0.8 (0.80–0.82)	69	1.2 (1.19–1.22)	21	0.4 (0.41–0.43)	< 0.001
Stroke/consequences of stroke	55	0.5 (0.49–0.50)	37	0.7 (0.65–0.67)	18	0.3 (0.33–0.34)	< 0.001
Liver cirrhosis	24	0.20 (0.19–0.20)	14	0.23 (0.22–0.23)	10	0.17 (0.16–0.17)	< 0.001

CHD – coronary heart disease; COPD – chronic obstructive pulmonary disease; MI myocardial infarction.

***Chi-square test for testing differences between men and women; **Unweighted number of participants; ***Weighted percentage.**

Table 3

Prevalence of multimorbidity according to demographic and socioeconomic characteristics and nutritional status

Parameter	All			Men			Women		
	chronic diseases		*p*-value*	chronic diseases		*p*-value*	chronic diseases		*p*-value*
	one or no	two or more		one or no	two or more		one or no	two or more	
Age (years)									
15–24	1,490 (98.2)	28 (1.8)		775 (97.8)	17 (2.2)		715 (98.6)	11 (1.4)	
25–34	1,580 (96.5)	48 (3.5)		841 (97.0)	24 (3.0)		739 (96.0)	24 (4.0)	
35–44	1,822 (93.3)	121 (6.7)	< 0.001	922 (92.8)	65 (7.2)	< 0.001	900 (93.8)	56 (6.2)	< 0.001
45–54	1,674 (84.7)	307 (15.3)		858 (87.3)	129 (12.7)		816 (82.3)	178 (17.7)	
55–64	1,656 (70.6)	719 (29.4)		814 (73.6)	307 (26.4)		842 (67.9)	412 (32.1)	
Level of education									
high	1,737 (92.2)	151 (7.8)		789 (92.4)	71 (7.6)		948 (92.1)	80 (7.9)	
intermediate	5,049 (88.5)	711 (11.5)	< 0.001	2,712 (89.4)	346 (10.6)	< 0.001	2,337 (87.4)	365 (12.6)	< 0.001
low	1,432 (81.2)	361 (18.8)		705 (85.5)	125 (14.5)		727 (77.6)	236 (22.4)	
Employment status									
employed	4,317 (91.3)	431 (8.7)		2,425 (91.3)	245 (8.7)		1,892 (91.2)	186 (8.8)	
unemployed	1,973 (88.1)	293 (11.9)	< 0.001	1,031 (88.2)	143 (11.8)	< 0.001	942 (88.0)	150 (12.0)	< 0.001
retired	482 (59.6)	341 (40.4)		198 (62.6)	127 (37.4)		284 (57.7)	214 (42.3)	
other inactive	1,426 (90.8)	157 (9.2)		546 (95.2)	27 (4.8)		880 (88.3)	130 (11.7)	
Marital status									
married	4,799 (86.0)	899 (14.0)		2,278 (86.3)	408 (13.7)		2,521 (85.7)	491 (14.3)	
never married	2,778 (96.5)	101 (3.5)	< 0.001	1,695 (96.1)	68 (3.9)	< 0.001	1,083 (97.0)	33 (3.0)	< 0.001
widowed	204 (62.7)	118 (37.3)		34 (60.6)	23 (39.4)		170 (63.1)	95 (36.9)	
divorced	421 (79.3)	104 (20.7)		194 (81.2)	42 (18.8)		227 (77.9)	62 (22.1)	
Income									
high	3,375 (90.6)	383 (9.4)		1,691 (90.8)	185 (9.2)		1,684 (90.5)	198 (9.5)	
intermediate	1,613 (88.0)	225 (12.0)	< 0.001	836 (91.1)	78 (8.9)	< 0.001	777 (85.0)	147 (15.0)	< 0.001
low	3,234 (85.2)	615 (14.8)		1,683 (87.1)	279 (12.9)		1,551 (83.3)	336 (16.7)	
Region of Serbia									
Belgrade	1,960 (90.1)	242 (9.9)		952 (91.5)	104 (8.5)		1,008 (88.7)	138 (11.3)	
Vojvodina	1,800 (87.0)	294 (13.0)		906 (87.1)	150 (12.9)		894 (86.9)	144 (13.1)	
Šumadija and Western	2,775 (89.1)	347 (10.9)	< 0.001	1,467 (90.8)	147 (9.2)	< 0.001	1,308 (87.5)	200 (12.5)	< 0.001
Southern and Eastern	1,687 (85.5)	340 (14.5)		885 (88.1)	141 (11.9)		802 (82.9)	199 (17.1)	

Table 3 (continued)

Parameter	All			Men			Women		
	chronic diseases		*p*-value*	chronic diseases		*p*-value*	chronic diseases		*p*-value*
	one or no	two or more		one or no	two or more		one or no	two or more	
Nutritional status									
normal weight	3,008 (93.2)	242 (6.8)	< 0.001	1,206 (93.5)	89 (6.5)	< 0.001	1,802 (93.0)	153 (7.0)	< 0.001
underweight	188 (92.2)	14 (7.8)		49 (96.6)	2 (3.4)		139 (90.8)	12 (9.2)	
overweight	2,319 (86.5)	384 (13.5)		1,423 (88.9)	193 (11.1)		896 (83.0)	191 (17.0)	
obesity	1,067 (74.0)	407 (26.0)		644 (80.5)	170 (19.5)		423 (65.9)	237 (34.1)	
Total	8,222 (88.0)	1,223 (12.0)		4,210 (89.4)	542 (10.6)		4,012 (86.7)	681 (13.3)	

All values are expressed as unweighted numbers of participants (weighted percentages). * Chi-square test.

Table 4

Association between demographic and socioeconomic variables and nutritional status with multimorbidity

	All*		Men**		Women**	
	OR	95% CI	OR	95% CI	OR	95% CI
Gender						
male	1					
female	1.14	1.13–1.15	–	–	–	–
Age (years)						
15–24	1		1		1	
25–34	2.38	2.32–2.44	1.70	1.64–1.76	4.03	3.86–4.21
35–44	3.77	3.68–3.86	3.11	3.01–3.22	5.66	5.42–5.90
45–54	8.30	8.10–8.51	5.18	5.01–5.36	15.78	15.14–16.44
55–64	13.04	12.72–13.36	11.00	10.64–11.38	18.88	18.12–19.68
Level of education						
high	1		1		1	
intermediate	1.24	1.22–1.25	1.47	1.45–1.49	1.06	1.05–1.08
low	1.80	1.77–1.82	1.94	1.91–1.98	1.68	1.65–1.70
Employment status						
employed	1		1		1	
unemployed	1.04	1.03–1.05	0.99	0.97–0.998	1.05	1.03–1.06
retired	2.51	2.48–2.53	2.21	2.17–2.24	2.87	2.82–2.91
other inactive	1.63	1.61–1.65	1.86	1.81–1.92	1.56	1.53–1.58
Marital status						
married	1		1		1	
never married	0.75	0.74–0.76	0.75	0.74–0.76	0.68	0.67–0.70
widowed	1.33	1.32–1.35	1.76	1.70–1.81	1.22	1.20–1.24
divorced	1.67	1.65–1.69	1.52	1.49–1.54	1.71	1.69–1.74
Income						
high	1		1		1	
middle	1.15	1.14–1.16	0.82	0.80–0.83	1.49	1.47–1.51
low	1.28	1.27–1.29	1.05	1.04–1.06	1.52	1.50–1.54
Region of Serbia						
Belgrade	1		1		1	
Vojvodina	0.80	0.80–0.81	1.14	1.12–1.15	0.63	0.62–0.64
Šumadija and Western	0.68	0.67–0.68	0.80	0.78–0.81	0.61	0.60–0.62
Southern and Eastern	0.92	0.91–0.93	1.10	1.08–1.11	0.82	0.80–0.83
Nutritional status						
normal weight	1		1		1	
underweight	1.57	1.53–1.61	0.64	0.59–0.69	2.11	2.05–2.18
overweight	1.55	1.54–1.56	1.42	1.40–1.44	1.58	1.56–1.60
obesity	2.88	2.85–2.90	2.35	2.32–2.39	3.33	3.29–3.37

OR – odds ratio; CI – confidence interval. *Logistic regression model was adjusted for gender, age, level of education, employment status, marital status, income, region, and nutritional status; **Logistic regression model was adjusted for age, level of education, employment status, marital status, income, region, and nutritional status.

1.04–1.06), while women with low income had 52% higher odds of multimorbidity (OR = 1.52; 95% CI: 1.50–1.54). The results showed that being overweight or obese was positively associated with multimorbidity. Obese men had 2.4 times higher chances for multimorbidity (OR = 2.35; 95% CI: 2.32–2.39), and obese women had a 3.3 times higher chance

(OR = 3.33; 95% CI: 3.29–3.37) for multimorbidity compared to men and women with an optimal BMI. Underweight was associated with higher odds of multimorbidity in women, while underweight in men was associated with lower odds of multimorbidity.

Discussion

The study was conducted on a representative sample of WAP in RS. To our knowledge, no prior studies assessed the burden of multimorbidity specifically amongst WAP in RS. It documented the burden of multimorbidity among WAP and the predictors of multimorbidity. Associations between multimorbidity and demographic and SE variables were found to be consistent in men and women, with some differences in the magnitudes of these relationships. The burden of multimorbidity was greater in women, the older, lower educated population, people with lower household incomes, widowed, divorced, and people who lived in the Belgrade region. Being overweight or obese was associated with higher odds of multimorbidity, in both men and women. Underweight was associated with higher odds of multimorbidity only in women.

The proportion of people with multimorbidity increased significantly with age, which is consistent with previous studies on multimorbidity [3, 14]. This was expected, since aging leads to multiple organ systems dysregulation and it is the most important risk factor for many CDs [20].

Multimorbidity was more prevalent among women than men. After controlling for age and other significant variables women had 14% higher odds of multimorbidity compared to men. Many other studies found that women have a higher risk of multimorbidity than men [2, 21]. A recent study by Bezerra et al. [21] reported a greater prevalence of multimorbidity in women in 16 out of 17 European countries.

Our study confirmed that the burden of multimorbidity is greater in socioeconomically deprived populations. The socioeconomically disadvantaged are more prone to having an unhealthy lifestyle that leads to the development of CD [22]. The importance of SE status for the development of multimorbidity is well documented in the research of Barnett et al. [3] who reported that multimorbidity occurs 10 to 15 years earlier in people living in the most deprived areas compared to the most affluent.

Multimorbidity disproportionately affects more people of lower SE status. This study included three indicators of SE status: level of education, employment, and income. All three indicators were significantly associated with multimorbidity, in both genders. There are several theories on how SE deprivation leads to multimorbidity, but the most frequently used are behavioral theories (health behaviors), materialist (access to health resources), and psychosocial (stress pathways) theories [23].

Multimorbidity prevalence was significantly higher in the least educated population. The educational level had an inverse association with multimorbidity after controlling for the effect of age and other covariates. A low level of education increases the risk of multimorbidity more in men than in women. These results are consistent with many other European studies [21, 24]. Education impacts health on several levels. It contributes to developing a range of skills important in using health care, making lifestyle choices, and in health-related behaviors. Individuals with higher levels of education tend to be more aware of health risks and are less likely to engage in risky behavior. They also have higher earnings, are wealthier, and have better access to social networks and support that improve health. Educated people are less likely to experience unemployment and economic problems. They also benefit from health-related characteristics of the environment in which they live, work, and study: access to healthy food, spaces and facilities for physical activity, access to health care, community economic resources, safer environments, and lesser exposure to environmental risk factors [25].

Differences in multimorbidity prevalences were also found for employment subgroups. According to employment status, the highest prevalence of multimorbidity was registered among retired persons, which could be explained by the fact that persons who left paid employment at working age have worse health than those who remained employed. The likelihood of having multimorbidity was 2.2 times higher among retired men and 2.9 times higher among retired women compared to their employed counterparts. An unemployed person had a higher prevalence of multimorbidity than an employed person, which is consistent with other studies [26, 27]. Unemployed women had a 5% higher chance for multimorbidity compared to employed women. After adjustment for other significant variables, unemployed men had a 1% lower chance of having multimorbidity compared to employed men, which was unexpected since unemployment is associated with worse health [28]. One of the reasons for unexpected results could be due to missing information on the duration of unemployment.

