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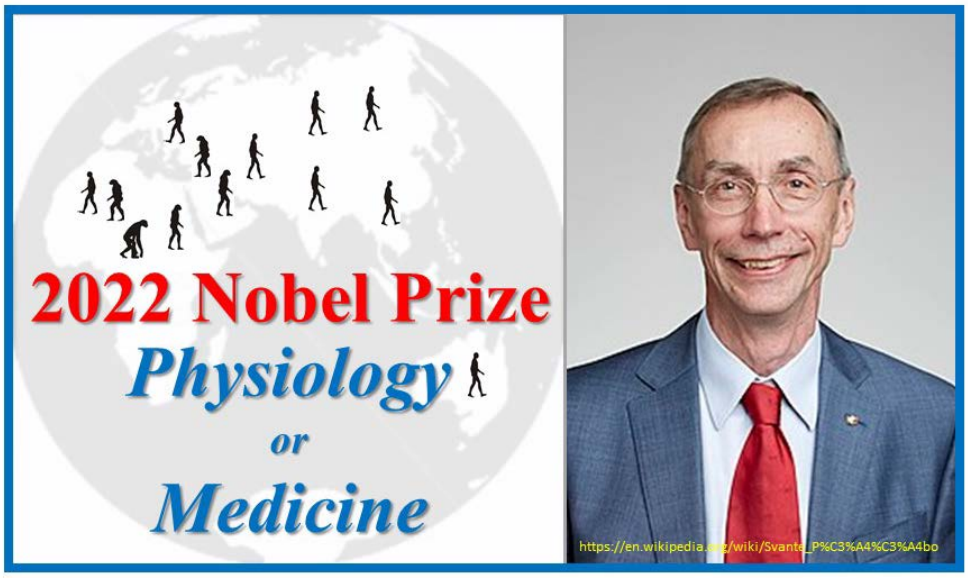

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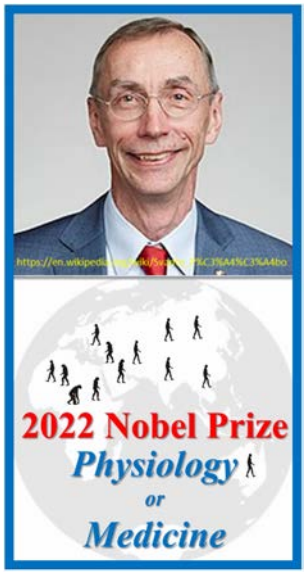

The winner of the Nobel Prize in Physiology or Medicine 2022 for discoveries concerning the genomes of extinct hominins and human evolution is Svante Pääbo, who succeeded in sequencing the genome of the Neanderthal, discovered a previously unknown hominin (*Denisova*) and discovered that gene transfer occurred from now-extinct hominins to *Homo sapiens* following the migration out of Africa (around 70,000 years ago). This ancient flow of genes to present-day humans has physiological relevance today, for instance, it affects how our immune system reacts to infections, which is especially important in the time of the pandemic that we have been living in for more than two years now.

Dobitnik Nobelove nagrade za fiziologiju ili medicinu 2022. godine za otkrića u vezi sa genomima izumrlih predaka čoveka i evolucijom čoveka je Svante Pebo koji je uspeo da sekvencionira genom neandertalca, otkrije ranije nepoznatog pretka čoveka (*Homo denisova*), kao i to da se transfer gena sa tih izumrlih predaka u *Homo sapiens* dogodio nakon migracije iz Afrike (pre oko 70 000 godina). Protok gena od drevnih vremena do današnjih ljudi ima fiziološku važnost, na primer utiče na to kako naš imunski sistem reaguje na infekcije, što je posebno važno u vremenu pandemije u kome živimo već duže od dve godine.

ORIGINAL ARTICLES

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The transition from conformal to advanced radiotherapy techniques in the treatment planning of gynecological cancer patients

Prelaz sa konformalne na napredne radioterapijske tehnike u planiranju lečenja obolelih od ginekoloških karcinoma

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Abstract

Background/Aim. The transition from standard to highly conformal radiation therapy techniques requires the implementation of complex advanced dosimetry. The aim of the study was to compare dosimetric parameters of the three-dimensional conformal radiotherapy (3DCRT) and volumetric modulated arc therapy (VMAT) plan, as well as complications after treatment in relation to dosimetric parameters in gynecological cancer patients. **Methods.** A total of 49 gynecological cancer patients were included in the study. All patients were planned for 3DCRT, but due to unacceptable doses to organs at risk (OARs), treatment plans for intensity modulated radiation therapy (IMRT), or VMAT, were generated for 21 patients. The patients were prescribed 50.4 Gy/28 fractions (4 patients) and 45 Gy/25 fractions (45 patients). The coverage of planning target volume (PTV) and doses to OARs were recorded. PTV margins were evaluated for both techniques according to the Van Herk formula. **Results.** ICRU 83 criteria were fulfilled in all 3DCRT/VMAT/IMRT plans providing optimal coverage of PTV. Doses to OARs, on average, the V45Gy in the small bowel in IMRT/VMAT plans was four times smaller than the same in 3DCRT plans. The V45 Gy of small bowels was, on average, 49.4 cm^3 in IMRT/VMAT plans, while in 3DCRT plans, it was 211.6 cm^3. In the case of the femoral head, a significant reduction in V30Gy (10.8% vs. 33.1%) and mean dose in the case of IMRT/VMAT plans was recorded (30.4 Gy in 3DCRT vs. 23.6 Gy). Rectum was planned with a significantly lower dose in terms of V30Gy (79.5% vs. 95.2%) in IMRT/VMAT plans. The bladder was better spared in VMAT plans in terms of V40Gy (51% vs. 91%), but the maximum dose was higher in VMAT plans than in 3DCRT (50.1 Gy to 48.1 Gy on average). For all OARs, there was a statistically significant difference registered at $p < 0.05$. Toxicities recorded in VMAT and 3DCRT patients included mainly radiation-induced cystitis and enteritis. Patients treated with 3DCRT generally had longer recovery time. The homogeneity index was 0.11 for VMAT plans and 0.09 for 3DCRT plans. **Conclusions.** Analysis of dosimetric parameters revealed significant differences in normal tissue doses for the same 3DCRT and VMAT patients, which confirmed the necessity for the implementation of advanced techniques for as many patients as possible.

Key words:
genital neoplasms, female; radiotherapy; radiotherapy, conformal; radiotherapy planning, computer-assisted; treatment outcome.

Apstrakt

Uvod/Cilj. Prelaz sa standardnih na visokokonformalne radioterapijske tehnike zahteva implementaciju kompleksne dozimetrije. Cilj rada bio je da se uporede dozimetrijski parametri trodimenzionog konformalnog plana (3DCRT) i lučnog zapreminski modulisanog plana zračenja (VMAT), kao i komplikacije nakon tretmana i veza sa dozimetrijskim parametrima kod bolesnica sa ginekološkim malignitetima. **Metode.** Ukupno 49 bolesnica sa ginekološkim malignitetima su bile uključene u studiju. Sve bolesnice su bile planirane za 3DCRT terapiju, ali zbog neprihvatljivih doza na organe pod rizikom (OPR), generisani su i isporučeni terapijski planovi u tehnici VMAT/radioterapija sa podesivim intenzitetom zračenja (IMRT) za 21 bolesnicu. Bolesnicama je propisana apsorbovana doza 50,4 Gy/28 frakcija (4 bolesnice) i 45 Gy/25 frakcija (45 bolesnica). Praćene su pokrivenosti planiranog ciljnog volumena (PCV) i doze na OPR. Margine PCV evaluirane su u obe tehnike prema formuli Van Herka. **Rezultati.** Kriterijumi ICRU 83 bili su ispunjeni u svim 3DCRT/VMAT/IMRT planovima i pokazali su optimalnu pokrivenost PCV. Doze od V45Gy

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na tankim crevima u IMRT/VMAT planovima bile su skoro četiri puta manje zapremine nego one u 3DCRT planovima (srednja vrednost zapremine obuhvaćene 45 Gy izodozom u IMRT/VMAT planu bila je 49,4 cm^3, dok je u 3DCRT planu iznosila 211,6 cm^3. U slučaju glave femura zabeleženo je značajno smanjenje doze V30Gy (10,8% vs. 33,1% kod 3DCRT) i smanjenje srednje doze u slučaju IMRT/VMAT planova (30,4 Gy vs. 23,6 Gy u 3DCRT). Rektum je u IMRT/VMAT planovima primio značajno manju dozu V30Gy (79,5% vs. 95,2% u 3DCRT). Bešika je bila bolje sačuvana u VMAT planu V40Gy (51% vs. 91% kod 3DCRT), ali su maksimalne doze veće kod VMAT planova nego kod 3DCRT (50,1 Gy Gy vs. 48,1). Za sve OPR registrovana je statistički značajna razlika ($p < 0,05$) između dve tehnike. Toksičnosti koje su praćene i kod 3DCRT i kod VMAT bolesnica bile su uglavnom, radijacioni cistitis i enteritis. Bolesnice lečene 3DCRT oporavljale su se duže od posledica zračenja. Indeks homogenosti bio je 0,11 za VMAT i 0,09 za 3DCRT planove. **Zaključak.** Analiza dozimetrijskih parametara otkrila je značajne razlike između doza na zdrava tkiva u 3DCRT i VMAT planovima, što potvrđuje neophodnost implementacije naprednijih tehnika zračenja za što veći broj radikalno lečenih bolesnika.

Ključne reči:
polni organi, ženski, neoplazme; radioterapija; radioterapija, konformalna; radioterapija, kompjutersko planiranje; lečenje, ishod.

Introduction

Cervical and uterine cancers are global public health care problems since they are the fourth most frequent cancers in females worldwide, after breast, colorectal, and lung cancer. Cervical cancer represents nearly 7% of all female cancers globally. Its incidence in developed and developing countries is not equal and varies from 2 to 75 per 100,000 women, while the mortality in developed and developing countries varies largely [1]. The average age at diagnosis worldwide is 53, and the average age at death from cervical cancer is 59. It is ranked highly as one of the top three cancers affecting women younger than 45.

The situation in the Republic of Serbia is somewhat similar for *cervix uteri*, but another cancer contributes to the cancer burden – *corpus uteri*. The Republic of Serbia had over 7 million inhabitants in 2017 [2, 3], of which 51.3% were women. Epidemiological data [2, 4] show that 5.8% of all new yearly cancers were *cervix uteri*, and 7% of all new cases were *corpus uteri*, and still increasing. Serbia is the third highest ranked country in Europe in the incidence of cervical and uterine cancer, after Moldavia and Bulgaria. Most newly diagnosed patients are 45–49 years old.

Treatment modalities of cervical or corporal cancer are multidisciplinary and include surgery, chemotherapy, and radiotherapy (RT). The main problem in RT treatment of any site is how to appropriately cover the planning target volume (PTV) with as high a dose as possible and at the same time minimally irradiate organs at risk (OARs), which are very often in the nearest vicinity of the PTV. Sometimes even PTV and close OARs overlap, and it is impossible to deliver the prescribed dose to PTV without delivering a significant dose to the OARs.

During the last 20 years in the developed world, intensity-modulated radiotherapy (IMRT) has become a standard RT treatment. In Serbia, due to a long-lasting economic crisis, RT patients were offered 2.5 dimensional (D) and 3D conformal radiotherapy (CRT) plans.

The concept of advanced techniques in RT is actually very old and consists of standard static fields, where the movement of a gantry is added and is synchronized with the movement of a multileaf collimator (MLC), as well as a controlled dose rate. Powerful com puters handle these complex movements, and the machine delivers highly conformal dose distribution to a patient. In recent years, not only has a huge investment of the Serbian government in RT improved access to RT services but also enabled the implementation of highly conformal techniques in RT, thus improving the overall outcome of patient treatment. The Oncology Institute of Vojvodina introduced advanced techniques into clinical practice in 2016 after cumbersome verification of advanced treatment modalities.

The paper deals with two different RT approaches to the treatment of cervical and uterine cancer, 3DCRT, as standard, and advanced treatment modality IMRT and volumetric modulated arc therapy (VMAT) as newly implemented one.

Methods

A total of 49 randomly selected patients from the treatment planning logbook included in the study were irradiated between January 2016 and December 2019. The patients were identified from the hospital registry together with their clinical and treatment data. Treatment planning data were taken from the treatment planning system.

All patients were initially planned for the 3DCRT technique. Due to heavy dose load to the small bowels, a subset of 21 patients was re-planned for VMAT or IMRT and treated, whilst another subset (the remaining 28 patients) was treated with 3DCRT according to the treatment plan. For the subset treated with VMAT/IMRT, a comparative analysis of their clinically applied VMAT and initially planned 3DCRT treatment parameters was presented in this work. For this subset of 21 VMAT/IMRT patients, daily imaging was performed, and after completing all treatments, clinical target volume (CTV) to PTV margins coming from interfraction motions recorded in the record and verify system were evaluated according to the Van Herk formula [5]. Additionally, these margins were also evaluated for a subset of 28 3DCRT treated patients, according to the same protocol.

All patients (both subsets) were followed-up in the following time frame: during the treatment or immediately

after treatment, one month after the treatment, and 6 months after the treatment (after the 6th month, patients were followed-up by their oncologists). The complications were collected from the hospital registry system [noted during their treatment and control examinations according to the Radiation Therapy Oncology Group (RTOG) toxicity grading system], evaluated and compared.

The Radiotherapy Clinic, Oncology Institute of Vojvodina, is equipped with two Versa HD linear accelerators (manufactured by Elekta, Crawley, UK). The 3DCRT treatment plans were generated by collapsed cone algorithm, while VMAT and IMRT plans were generated by the Monte Carlo calculation engine, both in the Monaco treatment planning system (Elekta, Crawley, UK).

The beams were verified according to the end-to-end dosimetry audit for 3DCRT and VMAT/IMRT [5], as recommended by the International Atomic Energy Agency. The beams are regularly calibrated and verified biannually in thermos-luminescence dosimeter (TLD) postal dose audits.

For 3DCRT plans, treatment strategy basically includes box technique with segments field-in-field, while VMAT plan includes one or two arc techniques or static IMRT 7 field techniques.

The dose was prescribed to the PTV according to the adopted clinical protocols of the Clinic and included a prescription of 45 Gy/25 daily fractions or 50.4 Gy/28 daily fractions. The treatment plans were evaluated based on the dose volume histograms and the International Commission of Radiation Units and Measurements (ICRU) recommendations 62 and 83. The dosimetric parameters were evaluated according to RTOG1203, which was designed to compare late toxicities in pelvic cervical and endometrial treatments with standard box and IMRT [6].

The toxicities between the two groups (3DCRT and VMAT/IMRT) were statistically compared using Fisher's exact test.

Results

Patient demographics

At the time of prescription and treatment in the selected group of patients, the distribution of the age of all women was as follows: there were no patients younger than 39 or older than 73. The mean age of the patients was 55.

The distribution of diagnosis was as follows: the group treated with 3DCRT – 68% of patients had a diagnosis of *cervix uteri* cancer (C53 according to International Classification of Diseases and Related Health Problems − ICD 10), while 32% had *corpus uteri* cancer (C54). The staging was evaluated according to the International Federation of Gynecology and Obstetrics – FIGO classification, where 46% of patients were FIGO I, 46% FIGO II, 7% FIGO III, and none in FIGO IV. In IMRT/VMAT group – 57% of patients had a diagnosis of *cervix uteri* cancer (C53), while 43% had *corpus uteri* cancer (C54). Staging: 38% of patients were FIGO I, 38% were FIGO II, 19% were FIGO III, and 5% were FIGO IV.

The patients in 3DCRT irradiated group were also treated with neoadjuvant chemotherapy (total of 4 patients) and concurrent cisplatin chemotherapy (18 patients). The comorbidities were detected for 16 patients out of 28 [*hypertensio arterialis* (10), diabetes mellitus (7), arrhythmia (3), and renal insufficiency (2)]. The patients in VMAT/IMRT irradiated group were also treated with neoadjuvant chemotherapy (4 patients) and concurrent cisplatin chemotherapy (11 patients). The comorbidities were detected for 14 patients out of 21 [*hypertensio arterialis* (9), arrhythmia (1), asthma bronchiale (1), epilepsy (1), varicose veins (1), and ulcerative colitis (1)].

Brachytherapy modality was used as combined therapy with external beam RT in all patients included in the study.

Radiotherapy treatment

According to the institutional protocol, all patients were advised to fill in the bladder and empty bowels and rectum before computed tomography (CT) scanning and every treatment. CT scanning was done on a 3 mm slice distance (CT simulator model Definition As Open, manufacturer Siemens, Germany). Patients were scanned in the supine position, with immobilizing cushions for knees and feet.

All patients were treated according to clinically adopted radiotherapy protocols, their staging, and the type of illness.

CTV included primary tumor, cervix, entire uterus, parametrial and paravaginal tissue, and proximal vagina. If there was minimal or no vaginal tumor extension, the upper half of the vagina was included. In patients with involvement of the upper vagina, the proximal two-thirds were included in CTV and the whole vagina if there was more vaginal involvement. Moreover, CTV included regional lymph nodes as common iliac, external and internal iliac, presacral, and nodes close to the medial edge of the obturator muscle. An additional margin of up to 1 cm was added to CTV to represent PTV.

OAR delineation was performed for the small bowels cavity, femoral heads, bladder, and rectum.

Treatment planning was performed as a four-field box for 3DCRT with a 15 MV beam, 2 arcs for VMAT, or 7 fields for IMRT treatment with a 10 MV beam. The calculation was done with a 3 mm grid size resolution. Since the 3DCRT, as well as VMAT/IMRT treatment plans, were made for the subset of patients treated with VMAT/IMRT, the PTV dosimetric evaluation was performed through the evaluation of ICRU83 parameters of both plans [7]: the doses (DS) to 50%, 95%, 98% and 2% of the considered volume (DS50%, DS95%, DS98% and DS2%, respectively) and homogeneity index (Table 1).

The conformity index was not compared as it is proven to be much better for highly conformal treatments such as VMAT/IMRT than for 3DCRT.

Table 1

PTV 95% reference coverage, D50%, D98%, D2%, and HI according to ICRU83 and data obtained from the dosimetric evaluation of 3DCRT and VMAT/IMRT plans of same patients

Variable	ICRU 83	3DCRT plans	VMAT/IMRT plans
PTV 95% reference coverage	> 95% D	97.0 ± 1.03	98.3 ± 1.3
DS50%	100–102% D	101.3 ± 0.6	101.2 ± 0.8
DS98%	< 95% D	95.04 ± 1.3	94.6 ± 1.5
DS2%	< 107% D	104.6 ± 0.7	105.9 ± 0.9
HI	Ideally 0	0.09 ± 0.017	0.11 ± 0.012

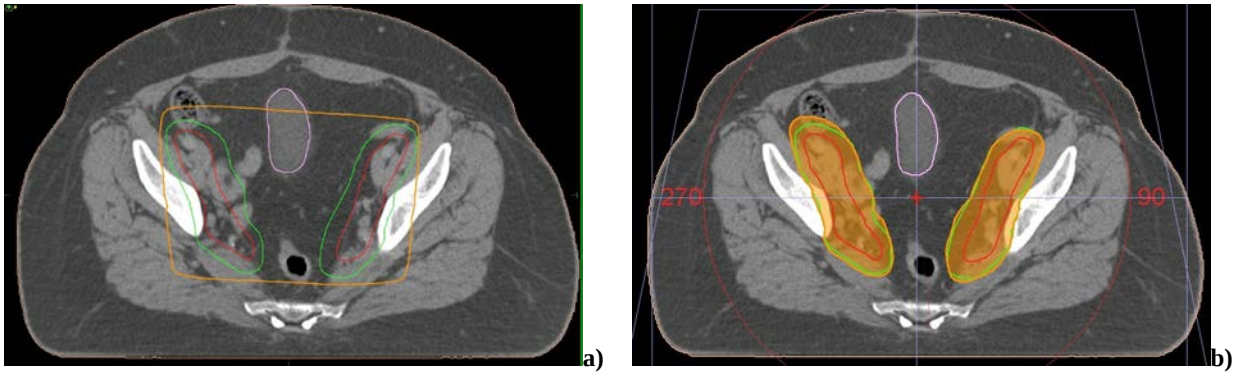
Values are expressed as mean ± standard deviation, minimum–maximum, or < of percentage dose (DS). ICRU – International Commission of Radiation Units and Measurements; 3DCRT – three-dimensional conformal radiotherapy; PTV – planning target volume; VMAT – volumetric modulated arc therapy; IMRT – intensity modulated radiation therapy; HI – homogeneity index.

Table 2

Organ at risk constraints according to RTOG1203 and data obtained from the dosimetric evaluation of 3DCRT and VMAT/IMRT plans of the same subset of patients

Organ at risk	RTOG 1203	3DCRT plans	VMAT/IMRT plans
Small bowel (the average volume of small bowel in the subset 492 cm^3)	Less than 30% of volume receives 40 Gy V40 < 30% V45 cm^3 < 195 cm^3	V40 = 304.2 ± 176 cm^3 (61.8 %) V45 = 211.6 ±143 cm^3	V40 = 140.8 ± 70 cm^3 (28 %) V45 = 49.4 ± 32 cm^3
Rectum	Less than 80% of the volume receives 40 Gy V40 < 80%	V30 = 95.2 ± 6.1 %	V30 = 79.5 ±12.3 %
Bladder	Less than 35% of the volume receives 45 Gy V45 < 35%	V45 = 65.5 ± 28.8 %	V45=27.0 ±12.3%
Femoral heads	Less than 50% of the volume receives 30 Gy V30 < 50%	L: V30 = 33.1 ± 22.2 % R: V30 = 30.6 ± 21.0 %	L: V30 = 10.8 ± 8.7% R: V30 = 9.7 ± 7.5%

Values are expressed as mean ± standard deviation.
RTOG – Radiation Therapy Oncology Group; 3DCRT – three-dimensional conformal radiotherapy; VMAT – volumetric modulated arc therapy; IMRT – intensity modulated radiation therapy.



a) b)

Fig. 1 – A 95% isodose distribution in: a) typical three-dimensional conformal radiotherapy (3DCRT) treatment plan; b) typical volumetric modulated arc therapy (VMAT)/ intensity modulated radiation therapy (IMRT) plan.

Organs at risk dosimetry evaluation

For the first subset of patients, planned both for 3DCRT and VMAT/IMRT and irradiated by VMAT, significantly lowered doses were registered in VMAT/IMRT treatment plans (Table 2).

Typical dose distributions are shown in Figure 1: a) in the same patient 3DCRT technique, and b) in VMAT/IMRT technique.

Clinical examination and follow-up comparison of the small bowel and bladder complications in two techniques – standard 3DCRT group vs. VMAT/IMRT group

All patients are followed-up during RT treatment delivery, one month after the last fraction and six months after the treatment. The parameters followed-up were radiation-induced cystitis and enteritis, both graded, and

other effects such as fistula or small bowel obstruction. The early effects on OARs were analyzed, and dose-volume dependence was determined for small bowels.

Table 3 shows acute effects in two examined groups treated with 3DCRT and VMAT/IMRT techniques.

It appeared that toxicity rates were significantly higher for the 3DCRT group at 1 and 6 months after treatment ($p < 0.05$), while it was the opposite during and immediately after treatment for the VMAT/IMRT group. The toxicities recorded within the VMAT/IMRT group after 1 month and after 6 months of follow-up were not statistically significant at $p < 0.05$, as well as for the group 3DCRT, but between groups, there is statistical significance recorded in toxicities.

Table 3

Acute effects (complications) of radiation treatment

Time of complication occurrence	No complications registered in the group		Radiation-induced cystitis		Radiation-induced enteritis		Small bowel obstruction	
	3DCRT subset (28 pts)	VMAT/IMRT subset (21 pts)	3DCRT subset	VMAT/IMRT subset	3DCRT subset	VMAT/IMRT subset	3DCRT subset	VMAT/IMRT subset
During or immediately after treatment	46.4	47.6	7.1 (GII)	23.8 (4.8 GIII, 9.6 GII, 9.6 GI)	25.0 (20 GII, 5 GIII)	19.0 (14.3 GI, 4.7 GII)	-	-
1 month after the last fraction	71.4	57	10.7 (7 GII, 3 GI)	14.3 (GI)	10.7 (GI)	9.5 (GI)	1 patient	-
6 months after treatment	75	61.9	17.8 (3.6 GIII, 3.6 GII, 10.6 GI)	9.5 (GI)	7.1 (GI)	6.3 (GI)	1 patient	-

Note: 3DCRT subset included 28 patients; VMAT/IMRT subset included 21 patients.
G – toxicity grading according to the Radiation Therapy Oncology Group (RTOG); 3DCRT – three-dimensional conformal radiotherapy; VMAT – volumetric modulated arc therapy; IMRT – intensity modulated radiation therapy.

Table 4

Average shifts during daily imaging in VMAT/IMRT and 3DCRT patients

Parameter	3DCRT	VMAT/IMRT
x (cm)	0.35 ± 0.22	0.32 ± 0.17
y (cm)	0.38 ± 0.22	0.37 ± 0.20
z (cm)	0.27 ± 0.22	0.30 ± 0.22
Calculated margins (cm)	x = 0.82; y = 0.78; z = 0.74	x = 0.75; y = 0.64; z = 0.75

Values are expressed as mean ± standard deviation.
3DCRT – three-dimensional conformal radiotherapy; VMAT – volumetric modulated arc therapy; IMRT – intensity modulated radiation therapy.

According to the daily shifts recorded in the RV system, which originated from daily kV-kV pairs or cone-beam CT (CBCT) imaging, for both groups of patients (3DCRT and VMAT), the PTV margins were calculated and evaluated according to the Van Herk formula [5] and instructions. The imaging was performed in the first three fractions and then weekly. The tolerance limit was 5 mm.

The results obtained are shown in Table 4.

Discussion

This study compared dosimetric parameters for the target and OARs in 3DCRT and VMAT/IMRT techniques in gynecological patients treated at our Institute between 2016 and 2019. It was found that the coverage and homogeneity of the PTV were similar for VMAT and 3DCRT treatment plans, which complies with literature data [6, 8–11].

VMAT/IMRT is not only a highly conformal treatment technique but also enables much better sparing of OARs, neighboring to the clinical targets. Target coverage in both VMAT/IMRT and 3DCRT was practically very similar, but this was not the case with OARs, where doses for small bowel, rectum, bladder, and femoral heads were significantly higher for 3DCRT plans than in VMAT/IMRT, and the doses also carried an accompanying risk of acute and later effect.

Parallel analysis of dose-volume histograms for two different techniques in the same subset of patients revealed that the V45Gy in the small bowel in IMRT/VMAT plans was four times smaller than the same of 3DCRT plans (49.4 cm^3 in IMRT/VMAT plans vs. 211.6 cm^3). In the case of the femoral head, a significant reduction in V30Gy (10.8 % vs. 33.1%) and mean dose in the case of IMRT/VMAT plans was recorded (30.4 Gy in 3DCRT vs. 23.6 Gy). Rectum was planned with a significantly smaller dose in terms of V30Gy (79.5% vs. 95.2%) in IMRT/VMAT plans. The bladder was better spared in VMAT plans in terms of V40Gy (51% vs. 91%), but the maximum dose was higher in VMAT plans than in 3DCRT (50.1 Gy to 48.1 Gy on average). For all OARs, a statistically significant difference was registered at $p < 0.05$.

The RTOG1203 trial, whose dosimetric limits were used in this work, concluded that pelvic IMRT/VMAT is associated with significantly fewer toxicities than standard RT from the patient perspective, which was confirmed here. Other literature data show that the use of advanced techniques instead of four field boxes (3DCRT) significantly reduces the grade of acute toxicity, as demonstrated in our work.

Acute effects were similar in both groups. Since doses for the OARs were smaller in VMAT/IMRT group, patients recovered faster than in the 3DCRT group, which correlates to data found in literature [9]. It is also important to mention that there were no GIII toxicities in VMAT/IMRT group during follow-up, which were fairly often seen in 3DCRT patients.

In 2010, Quantitative Analysis of Normal Tissue Effects in Clinic [8] summarized the dose-volume relationship for many OARs, but the data for small bowel were very limited, providing one high dose parameter for the dose-volume constraint. During many years of clinical experience, we have noticed that this Quantitative Analysis of Normal Tissue Effects (QUANTEC) 2010 parameter V45 < 195 cm^3 was not sufficient to predict toxicity to small bowels, so other parameters were used, in accordance with literature data [6, 9]. Literature data found a positive correlation between late effects of the small bowel and small bowel volume parameters in cases of cervical radiotherapy. But these parameters were different between the studies, review of the literature showed some studies recommended a V40 < 340 cm^3, while others recommended a V15 < 275 cm^3 [6, 9].

As for the small bowel and radiation-induced enteritis, in VMAT/IMRT patients, the small bowel reactions hardly went over GI (RTOG Toxicity Grade I), while most patients in 3DCRT had GIII and GII and recovered slower than the patients in the VMAT/ IMRT group. In recent years, we have seen that the recovery of 3DCRT patients can prolong from one to even more years, as proven in literature [11].

We noticed that radiation-induced cystitis appeared later in the subset of patients treated with 3DCRT and increased in terms of grade and number of cases as time passed up to 6 months after treatment (and later) than in VMAT/IMRT treated patients where cystitis appeared in lower grade during or after treatment and already at the first or second follow-up examination; it was not registered anymore. This could be explained by the accumulated volume/dose relationship to the bladder and the response of its epithelium to it [10].

Toxicities between two groups (3DCRT and VMAT/IMRT) were statistically compared using Fisher's exact test. The test showed that toxicity rates were significantly higher for the 3DCRT group 1 month and 6 months after the treatment ($p < 0.05$), while it was the opposite during and immediately after the treatment for the VMAT/IMRT group. That can be explained by higher stages of illness in VMAT/IMRT group, which contributed to the acute effects recorded in this group.

The margins of CTV to PTV are dependent on at least two factors: the position of the target inside the patient's body and its relationship to other organs, and the position of a patient in relation to the radiation beam. The size of the margin of CTV-PTV is a compromise between the risk of underdosing of CTV and also the risk of toxicity to healthy tissue around the CTV.

The margin is calculated by a widely used Van Herk's formula [5], based on the probability distribution of the cumulative dose over a range of patients.

Our data show that, currently, margins for CTV to PTV in VMAT/IMRT patients must remain the same as in 3DCRT (1 cm) due to daily setup movements. In future months, there will be a need to re-evaluate margins and correct them to smaller ones according to the improvements achieved in clinical practice in VMAT/IMRT cases. The reduction of margins will further decrease the dose burden on OARs, but it must be proven clinically acceptable.

Conclusion

The study showed that QUANTEC data used for 3DCRT is not detailed enough to support advanced treatments in the pelvic area, in this particular case-gynecological treatments. Clinical follow-up showed the origin of problems, which can be solved by the implementation of additional dose-volume parameters during treatment planning, thus creating the desired dose-volume histogram for a particular organ and, therefore, predicting the possible complication probability and rates. We clinically implemented multiple dose-volume parameters of all associated OARs, including small bowels, based on QUANTEC 2010, RTOG 1203, and other studies of pelvic IMRT/VMAT treatments.

In summary, we can conclude that VMAT/IMRT is a better treatment modality for gynecological malignancies in comparison to 3DCRT, significantly dosimetrically superior. Both techniques provide optimal coverage of the target volume, but OARs can be better spared in the advanced modality and, therefore, complications minimized, which complies with literature data. This advanced treatment modality should be an option for all radically treated patients.

The margins for CTV-PTV must be re-evaluated regularly to decrease the potential dose of OARs during advanced RT treatments.

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The role of thrombopoietin receptor agonists in the management of adult primary immune thrombocytopenia – a single center experience

Agonisti trombopoetinskih receptora u lečenju primarne imunske trombocitopenije odraslih – naša iskustva

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Abstract

Background/Aim. The availability of thrombopoietin receptor agonists (TPO-RA) for treating primary immune thrombocytopenia (ITP) has transformed its management over the last decade. The aim of this study was to assess the efficacy of TPO-RA in adults with chronic ITP treated at the University Clinical Center of Serbia. **Methods.** A total of 28 adult ITP patients (10 males and 18 females), who were given eltrombopag and/or romiplostim, were enrolled in the study. Data on demographic characteristics, ITP duration, previous therapeutic modalities, comorbidities, concomitant therapy both for comorbidities and ITP, indications for TPO-RA, bleeding episodes before and during TPO-RA, TPO-RA doses, adverse events, and response rates were collected from the patients' medical records. TPO-RAs were administered in patients with chronic refractory ITP when splenectomy was contraindicated/unfeasible and as preparation for splenectomy. Favorable treatment response was defined as a stable platelet count $\geq 50 \times 10^9$/L. **Results.** A total of 22 (78.57%) and 14 (50.0%) patients were treated with eltrombopag and romiplostim, respectively. A good treatment response (GTR) was achieved in 81.8% of the patients receiving eltrombopag and 71.4% of those treated with romiplostim. The non-responders to eltrombopag (4 patients) and those who had lost their response to eltrombopag (4 patients) were switched to romiplostim. Six of 8 patients achieved a GTR. At the time of TPO-RA initiation, 46.4% of the patients used concomitant ITP therapy, which was ceased in all those with a GTR. The following adverse effects of TPO-RA were registered: transaminitis and transient ischemic attack for eltrombopag – one patient each, and pulmonary embolism in one romiplostim-treated patient. **Conclusion.** Our study showed that TPO-RAs are an effective and safe treatment option since the majority of patients achieved stable remission without bleeding episodes.

Key words:
eltrombopag; purpura, thrombocytopenic, idiopathic; receptors, thrombopoietin; romiplostim; treatment outcome.

Apstrakt

Uvod/Cilj. Lečenje primarne imunske trombocitopenije (ITP) se zahvaljujući agonistima trombopoetinskih receptora (TPO-RA), značajno izmenilo tokom prethodne decenije. Cilj rada je bio da se utvrdi efikasnost TPO-RA u lečenju bolesnika sa hroničnom ITP u Univerzitetskom kliničkom centru Srbije. **Metode.** U studiju je bilo uključeno 28 odraslih bolesnika sa ITP (10 muškog pola i 18 ženskog pola) lečenih primenom eltrombopaga i/ili romiplostima. Prikupljani su demografski podaci, trajanje ITP, prethodni terapijski modaliteti, komorbiditeti, prateća terapija (kako za komorbiditete tako i za ITP), indikacije za uvođenje TPO-RA, krvarenje pre i tokom primene TPO-RA, prosečne doze TPO-RA, neželjeni događaji i stopa terapijskog odgovora na TPO-RA. Indikacije za primenu TPO-RA bile su hronična refraktona ITP, kontraindikovana/neizvodljiva splenektomija i priprema za splenektomiju. Povoljan odgovor na lečenje je bio definisan kao stabilan broj trombocita $\geq 50 \times 10^9$/L. **Rezultati.** Ukupno 22 (78,57%) bolesnika lečena su eltrombopagom, a 14 (50,0%) bolesnika romiplostimom. Dobar terapijski odgovor (DTO) postignut je kod 81,8% bolesnika lečenih eltrombopagom i kod 71,4% bolesnika lečenih romiplostimom. Bolesnici kod kojih nije postignut DTO na eltrombopag (4 bolesnika) i oni koji su izgubili DTO na eltrombopag (4 bolesnika) prevedeni su na romiplostim. Kod njih 6/8 postignut je DTO. U vreme uvođenja TPO-RA, 46,4% bolesnika je koristilo prateću terapiju za ITP, koja je kod svih ukinuta po postizanju DTO. U toku primene TPO-RA zabeleženi su sledeći neželjeni događaji: transaminitis i tranzitorni ishemijski

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atak kod po jednog bolesnika lečenog eltrombopagom i plućna embolija kod jednog bolesnika lečenog romiplostimom. **Zaključak.** S obzirom na to da je većina bolesnika tokom lečenja postigla stabilnu remisiju, bez epizoda krvarenja, naši rezultati su pokazali da su TPO-RA efikasni i bezbedni u lečenju odraslih bolesnika sa ITP.

Ključne reči:
eltrombopag; purpura, trombocitopenijska, idiopatska; receptori, trombopoetinski; romiplostim; lečenje, ishod.

Introduction

Primary immune thrombocytopenia (ITP) is an acquired immune-mediated disorder characterized by isolated thrombocytopenia, defined as a platelet count (PC) below 100×10^9/L and the absence of any other cause of thrombocytopenia [1, 2]. The primary manifestation of ITP is an increased bleeding tendency that varies from cutaneous purpura to more severe mucosal bleeding [3]. However, 16–21% of adults diagnosed with ITP are without bleeding symptoms [1]. Nevertheless, patients with ITP have a slightly increased risk of arterial and venous thrombotic events [4], and many of them suffer from fatigue and depression [5]. ITP is classified by disease duration as newly diagnosed (0–3 months), persistent (> 3–12 months), or chronic (> 12 months) [2]. The estimated incidence of ITP is approximately 2–4 *per* 100,000 adults/year [6].

ITP is characterized by both increased platelet destruction as well as inappropriately low platelet production. This is mediated by the proapoptotic action of glycoprotein-specific platelet autoantibodies and cytotoxic lymphocytes on megakaryocytes [1, 3]. The treatment goals aim to prevent severe bleeding episodes and to maintain PC > $20–30 \times 10^9$/L [7]. Moreover, any medication should have minimal toxicity and optimize the patient's quality of life [1].

ITP treatment options are numerous and may be categorized into first-line and second-line treatment modalities [7].

The availability of thrombopoietin (TPO) receptor agonists (TPO-RA) for the treatment of ITP has transformed its management over the last decade. TPO-RAs activate the same signaling pathways as endogenous TPO, leading to an increase in PC, cessation of bleeding, and improved quality of life in 80% of patients with chronic ITP, both splenectomized and non-splenectomized. That makes them the most effective drugs in second-line therapy [3, 7–9].

Romiplostim and eltrombopag are licensed by the European Medicines Agency for the treatment of ITP in adults when an insufficient response to corticosteroids or intravenous gamma globulins has been registered. Eltrombopag is licensed for ITP lasting more than 6 months, while such a restriction does not apply to romiplostim [10, 11]. However, in Serbia, both drugs are licensed exclusively for chronic ITP [12, 13]. Romiplostim is a peptibody that binds directly and competitively at the TPO binding site and is dosed as a weekly subcutaneous (sc) injection. In contrast, eltrombopag is a small molecule that binds to a transmembrane site on the TPO receptor and is given orally. Their effect is manifested after 1–5 weeks from treatment initiation [1, 3, 7, 8]. Both drugs are safe, well tolerated, and equally effective [1, 3, 7–9, 14]. If one of the two shows ineffectiveness or side effects, the chance of establishing a response to the other one is about 50% due to the absence of cross-resistance between them [15]. Rebound thrombocytopenia typically recurs upon abrupt discontinuation of TPO-RAs. However, several studies have shown that TPO-RAs induce remission and a stable response in 10–30% of patients after gradual discontinuation [16, 17]. In addition, successful short-term administration of TPO-RAs as part of the preparation for surgical interventions, including splenectomy, has been described [18].

The aim of this study was to assess the efficacy and safety of TPO-RAs in chronic ITP patients treated at the University Clinical Center of Serbia.

Methods

This retrospective observational study was conducted at the Clinic of Hematology of the University Clinical Center of Serbia in Belgrade and included the period from April 2013 to January 2020. A total of 28 adult ITP patients (10 males and 18 females) treated with eltrombopag and/or romiplostim were enrolled. The diagnosis of ITP was made according to the current guidelines [3, 7, 8]. The following data were obtained from patients' medical records: 1) demographics (age and sex); 2) ITP-related data: time from diagnosis, previous therapeutic modalities including splenectomy, PC, bleeding score, and concomitant therapy for ITP at the initiation of TPO-RA; 3) each patient's medical history: comorbidities, concomitant therapy for comorbidities; 4) TPO-RA related data: indication and TPO-RA doses, time to response, adverse events (AEs), response rate and whether TPO-RAs were switched. TPO-RAs were administered in patients with chronic refractory ITP when splenectomy was contraindicated/unfeasible and as a preparation for splenectomy. Chronic refractory ITP was defined according to the recommendations of the International Working Group [2]. A PC $\geq 50 \times 10^9$/L was considered a good treatment response (GTR). Bleeding was graded according to Khellaf et al. [19].

Eltrombopag was given orally at the starting dosage of 50 mg/day, while romiplostim was initiated at the dose of 1 mcg/kg/week sc. For both drugs, subsequent doses were adjusted according to the PC, up to the maximum of 75 mg/day for eltrombopag and 10 mcg/kg/week for romiplostim. All data were summarized using descriptive statistical methods.

Results

TPO-RAs were administered to 11 (39.3%) patients with chronic refractory ITP, to 12 (42.9%) patients in whom splenectomy was contraindicated/unfeasible, and to 5 (17.9%) patients as a preparation for splenectomy. The characteristics of the patients are shown in Table 1. The median number of previous treatments for ITP was four in the eltrombopag and five in the romiplostim group. Treatment modalities used before TPO-RA initiation are listed in Table 2.

A total of 22 (78.6%) patients received eltrombopag and 14 (50%) romiplostim. More than 70% of our patients had experienced some comorbidities (Table 1), mainly cardiovascular conditions, and 15 (53.6%) patients often

Table 1

Characteristics of the study population

Parameter	Eltrombopag* (n = 22)	Romiplostim (n = 14)
Age at TPO-RA initiation (years), median, (IQR)	58.5 (IQR: 53–69)	52.5 (IQR: 24–66.5)
Females/males, n (%)	13 / 9 (59.1/40.9)	12 / 2 (85.7/14.3)
ITP duration (months), median (IQR)	71 (IQR: 29–230.5)	97 (IQR: 21–248)
Splenectomized patients, n (%)	9 (40.9)	6 (42.9)
Patients with comorbidities n (%)	19 (86.4)	10 (71.4)
Patients who used therapy for comorbidities, n (%)	17 (77.3)	7 (63.6)
Platelet counts at TPO-RA initiation ($\times 10^9$/L), median (IQR)	11.5 (IQR: 7–19)	10 (IQR: 2–22)
Bleeding score, median (IQR)	0 (IQR: 0–2.25)	3.5 (IQR: 0–13)
ITP treatment modalities prior to TPO-RA (n), median (IQR)	4 (IQR: 3–4)	5 (IQR: 4–5)
Concomitant ITP medications at TPO-RA initiation, n (%)	14 (63.6)	10 (71.4)

*** Eight patients were initially treated with eltrombopag and afterward switched to romiplostim.**
n – number of patients; TPO-RA – thrombopoietin receptor agonist; IQR – interquartile range; ITP – primary immune thrombocytopenia.

Table 2

ITP treatment modalities administered before the initiation of TPO-RAs

Type of ITP treatment	Eltrombopag (n = 22)	Romiplostim (n = 14)
Corticosteroids	22 (100.0)	14 (100.0)
Intravenous gammaglobulins	8 (36.4)	10 (71.4)
Splenectomy	9 (40.9)	6 (42.9)
Rituximab	0 (0.0)	1 (7.1)
Azathioprine	19 (86.4)	11 (78.6)
Mycophenolate-mofetil	2 (9.1)	3 (21.4)
Cyclosporine A	0 (0.0)	2 (14.3)
Vinca alkaloids	12 (54.5)	8 (57.1)
Danazol/dapsone	4 (18.2)	3 (21.4)
Cyclophosphamide	5 (22.7)	4 (28.6)

All values are expressed as numbers (percentages).
ITP – primary immune thrombocytopenia; TPO-RAs – thrombopoietin receptor agonists; n – number.

Table 3

Treatment response characteristics

Parameter	Eltrombopag (n = 22)	Romiplostim (n = 14)
GTR, n (%)	18 (81.8)	12 (71.4)
GTR in splenectomized patients, n (%)	7/9 (77.8)	4/6 (66.7)*
Time to response (weeks), mean ± SD	2.2 ± 1.2	2.6 ± 1.2
Average TPO-RA dose, mean ± SD	54.19 ± 16.59 mg	6.41 ± 2.63 μg
Duration of response (months), median (IQR)	24.5 (IQR: 5.5–36)	13.5 (IQR: 8–37.5)
Follow-up (months), median (IQR)	25 (IQR: 6–36.5)	23.5 (IQR: 8–37.5)
Loss of response, n (%)	4/18 (22.2)	2/12 (16.7)
Switch to other TPO-RA, n (%)	8 (36.4)	0 (0)
Sustained response after TPO-RA discontinuation, n (%)	1 (4.5)	1 (7.1)
Adverse events, n (%)	2 (9.1)	1 (7.1)

***2 of 3 splenectomized patients who were switched to romiplostim, achieved good treatment response.**
n – number; GTR – good treatment response; SD – standard deviation; TPO-RA – thrombopoietin receptor agonist; IQR – interquartile range.

required antiplatelet or anticoagulant therapy. At the time of TPO-RA initiation, 46.4% of the patients were using concomitant ITP therapy (tranexamic acid, corticosteroids, and azathioprine).

An initial GTR was noted in 18 (81.8%) patients receiving eltrombopag and in 12 (71.4%) romiplostim-treated patients (Table 3). The non-responders (4 patients), as well as those who had lost their response (4 patients) while receiving eltrombopag, were switched to romiplostim. Six of them initially achieved a GTR. However, two of them lost their response after 5 and 8 months, respectively (Table 3).

