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Sadržaj

ORIGINALNI RADOVI

PREGLEDNI RADOVI

PRIKAZ BOLESNIKA

Contents

ORIGINAL ARTICLES

REVIEW

CASE REPORT

Iz uredništva

Poštovani saradnici,

Od ovog broja, 1. 2021. godine, odlučili smo da engleski jezik bude prioritetni jezik na kome ćemo objavljivati većinu radova u našem časopisu. Želja nam je da izvršimo internacionalizaciju našeg časopisa, jer publikovanje naučnih radova i nauka sama po sebi predstavlja globalnu aktivnost na međunarodnom nivou koji uvijek prevazilazi lokalne okvire.

Svjesni smo da to otežava objavljivanje radova za autore iz sredina kojima engleski jezik nije maternji. Ali to je i korak naprijed da se autori sa neengleskog govornog područja postepeno edukuju za objavljivanje naučnih radova na engleskom jeziku. Objavljivanje naučnih radova na engleskom jeziku predstavlja dio vizije Medicinskog fakulteta u Foči kako bi osnažio svoju naučnu aktivnost i internalizovao je. Sve to uredništvo časopisa Biomedicinskih istraživanja stavlja pred zadatak da ga uvrsti u međunarodne baze časopisa čime bi radovi objavljeni u našem časopisu postali vidljiviji i zanimljiviji međunarodnoj naučnoj zajednici kako za čitanje i citiranje, tako i za objavljivanje.

Od broja 2. 2021. godine prelazimo gotovo u cjelosti na elektronski model prijavljivanja, recenziranja i pripreme radova za njihovo konačno objavljivanje. Iz tog razloga će u narednom periodu biti objavljena nova uputstva za autore, a takođe i za recenzente radova u našem časopisu. Iskustva drugih časopisa su da prelazak na kreiranje časopisa potpomognuto korišćenjem savremene digitalne tehnologije znatno olakšava rad svim akterima koji su uključeni u ovaj proces i povećava kvalitet publikovanja časopisa.

Prof. dr Siniša Ristić
glavni i odgovorni urednik

Dear associates,

During the age of covid19 pandemic the medical professionals have learned more than ever about the importance of scientific networks. Joint endevours have led us from understanding the scale of the challenge to devising the means to overcome it. We can conclude that none of it would be possible without “lingua franca” of our times and digital technology advancement. The same is needed to understand other health issues that we encounter or to solve thoughtprovoking dilemmas of basic science. Therefore, we decided to raise our scientific voice higher by asking all our authors to prioritize writing for our journal in English. We believe that young researchers should be given more chance to publish their academic works, as much as we cherish the contributions from experienced scientists and clinical experts. Our journal aims to be recognizable for the diversity of themes and their practical applicability that readers may benefit from. Our hope is to bring about experiences and views not only from our midst of Southeast Europe, but also from other parts of the world. This is a complex task, both for authors and editors. We do not know what the future holds, but we are sure that it will be exciting.

Adrijan Sarajlija, MBI, MD, PhD
Advisory editor

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Original article

Effect of using HEART score in patients with chest pain at the emergency department of University Clinical Centre of the Republic of Srpska

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Summary

Introduction. Recent data show that 1/5 of patients with chest pain in emergency room (ER) have an acute coronary syndrome that requires admission and treatment. Current guidelines have endorsed the HEART score for admission, observation or discharge in individual patients. We aimed to assess performance of the HEART score at the University Clinical Centre of the Republic of Srpska.

Methods. Between March 1 and March 31, 2019, all patients with chest pain who presented at ER were evaluated. The HEART score for each patient was calculated, and patients were stratified based on the HEART score recommendation, i.e. low-, intermediate- and high-risk. Patients were followed 6 weeks for major adverse cardiac events (MACE).

Results. Out of a total of 144 included patients, 23 had low-risk (0–3) HEART scores, while 73 and 48 patients had intermediate-risk (4–6) and high-risk (7–10) HEART scores, respectively. The discordance among intuitive judgments by clinicians and the HEART score advice became typically obtrusive in patients with excessive (7–10) HEART score rankings: 25 out of 48 (52.1%) patients had been discharged, while the remaining 22 patients had been admitted and 1 person was observed. In population with HEART score rankings 4–6, MACE became recognized in 1/73 (1.4%) while in patients with excessive HEART score rankings (values 7–10), MACE befell in 5/48 (10.4%). Only one patient who was discharged experienced MACE. The ROC analysis of the HEART score revealed a value of 0.78, suggesting a good performance in discriminating between low- and high-risk patients.

Conclusion. Discordance between clinical decision and HEART score recommendation was not associated with severe clinical consequences.

Keywords: heart score, emergency room, chest pain

Introduction

Patients with chest pain are very common in an emergency department (ED) [1]. However, only 1/5 of patients with chest pain have an acute coronary syndrome (ACS) that requires admission and treatment. In the vast majority, the underlying condition is non-cardiac and these patients can be safely discharged from the ER and treated in an outpatient clinic [2]. However,

typical chest pain is present in only half of the patients with ACS [3] which is the reason that 2–6% of patients with ACS are not timely recognized [4,5]. In addition, clinicians tend to postpone their decisions and to confess those patients for medical observation, and to deal with them as patients with ACS. Consequently, over-diagnosing and needless remedy are common.

Recent trials have shown that risk scoring systems are superior to clinical assessment in identifying high-risk population [6–9]. Therefore, current guidelines have endorsed the HEART score which was designed to improve risk stratification of all-cause chest pain patients at the ED [10]. It consists of five parameters in the initial assessment of patients with chest pain: medical history, electrocardiogram, age, risk factors, and high sensitive troponin value [11]. Each of the parameters can be scored with 0, 1 or 2 points (Figure 1). Importantly, the HEART score is based on clinical experience with simple bed-side applicability, and provides physicians with a recommendation for admission, observation, or discharge in individual patients. The diagnostic utility of the HEART score has been confirmed in many studies [12–15].

In the present study, we aimed to assess performance of the HEART score in tertiary institution with real-world everyday population.

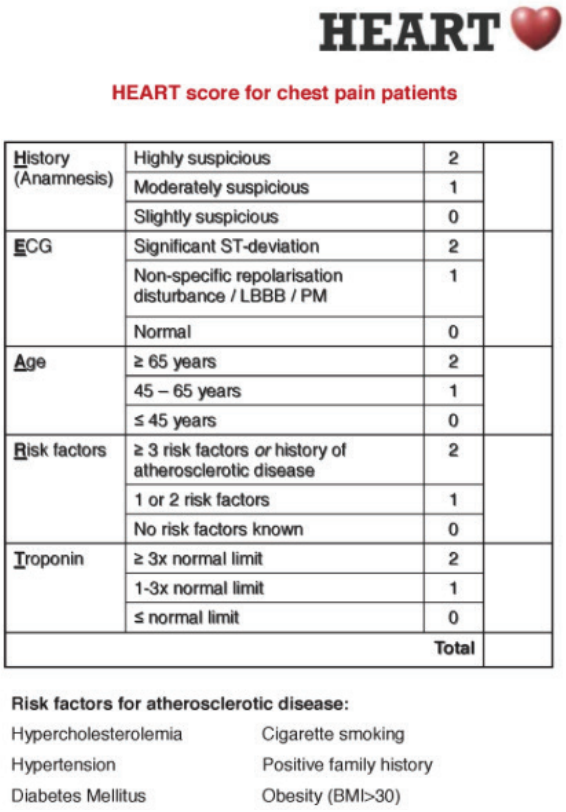

Figure 1. Original HEART score, with permission of the authors. BMI, body mass index; LBBB, left bundle branch block; PM, pacemaker

Methods

Between March 1 and March 31, 2019, all patients with chest pain who presented at ER of University Clinical Centre of the Republic of Srpska were evaluated. Presenters with symptoms of dyspnea or palpitations were not included. Patients with ST-segment elevation myocardial infarction at admission were excluded. The HEART score for each patient was calculated, and patients were stratified based on the HEART score recommendation. The study became authorized via way of means of nearby ethics committee. As this became an observational non-intervention study, knowledgeable consent strategies had been waived.

Data acquisition consisted of separate entries for classical factors of affected person history, cardiovascular threat factors, medication, physical exam and beyond clinical history. Laboratory values which include high-sensitive troponin T have been accrued for the time being of admission. Only the first troponin T value became used for the HEART score calculation. Follow-up have been retrieved from electronic and written records, which included discharge letters, revascularization reviews and some other applicable documentation. The diagnosis of acute myocardial infarction was made according to the applicable guidelines. Therefore, non ST-elevation myocardial

infarction was defined as a syndrome consisting of a rise of high-sensitive troponin values, typical patient history and persistent or transient ST-segment depression or T-wave inversion, flat T-waves, pseudo-normalization of T-waves, or no changes at presentation. As a percutaneous coronary intervention was considered any therapeutic catheter intervention in the epicardial arteries, while coronary artery bypass graft surgery was defined as any surgery in which epicardial arteries were operated on.

During the six-week follow-up of the initial presentation, following major adverse cardiac events (MACE) were collected: acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft surgery, coronary angiography revealing procedurally correctable stenosis managed conservatively, and all-cause death.

Statistical analysis was performed with SPSS (Version 25; MAC). Descriptive statistics are given as mean +/− standard deviation or median +/- interquartile range, based on the distribution of continuous variables. Categorical variables were presented as a number with a percentage. Differences between means were assessed through the Student's t-test when normally distributed or Mann Whitney U-test when not normally distributed. The probability of reaching an endpoint was calculated as the percentage of cases with an endpoint within a given category. Kaplan-Meier analysis was used to assess differences in outcome of low-, intermediate- and high-risk patients. The ability of the HEART score in discriminating between low- and high-risk was described by the Receiving Operating Curves (ROC) analysis. It estimates the probability that, of 2 randomly chosen patients, the patient with more favorable prognostic score will surpass the patient with less favorable prognostic score and ranges from 0.5 (no discrimination) to the theoretical maximum of 1.

Results

Out of 932 patients presented to the ER, 788 did not meet inclusion criteria. Out of a total of 144 included patients, 23 had low-risk (0–3) HEART scores, while 73 and 48 patients had intermediate-risk (4–6) and high-risk (7–10) HEART scores, respectively. There were higher incidence of arterial hypertension, type 2 diabetes mellitus and coronary artery disease within the high-risk group (Table 1). The discordance between „intuitive" judgments and HEART score advise was mostly prominent in patients with highest (7–10) HEART scores: 25 out of 48 (52.1%) patients were discharged, while the remaining 22 were immediately admitted and 1 patient was observed (Table 2).

During the follow-up (Figure 2), six (4.2%) patients have experienced MACE within six weeks: acute myocardial infarction was diagnosed in 4 patients of which 2 patients presented with STEMI, 2 patients underwent PCI, 0 patients had a CABG and 2 patients had coronary angiography revealing angiographically significant epicardial stenosis. In patients with intermediate HEART scores ranking of 4–6, MACE was found in 1/73 (1.4%). On the other side, in patients with highest HEART scores (values 7–10), MACE occurred in 5/48 (10.4%). One patient from the high-risk group, who was discharged, experienced MACE within 6 weeks. The ROC analysis of the HEART score revealed a value of 0.78 (95% confidence interval 0.74-0.82; p=0.01), suggesting a good performance of the HEART score in discriminating between low- and high-risk patients.

Discussion

Chest pain is one of the most common complaints among patients presented in the ER. Failure to identify a patient's chest pain cause put the risk of sustaining missed acute coronary syndrome which may lead to significantly

Table 1. Baseline characteristics

	HEART SCORE				
VARIABLE	**LOW (n=23)**	**MODERATE (n=73)**	**HIGH (n=48)**	**p-value**	**TOTAL (n=144)**
Arterial hypertension	6 (26.1%)	54 (74.0%)	42 (87.5%)	<0.001	102 (70.8%)
Type 2 diabetes mellitus	0 (0.0%)	11 (15.1%)	14 (29.2%)	0.008	25 (17.4%)
Coronary artery disease	4 (17.4%)	19 (26.0%)	34 (70.8%)	<0.001	57 (39.6%)
Dyslipidemia	0 (0.0%)	2 (2.7%)	2 (4.2%)	0.606	4 (2.8%)
Obesity	1 (4.3%)	0 (0.0%)	1 (2.1%)	0.263	2 (1.4%)
Smoking	3 (13.0%)	3 (4.1%)	2 (4.2%)	0.232	8 (5.6%)
High-sensitive Troponin T	8.7 ± 14.8	38.7 ± 71.9	327.6 ± 1516.2	0.164	130.2 ± 881.9
Serum Creatinine	58.5 ± 31.2	101.0 ± 85.3	86.0 ± 94.2	0.404	91.5 ± 84.4
Hemoglobine	145.2 ± 15.6	102.0 ± 55.2	113.7 ± 49.8	0.034	112.5 ± 51.1
Male	15 (65.2%)	34 (46.6%)	29 (60.4%)	0.167	78 (54.2%)
Age	68.8 ± 12.9	71.1 ± 12.0	66.2 ± 12.9	0.533	69.3 ± 12.3

Data given as mean ± standard deviation or n (%)

higher clinical consequences [16]. The appropriately use of the HEART score gives the clinician reliable prediction of outcome, and importantly, very soon after the patients arrival. Our study showed that no MACE occurred among those low-risk HEART score. When taken into account, low-risk HEART score criteria may fail in 2% of patients only who develop MACE [17]. One study reported that acceptable miss rate of MACE would be around 1% [17].