Our study showed that never-married men and women have lower odds of multimorbidity and that widowed and divorced men and women have higher odds of multimorbidity compared to their married counterparts. A Brazilian study also found that persons living with a partner had higher odds for multimorbidity compared to those living without a partner [29]. Contrary to our results, studies conducted in Europe, the UK, the US, and China demonstrated that persons who were not married (separated/divorced/widowed/never married) had higher odds of multimorbidity than those married. The authors also analyzed the duration of the marriage. Persons who had been married for 21 to 30 years had lower odds of experiencing multimorbidity than those whose marriage lasted less than ten years [30]. Research from Germany showed no association between marital status and multimorbidity [31]. Since results regarding the association between marital status and multimorbidity were heterogeneous across countries, there is a need for further research, for instance, the inclusion of cultural factors that influence the association between marital status and multimorbidity.

Results showed that the prevalence of multimorbidity increased with BMI, and it reached a value of 19.5% in obese men and 34.1% in obese women. Overweight and obesity were associated with higher odds of multimorbidity in both men and women. This is consistent with many other

studies that reported that obesity increases the risk for co-occurrence of two or more diseases [32]. Obesity is a risk factor for many CDs, which develop as a result of endocrine and metabolic dysregulation, most notably type 2 DM, cardiovascular diseases, and malignant diseases [33]. Since every fifth man (21.1%) and every sixth woman (15.9%) of working age in RS is obese, this means that a significant proportion of the population is exposed to an increased risk of multimorbidity, which can lead to an overload of the health care system. Besides obese people, overweight people also have an increased risk of multimorbidity compared with individuals with optimal BMI. The number of overweight individuals in RS is even greater than the number of obese individuals, both in men and women, which emphasizes the importance of the overweight-multimorbidity association. On the other hand, weight loss is one of the best behavioral changes that one can make in order to prevent and manage many CDs (AH, DM type 2, CHD, malignant diseases, etc.) [34], and this could also be a potential strategic target for the prevention of multimorbidity.

Multimorbidity was measured based on information on 13 CCs, which included not only indicators of the physical aspect of health disbalance (cardiovascular, respiratory, endocrine, musculoskeletal diseases, etc.) but also an indicator of mental health conditions, such as depression. This is very important for understanding properly the epidemiology and implications of multimorbidity [35]. Most previous studies included the general or elderly population, and relatively few studies have specifically focused on the WAP. Furthermore, one of the advantages of the study is that conclusions were made based on the representative sample for the population of RS. Another advantage is that the data used for BMI calculations were measured, not self-reported.

One of the limitations of the study is that the association between exposure (demographic, SE variables, NS) and outcome (multimorbidity) is established cross-sectionally, so conclusions about causal relationships cannot be made. The data on CDs were self-reported, which may not accurately reflect health status, and multimorbidity prevalence could be underreported.

Conclusion

Multimorbidity is an important public health problem among the WAP in RS due to its high prevalence, and vulnerable groups (women, poorly educated, lower income, etc.) carry a greater burden of multimorbidity. One of the possible strategic targets for multimorbidity prevention could be obesity since it is associated with higher odds of multimorbidity. Obesity prevention measures and health promotion activities should be more intensively promoted in the workplace, which will positively impact multimorbidity as well.

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Sociodemographic factors related to internet addiction among adolescents in Serbia

Socio-demografski faktori povezani sa zavisnošću od interneta kod adolescenata u Srbiji

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Abstract

Background/Aim. The term "internet addiction" (IA) describes a compulsive behavior associated with any online activity that disrupts everyday social interactions. The aim of this study was to determine risk factors and predictors of the development of IA in adolescents. **Methods.** Data were collected using an online form consisting of demographic data, questions related to the use of the Internet, and the Internet Addiction Test (version for adolescents). **Results.** A total of 1,669 respondents participated in this research; 1,040 of them (62.3%) were female, 590 (35.4%) were male, and 39 (2.3%) did not want to declare their gender. The average age of the respondents was 15.09 ± 1.757 years. Significant factors in the multivariate factor analysis model were age, addictive substance use frequency, purpose, and time spent on the Internet ($p < 0.05$). Analysis results indicate that the risk of developing IA was higher if an adolescent spent 1–3 hrs and more than 3 hrs daily on the Internet (2.8 and 8.2 times, respectively). With increasing age numbers for one unit (year), the risk of developing IA was decreasing by 11.3%. **Conclusion.** According to the findings of the present study, the relationship between IA and age, addictive substance use frequency, purpose, and time spent on the Internet has been proven. These results should be highlighted so that educators and all those who work with children could create targeted treatments to prevent the development of IA in adolescents.

Key words:
adolescent; internet use; surveys and questionnaires; technology addiction.

Apstrakt

Uvod/Cilj. Termin „zavisnost od interneta" (ZoI) opisuje kompulzivno ponašanje koje je povezano sa bilo kojom aktivnošću na internetu koje ometa svakodnevne društvene interakcije. Cilj rada bio je da se utvrde faktori rizika i prediktori razvoja ZoI kod adolescenata. **Metode.** Podaci u ovom istraživanju prikupljeni su uz pomoć *online* upitnika sačinjenog od demografskih podataka, pitanja u vezi sa upotrebom interneta i Testa za ispitivanje zavisnosti od interneta (verzija za adolescente). **Rezultati.** Ukupno 1 669 ispitanika učestvovalo je u ovom istraživanju; 1 040 (62,3%) ispitanika bilo je ženskog pola, 590 (35,4%) muškog i 39 (2,3%) ispitanika nije želelo da se izjasni po pitanju polne pripadnosti. Prosečno životno doba ispitanika bilo je 15,09 ± 1,757 godina. Značajni faktori u modelu multivarijantne faktorske analize bili su životno doba, učestalost upotrebe psihoaktivnih supstanci, svrha i vreme provedeno na internetu ($p < 0,05$). Rezultati analize pokazuju da su viši rizik od razvoja ZoI imali adolescenti koji su provodili 1–3 sata i više od 3 sata dnevno na internetu (2,8 i 8,2 puta, redom). Povećanjem broja godina za jednu jedinicu (godinu) rizik od razvoja ZoI smanjivao se za 11,3%. **Zaključak.** Ovim istraživanjem dokazana je povezanost između ZoI i životnog doba, učestalosti upotrebe psihoaktivnih supstanci, svrhe i vremena provedenog na internetu. Važno je istaći dobjene rezultate kako bi prosvetni radnici i svi oni koji rade sa decom kreirali ciljane programe prevencije razvoja ZoI kod adolescenata.

Ključne reči:
adolescenti; internet, korišćenje; ankete i upitnici; zavisnost od tehnologije.

Introduction

In recent years, the time spent on the Internet by adolescents has increased rapidly. Not only do schools and the educational system rely continuously more on internet activities, but the social lives of adolescents are also increasingly conducted through activities on social networks and other online applications [1]. The activities of young people

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on the Internet during the coronavirus disease 2019 (COVID-19) pandemic have significantly intensified due to the transition of the educational system to online teaching [2]. The term "internet addiction" (IA) describes a compulsive behavior associated with any online activity that disrupts everyday life and social interactions. As Shek et al. [3] stated, "problematic internet use" is a synonym for IA, which is defined as an individual's inability to maintain control over their internet use. It has become widely recognized as a serious health concern worldwide. One in eight Americans reports having trouble using the Internet [4], 2.4% of Chinese [5], 1.5% and 8.2% of Americans and Europeans [6], and 3.2% of United Kingdom citizens [7] said they were addicted to the Internet. Estimates from the World Bank indicate that Serbia has seen a rise in the number of Internet users in recent years. The percentage of people who used the Internet in 2009 was 38.1%; in 2012, it was 48.1%; however, in 2022, it was 84%. This pattern appears to be consistent with worldwide patterns of internet usage [8]. Research aimed at assessing the prevalence of internet use and IA among adolescents aged 14–18 years in the municipality of Novi Sad, Serbia, and the impact of sociodemographic variables on internet use showed that the estimated prevalence of IA was high (18,7%), mainly among younger adolescents. IA was found in every fifth adolescent [9]. These findings suggest a higher prevalence among adolescents compared to the general population, similar to a limited case study of IA in the City of Niš among the student population aged 19–23 who had a prevalence of internet use of 100% [10]. In a 2016, Dukanac et al. [11] showed that adolescents who developed IA have the lowest self-directedness and cooperativeness as character dimensions; however, they are more asocial than antisocial. Given that the internet-addicted adolescent group also scored highest in harm avoidance, the combination of the two factors may indicate genuine social anxiety, as well as a protective withdrawal into the virtual world and social avoidance. This group also had the highest scores in the two categories of exploratory curiosity, impulsivity, and novelty seeking. This aligns with the common conduct issue associated with the Internet, highlighting impulse control disorder independent of the cause of the disturbance in control. Advancements in technology may create new avenues for impulsivity to be expressed in virtual reality, or more likely, extended computer use and the consumption of violent content exacerbate irritation and encourage impulsive behavior [11]. According to previous research, the possible causes of excessive internet use are dysfunctional family, the degree of parental overprotection, parental control over the adolescent's free time, the adolescent's personality type (level of impulsivity, degree of self-control), aggressiveness, and neuroticism [6]. It has also been proven that the effects of excessive use of the Internet on the central nervous system are very similar to the effects of drug addicts, i.e., the response of the brain is very similar and is reflected in the increased secretion of dopamine. Adolescents are often shown to be the most vulnerable group in terms of developing IA; therefore, it is invaluable to examine the predictors and factors that influence the appearance and outcomes of this "new" form of addiction that has a negative impact on health, especially mental health.

In the present study, IA has been examined in adolescents in Serbia, with a correlation to differences in gender, place of residence, parent's education, and psychoactive substance use.

Methods

Sample

The sample of our research consisted of 1,669 adolescents aged 11 to 19 attending primary and secondary schools in Serbia. More detailed information on the sample structure will be provided in the Results section.

Instruments

For this study, we created an online form (Google Forms) that consisted of five parts. The first three sections of the full questionnaire were used for the current paper. The first part was related to the demographic data of the respondents (age, gender, class, level of professional education of the parents, success in the current and previous semesters, and use of psychoactive substances). The second part was related to the use of the Internet: from which device they connect to the Internet most often, how much time a day they spend on the Internet for school duties and extracurriculars, which group of applications they devote the most time to (social networks, games, viewing media, movies, music, etc.), whether their parents control them in this, and to what extent. The third part consisted of the Internet Addiction Test (IAT), a scale validated on the Croatian adolescent population that was used to diagnose IA with the author's permission and consent [12]. This scale consists of 20 questions, which can be answered with 6 answers: 0 – never, 1 – rarely, 2 – occasionally, 3 – often, 4 – very often, and 5 – always. The test taker can score 0–100 points. The classification of the points obtained is as follows: 0–19 = no signs of IA; 20–39 = low level of IA; 40–69 = medium level; 70–100 = high level of IA.

Design and procedure

In this research, a cross-sectional epidemiological study was used. The protocols used in this study followed the guidelines of the Declaration of Helsinki [13]. The study was approved by the Ethics Committee of the University of Kragujevac, Faculty of Medical Sciences, Serbia (No. 01-6816). Parents signed informed consent for minor participants (below 18 years of age). Schools were contacted randomly by email. After principal approval, a form link was forwarded to students. Responses were instantly available after form completion and presented in an Excel spreadsheet. Every response triggered a notification email in the researcher's mailbox.

Statistical analyses

Statistical data processing was performed using the standard SPSS software package, version 19.0. (SPSS Inc, version 19.0, Chicago, IL). The following descriptive statistics measures were used in this research: arithmetic mean, standard deviation, median, quartiles, frequencies, and percentages. The reliability of the measuring scales was tested using the Cronbach coefficient. The normal distribution of numerical variables was checked using the Shapiro-Wilk and Kolmogorov-Smirnov test. The correlation of category variables was examined using the Chi-square (χ^2) diagram for the contingent tables. Univariate and multivariate factor analyses were used for predicting IA.

Results

A total of 1,669 respondents participated in this research; 1,040 of them (62.3%) were female, 590 (35.4%) were male, and 39 (2.3%) did not want to declare their gender. The average age of the respondents was 15.09 ± 1.757 years, with the youngest respondent being 11 and the oldest 19 years old. Other sociodemographic data about the respondents can be found in Table 1.