During the observational period, the following AEs were noted (in one patient each): pulmonary thromboembolism in the romiplostim group and transaminitis and transitory ischemic attack in the eltrombopag group.

Discussion

In our study, the safety and efficacy of TPO-RA in adults with previously treated chronic ITP were evaluated. Eltrombopag was given almost twice as often as romiplostim since we were guided by patients' preferences. Our patients were of the average age of 58.5 years and had numerous comorbidities, mostly of cardiovascular nature (hypertension, ischemic heart disease, atrial fibrillation), often requiring antiplatelet or anticoagulant therapy. The median time from ITP diagnosis to TPO-RA initiation was 71 months [interquartile range (IQR): 29-230.5 months] for eltrombopag and 97 months (IQR: 21-248 months) for romiplostim, which is significantly longer than reported in other studies [20, 21]. This could be explained by the stringent criteria for TPO-RA initiation dictated by Serbian Public Health Insurance [22].

A GTR was achieved in 81.8% of patients treated with eltrombopag and 71.4% of patients treated with romiplostim, which is consistent with previously reported results [21, 23]. All of our patients had been treated with multiple therapeutic modalities before TPO-RA initiation (Table 2). Nevertheless, more than one-third of them underwent splenectomy, and more than two-thirds achieved a GTR after introducing TPO-RA. Our results are consistent with those in previous publications. Namely, GTR was achieved in 68% of splenectomized patients treated with romiplostim [23] and 61% of splenectomized patients treated with eltrombopag [24].

At the initiation of TPO-RAs, the majority of our subjects used concomitant ITP therapy, which was discontinued after the achievement of a GTR. The median PC was 11.5 × 10^9/L (IQR: 7-19 × 10^9/L) before eltrombopag and 10 × 10^9/L (IQR: 2-22 × 10^9/L) before romiplostim initiation. The time to response was 2.2 weeks for eltrombopag and 2.6 weeks for romiplostim, which is in line with previous studies [21, 25]. The median duration of response was 24.5/13.5 months, with a follow-up period of 25/23.5 months, respectively.

As reported previously, TPO-RAs are generally well tolerated [21, 25, 26]. The prevalence of AEs was 9.1% in our eltrombopag-treated and 7.1% in our romiplostim-treated patients. Thus, transaminitis and transient ischemic attack were registered in single eltrombopag-treated patients, while pulmonary embolism occurred in one romiplostim-treated individual.

The occurrence of thrombotic events in romiplostim-treated patients has been described earlier with an incidence of 6.5% [27]. However, it should be underlined that the studied patients were obese (38 kg/m^2), splenectomized, and experienced transient thrombocytosis of 800 × 10^9/L at the time of pulmonary embolism. On the other hand, AEs were noted in two patients treated with eltrombopag. One patient had transaminitis, and the other had a transient ischemic attack, which had been previously described as well [3, 28].

To avoid PC oscillation in our romiplostim-treated patients, we administered the same dose regardless of the PC. As a result, we observed stable disease remission with PC ≥ 50 × 10^9/L. This was maintained with romiplostim at a mean level of 6.41 μg/kg and eltrombopag (mean 54.9 mg). Our romiplostim dose was higher than that recorded by others (2.8–5.1 μg/kg) [25, 26]. In those studies, romiplostim was introduced earlier in the disease course, sometimes as a second line just after corticosteroids, while in our case, it was given to highly refractory patients, including those who failed with eltrombopag.

Long-term remission despite TPO-RA discontinuation has been reported [25]. Two (7%) of our patients achieved a sustained response after gradual discontinuation of TPO-RAs (one on romiplostim and the other on eltrombopag therapy), which is less than observed by others (10–30%), but the early introduction of TPO-RAs is associated with a higher frequency of treatment-free response [17].

Many studies have confirmed that switching TPO-RAs could be beneficial [15, 28, 29]. In our study, eight patients were changed from eltrombopag to romiplostim; a GTR was achieved in 50% of them, which supports earlier data [15, 28]. However, all of the patients who were initially treated with romiplostim achieved a GTR, and we registered no cases of romiplostim to eltrombopag switch. Additionally, four patients treated with eltrombopag lost their response after an initial GTR, but two of them achieved a GTR after switching to romiplostim. Among the patients who lost their response or remained refractory to both TPO-RAs, TPO-RAs were discontinued, and tranexamic acid was introduced. Moreover, corticosteroids and/or intravenous gamma globulins were administered in cases of bleeding.

Conclusion

On balance, our study showed that the patients treated with TPO-RAs achieved stable remission with minimal incidence of AEs and no serious bleeding events during the therapy. Moreover, we confirmed the efficacy of a TPO-RA switch since the response to the second TPO-RA was long-lasting in our group. Bearing in mind the aforementioned characteristics, wider use of TPO-RAs during the earlier course of the disease should be considered.

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The evaluation of epicardial adipose tissue and carotid *intima-media* thickness in patients with Behçet's disease

Procena debljine epikardnog adipoznog tkiva i sloja *intima-media* karotidne arterije kod bolesnika sa Behčetovom bolešću

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Abstract

Background/Aim. Epidemiological studies indicate that cardiovascular disease (CVD) is common in almost all patients diagnosed with autoimmune disease. This study aimed to examine whether epicardial adipose tissue (EAT) thickness (EATT) and carotid *intima-media* (CIM) thickness (CIMT) differ between patients with Behçet's disease (BD) and healthy individuals. **Methods.** A total of 40 healthy subjects as controls and 40 BD patients with musculoskeletal complaints were enrolled in this cross-sectional prospective study. Socio-demographic, clinical, and laboratory data were obtained and compared between groups. The Behçet's Disease Current Activity Form was used to assess disease activity. Both groups underwent echocardiography in order to measure EATT and CIMT. **Results.** The mean thickness of EAT (5.70 ± 1.05 mm; 2.50 ± 0.61 mm, respectively, $p < 0.001$) and CIM (0.68 ± 0.05 mm; 0.63 ± 0.06 mm, respectively, $p = 0.002$) were significantly increased in BD patients compared to the control group. A positive correlation was observed between EATT and age ($r = 0.500$, $p = 0.001$), the duration of the disease ($r = 0.330$, $p < 0.001$), waist circumference ($r = 0.316$, $p = 0.013$), and disease activity ($r = 0.31$, $p < 0.001$) in the patient group. CIMT was positively correlated with age ($r = 0.594$, $p = 0.001$) and the duration of the disease ($r = 0.585$, $p = 0.001$). The use of glucocorticoids or clinical manifestations (joint involvements, genital ulcer, skin lesions, inflammatory back pain, and major organ involvement) of the patients were not found to be associated with EATT or CIMT. **Conclusion.** EATT and CIMT are increased in patients with BD and are associated with disease activity. Echocardiographic measurement of EATT and CIMT is an objective, noninvasive, and available method that can evaluate the risk of subclinical atherosclerosis in patients with BD.

Key words:
adipose tissue; atherosclerosis; autoimmune diseases; behcet syndrome; carotid artery, common; pericardium; risk; tunica intima; tunica media; ultrasonography.

Apstrakt

Uvod/Cilj. Epidemiološke studije pokazuju da su kardiovaskularne bolesti (KVB) česte kod gotovo svih bolesnika sa dijagnozom autoimunske bolesti. Cilj ove studije bio je da ispita da li se debljina epikardnog masnog tkiva [*epicardial adipose tissue (EAT) thickness (EATT)*] i debljina sloja *intima-media* karotidne arterije [*carotid intima-media (CIM) thickness (CIMT)*], razlikuju između bolesnika sa Behčetovom bolešću (BB) i zdravih osoba. **Metode.** Ukupno 40 zdravih ispitanika u kontrolnoj grupi i 40 bolesnika sa BB sa tegobama mišićno-zglobnog sistema uključeno je u ovu prospektivnu studiju preseka. Dobijeni su socio-demografski, klinički i laboratorijski podaci koji su upoređeni između grupa. Za procenu aktivnosti bolesti korišćen je Behčetov obrazac trenutne aktivnosti bolesti. Obe grupe su podvrgnute ehokardiografiji radi merenja EATT i CIMT. **Rezultati.** Srednja vrednost EATT (5,70 ± 1,05 mm; 2,50 ± 0,61 mm, redom, $p < 0,001$) i CIMT (0,68 ± 0,05 mm; 0,63 ± 0,06 mm, redom, $p = 0,002$) su bile značajno povećane kod bolesnika sa BB u poređenju sa kontrolnom grupom. Pozitivna korelacija je pokazana između EATT i starosti ($r = 0,500$, $p = 0,001$), vremena trajanja bolesti ($r = 0,330$, $p < 0.001$), obima struka ($r = 0,316$, $p = 0,013$) i aktivnosti bolesti ($r = 0,31$, $p < 0,001$) u grupi bolesnika. CIMT je pozitivno korelisao sa godinama života ($r = 0,594$, $p = 0,001$) i vremenom trajanja bolesti ($r = 0,585$, $p = 0,001$). Upotreba glukokortikoida ili kliničke manifestacije (zahvaćenost zglobova, ulceracija na genitalijama, kožne lezije, upalni bol u leđima i zahvaćenost velikih organa) kod bolesnika nije bila povezana sa EATT ili CIMT. **Zaključak.** EATT i CIMT su povećane kod bolesnika

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sa BB i povezane su sa aktivnošću bolesti. Ehokardiografsko merenje EATT i CIMT je objektivna, neinvazivna i dostupna metoda koja može proceniti rizik od supkliničke ateroskleroze kod bolesnika sa BB.

Ključne reči:
masno tkivo; ateroskleroza; autoimunske bolesti; behčetov sindrom; a. carotis communis; perikard; rizik; tunica intima; tunica media; ultrasonografija.

Introduction

Behçet's disease (BD) is a rare multisystemic autoimmune inflammatory vasculitis characterized by a heterogeneous pattern of organ involvement with an unknown etiology [1]. The cardiovascular (CV) system may be affected on any level ranging from aneurysms to thrombosis and myocardial infarction, and has been found in between 2.4% and 6.4% of individuals with BD in two major datasets [2]. The presence of coronary artery disease (CAD) is associated with poor prognosis that may differ among geographic distribution [3, 4].

According to previous studies, it has been established that autoimmune inflammatory diseases are independent risk factors for atherosclerosis [5]. Understanding the cellular and molecular connections that govern the genesis and progression of atherosclerosis, as well as the designation of endothelial dysfunction as the primary lesion, is critical in these patients [6]. Since BD is mostly known as venous thrombotic disease, subclinical atherosclerosis was less investigated in patients with BD. In most previous cases, echocardiography (ECHO) was used to show the presence of thrombus and valvular lesions in patients with BD. Given the chronic inflammatory background of BD, premature atherosclerosis was addressed with conflicting evidence in the literature [7, 8].

Recent studies clarify the connection between the increased epicardial adipose tissue (EAT) thickness (EATT) and the development of CADs [9]. EAT is the visceral adipose tissue of the heart located between the visceral pericardium and the myocardium. Under physiological conditions, EAT acts as a buffer zone absorbing excess free fatty acids and protects the heart from exposure to high levels of acids. On the other hand, excessive EAT is an independent risk factor for occlusive coronary artery plaques, atherosclerosis, and ischemia [10–12]. The carotid *intima-media* thickness (CIMT) is also a widely used clinical marker to determine subclinical atherosclerosis measured simply and noninvasively with ECHO [13]. Measurement of CIMT is determined as a strong indicator of subclinical atherosclerosis and heart disease risk, and increased CIMT has been indicated for CV risk stratification [14].

So far, increased atherosclerosis is not a prominent reported feature of BD, unlike other inflammatory arthritis such as systemic lupus erythematosus, ankylosing spondylitis, psoriatic arthritis, and rheumatoid arthritis, whereas coronary aneurysms and stenotic lesions are the most frequent cardiac lesions observed in patients with BD [15]. This study aimed to compare the EATT and CIMT between BD patients and healthy controls and to assess the relationship between EATT and CIMT and various sociodemographic, clinical, and laboratory parameters in patients with BD.

Methods

This cross-sectional study was conducted with a total of 40 BD patients who complied with the criteria of the International study group for BD, were followed up with the diagnosis of BD, and did not have exclusion criteria. BD patients with any musculoskeletal complaints that were referred from outpatient clinics and admitted to orthopedic outpatient clinic, were investigated. As a control group, a total of 40 age and gender-matched healthy individuals were evaluated. Patients with a history of any reported CV disease (CVD) (myocardial infarction, atrial flutter or fibrillation, heart failure, valvular heart disease, CAD, transient ischemic attack, stroke, peripheral artery disease), any previous vascular complication, chronic obstructive pulmonary disease, with a diagnosis of any other significant systemic disease such as hypothyroidism, hyperlipidemia, diabetes mellitus (or fasting blood glucose > 125 mg/dL), or Cushing syndrome that could interfere with atherosclerosis were excluded from the study.

Relevant socio-demographic characteristics of BD patients and control subjects were questioned, and clinical data of BD patients, including smoking status and body mass index (BMI), were noted. The disease activity score was determined by the Behçet's Disease Current Activity Form (BDCAF) in patients with BD. The BDCAF is the most widely used instrument for evaluating the activity of BD, which is dependent on the history of symptoms. Scoring is based on an accurate history of clinical features that had been present over the preceding four weeks prior to the date of examination [16].

Laboratory parameters, including fasting blood glucose, creatinine, sedimentation, C-reactive protein, lipid profile, and other biochemical parameters, were recorded. Inflammatory joint involvements (arm, leg, shoulder, hip), oral ulcer, genital ulcer, skin lesions (pseudofolliculitis, erythema nodosum), inflammatory back pain, major organ involvement (ocular, neurologic, gastrointestinal, and vascular manifestations) and glucocorticoid treatment were noted from the medical charts. Systolic and diastolic blood pressure (mmHg) and waist circumference (cm) were also measured. An orthopedic surgeon recorded the baseline features of both BD patients and the control group. EATT and CIMT were assessed with ECHO by a single cardiologist blinded to the other assessments of all participants.

All procedures performed in this study were in accordance with the Helsinki declaration and its later amendments. This study was approved by Erzurum Regional

Training and Research Hospital ethics committee and patient recruitment took place in Erzurum Regional Training and Research Hospital. Informed consent was taken from all participants.

The measurement of EATT with transthoracic ECHO

Both groups in the study were examined with transthoracic ECHO (Vivid 7, GE-Vingmed Ultrasound AS, Horten, Norway) at the Cardiology Clinic ECHO Laboratory. After the 15-minute rest period, EATT measurements were done at the end of the diastole, using the ultrasound probe at the frequency of 2.5–3.5 MHz and with the 2-D and M mode method on the parasternal long axis in the left-lying position.

The measurement of CIMT with transthoracic ECHO

CIMT can be defined as the distance between the *media-adventitia* interface and the *lumen-intima* interface. Measurements were done using a duplex ultrasound system with a 10-MHz scanning frequency in the B-mode, pulsed Doppler mode, and color mode using the Vivid 5 device. CIMT was measured at three points, 10 mm proximal to the carotid bulb at the far wall of the right and left common carotid arteries. The CIMT of these three locations was used to obtain the mean thickness for each side.

Statistical analysis

IBM SPSS Statistics 22 (SPSS IBM, Turkey) program was used to analyze the results. Continuous variables were expressed as mean ± standard deviation. In comparisons between groups, the Mann-Whitney *U* test was used for continuous variables, and the Pearson's chi-squared test was used for categorical variables. Data were tested for normal distribution by the Kolmogorov-Smirnov test. The Pearson's correlation analysis was performed to examine the relationships between parameters. Statistical significance was evaluated at the level of $p < 0.05$.

Results

Our study was conducted on 80 cases, 44 (55%) male and 36 (45%) female, whose ages ranged from 20 to 66 years. Forty patients with BD were enrolled and 40 healthy individuals were evaluated as a control group. There were no differences between the groups regarding age, BMI, gender, waist circumference, active smoking rate, systolic or diastolic blood pressure, C-reactive protein, erythrocyte sedimentation rate, fasting plasma glucose, serum creatinine, or lipid profile. Mean EATT was determined as 5.70 ± 1.05 mm in the BD group and 2.50 ± 0.61 mm in the control group ($p < 0.001$). The mean value for CIMT was 0.68 ± 0.05 mm in the BD group and 0.63 ± 0.06 mm in the control group ($p = 0.002$) (Table 1). The mean disease duration was 7.8 ± 4.2 years, and the mean BDCAF score was 3.52 ± 1.04 in the BD group.

Table 2 shows the clinical features of BD patients. All patients had previous or present oral ulcers. BD patients were divided into groups regarding the presence of several clinical manifestations and glucocorticoid use. No significant association was observed between EATT, CIMT, and clinical characteristics of BD patients (Table 2).

EATT was positively correlated with age (r = 0.500, $p = 0.001$), the duration of the disease (r = 0.330, $p < 0.001$), waist circumference (r = 0.316, $p = 0.013$), and disease activity (r = 0.31, $p < 0.001$) in the patient group. CIMT was also positively correlated with age (r = 0.594, $p = 0.001$) and the duration of the disease (r = 0.585, $p = 0.001$). No significant correlation was found between EATT, CIMT, and other sociodemographic and clinical parameters (Table 3).

Table 1

Baseline characteristics of the study population

Characteristics	Behçet's disease (n = 40)	Control (n = 40)	*p*-value
Age (years)	37.55 ± 12.06	37.81 ± 15.17	0.973
Gender (male/female)	22/18	22/18	1.000
BMI (kg/m^2)	23.24 ± 11	21.90 ± 14.5	0.270
Waist circumference (cm)	77.69 ± 10.45	78.34 ± 9.85	0.849
Active smoking, n (%)	10 (25)	13(32)	0.350
Systolic blood pressure (mmHg)	124 ± 18	124 ± 13	0.504
Diastolic blood pressure (mmHg)	73.2 ± 6.1	74.1 ± 6.2	0.442
C-reactive protein (mg/dL)	2.87 ± 2.36	2.55 ± 1.95	0.356
Erythrocyte sedimentation rate (mm/hr)	21.5 ± 10.4	16.4 ± 70	0.767
Fasting plasma glucose (mg/dL)	86.50 ± 13.95	86.50 ± 15.87	0.126
Creatinine (mg/dL)	0.71 ± 0.11	0.71 ± 09	0.980
High-density lipoprotein cholesterol (mg/dL)	41.81 ± 7.0	42.86 ± 6.45	0.493
Low-density lipoprotein cholesterol (mg/dL)	125.52 ± 34.25	124.16 ± 33.387	0.528
Triglycerides (mg/dL)	131 ± 33	125 ± 66	0.741
Total cholesterol (mg/dL)	211.31 ± 47.38	203.70 ± 53.70	0.528
Epicardial adipose tissue thickness (mm)	5.7 ± 1.05	2.5 ± 0.61	**< 0.001**
Carotid *intima-media* thickness (mm)	0.68 ± 0.05	0.63 ± 0.06	**0.002**
BDCAF score	3.52 ± 1.04	–	–

Results are given as mean ± standard deviation or number of patients.
BMI – body mass index; BDCAF – Behçet's Disease Current Activity Form.
Bolded values are statistically significant.

Table 2

Comparison of epicardial adipose tissue thickness (EATT) and carotid *intima-media* thickness (CIMT) according to clinical features in patients with Behçet's disease

Parameters	Patients	EATT (mm)	*p*-value	CIMT(mm)	*p*-value
	n (%)	mean ± SD		mean ± SD	
Joint involvement (+)	22 (55)	5.80 ± 0.81	0.456	0.68 ± 0.07	0.675
Joint involvement (-)	18 (45)	5.57 ± 1.12		0.69 ± 0.08	
Genital ulcer (+)	17 (42.5)	5.71 ± 0.92	0.949	0.68 ± 0.08	1.000
Genital ulcer (-)	23 (57.5)	5.69 ± 1.02		0.68 ± 0.08	
Skin lesions (+)	9 (22.5)	5.70 ± 1.00	-	0.67 ± 0.09	-
Skin lesions (-)	31 (77.5)	5.70 ± 1.03		0.68 ± 0.03	
Inflammatory back pain (+)	10 (25)	5.78 ± 1.24	0.773	0.68 ± 0.08	1.000
Inflammatory back pain (-)	30 (75)	5.67 ± 0.97		0.68 ± 0.10	
Ocular involvement (+)	21 (52.5)	5.69 ± 1.02	0.907	0.70 ± 0.11	0.09
Ocular involvement (-)	19 (47.5)	5.73 ± 1.14		0.65 ± 0.07	
Neurologic involvement (+)	2 (5)	5.81 ± 0.90	-	0.68 ± 0.09	-
Neurologic involvement (-)	38 (95)	5.69 ± 1.07		0.68 ± 0.10	
Gastrointestinal involvement (+)	1 (2.5)	5.77 ± 1.26	-	0.67 ± 0.00	-
Gastrointestinal involvement (-)	39 (97.5)	5.69 ± 1.05		0.68 ± 1.02	
Glucocorticoid treatment (+)	22 (55)	5.54 ± 0.80	0.277	0.68 ± 0.10	0.810
Glucocorticoid treatment (-)	18 (45)	5.89 ± 1.20		0.69 ± 0.16	

SD – standard deviation.

Table 3

Correlation of demographic and laboratory findings with epicardial adipose tissue thickness (EATT) and carotid *intima-media* thickness (CIMT)

Parameters	EATT		CIMT	
	r	*p*-value	r	*p*-value
Age	**0.500**	**0.001**	**0.594**	**0.001**
BMI	0.018	0.802	0.016	0.823
Gender (male/female)	0.047	0.798	0.044	0.857
Disease duration	**0.330**	**< 0.001**	**0.585**	**0.001**
Systolic blood pressure	0.249	0,177	0.254	0.180
Diastolic blood pressure	0.244	0.151	0.235	0.203
Waist circumference	**0.316**	**0.013**	0.04	0.654
C-reactive protein	0.111	0.124	0.05	0.640
Erythrocyte sedimentation rate	0.034	0.644	0.152	0.117
Fasting plasma glucose	0.150	0.092	0.120	0.226
High-density lipoprotein cholesterol	-0.224	0.226	-0.225	0.203
Low-density lipoprotein cholesterol	-0.196	0.292	-0.197	0.217
Triglycerides	0.017	0.362	0.017	0.254
Total cholesterol	-0.042	0.827	-0.052	0.810
BDCAF score	**0.310**	**< 0.001**	0.090	0.254
CIMT	**0.27**	**0.002**		

BMI – body mass index; BDCAF – Behçet's Disease Current Activity Form.
Bolded values are statistically significant.

Discussion

Endothelial functions were found to be impaired in patients with chronic inflammatory musculoskeletal diseases compared to healthy individuals in the absence of conventional risk factors or overt CADs [17, 18]. Visceral adipose tissue is metabolically more active than subcutaneous adipose tissue and, therefore, more dangerous for the development of CVD. Recent studies have demonstrated that EATT and CIMT have emerged as markers of CVDs [19, 20]. This study revealed that these indicators of early atherosclerosis were increased in BD patients compared to healthy individuals.

Endothelial dysfunction can be detected with the measurement of flow-mediated dilatation of the brachial artery. A few previous studies observed that flow-mediated dilatation was impaired in patients with BD due to vasculitis, which is a cornerstone of CV involvement in BD [21, 22]. Chen et al. [23] conducted a retrospective case-control study with 476 Chinese patients with BD and stated that 19 (4%) of them (17 males) had CAD. In a prospective Korean study, Sun et al. [24] used speckle-tracking ECHO to investigate early cardiac symptoms of BD in individuals with no history of heart disease. Despite no obvious abnormalities on standard ECHO, patients had intrinsic left ventricular dysfunction.

Chronic inflammation can lead to atherosclerosis, and there has been a significant focus on the inflammatory component of atherosclerosis in the last decades. Increased atherosclerosis is an important contributor to CV complications in most autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis [25]. However, some researchers have observed that atherosclerosis is characteristically not increased in BD patients in contrast to the finding of this present study [26]. While the incidence of overt atherosclerosis does not seem to be increased in patients with BD, Hong et al. [27] reported that *intima-media* thickness was increased in BD, which might be due to nonatherosclerotic causes of endothelial hyperactivity. Another study showed that the atherogenic index of plasma value and CIMT in BD patients were significantly higher compared to the control group, and there was a strong positive correlation between these values [6]. Keser et al. [28] investigated EATT and brachial artery flow-mediated dilatation in BD patients using ECHO. They found that the mean EATT was higher, and flow-mediated endothelium-dependent dilatation was significantly lower in patients with BD compared to the controls. They reported that flow-mediated dilatation was negatively correlated with disease activity and age, and EATT was positively correlated with disease duration, waist circumference, and disease activity. Similarly, the present study revealed that the mean value for EATT and CIMT in patients with BD determined by ECHO were found to be significantly higher than that of the healthy control group. This result occurred despite there being no significant CV involvement of BD.

Another indicator of BD examined in this study was CIMT, and concordant with the study of Taşolar et al. [29], CIMT was significantly thicker in BD patients, and CIMT was defined as a well-known marker of subclinical atherosclerosis. Recently, a study conducted with 100 BD patients and 30 healthy individuals showed that the frequency of subclinical atherosclerosis in the BD patients was significantly higher than that in the control group [30]. The CIMT cut-off value for BD patients was determined as 0.54 mm in a meta-analysis, while this present study determined a mean CIMT of 0.68 mm in BD patients [31]. Hassan et al. [32] evaluated CIMT formation in 30 patients with BD by doppler ultrasonography to gain morphologic evidence of subclinical atherosclerosis. Their results showed an association between CIMT and disease activity. Another additional finding of this study that is worth discussing here is that the mean CIMT was significantly correlated with urea, creatinine, cholesterol, and triglycerides in BD patients compared with the controls. In this study, there was no association between the CIMT, EATT, and several parameters, such as BMI. This result was in accordance with the results of other studies recruited with obese patients that also found no connection between CIMT and BMI [33, 34]. In contrast, some other studies reported a statistically significant correlation between CIMT and BMI [35].

Studies conducted with several other rheumatologic diseases observed that EATT increases with age, similarly to our analysis [36, 37]. Anthropometric variables such as waist circumference and BMI are other clinical parameters that may be related to EATT. Ormseth et al. [38] found that EATT is associated with waist circumference and waist/hip ratio in patients with rheumatoid arthritis (RA) compared with the control group. Lima-Martínez et al. [39] did not report a relationship between EATT and waist circumference in RA patients, and EATT had been proposed to constitute a better marker of visceral adiposity when compared with BMI and waist circumference. The results of this study also postulated the positive correlation between EATT and increased waist circumference caused by the increase of visceral fat. EATT may be more compatible with waist circumference, which is more related to abdominal obesity than BMI. This fact demonstrates that EATT and waist circumference are together more descriptive in determining CVD risk than BMI.

There may be a positive correlation between EATT and systolic and diastolic blood pressure levels in some chronic diseases; however, contradictory data was also reported [36, 39]. This study could not demonstrate a significant relationship between EATT measurements with neither systolic nor diastolic arterial blood pressure levels, whereas the findings of this study did not show a significant relationship between the EATT or CIMT and laboratory parameters.

In a recent large-scale prospective study, sustained treatment of inflammation and decreased disease activity was shown to lower the risk of CV events [40, 41]. A recent review indicated that glucocorticoids act on the vessel wall, which may suppress or increase the development of atherosclerotic lesions. Glucocorticoids affect cells involved in the formation of atherosclerotic lesions that can either promote or prevent the creation of the lesions [42]. Regardless of the inflammatory diseases, corticosteroids were linked to an elevated risk of CVDs [43]. We investigated the relationship between several clinical manifestations and glucocorticoid use with CIMT and EATT. However, these associations were not statistically significant, perhaps due to the sample size.

Noninvasive parameters such as EATT and CIMT are useful to determine disease progression and identify BD subjects at high risk of CADs. BDCAF is an important tool and may significantly suggest CV manifestation in BD patients. In the present study, disease activity evaluated by using BDCAF was also found to be associated with both EATT and CIMT. This study affirms that subclinical atherosclerosis is not as uncommon as previously reported in the literature, and constant evaluation of the CV system in asymptomatic patients is needed. The exclusion criteria were important to omit possible comorbidities leading to atherosclerosis for the power of the study. There was also no statistically significant difference between groups regarding several features such as age, gender, smoking status, BMI, and systolic and diastolic pressure. The presence of a control group and an evaluation of several clinical and laboratory variables can be counted as the contribution of our study to the literature in terms of BD and subclinical atherosclerosis relationship. Moreover, EATT and CIMT measurements were made by a blind cardiologist with ECHO, which is a reliable imaging method. However, this cross-sectional study

was single-centered as a limitation. Thus, the correlation between EATT and CIMT and some socio-demographic, clinical, and laboratory parameters could not be detected. Another limitation was the lack of other predictors of endothelial dysfunction, such as the markers of oxidative stress. Long-term follow-up of BD patients should be considered in terms of atherosclerotic events for future studies.

Cardiac involvement in BD can be seen frequently without symptoms. EATT and CIMT have recently emerged as new markers of subclinical atherosclerosis. They seem to be increased in patients with BD, similar to RA patients, as a prototype for a high risk of CVD. Patients suffering from BD should be followed up routinely for CV manifestations, even with a bizarre presentation. The measurement of EATT and CIMT may be considered a diagnostic test to screen the development of atherosclerosis in the BD patient population and can afford an opportunity for the patients to receive early and appropriate treatment for CVDs.

Conclusion

EATT and CIMT are markers to assess cardiometabolic and CV risk, and they seem to increase in patients with BD. Subclinical cardiac involvement in patients with BD can be detected easily and quickly by echocardiographic examinations so that it can be treated early, preventing mortality and providing necessary approaches.

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The prevalence of depression/anxiety among patients with rheumatoid arthritis and its relationship with quality of life

Učestalost depresije/anksioznosti i njena povezanost sa kvalitetom života kod bolesnika sa reumatoidnim artritisom

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Abstract

Background/Aim. Rheumatoid arthritis (RA) is accompanied by numerous comorbidities, among which depression and anxiety (D/A) occupy a significant place. The aim of the study was to determine the prevalence of D/A in RA patients and the relationship with their quality of life (QoL). **Methods.** The study included RA patients treated at the Rheumatology Clinic of the Military Medical Academy in Belgrade, Serbia in the period from May to November 2016. Disease activity was assessed by the Disease Activity Score 28-SE (DAS28-SE). D/A was determined using the Hospital Anxiety and Depression Scale (HADS) questionnaire and European Quality of Life 5 Dimensions 3 Level Version (EQ-5D-3L) questionnaire Question 5. Three questionnaires were used to assess QoL: the general RAND 36-item Health Survey 1.0 (RAND36), the specific Rheumatoid Arthritis Quality of Life Questionnaire (RAQoL) and EQ-5D-3L. **Results.** Based on the HADS questionnaire, the prevalence of depression was 52% with the average HADS score value of 7.6 ± 3.2, while the prevalence of anxiety was 32% with the mean HADS score value of 5.8 ± 3.8. Question 5 of the EQ-5D questionnaire showed that the prevalence of D/A was 77.4%, of which 71.7% of patients had moderate D/A, while 5.7% of patients had severe D/A. Impairment in all the domains of QoL was found in tested patients, as assessed by all three questionnaires. The RAQoL questionnaire showed moderate QoL impairment, with a score value of 15.5 ± 7.9. The EQ-VAS score value was 58.6 ± 16.0, while the EQ-5D index was 0.6 ± 0.3. Univariate linear regression produced a statistically significant negative predictive value of QoL for the presence of D/A. Multivariate linear regression showed a statistically significant independent negative predictive value of QoL, as assessed by the RAQoL questionnaire (p = 0.010) and the mental QoL component of the RAND 36 questionnaire (p = 0.030) for the degree of depression. **Conclusion.** In RA patients, there is a significant prevalence of D/A as well as impairment of QoL in all domains. The tests performed have shown that QoL has a statistically significant negative predictive value for the presence of D/A.

Key words:
anxiety; arthritis, rheumatoid; depression; prevalence; prognosis; quality of life; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Reumatoidni artritis (RA) praćen je mnogobrojnim komorbiditetima među kojima depresija i anksioznost (D/A) zauzimaju značajno mesto. Cilj rada bio je da se proceni učestalost D/A kod bolesnika sa RA i njena povezanost sa kvalitetom života (KŽ). **Metode.** Ispitivanjem su obuhvaćeni bolesnici sa RA, lečeni na Klinici za reumatologiju Vojnomedicinske akademije u Beogradu, Srbija, u periodu od maja do novembra 2016. godine. Aktivnost bolesti je procenjivana pomoću *Disease Activity Score* 28-SE (DAS28-SE). Pomoću *Hospital Anxiety and Depression Scale* (HADS) i petog pitanja *European Quality of Life 5 Dimensions 3 Level Version* (EQ-5D-3L) upitnika određivani su D/A. Za procenu KŽ korišćena su tri upitnika: opšti *RAND 36-item Health Survey* 1.0 (RAND36), specifični upitnik KŽ osoba obolelih od RA (RAQoL) i upitnik EQ-5D-3L. **Rezultati.** Na osnovu HADS upitnika, učestalost depresije iznosila je 52%, sa prosečnom vrednošću HADS skora 7,6 ± 3,2, dok je učestalost anksioznosti iznosila 32%, sa srednjom vrednošću HADS skora 5,8 ± 3,8. Pomoću petog pitanja EQ-5D upitnika nađena je učestalost D/A od 77,4%, od čega je umerenu D/A imalo 71,7%, a izraženu 5,7% bolesnika. Kod

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ispitivanih bolesnika je pomoću sva tri upitnika utvrđena narušenost u svim domenima KŽ. Upitnikom RAQoL pokazana je umerena narušenost KŽ, sa vrednošću skora 15,5 ± 7,9. Vrednost EQ-VAS skora iznosila je 58,6 ± 16,0, dok je EQ-5D indeks iznosio 0,6 ± 0,3. Univarijantnom linearnom regresijom dobijena je statistički značajna negativna prediktivna vrednost KŽ za prisustvo D/A. Multivarijantnom linearnom regresijom pokazana je statistički značajna nezavisna negativna prediktivna vrednost KŽ, procenjena pomoću RAQoL upitnika (p = 0,010) i mentalne komponente KŽ RAND 36 upitnika (p = 0,030) za stepen depresije. **Zaključak.** Kod bolesnika sa RA postoji značajna učestalost D/A kao i narušenost KŽ u svim domenima. Sprovedenim ispitivanjima je pokazano da KŽ ima statistički značajnu negativnu prediktivnu vrednost za prisustvo D/A.

Ključne reči:
anksioznost; artritis, reumatoidni; depresija; prevalenca; prognoza; kvalitet života; ankete i upitnici.

Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by persistent synovitis and systemic inflammation leading to joint destruction, functional disability, and premature mortality. The disease is accompanied by numerous comorbidities that significantly impair quality of life (QoL). According to the results of the large multicenter COMOrbidities in Rheumatoid Arthritis (COMORA) study, which determined the prevalence of comorbidities in RA, depression was most common with 15%, followed by asthma with 6.6%, cardiovascular events (myocardial infarction, cerebrovascular insult) with 6%, solid tumors (except for basal cell carcinoma) with 4.5%, and chronic obstructive pulmonary disease (COPD) with 3.5% [1].

Despite the prevalence and significance of mental health disorders, they have been rarely investigated in rheumatology studies and clinical practice. Reports show that mental health has been studied in less than 8% of published works dealing with RA, while QoL is studied somewhat more frequently (in 19% of studies), mostly using the SF-36 questionnaire [2].

The prevalence of depression in RA ranges between 9.5% [3] and 41.5% [4], while the prevalence of anxiety ranges from 21% to 70% [5]. Follow-up studies have indicated that the cumulative risk of the occurrence of depression after 9 years of RA is 40% [6]. Depression and anxiety (D/A) in RA are associated with a higher degree of disease activity, reduced QoL, increased use of healthcare services, as well as reduced adherence to therapy. The correlation between depression and RA is multifactorial: it may be a result of social and economic factors, functional disability, and/or inflammation. There are various reasons for the large variation in the prevalence. It is very difficult to distinguish between patients with depressive disorder and those with a normal reaction since they live with a chronic, functionally limiting condition. Further, numerous symptoms of depression, such as fatigue, poor sleep, and loss of appetite, can be part of the clinical picture of RA itself. In addition to the foregoing, the prevalence is also affected by different methods used for diagnosing depression. A gold standard is a psychiatric interview and diagnosis through the Diagnostic and Statistical Manual (DSM) or International Classification of Diseases (ICD) criteria. However, alternative self-report questionnaires can also be useful due to their practicality in everyday work. Previous research has indicated that the recognition and appropriate treatment of D/A improve the response to treatment and considerably reduce symptoms associated with RA, improving the functional status and QoL [7]. That is why the aim of the study was to determine the prevalence of D/A in patients with RA and the correlation with their QoL.

Methods

The study included RA patients treated at the Rheumatology Clinic of the Military Medical Academy in Belgrade, Serbia from May to November 2016. The enrolment criteria were the following: RA diagnosis based on the American College of Rheumatology (ACR) 1987 classification criteria and ACR/European European Alliance of Associations for Rheumatology (EULAR) 2010, age ≥ 18 years. Patients with other systemic connective tissue diseases, fibromyalgia, previously verified cognitive disorder and psychiatric disease, and those who had used antidepressants in the previous month were excluded from the study. Structured questionnaires compiled based on literature data were used for the collection of information regarding subject and disease characteristics. The first part of the questionnaires included social and demographic characteristics of subjects: gender, age, place of residence (rural/urban environment), employment status, and level of education. The second part of the questionnaires referred to the clinical characteristics of RA: disease duration, therapy [use of methotrexate (MTX), other disease-modifying antirheumatic drugs (DMARDs), corticosteroids, and biological therapy].

To assess the activity of RA, the Disease Activity Score 28 (DAS 28-SE) was determined for all patients based on the total number of tender and swollen joints, the patient's assessment of disease activity on the VAS scale, and erythrocyte sedimentation rate (ESR) (mm/h). The assessment of the patient's functional ability was performed using the Health Assessment Questionnaire – Disability Index (HAQ DI), which contains a total of 20 questions scored from 0 to 3 (0 = without difficulties, 3 = I cannot do it).

When it comes to inflammation markers, the following tests were performed: ESR rate by Westergren (SE) and C-reactive protein (CRP) using the nephelometric method. D/A were diagnosed using two questionnaires: Hospital Anxiety and Depression Scale (HADS) and Question 5 of the

European Quality of Life 5 Dimensions 3 Level Version (EQ-5D-3L). The HADS [8] questionnaire contains 14 questions (7 for depression and 7 for anxiety). The patient assesses the degree of agreement using the Likert scale, from 0 to 3. The total score for each scale is from 0 to 21. A score exceeding 11 indicates the presence of D/A; 8-10 indicates borderline cases; 0-7 is a normal finding. The EQ-5D-3L [9] questionnaire contains five questions regarding various dimensions of health: mobility, self-care, usual activities, pain/discomfort, and D/A.

Three questionnaires were used for the assessment of QoL: general – the RAND 36-item Health Survey 1.0 (RAND36), specific – the Rheumatoid Arthritis Quality of Life (RAQoL), and EQ-5D-3L. The RAND contains 36 questions scored in 8 domains (physical functioning, social functioning, limitations due to physical problems, limitations due to emotional problems, mental health, vitality, pain, and general health perception). Each subscale is ranked from 0 to 100, where a higher score indicates better QoL. The RAND can be converted into two composite scores: physical health composite score (PCS) and mental health composite score (MCS) [10]. The RAQoL contains 30 questions regarding the mental and physical domains, which are answered with yes and no (1/0). The total score is 30, and a lower score indicates a better QoL [11]. The EQ-5D-3L comprises two parts – a descriptive system and a visual analog scale (VAS). The EQ-5D-3L descriptive system measures five dimensions of health: mobility, self-care, usual activities, pain/discomfort, and D/A. Each dimension has 3 levels: without problems (1), there are some problems (2), and severe problems (3). The EQ VAS represents a self-assessment of health on a vertical analog scale from the worst (0) to the best possible health (100). All five dimensions of the EQ-5D-3L questionnaire are converted into a general health index using scores derived from a general population sample. The German time-trade-off (TTO) was used in this research as this European population is most similar to the studied population in Serbia.

Statistical analysis

The correlation analysis was assessed based on Spearman's rank correlation coefficient. The investigation of the predictive value of inflammation parameters and main disease characteristics as the selected variables in the assessment of depression and QoL was carried out using linear and logistic regression analysis methods. Values of $p < 0.05$ were considered statistically significant differences. The obtained results were statistically processed using a statistical program for Windows, version 20.0 (Statistical Package for Social Science-SPSS Inc.).

Results

The study included 53 patients with RA with an average age of 54.6 ± 9.8 years. Women accounted for 86.8% (53/46). There were 35.8% of employed patients (53/19), while 42 patients (79.2%) had primary and secondary education. The main characteristics of subjects and RA are presented in Table 1. Regarding the treatment, 79.2% of patients (42/53) were treated with MTX, while 64.2% (34/53) received glucocorticoids. Biological therapy, mainly tocilizumab (61%), was given to 37.7% of patients (20/53). The average DAS 28 score was 4.0 ± 1.8. About a third of patients (26.4%) were in remission, and slightly more of them had high disease activity (34%). The functional status of the investigated group (HAQ DI) was mildly impaired, and the average value was 0.77 ± 0.77.

Table 1

Clinical features and antirheumatic treatment in patients with rheumatoid arthritis (n = 53)

Parameter (characteristics)	Values
Disease duration (years)	8.0 ± 7.2
Presence of RF and/or anti-CCP	46 (86.8)
Methotrexate use	42 (79.2)
Therapy corticosteroids	34 (64.2)
Therapy another BMD	11 (20.8)
Any biological therapy	20 (37.7)
Tender joint count TJC	5.8 ± 5.7
Swollen joint count SJC	2.2 ± 3.3
VAS	43.5 ± 18.9
DAS28-ESR	4.04 ± 1.79
DAS 28 < 2.6	14 (26.4)
DAS28 2.6–3.1	3 (5.7)
DAS28 > 3.1	15 (28.3)
DAS28 > 5.1	18 (34)
HAQ DI	0.77 ± 0.77

The values represent mean value ± standard deviation (SD) or number (percentages).
RF – rheumatoid factor; VAS – visual analog scale (0–10 cm); HAQ DI (Health Assessment Questionnaire); DAS28 – Disease Activity Score using 28 joints; ESR – erythrocyte sedimentation rate; TJC– tender joint count; SJC – swollen joint count; anti-CCP – anti-cyclic citrullinated peptides; BMD – bone mineral density.

The prevalence of D/A in patients with RA is shown in Table 2.

The prevalence of depression assessed by the HADS questionnaire was 52%. The average value of the HADS score for depression was 7.6 ± 3.2. As assessed by the same questionnaire, the prevalence of anxiety was 32%, with the average HADS score value of 5.8 ± 3.8.

The prevalence of D/A assessed by Question 5 of the EQ-5D questionnaire was 77.4%, of which 38 (71.7%) patients had D/A and 3 (5.7%) patients had severe D/A.

The RAQoL questionnaire indicated moderately impaired QoL with an average score value of 15.5 ± 7.9. A significant percentage of patients had difficulties in all the five QoL domains when the EQ-5D questionnaire was used, mainly in the domain of the presence of pain/discomfort and everyday functioning. The same questionnaire was used to analyze the impact of emotional problems on QoL. It was established that 77.4% (41/53) of patients had problems due to the presence of D/A. As part of the mentioned questionnaire, based on the personal QoL assessment by the patient, the average value on the VAS score of 58.6 ± 16.0 was obtained, while the average value of the general health index (EQ-5D index) was 0.6 ± 0.3 (Table 3).

The RAND36 questionnaire was used for an additional QoL assessment, and the values are presented in Table 4.

An analysis of the QoL and D/A degree ratio revealed a statistically significant negative predictive value of QoL, as assessed by the RAQoL and RAND36 questionnaires, with the presence of D/A (Table 5).

Multivariate linear regression showed statistically significant independent negative predictive value of QoL, as assessed by the RAQoL questionnaire and the mental QoL component of the RAND 36 questionnaire for the degree of depression (Table 6).

Table 2

Prevalence of depression/anxiety (D/A) in patients with rheumatoid arthritis (n = 53)

Parameter	Values
HADS	
depression score	7.6 ± 3.2
0–7	24 (48)
8–10	18 (36)
> 11	8 (16)
anxiety score	5.8 ± 3.8
0–7	34 (68)
8–10	11 (22)
> 11	5 (10)
EQ-5D-3L	
number of patients with D/A	41 (77.4)
number of patients with moderate D/A	38 (71.7)
number of patients with severe D/A	3 (5.7)

The values represent mean value ± standard deviation (SD) or number (percentages).

HADS – Hospital Anxiety and Depression Scale; EQ-5D-3L – European Quality of Life 5 Dimensions 3 Level Version.

Table 3

Quality of life (QoL) assessment using RAQoL and EQ-5D-3L questionnaires

Parameter	Values
RAQoL	15.5 ± 7.9
Present state of health (EQ-5D-3L), any problem	
mobility	41 (77.4)
self-care	34 (64.2)
usual activities	44 (83.0)
pain/discomfort	51 (96.2)
anxiety/depression	41 (77.4)
EQ VAS	58.6 ± 16.0
EQ-5D index value	0.6 ± 0.3

The values represent mean value ± standard deviation (SD) or number (percentages).