The results of our study are similar to those previously reported by Van Den Berg and Body showing easy clinical applicability of the HEART score in everyday routine [18]. Nowadays, many risk scoring systems were developed with an aim to aid clinicians in their decision-making [19]. The most common of these are the TIMI- and GRACE-scores, who were developed to stratify patients in coronary units. Although not designed for the purpose excluding the acute coronary syndrome in unselected population, these scores are applied at ER for the whole range of chest pain patients. On the contrary, the HEART score was specifically designed for the population with chest discomfort at ER. Probably the best value of the HEART score is easy, bed-side applicability, especially having in

Table 2. Adherence to the Heart score

Heart score	Discharge	Observe	Admission	Total
Low	19	1	3	23
Intermediate	54	5	14	73
High	25	1	22	48
Total	98	7	39	144

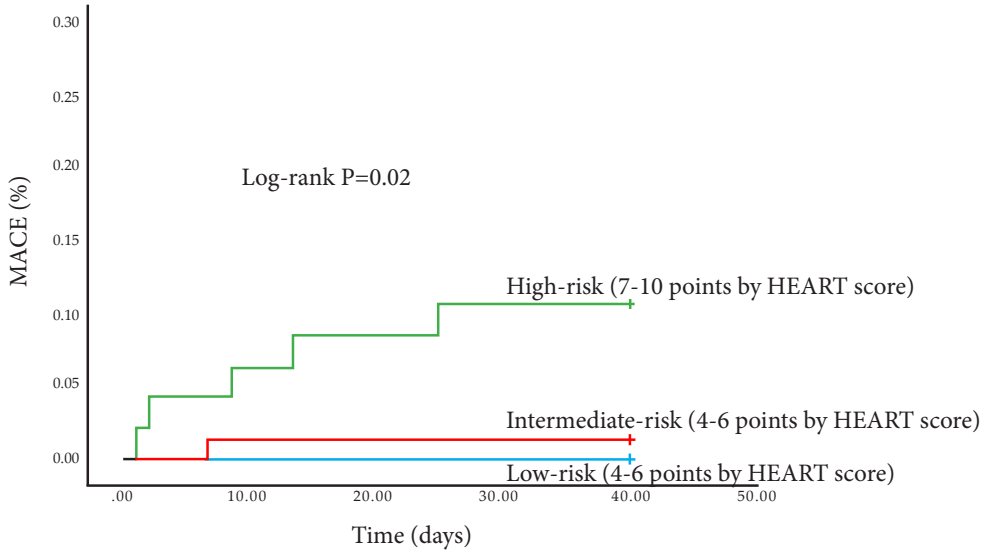

Figure 2. Kaplan-Meier analysis. The cumulative incidence rates of major adverse cardiac events between low-, intermediate- and high-risk group stratified by HEART score at 6-week follow-up

mind the fact that it uses admission data only, typically complete within 1h of initial assessment. There are some well-validated prediction models of death, for example the GRACE score. However, practical disadvantage of the GRACE score lies in fact that it can be calculated by computer only. The TIMI score, developed more than 25 years ago, was able to identify high-risk patients who may benefit from aggressive anticlotting agents, and was relatively easy to calculate. However, it was quite rough since it allowed binary choices only, and therefore ignored the fact that many variables have a 'grey area'.

Recently, a comparison of GRACE, TIMI and HEART scores in terms of predictive capabilities was done, and showed that the HEART score (c-index 0.83) is the best score to exclude acute coronary syndrome at ER, while GRACE (c-index 0.70) and TIMI (c-index 0.75) scores should be reserved for hospitalized patients [20]. Our analysis revealed the c-index of the HEART score of 0.78 meaning that the HEART score has retained its performance in our population.

Each element of the HEART score is important in forming predicition. The score follows clinical decision-making and can be used as a helpful tool to correctly stratify patients into low-, intermediate- or high-risk. However, probably the main issue represents usage of different cut-off values and low specificity of troponin measurements. In practice, this may result that some patients with slightly elevated troponins may be overclassified into the high-risk group. This may be the reason for high percentage (52.1%) of discrepancy between clinical decisions and HEART score recommendation. However, this had no severe clinical consequences.

In conclusion, we confirmed that the HEART score is a quick, easy and reliable predictor of MACE, without computer-required calculating. Low HEART scores (0–3), exclude short-term MACE with very high certainty, and these patients can be safely discharged. In contrast, patients with high HEART scores (7–10) may indicate more aggressive policies.

The present analysis had several limitations as well as advantages: (1) the low number of patients – however, our patients came

from everyday routine; (2) data collection was suboptimal – we were not able to sample more data on patients comorbidities; (3) since this was not a randomized study, it could not deal with possible confounders which may guide clinicians in the decision-making process.

Conclusion

The HEART score is a quick and reliable predictor of MACE. Discordance between clinical decision and HEART score recommendation was not associated with severe clinical consequences. However, there is no risk-scoring system that can replace careful multidisciplinary clinical decision-making. The HEART score seems to be a helpful tool in this process.

Funding source. The authors received no specific funding for this work.

Ethical approval. The Ethics Committee of the University Clinical Centre of the Republic of Srpska approved the study and informed consent was obtained from all the individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Efekat upotrebe HEART skora kod pacijenata sa bolom u grudima u Urgentnom centru Univerzitetskog kliničkog centra Republike Srpske

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Uvod. Najnoviji podaci pokazuju da 1/5 pacijenata s bolom u grudima koji se jave u Urgentni centar (UC) ima akutni koronarni sindrom koji zahtijeva prijem i liječenje. Trenutne smjernice prihvatile su HEART skor za prijem, posmatranje ili otpust kod pojedinačnih pacijenata. Cilj nam je bio da procijenimo značaj HEART skora na pacijente sa bolom u grudima u Univerzitetskom kliničkom centru Republike Srpske.

Metode. Analiza je obuhvatila period od 1. do 31. marta 2019. godine i uključila je sve pacijente sa bolom u grudima koji su se javili u UC. Za svakog pacijenta izračunat je HEART skor, a pacijenti su podijeljeni na osnovu preporuke HEART skora, tj. pacijenti sa niskim, srednjim i visokim rizikom. Pacijenti su praćeni šest nedjelja, zbog većih neželjenih kardiovaskularnih događaja (VNKD).

Rezultati. Od ukupno 144 uključena bolesnika, 23 su svrstana u grupu niskog rizika (HEART skor 0–3), dok 73 i 48 pacijenata je kategorisano kao srednje rizični (HEART skor 4–6), odnosno visoko rizični (HEART skor 7–10). Najveći nesklad između intuitivne procjene kliničara i preporuke HEART skora je primijećen u grupi visokog: čak 25 od 48 (52,1%) pacijenata je otpušteno, dok su preostala 22 pacijenta primljena, tj. jedan pacijent je posmatran. U populaciji srednjeg rizika, VNKD se desio kod 1/73 bolesnika (1,4%), dok je kod pacijenata s visokim rizikom konstatovano 5/48 VNKD (10,4%). Samo je jedan otpušteni pacijent doživio VNDK. ROC analiza HEART skora pokazala je vrijednost od 0,78, što sugeriše dobru prediktivnu sposobnost u razlikovanju pacijenata s niskim i visokim rizikom.

Zaključak. Nesklad između kliničke odluke i preporuke HEART skora nije povezan s ozbiljnim kliničkim posljedicama.

Ključne riječi: HEART skor, urgentni centar, bol u grudima

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Original article

Analysis of anatomy and configuration of the canal system of the maxillary second premolar in the population of Bosnia and Herzegovina

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Summary

Introduction. Successful endodontic treatment depends upon the clinician's knowledge and ability to recognize and diagnose the presence of anatomical and morphological variations of the root and canal system. The aim of this study was to establish the number of roots and root canal configurations of the maxillary second premolar in the population of Bosnia and Herzegovina.

Methods. The study sample was comprised of 150 maxillary second premolar teeth extracted for orthodontic or prosthetic reasons. Endodontic drills were used for trepanation of cavum dentis, and the number and patency of each root canal were determined by K- expander # 15. Then, the samples were decalcified, made transparent and colored, to enable 3D viewing of the canal system. Decalcified teeth were observed from two projections (clinical and approximal) and analyzed in detail with a magnifying glass under 3 × and 5 × magnification in order to determine the number of roots, number of canals, root canal configuration using Vertucci's classification and number of anastomoses between canals. Statistical significance was obtained using Chi-square test.

Results. The results obtained by decalcification of the teeth showed that, by radiographic analysis from the clinical projection, all the teeth had a single root. While, by the analysis from the approximal projection, 94.0% had one, 6.0% two roots. From the approximal projection, 70.7% with a single root canal and 29.3% with two root canals are visualized. The most common type of root canal configuration in the maxillary second premolars was Type I in both clinical (87.9%) and approximal projection (40.7%).

Conclusion. These results emphasize the importance of knowing the variations in root canal morphology, because excluding the possibility of morphological variations can lead to failure of endodontic therapy.

Key words: maxillary second premolar, root canal, decalcification

Introduction

The knowledge of the root canal morphology as well as of possible anatomical and morphological variations in the number of roots and the configuration of canal system, are the basic prerequisites for the success of an endodontic treatment [1–3].

Untreated root canals in irreversible pulp damage lead to persistence of microorganisms and necrotic tissue within the canal itself, which can cause the consequent development of a pathological process in periodontal tissue. Anatomic variations of the root canal system are the most frequent causes of the therapy negligence [2]. Previous studies have shown that premolars can differ significantly in the number of roots and root canals, as well as that maxillary premolars have very variable configuration of the internal canals, which can vary depending on the race and geographic origin. The maxillary second premolar is a tooth of complex morphological structure with frequent variations in the canal system and presents a challenge for all clinicians during endodontic treatment [2,4–10]. By the data available, the frequency of one-root maxillary second premolar varies from 69.6% to 90.3%, the frequency of two-root maxillary second premolar varies from 9.7% to 29.7%, while the frequency of three-root maxillary second premolar varies from 0 to 1.6% [7–10].

From the coronary part to the apex of the root, different variations of the canal can be observed, i.e. the canal may bifurcate, divide and/or merge at different levels of the root. In the maxilla, premolars show the greatest number of canal variations, especially the second premolar [4,7]. Studies describe different classifications of the root canals of permanent teeth, including the classification by Weine [11], Vertucci [1] and Gulabivala [12]. The Vertucci's classification is considered as the most frequently used, encompassing pulp space which can have eight different configurations: Type I (1), Type II (2-1), Type III (1-2-1), Type IV (2), Type V (1-2), Type VI (2-1-2), Type VII (1-2-1-2) and Type VIII (3). Maxillary second premolars are considered to be the most demanding teeth for endodontic treatment, due to the number of roots and canals, the course and longitudinal concavity of the root, different configuration of the pulp cavity and difficulties in visualizing of the apical foramen by radiographs [1,13,14]. Various in vivo and in vitro methods were used to examine the anatomy of the root canal. In vivo techniques include clinical assessment during treatment, retrospective analysis of a patient's dental history, conventional radiographic examinations as well as advanced radiographic techniques such as cone-beam computed tomography using a cone-shaped X-ray beam (CBCT) [15–17]. On the other hand, in vitro methods include cleaning and staining of tooth root canals [1,18], microscopic analysis of the root cross-section [11], methods based on filling and decalcification of canals, the analysis of conventional radiographic images and the use of three-dimensional modalities such as micro-computed tomography (μ-CT) [19,20]. Although different studies utilized various techniques for the root morphology assessment, it is registered that the most precise information can be gathered ex vivo, by demineralization and staining [1]. This technique makes the radiography with a canal instrument unnecessary, retaining the original shape and relationship of the canals and providing a three-dimensional view of the root canals [1,5,8]. The insertion of contrast dye into the canal system, followed by demineralization and the process of making the teeth transparent by immersing them into methyl salicylate, xylene or styrene, remains one of the most frequently used methods of testing the configuration of the canal system [1,8].

The aim of this study was to establish the number of roots and root canal configurations of the maxillary second premolar in the population of Bosnia and Herzegovina.

Methods

One hundred and fifty human maxillary second premolar teeth, extracted for orthodontic or prosthetic reasons, were collected as a sample for this study. Teeth were collected from patients aged from 20 to 40 who came to the Specialist Dentistry Center of the Faculty

of Medicine Foca, University of East Sarajevo, Bosnia and Herzegovina. The criteria for the teeth inclusion into research were: intact teeth, partially preserved dental crowns, teeth with fillings, teeth that have not been previously endodontically treated, teeth with no signs of pathological resorption and physical damage of the root, teeth with fully formed and developed roots.

After extraction, the teeth were rinsed under running water and mechanically cleaned of soft tissue residues and concretions (Figure 1a, 1b). All samples were stored in plastic containers with physiological saline in the freeze

The teeth were then rinsed under running water for a period of two hours. Endodontic drills with a long handle in sequences from larger to smaller (Micro Owner, Switzerland) were used for trepanation of cavum dentis.

The root orifices were found by an endodontic probe and the patency of each root canal was determined by K-expander # 15 (Kerr, Sybron Dental, USA) until the appearance of the tip of the instrument on the anatomical foramen. The number of canals was determined and recorded for each root at this point of the research.