Table 1

Sociodemographic characteristics

Question	Categories	Values
Do you go to primary or secondary school?	primary school	381 (22.8)
	secondary school	1,288 (77.2)
Do you have any siblings?	no	285 (17.1)
	yes	1,384 (82.9)
Which city is your place of residence (or closest)?	Belgrade	670 (40.1)
	Sombor	788 (47.2)
	Kragujevac	22 (1.3)
	Kruševac	82 (4.9)
	Niš	37 (2.2)
	other	70 (4.2)
What is the relationship of your parents?	divorced or separated	341 (20.4)
	married	1,253 (75.1)
	live together	75 (4.5)
What is your mother's education?	primary school	79 (4.7)
	secondary school	747 (44.8)
	college	188 (11.3)
	faculty	655 (39.2)
[1]What is your father's education?	primary school	99 (5.9)
	secondary school	833 (49.9)
	college	179 (11.3)
	faculty	548 (32.8)
What were your grades like last year?	acceptable (2, D)	22 (1.3)
	good (3, C)	193 (11.6)
	very good (4, B)	555 (33.3)
	excellent (5, A)	899 (53.9)
[2]Do you use any of these substances?	no, nothing	1,134 (68.0)
	e-cigars	217 (13.0)
	tobacco	100 (6.0)
	alcohol	198 (11.9)
	marihuana	4 (0.2)
	sleeping pills	13 (0.8)
	other	2 (0.1)
How often do you use the above-mentioned substances?	never	1,087 (65.1)
	rarely	176 (10.5)
	only when I go out with my friends	220 (13.2)
	every day	186 (11.1)

All values are given as numbers (percentages) of respondents.
Note: [1] Ten respondents did not answer this question, so the total number of respondents was 1,659 instead of 1,669; [2] One respondent did not answer this question, so the total number of respondents was 1,668 instead of 1,669.

Out of the total number of respondents, 1,618 (96.9%) use the Internet daily.

The distribution of answers to questions related to internet use is presented in Table 2.

After totaling/summing single IAT questions, a score for each participant was obtained. The average value of IAT was 28.90 ± 15.21, where the minimal score was 0 and the maximum 99. According to this IAT score, all participants were divided into categories as follows: 514 (30.8%) with no signs of IA, 773 (46.3%) with a low level of IA, 361 (21.6%) with a medium level of IA, and 21 (1.3%) with a high level of IA. Gender (χ^2 = 20.7. p = 0.002), attendance at primary or secondary school (χ^2 = 21.4, p < 0.001), and city of residence (χ^2 = 33.3, p = 0.004) were sociodemographic characteristics that were significantly related to IA level. Regarding the mother's education, significantly more participants without IA (39.3%), with low IA signs (42.3%), and with medium IA signs (34.9%) had a mother with university education (high education level) in relation to the ones with high IA signs (14.3%) (χ^2 = 20.7, p = 0.014). A similar result was observed concerning the father's education – respondents with high levels of IA had the lowest percentage of highly educated fathers compared to other groups (23.8%) (χ^2 = 12.1, p = 0.028). Moreover, the representation of students with excellent (A) grades was the least in the high IA level group (33.3%) relating to other groups (χ^2 = 42.3, p < 0.001). Usage of psychoactive substances that may result in developing addiction (χ^2 = 85.7, p < 0.001), same as the frequency of substance usage, was significantly related to IA level (χ^2 = 58.2, p < 0.001) as described in Table 3.

Table 2

Distribution of responses related to Internet usage

Question	Categories	Values
Usually, I access the Internet using?	computer	126 (7.5)
	smartphone	1,511 (90.5)
	laptop	32 (1.9)
Internet content mostly visited?	gaming	202 (12.1)
	social networks (Facebook, Instagram, Snapchat, TikTok, etc.)	1,060 (63.5)
	media content (YouTube, Spotify, Netflix, HBO, etc.)	371 (22.2)
	school activities	36 (2.2)
Do you use the Internet every day?	no	51 (3.1)
	yes	1,618 (96.9)
If used every day, approximately for how long?	max 1 hr	109 (6.5)
	1–3 hrs	617 (37.0)
	more than 3 hrs	943 (56.5)
Do your parents control the time you spend on the Internet?	no	700 (41.9)
	sometimes	694 (41.6)
	yes	275 (16.5)
Do you use the Internet for school activities?	never	81 (4.9)
	sometimes	748 (44.8)
	often	840 (50.3)

All values are given as numbers (percentages) of respondents.

Table 3

Sociodemographic characteristics related to the Internet addiction presence

Parameters	No signs	Low level	Medium level	High level	Chi-square/*p*-value
Gender					
female	299 (58.2)	504 (65.2)	227 (62.9)	10 (47.6)	
male	204 (39.7)	253 (32.7)	125 (34.6)	8 (38.1)	**20.7/0.002**
undeclared	11 (2.1)	16 (2.1)	9 (2.5)	3 (14.3)	
School					
primary	152 (29.6)	152 (19.7)	70 (19.4)	7 (33.3)	**21.4/< 0.001**
secondary	362 (70.4)	621 (80.3)	291 (80.6)	14 (66.7)	

Table 3 (continued)

Parameters	No signs	Low level	Medium level	High level	Chi-square/*p*-value
Siblings					
no	91 (17.7)	121 (15.7)	69 (19.1)	4 (19.0)	2.4/0.500
yes	423 (82.3)	652 (84.3)	292 (80.9)	17 (81.0)	
Nearest city of residence?					
Belgrade	184 (35.8)	344 (44.5)	138 (38.2)	4 (19.0)	**33.3/0.004**
Sombor	265 (51.6)	329 (42.6)	180 (49.9)	14 (66.7)	
Kragujevac	5 (1.0)	15 (1.9)	1 (0.3)	1 (4.8)	
Kruševac	26 (5.1)	34 (4.4)	22 (6.1)	0 (0.0)	
Niš	11 (2.1)	18 (2.3)	6 (1.7)	2 (9.5)	
Other cities	23 (4.5)	33 (4.3)	14 (3.9)	0 (0.0)	
Parents' relationship					
divorced or separated	91 (17.7)	155 (20.1)	90 (24.9)	5 (23.8)	9.6/0.143
married	403 (78.4)	583 (75.4)	253 (70.1)	14 (66.7)	
live together	20 (3.9)	35 (4.5)	18 (5.0)	2 (9.5)	
Mother's education					
primary school	26 (5.1)	32 (4.1)	18 (5.0)	3 (14.3)	**20.7/0.014**
secondary school	240 (46.7)	327 (42.3)	169 (46.8)	11 (52.4)	
college	46 (8.9)	87 (11.3)	51 (14.1)	4 (19.0)	
university degree	202 (39.3)	327 (42.3)	123 (34.9)	3 (14.3)	
[1]Father's education					
primary school	31 (6.0)	39 (5.0)	27 (7.5)	2 (9.5)	**12.1/0.028**
secondary school	262 (51.0)	344 (47.1)	196 (54.3)	11 (52.4)	
college	59 (11.5)	92 (11.9)	35(9.7)	3 (14.3)	
university degree	162 (31.5)	278 (36.0)	103(28.5)	5 (23.8)	
Last-year school grades					
acceptable (2, D)	4 (0.8)	9 (1.2)	7 (1.9)	2 (9.5)	**42.3/< 0.001**
good (3, C)	61 (11.9)	70 (9.1)	58 (16.1)	4 (19.0)	
very good (4, B)	161 (31.3)	245 (31.7)	141 (39.1)	8 (38.1)	
excellent (5, A)	288 (56.0)	449 (58.1)	155 (42.9)	7 (33.3)	
[2]Addictive substance use					
no	392 (76.3)	531 (68.8)	197 (54.6)	14 (66.7)	**85.7/< 0.001**
e-cigars	39 (7.6)	88 (11.4)	85 (23.5)	5 (23.8)	
tobacco	31 (6.0)	40 (5.2)	28 (7.8)	1 (4.8)	
alcohol	49 (9.5)	103 (13.3)	46 (2.7)	0 (0.0)	
marihuana	2 (0.4)	2 (0.3)	0 (0.0)	0 (0.0)	
sleeping pills	1 (0.2)	8 (1.0)	3 (0.8)	1 (4.8)	
other drugs	0 (0.0)	0 (0.0)	2 (0.6)	0 (0.0)	
Frequency of addictive substance use					
never	383 (74.5)	506 (65.5)	187 (51.8)	11 (52.4)	**58.2/< 0.001**
rarely	45 (8.8)	82 (10.6)	48 (13.3)	1 (4.8)	
only when I go out with friends	43 (8.4)	107 (13.8)	67 (18.6)	3 (14.3)	
every day	43 (8.4)	78 (10.1)	59 (16.3)	6 (28.6)	

All values are given as numbers (percentages) of respondents.
Note: [1] Twenty respondents did not answer this question, so the total number of respondents was 1,649 instead of 1,669; [2] One respondent did not answer this question, so the total number of respondents was 1,668 instead of 1,669.

Significant relatedness or connection (in terms of spending time) in all characteristics of internet use and IA level was observed ($p < 0.001$), as described in Table 4.

All respondents with high levels of IA signs declared using the Internet each day ($\chi^2 = 15.4$, $p = 0.002$), the same as the usage of the Internet for more than 3 hrs *per* day

(χ^2 = 250.1, p < 0.001). In the group of respondents who did not receive any warning from their parents regarding the time they spent on the In-ternet, the results were as follows: 38.5% of respondents had no signs of IA, 42.3% showed a low level of IA, medium level of IA was present in 44.3%, and 71.4% of respondents had a high level of IA) (χ2 = 14.9, p = 0.021) (Table 4).

After univariant logistic regression, all variables that showed significance (p < 0.05) were integrated into the multivariant model. This model clarifies the 17.6–24.8% variance of the dependent variable and has good predictive power according to the results of the Hosmer-Lemeshow tests (p > 0.05). The accuracy of this model was 73.8%. Significant factors in this model were age, addictive substance use frequency, purpose, and time spent on the Internet (p < 0.05). Other variables that were significant in the univariate model lost their significance in the multivariate model after impact assessments of other variables. Analysis results indicate that the risk of developing IA is higher if an adolescent spends 1–3 hrs and more than 3 hrs daily (2.8 and 8.2 times, respectively). With the age increasing by one unit (year), the risk of developing IA decreases by 11.3%. Other statistically significant variables are described in Table 5.

Table 4

Characteristics of Internet usage associated with Internet addiction

Question	No signs	Low level	Medium level	High level	Chi-square/ *p*-value
Usually, I access the Internet *via*?					
computer	43 (8.4)	41 (5.3)	40 (11.1)	2 (9.5)	
smartphone	457 (88.9)	724 (93.7)	311 (86.1)	19 (90.5)	**20.1/0.003**
laptop	14 (2.7)	8 (1.0)	10 (2.8)	0 (0.0)	
[1]Usually, I spend the time on the Internet for?					
gaming	58 (11.3)	73 (9.4)	65 (18.0)	6 (28.6)	
social networking (Facebook, Instagram, Snapchat, TikTok, etc.)	282 (56.2)	510 (66.0)	248 (68.7)	13 (61.9)	**75.0/< 0.001**
media (YouTube, Spotify, Netflix, HBO, etc.)	143 (27.8)	180 (23.3)	47 (13.0)	1 (4.8)	
for school activities	24 (4.7)	10 (1.3)	1 (0.3)	1 (4.8)	
Do you use the Internet every day?					
no	28 (5.4)	18 (2.3)	5 (1.4)	0 (0.0)	**15.4/0.002**
yes	486 (94.6)	755 (97.7)	356 (98.6)	21 (100.0)	
If used every day, how much time approximately would that be?					
1 hr max	76 (14.8)	28 (3.6)	5 (1.4)	0 (0.0)	
1–3 hrs	266 (51.8)	288 (37.3)	63 (17.5)	0 (0.0)	**250.1/< 0.001**
more than 3hrs	172 (33.5)	457 (59.1)	293 (81.2)	21 (100.0)	
Do your parents control the time you spend on the Internet?					
Do they warn you?					
no	198 (38.5)	327 (42.3)	160 (44.3)	15 (71.4)	
sometimes	214 (41.6)	331 (42.8)	145 (40.2)	4 (19.0)	**14.9/0.021**
yes	102 (19.8)	115 (14.9)	56 (15.5)	2 (9.5)	
Do you use the Internet for school activities and learning?					
never	22 (4.3)	31 (4.0)	24 (6.6)	4 (19.0)	
sometimes	206 (40.1)	344 (44.5)	187 (51.8)	11 (52.4)	**29.9/< 0.001**
often	286 (55.6)	398 (51.5)	150 (41.6)	6 (28.6)	

All values are given as numbers (percentages) of respondents.
Note: [1] Seven respondents did not answer this question, so the total number of respondents was 1,662 instead of 1,669.