RAQoL – Rheumatoid Arthritis Quality of Life; EQ-5D-3L – European Quality of Life 5 Dimensions 3 Level Version; EQ-5D index – general health index derived from all the five categories of the EQ-5D questionnaire; EQ VAS – Visual Analog Scale for the assessment of general health as part of the EQ-5D questionnaire.

Table 4

Quality of life (QoL) assessment using RAND36 questionnaires

Parameter	Values
Physical functioning	45.12 ± 19.20
Role limitations due to physical health	21.63 ± 30.13
Role limitations due to emotional problems	38.44 ± 37.63
Energy/fatigue	39.71 ± 22.83
Emotional well-being	59.62 ± 20.06
Social functioning	53.04 ± 24.82
Pain	40.19 ± 23.41
General health	46.83 ± 16.59
Health change	49.04 ± 29.69
PCS	38.44 ± 22.35
MCS	47.70 ± 26.33

The values represent mean value ± standard deviation.
PCS – Physical Component Summary (PCS) score RAND 36;
MCS – Mental Component Summary (MCS) score RAND 36.

Table 5

Univariate linear regression of the impact of (QoL) on the degree of depression (D)/anxiety (A)

Variables	RAQoL	EQ-5D	PCS RAND36	MCS RAND 36
D score (HADS)	0.596 (p = 0.000)	ns	-0.442 (p = 0.001)	-0.594 (p = 0.000)
A score (HADS)	0.379 (p = 0.008)	ns	-0.308 (p = 0.032)	-0.442 (p = 0.001)
EQ-5D (A/D)	ns		ns	ns

The given values represent the standardized regression coefficient β; ns – nonsignificant.
RAQoL – Rheumatoid Arthritis Quality of Life; HADS – Hospital Anxiety and Depression Scale; PCS – Physical Component Summary (PCS) score RAND 36; MCS – Mental Component Summary (MCS) score RAND36.

Table 6

Multivariate linear regression of quality of life (QoL) for the degree of depression*

Independent variables	Score D (HADS)	p
RAQoL	0.502	0.010
PSC RAND36	0.278	0.173
MCS RAND36	-0.426	0.030

The given values represent the standardized regression coefficient β.
RAQoL – Rheumatoid Arthritis Quality of Life; HADS - Hospital Anxiety and Depression Scale; PCS – Physical Component Summary (PCS) score RAND 36; MCS – Mental Component Summary (MCS) score RAND36.

Discussion

RA is a chronic inflammatory disease accompanied by numerous comorbidities, among which depression and anxiety occupy a significant place.

The results of our study based on the HADS questionnaire show that 52% of patients have some depression symptoms. This result complies with the previous studies in which the same questionnaire was used and which showed that more than 50% of RA patients had depressive disorders [4]. In other studies, the prevalence of depression in RA patients was 14-46%, depending on measuring instruments [12, 13]. In our study, it was found using Question 5 of the EQ-5D-3L questionnaire that even 77.4% of patients had D/A. With the same questionnaire, Arne et al. [14] obtained the prevalence of D/A of 48% in patients with RA. A possible reason for the lower presence of D/A could be the fact that in the said study, more patients (74.4%) had low disease activity in comparison with 32% of our subjects. Rathbun et al. [15], in their research, found a correlation between depression and the patient's assessment on the VAS and pain. Patient global VAS correlated with anxiety levels, which could partially explain the association between anxiety and worse disease activity outcomes in RA [16]. Numerous authors have shown that the correlation between depression and disease activity is bidirectional – active RA leads to the occurrence of depression, while depression impacts the activity of RA. In the work of Hider et al. [17], patients with depression had a higher DAS28 score, while patients with chronic depression had a slight decrease in the DAS28 score despite treatment with TNF inhibitors.

When it comes to anxiety, our research using the HADS questionnaire (HADS score > 8) showed that 32% of patients were anxious. Using the same questionnaire and HADS score > 8, Yokogawa et al. [18] found that 29.3% of patients were anxious. El-Miedany and el-Rasheed [19] found that the prevalence of anxiety in RA was 70%. The study was conducted based on a clinical interview as an instrument for anxiety disorder measurement. In any case, our results comply with data from previous studies, which have demonstrated the level of anxiety in RA of 21–70%, as assessed by various measuring instruments [5].

The reason for the high level of D/A is multifactorial. Upon investigating the impact of social and demographic characteristics of subjects, it was observed that women accounted for 86.8% of the studied group, and it is known that depression is more frequent in the female than the male population.

Investigating the level of education, it was found that about 80% of patients had elementary and secondary education. Salaffi et al. [20] have established that a lower level of education represents a risk factor for chronic musculoskeletal pain and physical functioning, while Evers et al. [21] have pointed to a correlation between a lower level of education and the degree of D/A in RA patients.

A large number of previous studies have demonstrated that a particular level of functional limitation determined using the HAQ is a strong predictor of depression in RA patients [22]. Our study did not demonstrate a correlation between functional limitation and the degree of D/A, as the average HAQ score value in our patients was 0.77 ± 0.77, which was significantly lower than the average HAQ score in patients for whom a correlation with D/A has been found.

It is known that the QoL of RA patients is considerably reduced, which we also obtained in our study using all three questionnaires. The QoL of our patients was reduced in all domains of the EQ-5D-3L questionnaire as well as all physical and mental health domains of the RAND36 questionnaire. These results comply with the results of a study conducted by West and Jonsson [23], who have demonstrated an adverse effect of RA itself on the patient's physical, emotional, and social functioning. The study of Salaffi et al. [20] has demonstrated that the QoL components relating to the patient's physical and functional condition are the most frequently affected domains of the SF36 questionnaire. Our results comply with the said research as, according to the EQ-5D questionnaire, the highest percentage of patients had difficulties in the domain of pain/discomfort (96.2%) and everyday functioning (83%), while according to the RAND 36 questionnaire, the highest degree of impairment was in the domain of limitations in physical functioning (21.6 ± 30.1). The presence of pain is actually the main characteristic of RA, which represents an important factor for determining QoL in the early period of the disease [24].

A meta-analysis showed that patients with RA with depression tended to have lower QoL than patients without depression [25]. Depressed patients with RA have more pain [26], high disease activity [27], and reduced QoL. Our study demonstrated a significant correlation between QoL and the degree of D/A. The multivariate regression analysis showed that the QoL assessed using the RAQoL questionnaire, as well as the MCS score of the RAND 36 questionnaire, is an independent predictor of the degree of depression. That is also in compliance with the studies of Covic et al. [28], who have discovered that physical limitations affect the patient's emotional condition, primarily depression. Numerous cross-sectional and longitudinal studies have demonstrated a significant association of somatic symptoms with the occurrence of D/A [29, 30].

Routine detection and treatment of D/A should be part of a future strategy to improve the overall treatment of RA [2], which requires a multidisciplinary approach in RA treatment [31].

Conclusion

In RA patients, there is a high prevalence of D/A as well as considerable impairment of QoL. The degree of QoL impairment is an independent negative predictor for the degree of depression. The correlation between psychological disorders and somatic symptoms is actually bidirectional, indicating the need for discovering and treating psychological disorders simultaneously with somatic symptoms.

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Post nephrectomy renal function: donor nephrectomy vs. radical nephrectomy

Renalna funkcija posle nefrektomije: donorska nefrektomija vs. radikalna nefrektomija

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Abstract

Background/Aim. Monitoring the renal function following donor nephrectomy (DN) or radical nephrectomy (RN) due to kidney tumors is considered essential. The aim of this study was to compare pre-operative and post-operative renal function in patients who underwent DN in relation to patients who underwent RN due to renal malignancy. **Methods.** A retrospective case-control study was performed, which included 199 patients divided into two groups: group 1 (105 patients) were patients who underwent DN due to living-related/unrelated kidney transplantation, while group 2 (94 patients) was a control group, and included patients who underwent RN due to clear cell renal cell carcinoma in the T1bNoM0 clinical stage, where this surgical procedure was the final form of treatment. **Results.** Preoperative estimated glomerular filtration rate (eGFR) according to the Chronic Kidney Disease−Epidemiology Collaboration (EPI) equation (eGFR EPI) in the DN group was 94.95 mL/min/1.73 m^2, while in the RN group, it was 71.00 mL/min/1.73 m^2. Patients who underwent RN tended to have eGFR EPI below 60 mL/min/1.73 m^2 after ten years of follow-up compared with patients who underwent DN. In the DN group, the average eGFR EPI was 80.40 mL/min/1.73 m^2, and in the RN group, it was 56.00 mL/min/1.73 m^2. A higher incidence of diabetes mellitus (DM) and arterial hypertension (AH) was also observed in the DN group of patients compared to the RN group (AH: 44.3% vs. 21.3%; DM: 22.6% vs. 9.6%, respectively). **Conclusion.** Comparative monitoring of these two groups showed that in both groups, the recovery of the renal reserve was achieved one year after nephrectomy due to the known adaptive mechanisms. Even though the initial renal reserve in a kidney donor is reduced after living kidney transplantation (nephrectomy, permanent loss of renal mass), kidney donors recover kidney function within the first year after surgery due to the adaptive mechanisms.

Key words:
glomerular filtration rate; kidney neoplasms; kidney transplantation; nephrectomy; tissue donors; treatment outcome.

Apstrakt

Uvod/Cilj. Posle donorske nefrektomije (DN) ili radikalne nefrektomije (RN) zbog tumora bubrega, praćenje bubrežne funkcije je od suštinskog značaja. Cilj rada bio je da se uporedi preoperativna i postoperativna bubrežna funkcija posle DN, u odnosu na bolesnike koji su bili podvrgnuti RN zbog maligniteta bubrega. **Metode.** Retrospektivnom studijom slučaj-kontrole obuhvaćeno je 199 ispitanika podeljenih u dve grupe: I grupa (n = 105) bili su ispitanici kojima je urađena DN zbog transplantacije bubrega živog srodnog/nesrodnog donora, dok je II grupa (n = 94) bila kontrolna grupa i obuhvatala je bolesnike kojima je urađena RN zbog svetloćelijskog karcinoma bubrežnih ćelija u kliničkom stadijumu T1bNoM0, gde je ta hirurška procedura bila i konačni vid lečenja. **Rezultati.** Preoperativno, procenjena stopa brzine glomerulske filtracije [*estimated glomerular filtration rate* (eGFR) prema jednačini *Chronic Kidney Disease−Epidemiology Collaboration* (EGFR EPI)] u grupi bolesnika sa DN iznosila je 94,95 mL/min/1,73 m^2, a kod bolesnika sa RN 71,00 mL/min/1,73 m^2. Bolesnici koji su bili podvrgnuti RN imali su tendenciju da eGFR EPI nakon deset godina praćenja ostane ispod 60 mL/min/1,73 m^2, u poređenju sa osobama koje su bile podvrgnute DN. Kod ispitanika I grupe (DN) prosečna eGFR EPI iznosila je 80,40 mL/min/1,73 m^2, a kod ispitanika II grupe (RN) 56,00 mL/min/1,73 m^2. Primećena je i veća učestalost pojave dijabetesa melitusa (DM) i arterijske hipertenzije (AH) u grupi posle DN u

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odnosu na grupu posle RN (AH: 44,3% vs. 21,3%; DM: 22,6% vs. 9,6%). **Zaključak.** Uporednim praćenjem obe grupe, može se primetiti da se, poznatim adaptivnim mehanizmima, kod njih ostvaruje oporavak bubrežne rezerve posle prve godine od nefrektomije. Bez obzira na to što se kod transplantacije bubrega živog davaoca kod davaoca bubrega smanjuje inicijalna bubrežna rezerva (zbog nefrektomije, trajnog gubitka renalne mase), davaocima bubrega se, zahvaljujući adaptivnim mehanizmima, funkcija bubrega oporavlja unutar prve godine od hirurške intervencije.

Ključne reči:
glomerulska filtracija; bubreg, neoplazme; transplantacija bubrega; nefrektomija; tkivo, davaoci; lečenje, ishod.

Introduction

Most of the world's leading kidney transplant centers focus their attention on donor/recipient selection, the transplantation process itself, post-operative follow-up of renal transplant patients, and long-term outcomes in recipients [1–6]. The situation is similar in Serbia [7]. However, in recent years, special attention has been given to living kidney donors due to long-term follow-up of these patients after nephrectomy and because, when compared with the general population, they have an increased occurrence rate of some diseases, such as ischemic heart disease [8, 9].

Donor selection and monitoring are not of crucial importance only as far as the quality of the kidney given to the recipient is concerned. From the medical point of view, it is of the utmost importance that we have not consciously or permanently caused impaired health of the donor, *primum non nocere*. Initially, donor nephrectomy (DN) inevitably leads to a decrease in renal function, manifested by increased proteinuria and blood pressure [9, 10]. Since kidney donors are "medically chosen" healthy individuals, and a reduction in the total reserve of kidney function occurs after the planned DN, the question arises of whether we are making patients out of healthy individuals this way. Making a good choice about whether one is an optimal kidney donor is a very important fact for the donor's long-term health. According to the studies published so far, renal function following DN expressed as glomerular filtration rate (GFR), decreases annually on average by -0.42 mL/min/1.73 m^2 [11–14]. Following DN, there is an immediate decrease in renal mass by 50% and in the estimated (eGFR) as well, which later reaches approximately 70% of the pre-donation value [11–13].

The aim of this study was to compare pre-operative and post-operative renal function in patients who underwent DN in relation to patients who underwent radical nephrectomy (RN) due to renal malignancy.

Methods

We conducted a retrospective observational, analytical, case-control study, which included 200 patients treated and followed up for ten years (2010–2020) at the Clinic for Nephrology and the Clinic for Urology of the Military Medical Academy in Belgrade, Serbia. The patients were divided into two groups: group 1 (105 patients) were patients who underwent DN due to related living-donor kidney transplantation, while group 2 (94 patients) was a control group and included patients who underwent RN due to clear cell renal cell carcinoma (ccRCC) in the T1bNoM0 clinical stage [tumor (T), node (N), metastasis (M) staging system], where this surgical procedure was the final form of treatment and, thereafter, patients were considered cured. The control RN group of patients was chosen with the assumption that it was their first malignancy, that their comorbidities included mild to moderate hypertension, and that those were the patients who suddenly, in a very similar way, diminished their kidney function after nephrectomy as well as donors. After the nephrectomy, patients were followed up for at least ten years.

As far as donors are concerned, these healthy people underwent the recommended medical screening before the intervention, i.e., the evaluating laboratory diagnostic tests, in order to exclude the patients with comorbidities that could significantly disrupt the renal functional reserve (diabetes mellitus, malignant hypertension, the existence of untreated malignancy, obesity, etc.). Thus, it could be stated that these were "medically chosen" individuals who, with their consent and the consent of the Ethics Committee of the Military Medical Academy, Belgrade (04/2019, from May 13, 2019), wanted to help their loved ones with organ donation.

In study participants, the GFR was used to monitor the remaining renal function, which is directly proportional to the reserve of the basic morphological and functional unit of the kidney, i.e., the nephron. Serum creatinine-based estimation equations [eGFR, Chronic Kidney Disease−Epidemiology Collaboration (CKD-EPI), Modification of Diet in Renal Disease (MDRD) Study] were used to estimate the GFR, which is also a recommendation based on the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines [13–15]. This way, patients' renal function was assessed using the data obtained from their pre-operative and post-operative laboratory parameters (urea, creatinine, urine).

The statistical analysis was conducted using the IBM SPSS Statistics 26.0 computer program. All continuous variables were described as the median [interquartile range (IQR) between 25th and 75th percentile] or mean ± standard deviation, according to the data distribution (Shapiro-Wilk test). The categorical variables were expressed as percentages and examined using the χ^2 test. Comparisons of nonparametric variables between two groups were performed by the Mann-Whitney *U* test. All analyses were evaluated at the level of statistical significance of $p < 0.05$. The research was approved by the Ethics Committee of Military Medical Academy, Belgrade, Serbia (date of approval May 13, 2019). All patients signed the informed consent.

Results

In the DN group, there were 38.1% males and 61.9% females (Table 1). In the RN group, there were 67% males and 33% females; statistically, there was a significant difference between these groups in terms of gender representation ($p < 0.001$). The average age was statistically significantly higher in the DN group, 55.6 years, than in the RN group of patients, 46.9 years (Table 1).

The median serum creatinine (Table 2), before surgery, was statistically significantly lower in the DN group (69 umol/L) compared with the RN group (92 umol/L) ($p < 0.001$). After a 10-year follow-up, there was a deterioration in serum creatinine in both groups. In the group 1, the median value of serum creatinine was 76.5 umol/L, while in the group 2, it was 115 umol/L. Compared to the increase in serum creatinine, the increase was around 10.8% in the group 1, in contrast to the group 2, where the increase was around 25%.

Compared to the pre-operative GFR value (Tables 3 and 4), it was statistically significantly higher, 94.9 mL/min/1.73 m^2 ("lower" renal functional reserve), in the DN group than in the RN group of patients, 71 mL/min/1.73 m^2 ($p < 0.001$). After ten years of follow-up, in the RN group, we observed that regardless of the formula used to calculate eGFR, it did not exceed 60 mL/min/1.73 m^2. Moreover, following our two groups of patients, we noticed an interesting fact – in the DN group, after 10 years of follow-up, there was a higher prevalence of hypertension (44.3% in the group 1 and 21.3% in the group 2, $p < 0.001$) and diabetes (22.6 % in the group 1, and 9.6% in the group 2, $p = 0.022$), compared to the RN group of patients (Table 5).

Table 1

Baseline characteristics of the patients who underwent nephrectomy

Variable	Group 1	Group 2	*p*-value
Number of patients	105	94	
Gender (male/female)	40 (38.1) / 65 (61.9)	63 (67.0) / 31 (33.0)	< 0.001*
Age at the time of intervention (years)	55.63 ± 7.35	46.93 ± 12.99	< 0.001**
Follow-up period (years)	11.47 ± 5.17	11.05 ± 2.01	0.419**

Values presented as numbers (percentages) or mean ± standard deviation.
***χ^2; ****Independent Samples Test.**
Group 1 included patients who underwent donor nephrectomy; Group 2 (control group) included patients who underwent radical nephrectomy due to clear cell renal carcinoma in the T1bNoMo clinical stage (TNM – tumor, node, metastasis staging system).

Table 2

Serum creatinine (sCR) in the patients who underwent nephrectomy

Time of sCR determination	Group 1	Group 2	*p*-value*
Before surgery	69.00 (59.00–78.00)	92.00 (76.50–112.00)	< 0.001
6 months after surgery	78.50 (66.00–89.25)	120.00 (101.50–134.25)	< 0.001
1 year after surgery	72.00 (64.25–88.00)	99.00 (89.00–113.75)	< 0.001
5 years after surgery	72.00 (65.50–89.00)	101.25 (90.00–118.25)	< 0.001
10 years after surgery	76.50 (70.00–86.00)	115.00 (98.00–117.00)	< 0.001

***Mann-Whitney test; values presented as median with interquartile range (25–75th percentile).**
Group 1 included patients who underwent donor nephrectomy; Group 2 (control group) included patients who underwent radical nephrectomy due to clear cell renal carcinoma in the T1bNoMo clinical stage (TNM – tumor, node, metastasis staging system).

Table 3

eGFR EPI in the patients who underwent nephrectomy

Time of eGFR EPI determination	Group 1	Group 2	*p*-value*
Before surgery	94.95 (83.12–102.82)	71.00 (59.00–91.75)	< 0.001
6 months after surgery	81.70 (66.35–95.22)	54.00 (47.00–63.75)	< 0.001
1 year after surgery	86.60 (69.22–97.20)	66.00 (58.00–74.00)	< 0.001
5 years after surgery	83.35 (69.00–91.97)	61.50 (53.42–69.00)	< 0.001
10 years after surgery	80.40 (63.60–86.40)	56.00 (48.50–60.00)	< 0.001

Values presented as median with interquartile range (25–75th percentile).
***Mann-Whitney test.**
eGFR EPI – estimated glomerular filtration rate according to the Chronic Kidney Disease−Epidemiology Collaboration (EPI) equation; Group 1 included patients who underwent donor nephrectomy; Group 2 (control group) included patients who underwent radical nephrectomy due to clear cell renal carcinoma in the T1bNoMo clinical stage (TNM – tumor, node, metastasis staging system).

Table 4

eGFR MDRD in the patients who underwent nephrectomy

Time of eGFR MDRD determination	Group 1	Group 2	*p*-value*
Before surgery	95.10 (79.02–105.65)	68.00 (59.00–91.00)	< 0.001
6 months after surgery	79.35 (65.10–93.25)	54.00 (47.00–63.00)	< 0.001
1 year after surgery	83.40 (67.75–96.30)	66.00 (58.00–74.00)	< 0.001
5 years after surgery	80.20 (67.10–89.50)	60.50 (53.25–69.00)	< 0.001
10 years after surgery	81.00 (63.08–84.05)	57.00 (49.50–61.00)	< 0.001

Values presented as median with interquartile range (25–75th percentile).
***Mann-Whitney test.**
eGFR MDRD – estimated glomerular filtration rate according to the Modification of Diet in Renal Disease (MDRD) Study equation; Group 1 included patients who underwent donor nephrectomy; Group 2 (control group) included patients who underwent radical nephrectomy due to clear cell renal carcinoma in the T1bNoMo clinical stage (TNM – tumor, node, metastasis staging system).

Table 5

The incidence of arterial hypertension (AH) and diabetes mellitus (DM) in the patients who underwent nephrectomy, 10 years after surgery

Parameter	Group 1	Group 2	*p*-value*
AH	47 (44.3)	20 (21.3)	0.001
DM	24 (22.6)	9 (9.6)	0.022

Values presented as numbers (percentages).
***χ^2-test.**
Group 1 included patients who underwent donor nephrectomy; Group 2 (control group) included patients who underwent radical nephrectomy due to clear cell renal carcinoma in the T1bNoMo clinical stage (TNM – tumor, node, metastasis staging system).

Discussion

The decrease in renal mass was accompanied by a consequent decrease in renal function, regardless of the reason that led to it; however, there were differences in the extent of renal reserve recovery. Inevitably, after unilateral nephrectomy in both groups, there was a loss of 50% of renal mass. The loss of renal mass occurs abruptly, immediately after the surgical intervention, i.e., renal mass and function fall to approximately half the pre-nephrectomy value. After a very short time, the remaining contralateral kidney begins to compensate for the loss of renal function through the so-called adaptive mechanisms [1, 11, 12, 15, 16].

It would be ideal to do a study with completely identical groups by age, gender, and previous diseases. Since the aim of our study was to assess renal function after nephrectomy, donor, or after radical due to RCC, we took two groups that share nephrectomy. In our study, the first group was comprised of healthy people in whom we performed a DN after assessing the total renal reserve in order to help a relative/non-relative (husband/wife); the second group of respondents were patients who underwent nephrectomy for medical reasons; acquired kidney failure is common in both groups. This decrease in GFR is directly dependent on the associated factors such as patient age, gender, pre-operative GFR, pre-operative presence of hypertension, hyperlipidemia, and diabetes [17, 18]. Certain adaptive mechanisms depend on pre-operative factors, which relate to the patients in both groups and certainly affect the degree of post-operative GFR recovery [19, 20]. Along with the decrease in the number of functional nephrons, the remaining nephrons hypertrophy in order to try to maintain the homeostasis of fluids and electrolytes in the body. Over time, recurrent and afterward chronic hyperfiltration, which is the result of a partial increase in glomerular pressure, leads to renal damage and/or accelerated exacerbation of the existing renal damage [21–24]. These short-term and long-term structural and functional adaptations of residual renal tissue must be taken into consideration when predicting the possibility of recovery and the final outcome in a patient with a single kidney.

In a study of renal function following a traumatic kidney loss, it has been reported that the remaining kidney recovered up to 70% of the initial GFR strength due to the adaptive mechanisms. It should be pointed out that any loss of nephrons, regardless of the reason, poses a risk of end-stage renal disease. For this reason, the patients who underwent RN due to a kidney tumor or DN are at risk of renal failure – chronic kidney disease (CKD). It is known that the surgical treatment of renal tumors increases the rate of CKD and that the chosen surgical method (classical or laparoscopic surgery) has a direct impact on post-operative renal function [14, 25–28]. RN has to be done regardless of the status of the contralateral kidney, but DN is not acceptable in case of the ill-functioning contralateral kidney (better kidney remains to the donor). DN is performed if the renal reserve is preserved, and a kidney with a smaller renal reserve is always taken.

Fehrman-Ekholm et al. [29] published a follow-up study concerning DN in 2001, which followed a total of 403 donors. In this study, it was stated that three donors developed stage 4 chronic renal failure, while one donor ended up with

stage 5 renal failure that required an active hemodialysis treatment. During the 12-year follow-up period, the mean GFR was 72 mL/min/1.73 m^2. It turned out that the prevalence of hypertension in these patients was not different from the same prevalence in the general population. In another study, Ellison et al. [30] showed that the frequency of the need for dialysis after DN was only 0.04%.

A study published in 2015 by Gazel et al. [31] followed a total of 200 patients, out of whom 70 patients underwent DN, and 130 patients underwent RN due to kidney tumors. After dividing the patients into groups with GFR below 60 mL/min/1.73 m^2 and those with GFR above 60 mL/min/1.73 m^2, they noticed that the GFR values of patients who underwent RN had a significantly stronger tendency to remain less than 60 mL/min/1.73m^2, compared to those patients who underwent DN ($p < 0.001$). Moreover, at the end of the follow-up period, approximately 20 months later, the decrease in GFR was 33.70% in the RN group and 34.29% in the DN group, and this difference was not statistically significant ($p = 0.783$).

Due to the adaptive hyperfiltration, the residual kidney after unilateral nephrectomy has a relatively rapid recovery within 6–12 months after nephrectomy [32–34]. It has been shown that the effective kidney flow increases by about 30% after only seven days after surgery and remains elevated for a longer period of time (> 10 years) [1–4]. It has also been found that after surgery, the residual mass and function have a remarkable ability to compensate for the loss [22–24]. In the published papers concerning healthy kidney donors, compared with the general population or appropriate controls, no differences were found in urinary albumin excretion, GFR, hypertension prevalence, quality of life, survival rate, and the risk of end-stage renal disease occurrence during a long-term follow-up [11, 12, 15].

If the value of serum creatinine was the only analyzed parameter in our patients after 10 years, in the group 1, we could observe an increase in serum creatinine by about 10.8% in contrast to the group 2, where the average increase in serum creatinine was 25%, which was directly related to the reduced functional reserve (in the group 1, GFR was higher than 80 mL/min/1.73 m^2, while in the group 2, GFR maximum was 60 mL/min/1.73 m^2). In two studies, the reduction in GFR following DN was 20–25% [20, 21]. In the aforementioned study published in 2015 by Gazel et al. [31], this reduction was 34.29%.

Regardless of the used formula, when comparing GFR after one year and after ten years after surgical treatment, it can be concluded that definitive recovery by the so-called adaptive mechanisms occurs within that first year and is practically maintained during the follow-up period of 10 years for both groups. The values of GFR EPI postoperatively after the first year were as follows: in the group 1, 86.60 mL/min/1.73 m^2 (69.22–97.20 mL/min/1.73 m^2) and in the group 2, 66.00 mL/min/1.73m^2 (58.00–74.00 mL/min/1.73 m^2), while the values of eGFR EPI after 10 years were as follows: in the group 1, 80.40 mL/min/1.73 m^2 (63.60–86.40 mL/min/1.73 m^2) and in the group 2, 56.00 mL/min/1.73m^2 (48.50–60.00 mL/min/1.73 m^2). The GFR recovery was better achieved in the group 1. That raises the question of whether the existence of malignancy, even if localized, affected the existing adaptive mechanisms.

In the group 1, after 10 years of follow-up, hypertension was present in 47 patients (44.3%), and in the group 2, in 20 patients (21.3%), which represents a statistically significant difference ($p = 0.001$). The difference in the numbers could be partially explained by the fact that mild and moderate hypertension was not a contraindication for donation in the group 1. Thus, in elderly patients and those who already suffered from mild to moderate hypertension, their condition only worsened in relation to the pre-operative one. On the other hand, the group 2 mainly consisted of younger people and males, so hypertension was not expected. After 10 years, in the group 1, diabetes was present in 24 patients (22.6%), and in the group 2, in 9 patients (9.6%), which is a statistically significant difference ($p = 0.022$).

In the group 1, even though these were carefully selected patients, it turned out that although statistically older, they had a better GFR than those in the group 2. The group 2 consisted of statistically younger patients diagnosed with RCC, predominantly males, without prior selection and exclusion of those with poorly regulated hypertension, diabetes, long-term history of smoking, and extreme obesity [35].

Limitations of the study

The limitations of this study are the retrospective follow-up model, as well as age and gender differences between investigated groups.

Conclusion

In the study group of healthy patients, one year after the intervention, the expected recovery of renal function was achieved after DN thanks to adaptive mechanisms, and a similar renal reserve was maintained after ten years of follow-up.

Comparing the group after DN with the group after nephrectomy due to RCC, we see that after ten years of follow-up, renal function was preserved in the first group after DN, unlike in the group after RN.

It can be concluded that our group of kidney donors was a carefully medically selected group of patients in whom, during the retrospective 10-year follow-up period, it was shown that the renal reserve did not worsen. That certainly confirms that a careful medical selection of "healthy" donors is conducted in our country. Thus, the principle of *primum non nocere* has been preserved.

A long-term evaluation of healthy kidney donors indicates that there is a decrease in creatinine clearance by about 30% (GFR) after 6–12 months post-DN, with a negligible risk of developing end-stage renal disease after kidney donation.

In subjects after DN after 10 years, there were more of those who had *de novo* diabetes compared to patients after RN due to RCC. The aforementioned could be explained by the fact that in the group 2, the patients were younger, while in group 1, older, marginal donors and patients with predia-

betes were accepted. The greater presence of diabetes in the group 1 after ten years after nephrectomy certainly accounted for obesity. It could be said that the donors "relaxed" after having fulfilled a great emotional goal by donating a kidney to their loved ones.

The patients who underwent RN in our hospital were younger males with a higher value of serum creatinine and, consequently, a lower volume of GFR pre-operatively. The inclusion of patients in the RN group is a forced choice, *per se*. In this group of patients, apart from monitoring the possible occurrence or recurrence of malignant disease, the risk factors that can accelerate CKD and other chronic diseases can be carefully reduced or treated through regular examinations.

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The influence of an anesthesiologist's postoperative visit on patient satisfaction with anesthesia for the reconstruction of the anterior cruciate knee ligament

Uticaj postoperativne vizite anesteziologa na zadovoljstvo anestezijom bolesnika podvrgnutih rekonstrukciji prednjeg ukrštenog ligamenta kolena

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Abstract

Background/Aim. When it comes to anesthesia, patient satisfaction (PS) is more difficult to assess than in any other medical specialty. The aim of this study was to construct a tool for assessing PS with anesthesia and then examine the effects of postoperative care provided by anesthesiologists on PS. **Methods.** The study included patients undergoing general anesthesia due to the reconstruction of the anterior cruciate knee ligament who were considered the American Society of Anesthesiologists (ASA) 1 and ASA 2 classes. Patients were divided into three groups: group 1 included 74 patients who had a postoperative visit performed by an attending anesthesiologist; group 2 included 70 patients who had a postoperative visit performed by a nurse anesthetist after surgery; group 3 included 74 patients who did not have postoperative visit during postoperative care by an anesthesiologist nor a nurse anesthetist. The tools used in the research were the Anesthesia Patient Satisfaction questionnaire specially designed for this study and the Post Anesthetic Recovery Scoring System (PAS). ANOVA and Pearson's correlation coefficient were used to estimate the statistical significance of the obtained results between the groups. **Results.** Association between an objective assessment of the postoperative status of patients on day zero and satisfaction with the anesthesiologist's patient management showed statistical significance ($p < 0.05$). Patients who had a postoperative visit by an anesthesiologist tolerated better preoperative and postoperative physical symptoms. Patients visited by an anesthesiologist were most satisfied with postoperative care ($p < 0.05$). **Conclusion.** The use of a highly reliable questionnaire for the evaluation of PS with anesthesia could improve the postoperative condition of patients and enable faster recovery during the postoperative period.

Key words:
anesthesiology; health personnel; patient satisfaction; postoperative care; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Zadovoljstvo bolesnika (ZB) je teže proceniti u oblasti anestezije nego u bilo kojoj drugoj medicinskoj specijalnosti. Cilj rada bio je da se ispita uticaj postoperativne vizite anesteziologa i korišćenja prethodno konstruisanog psihometrijskog instrumenta na ZB podvrgnutih rekonstrukciji prednjeg ukrštenog ligamenta kolena. **Metode.** Ispitanici koji su pripadali *the American Society of Anesthesiologists* (ASA) 1 i ASA 2 klasi su metodom slučajnog izbora bili podeljeni u tri grupe. Prvu grupu činila su 74 bolesnika koje je postoperativno na odeljenju obišao anesteziolog koji je davao anesteziju. Drugu grupu činilo je 70 bolesnika koje je posle operacije obišao medicinski tehničar koji nije učestvovao u anesteziji. Treću grupu činila su 74 bolesnika koja nisu imala postoperativnu vizitu anesteziologa ili medicinskog tehničara na anesteziji. U istraživanju su korišćeni sledeći instrumenti: Sociodemografski upitnik, Upitnik o ZB anestezijom i Postanestezijski sistem skoringa. Za procenu statističke značajnosti razlika dobijenih rezultata korišćena je analiza varijanse (ANOVA). Za proveru povezanosti varijabli korišćen je Pirsonov koeficijent korelacije. **Rezultati.** Subjektivno i objektivno stanje bolesnika posle operacije bilo je povezano sa ZBA ($p < 0,05$). Bolesnici koji su imali postoperativnu vizitu od strane anesteziologa su lakše podnosili preoperativne i postoperativne fizičke simptome i bili su zadovoljniji postoperativnom negom od ispitanika druge dve grupe ($p < 0,05$). **Zaključak.** Korišćenje veoma pouzdanog psihometrijskog instrumenta za procnu ZB

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anestezijom može da poboljša postoperativno stanje bolesnika i utiče na brži oporavak u postoperativnom periodu.

Ključne reči:
anestezija; zdravstveno osoblje; bolesnik, zadovoljstvo; postoperativna nega; ankete i upitnici.

Introduction

Anesthesiologists from around the world work daily to improve the quality of their work, building on their knowledge and skills and following the development of technology that facilitates work and broadens horizons. One of the most important requirements for improving the quality of work for anesthesiologists is an insight into patients' experience and satisfaction with anesthesia [1–6].

Patient satisfaction (PS), when it comes to anesthesia, is more difficult to assess than in any other medical specialty. Fear related to anesthesia affects patients more than fear of surgical procedures. Immediately after surgery, patients may have amnesia induced by premedication. A major problem is a relatively short time an anesthesiologist spends with their patients [2]. Assessing PS with anesthesia is a challenge because it is a multidimensional concept [1–3].

Detection of the adverse events during anesthesia is a relevant step in assessing the patient's satisfaction with anesthesia, but it is not the only indicative factor of the patient's contentment regarding the anesthesia. Patient morbidity and mortality are certainly important for assessing the outcomes but are not appropriate when it comes to the assessment of PS.

Patients seek emotional support from their anesthesiologists in order to feel safer [5]. Continuous monitoring, evaluation, and adaptation to changes in patients' expectations are the basis for continuous assessment of PS with anesthesia [3].

In 2014, the American Association of Anesthesiologists (ASA) issued recommendations on how to continuously monitor and assess PS with anesthesia [7]. They recommended that each hospital around the world construct a valid psychometric instrument for assessing PS with anesthesia [8]. The recommendation highlighted the importance of obtaining information about the operating procedure and patients' demographic data, as well as the construction of a psychometric instrument for assessing PS and its continuous use in the clinical setting. In Europe, the Joint Commission International (JCI) is in charge of monitoring and assessing the quality of healthcare. Part of the quality assessment is an insight into the PS provided with healthcare.

In the Republic of Serbia, the quality of healthcare is evaluated on an annual basis by the Ministry of Health. There is a specific set of guidelines assessing the quality of healthcare. The data collected are general data on every level of healthcare. When looking at tertiary healthcare institutions, the rulebook generally refers to the quality of surgical procedures without giving much consideration to anesthesia. The evaluation is performed with an assessment of patients' lethality rates, the length of hospital treatment, the total number of patients, and the need for patients to be treated in the intensive care unit (ICU) [9].

Aside from the general assessment of PS with the treatment in tertiary healthcare institutions, which is centered around surgery, there are no other assessment tools for PS and the quality of care provided in our country. Anesthesia, without which surgical work or any perioperative management would not be feasible, should be evaluated according to PS in order to provide better medical care in the future. With that in mind, creating a universal psychometric tool capable of assessing PS with anesthesia would be very beneficial for the field.

The aim of this study was to construct a tool for assessing PS with anesthesia and then examine the effects of postoperative care provided by anesthesiologists on PS with anesthesia after knee ligament reconstruction.

Methods

The research was a prospective clinical study that included patients undergoing reconstruction of the anterior cruciate ligament (ACL) of the knee in general anesthesia at the Clinic for Anesthesia and Intensive Therapy and the Clinic for Orthopedic Surgery and Traumatology of the University Clinical Center of Vojvodina, Serbia. The study was conducted from January to October 2014 and included 218 patients. The study included patients of both sexes, who signed an informed consent form, underwent the ACL reconstruction of the knee under general anesthesia, were over 18 years of age, spoke and wrote well in Serbian, and were classified as the American Society of Anesthesiologists (ASA) 1 or 2 patients according to the classification of the ASA. The study excluded patients who were not under general anesthesia for this type of surgical procedure, patients under 18 years of age, patients who did not speak or write well in Serbian, patients who previously experienced anesthesia for surgical procedures, and ASA 3 and ASA 4 patients.

Patients were divided into three groups by a method of random sorting. A randomization plan for treatment assignment to patients was generated using online randomization (https://www.randomizer.org/). We used simple randomization (Figure 1) based on a single sequence of random assignments, so each participant had an equal chance of being assigned to each group and had been assigned to a group independently of other participants [10–12]. The group 1 included 74 patients who had a postoperative visit performed by an attending anesthesiologist; the group 2 included 70 patients who had a postoperative visit performed by a nurse anesthetist after surgery; the group 3 included 74 patients who did not have a postoperative visit during postoperative care by an anesthesiologist nor a nurse anesthetist.

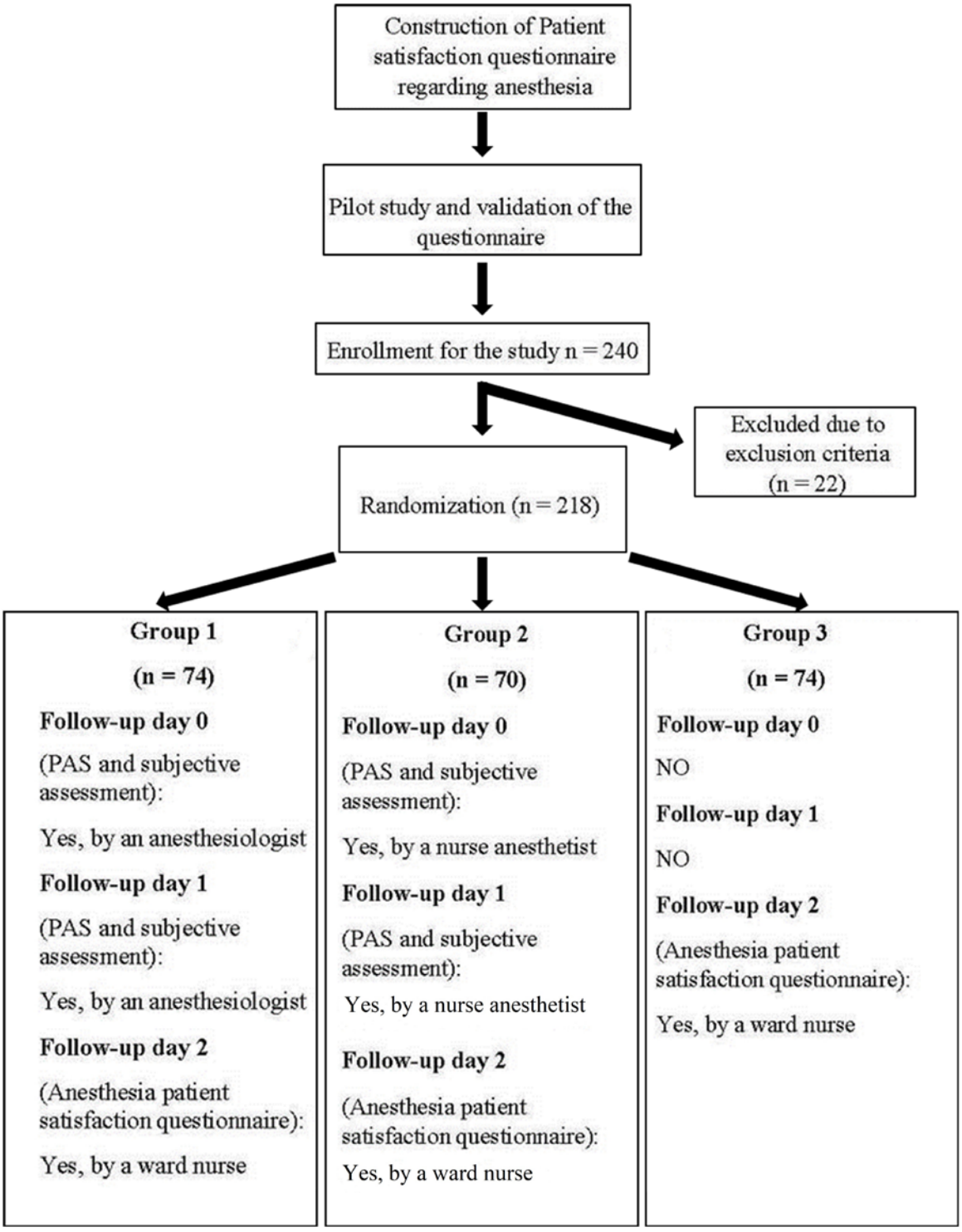

Fig. 1 – Randomization scheme diagram.

The tools used in the research were a sociodemographic questionnaire, Anesthesia Patient Satisfaction Questionnaire (APSQ), and Post Anesthetic Recovery Scoring System (PAS) [13, 14].

APSQ was constructed for the purposes of the study (Appendix 1) and previously validated in a pilot study that included 100 subjects. Based on the theoretical framework of the study, 21 items were formulated. Using factor analysis, the list of key factors was brought down to 4. The first factor was "satisfaction with the relationship between the anesthesiologist and the patient" which consists of ten items. The coefficient of reliability was measured to be 0.90 using Cronbach's alpha. The second factor, "perianesthesia comfort" consists of three items and relates to physical symptoms after surgery, which may be the result of anesthesia. The coefficient of reliability for the second factor

was measured to be 0.56 using Cronbach's alpha. The third factor, "dissatisfaction with postoperative care", consists of five items that focus on the professionalism and behavior of anesthesiologists and other team members before and after surgery. The coefficient of reliability for this factor was measured to be 0.80. The fourth factor, "fear of anesthesia", consists of three items and relates to the patient's stance on and fear of anesthesia. The coefficient of reliability for the fourth factor was measured to be 0.75. The coefficient of reliability for the entire questionnaire measured on the study sample population using Cronbach's alpha was 0.889. The "perianesthesia comfort" factor had weaker metrics when compared to the remaining three factors. Therefore, the questionnaire can be administered without the three items relating to factor two, and the high overall coefficient of reliability for the questionnaire makes it possible to implement a scoring system, which would indicate the PS with anesthesia. During this study, all four factors were used in order to have as much insight into PS.

During the first phase of the study, basic sociodemographic data were collected, and patients were interviewed. General balanced anesthesia was then administered to the patients during the second phase of the study. In the third phase of the study, one hour after awakening from anesthesia, in the recovery room, the group 1 was visited by the anesthesiologist during the postoperative care, while the group 2 was visited by a nurse anesthetist. The group 3 did not have a visit during postoperative care.

During the postoperative visit, we objectively (PAS score) and subjectively estimated the state of the patients (the groups 1 and 2). The second visit for the groups 1 and 2 was performed on the first day after the surgery, and subjective and objective assessment was also performed.

On the second postoperative day, all three groups of patients were given a questionnaire for measuring PS with anesthesia by a hospital ward nurse who was not involved in the postoperative care.

This study was approved by the Ethics Committee of the Faculty of Medicine in Novi Sad (issued on 26th June 2013) and the Ethics Commission of the University Clinical Center of Vojvodina, Serbia (issued on 11th June 2013, No 00-79/400).

Data were collected using a standardized questionnaire, verified by the author, coded, and entered into a specially created database on a personal computer. The basic descriptive statistical parameters used for qualitative and quantitative estimates of the results were arithmetic mean and standard deviation. Variance analysis (ANOVA) was used to estimate the statistical significance of the difference in obtained results between the groups. Pearson's correlation coefficient was used to examine the relationship between variables. We used factor analysis to determine the factor structure of the questionnaires used. For all tests, levels of statistical significance (*p*-values) were specified.