Further laboratory procedure included the preparation of the samples for the process

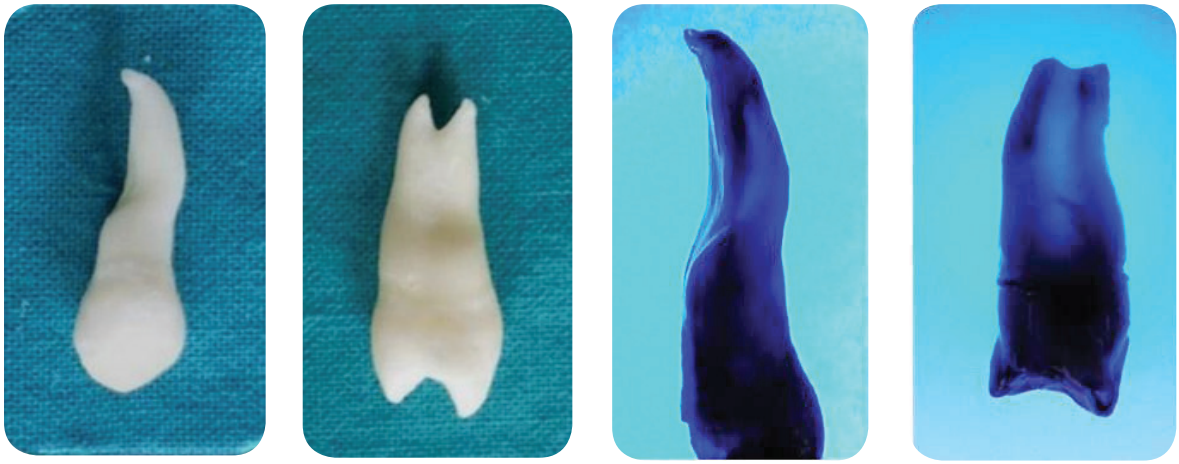

Figure 1. Extracted maxillary second premolar (a,b), decalcified tooth from the clinical projection (c), decalcified tooth from the approximal projection (d)

until the beginning of laboratory research, at a temperature below -7°C. Diamond and tungsten carbide round drills of a high-circulation water-cooled machine were used to remove caries and existing fillings (Meisinger, Germany).

The remnants of the coronal pulp were removed with an excavator and an ultrasound machine. Every specimen was immersed in a solution of 5.25% sodium hypochlorite for 48 hours, in order to decompose and easily remove organic detritus from root canals.

of decalcification in order to define anatomical features of the canal system. The samples were decalcified, made transparent and colored, to enable 3D viewing of the canal system (Figure 1c, 1d). The teeth were decalcified in 5% nitric acid for 72 hours. The acid solution was changed daily, and the end point of decalcification was determined by periodic radiographs. Thereafter, the teeth were washed under running water in order to remove the traces of nitric acid, dried and dehydrated using increasing concentrations of ethanol (70%

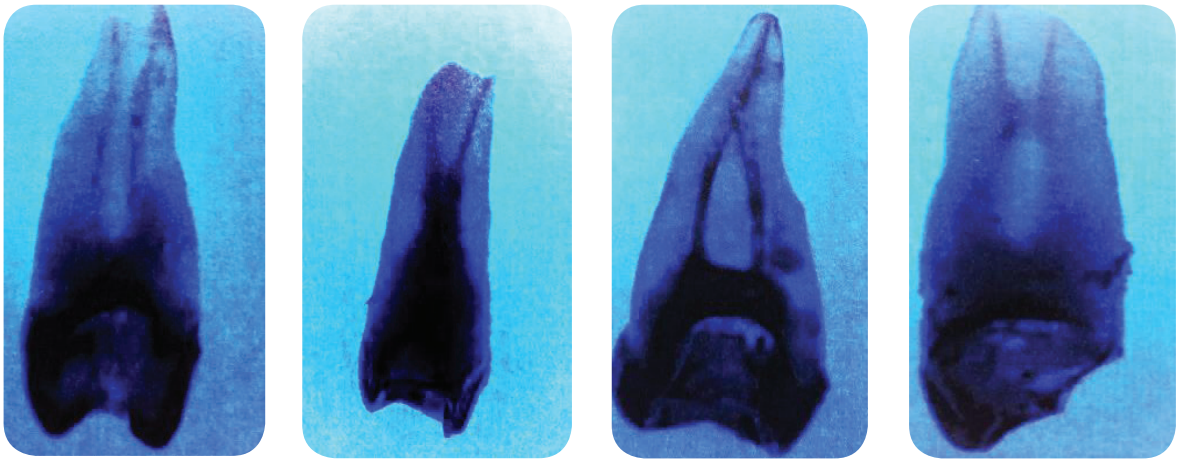

Figure 2. Different patterns of root canal systems by Vertucci classification (a, b, c), localization of anastomosis between canals in the medial third (d)

for 12 hours, then 90% for 8 hours and 100% for 1 hour). Decalcified teeth were placed into glass containers filled with methyl salicylate for 6 hours in order to reach transparency, while hematoxylin-eosin stain was injected into the access cavity.

Decalcified teeth were observed from two projections (clinical and approximal) and analyzed in detail with a magnifying glass under 3 × and 5 × magnification in order to determine the number of roots, canals, number of apical foramina and the location of anastomoses between canals (Figure 2a, 2b, 2c). The root configurations were categorized based on Vertucci's classification from 1984, as follows:

Type 1: a single canal is present from the pulp chamber to the root apex.

Type 2: two separate canals leave the pulp chamber and join at certain level of the apex to form one canal, which ends in one foramen.

Type 3: one canal leaves the pulp chamber, divides into two within the root, and then merges to exit through one canal.

Type 4: two separate and distinct canals are present from the pulp chamber to the apex.

Type 5: single canal leaves the pulp chamber but divides into two separate canals with two separate apical foramina.

Type 6: two separate canals leave the pulp chamber but join at the midpoint and divide again into two separate canals with two separate apical foramina before exiting from apex.

Type 7: one canal leaves the pulp chamber, divides and rejoins within the canal, and at the apex third finally divides again into two distinct canals with two separate apical foramina.

Type 8: three canals leave the pulp chamber and run independently towards the apex as three divides apex foramina.

In order to determine the location of anastomoses, decalcified teeth were observed from two projections (clinical and approximal) and analyzed in detail with a magnifying glass under 3 × and 5 × magnification in the cervical, middle, and apical thirds of the root canal (Figure 2d).

When statistical analysis of data is concerned, a statistical software program SPSS 15,0 was used (IBM Corp., Armonk, NY, USA). The relationship among the number of roots and canals and the Vertucci's classification was obtained using Chi-square test.

The significance level was defined as 5% (typically $p<0.05$).

Results

Within decalcified teeth, the analysis of the results obtained from the clinical projection of 150 extracted second maxillary premolars revealed that 100% of teeth possess a single root. However, observing from approximal projection 94% of teeth had a single root, and 6% had two roots. Statistical analysis showed a significant difference between the values obtained in the clinical and approximal projection ($p<0.05$) (Table 1). Also, Table 1 showed the distribution of the number of root canals of the second maxillary premolars. The radiograph analysis of decalcified teeth observed from clinical projection revealed that 94.7% had a single root canal, while 5.3% of teeth had two canals. On the other hand, the observation from the approximal projection showed that 70.7% of teeth had a single canal, while 29.3% had two canals. A highly statistically significant difference was observed in the number of root canals between clinical and approximal projection ($p<0.001$).

Table 2 presents the distribution of configuration types of root canals according to Vertucci's classification of the maxillary second premolars. While analyzing decalcified teeth, Type I was the most prevalent in both clinical (87.9%), and approximal projection (40.7%). The canal configuration Type II was detected in 8% of roots observed from clinical, or 30% observed from approximal projection. Type VI, VII and VIII by Vertucci were not detected.

The analysis of the present anastomoses in decalcified teeth between root canals in clinical projection revealed that 110 teeth did not possess anastomoses, while 24 (16.0%) teeth had anastomoses in the cervical third of the root. Anastomoses were detected in a certain number in the middle (8.7%) and apical third

Table 1. Distribution of morphologic configurations in the root canal system of maxillary second premolars

		Clinical projection N (%)	Approximal projection N (%)
Frequency of roots	one	150 (100)	141 (94.0)
	two	0 (0.0)	9 (6.0)
	total	150	
	p	p0.001	
Frequency of root canals	one	142(94.7)	106(70.7)
	two	8(5.3)	44(29.3)
	total	150	150
	p	p<0.001	

N - sample size

Table 2. Configuration of canal systems of the second maxillary premolars by Vertucci's classification

	Clinical projection N (%)	Approximal projection N (%)
Type I	132 (87.9)	61 (40.7)
Type II	12 (8.0)	45 (30.0)
Type III	0	4 (2.7)
Type IV	3 (2.0)	24 (16.0)
Type V	3 (2.0)	16 (10.7)
Type VI	0	0
Type VII	0	0
Type VIII	0	0
Total	150	150
p		p<0.001

N - sample size

(2.0%). Observed from approximal projection, the greatest number of detected anastomoses was located in the middle third of the tooth (42.0%), while smaller percentage of these was detected in cervical (8.0%) and apical part of the tooth (5.3%) (Table 3).

Table 3. Localization of anostomoses among the root canals of the maxillary second premolars

	Clinical projection N (%)	Approximal projection N (%)
None	110 (73.3)	67 (44.7)
Cervical third	24 (16.0)	12 (8.0)
Middle third	13 (8.7)	63 (42.0)
Apical third	3 (2.0)	8 (5.3)
Total	150	150
p		p<0.001

N - sample size

Discussion

The knowledge of the anatomy of the pulp chamber is of a crucial importance and leads to the very success of any endodontic treatment. The therapy of the root canals of the teeth possessing two or three roots is less successful comparing to one-root teeth [1]. Studies on the morphology of roots in different populations elicit the information related to the most frequent configurations as well as the possible variations in root canals [2–4]. Previous studies used different methods in order to estimate the internal morphology of teeth under laboratory and clinical conditions [4,9, 11,13,15,18–24]. Laboratory methods are extremely precise, while the technique of decalcification and staining is considered as the gold standard when estimating the morphology of the root canal [1,5,8]. This technique enables three-dimensional analysis of root canals, maintaining the original shape of a canal. It consists of nitric acid decalcification, alcohol dehydration and methyl salicylate cleaning, which makes teeth transparent [8]. This in vitro study used double projected decalcification and staining in order to acquire data related to the number of roots and the configuration of canal system of the maxillary second premolars in the population of Bosnia and Herzegovina. Upon the radiograph analysis from clinical projection of the extracted human maxillary second premolars, the obtained results showed that all the teeth in this study had 1 root. On the other hand, approximal projection revealed 94% of teeth with a single root, and 6% teeth possessing two roots. Previous studies in different populations reported similar results and the incidence of a single root teeth varied from 69.6% to 90.3% [1,5,10,14,16]. The findings of Pecora et al. [24] acquired from approximal projection and at a larger sample, which found the presence of a single root in 90% of the maxillary second premolars, also support this study. Moreover, while using approximal projection, this study revealed that 6% of the maxillary second premolars possess two roots, which is lot less comparing to several previous studies [7,10], and, in accordance with the results of the following studies: Vertucci [13], Pecora et al. [24], Singla MG and Pada BK [30]. The frequence of three-root canals was extremely low in previous studies (0.6%–1.6%). However, this study, using both projections, has not detected the presence of three roots in the maxillary second premolars [7,10,27]. The maxillary second premolars also display numerous variations in the number of roots in different geographical regions [10,13]. This study used the radiographic analysis from the clinical projection in order to prove that the upper maxillary premolars in 94.7% of cases possess a single root, in 5.3% two roots, while three-root canals were not detected. The approximal projection analysis interestingly revealed that the frequency of a single root canal was 70.7%, which shows higher percentage than previous studies [28,29], but lower than studies by Vertucci [13], Pecora et al [24] and

Caliskan et al. [30]. Nevertheless, comparing the prevalence displayed in this study (23.9%) to the results of the study by Yang et al [31], the presence of teeth with two canals was considerably higher (54.3%) While the frequency of the maxillary second premolars possessing three roots in previous studies was 0.2% to 3% [10,12,21,22,24,26], the presence of three canals was not detected in this study. These discrepancies may be explained by the usage of different research methodologies which reveal different results depicting wide variations in numbers of roots and root canals of the second maxillary premolars. In addition, there are differences in morphology of teeth among patients from different geographic and ethnic groups (different continents) and researchers need to be cautious when relying on the results acquired in researches conducted in different populations. The statistically significant difference in the number of roots and root canals from clinical and approximal projection can be explained by the fact that the superposition of roots or canals happened at clinical projection.

The most common configuration of root canals in the maxillary second premolars observed in this study was Type I in clinical (87.9%), as well as in approximal projection (40.7%). The greatest frequency of the canal configuration Type I was observed in Turkish population [5], as well as in Spanish [10], but Asian population showed difference [8], which complies with the results revealed by this study. The next most common type of canal system configuration in this study was Type II shown from approximal projection (30%). An interesting observation is that this percentage of Type II from approximal projection is similar to the findings of Udayakumar and Mylswamy who found Type II configuration of root canal system in the Indian population in 33.6% of cases [32]. The frequency of Type IV of root canal system configuration in this study was low from both clinical projection (2%), as well as from the approximal one (16%). Using CBCT analysis, the study by Abella et al. [10] also observed a higher percentage of Type IV Vertucci's configuration (19.8%), while Vertucci et al. [13] have reported the highest incidence of the Type IV (37.5%). Differences in results between mentioned studies and this study may be due to different methods used for root canal visualization, racial and geographic factors, as well as the characteristics of the samples. Namely, in this study, Type III configuration of the canal system was the least frequent, while Vertucci types VI, VII and VIII were not detected in any sample. These findings differ from the results obtained in previous studies [9,10,13].