Table 5

Univariate and multivariate factor analysis for Internet addiction prediction

Variable	Logistic regression			
	univariate		multivariate	
	OR	*p*-value	OR	*p*-value
Age, years				
11–19	1.105 (1.042–1.173)	**0.001**	0.887 (0.791–0.895)	**0.040**
Gender				
female	1	ref	1	ref
male	0.764 (0.615–0.948)	**0.015**	0.975 (0.748–1.271)	0.850
School				
primary	1	ref	1	ref
secondary	1.698 (1.338–2.155)	< **0.001**	1.501 (0.980–2.300)	0.062
Parents' relationship				
separated or divorced	1	ref	1	ref
married	0.768 (0.587–0.965)	**0.05**	0.899 (0.665–1.215)	0.487
City of residence				
Belgrade	1	ref	1	ref
Sombor	0.747 (0.597–0.936)	**0.011**	0.807 (0.600–2.138)	0.157
Addictive substance use				
none	1	ref	1	ref
e-cigars	2.411 (1.670–3.482)	< **0.001**	0.657 (0.295–1.461)	0.303
alcohol	1.606 (1.138–2.269)	**0.007**	0.607 (0.267–1.379)	0.233
How often do you use the addictive substances?				
never	1	ref	1	ref
rarely	1.584 (1.104–2.272)	**0.012**	2.087 (0.941–4.628)	0.070
only when going out with friends	2.239 (1.569–3.196)	< **0.001**	2.490 (1.106–5.605)	**0.028**
each day	1.809 (1.259–2.601)	**0.001**	2.022 (0.948–4.315)	0.069
What Internet content do you most often spend your time on?				
games	1	ref	1	ref
media (YouTube, Spotify, Netflix, HBO, etc.)	0.642 (0.444–0.929)	**0.019**	0.616 (0.399–0.951)	**0.029**
school activities	0.201 (0.094–0.429)	< **0.001**	0.261 (0.110–0.620)	**0.002**
Do you use the Internet every day?				
no	1	ref	1	ref
yes	2.836 (1.617–4.973)	< **0.001**	1.116 (0.581–2.142)	0.742
How much time do you spend on the Internet daily?				
1 hr max	1	ref	1	ref
1–3 hrs	3.039 (1.960–4.712)	< **0.001**	2.775 (1.706–4.512)	< **0.001**
more than 3 hrs	10.323 (6.644–16.042)	< **0.001**	8.205 (4.962–13.568)	< **0.001**
Do your parents control the time you spend on the Internet?				
no	1	ref	1	ref
yes	0.669 (0.498–0.898)	**0.008**	0.984 (0.695–1.393)	0.927

OR – odds ratio; ref – reference category; HBO – Home Box Office.

Discussion

The main aim was to determine which variables included in the research have the greatest predictive power for the development of IA. In this research, it was shown that 96.9% of the surveyed adolescents use the Internet daily, which is in line with previous studies conducted around the world [14]. Based on the IAT score, adolescents in our research were divided into the following groups: 30.8% had no signs of IA, 46.3% had a low level of IA, 21.6% had a moderate level of IA, and 1.3% had a high level of IA. These findings are consistent with those obtained on a population of 352 students in Croatia, where the results showed that 3.4% of high school students reported high levels of IA, while 35.4% of respondents reported some signs of addiction. It is important to note that for the assessment of IA in that research, an identical instrument was used as in our study [12]. Results for 31 nations were reported in a meta-analysis of 164 independent samples (n = 89,281) by Cheng and Li [15]. According to the findings of their research, the Middle East has the highest incidence of high IA (10.9%). The countries of North and West Europe have the lowest prevalence of IA

(2.6%), followed by Southeast European countries with a frequency of 6.1%.

In this study, a significant correlation was observed in all characteristics of internet use with the degree of IA because all respondents with a high level of IA stated that they use the Internet every day or use the Internet for more than 3 hrs a day, while 71.4% of respondents with a high level of IA stated that their parents do not control the time they spend on the Internet. Our findings are consistent with research conducted on a sample of 426 students in Saudi Arabia. The same instrument as in our study (20-item IAT) was used to measure IA, and it was shown that 40.8% of respondents used the Internet 5–7 hrs a day, mainly for social networking (88.5%) and to download media files. Approximately 6% were classified as internet addicts, and 42% had occasional problems. IA was significantly higher in those who used the Internet for more than 10 hrs a day [16]. In our research, after conducting univariate logistic regression, the following were identified as significant factors predicting addiction to the Internet: age, frequency of use of addictive substances, purpose of spending time on the Internet, and time spent on the Internet, which is in agreement with the results of other research [17]. The association between IA and frequency of substance use is expected because characteristic features of both physical and psychological addictions, including fluctuating emotions, tolerance, withdrawal, interpersonal conflict, and relapse, are evident in "behavioral" addictions, i.e., syndromes analogous to substance addiction but with a behavioral focus other than ingestion of a psychoactive substance [18]. The "self-medication hypothesis" states that patients typically utilize drugs to manage their cognitive deficiencies, lessen excruciating anxiety, and alter undesirable temperamental positions [19]. This may be seen in IA, which is a behavioral addiction, as well as in other behavioral issues that young people face, such as substance abuse. Nevertheless, the research from 2016 clearly showed the difference in the personality structure of chemical and behavioral, non-chemical addictions in adolescents. Adolescents abusing psychoactive substances had low harm avoidance and self-transcendence, while adolescents with IA were characterized by high novelty seeking (impulsivity and curiosity), low self-directedness, and the lowest cooperativeness [11]. The results of the aforementioned study from Serbia, in which, in addition to other instruments, the IAT consisting of 20 items for self-assessment of problems related to the use of the Internet was applied, showed that adolescents with the problem of IA have the most extreme results in personality dimensions, which could represent a significant psychopathological risk factor [11]. Some variables that showed significance in the univariate model lost it in the multivariate model after assessing the influence of other variables, and, in the final addiction prediction model, the results of the analysis showed that the risk of developing addiction to the Internet is greater if the child spends more than 1–3 hrs and more than 3 hrs a day on the Internet (2.8 and 8.2 times, respectively), and that with an increase in the number of years by one, the risk of IA decreases by 11.3%. These findings are expected and in accordance with the research conducted by Kolaib et al. [16].

Limitations and future directions

This study has some limitations that should be considered when interpreting the findings and conclusions. First, this is a descriptive, cross-sectional study, which limits the possibility of establishing cause-and-effect relationships between the examined variables. Second, the validation of the used test (IAT) of the Croatian population is not entirely adequate for the validation of the Serbian population. Additionally, response biases, which can often be difficult to eliminate in self-reported survey research like this one, may have influenced respondents' opinions, thus limiting the results of this study.

In addition to the mentioned limitations, it should be kept in mind that this research has important theoretical and practical significance when it comes to understanding the predictors of IA among adolescents. The preventative efforts ought to center on helping adolescents make good use of their own leisure time. The areas of emotional and social competence, responsible use of media content, and current technology should be the focus of prevention initiatives as well as treatment for youth with high levels of internet dependence. Early program interventions on appropriate and safe internet use are essential to lower the likelihood of high levels of IA during the adolescent years.

Conclusion

According to the findings of the present study, the relationship between IA and age, addictive substance use frequency, purpose, and time spent on the Internet has been proven. The largest number of respondents had a moderate addiction to the Internet. Adolescents with IA will more likely have lower grades in school. The use of psychoactive substances was not a significant predictor of developing IA.

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Conflict of interest

The authors declare no conflict of interest.

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Salivary kallikrein-8 as a favorable biomarker for stress response

Salivarni kalikrein-8 kao pogodan biomarker odgovora na stres

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Abstract

Background/Aim. Kallikreins (KLKs) are a group of serine protease enzymes capable of cleaving protein peptide bonds. Besides, they are proteolytic enzymes that mediate the conversion of kininogen (alpha 2-globulin) to bradykinin or kallidin. The aim of the study was to examine whether KLK8 might serve as a novel stress biomarker. **Methods**. Twenty-four students (17 female and 7 male) were included in the study. The general and dental health of the students were evaluated in the appropriate anamnesis format. Unstimulated samples were collected by Sarstedt® saliva collection tubes as recommended: 08.00–09.00 am, 12.00, and 2.00–3.00 pm on the exam day. KLK levels were measured by a KLK8 Human ELISA kit. **Results.** The salivary KLK8 levels in the morning (1.25 ± 0.26 pg/mL) were statistically significantly lower than the KLK8 levels pre-exam [at 12.00 (2.89 ± 0.85 pg/mL)] ($p = 0.0006$). There was also a significant difference in salivary KLK8 levels between pre- and post-exam (1.69 ± 0.39) time points ($p = 0.0005$). **Conclusion.** These results show that the differences in salivary KLK8 levels might be related to the degree of stress, indicating that KLK8 may serve as a novel stress biomarker.

Key words:
biomarkers; kallikreins; oral health; saliva; stress, psychological; students, dental.

Apstrakt

Uvod/Cilj. Kalikreini (KLK) su grupa serin proteaza, enzima koji su sposobni za cepanje peptidnih veza proteina. Pored toga, oni su proteolitički enzimi koji posreduju u konverziji kininogena (alfa 2-globulina) u bradikinin ili kalidin. Cilj rada bio je da se utvrdi da li bi KLK8 mogao biti novi biomarker za stres. **Metode.** Studijom su obuhvaćena ukupno 24 studenta (17 ženskog i 7 muškog pola). Opšte i dentalno zdravlje studenata bilo je procenjivano odgovarajućom anamnezom. Nestimulisani uzorci bili su prikupljani u epruvetama za sakupljanje pljuvačke Sarstedt® na sledeći način: u 08,00–09,00, u 12,00 i u 14,00–15,00 sati na dan ispita. Nivoi KLK mereni su *ELISA* metodom za humani KLK8. **Rezultati.** Jutarnji salivarni nivoi KLK8 (1,25 ± 0,26 pg/mL) bili su statistički značajno niži od nivoa KLK8 pre ispita [u 12,00 sati (2,89 ± 0,85 pg/mL)] ($p = 0,0006$). Takođe postojala je značajna razlika između salivarnih nivoa KLK8 pre i posle ispita (1,69 ± 0,39) ($p = 0,0005$). **Zaključak.** Navedeni rezultati pokazuju da razlike u nivoima salivarnog KLK8 mogu biti povezane sa stepenom stresa, što ukazuje na to da KLK8 može služiti kao novi biomarker stresa.

Ključne reči:
biomarkeri; kalikrein; oralno zdravlje; pljuvačka; stres, psihički; studenti stomatologije.

Introduction

The concept of stress, by definition, refers to the psycho-physiological response of a person to the physiological, mental, and social events that one encounters throughout daily life, which are defined as harmful [1, 2]. In the face of undesirable events, a person can respond to the event with physiological, emotional, cognitive, and behavioral changes. In addition to these adverse effects, stress can also cause severe changes in the hormonal system [3].

Depending on whether acute or chronic stress (CS) forms, changes can occur in the nervous, cardiovascular, endocrine, and immune systems [4, 5]. Although these changes constitute the stress response, they are usually adapted for the short term. In acute stress, glucocorticoids directly inhibit the hypothalamic-pituitary-adrenal (HPA) axis activity, while in CS, steroids can exert direct stimulating effects on the brain. The effects of acute and CS may be independent of each other [6]. Acute stress can trigger allergic reactions such as asthma, eczema, urticaria, gastrointestinal symptoms, high

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blood pressure, pain, and psychotic problems such as panic attacks [7]. CS increases adrenal glucocorticoid levels and can lead to harmful cognitive functions. CS can also cause psychological conditions such as depression and anxiety, muscle and bone problems, sleep disorders, cardiovascular diseases such as hypertension, and metabolic diseases such as obesity and diabetes [2].

The physiological response to stress consists of practical and interconnected systems to maintain bodily integrity, even in the most challenging conditions [8]. One of the most essential complex responses of the organism is the stress response, and this stress response can create adaptive processes [5, 9, 10]. All living organisms must sense and respond to conditions that underlie their homeostatic mechanisms [5, 11]. In addition, severe and prolonged stress responses may activate the nervous system's defense mechanism and damage the organism.