Results

The sample consisted of 218 subjects with a mean age of 29 years (18 to 50 years old). The majority (144) of respondents were male. The overall PAS score during the zero postoperative day was an average of 13.20 in the range of 11 to 14, where a lower score indicates a worse postoperative condition of the subject. The overall mean PAS score on the first postoperative day was 13.94 in a range from 12 to 14 (Table 1). Based on an objective estimate of the patient's health by the anesthesiologist, following the reconstruction of the ACL, it can be inferred that the patients were in good health.

The overall mean score of the subjective assessment of the postoperative condition during day zero (the indicators were pain, drowsiness, hunger and thirst, body tremors, fainting and headaches, nausea and vomiting, dyspnea, and subjective experience of whether they are feeling well) was 12.16 (maximum possible value for subjective assessment was 16, while patients assessed their condition with a maximum of 14 points). The mean subjective assessment score on day zero indicates that subjects were mostly satisfied with their health and did not experience any major issues.

On the first postoperative day, the overall mean score of subjective assessment was 7.95 in the range from 7 to 10 (Table 1). The maximum score of 10 during the first postoperative day implies the overall score of the indicators: pain, drowsiness, hunger and thirst, shortness of breath, and subjective assessment of whether they feel good. Indicators relating to fainting and headache, nausea and vomiting, as well as body tremors, were not measured on the first postoperative day. Patients assessed their subjective condition as excellent on day zero and day one.

Since the subjects reported their postoperative assessments immediately after the surgery and on the first postoperative day, as well as the fact that the PAS score was obtained, which is an objective measure of the patient's condition, a correlation analysis was performed on the data to determine whether there is a relationship between the subjective and objective scores of the patients' postoperative conditions.

Table 1

Mean values of Post Anesthetic Recovery Scoring System (PAS) score and subjective assessment of the subject's postoperative condition

Variable	Minimum	Maximum	Mean	SD
PAS score, zero postoperative day	11.00	14.00	13.20	0.87
PAS score, first postoperative day	12.00	14.00	13.94	0.30
Subjective assessment, zero postoperative day	7.00	14.00	12.16	0.92
Subjective assessment, first postoperative day	7.00	10.00	7.95	0.29

SD – standard deviation.

Based on Table 2, it can be observed that subjective and objective assessments of the postoperative condition of the patients are statistically positively correlated. This positive correlation tells us that subjective and objective assessments of the postoperative condition of the patient are as equally valid and relevant.

To investigate whether there is a relationship between the patient's subjective assessment of the postoperative condition and their satisfaction with anesthesia, we conducted a correlation analysis.

The results of the correlation analysis indicate a positive association between perianesthesia comfort and the subjective assessment of the postoperative state on day zero (Table 3). Based on the results of this analysis, we see that patients who were more satisfied with perianesthesia comfort also reported better postoperative health on day zero. On the other hand, patients who were not satisfied with perianesthesia comfort assessed their subjective condition as worse.

To investigate whether there was a relationship between an objective assessment of a patient's postoperative condition and PS with anesthesia, we conducted a correlation analysis.

Table 4 shows a statistically significant positive correlation between an objective assessment of the postoperative condition of patients on day zero and PS with the anesthesiologist's relationship with them. If the patients were more satisfied with the anesthesiologist's attitude toward them, the objective postoperative condition was better.

To check for statistically significant differences in anesthesia satisfaction between different groups of patients (the group 1, group 2, and group 3), a one-way analysis of the variance was conducted. The groups were used as independent variables in the analysis, while the four factors related to PS with anesthesia were used as dependent variables. When it came to satisfaction with the anesthesiologist's relationship with the patient factor, the group 1 was more satisfied than the groups 2 and 3. In addition, the group 2 was more satisfied with the relationship with the anesthesiologist than the group 3 (Table 5 and Figure 2).

There was no statistical significance when examining the perianesthesia comfort, but based on Figure 3, it can be observed that the group 1 more easily tolerated preoperative and postoperative physical symptoms and felt more comfortable than the patients of the other two groups.

When it comes to dissatisfaction with postoperative care, the group 1 was most satisfied with postoperative care in relation to the other two groups (Table 5 and Figure 4).

Table 2

Intercorrelation of subjective and objective assessment of the postoperative condition of the patient

Variable	PAS (zero postoperative day)	PAS (first postoperative day)
Subjective assessment (zero postoperative day)	**0.27****	
Subjective assessment (first postoperative day)		**0.50****

PAS – Post Anesthetic Recovery Scoring System.

$^{**}p < 0.01$.

Table 3

Correlation coefficients between the subjective assessment of patient's condition on the day zero and day one and satisfaction factors with anesthesia

Variable	Satisfaction with the relationship between the anesthesiologist and the patient	Perianesthesia comfort	Dissatisfaction with postoperative care	Fear of anesthesia
Subjective assessment (zero postoperative day)	0.16	**0.26****	-0.15	-0.06
Subjective assessment (first postoperative day)	-0.01	0.05	0.02	0.10

$^{**}p < 0.01$.

Table 4

Correlation coefficients between objective assessment of the postoperative condition of patients on the day zero and day one and satisfaction factors with anesthesia

Variable	Satisfaction with the relationship between the anesthesiologist and the patient	Perianesthesia comfort	Dissatisfaction with postoperative care	Fear of anesthesia
PAS				
zero postoperative day	**0.21***	0.11	- 0.15	0.03
first postoperative day	- 0.00	0.00	- 0.11	0.08

PAS – Post Anesthetic Recovery Scoring System.

$^{*}p < 0.05$.

Table 5

Differences between groups regarding satisfaction factors with anesthesia

Dependent variable	Differences in mean values	Standard error	*p*-value
Satisfaction with the relationship between the anesthesiologist and the patient			
group 1 vs.			
group 2	**0.51**	**0**.10	**0.00**
group 3	**1.03**	**0**.16	**0.00**
group 2 vs.			
group 1	**-0.51**	0.10	**0.00**
group 3	**0.51**	0.17	**0.01**
group 3 vs.			
group 1	**-1.03**	0.16	**0.00**
group 2	**-0.51**	0.17	**0.01**
Perianesthesia comfort			
group 1 vs.			
group 2	0.25	0.15	0.22
group 3	0.36	0.16	0.07
group 2 vs.			
group 1	-0.25	0.15	0.22
group 3	0.11	0.18	0.82
group 3 vs.			
group 1	-0.36	0.16	0.07
group 2	-0.11	0.18	0.82
Dissatisfaction with postoperative care			
group 1 vs.			
group 2	**-0.41**	0.17	**0.04**
group 3	**-0.61**	0.16	**0.00**
group 2 vs.			
group 1	**0.41**	0.17	**0.04**
group 3	-0.19	0.16	0.43
group 3 vs.			
group 1	**0.61**	0.16	**0.00**
group 2	0.19	0.16	0.43
Fear of anesthesia			
group 1 vs.			
group 2	-0.15	0.16	0.61
group 3	**-0.42**	0.16	**0.03**
group 2 vs.			
group 1	0.15	0.16	0.61
group 3	-0.26	0.17	0.26
group 3 vs.			
group 1	**0.42**	0.16	**0.03**
group 2	0.26	0.17	0.26

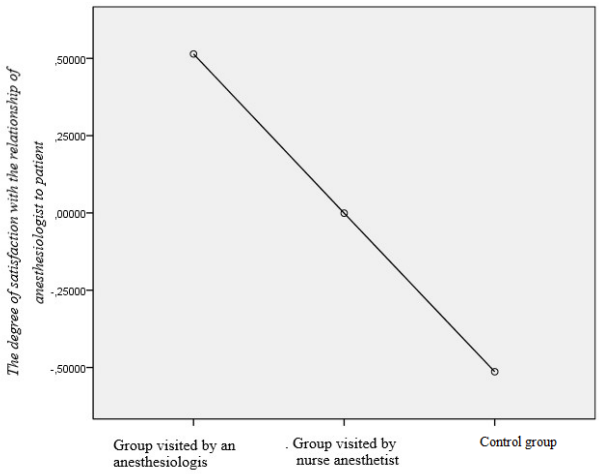

Fig. 2 –Tendency of differences between groups in the degree of satisfaction with the relationship of the anesthesiologist to the patient.

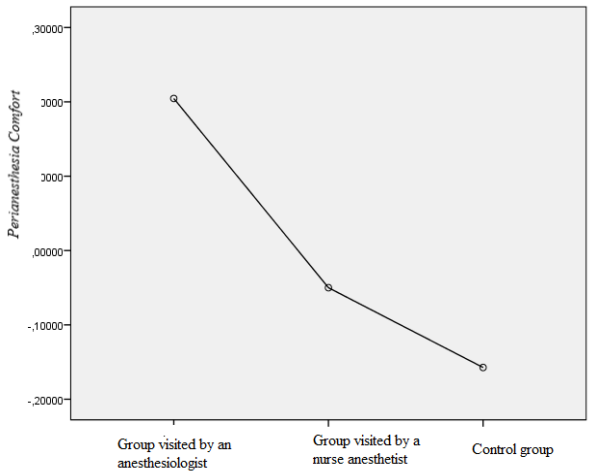

Fig. 3 – Tendency of group differences in perianesthesia comfort.

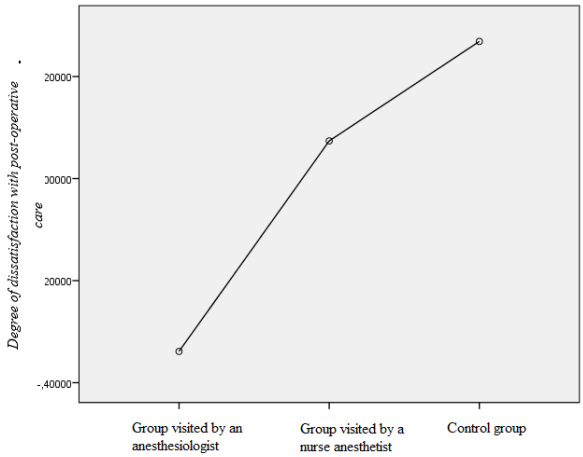

Fig. 4 – Tendency in the difference between groups in the degree of dissatisfaction with postoperative care.

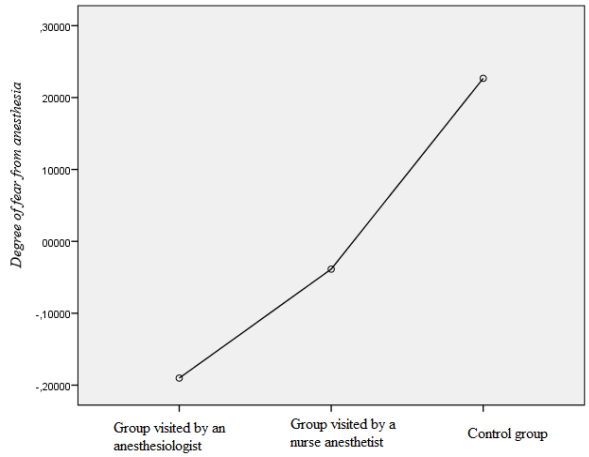

Fig. 5 – Tendency of differences between groups in the degree of fear of anesthesia.

Patients in the group 3 had a more pronounced fear of anesthesia than patients from the group 1 (Table 5). The two groups did not differ statistically from each other, but it can be seen that patients from the group 2 exhibited less fear of anesthesia than patients from the group 3 (Figure 5).

Discussion

Subjective and objective condition of patients after surgery is associated with PS with anesthesia. Patients who are objectively better in general condition are more satisfied with the relationship with their anesthesiologist, while the subjective assessment of the patients about postoperative recovery during day zero is more important for satisfaction on all factors, including satisfaction with anesthesia.

The overall PAS score during the zero postoperative day was an average of 13.20 in the range of 11 to 14, where a lower score indicated worse postoperative conditions. The overall average PAS score on postoperative day one was 13.94 in a range of 12 to 14. Based on the objective assessment of the postoperative condition of the patients by an anesthesiologist, it can be concluded that the respondents were in good general condition after surgery. Based on the patient population's age, ASA score, and the type of surgical intervention, it was expected to see patients in good postoperative condition. The high PAS score amongst the patients correlates well with data found in other literature [15–17].

The objective assessment of the postoperative condition of patients is extremely important for further treatment, as well as for early rehabilitation [18, 19]. Patients in good general condition – with minimal to no postoperative pain - will be able to tolerate more easily the early activation that comes on the first postoperative day after ACL reconstruction [20–22]. The way patients feel often correlates well with the objective assessment of their condition [23–25]. Patients who are feeling well do not have postoperative pain and side effects of anesthesia, hence their subjective assessment of their condition will be good [26–27]. In this study, subjective and objective assessment of the postoperative condition of patients is statistically significantly related, indicating the uniformity of assessments and contributing to a better

understanding of the assessment of the condition and satisfaction with anesthesia. This mutuality indicates that objective and subjective assessments of the patient's postoperative condition are equally important [28–30].

A statistically significant relationship exists between the objective assessment of the postoperative condition of patients on day zero and the "satisfaction with the relationship between the anesthesiologist and the patient". Patients who are more satisfied with the anesthesiologist's attitude towards them are also in a better objective postoperative state.

In this study, the subjective and objective condition of the patient after ACL reconstruction in general anesthesia correlates well with PS with anesthesia. Patients who are objectively in better condition are more satisfied with the relationship with the anesthesiologist, while the subjective condition of the patient on postoperative day zero has a bigger impact on all factors regarding satisfaction with anesthesia. Bost et al. [16] also showed a statistically significant correlation between the subjective and objective condition of the patient with the patient's satisfaction with anesthesia.

As in the study by Saal et al. [31], where significant differences in the level of satisfaction with anesthesia between groups were recorded, statistically significant differences were also found in our study. In our study, group 1 scored higher when it came to factors such as "satisfaction with the relationship between the anesthesiologist and the patient" and "perianesthesia comfort" when compared to the other two groups.

Subjects were also asked about their fear of anesthesia, with 52.8% of subjects stating they felt fear of anesthesia. More than half of the subjects felt fear of anesthesia prior to the surgery, and the control group (group 3) in the postoperative period had higher scores on the "fear of anesthesia" factor. This result validates data from literature where the level of fear of anesthesia is significantly higher in groups that did not have a postoperative visit by an anesthesiologist [32, 33]. Amongst the study population, group 3, which had no postoperative visit, had the highest score when it came to "dissatisfaction with postoperative care". In the study by Saal et al. [31], the experimental group, which had a postoperative visit by an anesthesiologist, scored higher regarding the "continuous care by an anesthesiologist and trust". There is no statistically significant difference between the groups visited by an anesthesiologist and medical technician from the department.

There was a statistically significant difference between groups when it comes to "satisfaction with the relationship between the anesthesiologist and the patient", "fear of anesthesia", and "dissatisfaction with postoperative care".

When it came to the "satisfaction with the relationship between the anesthesiologist and the patient", patients in the group 1 were more satisfied with this aspect than patients in the other two groups. Moreover, patients in the group 2 were more satisfied with the relationship with the anesthesiologist than in the control group. In the study by Saal et al. [31], a statistically significant difference was observed only when compared to the control group; no significant difference was observed between the groups that were visited by an anesthesiologist and medical technician. Ateleanu et al. [32] and Sultan et al. [34] observed a statistically significant difference in postoperative visits between anesthesiologists and other members of the anesthesiology team.

There was no statistically significant difference between groups regarding the assessment of "perianesthesia comfort". However, one can infer that the patients visited by an anesthesiologist handled pre- and postoperative symptoms more easily and consequently felt more comfortable than the other two groups.

When it comes to "dissatisfaction with postoperative care", the group 3 significantly differed from the other two experimental groups. Patients in the group 3 were more dissatisfied with postoperative care. The group 1, which was visited by an anesthesiologist, was the most satisfied with postoperative care of all groups. The additional attention provided by an anesthesiologist helps the patient feel safer and more satisfied with postoperative care. When looking at PS with anesthesia, the literature highlights the importance of the care provided by an anesthesiologist [33, 35, 36].

Patients in the group 3 felt a more pronounced fear of anesthesia when compared to patients in the group 1. The groups 1 and 2 did not differ significantly from each other. Patients in the group 2 showed lower levels of fear of anesthesia in comparison to patients from the group 3. The presence of an anesthesiologist, who explains to patients the anesthesia procedure and who is there to answer any possible questions, contributes to the reduction of anxiety and stress and represents a form of preoperative preparation which is very important for the patient's experience of surgery and later postoperative recovery [31, 34, 37].

Data from literature, which focuses on the importance of postoperative visits, indicates a statistically significant difference between patients who had a postoperative visit from anesthesiologists and those who did not [35–44].

The importance of a postoperative anesthesiologist's work is undeniable when considering PS with anesthesia [45].

Conclusion

Greater satisfaction with the relationship between the anesthesiologist and the patient, as well as with postoperative care and less pronounced fear of anesthesia in the subjects of the experimental group visited by an anesthesiologist, highlight the importance of proper communication with patients, i.e., communication of patients with the person they previously saw during the anesthesia procedure. A visit by the anesthesiologist who administered the anesthesia makes patients feel safer. That consequently increases the patient's level of satisfaction with the anesthesia and, even more so, the level of satisfaction with postoperative care and the relationship between the patient and the medical staff, more specifically with the anesthesiologist. We also constructed a highly reliable questionnaire for evaluating PS with anesthesia, which can be readily used in a clinical setting in our region. These results give us guidance for further work of the anesthesiologists in order to improve postoperative care and enable faster recovery, which is a consequence of the patient's greater satisfaction with anesthesia and postoperative care.

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Appendix 1

Question number	**Patient satisfaction questionnaire regarding anesthesia**
1	Are you satisfied with your anesthesiologist? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
2	Are you satisfied with anesthesia? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
3	Are you satisfied with the anesthesiologist's relationship with you? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
4	Are you satisfied with how much information regarding anesthesia you were given? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
5	Are you satisfied with the anesthesiologist's answers to the questions that you asked? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
6	Did you feel free to ask the anesthesiologist questions regarding anesthesia procedure? 1. I did not feel free to ask any questions; 2. I felt a little freedom to ask questions; 3. I am not sure if I felt free to ask questions; 4. I felt free to ask questions most of the time; 5. I felt completely free to ask any question regarding anesthesia procedure.
7	Was it significant to you that you were asked questions about your previous experience with anesthesia? 1. It did not have any significance; 2. It had little significance; 3. It was not significant, nor insignificant; 4. It was significant; 5. It was very significant.
8	Did you feel safe regarding anesthesia? 1. Unsafe; 2. Partly safe; 3. Not safe, nor unsafe; 4. Safe; 5. Very safe.
9	Did you feel relaxed regarding anesthesia? 1. Unrelaxed; 2. Partly relaxed; 3. Not relaxed, nor unrelaxed; 4. Relaxed; 5. Very relaxed.
10	Did you feel that your personal data were protected with regard to anesthesia? 1. I did not feel that my personal data were protected at all; 2. I did not feel that my personal data were protected most of the time; 3. I am not sure; 4. I felt that my personal data were protected most of the time; 5. I felt that my personal data were protected completely.

11	Were you satisfied with the professionalism of anesthesia team members? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
12	Did you feel fear of adverse events from anesthesia before talking to an anesthesiologist? 1 means no fear, 5 implies an intensive feeling of fear 1 2 3 4 5
13	Did you feel fear of adverse events from anesthesia after talking to an anesthesiologist? 1 means no fear, 5 implies an intensive feeling of fear. 1 2 3 4 5
14	How much are you satisfied with comfort in the recovery room after awakening from anesthesia? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
15	How comfortable did you feel on an operating table? 1 means completely uncomfortable, 5 means not uncomfortable at all. 1 2 3 4 5
16	How hard was it for you to tolerate pre-operative and perioperative fasting? 1 – it was extremely hard for me to tolerate the fasting 5 – I had no problem tolerating the fasting. 1 2 3 4 5
17	Did you experience postoperative nausea? 1 – I had severe nausea, 5 – I had no postoperative nausea at all 1 2 3 4 5
18	Are you satisfied with how much you waited between arriving at the operating theatre and the beginning of anesthesia? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
19	How much are you satisfied with the treatment of postoperative pain? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
20	How much are you satisfied with the visits from the anesthesiologist after the surgery? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
21	How much are you satisfied with the visits from other anesthesia team members after the surgery? 1. Dissatisfied; 2. Partly dissatisfied; 3. Not satisfied, nor dissatisfied; 4. Satisfied; 5. Very satisfied.
	What is your first memory after the surgery? _____ _____ Did you dream during anesthesia? Yes No

ORIGINAL ARTICLE

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Isolation with rubber dam: knowledge, training, and attitudes of final year dental students

Izolacija radnog polja upotrebom koferdama: znanje, veštine i stavovi studenata završne godine stomatologije

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Abstract

Background/Aim. Good undergraduate education is necessary to overcome the reluctance of dentists to use the rubber dam (RD). The aim of the study was to assess dental students' knowledge, training skills, attitudes, and opinions concerning the use of RD in order to isolate an operation field. **Methods.** A 34-item original questionnaire was distributed to 130 final-year students of the Faculty of Dental Medicine, University of Belgrade. The questions were divided into four segments: general information, RD-related knowledge and training skills, opinions and attitudes regarding the use of RD, and opinions on the intended future use of RD. **Results.** All students confirmed that they had theoretical lessons about RD and that its advantages were pointed out. During practical courses, 34% of students observed RD placement and 10% of them placed RD on their own or with assistance. Most (88%) of the students did not feel capable of using RD on their own. Less than half of the students (38%) believed that adequate isolation of the operating field is possible without RD. Sixty-four percent of students considered that RD was not uncomfortable for the patients. More than half of the students were willing to use RD in their future practice. Almost all of them planned to gain additional postgraduate training with RD. **Conclusion.** Students have solid theoretical knowledge about RD; they are aware of its importance and have a positive attitude toward RD use. However, their practical training and skills are poor and insufficient for independent RD use in order to isolate an operation field.

Key words:
education; health knowledge, attitudes; practice; rubber dams; students, dental; survey and questionnaires.

Apstrakt

Uvod/Cilj. Osnovni preduslov za prevazilaženje odbojnosti stomatologa prema upotrebi koferdama jeste kvalitetno obrazovanje, stečeno tokom osnovnih studija. Cilj rada bio je da se procene znanje, veštine, obučenost za rad, stavovi i mišljenje studenata u vezi sa upotrebom koferdama u izolaciji radnog polja. **Metode.** Ukupno 130 studenata završne godine Stomatološkog fakulteta popunjavalo je originalni upitnik od 34 pitanja, koja su bila podeljena u četiri segmenta: opšte informacije, znanje i obučenost za rad sa koferdamom, mišljenja i stavovi o upotrebi koferdama i mišljenja o korišćenju koferdama u budućem radu. **Rezultati.** Svi studenti su potvrdili da su imali teorijsku nastavu o izolaciji radnog polja koferdamom i da im je ukazano na njegove prednosti. Tokom praktičnih vežbi, 34% studenata je posmatralo postavljanje koferdama, dok je njih 10% samostalno postavilo koferdam. Većina (88%) studenata se nije osećala sposobnim da samostalno koristi koferdam. Manje od polovine (38%) studenata verovalo je u mogućnost odgovarajuće izolacije radnog polja i bez upotrebe koferdama. Da koferdam nije neprijatan za pacijente smatralo je 64% studenata. Više od polovine studenata bilo je spremno da koristi koferdam u svom budućem radu. Skoro svi studenti planirali su dodatno obučavanje za rad sa koferdamom posle završetka osnovnih studija. **Zaključak.** Studenti imaju solidno teorijsko znanje o upotrebi koferdama, svesni su njegovog značaja i imaju pozitivan stav prema njegovoj upotrebi. Njihova praktična obuka i veštine nedovoljne su i ne omogućavaju im da u cilju izolacije radnog polja samostalno postave koferdam.

Ključne reči:
obrazovanje; zdravlje, znanje, stavovi, praksa; koferdam; studenti stomatologije; ankete i upitnici.

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Introduction

The use of the rubber dam (RD) is universally acknowledged as an ideal method for performing dental treatments completely free of saliva and represents the crucial element for achieving an absolutely dry operating field [1–3]. It also provides retraction and protection of the soft tissues, better visibility and aseptic conditions of the operating field, reduction of infectious pathogens in the aerosol, and prevention of aspiration or ingestion of instruments or irrigants [4–7]. Dental practitioners are encouraged and required to use RD in their daily practice, as RD is considered an essential factor that significantly influences the success and durability of dental treatments [8–10].

Despite scientific evidence and official recommendations [11, 12], dentists seem reluctant to use RD, as many recent studies report a fairly low overall rate of RD usage [13–16]. The most common reasons reported for its underuse were inconvenience and difficulty in use, insufficient and inadequate training, prolonged time of treatment, cost of equipment, as well as the assumption that patients would not accept it [6, 13, 14, 17]. Interestingly, these obstacles were usually cited by dentists who did not use RD regularly [13, 18].

Among irregular RD users, the factors found to influence the decision to use RD included the type of treatment, material selection, and region of the mouth requiring treatment. Endodontic treatments are most frequently performed under RD [14, 17, 19]. Regarding restorative treatments, RD was more often used for composite than amalgam restorations, for treatment of posterior than of anterior teeth, and in the lower compared to the upper jaw [14, 17, 19].

Qualifying school [13] and graduate training intensity [20] also affect RD use. It was shown that recently graduated and younger dentists used RD more frequently than their older colleagues [21]. Moreover, there was a clear discrepancy in what dentists are taught in dental schools regarding RD use and how they practice using it after graduation [16]. Even final-year dental students believed their use of RD would decrease once they left school and began working in independent practice [22]. Previous studies reported students' insufficient theoretical knowledge about the importance of RD [23, 24], and possible negative perceptions associated with RD use [22, 25].

There is a general agreement that acquiring knowledge and skills for the proper use of RD should be a fundamental part of education in dental schools. Giving students a good theoretical background and allowing them to acquire manual dexterity during their studies should give them the confidence to use RD in the future. To the best of the authors' knowledge, there are no scientific data on the prevalence of RD use among Serbian dentists, nor are there data on whether and how RD is taught in dental schools. Therefore, the aim of the study was to assess knowledge and training skills, as well as attitudes and opinions towards the use of RD among the final, fifth-year dental students attending the Faculty of Dental Medicine, University of Belgrade, Serbia.

Methods

A 34-item original questionnaire, designed by the authors for the purpose of the study, was distributed to 130 fifth-year students of the largest public dental school in Serbia, the Faculty of Dental Medicine, University of Belgrade. The questionnaire included "open" and "closed" questions, divided into four segments: (1) general information regarding the students' attendance at practical and theoretical courses; (2) RD-related knowledge and training skills; (3) opinions and attitudes regarding the use of RD; (4) opinions on the intended future use of RD in their independent practice. The study protocol for this observational cross-section study was approved by the Ethics Committee of the Faculty of Dental Medicine, University of Belgrade, (no. 36/24, 23.10.2020). Students were allowed to decline participation in the study. All completed questionnaires were collected anonymously.

Descriptive analyses of the data gathered from the questionnaires were performed using the statistical program IBM SPSS for Mac (Version 21.0 Chicago, IL, USA).

Results

Out of 130 distributed questionnaires, 108 were adequately completed and returned and were included in the study (response rate of 83.08%).

General information

All (100%) students reported they were attending all practical courses regularly. Regarding theoretical classes, 44 (41%) students were regular attendants, while the rest (64, 59%) of them attended theoretical courses irregularly.

Knowledge and practical skills

All (44) students who regularly attended theoretical courses reported that they were taught about RD in classes and that the advantages of RD over relative isolation with cotton rolls and saliva ejectors were pointed out. Out of them, 21 (48%) considered that the topic was covered in detail, 18 (41%) students reported that it was covered superficially, and 5 (11%) of them claimed that the topic was only mentioned. Figure 1 shows the distribution of responses regarding subjects that had theoretical lessons about RD obtained only from the students who regularly attended theoretical courses.

During practical courses, 37 (34%) students observed RD placement, and 11 (10%) placed RD on their own or with the teacher's assistance. Nine students placed RD only once, while two students placed RD twice in different practical courses. In total, RD has been placed 14 times – five times in restorative dentistry, five times in pediatric dentistry, and four times in endodontics.

Ninety-five (88%) students answered that theoretical and practical training obtained during their studies was not sufficient for them to use RD on their own. A little more than half of them (56 students, 52%) have searched for more information about RD, mainly on the Internet.

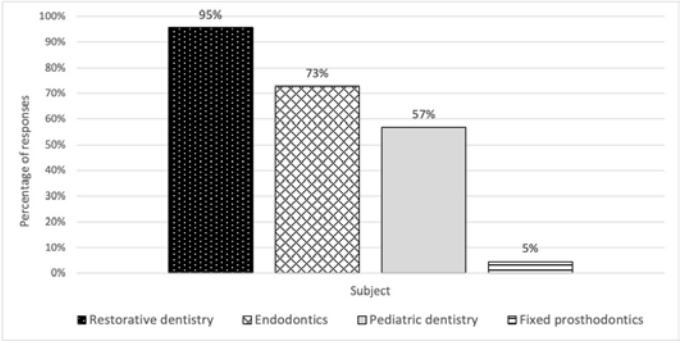

Fig. 1 – Distribution of students' responses regarding the subjects that had theoretical lessons about the rubber dam (only responses obtained from 44 students that regularly attended theoretical courses were taken into consideration).

Opinions and attitudes

The overview of the responses to "yes/no" questions related to the students' opinions and attitudes toward the use of RD is presented in Table 1.

The majority (55%) of students thought that RD use would decrease the duration of the treatment, while others (28%) thought it would increase or have no influence (17%) on the duration of the treatment.

When asked to express their opinion on the benefits of RD use, 64% considered it beneficial for dental interventions in both the upper and lower jaw, while the remaining students (36%) thought it was more beneficial for interventions in the lower jaw. Sixty-two percent of students agreed that it is equally important for the treatment of anterior and posterior teeth, whereas 38% thought it was more useful for posterior teeth. Half (50%) of the students reported that RD was useful for both composite and amalgam restorations, while 42% considered it useful only for composite restorations.

Figure 2 shows the students' opinions about the most difficult step during the RD application.

Table 1

The responses to "yes/no" questions related to the students' opinions and attitudes toward the use of the rubber dam

Question	Answer	
	yes n (%)	no n (%)
Do you think it is necessary to provide students with basic knowledge and practical training on the use of the rubber dam during their undergraduate studies?	105 (97)	3 (3)
Do you think that achieving adequate isolation of the operating field for either endodontic or restorative procedures is possible without the use of the rubber dam?	41 (38)	67 (62)
Do you think that the rubber dam has certain advantages compared to the isolation with cotton rolls and saliva ejectors?	108 (100)	0 (0)
Do you think that the success of the endodontic treatment is higher if the operating field is isolated with the rubber dam than with cotton rolls and saliva ejectors?	102 (94)	6 (6)
Do you think that rubber dam placement is a difficult and complicated procedure?	35 (32)	73 (68)
Do you think that rubber dam placement is more difficult than other procedures you regularly perform as part of your practical classes?	38 (35)	70 (65)
Do you think that the help of a dental assistant is necessary for rubber dam placement?	55 (51)	53 (49)
Do you think that dental treatment is less comfortable for patients if the rubber dam is used?	39 (36)	69 (64)
Do you think that significant financial resources are required for the rubber dam purchase?	36 (33)	72 (67)

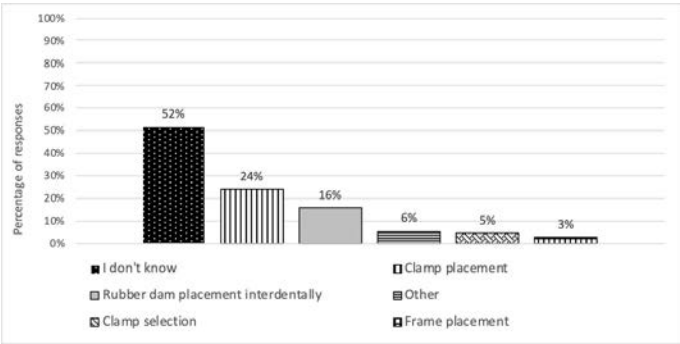

Fig. 2 – Distribution of students' responses on the most difficult step during the rubber dam application.

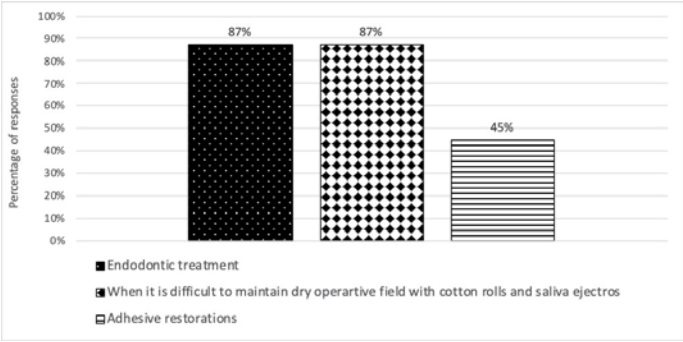

Fig. 3 – Students' responses regarding the type of operative procedure they would use the rubber dam for.

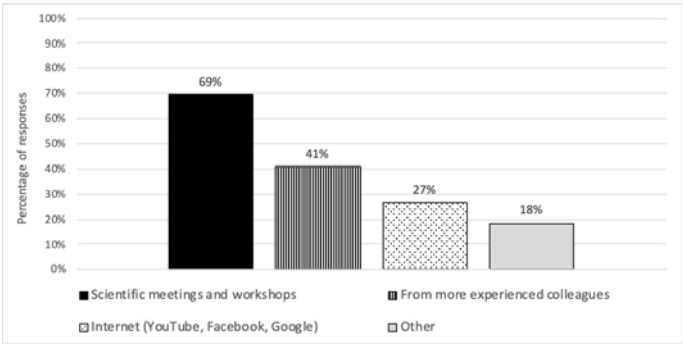

Fig. 4 – Students' responses regarding the plans for the postgraduate training in rubber dam placement.

Opinions on the intended future use

The majority (64%) of students reported they were willing to use RD in their future practice; 27% would decide whether to use RD or not, depending on the type of operative procedure or the situation they encounter (Figure 3); 9% of the students did not plan to use it at all. Almost all (84%) of them planned to gain additional postgraduate training in different ways (Figure 4).

Discussion

Although teaching students about RD has been part of dental school training for decades, there is worldwide scientific evidence showing its limited use among dental professionals [13, 14, 20, 26–28]. The most important measure proposed to overcome the reluctance of dentists to use RD is better undergraduate education and training [15]. Investigations among dental students, using questionnaires as a research instrument, are often conducted as a helpful tool to identify their knowledge and perceptions of RD, as well as to reveal potential problems in the educational process [22–25, 29].

In the present study, all students who regularly attended theoretical courses confirmed that they learned about RD and its advantages during lectures. Apart from restorative dentistry and endodontics, attention to RD was also given in pediatric dentistry lessons, indicating the necessity of using RD in pediatric patients as well. However, more than half of the participants have never observed or performed RD placement during clinical training. This discrepancy between what is taught and how clinical procedures are being performed may be confusing for future dentists. Considering the almost complete lack of practical training, it is not surprising that almost 90% of students did not believe they were capable of using RD on their own. All of the mentioned facts could explain RD's underuse in independent practice after graduation from dental school.

Various results could be found in the literature concerning the students' use of RD on adult patients. In Saudi Arabia, dental students used RD almost always [29], while in Ireland and the UK, the majority of students used RD occasionally [22]. When it comes to pediatric patients, more consistent findings were reported – RD was used rarely or never [22, 25, 29]. Interestingly, in the present study, almost an equal number of students placed RD during restorative dentistry, endodontics, and pediatric dentistry practical courses.

One of the segments that should be covered in dental schools is the legal aspect of RD placement. In case when RD isolation is not performed and an endodontic instrument is inhaled by the patient, a medicolegal aspect of negligence is impossible to defend [30]. Patient safety during dental treatment is essential from the practitioner's as well as the patient's point of view. Although it does not happen very often, there are some reports of inhaling and ingesting endodontic instruments during root canal treatment performed without RD isolation [31, 32].

The present study shows that, despite little experience with RD, all students seemed to be certain about the necessity of acquiring knowledge and skills for RD use during their studies. They were convinced that RD isolation has ad-

vantages compared to cotton rolls and saliva ejectors and that the success of endodontic treatment depends on RD use. However, around 40% of students still believed that adequate isolation of the operating field for either restorative or endodontic procedures is possible without the use of RD. These results support earlier findings, but it should be noted that significant differences existed between the schools when more than one school was investigated [22, 23, 25, 29]. Furthermore, various factors, such as clinical procedure, choice of material being placed, and the jaw in which treatment is performed, were found to influence the use of RD [14, 17, 19, 22, 25]. In this study, more than half of the participants believed that RD was beneficial for both the upper and lower jaw, anterior and posterior teeth, and composite and amalgam restorations.

Considering that one of the frequent reasons mentioned for RD underuse is its difficult application, around 70% of the students did not think it was a difficult and complicated procedure, nor that it was more difficult than other procedures they regularly perform. That is in contrast to the results of other studies [22, 23, 25, 29], probably because the students who participated in the present study observed the procedure but did not perform RD placement by themselves. Consequently, the vast majority of students were not sure about the most difficult step during RD placement. The second most frequent response was clamp placement, similar to a previous study that reported clamp selection and its adaptation as the most complicated step for students [25]. Conversely, in another research, most students were confident regarding which clamp to use, but the most cited difficulty in RD appliance was tight contacts [23]. Moreover, students had divided opinions on whether assistance was required for RD placement. In another study, students generally believed that assistance was not necessary for RD application [25], while Imbery and Carrico [28] reported that students particularly struggled with RD placement when they were working alone and that they preferred if the assistant was available.

There is a common belief among students [23, 25, 29] and dentists [6, 13, 14, 17] that patients have negative attitudes toward RD. In the present study, around 65% of students considered that the application of RD did not make dental procedures less comfortable for the patients, which is in line with the results reported by Mala et al. [22]. When patients were asked, the majority of them had a positive experience with RD and preferred it to be used at their next appointment [33–35]. RD was even acceptable to pediatric dental patients [36].

The students involved in the present research were not particularly worried about the time needed for RD application. Less than 30% of students thought that placing RD would increase the time of treatment, which is opposite to the opinions of the students from another research [25]. It was proved that it takes only 4 to 5 min [23, 33, 34] for students to apply RD and even less time for the dentists. Probably, calculating the time that would subsequently be saved throughout the procedure performed with RD, most of the students in this research considered that the overall time of the procedure would be shorter.

The financial aspect, i.e., the fact that the cost of the equipment and the treatment increase, is one of the widely discussed factors that might influence the use of RD [6, 13, 14, 17]. However, it is obvious that a technique with clear infection control has benefits, and medico-legal implications should not be excluded due to cost [14]. That was confirmed by a study where no respondent referred to cost as a reason for not using RD [17]. While most of the students in this study thought that RD purchase does not require significant financial resources, it could be assumed that finances would have a more significant impact on their attitude once they start working in the independent private practice.

Even though only a small number of students used RD during their studies, the encouraging fact is that around 60% of them plan to use it regularly in their future work, suggesting their positive attitude and commitment to its use. As expected [14, 17, 19, 22, 25], among those who intend to use RD only for certain clinical procedures, endodontic treatment would be the one that, in their opinion, requires RD application. Another anticipated situation that could potentially urge them to use RD is when it is difficult to maintain the operative field dry with cotton rolls and saliva ejectors. Nevertheless, final-year dental students that participated in this study did not feel they were sufficiently trained to use RD on their own in the future, as almost all of them plan to gain additional postgraduate training, mainly through scientific meetings and workshops and from more experienced colleagues.

Conclusion

Based on the results of this study, it could be concluded that students have solid theoretical knowledge about RD, are aware of its importance, and have a positive attitude and enthusiasm toward RD use. On the other hand, their practical training and skills are poor and seem to be insufficient for independent RD use. It is necessary to dedicate more attention to RD isolation techniques throughout undergraduate practical courses so that dental students can implement acquired knowledge and skills in their practice after graduating. To avoid confusion among students, teachers in dental schools should be consistent and eliminate the discrepancy between how they perform dental procedures in the clinic and what they teach in the classroom.

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Forecasting analysis of selected health- and economy-related indicators in South Eastern European and Balkan countries

Analiza predviđanja odabranih zdravstvenih i ekonomskih indikatora u zemljama Jugoistočne Evrope i Balkana

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Abstract

Background/Aim. Health indicators provide comparable information relevant to defining health goals. The aim of this study was to perform a forecasting analysis of the selected indicators, which could help anticipate the future necessities in the health economy and prevent the problems that would predictively grow in the future. **Methods.** Health indicators are collected from publicly available databases of the World Health Organization and Eurostat. We used the quantitative forecasting technique, commonly used for historical data, to predict several years in the future concerning selected health- and economy-related indicators. **Results.** Total health expenditures as a percentage of gross domestic product (GDP) by 2025 will increase in most of the 17 surveyed countries. The percentage of direct household budget payments (out-of-pocket) for health services will decrease in half of the countries, while values of GDP expressed in US$ will increase significantly compared to the last surveyed year (2017), except in Greece. The infant mortality indicator shows that the numbers will decrease in each surveyed country, while the urban population percentage will rise almost in each country except Estonia. Life expectancy will increase in each surveyed country. **Conclusion.** Health- and economy-related indicators must be monitored over time, as they provide significant information concerning the relevant issues in the health care system. Moreover, they may indicate changes that should be made in order to accomplish progress in each individual country.

Key words:
balkan peninsula; europe, eastern; forecasting; health care costs; health care economics and organization; health care evaluation mechanisms; health care reform; statistics; health status indicators.

Apstrakt

Uvod/Cilj. Zdravstveni indikatori pružaju uporedive informacije relevantne za definisanje zdravstvenih ciljeva. Cilj ovog rada bio je da se izvrši analiza predviđanja odabranih indikatora koji bi mogli da pomognu u predviđanju budućih potreba u ekonomiji zdravstva i sprečavanju problema koji bi u budućnosti mogli prediktivno da rastu. **Metode.** Zdravstveni pokazatelji su prikupljeni iz javno dostupnih baza podataka Svetske zdravstvene organizacije i Eurostata. Koristili smo tehniku kvantitativnog predviđanja koja se obično koristi za istorijske podatke, za predviđanje nekoliko godina u budućnost, uzimajući u obzir odabrane indikatore povezane sa zdravstvom i ekonomijom. **Rezultati.** Ukupni izdaci za zdravstvo kao procenat bruto domaćeg proizvoda (BDP) do 2025. godine povećaće se u većini od 17 analiziranih zemalja. Procenat plaćanja za zdravstvene usluge direktno iz budžeta domaćinstava smanjiće se u polovini analiziranih zemalja, dok će vrednosti BDP-a, izražene u američkim dolarima, znatno porasti u poređenju sa poslednjom analiziranom godinom (2017), osim u Grčkoj. Pokazatelj mortaliteta novorođenčadi pokazuje da će se brojevi smanjiti u svakoj analiziranoj zemlji, dok će procenat gradskog stanovništva rasti gotovo u svakoj zemlji, osim u Estoniji. Očekivani životni vek će se povećati u svakoj od pomenutih zemalja. **Zaključak**. Indikatori povezani sa zdravljem i ekonomijom moraju se pratiti tokom vremena, jer pružaju značajne informacije o relevantnim pitanjima u zdravstvenom sistemu. Štaviše, oni mogu ukazivati na promene koje bi trebalo izvršiti kako bi se postigao napredak u svakoj pojedinačnoj zemlji.

Ključne reči:
balkansko poluostrvo; evropa, istočna; predviđanje; zdravstvena zaštita, troškovi; zdravstvena zaštita, ekonomija i organizacija; zdravstvena zaštita, ocena kvaliteta; zdravstvo, unapređenje; statistika; zdravstveno stanje, indikatori.

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Introduction

A health indicator is a parameter used in order to gather information on certain priority topics concerning the health of the population or activities within the health system [1]. Health indicators provide comparable information across different geographical, organizational, or administrative territories and can track progress over time. They help monitor the key performance dimensions described in the health system performance measurement framework (HSPMF), which provides a common approach to health system management across the country [2].

Health indicators try to describe and monitor the health status of the population [3]. Attributes refer to health characteristics or qualities, while the concept of health itself encompasses physical, emotional, spiritual, environmental, mental, and social well-being [4, 5].

The reason why indicators are used in public health is to initiate health decisions. The ultimate goal is to improve the health of the population and reduce diseases and gender inequalities [6].

Health systems have a vital and lasting responsibility for human health throughout life. They are crucial for the healthy development of individuals, families, and societies everywhere [7, 8]. Real progress in health, according to the United Nations (UN) millennium development goals (UNMDG) and other national health priorities, vitally depends on a stronger health system based on primary health care [9, 10], like it was in former Yugoslavia.

Serbia and other Balkan countries share many historical specifics and a common heritage with the countries of Eastern Europe led by the Russian Federation [11]. This legacy is often attributed to the fact that the founding of the Semashko health care system was embedded in Bismarck's mixed model, accepted in the former Yugoslavia [12, 13]. The Bismarck system was established in 1893 in Germany, while the English Beveridge system was established in 1911 with many elements taken from the Russian Imperial model [14, 15].

In short, indicators play a key role in turning data into relevant information for public health decision-makers. Health indicators are relevant for defining the health goals that national health authorities should pursue [16].