Variations in the anatomy of root canals, i.e. the presence of anastomosis, significantly influence the outcome of the endodontic treatment. This study, using clinical projection, revealed the highest incidence of anastomoses in the cervical third of the root (16%), which is less than in previous studies [7,13,28], and in compliance with the findings of Sert and Bayirili [33]. The smallest number of anastomoses was detected in the apical third (2.0%). By the analysis of radiograph from the approximal projection the following was reported, smaller number of anastomoses in the cervical third (8.7%), while the apical third had 6.7%. The most frequent anastomoses were detected in the middle third (42.7%). Anastomoses have not been detected in 42% of cases. Comparing to our results, Singla and Pada [34] revealed that the most frequent anastomoses were detected in the cervical third (60%), then in the middle third (33.3%), and the least frequent anastomoses were detected in the apical third (6.7%). Differences in findings may be due to individual variations in the way of interpreting of the localization of anastomoses. The knowledge of the localization of anastomoses is of the highest importance and represents the great challenge during the endodontic treatment due to the fact that it can function as the reservoir of bacterial infection, so the inadequate root canal treatment and obturation, i.e. its negligence may lead to the failure in the endodontic treatment [33,34].

Conclusion

Based on the obtained results, it can be concluded that the approximal (mesiodistal) projection by radiography of the decalcified teeth is more reliable than the clinical (vestibuloral). This study revealed the highest incidence of the maxillary second premolars with a single root and a single canal – Vertucci's configuration canal Type I. Certain number of cases revealed two canals with different configurations of the canal system. These findings emphasize the importance of knowing the variations in root canal morphology, because excluding the possibility of morphological variations can lead to failure of endodontic treatment. Further research is recommended on a larger sample, with the application of modern computerized techniques for detecting different configurations of the root canal.

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Ethical approval. The Ethics Committee of the Faculty of Medicine Foca approved the study and informed consent was obtained from all individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Analiza anatomije i konfiguracije kanalnog sistema drugog maksilarnog premolara u populaciji Bosne i Hercegovine

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Kratak sadržaj

Uvod. Uspjeh endodontskog tretmana zavisi od znanja i sposobnosti kliničara da prepozna i dijagnostikuje prisustvo anatomskih i morfoloških varijacija korijenskog i kanalnog sistema. Cilj ovog istraživanja je bio da se prikaže broj korjenova i konfiguracija korijenskih kanala drugog maksilarnog premolara u populaciji Bosne i Hercegovine.

Metode. Uzorak se sastojao od 150 maksilarnih drugih premolara ekstrahovanih iz ortodontskih ili protetskih razloga. Za trepanaciju kavuma dentis korišćena su endodontska svrdla, a prohodnost svakog korijenskog kanala i njihov broj određeni su primjenom K-proširivača # 15. Zatim, uzorci su bili dekalcifikovani, napravljeni transparentnim i bojeni da bi se omogućio 3D prikaz kanalnog sistema. Dekalcifikovani zubi su posmatrani iz dvije projekcije (kliničke i aproksimalne) i detaljno analizirani sa lupom pod uvećanjem 3× i 5× u cilju utvrđivanja broja korjenova, broja kanala, konfiguracije korijenskih kanala primjenom Vertuči klasifikacije i broja anostomoza između kanala. Za statističku obradu podataka korišćen je Hi-kvadrat test.

Rezultati. Rezultati dobijeni dekalcifikacijom zuba drugih maksilarnih premolara su pokazali da su, analizom radiograma iz kliničke projekcije, svi zubi imali jedan korijen. Dok, analizom iz aproksimalne projekcije 94,0% imalo je jedan, a 6,0% dva korijena. Iz aproksimalne projekcije vizuelizuje se 70,7% sa jednim kanalom, a 29,3% sa dva kanala. Najčešći tip konfiguracije kanala korijena kod drugih maksilarnih premolara je bio tip I kako u kliničkoj (87,9%), tako i u aproksimalnoj projekciji (40,7%).

Zaključak. Ovi rezultati naglašavaju značaj poznavanja varijacija u morfologiji korijenskih kanala, jer isključivanje mogućnosti morfoloških varijacija može dovesti do neuspjeha endodontske terapije.

Ključne riječi: drugi maksilarni premolar, kanal zuba, dekalcifikacija

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Original article

The incidence of anxiety in patients with chronic subjective tinnitus

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Summary

Introduction. Tinnitus is a perception of a sound in the ears in the absence of acoustic stimulation whose pathophysiological mechanisms have not been evaluated yet. Approximately, 1–2% of people report distress which can negatively affect their daily performance. Our study aimed to assess the incidence of anxiety in patients with tinnitus.

Methods. The study was designed as a cross-sectional study. The participants were divided into two groups: a group of 73 patients with tinnitus (with two subgroups in relation to the duration of tinnitus - less than one year and more than one year) and a control group of 43 patients without tinnitus. We examined the presence of anxiety in all patients using the Burns Anxiety Inventory (BAI). The quality of life of all patients was estimated by Tinnitus Handicap Inventory (THI).

Results. In the group of patients with tinnitus, 56.2% of them had mild and 24.7% moderate hearing loss, while 27.7% of respondents from control group had mild and 8.5% moderate levels of hearing impairment. THI results showed that patients with tinnitus less than 1 year had a significantly (p= 0.002) higher level of disorders in daily life, compared with the group who had tinnitus for more than 1 year. The 30.8% of respondents had minimal anxiety, 26.7% borderline anxiety, 17.5% mild anxiety, the same percentage of respondents moderate, 5% severe, while 2.5% had extreme anxiety based on BAI.

Conclusion. Anxiety can be considered as potentially significant modulators of changes in brain structures observed in people with tinnitus.

Key word: tinnitus, anxiety, Tinnitus Handicap Inventory, Burns Anxiety Inventory

Introduction

Although a lot of progress in medicine has been made during last decades, tinnitus still remains a clinical puzzle and scientific enigma. Tinnitus is the perception of sound, or ringing in the ears (originated from the Latin word "tinnire"- to ring), in the absence of acoustic stimulation [1,2]. It was classified into objective tinnitus or somatosounds like "pulsatile tinnitus" (when specific

sound source is present) and subjective tinnitus as "non-pulsatile tinnitus" (the absence of a specific sound source) [2,3]. It was estimated that the tinnitus affects 8 to 25.3% of the population of the United States and among 5 to 15% of the general population and economic burden of health care of 7.5 billion dollars in some developed parts of the world [1,4]. Also, it has been known as multifactorial and heterogeneous disorder, most commonly defined as a phantom auditory sensation without an audible external source [5]. Like a persistent tinnitus, it is a symptom, rather than a disease entity, experienced by 10–15% of the population [6] and at least 7.5% of children and adolescents [5,7].

Approximately, one-third to half adults report a moderate annoyance, even though 1–2% report significant distress and disability which can negatively affect his/her daily performance or lead to insomnia, anxiety, depression, hearing problems, and usually can be associated with cognitive disorders (or data cognitive processing difficulty) [5,6]. As well as that, every fifth person in population has an emotional disability, while 0.5% of population are unable to lead a normal life [3]. Although there are people who compensate well the chronic, subjective tinnitus, depressive and anxiety disorders have been observed as typical emotional factors that are considered to be strong predictors of poor adaptation to tinnitus [1,3].

Pathophysiological mechanisms of tinnitus have not been evaluated yet. If we cut the auditory nerve, sound perceptions can still persist. This means that tinnitus is not just a reflection of cochlear damage. In addition to this, the results of previous studies Eggermont and Roberts [8], conducted only on animals, showed that the nervous changes can cause hearing loss correlated with clinical symptoms in humans. However, these assumptions have not been confirmed yet, as an increased rate of damage to the central neurons of the auditory tract has been observed, as one of more neural reasons for tinnitus [8].

Even though it is sometimes difficult to estimate the relationship between the duration of tinnitus and the intensity of symptoms with quality of life, our study aimed to assess the incidence of anxiety in patients with chronic subjective tinnitus and whether the severity of tinnitus and the degree of anxiety were related.

Methods

The research was designed as a cross-sectional study conducted from June to December 2011. Patients were divided into two groups. The first group consisted of 73 patients with unilateral or bilateral tinnitus who were at the first or control examination at the Department of Otorhinolaryngology and Maxillofacial Surgery - University Hospital Foca (21 of them) and the Clinic for Otorhinolaryngology and Maxillofacial Surgery of the Clinical Center of Serbia (52 of them). The second group consisted of patients who did not have tinnitus (control group) - 47 employees at the Faculty of Medicine in Foca and who met the general criteria for participation in the study. In the group of patients with tinnitus we classified respondents into two subgroups - patients who had tinnitus for less than one year and patients with tinnitus for more than one year. Also, we examined the presence of anxiety in all patients as well as its correlation with tinnitus.

The general criteria for inclusion of all respondents in the study were: 20–75 years of age, ability to answer the questions asked and consent to be included in the research. Exclusion criteria from the study were acute or chronic inflammatory diseases of the external and middle ear, patients who have occasional tinnitus and the presence of tinnitus in proven CNS disease (multiple sclerosis, tumors, etc.).

The examination was performed using the usual methods of approaching patients with tinnitus: anamnesis, clinical examination of

the ear (including otomicroscopy), nose and throat, functional examination of hearing, other examinations resulting from the above examinations. Tinnitus questions were standard for all patients who were examined.

Tonal liminal audiometry was performed by standard procedure on a SIBELMED AC50-B clinical apparatus in an acoustically isolated cabin, where the hearing threshold was determined for frequencies 250, 500, 1,000, 2,000, 4,000, 6,000 Hz for bone and air conduction.

All patients completed a specific questionnaire to assess quality of life in patients with tinnitus (Tinnitus Handicap Inventory - THI). It is one of the most used instruments worldwide, developed by Newman, Jacobson, and Spritzer in 1966 [9]. The instrument is composed of 25 questions divided into 3 subgroups: functional (11 items), emotional (9 items) and catastrophic (5 items) [10]. The THI evaluates how tinnitus interferes in daily activities and to quantify handicap in people who have tinnitus. It is a validated subjective self-administered test that aims to determine the degree of distress suffered by the tinnitus patient. Originally, the questionnaire is in English. According to the internationally accepted methodology for translation, validation and cultural adaptation of the HRQoL assessment questionnaire, a standard, "back translation" methodology was used to develop the Serbian version of the questionnaire [11,12].

Both groups of subjects, with and without tinnitus, completed the Burns Anxiety Inventory (BAI). This is one of the most common questionnaires for anxiety estimation widely used, easily understood by patients, which has been translated into more than 40 languages. The questionnaire consists of a list of thirty-three symptoms related to anxiety. They are divided into three categories: physical symptoms (16 items), anxious thoughts (11 items) and anxious feelings (6 items) [13]. The results of this questionnaire numerically show the general anxiety symptoms with which we assess the severity of anxiety (minimal, borderline, mild, moderate, severe and extreme anxiety), but also the success of treatment [14,15].

Data analysis was performed using the Software Package for Statistical Analysis – The IBM SPSS 21 (Chicago, IL, 2012). The $\chi 2$-square test of the nonparametric statistical tests was used, and of the parametric tests, the T test of independent samples was used. The correlation was done with the help of Pearson's correlation coefficient. Arithmetic means and standard deviations were used to display average values. The p-values of less than 0.05 were considered as statistically significant. The results were shown as figures and tables.

Results

The study involved 73 (60.8%) respondents diagnosed with tinnitus and 47 (39.2%) respondents without tinnitus. There were no significant differences between groups of respondents when it comes to gender and age. However, statistically significant difference was found in terms of level of hearing impairment (p=0.007), where majority of respondents from group with tinnitus had mild (56.2%) and moderate hearing loss (24.7%), while only 27.7% of respondents from group without tinnitus had mild and 8.5% had moderate level of hearing loss. Statistically significant difference (p=0.001) in type of audiometric curve was also found between two groups of respondents. Respondents without tinnitus significantly more often (68.1%) had straight audiometric curve in comparison to respondents with diagnosed tinnitus (28.8%), which is expected given that in this group majority of patients had normal hearing (59.6%) when compared to group with tinnitus (9.6%) (Table 1).