Kallikreins (KLKs) are the human origin family of 15 serine proteases differentiated by their physical properties [12, 13]. They have high substrate specificity defined by their ability to form bradykinin, a vasoactive protein from kininogen [14]. Some are expressed in very few tissues, while others are highly expressed. The protease effect is always unidirectional.

KLKs or KLK-related peptidases catalytic mechanism can be explained by substrate binding, formation of the acyl-enzyme intermediates, transition state stabilization, peptide bond cleavage, release of products, and regeneration of the enzymes that are all facilitated by the active site residues and surrounding protein structure. KLKs are synthesized as inactive zymogens (proenzymes) and are secreted into the extracellular environment. Once activated, they can cleave peptide bonds between the amino acids arginine and lysine in proteins and peptides, thereby exerting their proteolytic activity. This activation can occur through various mechanisms, including proteolytic cleavage by other enzymes or autoactivation [15]. They are expressed in multiple tissues, mainly secreted from the pancreas, salivary glands, pituitary, prostate, and testicles, and take part in several physiological processes. Their primary functions are tissue remodeling and angiogenesis [16], directly related to physical processes such as neurodegeneration [17] and inflammation [18] and others, such as neuronal plasticity, regulation of blood pressure, and electrolyte balance regulation of cell growth and differentiation. Furthermore, recent reports suggest that many other members of this family are related to ovarian and prostate cancers, as well as to diverse diseases of the central nervous system [13, 15].

The basis of salivation is the sympathetic and parasympathetic branches of the autonomic nervous system that innervate the salivary glands. The use of salivary biomarkers to assess stress states in humans has received much attention, as reactions of the sympathetic nervous system (SNS) [19, 20] induce stress markers. Salivary KLKs, a group of substrate-specific serine proteases, are found in secretory granules within salivary gland cells and are secreted into saliva in direct response to glandular sympathetic nerve stimulation and local norepinephrine release [21–27]. Due to this relationship between SNS activity and salivary KLK release, it was thought that the activities of human salivary KLKs might change during the exam stress associated with the response mediated by increased SNS activity [26, 28].

The aim of the study was to determine whether KLK8 would serve as a potential stress biomarker. Still, it was mainly a biological plausibility to hypothesize that level changes could reflect the physiological stress response. Besides, the selection depends on measurability, potential specificity, clinical implications, and previous research findings [29–31]. Salivary KLK levels were determined to confirm the occurrence of a stress response and its severity. Exam stress was evaluated to determine whether sensory afferent activation occurs, and we proposed that KLK8 could be used as a new stress biomarker.

Methods

Subjects

Twenty-four (17 female and 7 male) students with a mean age of 22.5 ± 0.95 years were included in the study. All described protocols were approved by the Ethics Committee of the Gazi University, Faculty of Medicine in Turkey (Ethical Approval No. 831, from November 12, 2018). Informed consent was obtained from all students. The presence of any disease that may affect the HPA axis was accepted as an exclusion criterion.

Saliva sampling

We used Sarstedt® (Sarstedt AG&Co. KG, Germany) saliva tubes explicitly designed to collect and store saliva samples. These tubes were made from a combination of polypropylene (PP) and low-density polyethylene (LD-PE), which provides superior sample quality and quantity performance.

Before collecting unstimulated saliva samples from young adults, they were instructed not to brush their teeth, smoke, eat, or drink anything besides water for at least an hour. Individuals were asked to keep cotton wool in their mouths for one minute to ensure it was completely saturated with saliva.

Biochemical analysis

Saliva was collected from the students in the morning (08.00–09.00 am), before the exam (12.00), and after the exam (2.00–3.00 pm) on the exam day.

Samples were centrifuged at 3,200 × g for 30 min at +4 °C, divided into Eppendorf tubes, and stored at -80 °C until the study was conducted.

Salivary kallikrein-8 concentration

The concentration of KLK8 in saliva (pg/mL) was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit KLK8 USCN CEA690Hu Wuhan

USCN Busines Co. Ltd., ELISA sensitivity: 1.73 pg/mL. The intra-assay and inter-assay coefficients of variation were less than 10% and 12%, respectively.

Statistical analysis

Data analysis was performed using the SPSS 18 and Jamovi programs. The means and standard deviations of the samples were used. Parametric one-way ANOVA and Tukey *post hoc* tests evaluated the data comparisons of the groups. Multiple comparisons were conducted using a *post hoc* test to pinpoint which groups contributed to this discrepancy. The values of $p < 0.05$ were considered significant.

Results

The students' mean age and body mass index (BMI) were 22.5 ± 0.95 years and 20.4 ± 0.93, respectively. BMI was calculated for the homogeneity of our group.

Figure 1 indicates the results of the salivary KLK8 levels of the students at different times of the day. The results of each time point in this study suggested differences in KLK8 concentrations in saliva samples which were low during the morning, increased before, and decreased after the exam.

Along with the perception of stress, salivary KLK8 levels were changed between morning, pre-, and post-exam (1.25 ± 0.26 pg/mL, 2.89 ± 0.85 pg/mL, 1.69 ± 0.39 pg/mL, respectively). Higher stress (academic exam) was associated with higher KLK8 levels. With a significance level of $p < 0.001$, it was observed that a notable difference existed between the averages of the groups being compared concerning the examined feature.

When morning salivary KLK8 levels were juxtaposed with pre-exam KLK8 levels, a difference of -1.64 in the averages of these groups was identified. Given a *p*-value of $p < 0.05$, it can be inferred that a significant distinction existed between the averages of the two groups. Notably, the mean KLK8 levels in samples taken before the exam were statistically higher than those collected in the morning. Due to the disparity between the averages in the *post hoc* test, it is evident that a difference existed between the averages of these two groups ($p < 0.001$).

When comparing the salivary pre- and *post*-exam KLK8 levels, the difference between the averages of these groups was found to be 1.20. With a resulting *p*-value of 0.0005, it was determined that there was a significant difference between them.

In the comparison between morning and *post*-exam salivary KLK8 levels, the difference between their averages was found to be -0.44. As the *p*-value was greater than 0.05, no significant difference was found between them. Looking at the average KLK levels in the morning (1.25 pg/mL) and *post*-exam (1.69 pg/mL), it is apparent that the morning levels were statistically lower than those taken before the exam. However, since there was no difference between the averages in the *post hoc* test, it could be inferred that the averages of these two groups are equivalent. Despite their apparent difference, there is no statistically significant distinction between them ($p = 0.164$) (Figure 1).

Discussion

The current study evaluated the differences in saliva KLK8 levels during stress status and suggested that KLK8 might serve as a new, favorable stress biomarker.

The results indicate a potential relationship between stress and salivary KLK8 levels, providing insights into the physiological responses to stress in academic exams.

As a biological fluid that can be analyzed for diagnostic purposes, saliva can be considered a stress-free alternative to blood draw because it is easy, non-invasive, and effortless compared to serum.

KLKs are a subgroup of serine proteases that undertake various physiological functions in human metabolism. Until recently, KLK-1, KLK-2, and KLK-3 were known as human KLKs. However, recent studies have identified up to 12 new

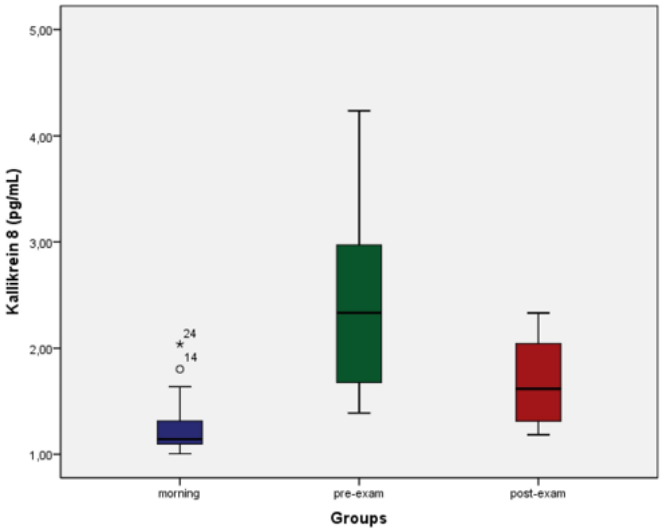

Fig. 1 – Salivary kallikrein concentration was measured at three different times of day.
***p* < 0.001.**
***Note*: numbers 14 and 24 indicate the outliers of sample 14 and 24 within the group.**

members of the KLK family, and some of these new members have been reported to have essential functions in nervous system injuries and diseases [32]. Many studies show that KLKs have the potential to be biomarkers of cancer and neurological diseases.

Based on the provided excerpt, it appears that Smith-Hanrahan [28] conducted a study investigating the association between salivary KLK output and the stress response to surgery. Saliva samples were collected to determine salivary KLK output. These samples were likely obtained using a saliva collection method, such as the passive drool technique or saliva collection devices, both before and several times after surgery. This suggests a longitudinal study design, where samples were collected at multiple time points to track changes in salivary KLK output over time, particularly in response to the stress of surgery. The study's results indicate that increased salivary KLK output is associated with the stress response to surgery [28].

Overall, the study by Smith-Hanrahan [28] suggests that salivary KLK output may be a valuable biomarker for assessing the stress response to surgery. However, it is essential to consider factors such as sample size, study population, and potential confounding variables when interpreting the results. Our findings, which aimed to investigate the effects of stress on salivary KLK8 levels at different intervals, showed that psychological factors (academic exam stress) could influence KLK8 saliva levels. Exam stress is one of the leading causes of emotional stress in students. Therefore, in this study, the salivary level of KLK8 was significantly lower in the pre-exam group than in the *post*-exam group, which indicated the effects of academic stress and was the first study that demonstrated the relationship between KLK8 and stress. The number of exams that young people experience is increasing and overwhelming and hurts mental and physical well-being. The direction of the KLK8 response to a stressor may be related to motivational and emotional factors. However, it is unclear which alterations in saliva mediate the observed change in KLK8.

Salivary biomarkers are well established in psychoneuroimmunology research as measures of stress. Our previous study showed a significant increase in cortisol and amylase levels, known as stress biomarkers, in response to pre- and *post*-exam stress [33]. Our analysis also revealed that the KLK8 level can be used as a new biomarker, as it directly correlates with increased stress. When we look at all these results, saliva sampling plays a potential role for noninvasive, real-time, and point-of-care biomarkers [34]. One of the critical benefits of Sarstedt Salivette® tubes is their ability to perform well with small volumes of samples, which is essential in ensuring that the sample collected is representative of the individual being studied. Additionally, the material used in the tubes is of high quality, ensuring the sample remains stable and protected throughout the collection and storage process. Their high analytical performance makes them a valuable tool in research studies.

Conclusion

Our research findings showed significant changes in KLK8 levels in the morning and before and after the exam, suggesting a relationship between KLK8 and stress response. In addition, this research suggested a possibility of salivary KLK8 being evaluated among stress biomarkers. Therefore, studies aimed at expanding the understanding of the various effects of psychosocial factors and processes on physiological responses to stress can use and benefit from this feasible and noninvasive type of measure. It would be interesting to conduct an additional study regarding this issue in a broader group based on the promising findings presented in this paper. Moreover, further validation studies are necessary to confirm its utility and establish its reliability and sensitivity in assessing stress levels.

Conflict of interest

The authors declare no conflict of interest.

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CASE REPORT

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Hodgkin transformation of chronic lymphocytic leukemia/small lymphocytic lymphoma

Hočkinova varijanta transformacije hronične limfocitne leukemije/limfoma malih limfocita

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Abstract

Introduction. In rare cases, chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma may transform into Hodgkin lymphoma, with about a hundred cases reported in the literature so far. We present a case of the Hodgkin variant of Richter transformation. **Case report.** After a one-year watch and wait period, a 60-year-old male with CLL developed B symptoms, generalized lymphadenopathy, and splenomegaly. Upon initial staging (Binet B/Rai 3), he was started on fludarabine, cyclophosphamide, and rituximab (FCR) regimen. After the third cycle of treatment, the right-sided axillary lymphadenopathy persisted and became painful, while the dimensions of the remaining organs affected by the disease decreased. Upon the finalization of the final sixth FCR cycle, the painful right-sided axillary lymphadenopathy persisted (though partially decreasing in size), with the development of local redness and swelling. A biopsy of the residual axillary node was performed, which revealed disease transformation into Hodgkin lymphoma. Upon multislice computed tomography-based staging (IV E clinical stage) and prognostic assessment (unfavorable prognosis), it was decided that the treatment be continued with doxorubicin, vinblastine, dacarbazine (AVD) regimen. The presented patient died two months after the diagnosis of Hodgkin transformation (HT) was established during the initial cycle of AVD. **Conclusion.** Although CLL is an indolent malignancy, in rare cases of HT, the prognosis is largely dismal. The available treatment strategies demonstrate suboptimal results, although novel immunotherapies may change the landscape of HT therapy in the near future.