Countries that have been chosen to be compared share similar historical backgrounds, but still, some of them are more successful than others in managing indicators related to different health systems [17]. Adapting the current health system and introducing the elements from the health system of other countries may be useful from the point of view of progress [18, 19].

The aim of this study was to perform a forecasting analysis of certain indicators which could help anticipate the future necessities in decision-making concerning the health economy and prevent problems that would predictively grow in the future.

Methods

The countries of interest that were surveyed are the following: Albania, Bosnia and Herzegovina, Bulgaria, Greece, Croatia, Montenegro, Northern Macedonia, Romania, Serbia, Slovenia, Turkey, Russia, Belarus, Lithuania, Latvia, Estonia, and Ukraine. The time range of surveyed indicators differs and goes from 1990 to 2019.

Health indicators were collected from publicly available databases of the World Health Organization and Eurostat, which deal with long-term evaluation and monitoring of indicators obtained from national authorities [20].

The presented research was an observational epidemiological study based on macro-aggregation data of national populations of entire countries. Since data is anonymous and does not belong to individual citizens, there is no issue of data privacy protection, i.e., the research does not require consideration by the Ethics Committee.

Following selected health- and economy-related indicators were taken into account: total health expenditure as % of gross domestic products (THE % of GDP); private households' out-of-pocket payments on health as % of total health expenditure (OOP % THE); gross domestic product US$ per capita (GDP US$); estimated infant mortality per 1,000 live births (infant mortality); estimated life expectancy at birth (life expectancy); percent of urban population (% of urban population). These indicators are the most consistent and used in order to follow the country's progress in health care protection [21,22.]

Forecasting is the process of making predictions of the future based on past and present data, most commonly by analysis of trends. Quantitative forecasting technique is used, which is commonly utilized for historical data, as is the case in our research, and it belongs to medium-termed forecasting analysis, anticipating several years in advance (by the year 2025). Prediction is similar but a more general term. Both might refer to formal statistical methods employing time series, cross-sectional or longitudinal data, or alternatively to less formal judgmental methods [23]. Forecasting analysis was performed by combining excel analysis and the SPSS program.

Results are expressed in decimal numbers, showing how many times certain indicators increased or decreased in comparison to the last surveyed year.

Results

Forecasting analysis of the THE % of GDP by 2025 shows that this indicator tends to increase in most of the monitored countries, especially in Serbia (by 2.94), while it will decrease in a smaller number of countries, such as Northern Macedonia, by more than 2.4, comparing with the last surveyed year (Figure 1, Table 1).

Forecasting analysis of the OPP % THE by 2025 shows that this indicator will decrease mostly in Bosnia and Herzegovina (by 19.5), as well as in Albania (by 14.8), while it tends to increase mostly in the Russian Federation by almost 17 while comparing with the last surveyed year (Figure 2, Table 1). It is interesting to find that this indicator for Slovenia is noticeably constant for the entire observed period, from 1995 to 2014, with a very similar projection by 2025.

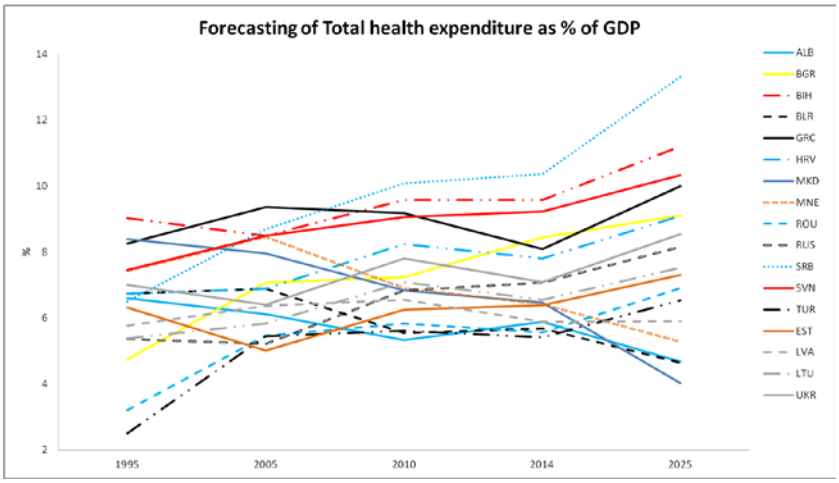

Fig. 1 − Total health expenditures % of GDP for observed countries, forecasting analysis by 2025.
Albania – ALB; Bosnia and Herzegovina – BIH; Bulgaria – BGR; Greece – GRC; Croatia – HRV; Montenegro – MNE; Northern Macedonia – MKD; Romania – ROU; Serbia – SRB; Slovenia – SVN; Turkey – TUR; Russia – RUS; Belarus – BLR; Lithuania – LTU; Latvia – LVA; Estonia – EST; Ukraine – UKR.

Table 1

Indicators of interest (% of GDP, % of OOP, GDP US$, infant mortality, life expectancy, and % of urban population) for each country showing the last year's available values, predicted values for the year 2025, and differences obtained

Country	% of GDP			% OOP			GDP US$			Infant mortality			Life expectancy			% Urban pop.		
	Last	Predict	dif	Last	Predict	dif	Last	Predict	dif	Last	Predict	dif	Last	Predict	dif	Last	Predict	dif
Albania	5.90	4.69	-1.20	49.9	35.10	-14.8	4538	5991	1454	10.3	-1.7	-12.0	79.1	81.8	2.7	52.2	65.7	13.5
Bulgaria	8.44	9.11	0.67	44.2	53.90	9.7	8228	11212	2985	5.6	2.4	-3.2	75.1	76.9	1.8	72.3	77.0	4.7
Bosnia	9.57	11.20	1.62	27.9	8.45	-19.5	5148	6794	1646	5.4	-0.2	-5.6	77.4	79.2	1.9	39.2	42.5	3.3
Belarus	5.69	4.64	-1.05	32.0	39.10	7.1	5728	9505	3777	2.5	-1.9	-4.4	74.5	78.4	3.9	74.6	80.0	5.4
Greece	8.08	9.99	1.91	34.9	23.71	-11.2	18613	17529	-1085	3.7	1.6	-2.1	81.7	83.1	1.4	76.3	80.9	4.6
Croatia	7.80	9.10	1.30	11.2	9.18	-2.0	13383	15890	2507	4.0	1.9	-2.1	78.6	80.1	1.5	57.5	62.6	5.1
Macedonia	6.48	4.03	-2.44	36.7	27.60	-9.1	5415	7222	1808	5.6	1.9	-3.7	76.6	77.8	1.2	/	/	/
Montenegro	6.42	5.28	-1.14	42.8	53.37	10.5	7783	10232	2499	2.4	-2.0	-4.4	76.7	78.8	2.1	63.1	66.2	3.1
Romania	5.57	6.90	1.34	18.9	17.62	-1.2	10818	14264	3446	5.8	0.1	-5.7	75.6	77.6	2.0	53.8	57.0	3.2
Russia	7.07	8.16	1.09	45.8	62.69	16.8	10743	17610	6867	7.4	-1.0	-8.4	68.9	75.6	6.7	73.7	75.3	1.6
Serbia	10.37	13.30	2.94	36.6	45.33	8.7	5900	7767	1867	4.8	-0.1	-4.9	76.0	77.4	1.4	55.2	57.4	2.2
Slovenia	9.23	10.34	1.11	12.1	12.42	0.4	23597	27245	3648	2.1	-0.1	-2.2	81.6	84.0	2.4	50	50.5	0.5
Turkey	5.41	6.54	1.12	17.8	6.53	-11.2	10546	15618	5072	9.1	-6.2	-15.3	79.1	83.1	4.0	70.7	77.7	7.0
Estonia	6.38	7.30	0.92	20.7	22.78	2.1	19705	26249	6545	1.6	-2.7	-4.3	79.0	82.4	3.4	68.1	67.5	-0.6
Latvia	5.88	5.91	0.03	35.1	26.51	-8.6	15594	20628	5034	3.4	-1.0	-4.4	75.7	78.1	2.4	67.7	67.9	0.2
Lithuania	6.55	7.53	0.97	31.3	33.12	1.9	16681	22613	5932	3.3	0.1	-3.2	76.5	78.6	2.1	66.8	67.3	0.5
Ukraine	7.10	8.54	1.44	46.2	47.43	1.2	2640	3699	1060	7.1	3.0	-4.1	73.4	76.7	3.3	68.7	72.1	3.4

Last − the last available year of observation; Predict − predicted value in the year 2025; dif − the difference between predicted and last year's available, shown as a negative or positive difference.
% of GDP – total health expenditure as % of GDP; % OOP – private households' out-of-pocket payments on health as % of total health expenditure; GDP US$ – gross domestic product (GDP), US$ per capita; Infant mortality – estimated infant mortality per 1,000 live births; Life expectancy – estimated life expectancy at birth; % of urban pop. – % of the urban population.

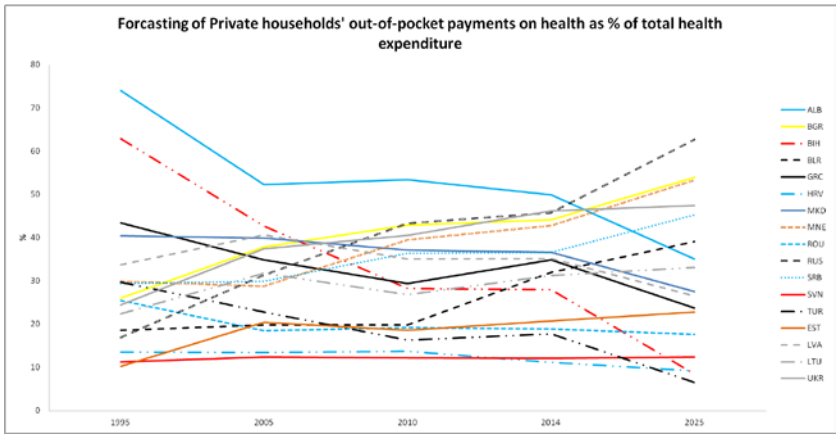

Fig. 2 − Private households' out-of-pocket payments on health as % of total health expenditure for surveyed countries, forecasting analysis by 2025.
Albania – ALB; Bosnia and Herzegovina – BIH; Bulgaria – BGR; Greece – GRC; Croatia – HRV; Montenegro – MNE; Northern Macedonia – MKD; Romania – ROU; Serbia – SRB; Slovenia – SVN; Turkey – TUR; Russia – RUS; Belarus – BLR;

In general, the values of GDP, compared to the first surveyed year, increased significantly (Figure 3, Table 1). Forecast analysis of the value of GDP US$ by 2025 shows that this indicator will decrease only in Greece (by $1,085), while this sum of money will increase in the rest of the surveyed countries, mostly in the Russian Federation (by $6,867) and Estonia (by $6,545) in comparison to the last surveyed year.

The projection of infant mortality shows that it will decrease in each surveyed country in South Eastern Europe and the Balkan peninsula by 2025 (Figure 4, Table 1). There will be a large reduction in infant mortality per 1,000 live births in Albania (by 12.0, respectively) in comparison to the last surveyed year, with the largest decrease in Turkey (by 15.3).

Estimated life expectancy at birth will increase in each surveyed country by the year 2025, with the largest increment in the Russian Federation and Belarus (by 6.7 and 3.9, respectively), while the smallest one will be in Macedonia and Greece (by 1.2 and 1.4) (Figure 5, Table 1). That indicates that the number of elderly people will continue to grow in this region.

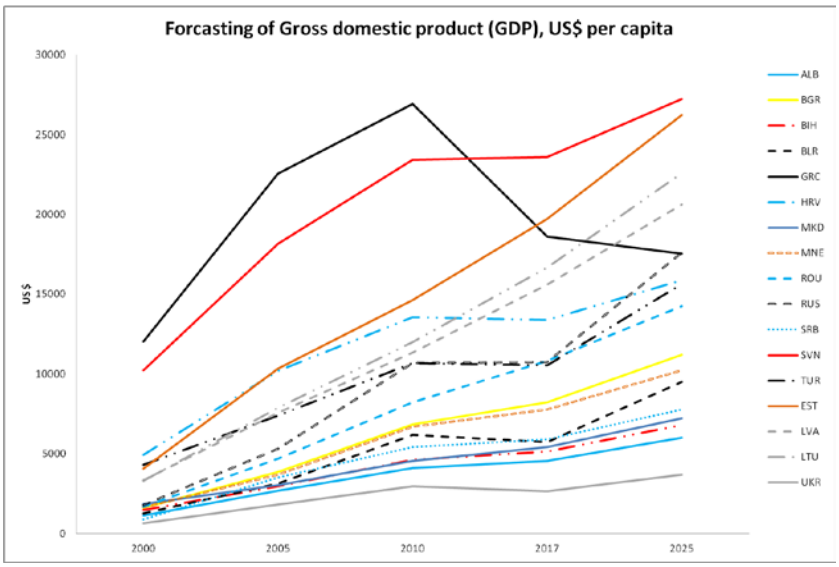

Fig. 3 – Gross domestic product (GDP), US$ per capita for surveyed countries, forecasting analysis by 2025.
Albania – ALB; Bosnia and Herzegovina – BIH; Bulgaria – BGR; Greece – GRC; Croatia – HRV; Montenegro – MNE; Northern Macedonia – MKD; Romania – ROU; Serbia – SRB; Slovenia – SVN; Turkey – TUR; Russia – RUS; Belarus – BLR; Lithuania – LTU; Latvia – LVA; Estonia – EST; Ukraine – UKR.

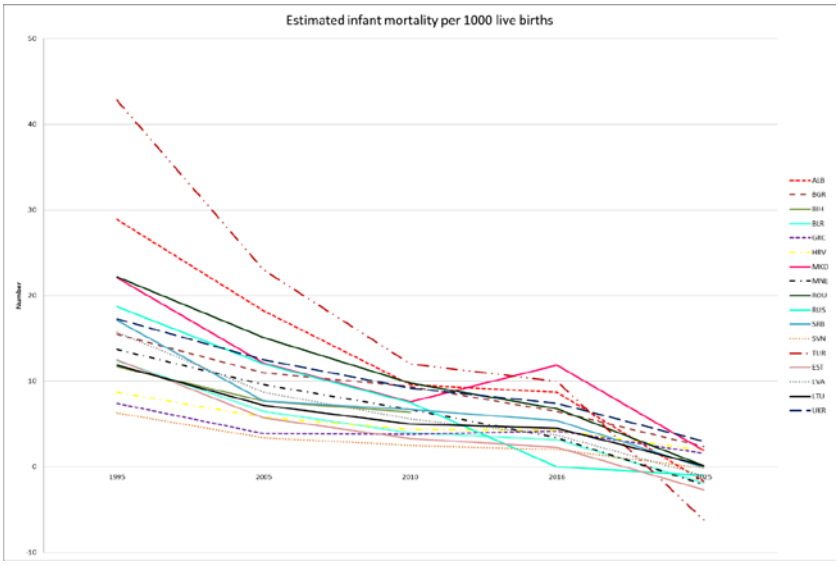

Fig. 4 − Estimated infant mortality per 1,000 live births for surveyed countries, forecasting analysis by 2025.
Albania – ALB; Bosnia and Herzegovina – BIH; Bulgaria – BGR; Greece – GRC; Croatia – HRV; Montenegro – MNE; Northern Macedonia – MKD; Romania – ROU; Serbia – SRB; Slovenia – SVN; Turkey – TUR; Russia – RUS; Belarus – BLR; Lithuania – LTU; Latvia – LVA; Estonia – EST; Ukraine – UKR.

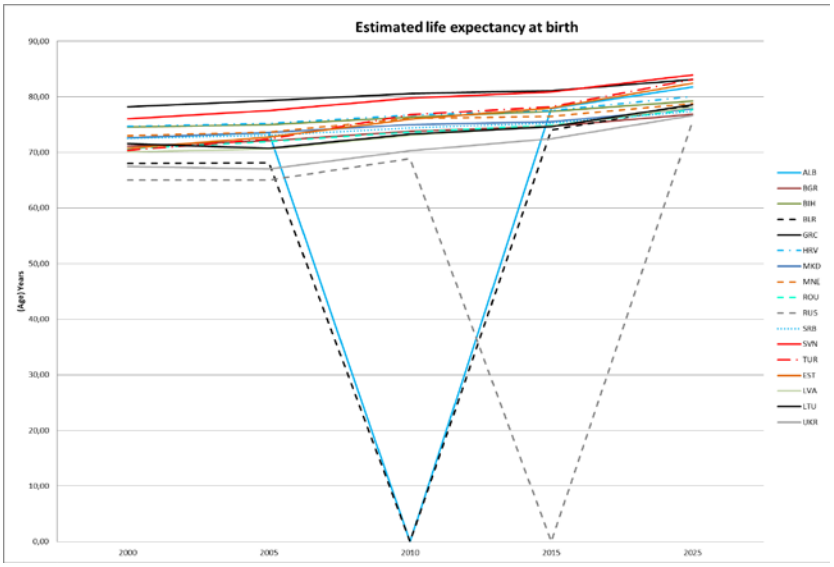

Fig. 5 − Estimated life expectancy at birth for surveyed countries, forecasting analysis by 2025.
Albania – ALB; Bosnia and Herzegovina – BIH; Bulgaria – BGR; Greece – GRC; Croatia – HRV; Montenegro – MNE; Northern Macedonia – MKD; Romania – ROU; Serbia – SRB; Slovenia – SVN; Turkey – TUR; Russia – RUS; Belarus – BLR; Lithuania – LTU; Latvia – LVA; Estonia – EST; Ukraine – UKR.

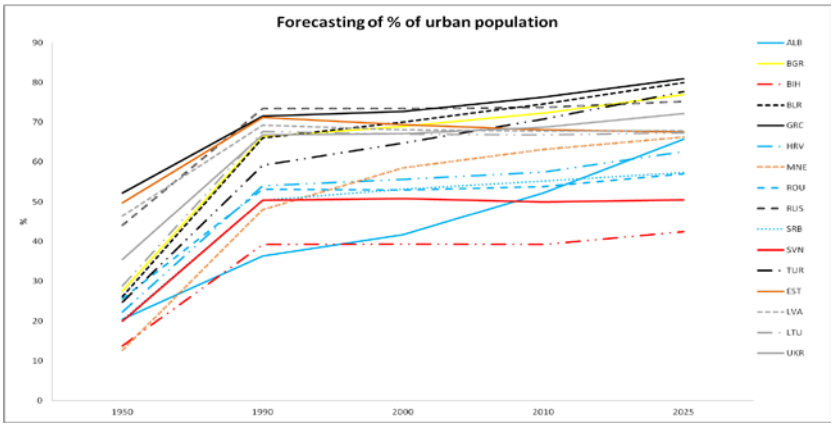

Fig. 6 − Percent of urban population for surveyed countries, forecasting analysis by 2025.
Albania – ALB; Bosnia and Herzegovina – BIH; Bulgaria – BGR; Greece – GRC; Croatia – HRV; Montenegro – MNE; Northern Macedonia – MKD; Romania – ROU; Serbia – SRB; Slovenia – SVN; Turkey – TUR; Russia – RUS; Belarus – BLR; Lithuania – LTU; Latvia – LVA; Estonia – EST; Ukraine – UKR.

Percentage of the urban population until 2025 will rise almost in every country except Estonia, which will have a slight decrement (by 0.6) (Figure 6, Table 1). The largest enhancement of the urban population, expressed in percentages, is estimated to take place in Albania (by 13.5) and Turkey (by 7) in comparison to the last surveyed year. There are no data concerning Northern Macedonia's urban population during the whole surveyed period.

Discussion

Global aging becomes more and more of a reality as time passes. Progress in both the medical and non-medical spheres of living, such as the development of the drug industry, medical technologies, and industry development, as a whole, explains by itself the fact that elderly people are becoming the leading age group nowadays [24, 25]. The share of the world population aged 65 and older will double, and the fast-growing group of people over the age of eighty will become four times larger by 2040 [26]. The expected years of life would change in parallel with all previously mentioned developments in human society [27]. All of this makes it even more important to address the issue of healthcare investments. All people must be treated equally by the health sector regardless of age, gender, and ethnicity [3]. A large percentage of older people is living in developing countries, and by 2025 it will even increase [28, 29]. Moreover, globally, the percentage of people living in urban areas will grow,

which is in line with the predictions from our research. In parallel with the demographic and economic transition, investments in the health sector are expected to grow, even sharply [30, 31]. The forecasting analysis shows increased investments in health, according to the obtained economic indicators. Namely, the ability to predict what impact these changes will have on the overall healthcare costs, both in public and private health sectors, is of key importance.

Health spending per capita for the period from 2015 to 2030 is projected to grow more than 4% per year in the Slovak Republic, Turkey, and the Republic of Korea [32]. On the contrary, in Lithuania, Chile, and Latvia, growth rates are projected to be over two percentage points lower than historical rates [1, 31]. The Organization for Economic Co-operation and Development (OECD) countries reported some of the highest growth rates in health spending per capita from 2000 to 2015 [33]. Our research also shows some variation concerning per capita spending.

Health expenditure as a share of GDP in the world is projected to rise to 10.2% by 2030, in comparison to 8.8% in 2015 [34, 35]. The only countries for which a slight decrease is expected to happen are Latvia, Hungary, and Lithuania [36]. Most countries are expected to experience a moderate increase in health expenditure as a share of GDP [37]. Based on the results of our research, it can be concluded that several countries are likely to have reduced percentage of investment by the year 2025, like Northern Macedonia, Albania, and Bosnia and Herzegovina, but countries such as Serbia are expected to have large increment.

People with the same health issues may end up spending differently due to different types of insurance, socioeconomic status, or comorbid diseases that can make treatment unsuccessful [38–42]. According to the data from The World Bank in 2018, Ukraine had 49.35% of OOP spending on health; Albania 44.58%; Russia and Serbia 38.31%, and the least among all surveyed countries – Slovenia and Croatia with 12% [43]. Our forecast analysis showed that most of the surveyed countries would experience a drop in the percentage of OOP share on health, especially Albania and Bosnia and Herzegovina, which is not the case with the Russian Federation. That means that health systems differ, and some countries cope better with health expenditures than others [44, 45].

The infant mortality rate is considered one of the indicators for describing both demographic conditions and socioeconomic well-being of a country [46–48]. The millennium development goal 4 (MDG 4) of the UN had set the goal of reducing high rates of infant mortality by two-thirds, to be reached by 2015, by using 1990 as the benchmark year [46].

Institut national d'études démographiques (INED) calculated the infant mortality rate (per 1,000 live births) in Europe and other developed OECD countries [49]. The results from 2018 showed that the largest infant mortality had Mexico, Colombia, Kosovo, and Albania (8.9 per 1,000); Romania (6 per 1,000) and Bulgaria (5.8 per 1,000) had moderate values, while Estonia (1.6 per 1,000), Slovenia (1.7 per 1,000), and Liechtenstein with no child mortality, were at the bottom of the list. By analysis of the last available data on infant mortality indicators, Estonia and Slovenia were the countries that had the smallest numbers in infant mortality (1.6 and 2.1 per 1,000). All surveyed countries in our research are expected to reduce infant mortality by 2025.

Population aging is increasing not only in low-mortality industrialized countries but also in several Eastern European countries, including Russia [50]. These countries have a slower pace of mortality improvement in several stages of life span compared to low-mortality countries, which delayed the aging problem [51]. Due to the evolved medical and pharmaceutical technology, as well as more accessible medical care, life expectancy should be prolonged [52, 53]. Our forecast analysis indicated that life expectancy would rise in each of the surveyed countries, and longevity would be the highest in the Russian Federation and Belarus.

In 2015, the UN reported that 54% of the global population lived in urban areas [54]. The level of Europe urbanization is expected to increase to approximately 83.7% in 2050 [55]. By 2030, urban residential areas are expected to expand a lot [56]. The highest growth (in percent) of urban residential areas, about 6%, is expected in Romania and Belgium [57]. Urban construction will certainly affect the relocation of residents from rural to urban areas; therefore, the percentage of people living in urban areas is expected to rise [58, 59]. Our forecast showed that this might happen and that in 2025 the growth in the percentage of the urban population in each surveyed country is realistic, except Estonia. The largest increment in the percentage of growth can be expected in Albania and Croatia. All age groups move to urban areas, the younger ones in order to get proper education, the middle age group are looking for a job, and the older ones are often placed in nursing homes. All of this brings problems for the authorities and the population which need to be solved.

Conclusion

Seventeen selected South Eastern European and Balkan countries we compared showed similar pathways in the progress of selected indicators, but some of them are expected to have more success than others. Total health expenditures as a percentage of GDP by 2025 will increase in most of the surveyed countries. The percentage of household payments OOP will decrease in half of them, while values of GDP expressed in US$ will increase significantly compared to the last surveyed year (2017), except in Greece. The infant mortality indicator shows decrement numbers in each surveyed country by 2025, while estimated life expectancy at birth will increase by the same year. Our forecast also showed that the growth in the percentage of the urban population in each surveyed country is realistic, except for Estonia.

Therefore, health- and economy-related indicators should be surveyed over time, as they enable significant and relevant information concerning the contemporary issues in health systems, also indicating where changes should be made and allowing further progress in the health care of the individual country.

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Randomization methods and cluster size in cluster randomized trials conducted in elementary and high schools

Tehnike randomizacije i veličina klastera u klaster randomizovanim studijama sprovedenim u osnovnim i srednjim školama

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Abstract

Background/Aim. Randomization allows for study groups to be formed so that they are similar in all characteristics except outcomes. The aim of this study was to examine the frequency of randomization methods and their effect on achieving baseline balance in cluster randomized studies conducted in schools. **Methods.** A literature search of the Medline bibliographic database showed that the total number of collected articles in the full text was 343, out of which 81 were eligible for inclusion. Each publication was reviewed by two independent reviewers, and data were extracted and analyzed. **Results.** Stratification was the most commonly applied randomization method, reported in 28 trials (34.6%). There was no statistically significant difference in the number of subjects and clusters, as well as in cluster size between trial groups in studies in which simple randomization was applied. However, there was a statistically significant difference in the number of subjects and clusters between groups in trials in which restricted randomization methods were used. Yet, there was no difference in the cluster size. **Conclusion.** Although there is no difference in the size of clusters between trial arms, either at the level of the entire sample or in relation to randomization methods applied, additional research should be conducted on a larger sample in order to establish the effect of randomization methods on baseline balance, when the size of clusters is in question.

Key words:
random allocation; research; research design; sample size; schools.

Apstrakt

Uvod/Cilj. Formiranje grupa u istraživanjima tako da budu slične u svim karakteristikama izuzev ishoda, obezbeđuje se postupkom slučajne raspodele. Cilj ove studije bio je da ispita učestalost tehnika slučajne raspodele i njihov uticaj na postizanje ravnoteže na početku istraživanja u studijama sa grupama formiranim pomenutom metodom, koje su sprovedene u školama. **Metode.** Pretraživanjem bibliografske baze podataka *Medline* ukupan broj prikupljenih radova je bio 343, od kojih je 81 ispunilo kriterijume za uključenje u studiju. Svaku publikaciju su pregledala dva nezavisna istraživača, podaci su ekstrahovani i analizirani. **Rezultati.** Najčešće primenjena tehnika slučajne raspodele bila je stratifikacija koja je opisana u 28 (34,6%) studija. U studijama u kojima je primenjena prosta metoda slučajne raspodele nije bilo statistički značajne razlike u broju ispitanika i klastera kao i veličini klastera između ispitivanih grupa. U studijama u kojima su primenjene tehnike restriktivne slučajne raspodele postoji statistički značajna razlika u broju ispitanika i klastera između grupa, ali ne i u veličini klastera. **Zaključak.** Iako u veličini klastera ne postoji razlika između ispitivanih grupa kako na nivou celog uzorka tako i u odnosu na tehnike slučajne raspodele, trebalo bi sprovesti dodatna istraživanja na većem uzorku kako bi se utvrdio uticaj primenjenih tehnika slučajne raspodele na prisustvo ravnoteže na početku istraživanja kada je u pitanju veličina klastera.

Ključne reči:
slučajni izbor, metod; istraživanje; istraživanje, dizajn; uzorak, veličina; škole.

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Introduction

Randomized controlled studies in which randomization is conducted at the level of clusters, where all subjects within the same cluster, such as hospitals or general practitioners, are subjected to the same treatment, are called cluster randomized trials (CRTs) [1]. Clusters may be groups of subjects, hospitals, schools, geographic regions, etc.

Compared with individually randomized studies, cluster randomized studies are of a more complex design and require more subjects to achieve adequate statistical power and the application of a more complex method of analysis [2]. Compared with an individually randomized trial testing the same hypothesis, cluster randomization requires a significantly larger sample size [3].

The main result of a such design application is that the outcome for one patient cannot be considered independently from other patients (as in individual randomized studies). Patients in the same cluster will probably have similar outcomes [4].

The formation of study groups so as to be similar in all characteristics except in the outcome is achieved through randomization. Baseline balance among groups shall ensure that all differences obtained at the end of the trial are attributed to the effect of study treatment, not the existing differences.

In cluster-randomized studies, it is necessary to achieve balance, both at the level of individual subjects and at the level of clusters [5]. Due to cluster size, a large number of clusters are often difficult to randomize into every study group, while a small number of clusters is not enough to provide adequate balance among study groups [6]. Furthermore, the necessary number of cases depends on the size of the clusters: 100 clusters each containing 10 probands lead to greater statistical power than 10 clusters of 100 probands each [7]. Regarding the use of the randomization method in CRTs, some authors believe that adequate balance cannot be achieved by the application of simple randomization, especially if the number of randomized clusters is small [8]. That is the main reason why a matched or stratified design of the study is used [6], although certain authors [2, 6, 9] favor stratification when studies of such design are in question.

In a systematic review of CRTs in the field of primary health care, published 1997–2000, Eldridge et al. [10] quote that in 54% of studies, matching and stratification were applied during randomization. In a systematic review of group randomized trials in the field of cancer prevention, published 2002–2006, Murray et al. [11] quote that simple randomization is applied in 40% of studies, matching is applied in 20% of studies, stratification in 35% of studies, while a combination of matching and stratification is applied in 5.3% of studies. In a systematic review of Rutterford et al. [12] that included 300 CRTs published 2000–2008, the stratification method is applied in 39% of studies, simple randomization in 37% of studies, while matching is applied in 19% of studies, and minimization in 5% of studies.

The aim of this study was to investigate the frequency of randomization methods and their relation with the size of the cluster in terms of achieving baseline balance in CRTs conducted in schools.

Methods

A literature search of the Medline bibliographic database was conducted until March 31, 2020, using following key words in the title of the paper: "cluster randomised trial", "cluster randomized trial", " randomised cluster trial", "randomized cluster trial", "field randomised trial", "field randomized trial", "randomised field trial", "randomized field trial", "community based randomised trial", "community based randomized trial", "randomised community based trial", "randomized community based trial", "community randomised trial", "community randomized trial", "randomised community trial", "randomized community trial", "group randomised trial", "group randomized trial", "randomised group trial", "randomized group trial", "place based randomised trial" "place based randomized trial", "randomised place based trial", "randomized place based trial", "randomised place trial", "randomized place trial", "place randomised trial", "place randomized trial", "prevention randomised trial", "prevention randomized trial", "randomised prevention trial", "randomized prevention trial", "randomised prevention trial". Study inclusion criteria were: prospective CRTs that include two study groups, with schools as randomization units and students as observation units. Exclusion criteria were: studies in which randomization is not performed at the level of clusters, cluster randomized studies in which randomization units are not schools, and pilot trials. After reading through the published titles and abstracts, all the ones which met the inclusion criteria were downloaded *in extenso*. The total number of collected articles in the full text was 343, out of which 81 (Appendix 1) were eligible for inclusion [13-93]. Each publication was reviewed by two independent reviewers and data about randomization methods, the number of subjects and clusters at the beginning of the trial were extracted. The size of the cluster was obtained by dividing the total number of randomized subjects by the number of randomized clusters (Figure 1).

Data analysis

For primary data analysis, descriptive methods and methods for testing statistical hypotheses were used. The measure of central tendency (median), a measure of variability [interquartile range (IQR)], and relative numbers were used from descriptive statistical methods. Statistical hypotheses were tested by the Wilcoxon test. Statistical data analysis was performed using IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL, USA). The criterion for statistical significance was $p < 0.05$.

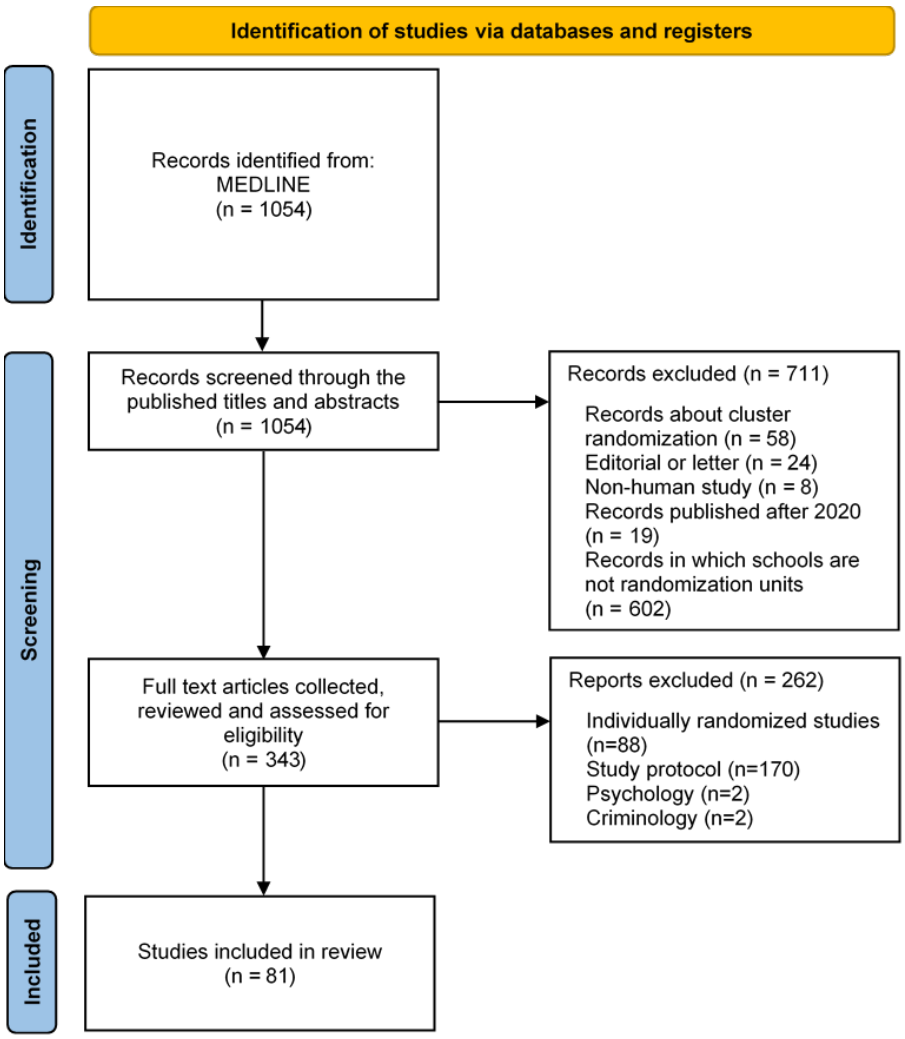

Fig. 1 − Identification of cluster randomized trials from PubMed citations indexed in March 2020.

Results

The most often applied randomization method was the method of stratification reported in 28 (34.6 %) studies. The following were simple randomization reported in 18 (22.2 %) studies, matching in 12 (14.8%) studies, and block randomization in 8 (9.9%) studies. In 9 (11.1%) studies, it was not reported which randomization methods were used. The frequency of other randomization methods was less than 5% (Table 1).

When the entire sample was considered, there was a statistically significant difference in the number of subjects and clusters between the intervention and control group, while there was no statistically significant difference in the size of clusters between groups. Studies where a simple randomization method was applied demonstrated the absence of a statistically significant difference between study groups in the number of subjects and clusters, as well as in the size of clusters, while studies with restrictive randomization methods demonstrated a statistically significant difference between study groups in the number of subjects and clusters but not in the size of clusters (Table 2).

Table 1

Randomization methods in cluster randomized trials conducted in schools as randomization units (n = 81)

Allocation techniques	n (%)
Stratification	28 (34.6)
Simple randomization	18 (22.2)
Matching	12 (14.8)
Not reported	9 (11.1)
Block randomization	8 (9.9)
Balanced randomization	3 (3.7)
Matching and stratification	1 (1.2)
Block and stratification	1 (1.2)
Restricted randomization	1 (1.2)

Table 2

The association of randomization methods and cluster size at baseline

Cluster size at baseline (n = 72)	Intervention group*	Control group*	*p***
Number of participants	813 (394–2.710)	823 (380–2.864)	0.020
Number of clusters	12.5 (7.75–34)	12 (8–31)	0.001
Cluster size	59.2 (33.8–160.4)	62.5 (33.9–158)	0.736
Baseline simple randomization studies (n = 18)			
Number of participants	314 (113–691)	314 (108–718)	0.088
Number of clusters	10 (6–13)	10 (7–12)	0.953
Cluster size	45 (28.9–62.8)	42.6 (24.9–65)	0.365
Baseline restricted randomization studies (n = 54)			
Number of participants	1,115 (669.5–4.253)	1,093 (628.5–4.299)	0.012
Number of clusters	20 (10–35)	16 (10–33.5)	< 0.001
Cluster size	76.8 (41.8–168.7)	74.7 (40.3–178)	1.000

***number of subjects and clusters in trial arms; **Wilcoxon test**
Note: Values are given as median and IQR (interquartile range 25–75 percentiles).

Discussion

The results of this trial show the possible presence of bias during randomization. The difference in the number of subjects and clusters between study groups during randomization is slight but statistically significant. According to the literature, there is a much greater probability of not achieving the balance between trial arms, especially if the number of clusters is small [94] like in the studies from this research. Without withstanding the aforementioned, there was no statistically significant difference in the number of subjects and clusters between study groups in studies where simple randomization was applied, which leads to the conclusion that the baseline balance was achieved although the randomization method, otherwise not recommended in CRTs, was applied.

In the bibliography, restrictive randomization methods are recommended for CRTs because they may improve the chances of achieving balanced study groups [95]. Author Lewsey [96] quotes that, when CRTs are in question, matching and stratification are especially popular methods, and also quotes that the most commonly used factors of stratification are the size of the cluster, cluster-level socio-economic status, geographic location, and categorized levels of individual-level prognostic factors. On the other hand, this trial showed a significant difference in the number of subjects and clusters between trial arms in studies that applied certain restrictive randomization methods. The number of subjects and clusters was significantly higher in intervention groups.

Although CRTs are of complex design, in certain cases, they are the only choice, for instance, if the nature of the intervention requires it to be performed in the entire community or to prevent contamination if subjects from both study groups come from the same population. The application of adequate randomization methods in these studies has a great impact on the quality of the trial. Several authors [6, 2, 9] recommend stratification, which is the most frequently applied method in one-third of all studies in this research. We can find a similar result in the research of Varnell et al. [97], while in the systematic review of CRTs in the field of oral health, stratification was reported to be the most frequently used randomization method in 48% of studies [98].

Although certain authors [6, 12] believe that balance in CRTs cannot be achieved by application of simple randomization, its frequency of 22.2% in this trial is rather high. In the bibliography, there is a trial where simple randomization was applied in more than half of the studies covered by systematic review [99], but there are also trials where the frequency of this method is similar to our results [98].

As for individually randomized controlled trials, the goal of randomization in group randomized trials is to achieve a balance of baseline covariates. In contrast to individually randomized trials, another form of baseline balance applies to group randomized trials, namely, baseline balance of group sample size [100]. In the case of CRTs, the most efficient design is achieved when the sizes of clusters are equal [101]. The results of this trial showed that there were no differences in the size of clusters between study groups. However, the possible presence of bias can be seen through the presence of differences in the number of subjects and clusters in the randomization process. The difference already existing between subjects and clusters at baseline may increase if a loss of subjects and/or clusters occurs during the study. For this reason, we believe that additional investigation is necessary.

The limitation of this study is that it included only studies conducted in schools as randomization units. There is a heterogenicity between trials that has not been investigated, which also represents a limitation of this trial. Moreover, the only balance measuring factor we took into consideration was the size of the cluster that represents a number of subjects and clusters in trial arms, without the presence of balance in prognostic factors.

Conclusion

The most frequently applied randomization method is stratification, although the frequency of simple randomization is also high. In studies where a simple randomization method

was applied, there was no difference in the number of subjects and clusters between study groups, unlike in studies where some restrictive randomization methods were applied. Even though there was no difference in the size of clusters between study groups, either with respect to the entire sample or the randomization method applied, additional research should be conducted on a larger sample in order to determine the effects of the randomization method on achieving baseline balance, when cluster size is in question.

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Appendix 1

Trials included in the analysis

Study	Publication year	Study power	Randomization methods	Intervention group	Control group	Intervention group	Control group
				number of participants randomized	number of participants randomized	number of clusters randomized	number of clusters randomized
Pereira et al. [13]	2012	Described	Stratification	176,843	171,240	388	375
Barreto et al. [14]	2011	Described in previous report	Stratification	176,843	171,240	388	375
Stephenson et al. [15]	2008	Described	Stratification	4,516	4,250	14	13
Cunha et al. [16]	2008	Described	Stratification	72,980	79,458		
Henderson et al. [17]	2007	Described	Balanced	2,080	2,135	13	12
Cooper et al. [18]	2006	Described	Block randomization	1,164	1,209	34	34
Rodrigues et al. [19]	2005	Described in previous report	Stratification	176,843	171,240	386	375
Madsen et al. [20]	2013	Described	Not reported	82	74	4	3
Sancho-Garnier et al. [21]	2012	Described	Stratification	798	567	39	31
Tol et al. [22]	2012	Described	Simple randomization	199	200	12	12
James-Burdumy et al. [23]	2012	Not reported	Block randomization	6,400	4,590	20	16
Ezendam et al. [24]	2012	Described in previous report	Stratification	485	398	11	9
Hartmann et al. [25]	2010	Not reported	Simple randomization			16	11
Walsh et al. [26]	2010	Described	Stratification	2,270	2,461		
Hunter et al. [27]	2010	Described	Block randomization	1,115	1,376	11	11
Wen et al. [28]	2010	Described	Matching	1,339	1,004	2	2
Berg et al. [29]	2009	Described	Stratification	375	378	17	17
Wolfe et al. [30]	2009	Not reported	Stratification	968	754	10	10
Ringwalt et al. [31]	2009	Described	Matching	3,990	4,348	20	10
Tol et al. [32]	2008	Described	Simple randomization	237	258	7	7
Martínez Vizcaíno et al. [33]	2008	Described	Simple randomization	691	718	10	10
Naldi et al. [34]	2007	Described in previous report	Stratification	5,676	5,554	62	60
Martiniuk et al. [35]	2007	Described	Block and stratification	403	380	12	12
Rapp et al. [36]	2006	Described	Simple randomization	605	629	16	16
Martiniuk et al. [37]	2003	Described	Simple randomization	197	271	8	11
Aveyard et al. [38]	2001	Described in previous report	Balanced	4,660	4,641	27	26
Priest et al. [39]	2014	Described	Stratification	8,859	7,386	34	34
Halliday et al. [40]	2014	Described	Stratification	2,710	2,523	51	50
Isensee et al. [41]	2014	Described	Stratification	2,437	2,335	26	22
Ebenezer et al. [42]	2013	Described	Block randomization	813	808	49	49
Martínez-Vizcaíno et al. [43]	2014	Described in previous report	Simple randomization	769	823	10	10
Bere et al. [44]	2014	Described	Not reported	585	1,365		
Primack et al. [45]	2014	Described	Stratification	554	578	31	33
Barreto et al. [46]	2014	Described in previous report	Stratification	176,843	172,240	388	375
Muhumuza et al. [47]	2014	Described	Stratification	2,523	3,036	6	6
Tol et al. [48]	2014	Described	Stratification	153	176	7	7
Santos et al. [49]	2014	Described	Block randomization	340	347	10	10
Freeman et al. [50]	2013	Described	Stratification			20	20
O'Leary-Barrett et al. [51]	2013	Not reported	Not reported	1,529	1,114	11	8
Lewis et al. [52]	2013	Not reported	Matching			7	7
Peskin et al. [53]	2014	Described	Balanced	598	847	5	5

Coleman et al. [54]	2012	Described	Matching	647	626	4	4
Peterson et al. [55]	2009	Described	Matching	1,058	1,093	25	25
Telford et al. [56]	2013	Not reported	Not reported	394	314	13	16
Telford-2013 et al. [57]	2013	Not reported	Simple randomization	394	314	13	16
LaBrie et al. [58]	2008	Not reported	Not reported	603	559	12	8
Sloboda et al. [59]	2009	Described	Not reported	10,028	7,292	41	42
Gmel et al. [60]	2012	Described	Matching and stratification	973	885	57	56
Waters et al. [61]	2018	Described	Simple randomization	3,433	3,601	12	11
Mallick et al. [62]	2018	Described	Stratification	223	231	5	5
Kittayapong et al. [63]	2017	Described	Not reported	1,297	1,017	5	5
Marcano-Olivier et al. [64]	2019	Described	Simple randomization	86	90		
Nawi et al. [65]	2015	Described	Simple randomization	47	50	4	2
Rathleff et al. [66]	2015	Described	Simple randomization	62	59	2	2
Sutherland et al. [67]	2016	Described	Block randomization	837	631	5	5
Baker-Henningham et al. [68]	2019	Described	Not reported	108	112	7	7
Halliday et al. [69]	2020	Described	Stratification	4,850	4,721	29	29
Nsangi et al. [70]	2020	Described	Stratification	6,383	6,256	60	60
Chang Wu et al. [71]	2018	Described	Simple randomization	365	565	7	9
Morgan et al. [72]	2018	Not reported	Matching	118	79	34	26
Bundy et al. [73]	2017	Described in previous report	Simple randomization	113	108	6	6
Rozi et al. [74]	2019	Described	Stratification	738	589	10	8
Andersen et al. [75]	2015	Described	Stratification	2,381	1,786	53	44
Gerald et al. [76]	2019	Described	Matching	224	169	10	10
Penalvo et al. [77]	2015	Described	Stratification			12	12
Schonfeld et al. [78]	2015	Not reported	Block randomization	692	702	12	12
Sutherland et al. [79]	2016	Described	Block randomization	696	537	5	5
Kaufman et al. [80]	2016	Described	Stratification	565	661	13	13
Sanchez et al. [81]	2019	Described	Not reported	3,243	3,148	38	34
Dalma et al. [82]	2019	Described	Stratification	6,831	5,587	36	30
Valente et al. [83]	2020	Described	Simple randomization	3,340	3,318	38	34
Andrade et al. [84]	2016	Described	Matching	700	740	10	10
Vik et al. [85]	2015	Described	Matching	1,713	1,681	31	31
Chard et al. [86]	2019	Not reported	Stratification	2,021	1,972	50	50
D ziaugyt_e et al. [87]	2017	Described	Simple randomization	112	94	2	2
Okely et al. [88]	2017	Described	Matching	771	747	12	12
Asdigian et al. [89]	2017	Not reported	Simple randomization	314	321	6	7
Peterson et al. [90]	2016	Described	Matching	1,058	1,093	25	25
Bauer et al. [91]	2020	Described	Matching	639	723	8	8
Potter et al. [92]	2016	Not reported	Restricted	1,775	1,469		
Praena-Crespo et al. [93]	2016	Described	Simple randomization	2,856	2,864	47	50

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Assessment of dental anodontia among Ferdinand I Military Technical Academy students in Romania

Procena bezubosti kod studenata Vojno-tehničke Akademije Ferdinand I u Rumuniji

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Abstract

Background/Aim. Data regarding tooth absence among Romanian military personnel are lacking. The aim of this study was to determine the prevalence of dental anomalies among military students at the Military Technical Academy in Bucharest, Romania. **Methods.** A cohort of 318 military students was enrolled in the study. Each participant underwent an extensive evaluation of their oro-dental health status based on guidelines of the European Global Oral Health Indicators Development II Project (EGOHID II). **Results.** Anodontia was discovered in 6 participants (prevalence rate was 1.9%), namely 4 women (prevalence among women was 4.6%) and 2 men (prevalence among men was 0.87%). Five of the six patients had never been previously diagnosed with anodontia. The most commonly affected teeth were second premolars (n = 8), followed by first premolars (n = 4) and second permanent molars (n = 2). Premolar anodontia was equally common in the maxilla and the mandible; both instances of molar anodontia were in the mandible. None of the participants with anodontia had remaining temporary teeth. A brief overview and two cases of diagnosed patients, who presented with inferior bilateral second molar anodontia and quadruple canine inclusion and a quadruple second premolar anodontia, are given. **Conclusion.** Military students in Romania would benefit from systematic dental evaluation and long-term monitoring prior to enrollment in the Military Academy.