We examined the quality of life of patients with diagnosed tinnitus with Tinnitus Handicap Inventory, which measures

Table 1. Comparison of socio-demographic characteristics, level of hearing impairment and type of audiometric curve between groups of respondents

Groups		Group with tinnitus, % or M ± SD	Group without tinnitus, % or M ± SD	p
Gender	Male	53.4	44.4	0.153*
	Female	46.6	59.6	
Age, years		54.3±14.7	50.2±8.8	0.381**
Level of hearing impairment	Normal hearing	9.6	59.6	**0.007***
	Mild HL	56.2	27.7	
	Moderate HL	24.7	8.5	
	Severe HL	x6.8	2.1	
	Profound HL	2.7	2.1	
Type of audiometric curve	Upward	2.7	2.1	**0.001***
	Straight	28.8	68.1	
	Downward	68.5	29.8	
Total, number (%)		**73 (60.8)**	**47 (39.2)**	

*Chi-square test, **student t test; M – mean, SD – standard deviation; HL - hearing loss

Table 2. Comparison of level of tinnitus handicap with duration, frequency and intensity of tinnitus

Level of Tinnitus Handicap		Slight	Mild	Moderate %	Severe	Catastrophic	p
Duration of tinnitus	≤ 1 year	7.3	24.4	46.3	17.1	4.9	0.002*
	> 1 year	71.9	6.3	12.5	0.7	9.4	
Frequency of tinnitus	Low	0	0	50	50	0	0.132**
	Moderate	37.5	37.5	25	0	0	
	High	36.5	14.3	31.7	9.5	7.9	
Intensity of tinnitus	Low	44.4	5.6	27.8	16.7	5.6	0.432*
	Moderate	35.4	22.9	29.2	8.3	4.2	
	High	14.3	0	57.1	0	28.6	
Total, number (%)		26 (35.6)	12 (16.4)	23 (31.5)	7 (9.6)	5 (6.8)	

*Chi-square test, **Fisher test; #student t test; M – mean, SD – standard deviation; HL - hearing loss.

the interference of tinnitus of respondents on their daily activities and quality of daily life. Majority of respondents (35.6%) had slight disturbance because they had tinnitus, 16.4% had mild, 31.5% had moderate, 9.6% had severe, while 6.8% of respondents had disturbed sleep patterns and difficulty with any daily activities. We categorized patients in terms of duration of tinnitus into two groups, tinnitus present within a year, and tinnitus present more than one year. Respondents who had tinnitus less than one year had significantly (p=0.002) higher level of disturbances in daily life, where mild, moderate and severe disturbances were more often present, when compared to group that had tinnitus that lasted more than one year. Statistically significant difference between groups divided by tinnitus handicap score was not found when it comes to level of frequency and intensity of tinnitus (Table 2).

The Burns Anxiety Inventory was used to assess the level of anxiety among respondents, both, in group with and without tinnitus. The majority of respondents had minimal anxiety (30.8%), 26.7% had borderline anxiety, 17.5% had mild level of anxiety, the same percentage of respondents had moderate level, 5% had sever anxiety, while 2.5% of respondents had extreme anxiety or panic. Chi-square test showed that between groups with and without anxiety high statistically significant difference was found ($\chi 2$ = 7.318; p=0.001) when it comes to level of anxiety among respondents. Borderline (28.8%:23.4%), mild (17.8%:17%), moderate (23.3%:8.5%), severe (6.8%:21%) and extreme anxiety (4.1%:0%) were significantly frequent in the group with tinnitus when compared to the group without tinnitus (Figure 1).

We tested correlation between used tests in our research (Tinnitus Handicap Inventory and Burns Anxiety Inventory). A strong and positive statistically significant correlation (r=0.617; p=0.0001) was found between quality of life tested by THI and level of anxiety tested by BAI, which means that respondents who felt handicapped in everyday life

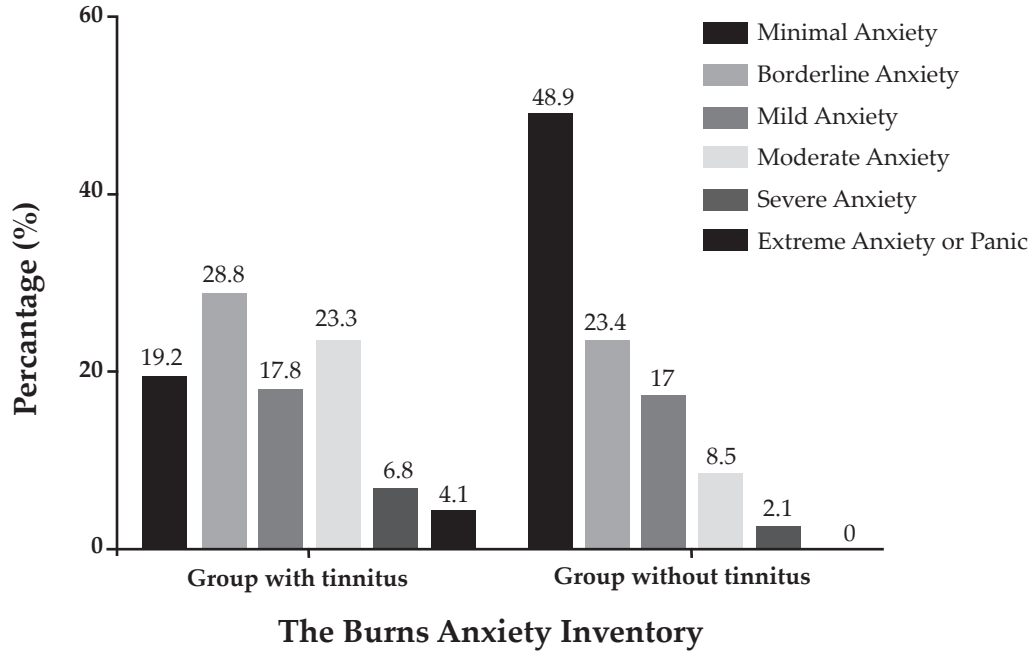

Figure 1. Level of Anxiety measured by the Burns Anxiety Inventory in respondents with and without tinnitus

because of effects of tinnitus also had higher level of anxiety (Table 3).

Table 3. Correlations between the Tinnitus Handicap Inventory and Burns Anxiety Inventory

	Tinnitus Handicap Inventory	Burns Anxiety Inventory
Tinnitus Handicap Inventory		
Burns Anxiety Inventory	**0.617***	

Pearson's correlation test was used, significance level 0.05, r2 values are presented in the table
*– p<0.001

When we divided patients with tinnitus into groups according to duration of tinnitus we found significant difference (p=0.001) between groups when it comes to level of anxiety measured by The Burns Anxiety Inventory. Patients who had tinnitus for less than one year also had higher level of mild, moderate, severe and extreme level of anxiety, when compared to the patients who had tinnitus for more than one year (Table 4).

Table 4. Comparison of level of anxiety measured by the Burns Anxiety Inventory with duration of tinnitus

Duration of tinnitus	≤ 1 year	> 1 year	p
Level of anxiety measured by BAI			
Minimal anxiety	7.3	34.4	**0.001***
Borderline anxiety	17.1	43.8	
Mild anxiety	26.8	6.2	
Moderate anxiety	34.1	9.4	
Severe anxiety	9.8	3.1	
Extreme anxiety	4.9	3.1	
Total, number (%)	**41 (56.2)**	**32 (43.8)**	**73 (100)**

*Chi-square test, BAI (The Burns Anxiety Inventory)

Discussion

Although tinnitus, as a phenomenon of sound perception in the absence of external auditory stimulus, is a common symptom in individuals of heterogeneous age groups, it is not still known whether the presence of tinnitus in patients leads to changes in the quality of life and mental disorder, or whether it is influenced by intensity and frequency of tinnitus as well as its duration, age, sex and degree of hearing impairment [14]. Potential reasons for tinnitus are variable disorders in several parts of the central and peripheral auditory tract and the brain. Recent studies have shown a close connection between tinnitus and mental disorders with an increase in the rate of comorbidity - depression, loss of libido and self-confidence with the appearance of anxiety [1].

In our study involved 73 (60.8%) respondents diagnosed with tinnitus and 47 (39.2%) respondents without tinnitus. We did not find significant differences between the groups of respondents when it comes to gender and age. However, some authors report that increase with age is one of the factors that determines the presence of tinnitus, and it is relatively more common in the individuals over 40 [14–16] while others describe predominance among men (16–18). Reasons for this are thought to be numerous, including study design, sample size, socio-demographic characteristics, ethnicity, work environment, short-term exposure to noise, stress, being a soldier, systemic and metabolic diseases like osteoarthritis, thyroid disease, rheumatoid arthritis, hyperlipidemia etc. [15,16]. On the other hand, in terms of level of hearing impairment, statistically significant difference has been found. The majority of respondents from the group with tinnitus had mild (56.2%)

and moderate hearing loss (24.7%), while only 27.7% of respondents from the group without tinnitus had mild and 8.5% had moderate level of hearing loss. Also, the respondents without tinnitus significantly more often had normal hearing (straight audiometric curve in 68.1%) in comparison to respondents with diagnosed tinnitus (28.8%), which is expected given that in this group majority of patients had normal hearing (59.6%) when compared to the group with tinnitus (9.6%).

Also, we estimated the incidence of anxiety and the degree of association with tinnitus in a patient with chronic subjective tinnitus. We used the BAI to assess the degree of anxiety, and to estimate discomfort caused by tinnitus, the THI questionnaire has been used. Our results have shown that patients which had tinnitus for less than one year had significantly higher level of disturbances in daily life, where mild, moderate and severe disturbances were more often present, when compared to the group which had tinnitus that lasted more than one year. Statistically significant difference between groups divided by tinnitus handicap score was not found when it comes to level of frequency and intensity of tinnitus. The score of the THI is consistent with the reports of other authors who used the same questionnaire to evaluate the burden of patients with tinnitus [19,20]. In relation to the control group by the results of THI test, which coincides with the reports of other authors, we concluded that very severe and severe tinnitus reduce working ability, disrupt sleep, cause emotional disorders and significantly affect the quality of life in general [20]. In the group with tinnitus according to the results of BAI, the largest number of subjects had borderline anxiety, 28.8%, moderate anxiety was found in 23.3% of subjects, mild anxiety was found in 17.8%, severe anxiety 6.8% and 3 subjects had extreme anxiety or 4.1%. The results of our study have shown that there is a statistically highly significant difference in the TAS between the group with tinnitus and the group without it, which indicates that the presence of noise has had an impact on the difficulties examined by this test.

Even though the auditory cortex is not thought to be directly related to anxiety disorders, changes in the activity of the major central neurotransmitter gamma-Aminobutyric acid (GABA) indirectly affect the mechanism of anxiety disorder. However, Shulman et al., then Lockwood et al. have connected the tinnitus to hyperreactivity of the subcortical structures [3]. Due to the influence of stress, noise and other environmental factors, glucocorticosteroid and mineralocorticoid hormones were secreted in order to maintain homeostasis and defend the organism from harmful influences. Released hormones, by binding to their receptors, indicate changes in the auditory pathway. There are numerous controversies about stress-induced neuroplasticity as one of the causes of tinnitus, but strong evidence in the literature, as explained by Mazurek et al., still does not exist [1,21]. However, Shulman et al., then Lockwood et al. connected the tinnitus to hyperreactivity of the limbic system and subcortical structures [3]. A directly related structure of the dorsal nuclear complex, with parts of the brainstem that do not belong to the auditory network, but have an impact on emotions and their control have been proven. In general, that hyperactivity of certain parts of the brainstem, especially the two subcortical structures - the locus ceruleus and the raphe nucleus, is directly related to anxiety disorder as the cause of tinnitus [21].

Also, a statistically highly significant difference in the scores of the BAI was proved between subjects in whom the noise lasted less than one year and subjects in whom the noise lasted more than one year, which means that respondents who felt handicapped in everyday life because of effects of tinnitus also had higher level of anxiety. As well as that, we found strong and positive statistically significant correlation between quality of life tested

by the THI questionnaire and the Burns anxiety questionnaire. These correlations have been reported in other studies [22]. Furthermore, there are many cross-sectional studies in the literature that have explained the association between tinnitus and anxiety disorders, but also one longitudinal study by Sheue-Jane Hou et al. [15]. They assessed the incidence of tinnitus in patients with and without the presence of anxiety and, through several explanations, indicated a positive correlation between tinnitus and mental disorders. Some of those explanations were, that in people who have tinnitus, anxiety disorder occurs first, as well as that tinnitus and anxiety are causally related. In addition, dysfunction of the central nervous system or genetic factors results in both diseases. This means that the hypothalamus, the limbic system, the locus coreuleus, the dorsal cochlear nucleus and the hypothalamic-pituitary-adrenal axis, as structures which are not under the control of our consciousness, are thought to be important parts of puzzle involved in the pathogenetic mechanisms of anxiety and tinnitus [1,15].

The results of our study indicate that anxiety is a possible cause of tinnitus, but due to the still insufficiently clear mechanisms, it is difficult to determine whether the tinnitus or a psychiatric disorder first occurred. A significant increase in the presence of tinnitus was observed in older women with anxiety disorders, as well as the fact that the anxiety is more often present in people in whom tinnitus lasts longer and greatly negatively affects the quality of life.

Explaining the mechanisms of occurrence and the cause-effect relationship between tinnitus and mental disorders require more thorough research in the field of neurobiology and genetics. One of the main criteria, in order to establish pharmacological and cognitive-behavioral therapeutic approaches in people with chronic subjective tinnitus, is their personal experience of discomfort and burden of the disease. Associated with mental comorbidities, such as insomnia, irritability, anxiety or depression, have only an additional negative impact on the quality of life.

We consider that a limitation of our study is the small size of control group in comparison to the size of group with tinnitus, which can have an impact on the outcome of anxiety.

Conclusion

Anxiety and other mental disorders can be considered as potentially significant modulators of changes in brain structures observed in people with tinnitus. Since both conditions, tinnitus and anxiety, significantly disrupt and impair the quality of life, they should be treated simultaneously but also individually, in order to achieve the best results for each patient and improve the quality of life. Therefore, the psychiatric evaluation should play a central role in a multidisciplinary approach in these patients.

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Ethical approval. The Ethics Committee of the Faculty of Medicine in Foca approved the study and informed consent was obtained from all individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Učestalost anksioznosti kod pacijenata sa hroničnim subjektivnim tinitusom

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Uvod. Tinitus je percepcija zvuka u ušima u odsustvu akustične stimulacije čiji patofiziološki mehanizmi još nisu dovoljno istraženi. Kod prosečno 1–2% ljudi negativno utiče na svakodnevne aktivnosti. Cilj naše studije bio je da se proceni učestalost anksioznosti kod pacijenata sa tinitusom.