Key words:
antineoplastic combined chemotherapy protocols; immunohistochemistry; histological techniques; hodgkin disease; leukemia, lymphocytic, chronic, b-cell; multidetector computed tomography; treatment outcome.

Apstrakt

Uvod. Hronična limfocitna leukemija (HLL)/limfom malih limfocita se u retkim slučajevima može transformisati u Hočkinov limfom, a u literaturi je do sada opisano oko sto ovakvih slučajeva. Prikazujemo bolesnika sa Hočkinovom varijantom Rihterove transformacije. **Prikaz bolesnika.** Godinu dana nakon početka kontrolnog praćenja, kod 60-godišnjeg muškarca sa HLL došlo je do razvoja B simptoma, generalizovane limfadenopatije i splenomegalije. Posle početnog određivanja stadijuma bolesti (Binet B/Rai 3), započeto je lečenje prema protokolu fludarabin, ciklofosfamid, rituksimab (FCR). Nakon trećeg ciklusa lečenja i dalje se održavala i postajala bolna, desnostrana pazušna limfadenopatija, dok su se dimenzije preostalih, bolešću zahvaćenih organa, smanjile. Po okončanju poslednjeg, šestog ciklusa FCR terapije, bolna desnostrana pazušna limfadenopatija se održavala (iako se delimično smanjila u veličini), sa razvojem lokalnog crvenila i otoka. Biopsijom zaostalog pazušnog nodusa dokazana je transformacija bolesti u Hočkinov limfom. Nakon određivanja stadijuma bolesti na osnovu nalaza multislajsne kompjuterizovane tomografije (IV E klinički stadijum) i procene prognoze (nepovoljna prognoza), odlučeno je da se lečenje nastavi prema protokolu doksorubicin, vinblastin, dakarbazin (AVD). Bolesnik je preminuo dva meseca nakon potvrde dijagnoze Hočkinove transformacije (HT), u toku sprovođenja prvog AVD. **Zaključak.** Mada je HLL sporo progresivna maligna bolest, u retkim slučajevima sa HT prognoza je nepovoljna. Dostupne terapijske mogućnosti daju suboptimalne rezultate, mada bi u skorijoj budućnosti inovativne imunološke terapije mogle izmeniti tok lečenja bolesnika sa HT.

Ključne reči:
lečenje kombinovanjem antineoplastika, protokoli; imunohistohemija; histološke tehnike; hočkinova bolest; leukemija, b ćelije, hronična; tomografija, kompjuterizovana, multidetektorska; lečenje, ishod.

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Introduction

Chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) is an indolent B-cell malignancy and the most common malignant disease in hematology [1].

CLL/SLL complications significantly contribute to both the morbidity and mortality of the affected patients. While infectious complications occur in 80% of CLL/SLL patients, other complications [secondary malignancies, autoimmune cytopenias, and Richter transformation (RT)] are less common [2–5]. An uncommon CLL/SLL complication, which is a result of disease transformation into more aggressive lymphomas or leukemias, is known as RT [5, 6].

We present a case of the Hodgkin transformation of CLL/SLL in a 60-year-old male patient.

Case report

In February 2022, a complete blood count performed on a 59-year-old male prior to eye surgery revealed isolated lymphocytosis. Upon further investigation, a diagnosis of CLL/SLL was established based on morphological and flow cytometric findings of peripheral blood (Matutes CLL score 4). The disease was initially staged as Binet A (Rai 0), requiring only regular follow-up.

The first symptoms developed in March 2023 with the onset of fatigue and night sweats, as well as generalized indolent lymphadenopathy (the largest of which were axillary nodes, ~ 5 cm in size) and splenomegaly (ultrasound bipolar diameter – 175 mm).

The patient was hospitalized and treated at the Hematology, Allergology, and Clinical Immunology Clinic, University Clinical Center Niš, Serbia, once each month between April and December 2023.

Being a fit patient with a symptomatic Binet B (Rai 3) stage CLL [lymphocytes (Ly) 7.7 × 10^9/L, reference range (RR): 1.0–4.0 × 10^9/L; white blood cells (WBC) 13.8 × 10^9/L, RR: 4.0–10.0 × 10^9/L], developing anemia [hemoglobin (Hgb) 104 g/L, RR: 110–170 g/L] (Table 1), with progressive lymphadenopathy, he was started on fludarabine, cyclophosphamide, rituximab (FCR) (six cycles) treatment in April 2023. After the third cycle of therapy, our patient experienced painful right-sided axillary lymphadenopathy (ultrasound: 31 × 9.5 mm, and a conglomerate 42 × 22 mm in size), while other peripheral lymph nodes slightly decreased in size, and the spleen became impalpable.

Upon the fourth cycle of therapy, he developed fever (39.4 °C) and night sweats, in addition to the persistent painful axillary lymphadenopathy (~ 5 cm), which was treated with antibiotics and supportive therapy and did not require surgery.

The sixth cycle of therapy was finalized in September 2023.

The right-sided axillary lymphadenopathy persisted, although partially decreasing in size, with the pain, redness, and swelling spreading to the right arm, causing its weakness. In October 2023, an excisional biopsy of the rest axillary lymph node was performed. Histological (hematoxylin and eosin) (Figure 1) and immunohistochemistry findings (Figure 2) supported the diagnosis of the Hodgkin transformation of CLL/SLL. A whole body computed tomography scan was performed in November 2023, revealing bilateral cervical, right-sided axillary (64 × 22 mm conglomerate), mediastinal, retroperitoneal, and pelvic lymphadenopathy, splenomegaly, focal changes in spleen and liver, as well as pleural, pericardial, and abdominal effusions (Figure 3). Drainage from the biopsy site persisted for 48 hrs, and since the fever developed, a swab was taken. Corynebacterium spp. were isolated, and antibiotic treatment (linezolid) followed.

On the day of the final admission (on December 12, 2023) to the Clinic, the patient brought the latest laboratory results (six days prior, from another laboratory) [Ly 0.31 × 10^9/L, RR: 1.19–3.35 × 10^9/L; WBC 3.83 × 10^9/L, RR: 4.0–10.7 × 10^9/L; Hgb 76 g/L, RR: 130–170 g/L; Platelets 172 × 10^9/L, RR: 150–400 × 10^9/L; C-reactive protein (CRP) 174.08 mg/L, RR: < 5.0 mg/L; erythrocyte sedimentation rate (ESR) 140 mm/1st hr, RR: < 20 mm/1st hr].

Being a fit, 60-year-old patient with an unfavorable advanced (IV E Ann Arbor clinical stage) Hodgkin transformation of CLL/SLL, it was decided that upon the resolution of the febrile syndrome treatment be continued with doxorubicin, vinblastine, dacarbazine (AVD) regimen.

Table 1

The dynamics of hematological and biohumoral parameters throughout treatment (April–December 2023)

Parameter	Type, month/day and cycle (C) of therapy								
	FCR 04/28 C1	FCR 05/28 C2	FCR 06/23 C3	FCR 07/24 C4	FCR 08/24 C5	FCR 09/22 C6	– 10/12 –	– 11/23 –	AVD 12/16 C1
White blood cells (×10^9/L)	13.8	12.7	8.9	10.6	18.4	4.0	5.1	7.7	3.1
Hemoglobin (g/L)	104	108	109	101	119	97	112	82	87
Platelets (×10^9/L)	269	181	259	198	279	202	229	112	27
Lymphocytes (×10^9/L)	7.7	3.4	–	1.4	3.3	0.7	1.3	1.2	1.1
Neutrophils (×10^9/L)	5.3	8.3	4.5	8.0	13.2	2.9	2.4	3.4	0.9
C-reactive protein (mg/L)	–	55.8	–	67.9	–	127.5	14.4	125.4	208.6
Albumin (g/L)	–	39	–	35	–	33	36	24	22
Lactate dehydrogenase (U/L)	–	471	–	574	–	506	503	513	445

FCR – fludarabine, cyclophosphamide, rituximab; AVD – doxorubicin, vinblastine, dacarbazine.
Note: The first line therapy of chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) was performed in the period from April–September; Rest axillary lymph node biopsy was performed in October; Diagnosis of Hodgkin lymphoma (HL) transformation of CLL/SLL was made in November; HL therapy was initiated in December.

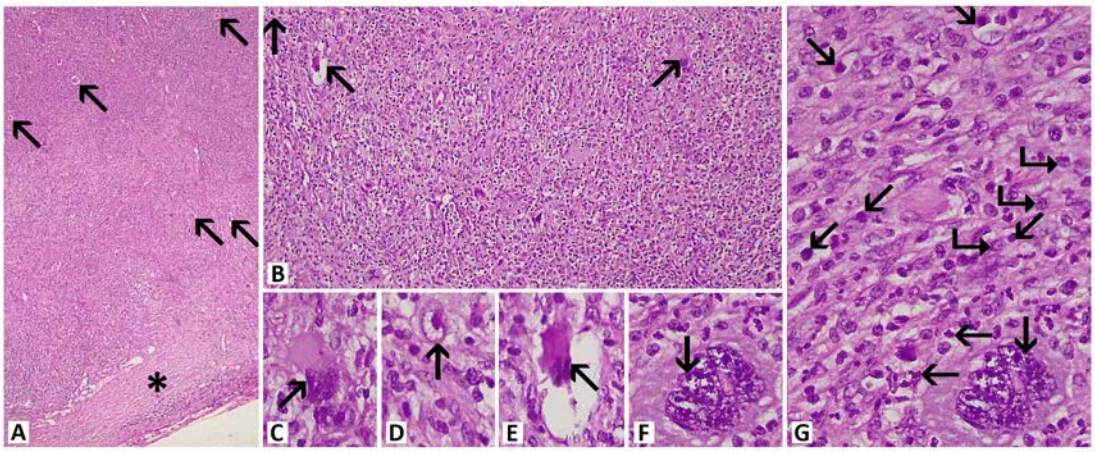

Fig. 1 – Morphological features which support the diagnosis of the Hodgkin transformation (nodular sclerosis) in a patient with prior chronic lymphocytic leukemia/small lymphocytic lymphoma: A) Lacunary Reed-Sternberg cells (RSC) (↖) are visible on low power field inside the parenchyma of an enlarged lymph node, which has a thickened capsule (*). Lymph node parenchyma is separated by irregular gracile bands of fibrous connective tissue [Hematoxylin and Eosin (HE), × 40]; B) Neoplastic tissue is heterogenous, with numerous (5% of total cellularity) large, neoplastic, lacunary (↖), pleiomorphic, mononuclear (↑), and polynuclear (↗) variants of RSC, inside an inflammatory milieu (HE, × 100); C) Polynuclear RSC (↗) – detail; D) Mononuclear Hodgkin cell (↑) – detail; E) Lacunary RSC (↖) – detail; F) Pleiomorphic RSC (↓) – detail; G) The heterogeneous inflammatory milieu consisting of small lymphocytes (↙), eosinophils (↘), neutrophils (←), and histiocytes (↳). C-G (HE, × 200).