Key words:
anodontia; military personnel; prevalence; romania; students.

Apstrakt

Uvod/Cilj. Podaci o nedostatku zuba kod rumunskog vojnog osoblja ne postoje. S obzirom na to, cilj rada bio je da se ispita prevalenca anomalije zuba među studentima Vojno-tehničke akademije u Bukureštu, Rumunija. **Metode.** Studijom je bilo obuhvaćeno ukupno 318 vojnih studenata. Svaki učesnik bio je podvrgnut sveobuhvatnoj proceni orodentalnog zdravstvenog stanja, na osnovu smernica *European Global Oral Health Indicators Development II* (EGOHID II) projekta. **Rezultati.** Anodoncija je otkrivena kod 6 učesnika u studiji (stepen prevalence iznosio je 1,9%) i to kod 4 žene (prevalenca među ženama iznosila je 4,6%) i dva muškarca (prevalenca među muškarcima iznosila je 0,87%). Kod pet od 6 ispitanika, anodoncija nikad ranije nije bila dijagnostikovana. Najčešće zahvaćeni zubi bili su sekundarni premolari (n = 8), zatim prvi premolari (n = 4) i sekundarni stalni premolari (n = 2). Premolarna anodoncija bila je jednako zastupljena i u maksili i u mandibuli, a oba slučaja molarne anodoncije bila su u mandibuli. Nijedan od učesnika u ispitivanju sa anodoncijom nije imao preostale privremene zube. Dat je kratak prikaz i dva slučaja dijagnostikovanih pacijenata koji su imali bilateralno nedostatak donjeg drugog kutnjaka i četvorostruku inkluziju očnjaka i četvorostruku anodonciju sekundarnog prednjeg kutnjaka. **Zaključak.** Pre upisa na Vojnu akademiju, vojni studenti u Rumuniji bi mogli imati koristi od sistemske stomatološke procene i dugotrajnog praćenja.

Ključne reči:
bezubost; kadar, vojni; prevalenca; rumunija; studenti.

Introduction

Romania, being a strategic partner of the North Atlantic Treaty Organization (NATO), is an active participant in peacekeeping operations which requires Romanian military troops to remain combat-ready. Military readiness depends on physical training, mental training, and health, including oro-dental health [1–3]. Specialized dental emergency care can

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not be administered to personnel located in isolated areas for combat missions or combat exercises. Moreover, the medical evacuation of a soldier in the event of such an emergency requires substantial logistical and human resources. Hence, it is important to monitor the oro-dental health status of troops. To this end, NATO has published military oral health standards for NATO member states with a dental fitness classification system [4].

Data regarding tooth loss among Romanian military personnel are lacking. Teeth can be missing for a variety of reasons, including extraction due to caries, trauma, or anodontia [5, 6]. Incomplete dentition can disrupt patients' quality of life due to several functional impacts, including impaired masticatory and phonetic function, as well as psychosocial impacts, due to discomfort, pain, or shame related to poor aesthetics [7]. It is important that oro-dental disease be timely diagnosed to enable the provision of appropriate interdisciplinary treatments that can correct functional and aesthetic deficiencies.

Individuals may have anodontia of both temporary and permanent teeth [8–10], or may, more commonly, have anodontia of a permanent tooth despite having had complete temporary dentition [11–15]. Ideally, anodontia should be recognized early in children's radiographs. However, many young adults joining the Romanian military may not have received regular dental care during childhood. Thus, it is likely that there are military academy students with undiagnosed congenital anodontia or with missing teeth due to trauma or tooth decay. The aim of the study was to assess the oral health status of Romanian military students, with particular attention to the identification of missing teeth due to anodontia.

Methods

Participants

Appropriate enrolment to achieve a representative sample was estimated to be 261 participants in Open-Epi, version 2.3 [16], under the following settings: population size of 805; hypothesized proportion for outcome factor in the population $p = 0.5$, with a maximum variance $\sigma(p) = p(1\text{-}p)$; margin of error $\Delta(p) = \pm 5$ percentage points; and an assumed type I error rate of 0.05 with a 95% confidence level. Year 1 (n = 327), year 2 (n = 259), and year 3 (n = 219) students enrolled in military-technical programs at the Ferdinand I Military Technical Academy, a polytechnic university in Bucharest, Romania, was invited to participate in this study. Because participation in the study was voluntary, following random selection, the sample should be considered a sample of convenience. The inclusion criteria in this study were: being a student in the 1st, 2nd, or 3rd year at the Academy and completing the informed consent form to participate in the study. Before being asked for consent, prospective participants were informed of the purpose and stages of the study, potential study benefits for participants and for medical research, and the implications of providing informed consent to participate in the study.

Clinical evaluation

Participating students were subjected to an examination at the Academy's dental office between March and May 2019. Prior to clinical evaluation, the following information was recorded for each participant: age, gender, time since last dental examination, time it takes to get to the dentist, use of fluoride toothpaste, typical number of snacks between main meals, tobacco and alcohol consumption, type of high school graduated (military or civilian), and environment of origin (urban or rural).

All examinations were performed by a single experienced examiner, according to the recommendations of the European Global Oral Health Indicators Development (EGOHID) II Project, after the examiner was informed about the aspects of examination that would be important for the study, including affirming informed consent, the need for good-quality materials, and adherence to the 2008 EGOHID II Full Standard Clinical Survey Form v22a. The clinical examination and registration of the collected data were performed in accordance with the recommendations of the EGOHID II Project Guide.

For each clinical examination, a dental mirror, dental probe, dental tweezer, light, air spray, cotton rolls, cotton balls, toothbrushes, and possibly dental floss were used. Before their examinations, subjects were asked to remove any removable braces or dentures. The examiner made sure that the teeth to be examined were clean, intervening where necessary by cleaning teeth with a dry toothbrush. Floss was used to remove interdental bacterial plaque. To perform a thorough examination, the teeth were dried for 5 seconds with air spray or a cotton ball, and the teeth were illuminated with a light source from the dental unit.

The examiner searched for signs of oro-dental disease and dental anomalies, according to the EGOHID II Dental Disease Assessment form, similar to the sample form shown in the EGOHID Project Report [17]. Causes of missing teeth were coded following EGOHID II guidelines outlined in the aforementioned report as follows: CODE 97, extracted due to caries; CODE 98, missing for any other reason; and CODE 99, unerupted (visible in an X-ray). Additionally, missing teeth that had been rehabilitated with an implant were noted with CODE P. Dental sites of anodontia were identified according to mouth quadrant (1-4, upper right and left, lower right and left, respectively) and dental site within the quadrant (numbered from central incisor backward). When anamnesis indicated that a missing tooth was never present in the arch, a radiological evaluation was performed or presented by the patient to confirm or rule out anodontia. Third molars were not considered in this study. The clinical examinations took, on average, 12 min *per* patient (range, 10~15 min).

Results

Participants

Out of the 805 students invited to participate, 318 students gave informed consent and enrolled in this study (39.5% enrolment rate), including 133 first-year (41.8% enrolment rate), 88 second-year (30.5% enrolment rate),

and 97 third-year (27.7% enrolment rate) students. This cohort could be considered statistically representative according to our OpenEpi analysis, which suggested the need for at least 261 subjects. Reasons given for choosing not to participate were the following: a busy daily schedule, fear of going to the dentist (despite knowing no therapeutic maneuvers would be performed at the examination), lack of interest in participating in biomedical research, and lack of interest in becoming more informed about one's own oral health status.

Demographically, the majority of respondents were men [231 males (72.6%); 87 females (27.4%)], and the cohort had a mean age of 20.2 years, with ages ranging from 18 [n = 8 (2.2%)] to 24 years [n = 1 (0.28%)]. The highest percentage of subjects was 21 years old [n = 123 (33.9%)]. A third of the cohort derived from rural origins [rural, n = 106 (33.3%); urban, n = 212, (66.7%)], and about a third graduated from military high school [military school graduates, n = 108 (34.0%); civilian school graduates, n = 210 (66.0%)].

Dental findings

Cohort

Anodontia was observed in 6 (1.88%) of the 318 participants, including 4 women (prevalence among women was 4.6%) and 2 men (prevalence among men was 0.87%). The most affected teeth were the second premolars (n = 8), followed by the first premolars (n = 4) and permanent molars (n = 2). There were six absent premolars from the maxilla and six absent premolars from the mandible. The sites of both anodontia-affected permanent molars were located in the mandible. None of the participants had a temporary molar corresponding to the premolar(s) affected by anodontia. The dental sites affected by anodontia are shown in Table 1.

Notably, five of the six participants in whom anodontia was discovered received their diagnosis of anodontia for the first time in the course of this study. Only one of them had been receiving ongoing dental monitoring, and the rest had not heard of this type of abnormality.

Treated case

Teeth surrounding sites affected by anodontia often exhibited interdental tremas and dental rotations that would be expected to disrupt optimal dento-maxillary functioning. For instance, in the case of a female patient (Figure 1) who had anodontia of the permanent lower second molars (sites 3.7 and 4.7), impaction of her bilateral and bimaxillary permanent canines was observed. The patient's lower canines (sites 3.3 and 4.3) were surgically-orthodontic straightened, and the same treatment was recommended for her upper canines (sites 1.3 and 2.3). This patient's radiological examination revealed additional anodontia of the wisdom teeth in all four quadrants.

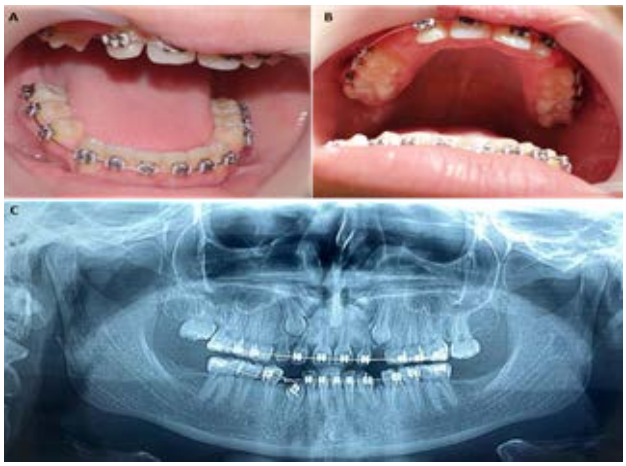

Fig. 1 – Images of a 19-year-old female patient with anodontia presenting with impaction, *tremas*, and maxillary diastema: A) Clinical appearance of the mandibular arch with bilateral anodontia of the permanent second molars, at sites 3.7 and 4.7; B) Clinical appearance of the maxillary arch of the same patient, with bilateral inclusion of permanent canines, at sites 1.3 and 2.3; C) Radiograph demonstrating anodontia of dentition at sites 3.7 and 4.7.

The coexistence of anodontia together with dental inclusions can affect the positioning of patients' teeth in both arches. Notably, this patient presented with excessive *tremas* in both arches, together with a midline maxillary *diastema*, which was corrected by orthodontic treatment. No skin, hair, nails, eyes, or bone abnormalities were observed during her clinical examinations, indicating that her anodontia was non-syndromic. Because the patient was adopted and not in contact with her biological family, it could not be determined whether she may have inherited the observed dental abnormalities.

Table 1

Summary of anodontia cases identified

Patient sex/age (years)	Anodontia site(s)[a]										No. sites
	1.4	1.5	2.4	2.5	3.5	3.4	3.7	4.4	4.5	4.7	
F/19						×					1
F/19		×		×	×				×		4
F/19					×				×		2
F/19							×			×	2
M/20		×		×				×			3
M/20	×		×								2

[a]Sites are identified as "quadrant locus": quadrants 1, 2, 3, and 4 are upper right, upper left, lower right, and lower left, respectively; teeth in each quadrant are numbered from the central incisor to molars, such that .4 and .5 indicate first and second premolars, respectively, and .7 indicates a second molar.

Untreated case

Another example is a 19-year-old female participant that had anodontia at the level of all four second premolars (Figure 2). In this case, the radiograph was performed four years before the clinical examination but showed the absence of premolars. Furthermore, in this case, there was a non-syndromic anodontia. This patient did not benefit from orthodontic treatment.

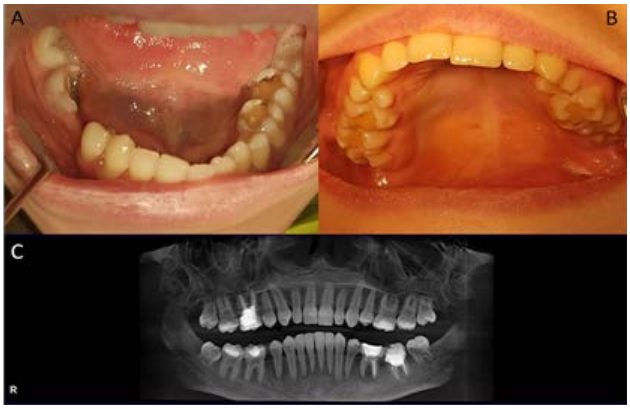

Fig. 2 – Images of a 19-year-old female patient with non-syndromic quadruple second premolar anodontia without any other dental abnormalities: A) Clinical appearance of her mandibular arch with bilateral anodontia of the permanent second premolars, at sites 3.5 and 4.5; B) Clinical appearance of her maxillary arch with bilateral anodontia of the permanent second premolars, at sites 1.5 and 2.5; C) The radiograph shows the absence of premolars.

Discussion

The prevalence of anodontia observed in the present study cohort (1.88%) was within the range of previously reported anodontia incidence rates (0.15–13.4%) [8, 10, 18–23], while being slightly lower than that reported in a prior study conducted in Romania from 2008 to 2015 (3%) [24]. Although women constituted only 27.4% of our cohort, they accounted for two-thirds of the anodontia cases observed, consistent with prior studies reporting a higher risk of anodontia in females than males [8, 10, 12].

The predominance of second premolars being affected by anodontia in our study is consistent with prior studies reporting that second premolars and upper lateral incisors were the teeth most often affected by anodontia [25, 26] with the most often affected teeth within a series of like-type teeth being the distal tooth in each series (i.e., lateral incisor, second premolar, and third molar) [8, 27, 28]. We observed bilateral, rather than unilateral, anodontia of the second premolars more frequently than reported in previous studies [10, 29].

In general population, anodontia of the second molars is quite rare, with reported prevalence rates ranging from 0% to 3.4% [19, 21, 22, 30, 31]; in our study, we observed a 0.31% (n = 1) prevalence. Previously, anodontia of the second permanent molars has been reported to be accompanied by other dental anomalies [10]. In the case observed here, the patient presenting with anodontia of the permanent lower second molars had comorbid inclusion of all four permanent canines.

Only one of the six patients diagnosed in our study had received a prior diagnosis of anodontia. Of the six patients in whom anodontia was identified, only one was selected to proceed with orthodontic treatment. That patient (presented in Figure 1) had anodontia of the second permanent molars of the lower arch. None of the patients in whom anodontia of the premolars was discovered accepted to receive specialized therapy to correct associated dento-maxillary dysfunctions.

Anodontia was found more frequently in women than men, with an overall prevalence of almost 1 in 50. Second premolars were the most common teeth affected, followed by first premolars and second permanent molars. Both arches and both sides were affected at similar rates.

The lack of early treatment for dental anomalies that impair dento-maxillary function, such as anodontia, can result in worsening of oro-dental status over time, and thus costlier corrective treatment [32].

Limitations of the study

There are two major limitations of this study. First, the study focused on a single military academy with young adult students in Romania; thus, the findings may not reflect the general population. Second, the sample of anodontia cases discovered was small. Given that data regarding the oral health status of Romanian military students and personnel are lacking, a similar analysis should be conducted for the Romanian armed forces at large. Furthermore, a longitudinal study conducted with periodic evaluations would be warranted to reveal how the treatment needs of military personnel change over time and thus provide information regarding the logistical and human resources that should be involved.

Conclusion

The participants' lack of prior knowledge of their diagnoses indicates that the dental needs of members of the Romanian military academy are not yet being well met. Timely therapeutic intervention may be facilitated by widespread early dental evaluations.

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The influence of various coatings of hydroxyapatite bone carrier on the success of bone regeneration in rabbit calvarial defects: histomorphometric and histological analysis

Uticaj različitih materijala koji oblažu hidroksiapatit koštanog nosača na uspeh regeneracije koštanih defekta kalvarije zeca: histomorfometrijska i histološka analiza

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Abstract

Background/Aim. The materials used nowadays for bone replacement do not fully meet the requirements for complete regeneration, which is why new ones are being tested. Despite numerous attempts to improve bone tissue regeneration, no fulfilling material has been found yet. This study investigated the influence of poly-lactide-co-glycolide (PLGA) and polyethyleneimine (PEI) as coatings for hydroxyapatite (HAP) bone carriers on bone tissue regenerative potential in rabbits' calvarial defect. **Methods.** Calvarial defects measuring 6 mm in diameter were made in 19 skeletally mature rabbits. Defects were filled with one of the following materials: PLGA coated HAP (HAP + PLGA), PEI coated HAP (HAP + PEI), and bovine HAP – Bio-Oss® (positive control). Unfilled defects represented negative control. Histological analysis was performed in order to determine the inflammatory response of the host tissue. The formation of the new bone was evaluated using histomorphometric analysis. All analyses have been conducted in samples obtained 3, 6, and 9 weeks after implantation. **Results.** Three weeks post-implantation, a trend toward increased healing in the HAP + PLGA group compared to other investigated materials was noticed, with no statistically significant difference between the study groups ($p > 0.05$). However, after 6 and 9 weeks, significant healing was observed in favor of the HAP coated with PLGA compared to other groups ($p < 0.05$). Within this group, greater bone healing was observed compared to the HAP + PEI and Bio-Oss® groups. **Conclusion.** PLGA demonstrated greater coating potential compared to PEI with respect to osteogenesis improvement in bone reconstructive surgery.

Key words:
bone regeneration; histological techniques; materials testing; polymers; rabbits.

Apstrakt

Uvod/Cilj. Materijali koji se u današnje vreme koriste za nadoknadu koštanog tkiva ne dovode do kompletne regeneracije, zbog čega se ispituju novi. Uprkos mnogobrojnim pokušajima da se poboljša regeneracija koštanog tkiva, još uvek nije pronađen materijal koji ispunjava sve kriterijume. Cilj rada bio je da se utvrdi uticaj poli(laktid-ko-glikolida) (PLGA) i polietilenimina (PEI), kao premaza za oblaganje hidroksiapatita (HAP), na regenerativni potencijal koštanog tkiva u defektu kalvarije zeca. **Metode.** Kod 19 zečeva „zrelog" skeleta načinjeni su defekti kalvarije dijametra 6 mm. Defekti su potom ispunjeni jednim od sledećih materijala: HAP obložen PLGA (HAP + PLGA), HAP obložen PEI (HAP + PEI) i goveđi HAP – Bio-Oss® (pozitivna kontrola). Prazni defekti su predstavljali negativnu kontrolu. Inflamacijska reakcija tkiva domaćina je ispitana histološkom analizom. Formiranje nove kosti je procenjivano

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histomorfometrijskom analizom. Analizirani su uzorci dobijeni 3, 6 i 9 nedelja nakon implantacije. **Rezultati.** Tri nedelje nakon implantacije, uočena je tendencija boljeg zarastanja u HAP + PLGA grupi, bez statistički značajne razlike između ispitivanih grupa ($p > 0.05$). Međutim, 6 i 9 nedelja nakon implantacije, primećeno je značajno formiranje koštanog tkiva u korist HAP + PLGA grupe ($p < 0,05$). Oblaganje HAP sa PLGA dovelo je do boljeg koštanog zarastanja u poređenju sa HAP+PEI i Bio-Oss®. **Zaključak.** U pogledu stimulisanja osteogeneze u rekonstruktivnoj hirurgiji kostiju, PLGA je pokazao veći potencijal prekrivanja defekta od PEI.

Ključne reči:
kost, regeneracija; histološke tehnike; materijali, testiranje; polimeri; zečevi.

Introduction

Bone regeneration is an important issue in oral and maxillofacial surgery. Autogenous bone still presents a gold standard for bone defect repair. On the other hand, several drawbacks regarding the use of autogenous bone grafts have been described, such as donor site morbidity, a limited amount of harvested bone, and a relatively high resorption rate of the construct. Having in mind these drawbacks, synthetic bone substitutes and xenografts have been introduced [1–3]. An ideal bone substitute should be non-irritable and non-toxic, providing an adequate microenvironment for adhesion, proliferation, and differentiation of the cells [4]. In addition, requirements for graft material include not easy achievable mechanical stability and high porosity [5]. Likewise, the ideal bone substitute is expected to resorb completely, in a proper period, synchronized with new bone synthesis [6]. Geistlich Bio-Oss® is the most investigated bone substitute, characterized by desirable clinical results in comparison to other commercially available products. Despite positive clinical outcomes obtained with Bio-Oss®, this material does not provide complete bone regeneration [7, 8]. Furthermore, it has been shown that some particles remain within connective tissue for years [9, 10].

As an effort to obtain a material with degradation levels synchronized by new bone formation, a novel bone tissue substitute (scaffold) based on calcium hydroxyapatite (HAP) was synthesized [11, 12]. In order to activate the surface, HAP can be layered with various surface-active substances, such as poly-lactide-co-glycolide (PLGA). It had been suggested that PLGA coating did not induce any inflammatory effects 12 weeks after implantation. In addition, accelerated cell adhesion when HAP was coated with PLGA (HAP + PGLA) has been documented [12]. Through activation of the Runx2 = CBFA-1 transcriptional activator, HAP + PLGA promotes osteogenic differentiation of preosteoblastic cell lineage. This combination can be used as a tissue engineering scaffold material and delivery carrier of pro-osteogenic bone morphogenetic protein 2 (BMP-2) and the pro-angiogenic gene of vascular endothelial growth factor [13]. The issue of whether new bone formation could be obtained in a shorter extent of time remains unclear. Even though coating provides certain advantages, a choice of adequate coating material is still at the center of researchers' interests.

Another option for bone substitute coating is polyethyleneimine (PEI) [14, 15]. This material belongs to the next generation of gene-activated scaffolds, which might include multiple genes to promote synergistic cell-mediated protein production and facilitate the neo-vascularisation of the damaged bone [16]. Linear PEI-enriched scaffolds have promoted cell growth by mimicking the biological function of the native extracellular matrix [17]. Modified PEI also exhibits a number of key advantages, like low immunogenic, low cytotoxic, and non-carcinogenic properties, and is considered safe for clinical use [18]. In addition, PEI contains a large number of amino nitrogen atoms in the molecular chain, leading to a strong affinity to cells [19, 20].

The aim of this study was to assess the influence of PLGA and PEI when used as HAP coatings on osteogenesis improvement. The ultimate goal was to determine the ratio of the newly formed bone in rabbits' calvarial defects after implantation of HAP + PLGA and HAP coated with PEI (HAP + PEI).

Methods

Materials synthesis

HAP synthesis and PLGA coating were performed as reported previously [11]. In short, powders of calcium and $(NH_4)_2HPO_4$ (p.a. Merck) were used for the hydrothermal synthesis of HAP. The precursor solutions were prepared as a combination of corresponding mixtures of $Ca(OH)_2$ and the aqueous solution of $(NH_4)_2HPO_4$. Afterward, the surface-active substance poly(ethylene-vinyl acetate)/poly(ethylene-) (PEVA/PEVV) was added for further hydrothermal processing in the autoclave at a temperature of 120 °C for 2 h. The obtained particles were filtrated through a filter with a pore size of 200 nm. HAP granules were obtained using a polyurethane foam template and HAP suspension. After immersion of the template in the HAP suspension and its drying, the composition was thermally treated to pyrolyze polyurethane template, followed by sintering of porous HAP after thermal treatment at 1,200 °C. Finally, HAP + PLGA coating was obtained by pouring the PLGA solution in chloroform over the HAP granules.

Coating with PEI included presumably slight PEI modification. Briefly, the solution of modified PEI was prepared by dissolving branched PEI (3 g) in 15 mL water by heating and stirring. Carbon dioxide (CO_2) was bubbled into this solution at ambient temperature, and stirring was continued for 5 h until the reaction was complete. The contents were transferred to an Eppendorf tube, freeze-dried to form solid PEI-CO_2, and later dissolved in ethanol. HAP + PEI coatings were obtained by immersion of HAP granules in a prepared solution. Amino content and subsequently cytotoxicity of PEI were reduced by modifying with CO_2.

Study design and surgical procedures

A total of 19 adult skeletally mature male rabbits weighing 2–3 kg were included in the study. Experiments

were performed in accordance with the European Union Directive 2010/63/EU for animal experiments, which was approved by the Ethics Committee of the Faculty of Veterinary Medicine, the University of Belgrade (number of the study: 323-07-08477/2015/3, issued on March 8, 2016). Total anesthesia was maintained after premedication with an intramuscular (IM) injection of xylazine 2%, (CP-Pharma, Burgdorf, Germany) 5 mg/kg, with the combination of 35 mg/kg ketamine (Laboratorio Sanderson S.A., Santiago, Chile) and 0.75 mg/kg acepromazine (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Mo 64506 USA). The average duration of anesthesia was 100 min. IM injection of 500 mg/kg penicillin-Streptomycin (Penstrep™) was administered. The four circular calvarial defects of 6 mm in diameter were created in the parietal bones of each animal. The first defect was filled with HAP + PLGA (also known as ALBO-OS), the second defect with HAP + PEI, the third defect was filled with Geistlich Bio Oss® as a positive control, and the fourth defect was left empty as a negative control. The first six rabbits were sacrificed after 3 weeks, the other 6 rabbits after 6 weeks, and seven rabbits were sacrificed 9 weeks following the implantation. The biopsy specimens were obtained from each animal with an oscillating saw, including the entire cranial vault for histology and histomorphometric analysis. In addition, the *dura mater*, *galea*, and *periosteum* remained intact in all animals.

Histological analysis

All specimens were optimally decalcified using formic acid. Each specimen was embedded in paraplast and sectioned in 4 μm thick slices by rotary microtome (Leica SM2000R, Leica Microsystems, Wetzlar, Germany). Thereafter, the preparations were de-waxed, processed to hematoxylin-eosin (HE) and Goldner's trichrome staining technique, and qualitatively analyzed under a light microscope to determine the level of host tissue inflammatory response.

Histomorphometric analysis

The histological parameters were evaluated at ×40 magnification under a microscope (Leitz Labor Lux S Fluorescence Microscope, Ernst Leitz Wetzlar GMBH, Germany), with the exception of inflammatory cell infiltrates, which were counted on a total magnification of ×400. Using a digital color camera (Leica DFC295, Germany), 2D images were captured at ×40 magnification and merged to create a single image for each histological section. Thereafter, images were analyzed using software (Leica University Suite, version 4.3, Leica Microsystems, Germany) running on a personal computer. Four sections from the central defect region and four from the peripheral defect region were analyzed with a spacing of 50 μm between sections. The following parameters were measured: total bone volume in percentages (TB%), mineralized bone in percentages (MB%), nonmineralized bone in percentages (NMB%), connective tissue in percentages (CT%), and blood vessels in percentages (BV%). Within the connective tissue, macrophages, giant cells, plasma cells, lymphocytes, and neutrophil granulocytes were counted.

Statistical analysis

Statistical analysis was performed using SPSS for Windows - version 18.0 software (SPSS, Inc., Chicago, IL, USA). All data were presented as the mean ± standard deviation (SD). Two-way ANOVA was performed at a 95% level of significance, followed by Tukey *post-hoc* comparisons.

Results

Histological and histomorphometric analysis after 3 weeks post bone replacement material implantation

In all specimens, 3 weeks after implantation, the demarcation line and area of the defect filled with connective tissue containing the graft particles could be clearly noticed (Figures 1 and 2). In addition, islands of new bone tissue mainly

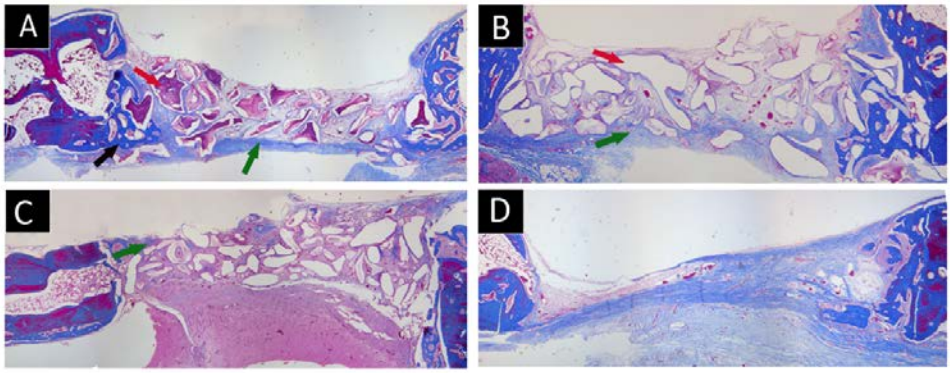

Fig. 1 – Histological specimens obtained after 3 weeks of regeneration: HAP + PLGA (A), HAP + PEI (B), Bio-Oss® grafted defects as a positive control (C) and non-grafted calvarial defect as negative control (D).
Initial signs of ossification, with newly formed bone tissue (black arrows), occurred at the most peripheral parts of particles of the grafted material (red arrows). Particles of grafted material and newly formed bone were surrounded by non-inflamed connective tissue (green arrows), with a minimal number of inflammatory cells or without them. The empty defect of control specimen (D) was filled with connective tissue. (Goldner's Trichrome staining, ×40 magnification).
HAP– hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

localized at the particle surfaces or near them were detected. In the HAP + PLGA group, particles of the graft were almost completely surrounded by new bone tissue, with trabeculae and osteoblasts.

Histomorphometric analysis of specimens after 3 weeks is shown in Table 1. Although the HAP + PLGA group showed a higher percentage of total bone area compared to the Bio-Oss® group, the difference was not statistically significant ($p > 0.05$). Likewise, there was no statistically significant difference in any other parameter 3 weeks after implantation (Figures 1 and 2, Table 1).

Inflammatory infiltrate (macrophages, giant cells, lymphocytes, plasmocytes, neutrophils) was mainly localized in close proximity to the particles of the material. The number of cells ranged from 0 to 10 in the field of view under the light microscope magnification of ×400 (Table 2).

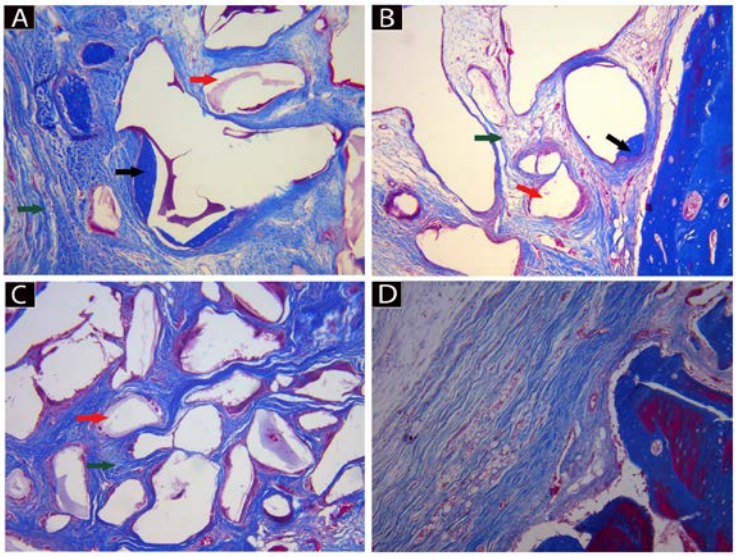

Fig. 2 – Photomicrographs of bone defects grafted with HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C) and non-grafted (empty) calvarial defect (D), obtained 3 weeks after implantation.
Although the amount of the newly formed bone differed in various test groups (with almost complete absence in empty defect), the histological structure of the new bone was very similar in all groups. Bone resembled lamellar structure with Haversian canals (black arrows) and numerous osteocytes in lacunae. New bone was in close contact with graft particles (red arrows) and surrounded by young connective tissue (green arrows). Although connective tissue was infiltrated with a minimal number of inflammatory cells, vascular stasis was observed in some specimens. (Goldner's Trichrome staining, ×100 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 1

Histomorphometric analysis of the specimens 3 weeks after implantation

Variable	Bio-Oss®	HAP+PEI	HAP+PLGA	Empty defect
Graft	47 ± 2	25 ± 5 ($p < 0.001$)	47 ± 3	10 ± 1
TB	13.2 ± 0.3	8.5 ± 0.10 ($p < 0.001$)	18 ± 3	8.4 ± 0.7
MB	10 ± 0.5	6.6 ± 0.4	13 ± 3	4 ± 0.3
NB	3.2 ± 0.4	1.4 ± 0.3 ($p < 0.001$)	5 ± 1	1.30 ± 0.13
CT	39 ± 2	66 ± 6 ($p < 0.001$)	34.5 ± 1.4	85 ± 0.4
BV	0.5 ± 0.7	0.4 ± 0.40	0.68 ± 0.16	0.26 ± 0.02

All values are expressed as percentages (mean ± standard deviation).
TB – total bone; MB – mineralized bone; NB – nonmineralized bone; CT – connective tissue; BV – blood vessels; HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

Table 2

Amount of inflammatory cells (ICs) under the light microscope, 3 weeks after implantation (magnification ×400)

ICs (number)	Bio-Oss® (n = 4)	HAP + PEI (n = 3)	HAP + PLGA (n = 4)	Empty defect (n = 1)
0–5	0 (0)	0 (0)	1 (25)	1 (100)
6–10	4 (100)	3 (100)	3 (75)	0 (0)
> 11	0 (0)	0 (0)	0 (0)	0 (0)

All values are expressed as numbers (percentages).
HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

Histological and histomorphometric analysis after 6 weeks post bone replacement with implanted material

The results of histological analysis 6 weeks after implantation showed that defects were filled by connective tissue with still unabsorbed particles of the graft and new bone tissue. The amount of newly formed bone was the largest in the HAP + PLGA group, followed by the HAP + PEI and Bio-Oss® groups, while the lowest amount was observed in empty defects. In all tested groups, the newly formed bone had a lamellar structure with osteocytes which indicated bone vitality. In the majority of the samples, the absence of inflammatory cells in connective tissue was detected (Figures 3 and 4).

Histomorphometric analysis of the specimens obtained after 6 weeks demonstrated that the amount of newly formed bone in the HAP + PLGA group was 18.9 ± 1.3%, which was statistically higher compared to the other groups ($p < 0.05$) (Table 3).

The decreasing tendency of inflammatory reaction has been noted. In 4 out of 12 cases, no inflammatory cells were noticed. In the rest of the specimens, the number of cells was minimal (Table 4).

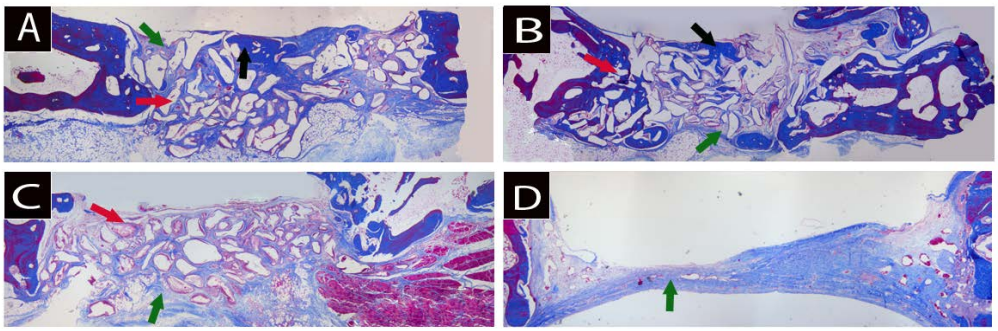

Fig.3 –Histological specimens of HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C) grafted defects, and non-grafted calvarial defect (D) obtained 6 weeks after implantation. The amount of newly formed bone tissue was larger compared to that after 3 weeks of regeneration, especially in the HAP+ PLGA group. Samples of the tested groups contained islands of newly formed bone (black arrows), residual particulate bone graft (red arrows), and connective tissue (green arrows). Newly formed bone tissue had both lamellar and woven structures, which indicated the occurrence of intensive bone remodeling. Empty defect contained connective tissue without bone formation. (Goldner's Trichrome staining, ×40 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine

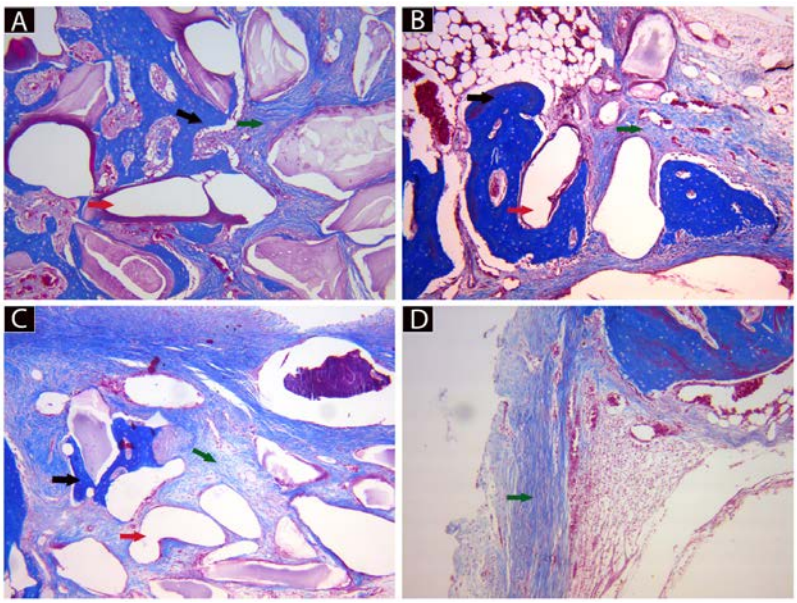

Fig. 4 – Photomicrographs of bone defects grafted with HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C), and non-grafted calvarial defect (D) obtained 6 weeks after implantation.
Defects were filled by still unabsorbed graft particles (red arrows), non-inflamed connective tissue (green arrows), and newly formed bone in close relation to particles (black arrows). The main difference in findings compared to the previous healing period (3 weeks) was an increased amount of new bone and decreased amount of graft particles due to intensive resorption via dissolution. (Goldner's Trichrome staining, ×100 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 3

Histomorphometric analysis of the specimens 6 weeks after implantation

Variable	Bio-Oss®	HAP+PEI	HAP+PLGA	Empty defect
Graft	52 ± 3	51.2 ± 1.4	52 ± 3	0
TB	15 ± 3	16.4 ± 1.2	18.9 ± 1.3 ($p < 0.001$)	6.6 ± 0.3
MB	10 ± 3	9 ± 0.8	14.7 ± 1.7 ($p < 0.005$)	2.64 ± 0.11
NB	4.5 ± 0.7 ($p < 0.001$)	7.5 ± 1.3	4.3 ± 0.7	3.92 ± 0.16
CT	33 ± 3	32 ± 3	29 ± 3	93 ± 3
BV	0.46 ± 0.19	0.6 ± 0.10	0.4 ± 0.3	0.40 ± 0.18

All values are expressed as mean ± standard deviation.
TB – total bone; MB – mineralized bone; NB – nonmineralized bone; CT – connective tissue;
BV – blood vessels; HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

Table 4

The number of inflammatory cells (ICs) under the light microscope 6 weeks after implantation (magnification ×400)

ICs (number)	Bio-Oss® (n = 4)	HAP+PEI (n = 3)	HAP+PLGA (n = 4)	Empty defect (n = 1)
0–5	1 (25)	0 (0)	2 (50)	1 (100)
6–10	3 (75)	3 (100)	2 (50)	0 (0)
> 11	0 (0)	0 (0)	0 (0)	0 (0)

All values are expressed as numbers (percentages).
HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide;
PEI – polyethyleneimine.

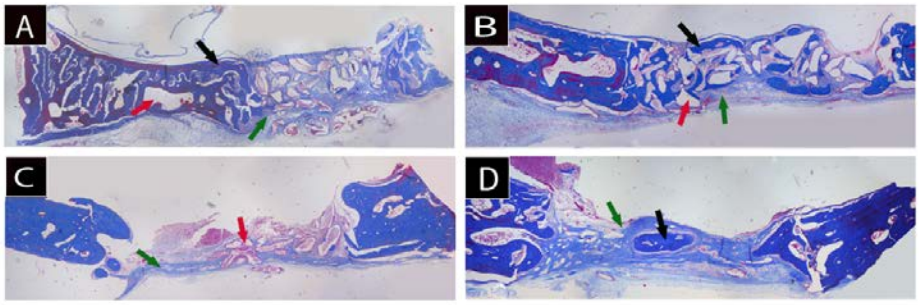

Fig. 5 – Histological specimens of HAP + PLGA (A), HAP + PEI (B), Bio-Oss® **(C) grafted defects, and non-grafted calvarial defect (D) obtained 9 weeks after implantation.**
It could be noticed that, particularly in the HAP + PLGA group, almost complete regeneration has occurred, with a large amount of newly formed bone (black arrows) with lamellar structure and osteocytes. Graft particles (red arrow) and newly formed bone were surrounded by young connective tissue (green arrows) with the absence of inflammatory infiltrate. Unfilled defects predominantly resulted in connective tissue formation, with signs of spontaneous bone regeneration in the form of small islands of newly formed bone. (Goldner's Trichrome staining, ×40 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 5

Histomorphometric analysis of the specimens 9 weeks after implantation

Variable	Bio-Oss®	HAP+PEI	HAP+PLGA	Empty defect
Graft	43 ± 0.7	46 ± 3	39 ± 3	0
TB	20.4 ± 0.9	21.9 ± 0.3	34 ± 3 ($p < 0.001$)	11.94
MB	13.7 ± 05	16.9 ± 0.8	28 ± 4	3.30
NB	6.3 ± 0.6	4.5 ± 0.9	5.9 ± 0.3	8.64
CT	35.9 ± 1.4 ($p < 0.001$)	31 ± 3	27.3 ± 0.7	87.69
BV	0.5 ± 0.5	0.4 ± 0.7	0.5 ± 0.3	0.37

All values are expressed as mean ± standard deviation.
TB – total bone; MB – mineralized bone; NB – nonmineralized bone; CT – connective tissue; BV – blood vessels; **HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.**

Histological and histomorphometric analysis 9 weeks post bone replacement with implanted material

The results of histological and histomorphometric analysis 9 weeks after implantation showed the highest amount of newly formed bone in the HAP + PLGA group and the lowest in the Bio-Oss® group and empty defect. The difference between the HAP + PLGA group and the Bio-Oss® group was found to be statistically significant ($p < 0.001$) (Figures 5 and 6, Table 5).