Metode. Studija je sprovedena kao studija preseka. Učesnici su podeljeni u dve grupe, grupa od 73 pacijenta sa tinitusom (sa dve podgrupe u odnosu na dužinu trajanja tinitusa - manje od jedne godine i više od jedne godine) i kontrolna grupa od 43 pacijenta bez tinitusa. Ispitivali smo prisustvo anksioznosti uz pomoć Burnsovog upitnika za anksioznost (eng. Burns Anxiety Inventory, BAI). Kvalitet života svih pacijenata je procenjivan uz pomoć Tinitus hendikep upitnika (engl. Tinnitus Handicap Inventory, THI).

Rezultati. U grupi sa tinitusom 56,2% je imalo blagi i 24,7% umereni gubitak sluha, dok je 27,7% ispitanika iz kontrolne grupe imalo blagi i 8,5% umereni nivo oštećenja sluha. Rezultati THI pokazali su da su pacijenti sa tinitusom kraćim od jedne godine imali značajno ($p = 0,002$) viši nivo poremećaja u svakodnevnim aktivnostima u poređenju sa grupom koja je imala tinitus duže od jedne godine. Minimalnu anksioznost je imalo 30,8% ispitanika, 26,7% graničnu anksioznost, 17,5% blagu anksioznost, isti procenat ispitanika umerenu, 5% tešku, dok je 2,5% imalo ekstremnu anksioznost na osnovu BAI.

Zaključak. Anksioznost se može smatrati potencijalno značajnim modulatorima promena uočenim u moždanim strukturama kod ljudi sa tinitusom.

Ključne reči: tinitus, anksioznost, tinitus hendikep upitnik, Burnsov upitnik anksioznosti

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Original article

Expression of tumor necrosis factor alpha, interleukin-1 and matrix metalloproteinase-9 and pathomorphological changes in acquired middle ear cholesteatoma

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Summary

Introduction. The inflammatory mediators play a central role in the pathogenesis of the inflammatory process of the middle ear and cholesteatoma from the aspect of initiating and maintaining the inflammatory response to infection and lesion. The aim of the study was to examine if the presence of acquired cholesteatoma could predict pathomorphological changes of the tympanic cavity mucosa in relation to the control tissue of the inflamed middle ear mucosa and to examine and compare the expression levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and matrix metalloproteinase 9 (MMP-9) with pathomorphological changes in the middle ear mucosa in chronic otitis media (COM), with and without acquired cholesteatoma (AC).

Methods. The immunohistochemical study included 178 patients of both sexes, aged 5 to 75, who underwent microsurgical treatment of COM from 2015 to 2018. Patients were divided into two groups based on the presence or absence of AC of the middle ear: 97 with cholesteatoma (CCOM) and 81 without cholesteatoma (COM). Samples of the perimatrix of AC and inflamed middle ear mucosa were taken intraoperatively. The condition of the tympanic cavity mucosa was examined by otomicroscopy exploration intraoperatively. The expression levels of TNF-α, IL-1 and MMP-9 were determined by immunohistochemical analysis.

Results. The difference in the percentage distribution of patients according to the condition of the tympanic cavity mucosa between both groups was statistically significant ($p < 0.01$) where in the COM group the highest frequency was 43.2% of patients with mucosal hypertrophy, and in the CCOM 56.7% with granulations. With highly positive expression of TNF-R2 and IL-1, a higher probability of the presence of mucosal hypertrophy and granulations can be expected, and with highly positive expression of MMP-9 the presence of granulations.

Conclusion. Acquired middle ear cholesteatoma is a statistically significant predictor of the occurence of mucosal hypertrophy and granulations in the tympanic cavity in relation to the control tissue of the inflamed middle ear mucosa. The high expression of TNF-R2, IL-1 and MMP-9 shows a statistically significant association with the presence of granulations and mucosal hypertrophy in acquired middle ear cholesteatoma which may have clinical significance in the evaluation and prognosis of the disease.

Keywords: cholesteatoma, inflammatory mediators, middle ear mucosa

Introduction

Cholesteatoma is defined as a cystic, expansive lesion of the temporal bone, consisting of a multilayered squamous epithelium with desquamated keratin, whose main feature is characterized by progressive growth with destruction of the surrounding bone due to the pressure effect and activation of the osteoclasts. Cholesteatoma may be classified as either congenital or acquired [1,2]. The development of acquired cholesteatoma is characterized by a cascade of reactions at the molecular level, which include the induction of MMP, the release of oxygen radicals and other inflammatory mediators, leading to the destructive effects of cholesteatoma, based on proteolytic activity, bone resorption and recruitment of inflammatory cells [3].

The inflammatory mediators have a leading role in the pathogenesis of the inflammatory process of acquired middle ear cholesteatoma from the aspect of initiating and maintaining the inflammatory response to infection and lesion. They may be one of the reasons why in some patients there is a progression from the acute to the chronic phase of the inflammatory process of the middle ear, as well as the development of cholesteatoma [4–6]. In most cases, in the induction of the development of cholesteatoma, disorders of internal molecular regulation participate together with external stimuli in the form of proinflammatory cytokines, growth factors and/or bacterial toxins. The pathogenetic reason for the uncontrolled proliferation of cholesteatoma tissue has not been fully clarified until now [7,8].

The main physiological effect of TNF-α, as one of the major inflammatory cytokines, is to promote the immune and inflammatory response of recruited neutrophils and monocytes at the site of infection with their concomitant activation, resulting in its numerous effects in the body [9]. The correlation of the levels of TNF-α expression in the tissue of acquired cholesteatoma with the level of inflammation has been proven. However, precise mechanisms in the process of their action are still the subject of scientific research [10].

Interleukin-1 cytokines (IL-1α, IL-1β and IL-1Ra) have a significant role in immune regulation and inflammatory processes by inducing the expression of many effector proteins, such as cytokines/chemokines, nitric oxide synthases and matrix metalloproteinase [11]. Cholesteatoma epithelial cells and inflammatory cells of the tissue of the surrounding granulations can produce IL-1. IL-1α and IL-1β levels are significantly higher in cholesteatoma than in normal squamous epithelium. IL-1 is involved in the process of bone resorption, and has been shown to stimulate keratinocyte proliferation [12,13].

MMPs belong to the family of zinc metalloenzymes that are secreted as latent proenzymes and activated during proteolytic degradation processes [14]. MMP-9 is produced in a variety of cells, including epithelial cells, fibroblasts, keratinocytes, osteoblasts, dendritic cells, macrophages, granulocytes, and T cells [15]. MMP-9 has a key role in inflammatory cell migration and destructive behavior of cholesteatoma although serum MMP-9 levels do not necessarily directly reflect the extent of local tissue inflammation [16].

The aim of the study was to examine if the presence of acquired cholesteatoma could predict pathomorphological changes of the tympanic cavity mucosa in relation to the control tissue of the inflamed middle ear mucosa and to examine and compare the expression levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and matrix metalloproteinase 9 (MMP-9) with pathomorphological changes in the middle ear mucosa in chronic otitis media (COM), with and without acquired cholesteatoma (AC).

Methods

The immunohistochemical study included 178 patients of both sexes, aged 5 to 75, who underwent microsurgical treatment of COM at the Ear, Nose and Throat Department, University Clinical Center of the RS (UCC RS), Banja Luka, from 2015 to 2018. The study was approved by the Ethics Committee of the UCC RS. Patients were divided into two groups based on the presence or absence of AC: 97 with cholesteatoma (CCOM) and 81 without cholesteatoma (COM). The diagnosis of the disease is based on the history of the disease, clinical examination of the patients and additional diagnostic procedures. Closed and open tympanoplasty techniques with modifications were applied. Samples of the perimatrix of AC (n = 97) (CCOM) and inflamed mucosa (n = 81) (COM) of the middle ear were taken from the patients during the microsurgical procedure. The condition of the tympanic cavity mucosa was examined by otomicroscopy exploration intraoperatively, which is classified into the following categories: eutrophy, hypertrophy, granulations and polypoid altered mucosa.

The patients with diagnosed COM with and without AC, who were referred for middle ear surgery, were included. The patients with congenital cholesteatoma, malignant tumor of the middle ear, otitis externa, previous history of ear surgery, as well as samples of cholesteatoma without perimatrix, were excluded from the study. Samples obtained during the microsurgical procedure were fixed in 10% formalin and then molded into paraffin blocks from which 4 μm thick semester sections were obtained. After dewaxing, the samples were stained by routine hematoxylin-eosin method and then analyzed under a light microscope. Samples were treated with citrate buffer by heating in a microwave oven for 20 minutes to unmask the antigen. After blocking endogenous peroxidase with hydrogen peroxide in methanol, the samples were washed in tris-buffered saline solution with pH of 7.6.

For the immunohistochemistry analysis of TNF-α (TNF-R2) and IL-1, Rabbit TNF-R2 Polyclonal Antibody (TNFR2 Polyclonal Antibody, Product#PA1-21148, Thermo Fisher, USA) and IL-1 alpha Polyclonal Antibody (IL-1 alpha Polyclonal Antibody, Product# PA5-25921, Invitrogen, Thermo Fisher, USA) were used, and for the analysis of MMP-9, Mouse anti-MMP-9 Monoclonal Antibody (MMP9 Monoclonal Antibody, Clone 2C3, Product # MA1-12894, Invitrogen, Thermo Fisher, USA). Immunohistochemical identification of the examined mediators was performed using the EnVision technique. 3,3′-Diaminobenzidine was used as the chromogenic substrate, while contrast was performed with hematoxylin. Imunohistochemistry analysis was performed manually in the competent laboratory of the Department of Clinical Pathology. Original reagents were used.

In immunohistochemically processed samples, expression intensities were observed in each individual sample, while the analysis of immunohistochemical reactions was based on quantitative (0 - absent, 1 - present) and semiquantitative determination of expression intensity by light microscope with a grading scale from 0 to 3 (0 - absent, 1 - weak, 2 - moderate, 3 - high intensity).

In relation to the percentage of stained cells, it was divided into 4 categories. Results were considered negative if there was no staining and were marked with 0, weakly positive in ≤25% of positive cells with label 1, moderately positive in ≥25−50% of positive cells with label 2, and highly positive with label 3, if ≥50% of positive cells were present. Determination of the total result of the immunohistochemical reaction was calculated based on the product of the results of the expression intensity and the percentage of stained cells. The overall results of the analysis were considered negative in R≤1 and were marked with 0, weakly positive in R≥2≤3 with a notation of 1+, moderately positive in R≥4≤6 with a notation of 2+, and very

positive in R = 9 with the notation 3+.

The data were analyzed at the level of descriptive statistics, by calculating the absolute and relative distributions of COM and CCOM patients, with mediator expression levels, in relation to the observed factors, and by calculating the arithmetic mean and standard deviation for patient age. For the purpose of calculating the difference in the distribution of the patients of the COM and CCOM groups, the Pearson χ^2-test was used, and Mann-Whitney U - the nonparametric test was used for calculating the differences in the age of the subjects between the groups. Categorical logistic regression was performed to evaluate the influence of the two variables: presence or absence of the cholesteatoma (CCOM/COM) and the level of TNF R2, IL-1 and MMP-9 expression respectively on the occurrence probability of the tympanic cavity mucosa pathological changes. The results are considered significant if p <0.05. Statistical data processing was performed using SPSS version 21.0 data processing tools (IBM, USA).

Results

Out of the total of 151 (84.8%) patients with pathologically altered tympanic cavity mucosa, the highest frequency was shown by 76 (42.7%) patients with granulations and 67 (37.6%) with mucosal hypertrophy. The difference in the percentage distribution of patients according to the condition of the tympanic cavity mucosa between both groups was statistically significant (p <0.01) where in the COM group the highest frequency was shown by 43.2% of patients with mucosal hypertrophy, and in the CCOM by 56.7% of patients with granulations (Table 1).

The results of the levels of the TNF-R2, IL-1 and MMP-9 expression among the patients classified in two groups based on the cholesteatoma presence and on the tympanic cavity mucosa pathological changes are shown in Tables 2, 3 and 4 respectively.

A categorical logistic regression, which included the CCOM/COM groups and TNF-R2 expression results, showed that both predictors had a statistically significant effect on the occurrence probability of pathologically altered mucosa of the tympanic cavity (p< 0.01). This result implies that a higher probability of the mucosal hypertrophy and granulation in the presence of cholesteatoma (CCOM group) and weak (R = 1 +) and moderately positive (R = 2 +) expression of TNF-R2 can be expected (Table 5).