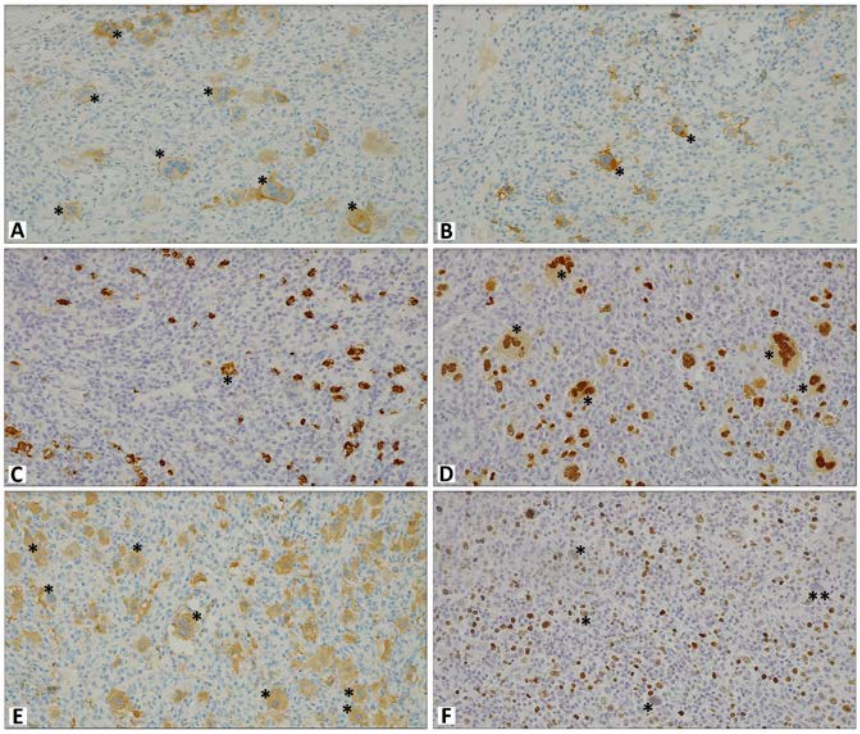

Fig. 2 – Immunohistochemistry (IH) features of the Hodgkin transformation in a patient with prior chronic lymphocytic leukemia/small lymphocytic lymphoma. The immunophenotype of Hodgkin-Reed-Sternberg cells (HRSCs) of the presented patient: $CD30^{+}$, $CD15^{+}$, $MUM1^{+}$, $Pax5^{+}$, $Fascin^{+}$, $EMA^{+/-}$, $LMP1^{-}$, $CD3^{-}$, $CD20^{-}$. A) CD30 membrane positivity of HRSCs (*); B) CD30 positivity of the Golgi zone (*) of HRSCs; C) CD15 positivity of the cytoplasm of a HRSCs (*); D) Weak Pax5 positivity of HRSCs (*); E) Fascin positivity of HRSCs (*); F) The majority of HRSCs demonstrate Ki67 nuclear positivity (indicating proliferation) (*), while other HRSCs are ki67 negative (**). A-F (IH, × 100).

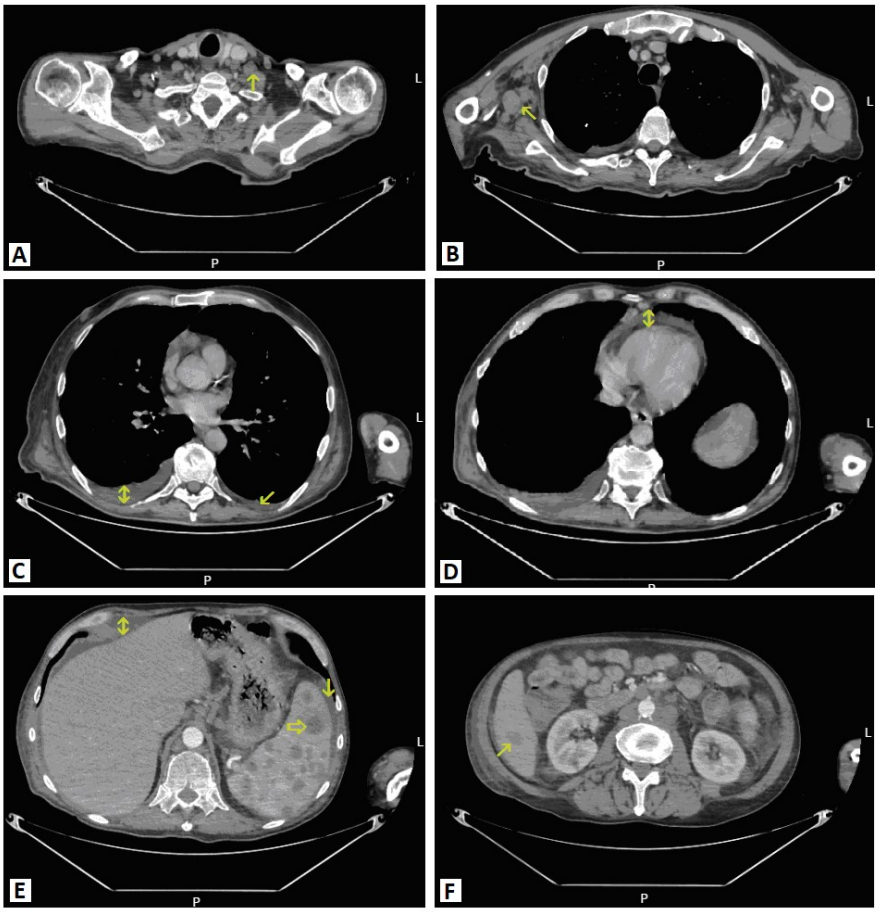

Fig. 3 – Multislice computed tomography of the patient with the Hodgkin transformation of chronic lymphocytic leukemia: A) Bilateral cervical lymphadenopathy, with the largest lymph node on the left (↑); B) Lymph node conglomerate in the right axilla with signs of central necrosis (↖); C) Both right-sided (↕) and left-sided (↙) pleural effusion; D) Pericardial effusion (↕) with the density of simple fluid next to the right ventricle of the heart; E) Perihepatic (↕) and perisplenic (↓) collection of simple fluid (ascites) as well as an enlarged spleen (170 mm) with numerous focal hypodense lesions, the largest of which is located near the lower pole of the spleen (⇨); F) The largest hypodense lesion in the S6 segment of the liver (↗).

Prior to the initiation of the first cycle of AVD treatment, on December 16, 2023, further laboratory analyses were performed [alkaline phosphatase (ALP) 202 U/L, RR: 30–120 U/L; total bilirubin 28.2 μmol/L, RR: 5.0–21.0 μmol/L; direct bilirubin 13.1 μmol/L, RR: 0.0–3.4 μmol/L; cholesterol 1.59 mmol/L, RR: 3.9–5.2 mmol/L; CRP 208.6 mg/L, RR: < 5.0 mg/L; albumin 22 g/L, RR: 35–52 g/L; lactate dehydrogenase (LDH) 445 U/L, RR: 220–450 U/L; aspartate transaminase 31 U/L, RR: 10–37 U/L; alanine transaminase 34 U/L, RR: 10–42 U/L].

Unfortunately, the patient died due to disease progression on December 23, 2023, during the initial cycle of AVD, about two months after the diagnosis of Hodgkin lymphoma (HL) was established.

Discussion

RT develops in about 2–10% of CLL/SLL patients [7, 8]. RT is named after the American pathologist Dr. Maurice Richter, who, in 1928, first published a case report of a male with lymphocytic leukemia who had died in just one month upon rapidly developing progressive lymphadenopathy and organomegaly [5, 6, 9, 10]. However, it was not until 1964 when Dr. Lortholary and colleagues correctly suggested that the disease transformation might be related to the underlying CLL and proposed the term “Richter syndrome” to name this phenomenon [5, 6, 10].

Even though the majority of CLL/SLL transformation cases consist of disease transformation into diffuse large B-cell lymphoma (80–90%), other less frequent variants have also been described, such as transformation into HL (10–20%), however, all other variants (Burkitt lymphoma, multiple myeloma, plasmablastic lymphoma, lymphoblastic lymphoma, T-cell lymphoma, hairy cell leukemia, prolymphocytic leukemia) comprise less than 1% of RT cases [5, 6, 8, 10, 11].

CLL/SLL causes a secondary immunodeficiency (associated with impaired immune surveillance), which, together with specific aberrations (TP53 mutation; 17p deletion) pre-

sent in some CLL/SLL cases, provides a setting for the development of secondary hematological and non-hematological malignancies [1, 12, 13]. Moreover, antineoplastic chemoimmunotherapy (such as FCR) is also a major contributing factor to the development of cancer in CLL/SLL patients [4].

One might imagine it would be appropriate to determine whether a newly diagnosed hematological malignancy in a CLL/SLL patient is a coexisting *de novo* disease (clonally independent) or a result of a true RT, in which an aggressive lymphoproliferative disease is clonally related to the underlying CLL/SLL [11].

However, in the case of the Hodgkin transformation of CLL/SLL, it is impossible to make such a distinction in routine clinical practice.

The malignant Hodgkin-Reed-Sternberg (HRS) cells are scattered throughout the affected lymph nodes, comprising no more than 1–5% of total lymph node cellularity, making microdissection (which would be essential for performing genomic studies) substantially difficult [14, 15].

Another reason for the lack of insight into the genetic mechanisms of Hodgkin transformation of CLL/SLL is the fact that the Hodgkin transformation is a rare clinical entity, with only about a hundred reported cases in the literature so far [8, 16].

This is also the main reason for insufficient studies regarding the efficacy of various treatment options in this group of patients.

Recent studies suggest that the Hodgkin transformation of CLL/SLL might be a two-step process, beginning with isolated HRS cells forming inside an environment containing scarce inflammatory cells (CLL-HRS), which is going to be replaced with mixed inflammatory background later on (CLL-HL) [17].

However, based on the findings of immunohistochemical studies, it seems that there are no major differences between the immunophenotype of HRS cells found in the Hodgkin transformation of CLL/SLL, and those found in classic HL (cHL) [17, 18].

Thus, all HL cases in patients with a previous CLL/SLL diagnosis are considered RT [17].

Excisional lymph node biopsy with subsequent morphological and immunohistochemical microscopic analyses is considered the gold standard for diagnosing the Hodgkin variant of RT [5].

Although LMP1 positivity of HRS cells is found in 60–80% of patients with Hodgkin transformation of CLL/SLL [17], this was not the case with our patient, whereas typical (although not obligatory) CD20 negativity was observed.

Typical clinical manifestations of RT (worsening of B symptoms, rapid enlargement of the lymph nodes, rapid organomegaly, increase in LDH levels) were mostly absent in our patient, with the exception of rapid splenomegaly. On the other hand, the patient had extranodal affection of both the spleen and liver, which may be attributed to the Hodgkin transformation since such findings have been previously reported in the Hodgkin transformation of CLL/SLL [19]. We did not perform biopsies of the affected extranodal sites because we strived to initiate treatment as soon as possible, as we expected to achieve a therapeutic response of affected extranodal sites upon treatment. The mechanisms of hepatic granulomatosis present in HL are not fully understood; however, the results of certain studies suggest the importance of the secretory activity of HRS cells in the development of granulomas and fibrosis (delayed hypersensitivity) [19, 20]. In December 2023, the presented patient had a moderate increase in serum ALP (202 U/L), which could be attributed to the existing hepatic lesions. The increase in serum ALP levels had already been reported in a case of the Hodgkin transformation of CLL/SLL with hepatic involvement, indicating an unfavorable prognosis [19]. Some other hepatic parameters were also abnormal in case of our patient, such as elevated levels of both total and direct bilirubin (28.2 μmol/L and 13.1 μmol/L, respectively), as well as hypocholesterolemia (cholesterol 1.59 mmol/L) and hypoalbuminemia (albumin 22 g/L) causing pericardial, bilateral pleural, and abdominal effusions, indicating impairment of both excretory and synthetic functions of the liver. The levels of both LDH and transaminases were normal. There was an increase in the level of inflammatory markers (CRP 174.08 mg/L, ESR 140 mm/1st hr, prior to the final admission to the Clinic on December 12, 2023), which could have been attributed to both HL and the resolution of a prior postoperative wound infection.

Our patient had hardly any risk factors associated with the development of RT (such as young age, markedly elevated LDH, and multiple CLL relapses) except for anemia [19].

On the contrary, at the moment of establishing the RT diagnosis, our patient had almost all of the established cHL risk factors (age $\geq$ 50 years, elevated ESR, at least four supradiaphragmatic nodal areas, extranodal disease, at least three nodal areas on both sides of the diaphragm) except for a large mediastinal tumor mass [21].

Based on the clear survival benefit, it is justifiable that patients with the Hodgkin transformation of CLL/SLL should be treated with chemotherapy protocols used for cHL since the efficacy of CLL-based regimens is far inferior in these circumstances [1, 16, 17].