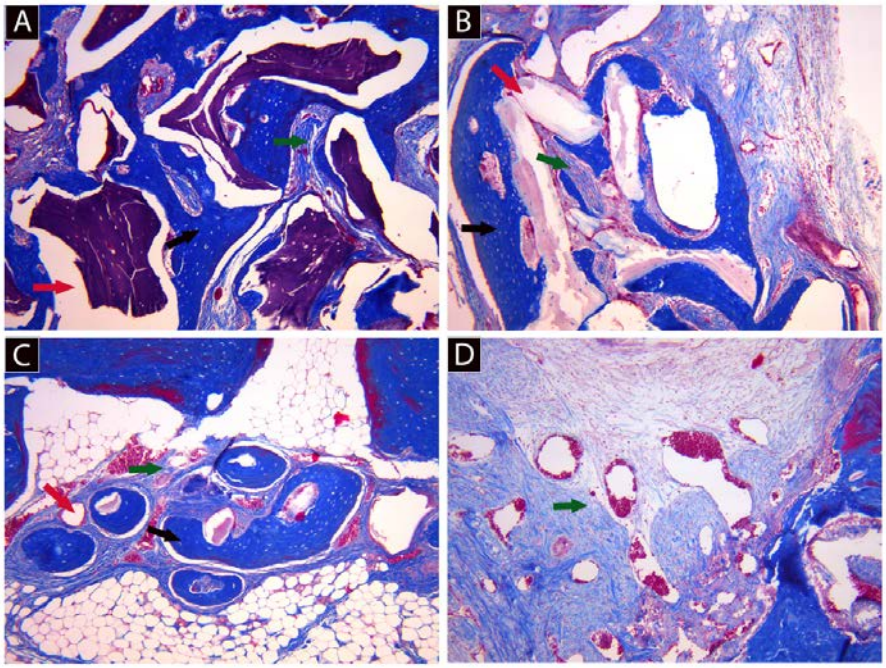

Fig. 6 – Photomicrographs of bone defects grafted with HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C), and non-grafted calvarial defect (D) obtained 9 weeks after implantation.
It could be observed that, in defects treated with HAP + PLGA and Bio-Oss, bone regeneration occurred at a significantly higher level compared to defects treated with HAP+PEI. In empty defects, bone regeneration almost completely failed, with the exception of a minor bone island in the central part of the defect. The resorption of particles with HAP+PLGA and Bio-Oss® was also remarkable. Connective tissue displayed remarkable venous stasis. (Goldner's Trichrome staining, ×100 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 6

The number of inflammatory cells (ICs) under the light microscope 9 weeks after implantation (magnification ×400)

ICs (number)	Bio-Oss® (n = 4)	HAP+PEI (n = 3)	HAP+PLGA (n = 4)	Empty defect (n = 1)
0–5	3 (75)	2 (66)	4 (100)	1 (100)
6–10	1 (25)	1 (33)	0 (0)	0 (0)
> 11	0 (0)	0 (0)	0 (0)	0 (0)

All values are expressed as numbers (percentages).
HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

Nine weeks after the implantation of the materials, in 10 out of 12 specimens, no histological signs of inflammatory reaction were discovered (Table 6).

Discussion

The present study evaluated the regenerative potential of various coatings for HAP bone substitutes. In the HAP + PLGA group, the greatest amount of newly formed bone was noticed, accompanied by the lowest number of inflammatory cells in all the investigated time cut-offs.

Obtained results are the extension of the previous investigation that evaluated HAP + PLGA influence on the calvarial defect healing in the period of 12 weeks after implantation [12]. The objectives of the current study were to determine whether bone healing could be achieved in three to nine weeks post-implantation, as well as to assess the influence of PEI coating on the regeneration capacity of previously designed HAP scaffold. It has been demonstrated that the type of coating exhibits a considerable impact on the regeneration potential of the bone substitute. For instance, it should be observed that the newly formed bone ratio 6 weeks following the implantation was three times higher in the PLGA than in the PEI coated group (4.3 ± 0.7% vs. 1.4 ± 0.3%, respectively). Similarly, the rate of mineralized bone at 9 weeks' cut-off was almost two times higher in the HAP + PLGA compared to the HAP + PEI group (28 ± 4% vs. 16.9 ± 0.8%, respectively). The results of the current study are promising: when compared to the "gold standard", Bio-Oss®, ~50% more mineralized bone was observed in the HAP + PLGA group after six weeks and ~70% more mineralized bone at week 9.

Figures 1 and 2 demonstrate bone tissue samples three weeks after implantation, with observable islands of the new bone tissue near the graft particles, indicating osteoconductivity of the tested materials. Although a significant difference between study samples at week 3 was not found, it is interesting to note that the greatest amount of osteoid was noticed in the HAP + PEI group, two times higher than in the Bio-Oss® group (Table 1). This observation points out that intensive osteogenesis occurred in these specimens. In the HAP + PLGA group, particles of the graft were completely surrounded by newly formed bone with osteoblasts, which clearly describes active osteogenesis. The main histological parameter of biocompatibility was the number of inflammatory cells. The estimated number ranged from 6 to 11 in all samples obtained after 3 weeks (Table 2). This result presents a mild inflammatory reaction.

Six weeks after implantation, the highest amount of new bone was detected in the HAP + PLGA group (Figures 3 and 4, Table 3), which can be explained by a weak immune response and consequent acceleration in creating new bone tissue. It seems that the nano-topology of the HAP + PLGA coating is suitable for cell adhesion. In tested specimens, the new bone had a lamellar structure with osteocytes which indicated bone vitality. According to the number of inflammatory cells listed in Table 4, a mild inflammatory reaction was present six weeks after implantation.

Nine weeks after the implantation, the best result was obtained in the HAP + PLGA coating group, as shown in Figures 5 and 6. That can be clarified by an inter-group comparison of dynamics in new bone tissue forming: after 3 weeks, the rate of the newly formed bone was approximately identical in the HAP + PLGA, HAP + PEI, and Bio-Oss® groups (13.2%, 16.4%, and 18%, respectively); at week 6, more new bone tissue was detected in the HAP + PLGA and Bio-Oss® groups (18.9% and 15%, respectively) than in the HAP + PEI group (8.5%); finally, at week 9, the rate of newly formed bone in the HAP + PLGA group obviously confirms its superiority over the HAP + PEI and Bio-Oss® groups (Table 5). Nine weeks after implantation, a number of observed inflammatory cells (Table 6) indicated a mild inflammatory reaction again.

The HAP used in this study reaches an adequate balance between material resorption and new bone formation. It was previously demonstrated that HAP coated with PLGA promotes adequate bone healing 12 weeks after implantation [16, 21, 22]. This study went a step further by introducing PEI as a novel coating substance. PEI is a typical poly-cationic polymer that contains a high density of protonated secondary amines. Even though cytotoxic effects of free PEI on many cells were documented, the protonated form has been most widely used as a gene delivery agent due to its high charge density [23]. In our investigations, CO_2-modified PEI coatings were used in order to decrease the toxic properties of PEI [19, 24].

Although the results obtained for HAP coated with PEI are inferior when confronting HAP + PLGA, HAP + PEI showed superior healing capacity in comparison to Bio-Oss® 6 and 9 weeks following implantation.

Concerning the slight toxicity of modified polyethylene, this material probably triggers the response of surrounding immune cells as well as the initial period of inflammation. Furthermore, as a result of high networking and adhesion properties, it supports osteoblast propagation *via* interaction between PEI and bone morphogenetic protein (BMP)-2.

Comparing the unmodified and CO_2-modified PEI form, its cytotoxicity is remarkably less expressed in the latter. A high positive charge of unmodified PEI can damage the cell membrane and disturb critical intracellular mechanisms. CO_2 alteration mitigates this cascade without disrupting the activity of protein kinase C [24–27].

When a mixture of unmodified PEI and carbonic acid is formed, a part of the positive charge is neutralized by negatively charged acid anions, which ultimately reduces material toxicity. Liu et al. [25] demonstrated that modified PEI promotes the differentiation of multipotent stem cells to several tissue-specific pathways, including bone tissue. Furthermore, a positive impact on growth factors such as transforming growth factor and BMP with an indirect effect on osteogenesis has been shown [28].

In addition, all the abovementioned results stand in line with the outcomes achieved by Tang et al. [29], who used a bio-inspired trimodal macro/micro/nano-porous scaffold loaded with recombinant human (rh) BMP-2 (TMS/rhBMP-2). They assumed that osteogenic promotion of TMS/ rhBMP-2 mainly occurred in the first 8 weeks after implantation. Later on, tissue maturity mostly depended on the self-remodeling of the newly formed bone tissue. In the current study, the greatest amount of newly formed bone was found in the HAP + PLGA group after 6 weeks of regeneration, with the same trend prolonged to week 9 in both studies. Extensive angiogenesis and osteogenesis noticed in our specimens after 3 weeks of regeneration are in agreement with the primary bone formation stage in the study by Tang et al. [29]. In the present study, lamellar bone was formed after 9 weeks of regeneration, which is close to 8 weeks found in the investigation by Tang et al. [29]. Exogenous rhBMP-2 was important but probably not the crucial factor for the bone regeneration process in the mentioned study. Results of our experiments for periods of 6 and 9 weeks after implantation indicate that the precise biological mechanism of bone-forming after implantation remains unresolved. It is well known that bone regeneration implies biological events, including bone induction and conduction, as well as several cell types and signaling pathways. Bone grafting includes osteoinduction (BMPs and other growth factors), osteogenesis (osteoprogenitor cells), and osteoconduction (scaffold) [30]. The used scaffold has a key role in supporting cell growth and tissue formation, as well as in providing appropriate microenvironment and structural integrity; additionally, it can support cellular colonization and tissue regeneration. A suitable scaffold should also support direct cell growth and tissue formation by many growth factors, cytokines, and signal molecules. Biological mechanisms, which occur after scaffold implantation, e.g., scaffold biodegradation, still have to be elucidated.

Conclusion

The efforts of the current research were focused on modifying the surface topography of novel HAP using PLGA and PEI coating in order to accelerate new bone tissue forming. Results of the study suggest that PLGA presents a superior coating option capable of considerably improving the bone regenerative potential of the synthetic HAP.

Acknowledgement

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CASE REPORTS

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Diagnosing a hematopoietic malignancy during shoulder arthroplasty

Dijagnostikovanje hematopoetskog maligniteta tokom artroplastike ramena

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Abstract

Introduction. Determining the cause of shoulder pain is usually a challenge as many problems, such as rheumatoid arthritis, osteoarthritis, osteonecrosis, rotator cuff arthropathy, traumatic arthritis, fractures, conditions of cervical vertebra, and neoplasms, can produce similar symptoms. The diagnosis is usually regarded as incidental, however, chronic lymphocytic leukemia (CLL) diagnosis by the histopathological evaluation performed on lymph nodes excised during a shoulder procedure has not been reported in the literature, to the best of our knowledge. We present a CLL-small lymphocytic lymphoma (SLL) case diagnosed incidentally during reverse shoulder arthroplasty. **Case report.** A 69-year-old female with a history of left proximal humerus fracture a year prior was presented to our outpatient clinic with left shoulder pain. A proximal humerus non-union was revealed radiographically. The patient underwent reverse shoulder arthroplasty. During surgery, a 2.5 × 1.5 × 1 cm sized lymph node was observed near the left cephalic vein. The lymph node biopsy result was reported to be CLL-SLL. The patient had no early postoperative complications and was discharged two days after. At her final evaluation, she was free of orthopedic complaints and was consulted with the hematology department to receive CLL-SLL treatment. **Conclusion.** Elderly patients presenting with non-union should be questioned for vague symptoms; lymph nodes surrounding the non-union site should be examined thoroughly. Any suspicious lymphadenopathy encountered during orthopedic surgery should be excised and sent for pathological evaluation.

Key words:
diagnosis; fractutres, bone; histological techniques; humerus; leukemia, lymphocytic, chronic, b-cell; lymph nodes; orthopedic procedures.

Apstrakt

Uvod. Utvrđivanje uzroka bola u ramenu predstavlja izazov zbog mnogih oboljenja i stanja koja mogu izazvati slične simptome (reumatoidni artritis, osteoartritis, osteonekroza, artropatija rotatorne manžetne, traumatski artritis, prelomi, različita stanja vratne kičme i neoplazme). Dijagnoza se obično postavlja slučajno, međutim, u literaturi do sada, po našem saznanju, nije objavljen slučaj postavljene dijagnoze hronične limfocitne leukemije (HLL) na osnovu histopatološke procene limfnih čvorova uklonjenih tokom operacije ramena. Prikazujemo slučaj HLL-limfoma malih limfocita (LML) koji je slučajno otkriven tokom reverzne atroplastike ramena. **Prikaz bolesnika.** Bolesnica, stara 69 godina, javila se na pregled na ortopedsku kliniku zbog bola u levom ramenu, sa podatkom o prelomu proksimalnog humerusa prethodne godine. Radiografski je ustanovljeno nesrastanje proksimalnog humerusa i urađena je artroplastika ramena. Tokom operacije u blizini leve cefalične vene je uočen limfni čvor veličine 2,5 × 1,5 × 1 cm čijom biopsijom je utvđena HLL-MLL. Bolesnica nije imala rane postoperativne komplikacije i otpuštena je posle dva dana. Na završnoj proceni zaključeno je da je bez ortopedskih tegoba i upućena je na hematološku kliniku radi daljeg lečenja HLL-MLL. **Zaključak.** Ukoliko imaju nejasne simptome, treba pažljivo ispitati starije bolesnike, kod kojih nije došlo do zarastanja preloma. Limfne čvorove oko mesta koje nije zaraslo treba pažljivo ispitati i svaki sumnjivi limfni čvor zapažen tokom ortopedske hirurgije treba odstraniti i uputiti patologu na analizu.

Ključne reči:
dijagnoza; prelomi; histološke tehnike; humerus; leukemija, b-ćelije, hronična; limfne žlezde; ortopedske procedure.

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Introduction

Determining the cause of shoulder pain is usually a challenge as many problems, such as rheumatoid arthritis, osteoarthritis, osteonecrosis, rotator cuff arthropathy, traumatic arthritis, fractures, conditions of cervical vertebra, and neoplasms, can produce similar symptoms [1]. A reverse shoulder arthroplasty is a popular option in massive irreparable rotator cuff tears, multi-fragmented proximal humerus fractures, and revision shoulder arthroplasty [2]. The preferred approach for shoulder arthroplasty is usually deltopectoral, which involves visualization of the cephalic vein [3]. The cephalic vein's anatomic course is known to be in close relation with the lymphatic system, and the shoulder region is rich in lymph nodes [4, 5]. Chronic lymphocytic leukemia (CLL) is the most common leukemia type, and patients are usually asymptomatic upon admission. Increased white blood cell count and lymphocyte count are usually the findings that lead to the diagnosis. Fever of unknown origin, weight loss, and palpable lymph nodes are less common clinical findings in CLL [6].

The diagnosis is usually regarded as incidental, however, CLL diagnosis by the histopathological evaluation performed on lymph nodes excised during a shoulder procedure has not been reported in the literature, to the best of our knowledge. We present a CLL–small lymphocytic lymphoma (SLL) case diagnosed incidentally during reverse shoulder arthroplasty.

Case report

The case review was conducted according to all guidelines outlined in the Declaration of Helsinki. Written informed consent for publication was obtained from the patient's nearest relative.

A 69-year-old female with a history of type 2 diabetes mellitus, hypertension, and congestive cardiac failure was presented to our outpatient clinic with left shoulder pain. Left proximal humerus fracture was diagnosed a year prior, and an ongoing limited range of motion was noticed. After the radiographic evaluation, a proximal humerus non-union was revealed (Figure 1A). Reverse shoulder arthroplasty was opted as a treatment strategy by evaluating the status of the joint and functional expectations of the patient. In preoperative evaluation, there were no fever, cough, or other symptoms that could be relatable to a chronic inflammatory condition. Routine preoperative bloodwork was unremarkable.

The patient underwent reverse shoulder arthroplasty with a deltopectoral approach in a beach-chair position. During surgery, a 2.5 × 1.5 × 1 cm sized lymph node with an irregular surface and off-white color was observed near the left cephalic vein (Figure 2). The lymph node was excised and sent for pathologic evaluation. Surgery was carried on with no complications (Figure 1B). The patient had no early postoperative complications and was discharged two days after.

In the histopathological examination of the excisional lymph node biopsy, there were a diffuse infiltration and effacement of normal lymph node architecture with small-medium sized lymphocytes with scant cytoplasm, clumped chromatin, and indistinct or absent nucleoli, which are representative of prolymphocytes and paraimmunoblasts. Immunohistochemical stainings revealed the B lymphocytic infiltration with diffuse CD20 positivity. These cells showed CD5 and LEF-1 coexpressions and cyclin D1 and SOX11 negativity. CD23 was positive in dendritic cells of residue lymphoid follicles. CD3 staining was observed in residue T-lymphocytes. The lymph node biopsy result was reported to be CLL-SLL (Figure 3). The patient was free of

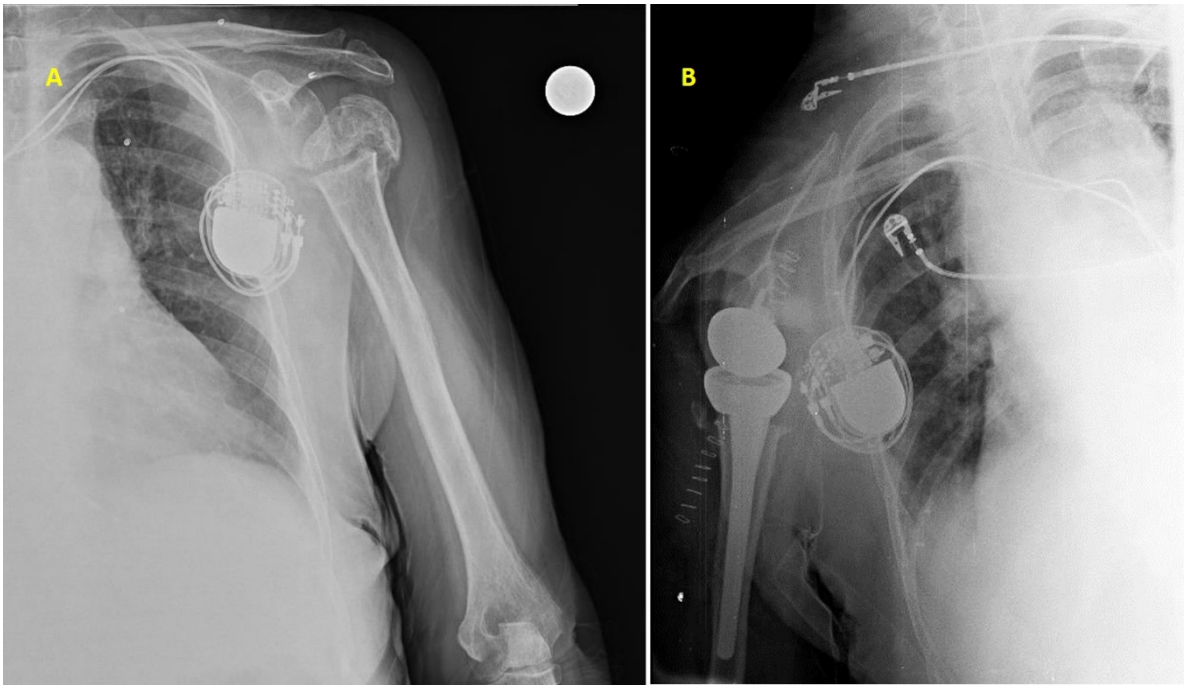

Fig. 1 – A) Patient on admission had non-union at proximal humerus; B) Postoperative radiograph after reverse shoulder arthroplasty is shown.

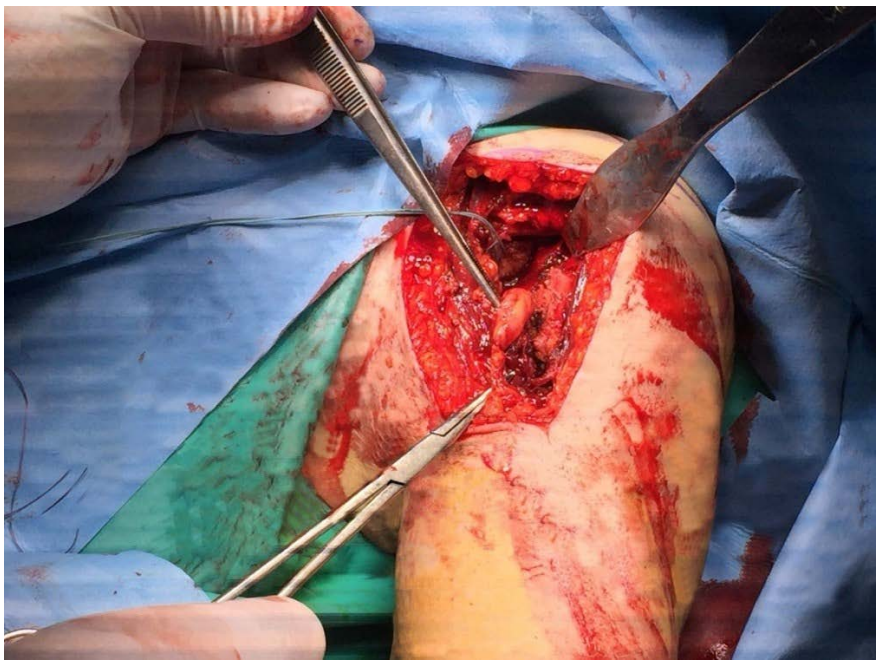

Fig. 2 –Intraoperatively, a 2.5 × 1.5 × 1 cm sized lymph node with an irregular surface and off-white color was observed near the left cephalic vein.

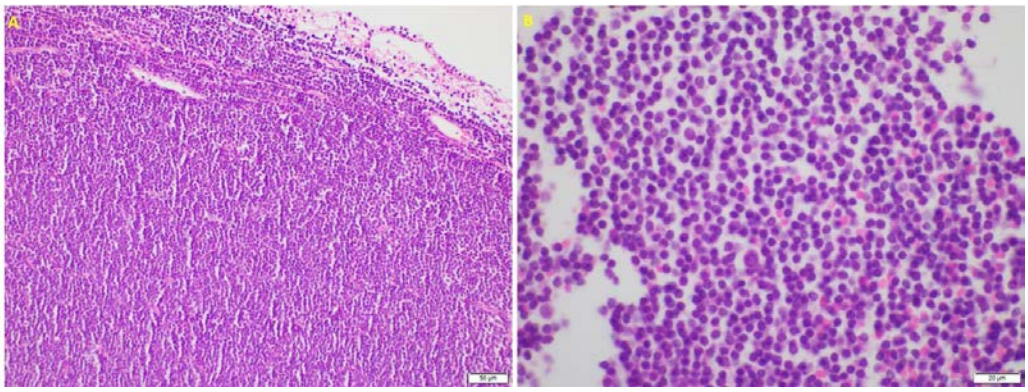

Fig. 3 – Hematoxylin and eosin stained diffuse, small prolymphocytes: A) magnification 40 ×; B) magnification 100 ×.

orthopedic complaints at her final evaluation and was consulted with the hematology department to receive CLL-SLL treatment. In the follow-up performed in the hematology department, two 1 × 1.5 cm eraser-like lymphadenopathies (LAP) in the right anterior cervical chain, 1 × 1 cm LAP in the left posterior cervical chain, two 1 × 1 cm LAP in the right axillary region, and 1.5 cm LAP in both inguinal areas were detected. No hepatosplenomegaly was detected during the follow-up.

Discussion

The most affected group in CLL is the geriatric population [7]. Diagnosis of CLL is usually incidental as it lies dormant for long periods of time. The identification of incidental lymphocytosis or lymphadenopathy, seen in 50% to 90% of patients, is the most common clue for diagnosis. However, in the reported case, we have not palpated lymphadenomegaly or spotted lymphocytosis upon admission to the orthopedic outpatient clinic. Diagnosis relied on incidental lymph node biopsy performed after clinical suspicion. Some authors argue that malignancies may interfere with fracture healing and should be regarded as a factor of bone destruction thus, non-union in the presented case may be due to malignancy itself [8].

There are reports of incidental CLL diagnosis from lymph node biopsies during pelvic and abdominal procedures such as: radical prostatectomy [9, 10], in which the rate of incidental diagnosis has been reported to be 0.3% [1]; splenic rupture [7]; chronic cholecystitis [11]. Incidental biopsies resulting in CLL diagnosis have also been reported in tissue samples from the lungs, oral cavity, and orbital sinus [12–14]. Extremity involvement is exceedingly rare, where reported cases were diagnosed with biopsies following pathologic fractures of the proximal femur and radius [15]. In rare cases, patients present with shoulder pain can have underlying

lymphoma. However, almost all reported patients were diagnosed with non-Hodgkin lymphoma [5, 16].

Conclusion

Several conclusions can be drawn by an orthopedic surgeon from this report. Firstly, elderly patients presenting with non-union should be questioned for B symptoms such as fever, night sweats, and weight loss. These symptoms are usually vague and could be easily missed by even experienced surgeons. Secondly, lymph nodes surrounding the non-union site should be examined thoroughly. Finally, despite extremity involvement being exceedingly rare, any suspicious LAP encountered during orthopedic surgery should be excised and sent for pathological evaluation. A strong collaboration with the pathologist is necessary to increase the accuracy of diagnosis. Careful management following the identification of a pathologic lymph node during dissection may lead to an early diagnosis and directly affect ultimate survival.

Conflicts of interest

The authors report no conflict of interest.

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CASE REPORT

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Early-onset ischaemic stroke in a patient with the novel *F2 c.1824C>T* gene variant and *PAI-1 4G/4G, MTHFR 677TT* genotype

Ishemijski moždani udar u mlađem životnom dobu kod bolesnika sa novom *F2 c.1824C>T* genskom varijantom i *PAI-1 4G/4G, MTHFR 677TT* genotipom

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Abstract

Introduction. Ischemic stroke (IS) is a heterogeneous disorder caused by several genetic and environmental risk factors. It was suggested that coagulation disorders cause 1-4% of cases with IS, especially in patients with early onset of IS. **Case report.** We describe a case of a young adult male who developed an unprovoked IS. Biochemical, immunological, and thrombophilia screening, as well as DNA sequencing, were performed in order to reveal molecular pathology underlying the stroke of the patient. Thrombophilia testing showed that patient was a homozygous carrier for *PAI-1 4G/5G* and *MTHFR C677T* mutations. Additional genetic analysis revealed the presence of the recently reported *F2 c.1824C>T* gene variant, located in the last exon of the prothrombin gene and has previously been shown to cause hyperprothrombinemia, hypofibrinolysis, and altered fibrin clot phenotype. **Conclusion.** Our results suggest that the newly reported *F2 c.1824C>T* gene variant might have a synergistic effect with *PAI 4G/4G* and *MTHFR 677TT* genotype in the formation of altered fibrin clot phenotype characterized by thin, densely packed fibrin fibers, which makes clot less susceptible to fibrinolysis and greatly increases the risk for early ischemic stroke onset.

Key words:
fibrin; genes; genetic variation; genotype; sequence analysis, dna; stroke; thrombophilia.

Apstrakt

Uvod. Ishemijski moždani udar (IMU) je heterogeni poremećaj koji može biti uzrokovan genetskim faktorima rizika i faktorima sredine. Poremećaji koagulacije mogu biti uzročnici u 1-4% slučajeva IMU, naročito kod bolesnika kod kojih se IMU dogodi u mlađem životnom dobu. **Prikaz bolesnika.** Prikazan je slučaj bolesnika koji je u mlađem životnom dobu razvio IMU nepoznatog uzroka. Urađeni su biohemijski, imunološki i testovi za trombofiliju kao i sekvenciranje DNK sa ciljem da se utvrdi molekularna patologija koja je mogla biti u osnovi moždanog udara kod tog bolesnika. Testovima za trombofiliju utvrđeno je da je bolesnik homozigotni nosilac mutacija *PAI-1 4G/5G* i *MTHFR C677T*. Dodatnom genetičkom analizom otkriveno je prisustvo nedavno opisane *F2 c.1824C>T* genske varijante, koja se nalazi u poslednjem egzonu gena za protrombin i za koju je prethodno pokazano da izaziva hiperprotrombinemiju, hipofibrinolizu i izmenjeni fenotip fibrinskog ugruška. **Zaključak.** Naši rezultati ukazuju na to da bi nova *F2 c.1824C>T* genska varijanta mogla imati sinergistički efekat sa *PAI 4G/4G* i *MTHFR 677TT* genotipom u nastanku fibrinskog ugruška sa izmenjenim fenotipom, koji se odlikuje tankim, gusto upakovanim fibrinskim vlaknima, što čini ugrušak manje podložnim fibrinolizi i povećava rizik od nastanka IMU u ranijem životnom dobu.

Ključne reči:
fibrin; geni; geni, varijacije; genotip; dna, analiza sekvenci; moždani udar; trombofilija.

Introduction

Ischemic stroke (IS) is a heterogeneous disorder that counts for 3/4 of all stroke cases and could be provoked by multiple risk factors, both genetic and environmental [1, 2]. A great number of genetic mutations have been shown to have a role in the etiology of several subtypes of stroke [2], but it is still challenging to identify specific causative mutations [3].

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Studies performed on twins and siblings have shown that IS onset is greatly affected by inherited risk factors [4]. The majority of genetic factors for IS have rather polygenic than monogenic influence. Additionally, IS could have a wide range of phenotypes, which could differ in their genetic background. Almost all human studies to date have employed a candidate gene approach [4].

Previous studies have suggested that coagulation disorders are the major cause of only 1% to 4% of all IS, but may be relevant for the pathogenesis of subgroups of stroke patients such as strokes in young ones [5, 6]. The perturbation of the coagulation cascade, due to the gene variants found in several genes involved in hemostasis [fibrinogen, prothrombin, FV, FVII, FXIII, thrombomodulin, plasminogen activator inhibitor-1 (PAI-1), thrombin activatable fibrinolysis inhibitor (TAFI)] are associated with increased coagulability and thrombotic risk [4]. The most frequently studied genetic variants in the pathogenesis of IS are *FV G1691A* (FV Leiden), *F2 G20210A*, and the *MTHFR C677T* [7].

One of the potential candidate genes involved in the pathogenesis of IS is the prothrombin *(F2*) gene. Its unusual non-canonical architecture makes the 3' end of the prothrombin gene sensitive to gain-of-function mutations associated with increased prothrombin expression [8]. Recent studies reported two novel gain-of-function variants in this region of the prothrombin gene, *F2 c.1787G>A* (prothrombin Belgrade) and *F2 c.1824C>T*, which are recognized as significant risk factors for the IS occurrence [9, 10].

We present a case of a patient who suffered from early-onset unprovoked IS. Since the cause of this IS remained unknown after routine testing, we performed additional genetic analyses, which revealed that the patient is a heterozygous carrier of the recently described *F2 c.1824C>T* gene variant.

Case report

A 42-year-old, right-handed male, nonsmoker, was initially presented to the emergency department of the local hospital due to the sudden onset of left-sided weakness. Upon arrival, he was alert and cooperative, while left facial palsy and severe left-sided hemiparesis were noted. The findings of the other neurological examination were normal. Computed tomography (CT), performed on admission, revealed no evidence of acute cerebral infarction or any other brain pathology (Figure 1a). Brain magnetic resonance imaging (MRI) performed on day 4 showed a lesion in the region of the right thalamus with characteristics of possible ischemia and less likely large demyelinating plaque (Figure 1b1). MRI of the cervical spinal cord was normal (Figure 1b2). Radiographic examination of the heart and lung and carotid ultrasonography were also normal. A cardiac evaluation, including echocardiography, revealed no source of embolism. A cerebrospinal fluid (CSF) examination was performed and revealed normal cell counts, glucose levels, and a mild increase of protein levels – 0.83 mg/L (normal range is 0.15–0.6 g/L). The levels of hematological and biochemical parameters and the levels of vitamin B12 and angiotensin-converting enzyme (ACE) were regular. Antibodies to *Borrelia burgdorferi*, human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B surface (HBS) were negative. The origin of the brain lesions was unknown; the patient was treated conservatively with acetylsalicylic acid (100 mg/day) and simvastatin (10 mg/day). His condition gradually improved over the four weeks until he was able to walk independently. The patient was admitted to our hospital 90 days after the beginning of symptoms to define the nature and etiology of the thalamic lesions. Physical examination was normal; the left-sided weakness was stationary during a three-month follow-up. The estimated National Institute of Health Stroke Scale (NIHSS) score was 3 on admission, and multi-slice computed tomography (MSCT) with contrast showed hypodense lesions restricted to the right putamen and corona radiate (Figure 1c1). The liquid density lesions represent the porencephaly cavity resulting in the area of the prior ischemia. MSCT- angiography was done (Figure 1c2), and brain blood vessels were normal. Ophthalmic examination and visual evoked potentials (VEP) showed no particularities.

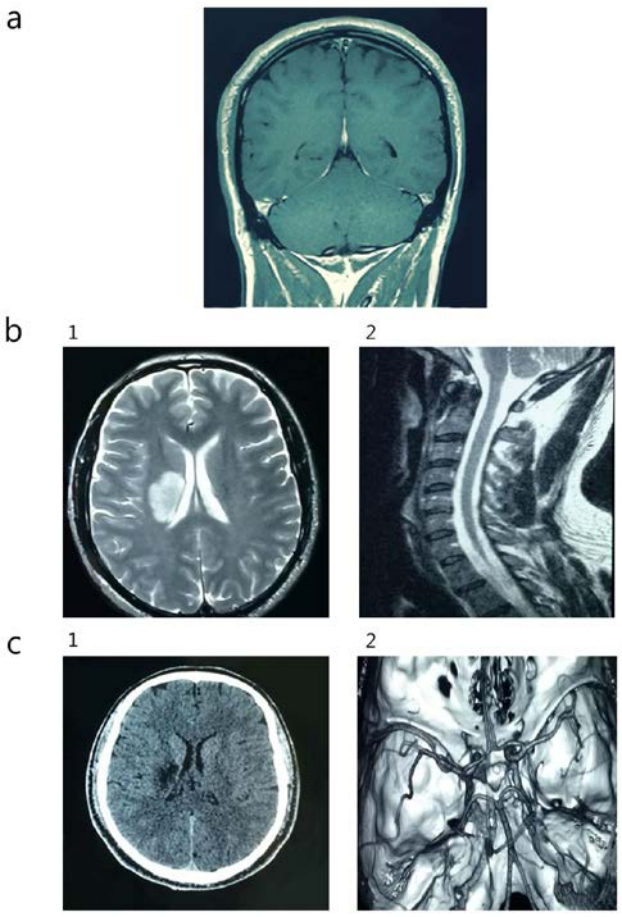

Fig. 1 – Brain scan and cervical spinal cord of stroke patient: a) brain computed tomography (CT) performed on patient admission; b) scans performed on day 4 after stroke onset [b1 − brain magnetic resonance imaging (MRI); b2 − MRI of the cervical spinal cord]; c) scans performed 90 days after stroke [c1 − brain multi-slice CT (MSCT) with contrast; c2 − MSCT angiography].

A cardiac evaluation, including transesophageal echocardiography (that demonstrated mitral valve prolapse with minimal mitral regurgitation, the aortic valve was intact, and the atrial septal defect was not seen) revealed no valid source of embolism. Biochemical and immunological results (antinuclear antibodies, antineutrophil cytoplasmic antibodies, and anticardiolipin antibodies) were within normal range. Thrombophilia screening showed normal results for prothrombin time (PT), activated partial thromboplastin time (APTT), lupus anticoagulant (LA), the biological activity of antithrombin, protein C, and protein S. Additionally, F2 activity of 1.07 (reference range 0.80–1.40) and D-dimer level of 0.07 mg/L (reference range < 0.25 mg/L) were obtained. Since the cause of the patient's stroke was unknown, we performed routine polymerase chain reaction (PCR) tests that included genotyping for *FV* Leiden, *F2 G20210A*, *MTHFR C677T*, and *PAI-1 4G/5G* mutations. PCR tests showed that the patient is a homozygous carrier of *PAI-1 4G/5G* and *MTHFR C677T* mutations, non-carrier for FV Leiden and *F2 G20210A* mutations.

As previously shown that certain gain of function variants within the 3' end of the *F2* gene could be associated with the early onset of IS [10], we performed additional analysis: DNA sequencing of this region in the *F2* gene and screening for variants, as well as determination of patient's plasma prothrombin level by Western blot analysis. DNA sequencing analysis, performed as described previously [11], revealed the presence of the *F2 c.1824C>T* gene variant, located in the last exon of the prothrombin gene in the heterozygous state. In Western blot analysis, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of proteins in the patient's and control's plasma samples. Standard human plasma (Siemens, Germany) was used for the normalization, with the given referent value of 100, and prothrombin deficient plasma (HemosIL, USA) was used as a negative control. As an additional control, the plasma of a symptomatic heterozygous carrier of *F2 G20210A* mutation suffered from deep venous thrombosis, and the plasma of a healthy volunteer, who was non-carrier for all known mutations in the 3' end of the prothrombin gene (confirmed by direct sequencing), was used. The plasma dilution, protein transfer, membrane blocking, antibodies used for protein detection, and protein quantification were performed as described previously [10]. Results of Western blot analysis showed that examined stroke patients had elevated prothrombin levels (170.83 ± 34.68%) compared to standard plasma (referent value 100%). The patients' plasma prothrombin levels were similar to the prothrombin level of the heterozygous carrier for *F2 G20210A* mutation (163.64 ± 21.74%) and higher than the prothrombin level in the healthy volunteer (105.49 ± 13.54%, $p < 0.0001$) (Figure 2).

Written approval from the Ethic Committee of the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade (registration number: O-EO-004/2015, registration date: July 29, 2015), in accordance with the Declaration of Helsinki for experiments involving humans, was obtained. The patient signed the informed consent form.

Discussion

Here we present a patient with early-onset IS of unknown etiology. Thrombophilia testing showed that the patient was a homozygous carrier for *PAI-1 4G/5G* and *MTHFR C677T* mutations. Additional analysis revealed the presence of recently reported *F2 C.1824C>T* gene variant in heterozygous state [10] and elevated plasma prothrombin level.

Impaired fibrinolysis and decreased fibrin network permeability are shown to represent substantial prothrombotic mechanisms contributing to the IS onset [11].

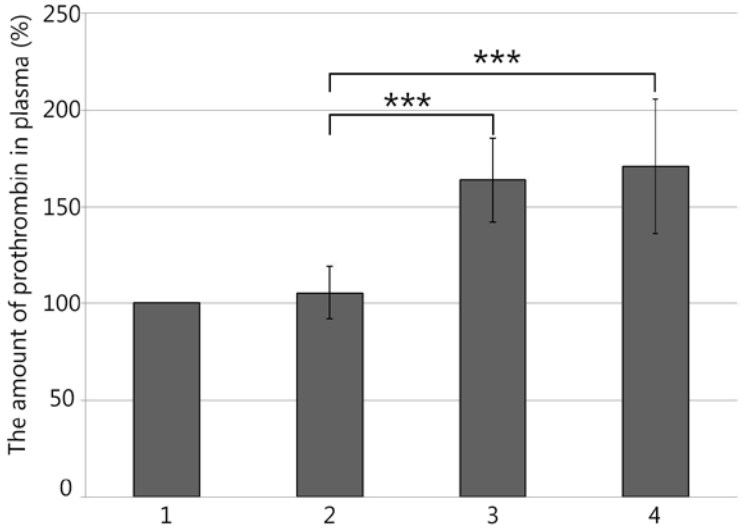

Fig. 2 – Relative quantification of plasma prothrombin level by densitometry of Western blot bands (Image Studio Lite™, LI-COR).
1: Standard human plasma (assigned referent value of 100%); 2: Plasma sample of a healthy volunteer (non-carrier of mutations in 3' end of the prothrombin gene);
3: Plasma sample of the heterozygous carrier of *F2 G20210A* mutation;
4: Plasma sample of stroke patient with novel *F2 c.1824C>T* gene variant.
***** $p <$ 0.0001 compared to healthy volunteer.**

PAI-1 has a crucial role in the inhibition of two types of plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (u-PA), thus representing one of the most potent inhibitors of plasma fibrinolytic activity [12]. The common *PAI-1 4G/5G* polymorphism within the promoter region of the *PAI-1 gene* influences the transcription of the gene and expression level of PAI-1 protein. The additional guanine in the DNA strand of the promoter region (5G allele) creates a repressor protein binding site absent in the 4G allele. This reflects on PAI-1 expression and its plasma concentrations with 4G homozygotes having the highest and the 5G homozygotes having the lowest PAI-1 concentrations [13]. High levels of PAI-1 in 4G/4G carriers lead to suppressed fibrinolysis and consequent pathological fibrin deposition and tissue damage [14]. The results on the association of *PAI-1 4G/5G* polymorphism with IS are conflicting; however, the meta-analysis, which included population-based association studies from 1966 to 2006, showed that *PAI-1 4G/4G* genotype is likely to be associated with IS [15].

Elevated plasma homocysteine (hyperhomocysteinemia) and homozygosity for the *MTHFR C677T* variant have been associated with the increased risk for IS; however, the exact mechanism is not fully determined [16, 17]. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate pathway and homocysteine conversion to methionine. The presence of the *C677T* variant leads to reduced enzyme activity and re-methylation of homocysteine to methionine, resulting in elevated homocysteine plasma levels [17]. Homocysteine or its metabolites interact with plasma coagulation proteins and affect their function *in vivo* [16]. It has been shown that elevated homocysteine level alters coagulation FV *in vitro* and inhibits its cleavage by activated protein C [18]. Hyperhomocysteinemia might also promote fibrinogen modification, thereby impairing the activity of fibrinolytic enzymes and fibrin polymerization. That results in altered fibrin clot structure, composed of thinner and tightly packed fibers resistant to fibrinolysis [16], which correlates with the fibrin clot phenotype observed in IS patients [17].

The third most common genetic risk factor associated with IS are gain of function variants in the *F2* gene, which cause elevated prothrombin level, hypercoagulability, and a tendency towards hyperproduction of fibrin clot [19, 20]. One of the most commonly tested prothrombotic variants in IS patients is *F2 G20210A* mutation [19, 21]. Taking into account that thrombophilia testing showed our patient is not a carrier of this variant, we decided to look into novel potential variants in the *F2* gene associated with early IS onset and did the sequencing of 715 bp within its 3' end, where a recently described *F2 c.1824C>T* gene variant was detected. The *F2 c.1824C>T* represents a synonymous *F2* variant leading to the CGC to CGT codon replacement on the Arg608 position in the protein [10]. The study by Pruner et al. [10] showed an increased frequency of *c.1824C>T* gene variant in patients who suffered IS compared to healthy controls (4.8% vs. 0.9%), and 5.4-fold greater risk for IS occurrence in variant carriers, *in vitro* examination demonstrated that *F2 c.1824T* allele is associated with elevated expression of prothrombin mRNA [10]. In addition, an *ex vivo* study indicated that *F2 c.1824C>T* variant leads to hyperprothrombinemia, hypofibrinolysis, and the formation of denser and thinner fibrin fibers within the clot, which makes it resistant to fibrinolysis [10]. The properties of the fibrin clot are shown to have clinical relevance in patients with acute IS. Undas et al. [22] enrolled 45 patients with acute IS and showed that the detected fibrin clots are less susceptible to fibrinolysis and that fibers form a less porous fibrin network. However, in comparison to the increased fiber thickness in acute IS patients [22], Pruner et al. [10] showed that fibrin clot in *F2 c.1824C>T* patients is structured from densely packed, but thinner fibers, which implicates that the fiber thickness could be *F2 c.1824C>T* specific.

The result of the elevated plasma level of prothrombin detected in our IS patient is in concordance with the study which examined the prothrombin level in carriers of *F2 c.1824C>T* who suffered from IS or venous thromboembolism (VTE) [10]. The increase in prothrombin level that we detected in the presence of the *F2 c.1824C>T* variant is similar to the one detected previously in IS and VTE patients. When compared to the effect of *F2 G20210A*, which increased prothrombin plasma level by approximately 60%, *F2 c.1824C>T* variant could be a potentially new, equally potent prothrombotic risk factor that leads to the development of a prothrombotic phenotype [10]. In this case study, we did not investigate the fibrin clot structure and thickness of fibrin fibers in our IS patient as did the Undas et al. [22]. However, electron microscopy scanning of fibrin clot structure from *F2 c.1824C>T* carriers, *F2 G20210A* and healthy noncarriers by Pruner et al. [10] revealed that fibrin fibers in plasma are denser and thinner in case of *F2 c.1824C>T* compared to clots in *F2 G20210A* and healthy noncarriers.