A categorical logistic regression, which included the CCOM/COM groups and IL-1 expression results, showed that both predictors had a statistically significant effect on the occurrence probability of pathologically altered

Table 1. The patients according to the condition of the tympanic cavity mucosa

			Group		Total
			CCOM	COM	
The tympanic cavity mucosa	Eutrophic	N	3	24	27
		%	3.1%	29.6%	15.2%
	Hypertrophic	N	32	35	67
		%	33.0%	43.2%	37.6%
	Granulations	N	55	21	76
		%	56.7%	25.9%	42.7%
	Polypoid modified	N	7	1	8
		%	7.2%	1.2%	4.5%
Total		N	97	81	178
		%	100.0%	100.0%	100.0%

Chi-square test: γ^2=35.23, p= 0,000; p<0.01

Table 2. Different levels of the TNF-R2 expression among the patients classified in two groups based on the cholesteatoma presence and on the tympanic cavity mucosa pathological changes in every group

Inflammatory mediator	Group	Level of expression	Tympanic cavity mucosa			
			Eutrophic	Hypertrophic	Granulations	Polypoid altered
			N (%)	N (%)	N (%)	N (%)
TNF-R2	CCOM	R = 0	1 (8.3%)	3 (25.0%)	8 (66.7%)	0 (0.0%)
		R = 1+	1 (5.9%)	6 (35.3%)	9 (52.9%)	1 (5.9%)
		R = 2+	0 (0.0%)	17 (41.5%)	22 (53.7%)	2 (4.9%)
		R = 3+	1 (3.7%)	6 (22.2%)	16 (59.3%)	4 (14.8%)
	COM	R = 0	4 (57.1%)	3 (42.9%)	0 (0.0%)	0 (0.0%)
		R = 1+	9 (31.0%)	14 (48.3%)	6 (20.7%)	0 (0.0%)
		R = 2+	10 (25.6%)	15 (38.5%)	14 (35.9%)	0 (0.0%)
		R = 3+	1 (16.7%)	3 (50.0%)	1 (16.7%)	1 (16.7%)

CCOM - chronic otitis media with cholesteatoma, COM - chronic otitis media, TNF-R2 - tumor necrosis alpha receptor, R = 0 - negative result; R = 1+ - weakly positive result; R = 2+ - moderately positive result; R = 3+ - very positive result

Table 3. Different levels of the IL-1 expression among the patients classified in two groups based on the cholesteatoma presence and on the tympanic cavity mucosa pathological changes in every group

Inflammatory mediator	Group	Level of expression	Tympanic cavity mucosa			
			Eutrophic	Hypertrophic	Granulations	Polypoid altered
			N (%)	N (%)	N (%)	N (%)
IL-1	CCOM	R = 0	0 (0.0%)	4 (33.3%)	8 (66.7%)	0 (0.0%)
		R = 1+	1 (9.1%)	4 (36.4%)	6 (54.5%)	0 (0.0%)
		R = 2+	1 (2.8%)	11 (30.6%)	22 (61.1%)	2 (5.6%)
		R = 3+	1 (2.6%)	13 (34.2%)	19 (50.0%)	5 (13.2%)
	COM	R = 0	1 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
		R = 1+	5 (71.4%)	2 (28.6%)	0 (0.0%)	0 (0.0%)
		R = 2+	7 (25.0%)	19 (67.9%)	2 (7.1%)	0 (0.0%)
		R = 3+	11 (24.4%)	14 (31.1%)	19 (42.2%)	1 (2.2%)

CCOM - chronic otitis media with cholesteatoma, COM - chronic otitis media, IL-1 - interleukin -1, R = 0 - negative result; R = 1+ - weakly positive result; R = 2+ - moderately positive result; R = 3+ - very positive result

mucosa of the tympanic cavity ($p<0.01$). This result implies that a higher probability of the mucosal hypertrophy and granulation in the presence of cholesteatoma (CCOM group) and moderately (R = 2 +) and very positive (R = 3 +) expression of IL-1can be expected (Table 6).

A categorical logistic regression, which included CCOM/COM groups and MMP-9 expression results, showed that both predictors had a statistically significant effect on the occurrence probability of the pathologically altered tympanic cavity mucosa ($p<0.01$). This result implies that a higher probability of mucosal hypertrophy and granulation can be expected in the presence of cholesteatoma (CCOM group) and very positive (R = 3 +) expression of MMP-9. With moderately positive (R = 2 +) expression of MMP-9, a higher probability of the presence of granulations can be expected (Table 7).

Table 4. Different levels of the MMP-9 expression among the patients classified in two groups based on the cholesteatoma presence and on the tympanic cavity mucosa pathological changes in every group

Inflammatory mediator	Group	Level of expression	Tympanic cavity mucosa			
			Eutrophic	Hypertrophic	Granulations	Polypoid altered
			N (%)	N (%)	N (%)	N (%)
MMP-9	CCOM	R = 0	0 (0.0%)	0 (0.0%)	1 (50.0%)	1 (50.0%)
		R = 1+	1 (3.7%)	12 (44.4%)	12 (44.4%)	2 (7.4%)
		R = 2+	0 (0.0%)	10 (27.8%)	23 (63.9%)	3 (8.3%)
		R = 3+	2 (6.3%)	10 (31.3%)	19 (59.4%)	1 (3.1%)
	COM	R = 0	17 (43.6%)	18 (46.2%)	4 (10.3%)	0 (0.0%)
		R = 1+	5 (19.2%)	13 (50.0%)	8 (30.8%)	0 (0.0%)
		R = 2+	2 (14.3%)	4 (28.6%)	8 (57.1%)	0 (0.0%)
		R = 3+	0 (0.0%)	0 (0.0%)	1 (50.0%)	1 (50.0%)

CCOM - chronic otitis media with cholesteatoma, COM - chronic otitis media, MMP-9 - metalloproteinase matrix 9, R = 0 - negative result; R = 1+ - weakly positive result; R = 2+ - moderately positive result; R = 3+ - very positive result

Table 5. The influence of the presence/absence of the cholesteatoma and the level of TNF-R2 expression on the tympanic cavity mucosa pathological changes

Variables	Standard coefficients		df	F	p
	Beta	S.E.			
CCOM/ COM	-.414	.073	2	32.195	.000**
TNF-R2	.173	.076	4	5.229	.001**

*** p< 0.01, CCOM - chronic otitis media with cholesteatoma, COM - chronic otitis media without cholesteatoma, TNF-R2 - tumor necrosis alpha receptor, Beta - standard partial regression coefficient; S.E. - standard regression error; df - number of degrees of freedom; F - variance ratio test; Dependent variable: the tympanic cavity mucosa pathological changes*

Table 6. The influence of the presence/absence of the cholesteatoma and the level of IL-1 expression on the tympanic cavity mucosa pathological changes

Variables	Standard coefficients		df	F	p
	Beta	S.E.			
CCOM/ COM	-.473	.059	1	64.357	.000**
IL-1	.222	.065	3	11.705	.000**

*** p< 0.01, CCOM - chronic otitis media with cholesteatoma, COM - chronic otitis media without cholesteatoma, TNF-R2 - tumor necrosis alpha receptor, IL-1 - interleukin -1, Beta - standard partial regression coefficient; S.E. - standard regression error; df - number of degrees of freedom; F - variance ratio test; Dependent variable: the tympanic cavity mucosa pathological changes*

Table 7. The influence of the presence/absence of the cholesteatoma and the level of MMP-9 expression on the tympanic cavity mucosa pathological changes

Variables	Standard coefficients		df	F	p
	Beta	S.E.			
CCOM/COM	-.386	.091	1	18.028	.000**
MMP-9	.333	.116	3	8.296	.000**

*** p< 0.01, CCOM - chronic otitis media with cholesteatoma, COM - chronic otitis media without cholesteatoma, MMP-9 - metalloproteinase matrix 9, Beta - standard partial regression coefficient; S.E. - standard regression error; df - number of degrees of freedom; F - variance ratio test; Dependent variable: the tympanic cavity mucosa pathological changes*

Discussion

In 151 (84.8%) patients in the total sample, a pathological finding was observed on tympanic cavity mucosa. The difference in the distribution of patients according to the examined pathomorphological change was statistically significant ($p<0.01$), where mucosal hypertrophy in the COM group (43.2%) and granulations in the CCOM group (56.7%) showed the highest frequency. Highly positive TNF-R2 and IL-1 expression was recorded in both groups, while in terms of MMP-9 results, the highest frequency in the CCOM group was shown by high-positive expression, and in the COM group by negative expression (Figure 1). The predictor model, which included the level of expression of TNF-R2, IL-1 and MMP-9 in relation to cholesteatoma tissue and control tissue of inflamed tympanic cavity mucosa, proved a statistically significant prediction of cholesteatoma presence on pathological changes of tympanic cavity mucosa, respectively the occurrence of mucosal hypertrophy and granulations. There was also a statistically significantly higher probability of the presence of granulationss in the highly positive TNF-R2, IL-1 and MMP-9 expression and mucosal hypertrophy in the highly positive TNF-R2 and IL-1 expression. These data corroborate literature data.

Akimoto et al. state that most AC are associated with an inflammatory cell infiltrate. The authors find the presence of granulationss in the acquired, in contrast to congenital cholesteatoma, but also a statistically significant correlation of TNF-α expression levels with the degree of infection and bone resorption, in contrast to IL-1 expression levels. In the same study, no statistically significant correlation was found between the expression levels of TNF-α and IL-1 and the presence of granulationss in AC [17].

Marenda i Aufdemorte immunolocalized a high levels of TNF-α, TGF- β 1 and 2, and IL-1 and IL-6 expression in the epithelial and subepithelial layers of AC of human origin. The autors proved that elevated levels of these inflammatory mediators in the epithelial layer and stroma of AC significantly correlate with the presence of granulation tissue, as well as with degree of erosion of the auditory ossicles and surrounding bone [18].

Kuczkowski et al. found high levels of IL-1, TNF-α and IL-6 expression in granulations and cholesteatoma tissue. The authors point out that a strong positive association between the levels of these cytokines and the degree of osteolysis of bone indicates the destructive behavior of cholesteatoma or granulation tissue [19]. Sudhoff et al. point out that cholesteatoma epithelium shows a high staining intensity of

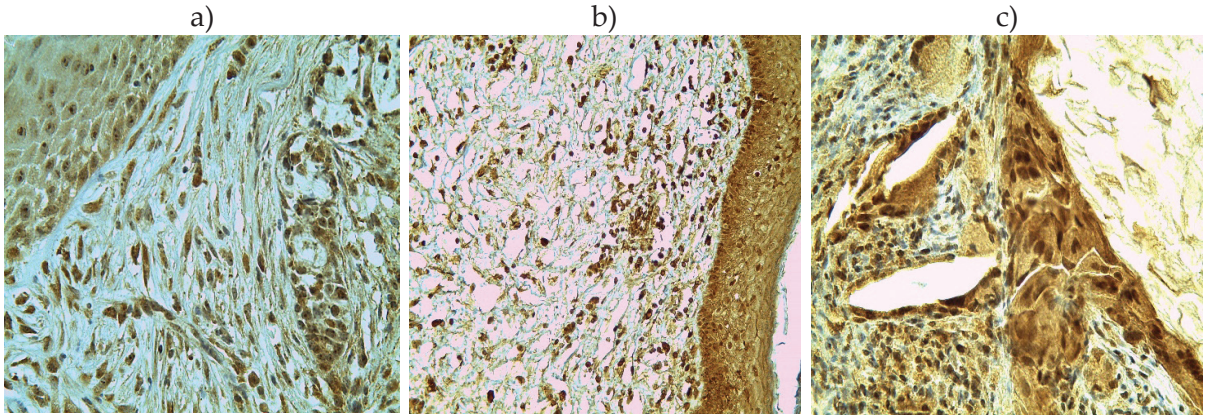

Figure 1. A high expression of stromal and inflammatory cells in environment of cholesteatoma (IHC): anti-TNF-R2x400 (a), anti-IL1x400 (b) and anti-MMP9x400 (c)

IL-1, TGF- α, and the epidermal growth factor receptor (EGF-R) in contrast to middle ear mucosa. The authors also found high concentrations of lymphocytes and macrophages in the surrounding stroma of cholesteatoma, most of which expressed IL-1, TGF- α and EGF-R [20]. Yetiser et al. demonstrate that there are no statistical differences in TNF-α and IL-1 expression levels between granulations and external auditory canal skin tissue, while Kim et al. in their study found significantly higher levels of IL-1 mRNA expression in granulations versus healthy skin tissue and cholesteatoma [21,22]. Schmidt et al. point out that MMP-9 expression occurs mainly in the suprabasal, and less frequently in the basal layers of the cholesteatoma epithelium, as well as in inflammatory cells of the perimatrix, in contrast to keratinocytes and granulation tissue [23]. Schönermark et al. found the expression of MMP-9 and MMP-2, limited only to the basal and suprabasal epithelial cell layers of the cholesteatoma as opposed to the mucosal layer of the tympanic cavity and the tympanic membrane. The autors point out that members of the MMPs family could have an active role in the molecular mechanisms of cholesteatoma invasion of the temporal bone, wich provides new insights into the pathophysiology of the disease and potential therapeutic approaches [24].

Conclusion

Acquired middle ear cholesteatoma is a statistically significant predictor of the mucosal hypertrophy and granulations occurrence in the tympanic cavity in relation to the control tissue of the inflamed middle ear mucosa. The high expression of TNF-R2, IL-1 and MMP-9 shows a statistically significant association with the presence of granulationss and mucosal hypertrophy in acquired middle ear cholesteatoma which may have clinical significance in the evaluation and prognosis of the disease.

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Ethical approval. The Ethics Committee of the University of Banja Luka, Faculty of Medicine, approved the study and informed consent was obtained from all the individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflicts of interest.

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Ekspresija faktora nekroze tumora alfa, interleukina-1 i matriks metaloproteinaze-9 i patomorfološke promjene kod stečenog holesteatoma srednjeg uha

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Uvod. Medijatori inflamacije imaju centralnu ulogu u patogenezi upalnog procesa srednjeg uha i holesteatoma sa aspekta pokretanja i održavanja upalnog odgovora na infekciju i leziju. Cilj istraživanja je bio da se ispita da li prisustvo stečenog holesteatoma može da predvidi patomorfološke promjene sluznice kavuma timpani u odnosu na kontrolno tkivo inflamirane sluznice srednjeg uha, te da se ispitaju i uporede nivoi ekspresije faktora nekroze tumora-alfa (TNF-α), interleukina-1 (IL-1) i matriks metaloproteinaze-9 (MMP-9) s patomorfološkim promjenama na sluznici srednjeg uha kod hroničnog otitis media (HOM), sa i bez stečenog holesteatoma (SH).