Besides being the most commonly used protocol for the treatment of Hodgkin transformation of CLL/SLL, doxorubicin, bleomycin, vinblastine, and dacarbazine (ABVD) seems to be also the most efficient regimen in this indication, with an estimated median overall survival of 13.2 years [16]. It is not clear whether brentuximab vedotin–AVD is less efficient than ABVD since brentuximab vedotin–AVD was usually the treatment of choice for older, frail patients with a higher International Prognostic Score (IPS), who were more likely to have a poor prognosis regardless of the chosen treatment option (brentuximab vedotin–AVD vs. ABVD), whereas the younger, more fit patients, with a lower IPS, usually received ABVD, being a more aggressive option [16]. Other less used and less efficient therapeutic alternatives include brentuximab vedotin monotherapy, cyclophosphamide, vinblastine, procarbazine, and prednisone (CVPP), nitrogen mustard, vincristine, procarbazine, prednisone (MOPP), rituximab, cyclophosphamide, vincristine, prednisone (R ± CHOP), and

FCR regimens [8, 11, 16]. The strongest predictor of survival of the patients with the Hodgkin variant of RT is achieving complete remission upon ABVD treatment [22]. Unlike for cHL, in the case of Hodgkin transformation of CLL/SLL, most clinicians avoid high-dose chemotherapy (HDT) followed by hematopoietic stem cell transplantation (HSCT) (HDT + HSCT) upon achieving the first complete remission (CR1), since these patients are usually elderly (> 70 years), with significant comorbidities, making them unfit for such aggressive treatment, whereby performing HDT + HSCT after CR1 demonstrated no survival benefit in patients with a Hodgkin variant of RT [16].

Based on the achievements reported in individual cases, there may be reasons to assume that novel immunotherapies (such as brentuximab vedotin or checkpoint inhibitors) could change the treatment paradigm and prognosis of affected patients in due time [18].

Since fluorescence *in situ* hybridization of bone marrow blood was not performed prior to the initiation of FCR, the 17p deletion/TP53 mutation status of our patient remains unknown. Although fludarabine may promote the development of the Hodgkin variant of RT due to immunosuppression, this complication has been reported in CLL/SLL patients on Bruton's tyrosine kinase (BTK) inhibitor treatment as well [16, 19, 23]. Therefore, we could speculate that the Hodgkin variant of RT would have occurred in our patient regardless of the therapeutic option chosen for the first-line treatment of CLL/SLL (FCR vs. BTK inhibitor). According to the indications provided by the Republic Fund of Health Insurance (Belgrade, Serbia), ibrutinib is currently the sole BTK inhibitor available for first-line therapy of CLL/SLL only for patients with a documented 17p deletion/TP53 mutation [24].

The Hodgkin transformation of CLL/SLL was considered to have a more unfavorable prognosis than *de novo* cHL [11, 16]. However, based on the results of the largest retrospective multicentric study performed on a cohort of 94 cases of the Hodgkin transformation of CLL/SLL, it seems that there are no major differences prognostic-wise [16].

IPS [male gender; age $\geq$ 45 years; IV Ann Arbor clinical stage (CS); albumin level < 40 g/L; Hgb < 105 g/L; Ly < 0.6 × 10^9/L and/or Ly < 8%; WBC $\geq$ 15 × 10^9/L; each awarded 1 point] and Richter Syndrome Score (RSS) [Eastern Cooperative Oncology Group performance status (ECOG-PS) > 1; LDH level 1.5 times greater than the upper limit of normal; thrombocytopenia; tumor mass at least 5 cm in size or larger; more than one prior line of therapy – each awarded 1 point] are used for the prognostic assessment of the Hodgkin variant of RT [5, 16, 25, 26].

Our patient had developed several episodes of febrile neutropenia during the course of FCR treatment and was therefore treated with granulocyte colony-stimulating factor (CSF-G) on multiple occasions. He was also a chain-smoker for almost four decades. Since he was an elderly heavy smoker who would potentially require CSF-G support during the course of further treatment, it was decided that bleomycin be omitted from the ABVD in order to avoid lung toxicity [21, 27, 28].

Since our patient had a high IPS = 6 (male, 60 years old, IV Ann Arbor CS, albumin 22 g/L; Hgb 76 g/L; Ly 0.31 × 10^9/L) and an intermediate RSS = 2 (ECOG-PS = 2, lymph node conglomerate in the right axilla 64 × 22 mm in size), even though without markedly elevated LDH, we considered him to be a patient with an unfavorable prognosis.

Although patients with the Hodgkin transformation of CLL/SLL may have similar survival rates compared to the patients with *de novo* cHL, Stephens et al. [16] reported that 5% of patients with the Hodgkin variant of RT die within two months of RT diagnosis, which was the case with our patient as well.

Conclusion

For most cases, CLL currently remains an incurable B-cell malignancy with a generally favorable prognosis, being a slowly progressive disease with an adequate response to the existing therapeutic options. However, in a small portion of patients who develop RT, the prognosis is largely dismal. The treatment of the Hodgkin transformation of CLL remains an unmet need, although novel immunotherapies may potentially alter the outcome of such patients in the near future.

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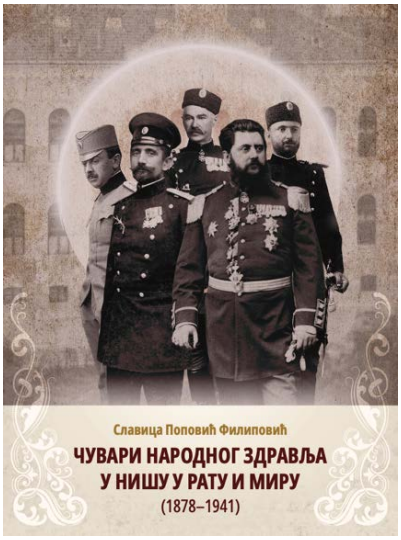
BOOK REVIEW/PRIKAZ KNJIGE

The guardians of public health in Niš in war and peace (1878–1941)

Čuvari narodnog zdravlja u Nišu u ratu i miru (1878–1941)



Author/Autor: Slavica Popović Filipović
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ISBN: 978-86.335-0804-9

"The Guardians of Public Health in Niš in War and Peace (1878–1941)" was written by Slavica Popović Filipović, B.A. in the English language and literature, translator, publisher, researcher, and writer of the history of Serbian medicine and the world and Serbian philanthropy. She authored 10 books and over 100 research papers in the above fields.

This book was published on the occasion of the 145th Anniversary of the Military Hospital in Niš. It is dedicated to all public health workers in Niš, in war and peace, who participated in the development and prosperity of the Serbian medicine and Serbian Medical Corps. All events from 1878 until 1941 are presented chronologically and laid out clearly, including the founding of various institutions, a listing of important dates, biographies of outstanding individuals, original reports, and personal writings of the contemporaries.

The book has 594 pages and it is divided into 11 Chapters: The Great Military Hospital of Niš; the Niš District Hospital and Municipal Physicians in Niš; From the First Royal Serbian Military L. Pasteur Research Center to the modern Institute for Public Health; The Leaders of the Serbian Military Medical Corps; First Women Physicians and Pharmacologists; Foreigners in the Serbian Medical Corps and public health in Niš and its district; Medical Assistants in the Morava Permanent Military Hospital in the Great War; Volunteer Nurses, Qualified Nurses, and humanitarian women workers; Establishment of the first Pharmacies and development of the Pharmaceutical guild in Niš; The "Morava" Permanent Military Veterinary Hospital and the Serbian Red Cross Committee in Niš.

The Chapter "Leaders of the Serbian Military Medical Corps" is dedicated to the following outstanding individuals: Dr. Sava V. Popovic, General of the Medical Corps; Prof. Dr. Mihailo V. Petrovic and Dr. Vladimir Stanojevic, both Generals of the Medical Corps; Dr. Jovan Hadzi-Bogdanovic and Dr. Dragutin S. Petkovic, both Colonels of the Medical Corps; Dr. Aleksa Savich and Dr. Milan Petrovic, both Lieutenant Colonels of the Medical Corps. The book has 313 original photos (many from personal family albums, first time published). There are about 1,000 listed references from Serbian history, foreign sources of literature, and contemporary international newspapers.

"The Guardians of Public Health in Niš in War and Peace (1878–1941)" presents an objective, unbiased, and analytical approach to the history of not just the Military Hospital in Niš but of the whole health system in Niš and its environs from 1878 until 1941. Included are many important details from the history of Niš and Serbia, cultural events, health conditions, and wartime epic suffering lasting almost 65 years. It was also a picture of a difficult, dynamic, and turbulent life in Serbia at that time when there were three regional wars and one world war – the period of immense

wartime suffering, cataclysmic upheavals, and historic changes at the local, state, and global – international level. All that impacted the work of the health professions, their active participation, and the whole Serbian people.

Only ten days after the end of the Second Serbian-Turkish War, on January 22, 1878, Dr. Vladan Djordjević initiated the formation of the Great Military Hospital in Niš, today's Military Hospital in Niš. Under his guidance, the first military medical hospital was established with 1,000 beds spread over five sections. From those first formative days of the well-organized medical services up to today, during 145 years of its operation, many generations of health workers of different profiles have contributed by their dedicated work and creativity to the high reputation of the Military Hospital in Niš.

This significant volume, like the majority of the books published by this author so far, aims to enlighten, uncover, and bring closer to the health professionals and the general public the rich tradition of Serbian medicine by using positive examples to encourage the formation and strengthening of appropriate professional, ethical, and patriotic principles within present and future generations of physicians, and other health professionals, as well as the wider community. That way, we shall appreciate better and face the difficulties, problems, and challenges we are facing today.

This manuscript also sends a message that transcends time through epochs: no civilization should suffer such a great tragedy as war, its consequences, and deep wounds. This is something that both present and future generations should be well aware of. All our potential energy, strengths, skills, and creativity should be used for general well-being and towards a better and more humane world.

"The Guardians of Public Health in Niš in War and Peace (1878–1941)" will be of great benefit to historians, physicians, medical students, professional soldiers, as well as a wider general public not only around the city of Niš but also throughout Serbia. In her extensive and all-encompassing book, the author included the writings of the past times but left room for further new research.

Brigadier General (ret.)
Veljko Todorović, MD, Ph.D.

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Rukopis se piše sa proredom 1,5 sa levom marginom od **4 cm**. Koristiti font veličine 12, a načelno izbegavati upotrebu **bold** i *italic* slova, koja su rezervisana za podnaslove. Originalni članci, opšti pregledi i metaanalize i članci iz istorije medicine ne smeju prelaziti 16 stranica (bez priloga); aktuelne teme – deset, seminar praktičnog lekara – osam, kazuistika – šest, prethodna saopštenja – pet, a komentari i pisma uredniku – tri, izveštaji sa skupova i prikazi knjiga – dve stranice.

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a) Poželjno je da naslov bude kratak, jasan i informativan i da odgovara sadržaju, podnaslove izbegavati.

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Na drugoj stranici nalazi se strukturisani apstrakt (250-300 reči za originalne članke i meta-analize) sa naslovom rada. Kratkim rečenicama na srpskom i engleskom jeziku iznosi se **Uvod/Cilj** rada, osnovne procedure **– Metode** (izbor ispitanika ili laboratorijskih životinja; metode posmatranja i analize), glavni nalazi **– Rezultati** (konkretni podaci i njihova statistička značajnost) i glavni **Zaključak**. Naglasiti nove i značajne aspekte studije ili zapažanja. Strukturisani apstrakt za kazuistiku (do 250 reči), sadrži podnaslove **Uvod, Prikaz bolesnika** i **Zaključak**). Ispod apstrakta, „Ključne reči“ sadrže 3–10 ključnih reči ili kratkih izraza koje ukazuju na sadržinu članka.

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Tekst sadrži sledeća poglavlja: **uvod**, **metode**, **rezultate** i **diskusiju**. **Uvod.** Posle uvodnih napomena, navesti cilj rada. Ukratko izneti razloge za studiju ili posmatranje. Navesti samo važne podatke iz literature a ne opširna razmatranja o predmetu rada, kao ni podatke ili zaključke iz rada o kome se izveštava.

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Rezultate prikazati logičkim redosledom u tekstu, tabelama i ilustracijama. U tekstu naglasiti ili sumirati samo značajna zapažanja.

U **diskusiji** naglasiti nove i značajne aspekte studije i izvedene zaključke. Posmatranja dovesti u vezu sa drugim relevantnim studijama, u načelu iz poslednje tri godine, a samo izuzetno i starijim. Povezati zaključke sa ciljevima rada, ali izbegavati nesumnjive tvrdnje i one zaključke koje podaci iz rada ne podržavaju u potpunosti.

Literatura

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Primeri referenci:

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Balint B. From the haemotherapy to the haemomodulation. Beograd: Zavod za udžbenike i nastavna sredstva; 2001. (Serbian)

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Tabele

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

Ilustracije

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

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

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

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

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