Fibrin clot phenotype is dictated by the number of variables involving pH, ionic strength, and concentrations of calcium and fibrinogen [23]. However, thrombin concentration during clot formation has a crucial role in the density and stability of the fibrin clot. Higher thrombin concentrations produce thin fibrin fibers that are densely packed, less susceptible to fibrinolysis, and associated with thrombosis, whereas lower concentrations lead to the production of thick, loosely-woven, permeable clots and bleeding disorders [23]. Fibrin clot structure differs in terms of stability depending on the type of stroke, characterized as clots more prone to lysis in acute intracerebral hemorrhage, as opposed to more stable clots in the case of IS [24]. Taking into account that our IS patient had elevated prothrombin level and previous results that the *F2 c.1824C>T* variant leads to the production of denser clots [10], we could hypothesize that this mechanism of clot formation is involved in the pathogenesis of early-onset IS, but further studies concerning the association of fibrin clot phenotype and early-onset IS are necessary.

Genetic predisposition for cerebral ischemia may result from an additive effect of several genes or synergistic co-effects. Several studies have shown that *PAI-1 4G/4G* and *MTHFR 677TT* genotype could affect the fibrin clot microstructure. High levels of PAI-1 lead to suppressed fibrinolytic activity, while high homocysteine levels modify

fibrinogen to be more resistant to cleavage by fibrinolytic enzymes [4, 16, 25, 26]. Altogether, the conjunct effect of PAI-1 4G/4G and *MTHFR 677TT* genotypes observed in our patient might influence the susceptibility of the fibrin clot to lysis and cause reduced clot permeability.

Conclusion

Based on our findings, we hypothesize that *PAI-1 4G/5G* and *MTHFR C677T* variants, in synergy with hyperprothrombinemic and hypofibrinolytic *F2 c.1824C>T* variant, could lead to the formation of altered fibrin clot phenotype characterized by densely packed, fibrinolysis resistant fibrin fibers, which could contribute to the early-onset IS in our patient.

Acknowledgement

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Conflict of interest

All authors declare no conflict of interest.

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CASE REPORT

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Successful treatment of synchronous hairy cell leukemia and diffuse large B-cell lymphoma in a patient with severe hypercalcemia and extensive osteolytic lesions

Uspešno lečenje bolesnika istovremeno obolelog od leukemije vlasastih ćelija i difuznog B krupnoćelijskog limfoma sa teškom hiperkalcemijom i ekstenzivnim osteolitičkim lezijama

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Abstract

Introduction. Although secondary malignancies usually occur at different times after hairy cell leukemia (HCL) treatment, the occurrence of HCL and other malignancies at the same time is very rare. Synchronous HCL and diffuse large B-cell lymphoma (DLBCL) have not been described so far. **Case report.** The report presents a 62-year-old female patient with intense constitutional symptoms, hypercalcemia, pancytopenia, and osteolytic destruction of the left shoulder joint. Immunohistochemical analysis of the bone marrow revealed the presence of two cell populations: a population of HCL cells and a population of DLBCL cells with the expression of CMYC and BCL-2 proteins ("double expressor" DLBCL) and high proliferative activity (Ki-67+cells > 90%). Fluorescence *in situ* hybridization (FISH) analysis showed amplification of the *BCL-2* gene. In addition, *BRAF* gene V600E mutation was detected. After intensive treatment with immunochemotherapy, radiotherapy, and bisphosphonates, the patient achieved complete remission, lasting for more than two years. **Conclusion.** As the association of HCL and lymphoma is very rare, diagnosis of synchronous occurrence of two lymphoproliferative diseases is a diagnostic and therapeutic challenge. It remains unclear whether DLBC and HCL originated from two different malignant clones or DLBCL developed by the transformation of HCL as the result of clonal evolution of the B-cell clone.

Key words:
lymphoma, large b-cell, diffuse; drug therapy; gene expression; leukemia, hairy cell; hypercalcemia; immunotherapy; magnetic resonance imaging; mutation; tomography, emission-computed.

Apstrakt

Uvod. Iako se sekundarni maligniteti obično javljaju u različito vreme nakon lečenja leukemije vlasastih ćelija (LVĆ), istovremena pojava LVĆ i drugih maligniteta je veoma retka. Istovremeno pojavljivanje LVĆ i difuznog krupnoćelijskog B limfoma (DKBL) do sada nisu opisani. **Prikaz bolesnika.** U radu je prikazana bolesnica stara 62 godine koja je imala intenzivne konstitucijske simptome, hiperkalciemiju, pancitopeniju i osteolitičku destrukciju levog ramenog zgloba. Imunohistohemijska analiza koštane srži otkrila je prisustvo dve ćelijske populacije: populaciju LVĆ i populaciju DKBL ćelija sa ekspresijom CMYC i BCL-2 proteina („dvostruki ekspresor" DKBL) i visokom proliferativnom aktivnošću (Ki-67+ ćelije > 90%). *Fluorescence in situ hybridization* (FISH) analiza je pokazala amplifikaciju *BCL-2* gena. Pored toga, otkrivena je mutacija V600E kod *BRAF* gena. Nakon intenzivnog lečenja imunohemoterapijom, radioterapijom i bifosfonatima, bolesnica je postigla potpunu remisiju, koja je trajala više od dve godine. **Zaključak**. Kako je povezanost LVĆ i limfoma veoma retko, istovremena dijagnoza dve limfoproliferativne bolesti predstavlja dijagnostički i terapijski izazov. Ostaje nejasno da li su DKBL i LVĆ vodile poreklo od dva različita maligna klona ili se DKBL razvio transformacijom LVĆ kao rezultat klonalne evolucije B-ćelijskog klona.

Ključne reči:
limfomi, b-krupnoćelijski, difuzni; lečenje lekovima; geni, ekspresija; leukemija vlasastih ćelija; hiperkalciemija; imunoterapija; magnetska rezonanca, snimanje; mutacija; tomografija, kompjuterizovana, emisiona.

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Introduction

Hairy cell leukemia (HCL) is an uncommon type of hematological malignancy which constitutes 2% of all leukemias. The prognosis of HCL has considerably improved thanks to new effective chemotherapeutic agents. However, literature data showed that HCL patients have a higher risk of developing second malignancies than the general population. In some studies, second malignancies were the primary cause of death in patients with HCL [1]. Although secondary malignancies usually occur at different times after HCL treatment, the occurrence of HCL and other malignancies at the same time is very rare. Simultaneous occurrence of HCL and several other lymphoproliferative diseases (follicular lymphoma [2], T cell lymphoma [3], B-cell chronic lymphocytic leukemia [4], multiple myeloma [5]) has already been reported. However, no one has described simultaneous HCL and diffuse large B-cell lymphoma (DLBCL) so far.

Case report

A 62-year-old female patient was admitted to our hospital in August 2018 with fatigue, fever, weight loss (15 kg/two months), and left shoulder pain. Physical examination showed poor general condition [Eastern Cooperative Oncology Group (ECOG) 3], fever (38 °C), pallor of the skin, and weakness of the left hand. Skeletal radiography showed osteolytic lesions in the glenoid region of the scapula, whilst chest X-ray showed consolidation in the area of the left lower lobe of the lung. Computed tomography of the thorax showed non-homogeneous consolidation in the S3 zone of the left lung with a negative bronchogram and hilar lymphadenopathy. Blood counts showed pancytopenia [hemoglobin 74g/L (normal range 115–160 g/L); platelets 84×10^9/L (normal range 150–450 $\times 10^9$/L); white blood cells 3.8×10^9/L (normal range 4.0–10.0 10^9/L), with 48% neutrophils (normal range 44–72%); 44% lymphocytes (normal range 20–46%); 6% monocytes (normal range 2–12%); 2% eosinophils (normal value < 7%) and 3 erythroblasts/100 leukocytes] in differential count. Biochemistry analysis showed hypercalcemia (3.3 mmol/L, normal value < 2.35 mmol/L), elevated potassium (5.42 mmol/L, normal value < 4.4 mmol/L), C- reactive protein (147.2 mg/L, normal value < 1.5 mg/L), lactate dehydrogenase (4,008 U/L, normal value < 343 U/L) and β_2-microglobulin (4.48 mg/L, normal range 0.8–2.2 mg/L). The erythrocyte sedimentation rate was significantly accelerated (140 mm/hr, first hour). Needle biopsy of lung and shoulder was unsuccessful several times. However, morphological and immunohistochemical analysis of the bone marrow revealed the presence of two cell populations: a population of HCL cells ($CD103^+$, $TRAP^+$, $annexin^+$) and a population of DLBCL cells with the expression of CMYC and BCL-2 proteins ("double expressor" DLBCL), expression of MUM1 and BCL-6 antigens, and high proliferative activity ($Ki\text{-}67^+$cells > 90%) (Figure 1). Flow cytometry immunophenotyping of bone marrow cells revealed a clonal population of atypical mature B-cells (16% of cells) with immunoglobulin-kappa light chain expression in combination with heavy immunoglobulin D or immunoglobulin M chains and high expression of CD19, CD103, CD11c, CD305, and FMC7 antigen. The same population was detected by flow cytometry in the peripheral blood (6% of cells). Fluorescence *in situ* hybridization (FISH) analysis showed amplification of the *BCL-2* gene, whereas *CMYC* gene amplification was not detected. In addition, *BRAF* gene V600E mutation was detected. Nuclear magnetic resonance of the left shoulder showed complete destruction of the left shoulder joint, with an osteolytic lesion of the left scapula in the glenoid and coracoid region and focal infiltration of surrounding soft tissue (Figure 2). Positron emission

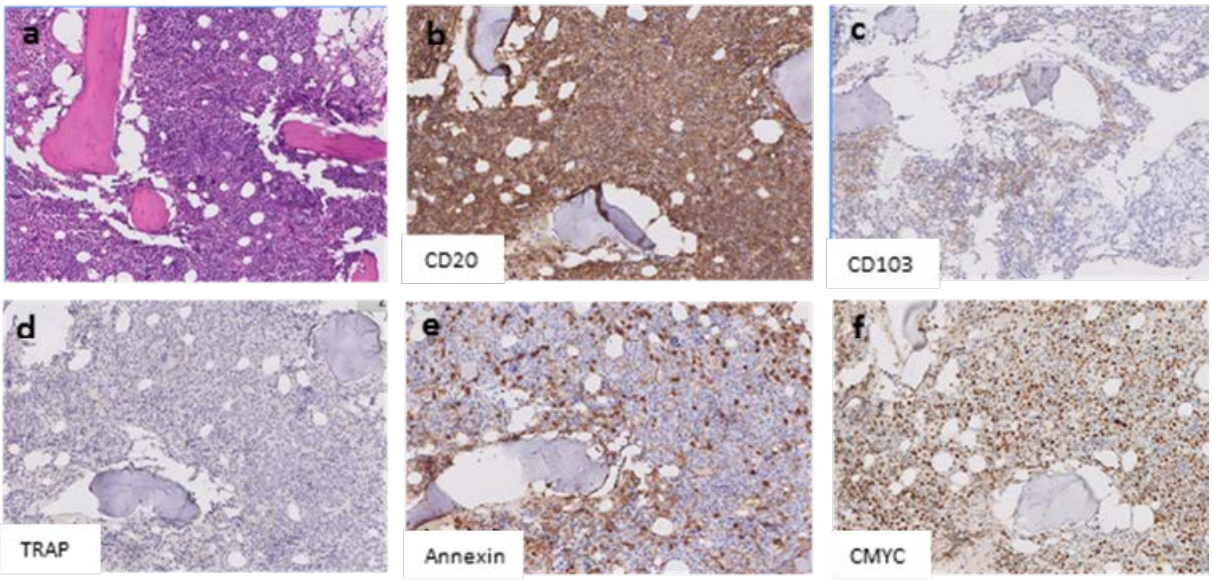

Fig. 1 − Presence of two populations of cells in bone marrow: a population of hairy cell leukemia cells and a population of diffuse large B-cell lymphoma cells (a − hematoxylin eosin, ×40). Immunohistochemical stains of formalin-fixed, paraffin-embedded sections for: b) CD20 (x40), c) CD103 (x40), d) TRAP (x40), e) annexin (x40), f) CMYC (×40).

tomography-computed tomography (PET-CT) scan showed hypermetabolic lesions on left axillary and retroperitoneal lymphadenopathy and multiple bone lesions (highest metabolic activity of SUV 25.52 noted in left scapula). We concluded that the patient has synchronous HCL and "double expressor" DLBCL in IVB clinical stage with a high international prognostic index (IPI) and high national comprehensive cancer network (NCCN)-IPI score. Treatment consisted of immunochemotherapy and bisphosphonates (zoledronic acid). Although lymphoid cells were not detected in cerebrospinal fluid using flow cytometry, because of the high central nervous system (CNS)-IPI score, CNS prophylaxis with intrathecal methotrexate injections was applied. After eight cycles of rituximab, cyclophosphamide, hydroxydaunorubicin hydrochloride (doxorubicin hydrochloride), vincristine (Oncovin), and prednisone (R-CHOP) chemotherapy, local irradiation of residual area with PET positivity (left shoulder soft tissue) was applied. Therapy resulted in complete hematological remission, lasting almost two years after completion of the treatment (last follow-up, December 2020).

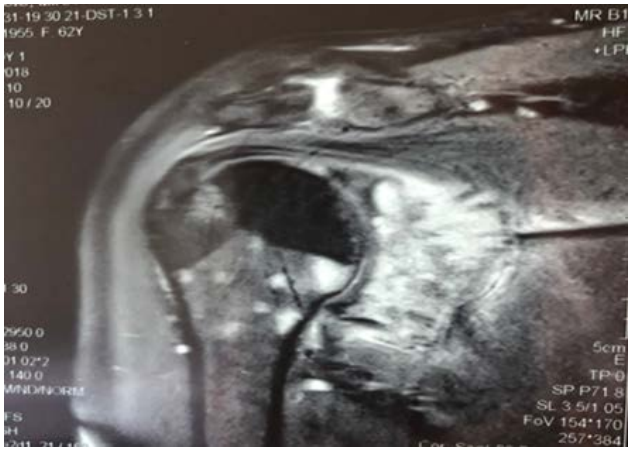

Fig. 2 – Nuclear magnetic resonance (NMR) of the left shoulder showing complete destruction of the left shoulder joint, with an osteolytic lesion of the left scapula.

Discussion

The presented patient is the first reported case of synchronous occurrence of HCL and DLBCL. The patient also had a very uncommon clinical presentation since the association of osteolytic lesions and hypercalcemia are extremely rare initial symptoms of lymphoma (approximately 2%) [6]. Hypercalcemia in malignancies can be mediated by several different mechanisms. Parathyroid hormone (PTH)-like substances [PTH-related protein (PTHrP)] secreted by malignant cells, secreting other cytokines that activate osteoclasts, the ectopic activity of 1-alpha-hydroxylase and the production of 1,25-dihydroxycholecalciferol as well as the excessive production of PTH are some of the well-characterized mechanisms of hypercalcemia [7]. Hypercalcemia caused by malignancy is most often related to PTHrP [8] but, in some cases, is associated with extensive bone metastases, which was the case in our patient. It was previously considered that hypercalcemia is a result of the direct destruction of bone by metastases. However, it was found that hypercalcemia was a consequence of the local release of cytokines from the tumor cells, which activate osteoclasts and stimulate bone resorption, usually through receptor activator of NF-kB/ ligand (RANK/RANKL) [9]. Patients with malignancy hypercalcemia have limited survival of several months, thus it is considered a marker of poor prognosis [7]. It is unclear whether this poor prognosis is related to the advanced stage of malignancy-associated hypercalcemia or is it just a simple marker of underlying cancer. Our patient had numerous osteolytic lesions and advanced disease, however, a complete therapeutic response was achieved owing to optimal therapy.

Nevertheless, it remains unclear whether DLBC and HCL emerged from two different malignant clones or DLBCL developed by the transformation of HCL as the result of clonal evolution of the B-cell clone. Based on phenotype and molecular features of HCL cells, malignant transformation occurs at the level of germinal or post-germinal center cells [10]. That is confirmed by the presence of somatic mutations of variable regions of the immunoglobulin genes, which are the hallmark of a germinal center origin [11]. Lymphoma cells of DLBCL in our patient originated from transformed germinal center B-cells (i.e., GBC type). That altogether supports a thesis of the common origin of synchronous HCL and DLBCL in this case. On the other hand, the hypothesis on the existence of two different malignant clones can be supported by common findings of amplification of the *BCL-2* gene in DLBCL patients and V600E mutation of the *BRAF* gene, a genetic alteration invariably associated with hairy cell leukemia [12].

As the association of HCL and lymphoma is very rare, diagnosis of synchronous occurrence of two lymphoproliferative diseases is a diagnostic and therapeutic challenge. Establishing an adequate diagnosis in cases of double malignancies is particularly important because it allows the implementation of an optimal therapeutic approach which is usually directed to more aggressive diseases. Such an approach resulted in the complete remission of disease in our patient. It is important to emphasize that our patient achieved complete clinical remission after immuno-chemotherapy treatment (i.e., rituximab-CHOP protocol), which can be explained by the responsiveness of malignant B-cell clone of HCL to monoclonal anti-CD20 antibody [13].

Conclusion

As the association of HCL and lymphoma is very rare, diagnosis of synchronous occurrence of two lymphoproliferative diseases is a diagnostic and therapeutic challenge. It remains unclear whether DLBC and HCL originated from two different malignant clones or DLBCL developed by the transformation of HCL as the result of clonal evolution of the B-cell clone.

Conflict of interest

The authors declare no conflict of interest.

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Medicine in the Hippocratic and post-Hippocratic age

Medicina u doba Hipokrata i posle Hipokrata

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greece; history, ancient; history of medicine; physicians; therapeutics.

Ključne reči:
grčka; istorija, drevna; istorija medicine; lekari; lečenje.

Introduction

From a historical aspect, medicine is divided into pre-Hippocratic, Hippocratic, and post-Hippocratic medicine. Hippocratic medicine represents a period in which medicine developed under the influence of Hippocrates. The Hippocratic period includes the time when he lived, but also several centuries later. People who studied and practiced medicine according to his doctrine formed a kind of direction in medicine called Hippocratic medicine. It is not possible to precisely define the date range of the actuality of Hippocratic medicine, but its influence on medicine today is realistically immeasurable [1].

Hippocrates was a Greek physician who lived from 460 to 377 BC and who belonged to the period of the highest scope of the classical Hellenic science of medicine. His father was Heraclides. He worked within the Medical School in Kos. He traveled extensively and died in Thessaly [1]. Hippocrates's portrait is shown in Figure 1 [2].

Hippocrates was the first to separate medicine from superstition, which was a kind of turning point in medicine. Because of that, he became a legend of medicine. The title "father of medicine" rightly belongs to him since it represents the prototype and ethical ideal of the doctor, who spoke about human life and the doctor's attitude towards the patient. He influenced medical practice centuries in advance. He put the patient at the center of the treatment process and the patient's well-being, that is, the welfare of the patient, as a priority in treatment. He believed that people were a part of nature and diseases were a natural phenomenon. According to that belief, people were treated with natural methods. He believed that a man was treated, not a disease. He was the first doctor to ask a person how they felt. There are a lot of

Fig. 1 – Hippocrates: portrait. Available from: https://schoolhistory.co.uk/early-modern/hippocrates/ [2].

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valid terms and methods of treatment that he left us. He was the originator of the concept of humoral medicine, which marked the diagnosis and understanding of pathology until the end of the New Century. He individualized medical therapy and pioneered medical propaedeutic. Medical ethics, as we call it, began with him. He practiced surgery, orthopedics, pharmacology, diet therapy, and philosophy. A well known Hippocrates's aphorism is: "Human life is short, medical skill is enormous; a favorable moment passes quickly; experience is deceptive, making a decision is difficult" [1].

After Hippocrates, medicine was marked by the Dogmatic and Alexandrian schools. The most valuable representatives of the Dogmatic school were Diocletian and Praxagoras. The Alexandrian school was marked by Herophilus and Erasistratus. Aristotle made a great contribution to medicine in this period. Another physician who marked the Hippocratic period was Galen. Galen was a Roman physician who was claimed to have lived between 125 and 215 AD. His life expectancy was almost three times longer than expected in the Old Century, which raises doubts about the reliability of this information. Like Hippocrates, Galen developed the idea of humoral medicine, which practically meant that human health was defined by body fluids. Galen influenced medicine so much that his name bears not only the vein but also the methods of preparing medicines in the pharmacy, and the prepared medicines are called Galena preparations. His teaching was considered dogmatic for 15 centuries. Roman medicine did not leave anything original on its own, except for a few personal discoveries. It mainly elaborated on the ideas and methods of Greek medicine. Notable Roman physicians in this period were Themison, Soranus, and Celsus [1, 3].

It is important that the doctors of that time had open access to museums and other selections of archeological discoveries. The concept of pathogenic factors varied depending on the cultural context and historical period. In this sense, subdisciplines developed, such as paleopathology. Old methods and treatments that we neglected and overlooked may prove useful. Modern medicine should take into account past experiences in treatment, at least as a basis for research [4].

Ancient Greek medicine of the Hippocratic period

Greek medicine was a turning point in human treatment. The ancient Greeks separated medicine from superstition and divine manipulations and turned it towards a naturalistic concept. That paved the way for the development of scientific medicine, and in the 5th century BC, Greek doctors already had their own association. There were permanent doctors (demiurges) and traveling doctors (periodeutes). Demiurges performed interventions in surgeries, where patients sometimes stayed in recovery without charge. The concept of hospitals did not exist at the time, but it can be said that this was its beginning. Periodeutes went to places where there were no doctors, and they charged according to the agreement. In treatment, they used herbs, examination, observation, and surgery, not prayers and spells. Nevertheless, Hippocrates made medicine a science [1, 3, 4]. The most famous schools of medicine were in Kos, Kroton, Cnidus, and Alexandria [1, 5].

Nemesius was the first to theorize about the division of the brain in the 4th century BC. He believed that feelings and imagination were located in two lateral chambers in the frontal lobe of the brain, and he called it the first cell. The mind was the function of the second cell, and in the posterior part of the brain was the third cell, which was in charge of remembering [6].

Chrysippus of Soli, who lived between 279 and 206 BC, was the first to mention cataract surgery [7].

Theophrastus is considered the father of botany. He wrote *De Causis Plantarum* and *De Historia Plantarum*, important collections of herbal medicines [8].

Hippocratic teaching

Hippocrates claimed that people were a part of nature. Hence, the disease had natural causes. He believed that climate, atmosphere, pollution, miasmatic fumes, food, colds, and other external influences significantly affected human health. He assessed the signs and symptoms by following human feelings as well as objective factors. He adjusted the therapy to the patient. A significant part of his treatment was diet therapy. Many aspects of which medicine is proud today, we really owe to him. The saying "Let food be your medicine" is attributed to him. He viewed people as beings who functioned in harmony with their environment. A person was treated as a complete being, and everything related to his external and internal interactions was seen as a factor of illness. Therefore, Hippocrates is considered the originator of the concept of holistic medicine. Today, holistic medicine is seen as the ideological forerunner of complementary medicine. He laid important foundations of medical ethics. Hippocrates did not distinguish veins from arteries. All blood vessels were called *phlebes*, hollow tubes. He understood the brain in the same way as Alcmaeon: its function was to analyze information from the outside world, which created our thoughts and feelings [1, 8].

By the term 'neura', he meant fibers such as veins, nerves, tendons, and ligaments, and he believed there were also hollow neurons such as pores, which could be opened to enter fluid if necessary. He claimed that the compression of the leg caused pallor or blue discoloration of the extremities. He described how a carotid artery injury caused contralateral hemiplegia. He introduced inspection, palpation, and the primitive variant of auscultation [5–8].

Hippocratic humoral medicine

Hippocrates based his teaching on Empedocles's idea of four elements: water, fire, air, and earth. Thus, humans were a phenomenon, a consequence of the activity of four fluids: blood, phlegm, and yellow and black bile. These liquids had four properties: hot, cold, dry, and wet. He believed that these aspects were governed by the force of physics. All

harmful liquids tended to be expelled from the body after cooking (pepsis), which gave a fever. All these aspects formed the disease, as well as the symptoms. He observed the appearance and behavior of the person and considered the examination to be the main moment in the treatment.

The famous *facies hippocratica*, *digiti hippocratica*, and much more were a consequence of his observation of patients. Practically, he laid the foundations of propaedeutics as the point of humoral medicine. Humoral means wet. In his treatment, he relied heavily on herbal medicines as essential elements of his work. Not only did he base his entire doctrine on that, but he also significantly influenced his students, particularly Galen, and Galen was practically the basis of Avicenna's work. Avicenna (Ibn Sina) gave his concept of four types of persons (choleric, phlegmatic, sanguine, and melancholic) based precisely on humoral medicine. The repercussions of this physical classification have not subsided to date, especially in psychiatry and psychology, even though they are no longer part of standardized diagnostics [8].

We learn about Hippocratic teaching from a work called the "Hippocratic Corpus" (*Corpus Hippocraticus)* [8] (Figure 2).

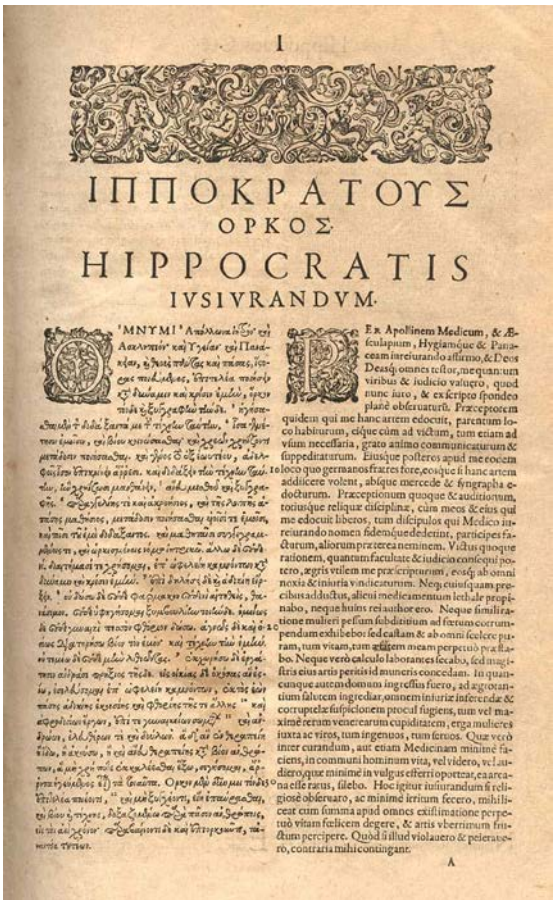
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Fig. 2 – Corpus Hippocraticus. Available from: https://schoolhistory.co.uk/early-modern/hippocrates/ [2].

The "Hippocratic Corpus" is believed to have been compiled in the Library of Alexandria in 250 BC. It is a collection of 70 works in the ancient Greek language, which mostly deal with medical issues. In addition to medicine, there are also philosophical texts. Authorship was not identified in the Old Century as it is today. Authorship had nothing to do with the creator but authority, which gave the concepts within which the work was created. If you established yourself as an authority, the work would be attributed to you.

Therefore, when we come across something that Hippocrates said, the chances that these were really his words are not that big. Entire texts were attributed to him. However, everything that was attributed to him was completely within the framework of his doctrine [8].

The works that are known to be his are: "Of the Epidemics", "The Book of Prognostics", "On Airs, Waters, and Places", "On the Sacred Disease (Epilepsy) ", "On Ancient Medicine", "Aphorisms", and "The Hippocratic Oath" [1, 3, 8].

Hippocrates's contribution to the understanding of specific diseases

The Hippocratic method for manual repositioning in glenohumeral dislocation and shoulder fractures is known and described in the section "On fractures": The upper arm is fixed in extension and under load while the patient is sitting. After returning the humerus, unless the head is broken, the bandages are dipped in oil, and the fracture is bandaged. The arm remains fixed for 40 days with diet therapy. The problem of poor healing was also pointed out. That clearly indicates that the students of the Hippocratic school understood the dynamics of articulation [3, 8].

The Hippocratic School saw diseases of the urinary tract as a problem in the swelling of the semen. It was thought that both men and women could suffer from it and that sometimes sex could be the cause. However, they had trouble distinguishing sexually transmitted diseases from other urinary tract diseases. Interestingly, they linked urinary tract disease to decay or spinal cord phthisis. The connection between seminal diseases and the condition of the spinal cord has survived to this day, even in popular culture and jargon. Here is an extract from the "Hippocratic Corpus", which illustrates how much Hippocratic doctors actually relied on inspection: "Most often it attacks the youth and those who like intercourse. They do not have a fever but still excrete something. If you ask the patient, he will say that he feels something coming from his head, down his spine, like ants. When he urinates or has a bowel movement, watery liquid comes out. He will not make children, and at night he urinates whether he has intercourse with a woman or not. When he walks or runs, especially uphill, he is out of breath, feels weak and dizzy, and it rings in his ears. When he finally gets a fever, he dies of it" [9].

In the section "On joints", from the book "Mohlikon", nasal injuries and the procedure of repositioning the nose were described [10]. Traction therapy of scoliosis is still used today in the form it was proposed by the Hippocratic school [11]. The treatment of lower jaw dislocation was taken over from the Hippocratic school [12]. The term and description of clubbed fingers came from Hippocrates [13]. Hippocrates considered urine a blood filtrate that had passed through the kidneys. In

the "Aphorisms", he stated that bubbles in fresh urine were a sign of long-term kidney disease, and urine sediment was a sign of fever [14].

Hippocrates coined the term *chylos*, and in the book "Peri Adenoma", he tried to explain lymphatics, calling them a branched and complex network [15]. He associated sciatica with claudication and thought it was more common during summer, and prescribed rest and diet to the patient [16]. He is thought to have performed abortions with a special instrument for that [17].

Ancient Greek medicine after Hippocrates

Hippocrates practically defined Greek medicine after the period of his work. His work was continued by his sons and a son-in-law. After him, two important schools of medicine stood out: Dogmatic and Alexandrian [18].

Dogmatic School

The Dogmatic School of Medicine considered the Hippocratic teachings a dogma, which was, ironically, diametrically opposed to the Hippocratic principle. The main teachers of the dogmatic school were Diocles, Praxagoras, and Crisipus. The Dogmatic School was the ideological and personnel predecessor of the Alexandrian school. Diocles wrote his first work on anatomy, the book "Anatomy". He practiced embryology and believed that both mother and father participated in the creation of embryos. They called him another Hippocrates. Praxagoras was Diocles's successor. He was the first Greek physician to deal with the pulse and the first to distinguish between arteries and veins. As Herophilus, who was his student, Praxagoras thought there was air in the arteries [1, 18].

Aristotle was the son of the philosopher and mathematician Nicomachus. He was not a doctor but he knew about medicine and wrote about it. He claimed that the basis for a human being was determined by the father and the mother only gave what was needed for growth. In addition to the known four elements, he added the fifth – "the principle of life" [1, 18]. Aristotle thought that the heart was the center of emotions and intelligence. He thought that we learned from memory and with the help of feelings. In his book *Historia Animalium*, he gave the first description of the lymphatic system. He believed that some of the fibers between nerves and veins contained *sanies*, a colorless fluid [1, 15, 18].

Alexandrian school

The Alexandrian School of Medicine was under the patronage of a pharaoh from the Ptolemaic dynasty. That means they were allowed vivisections and, in a short period of time, even dissecting people.

That influenced the accelerated development of clinical knowledge, primarily in the field of anatomy. The two most famous students were Herophilus and Erasistratus. The Alexandrian School also left vague anatomical descriptions concerning the lymphatic system [15, 18].

Herophilus of Chalcedon is considered the father of anatomy as we know it today. He was born in 325 and died in 255 BC. He was a big name of the Alexandrian School of Medicine. He dissected human corpses, which led to comprehensive anatomical knowledge at the time. However, this practice was not allowed for a long time. He described the brain and spinal cord, as well as the seven nerves that came out of the brain. He explained the structure of the eye, described the intestines and gave the name to the duodenum. He distinguished between porous sensory fibers and hard fibers that moved muscles [15, 18]. Herophilus wrote the work "On the Eyes". He is thought to have discovered the optic nerve. He also practiced obstetrics and performed abortions with an instrument called 'fetal destroyer'. Demosthenes Philalethes, who wrote the work *Ophthalmicus* in the first century, was a student of Herophilus [7, 18].

Crisipus was at the head of the Cnidian School. He worked between 320 and 280 BC. He wrote about the importance of vegetables in medicine, and he especially appreciated the cabbage compresses. He wrote the work "The Treatment of Vision". Unfortunately, that work has been lost. His grandfather and son bore the same name and were famous physicians [7, 18].

Erasistratus of Chios was born in 304 and died in 250 BC. Erasistratus relied on Herophilus's anatomy, but his personal work was more based on physiology. He described 4 chambers of the brain. He claimed that the fourth ventricle was below the third and that it was used for communication. That was the first mention of a cerebral aqueduct in the literature. He compared the cerebral convolutions with the convolutions on the intestines, which indicated that he had seen them. He described the heart valves and thought they moved the blood in one direction. He claimed that the heart worked on the principle of a pump. He considered the ends of the arteries to be the beginnings of the veins. He thought there was only blood in the veins and that the arteries contained breath [5, 18]. Erasistratus rejected the humoral theory [1, 18].

Roman medicine

Roman medicine was practically a continuation of Greek medicine. The Romans were often treated by Greek doctors. Ancient Rome relied on hygiene. The brothels were outside the towns, and the prostitutes were strictly controlled. Cemeteries and cremation sites were also outside the cities. They introduced sewerage and public baths. Spa healing literally originated from the Romans. In ancient Rome, being a doctor was a shameful occupation. They had the status of slaves. The only hospitals were *valetudinaria*, and these were places for the recovery of slaves so that their value would not fall. The first significant physician, who received civil rights, was Archagathus [1, 18].

Asclepiades of Bithynia was a famous Roman physician of Greek origin. He rejected the humoral theory and believed that solid parts of the body were more important than fluids in the human body [18]. He believed that the human body was composed of pores and channels. He understood disease as the stagnation of atoms. That was called solidarity medicine [1]. Asclepiades was among the first to advocate a naturalistic approach to healing. He strongly opposed superstition in treatment and believed that the main medicines were heat, cold,

sun, and plants. He made balsams, medicines for the eyes and ears, vaginal suppositories, etc. [8, 18].

Themison of Laodicea worked in the second half of the first century. His teacher was Asclepiades. He also adhered to solidarity medicine. As with Asclepiades, solidarity medicine meant that health was determined by solid parts of the body. It was important to strengthen the tone of the organism in order not to burden the sick person. For this purpose, baths, astringents, carrying in a stretcher, and massage were given. Themison relied on the systematic determination of the causes and consequences of the disease. For this reason, he founded the Methodic School. The Methodic School respected the solidarity medical principle [1, 18].

Aulus Cornelius Celsus was a Roman physician who lived between 25 BC and 50 AD. He wrote a collection of eight books, *De Medicina* (Figure 3) [19].

Fig. 3 – De medicina, Celsus. Available from: https://www.historyofinformation.com/detail.php?id=1765 [19].

It was the first saved manuscript in Latin, printed in 1478. He significantly influenced the spread of ancient medical thought in Renaissance Europe. He based his work on Hippocratic medicine [3]. He was the first to identify four signs of inflammation: tumor, rubor, dolor, and calor [17].

His record of genital infections was interesting, and in the thirties of the new era, he described *profusio seminis*. He claimed that he based his medical information on Greek sources. Here is an extract for which he attributed the data to an anonymous Greek source: "In those who suffer from gonorrhea, we notice reluctant, constant swelling (*rhein*) of the semen (*gonos*), without pleasure. They decay, lose color and strength, get a fever with the loss of appetite, and their pulse weakens." As a remedy, he cited cold compresses with vinegar, rest, and not to lie on the back. He also left records about cataracts and other eye diseases. He described the decapitation of the fetus with a metal hook during abortion [7, 9, 18].

Soranus of Ephesus was the author of the first gynecology. He is considered to have been the greatest obstetrician of the Old Age. He wrote "The Biography of Hippocrates" [9, 18].

Aretaeus of Cappadocia wrote the work "On Acute and Chronic Diseases" in the second century. Aretaeus claimed that apoplexy was the cause of paralysis. Furthermore, if the left side of the head was injured, the right side would be paralyzed and vice versa [9, 18].

Rufus of Ephesus, who lived in the first and the second century of the new era, described the mesenteric, inguinal, and axillary lymph nodes and the thymus [9, 15, 18].

Dioscorides was a Greek pharmacologist of the first century. He wrote the book *Materia medica*, which is considered to be the most important pharmacological book of the Old Century. It consists of five parts. It describes the procurement, preparation, and application of medicines from parts of plants, seeds, oils, metals, aromas, fruits, animals, waxes, milk, cereals, and minerals. He ground the ingredients in a special oven [8, 9, 15, 18].

In the first and second century, unsigned Egyptian papyri of medical content were created based on the teachings of Hippocrates [7–9, 18].

Galen

Galen was born in Pergamon, today's Bergama in Turkey. The exact year of birth and death are unknown but it is believed that he was born in 129 and died around 215 AD [18] (Figure 4).

Fig. 4 – Galen of Pergamum: an eighteenth-century portrait by George Paul Bush (The history of medicine, public domain image files).

He worked as a famous doctor for 50 years and as a surgeon of the gladiator Marcus Aurelius. He studied medicine in Alexandria and Smyrna [18]. He considered the brain to be the center of emotions and intelligence. Since it was forbidden to dissect people in Rome, the fact that he was watching the dissection of corpses in Alexandria for four years was of undeniable importance for his knowledge [3, 18]. Galen was a medical authority for almost 15 centuries, until the 17th century. Of the 600 texts that he wrote on over 1,300 pages, only a third survived. He introduced the experimental method into medicine, as well as research. He was known for his medical ethics. He contributed a lot to pharmacology and philosophy. He was notable as a good diagnostician. He relied heavily on the pulse in his diagnosis. He began to systematize the organs. He realized that blood was carried by the body through the vessels. He was the first to mention anastomoses between arteries and veins, but he thought it was a way of communication between arteries and veins globally, but he could not know where they were. He was called the "divine Galen" [19]. He believed that three pneumas went through the liver, heart, and brain, which were separated from the blood vessels: natural, vital, and animal spirit. Natural went through the heart, animal through the liver, and vital through the brain [6, 9, 20]. He significantly influenced the development of Persian medicine. He later advocated Islamic medicine as well. In Avicenna's "Canon of Medicine", Galen was cited over 300 times [21]. He believed that vital pneuma was created in the heart and carried by arteries to the brain, and then transformed into the animal one in the retiform plexus (a formation he claimed to have been localized near the pineal gland) [20, 21].

Pneuma is actually what we would now call a ghost. Animal pneumas were stored in the ventricles of the brain. If necessary, it was the drive for the transmission of motor and sensory information to the periphery [18, 21]. Galen believed that we received external information from the five senses – sight, hearing, touch, smell, and taste, which processed common sense and formed perception and thought. That process, according to him, took place in the brain chambers. By "internal processing", the thought would further have gone into the processes of memory, evaluation, cognition, and imagination. Practically, he laid the foundations of neuropsychiatry. That was one of the first descriptions of what we now call the perceptual process [22]. Galen was a proponent of humoral medicine. Here is how he described the stroke: "The phlegm is cold and moist. When the blood vessels attract the phlegm into themselves, the body must calm down. If the phlegm prevails, the blood cools and gelatinizes. Then the person dies. The accumulation of phlegm blocks the animal pneuma. This causes dizziness, loss of consciousness, and possibly death. That is why, as we get older, there is more and more phlegm." That is why he considered cleansing from mucus or phlegm a priority, so hygiene and proper nutrition were a priority [22]. He associated everything with stroke because stroke mainly occurred in old age, even today. What he considered old was not the same as what we consider today because life expectancy is getting longer [22].

Galen made a huge contribution to pharmacology. He acquired part of his knowledge from Dioscurides's book *De Materia Medica*. He wrote "On the Mixtures and Powers of Simple Drugs", "On the Composition of Drugs According to Kind", and "On the Composition of Drugs According to Places" [8, 23]. He considered that breast cancer was a consequence of the accumulation of black bile and treated it with opium. He took it from Hippocrates [23]. In the books *De usu partium* and *De Anatomicis administrationibus*, Galen described mesenteric lymph nodes, thymus, and pancreas. He distinguished between the milky and clear contents of the lymph nodes. He thought that Hippocrates's chyle was transmitted from the intestine to the liver through small branches of the portal vein in order to turn it into blood, which went to all tissues. He called this process *anadosis*. He thought, relying on Herophilus, that the pancreas and mesenteric lymph nodes fed the intestines [15, 23]. He discovered that there was blood in the veins [15]. He was the first to notice a double pulsus bisferiens and used it to diagnose [24]. Interestingly, he called polyuria urine diarrhea[14]. Galen considered inflammation to be a struggle with the disease, and as a fifth sign, he added reduced function (*functio laesa*) [17, 24]. He put a lot of effort into experimental work in medicine. He tied *nervus (n.) laryngeus recurrens* to prove the assumption that it had a role in a pig's squealing. Due to such research in the creation of the voice, in his honor, the nerve loop between the posterior branch of *n. laryngeus recurrens* and the internal branch of *n. laryngeus superior* was called the *Ansa Galeni*. In fact, the scale of his work on dissecting animals instead of humans throughout his career led him to warn students of the potential differences in anatomy [24]. That also led to his errors in anatomical descriptions, unprovoked until the New Century [5]. There was also a medical scholar named Pseudo-Galen. He lived around the time when he did, too, and got his name in that way. He wrote "Introduction Sive Medicus" [7, 22].

Medicine today, after Hippocrates, is becoming a complex science and the "art of healing" *(ars medicina)*, based on scientific principles, biomedical research, contemporary medical technologies for modern diagnostics, and actual treatment [23, 24].

Conclusion

The history of medicine is a science that deals with the traces of the past, which exist in the present. The history of Hippocratic medicine is not exact. A lot of works have been lost; we learn about many authors from the mentions of other authors, so we have to take their word for it. Doctors of that time, for the most part, were not allowed to dissect corpses. That is why there are a lot of anatomical errors. Even Galen's mistakes were widely discussed in world literature. However, due to the lack of imaging methods and limitations in dissection, the doctors of Hippocratic medicine left us extraordinary anatomical records. If it had not been for Hippocrates, for example, we would not have propaedeutic today because he was the one who laid the foundations of what is the essence of medical science today: rationalism, nature,

ethics, patient care, observation skills, and clinical experience. For centuries, Hippocrates and Galen's knowledge was considered the absolute truth. We learned a lot that allowed us to refute them. Precisely, constant suspicion and constant improvement of knowledge are the key elements to the progress of medicine. That is why it is important to know the history of medicine, especially the entire Hippocratic and post-Hippocratic period. The skill of observing the physicians of the Hippocratic period points us to things that today, perhaps due to reliance on image and laboratory methods, we professionally miss. Furthermore, there are forgotten pieces of information, which we can use again, or at least research further. It is enough to understand even why mistakes were made sometimes. Today, malicious people are abusing the concept of holistic medicine and healthy eating to disavow the achievements of evidence-based medicine and promote pseudoscience and financial gain. In that way, people are deterred from visiting doctors, and trust in the medical profession and science is being actively destroyed. The duty of medicine as a science in the coming period dictates that we protect Hippocrates's legacy. We must not allow Hippocrates's teaching to be tarnished by someone's lucrative motives, even to the detriment of the patient's existence. The oath is the least that Hippocrates left us and intended in post-Hippocratic medicine.

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2. ***Apstrakt i ključne reči***

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.

3. ***Tekst članka***

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.

Metode. Jasno opisati izbor metoda posmatranja ili eksperimentnih metoda (ispitanici ili eksperimentne životinje, uključujući kontrolne). Identifikovati metode, aparaturu (ime i adresa proizvođača u zagradi) i proceduru, dovoljno detaljno da se drugim autorima omogući reprodukcija rezultata. Navesti podatke iz literature za uhodane metode, uključujući i statističke. Tačno identifikovati sve primenjene lekove i hemikalije, uključujući generičko ime, doze i načine davanja. Za ispitivanja na ljudima i životinjama navesti saglasnost nadležnog etičkog komiteta.

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

U radu literatura se citira kao superskript, a popisuje rednim brojevima pod kojima se citat pojavljuje u tekstu. Navode se svi autori, ali ako broj prelazi šest, navodi se prvih šest i *et al*. Svi podaci o citiranoj literaturi moraju biti tačni. Literatura se u celini citira na engleskom jeziku, a iza naslova se navodi jezik članka u zagradi. Ne prihvata se citiranje apstrakata, sekundarnih publikacija, usmenih saopštenja, neobjavljenih radova, službenih i poverljivih dokumenata. Radovi koji su prihvaćeni za štampu, ali još nisu objavljeni, navode se uz dodatak „u štampi“. Rukopisi koji su predati, ali još nisu prihvaćeni za štampu, u tekstu se citiraju kao „neobjavljeni podaci“ (u zagradi). Podaci sa interneta citiraju se uz navođenje datuma pristupa tim podacima.

Primeri referenci:

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Tabele

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

Ilustracije

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

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

Skraćenice i akronimi

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

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

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