Metode. Imunohistohemijska studija je uključila 178 ispitanika oba pola, od 5 do 75 godina, koji su podvrgnuti mikrohirurškom liječenju (HOM) od 2015. do 2018. godine. Ispitanici su podijeljeni u dvije grupe na osnovu prisustva ili odsustva stečenog holesteatoma srednjeg uha: 97 s holesteatomom (HHOM); i 81 bez holesteatoma (HOM). Uzorci perimatriksa SH i inflamirane sluznice srednjeg uha su uzeti intraoperativno. Intraoperativnom otomikroskopskom eksploracijom je ispitano stanje sluznice kavuma timpani. Nivoi ekspresije TNF-α, IL-1 i MMP-9 su određeni imunohistohemijskom analizom.

Rezultati. Razlika u procentualnoj distribuciji ispitanika prema stanju sluznice kavuma timpani između obje grupe se pokazala kao statistički značajna ($p<0,01$) gdje je u HOM grupi najveću učestalost pokazalo 43,2% ispitanika s hipertrofijom sluznice, a u HHOM 56,7% s granulacijama. Visokopozitivna ekspresija TNF-R2 i IL-1 je pokazala statistički značajnu povezanost s prisustvom hipertrofične sluznice i granulacija, a visokopozitivna MMP-9 granulacija.

Zaključak. Stečeni holesteatom srednjeg uha je statistički značajan prediktor nastanka hipertrofične sluznice i granulacija u kavumu timpani u odnosu na kontrolno tkivo inflamirane sluznice srednjeg uha. Visoki stepen ekspresije TNF-R2, IL-1 i MMP-9 pokazuje statistički značajnu povezanost s prisustvom granulacija i hipertrofične sluznice kod stečenog holesteatoma srednjeg uha, što može imati klinički značaj u evaluaciji i prognozi bolesti.

Ključne riječi: holesteatom, medijatori inflamacije, sluznica srednjeg uha

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Original article

Relationship between auditory discrimination of Serbian language phonemes and dysgraphia in different forms of written expression

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Summary

Introduction. Writing is the most complex human ability and the most direct form of communication. Auditory discrimination is the ability to distinguish different sounds of language. After the age of seven, difficulties in auditory discrimination, even of similar sounds, are considered a pathological phenomenon. The aim of the research was to determine whether difficulties in auditory discrimination of phonemes are related to the manifestation of dysgraphia in children of younger school age.

Methods. The research was conducted at the Elementary School "Vuk Karadzić" in Priboj, during 2020, with the previous consent of the school principal, as well as the students' parents. The research sample included fifty children of the third and fourth grade, aged 9 and 10. For the purpose of this research, two tests were used: the Phonemic Discrimination Test (Kostić, Vladisavljević, Popović, 1983) and the Dysgraphic Handwriting Assessment Test (Ajuriaguerra, Auzias. 1971).

Results. There was no significant difference in achievement in the Phonemic Discrimination Test between boys and girls. Half of the tested students achieved the maximum score in the Phonemic Discrimination Test and they were fairly equal in their achievement in the Phonemic Discrimination Test. Girls generally had harmoniously developed handwriting, while more than half of the boys in the categories had inconsistent handwriting or dysgraphic handwriting when it came to the forms of dictation, free topic and transcription. No statistically significant correlations were found between the results in the Phonemic Discrimination Test and the Dysgraphic Handwriting Assessment Test, $p > 0.05$.

Conclusion. Based on the assessment of writing ability and auditory discrimination in young school children, no statistically significant association was found between auditory discrimination of sounds and manifestations of dysgraphic handwriting in all three forms of written expression (dictation, free topic, transcription).

Key words: students, writing, auditory discrimination, dysgraphia

Introduction

Communication is an essential part of human life and all living beings have the need to communicate [1,2]. Literature most often mentions the categorization of communication into verbal and nonverbal [3]. Verbal communication occurs in the forms of listening, speaking, reading and writing. It is considered to be the most successful mean of social influence as it can

most completely express thoughts, the most diverse and complex contents and present the most complete and precise ideas and knowledge [4].

Writing is the most complex human ability which integrates almost all brain functions and it is more complex than reading. Today, writing is the most direct form of communication, despite the widespread use of technology [5]. Learning the writing process begins with visual, auditory and sensory-motor perception, but also by practicing graphic forms of letters within the acquiring alphabet. Basic skills needed to master writing are the differentiation of small muscles and the ability to control hand and finger movements, visuomotor integration, the ability to hold writing utensils, to perform basic strokes needed to draw a line, visual perception and discrimination, visual analysis of letters and words, as well as the orientation in space [6]. Mistakes that a child makes at the level of letters and syllables are omitting, moving, adding a superfluous letter or syllable, substituting and mixing, perseveration and anticipation [6,7]. Mistakes of incorrect disassembly and composition of words and disturbance of their boundaries, as well as errors at the sentence level indicate difficulties in individualizing separate words in oral speech. They are common in children with underdeveloped speech or with somewhat reduced cognitive abilities [8].

Auditory (phonemic) discrimination is the ability to distinguish the sounds of a particular language. It allows the child to distinguish the sounds from each other and to control the harmonization of his own sound production with the production of sounds from the speech environment [5,9]. It gradually develops at preschool age, and the ability to recognize sounds in words is a prerequisite for learning to read and write. These deficiencies in phonemic discrimination during period of phonological development may jeopardize the establishment and organization of spoken sounds. Based on this, it can be assumed that the voice production disorder is related to difficulties in phonemic discrimination [9,10]. After the age of seven, difficulties in auditory discrimination, even of similar sounds, are considered to be a pathological phenomenon. In a number of children, auditory discrimination and speech reproduction are endangered, which puts them at risk of social and academic difficulties. Detecting these difficulties as early as possible is of great importance because appropriate training can be organized for the children [10].

The aim of the study was to determine whether difficulties in auditory discrimination of phonemes are related to the manifestation of dysgraphia in children of younger school age. We hypothesized that students with detected difficulties in auditory discrimination would be more likely to have dysgraphic handwriting in all forms of written expression.

To our knowledge, this is the first study in Serbian language that examines the possible connection between these variables.

Methods

The research was conducted in the elementary school "Vuk Karadzic" in Priboj, during 2020, with the previously obtained consent of the school principal.

The research sample included fifty children of the third and fourth grade, of which 28 were male and 22 were female. The age of the respondents was nine and ten.

The criteria for inclusion in the research were average intellectual abilities and signed parental consent. All students were tested before starting school. The use of this data was approved by the school principal, as well as the students' parents who confirmed that by giving their written consent. Available data in students were whether their achievement on the test was below average, average or above average. The study inclusion criteria involved

students with IQ of 90 and >90. Children who had below-average achievement when enrolling in school did not take this test included in the study.

The writing test was conducted in class, for all students at the same time. The students were first explained the examination procedure. Every student was given a sheet of paper without lines and a pencil with a task to write the text at the dictation of the examiner. The text was dictated at a rhythm appropriate for the age of the children. After finishing writing dictation, the students were tasked with writing an essay on an agreed topic. The final task was to copy the previously dictated text.

The Phonemic Discrimination Test was done individually with each student, in a separate room as it was necessary to isolate the student from all distractors. The Phonemic Discrimination Test lasted for about 15 minutes, while the Dysgraphic Handwriting Assessment Test, for all three forms of written expression, lasted about 30 minutes. Before administering each test, students were given detailed instructions, with the possibility of asking the examiner for additional clarifications.

Two tests were used for the purposes of this research:

1. Phonemic Discrimination Test (Kostić, Vladisavljević, Popović, 1983) - The Phonemic Discrimination Test aims to determine the extent to which respondents of certain age have the ability to discriminate sounds. This test consists of pairs of cards, containing images of objects whose names have a very similar sound context. The differences are usually in one phoneme. The test is composed of 20 pairs of cards - objects, including words with phonemes whose distinctive characteristics are very similar to each other. They do not include all sounds, but only those which are more difficult to discriminate at these developmental ages. The examiner shows two pictures to students while saying the name of the object from one of those pictures. The student is asked to show the object whose name he has just uttered. If the student does the task correctly, he gets one point, if he makes a mistake, he does not get any points. The total number of points is 40. The average score is between 20 and 30 points. Above the average is between 30 and 40 points. The examination is individual [11].

2. Dysgraphic Handwriting Assessment Test (Ajuriaguerra, Auzias, 1971) aims to detect dysgraphia in written text. It contains 25 features, divided into three groups. The first group consists of seven features identifying poor spatial organization of the handwriting as a whole, the second group has thirteen features that represent clumsy spelling, and the third group consists of five features assessing errors in the form and proportions of letters. During the scoring, 0, 0.5 or 1 point can be given for each feature. The obtained point is multiplied by the coefficient given with each feature. The quality of the handwriting is assessed based on the total number of points. A score below 10 indicates harmoniously developed handwriting, a score between 10 and 13.5 indicates inconsistent/difficult to read handwriting, a score between 14 and 18 points represents dysgraphic handwriting, and a score of 19 or more points is an indicator of expressed dysgraphic handwriting [12].

The results were recorded for each child individually, after which the analysis of the obtained data was conducted.

Descriptive statistics was used in the analysis and description of the obtained data, while for the variables that were quantitatively expressed (results in the Phonemic Discrimination Test and the Dysgraphic Handwriting Assessment Test) it was determined that the assumption of normal distribution of results (Kolmogorov-Smirnov test) was not met. A nonparametric Mann-Whitney U test was applied to analyze the differences between boys and girls, as well as between students of third and fourth grade. The Spearman correlation

coefficient was used to determine the relationship between the two tests. The results are presented in tables. Statistical processing and analysis was done using SPSS ver. 20

Results

The research included 50 students, of which 28 were boys (56%) and 22 were girls (44%).

Of the entire sample, 66% of students were in the third grade, while 34% of students attended the fourth grade.

Based on the results shown in Table 1, it can be seen that there is no difference in achievement in the test between boys and girls. Fourth graders scored slightly better compared to third graders.

Half of the tested students achieved the maximum score in the Phonemic Discrimination Test, 8% had a score of 39, and every fourth the score of 38. About 15% of students scored 37 or less points, with a minimum score of 32. The average number of points in the test is 38.8, with the standard deviation of 1.7, which has shown that there were no substantial deviations, or that the students achieved fairly similar results in the Phonemic Discrimination Test.

It can be noticed that girls generally have harmoniously developed handwriting, while more than half of the boys in the categories have inconsistent or dysgraphic handwriting when it comes to the form of dictation (Table 2).

Table 1. Descriptive statistics in the Phonemic Discrimination Test in relation to gender and grade

		N	MIN	MAX	Mean	SD
Gender	boys	28	32	40	38.8	1.8
	girls	22	34	40	38.8	1.7
Grade	third	33	32	40	38.5	1.8
	fourth	17	34	40	39.4	1.5

N-number of subjects, MIN-minimum score, MAX-maximum score, Mean-arithmetic mean, SD-standard deviation

In terms of grades, dysgraphia is more present in younger students, and it is absent in fourth grade students.

Two thirds of students (66%) showed harmoniously developed handwriting on dictation, 16% showed inconsistent handwriting, while every tenth student had dysgraphic handwriting. Distinctly dysgraphic handwriting was found in 8% of students. So, a third of students had a problem with handwriting. The average score on this test is 9.8, with the standard deviation of 5.3.

Based on the results shown in Table 3, it can be seen that girls generally have harmoniously developed handwriting in the form

Table 2. Distribution of students in the Dysgraphic Handwriting Assessment Test in relation to gender and grade (form of dictation)

Dictation		Harm. dev. handwr.	Inconsist./ illeg. handwr.	Dysgr. handwr.	Dist. dysgr. handwr.	Total
Gender	boys	13	6	5	4	28
	girls	20	2	0	0	22
Grade	third	21	3	5	4	33
	fourth	12	5	0	0	17

Harm. dev. handwr. - Harmoniously developed handwriting; Inconsist. / illeg. handwr. - Inconsistent/illegible handwritng; Dysgr. handwr. - Dysgraphic handwriting; Dist. dysgr. handwr. - Distinctly dysgraphic handwriting

Table 3. Distribution of students in the Handwriting Dysgraphic Handwriting Assessment Test in relation to gender and grade (form - free topic)

Free topic		Harm. dev. handwr.	Inconsist./ illeg. handwr.	Dysgr. handwr.	Dist. dysgr. handwr.	Total
Gender	boys	13	6	6	3	28
	girls	19	3	0	0	22
Grade	third	20	4	6	3	33
	fourth	12	5	0	0	17

of free composition, while more than half of the boys in the categories have inconsistent handwriting or dysgraphia. In terms of grades, dysgraphia is more present in younger students, while it is absent in fourth grade students.

About two thirds of students showed harmoniously developed handwriting in the form of writing on a free topic, 18% of them have inconsistent, and 12% of students dysgraphic handwriting. Distinctly dysgraphic handwriting was found in 6% of students. The average score in this test is 10, with the standard deviation of 4.9. Therefore, the variability of results is high among students.

Based on the results shown in Table 4, it can be seen that girls generally have harmoniously developed handwriting, while more than half of the boys in the categories have inconsistent handwriting or dysgraphia in the form of transcription. In terms of grades, dysgraphia is more present in younger students, while it is absent in fourth grade students.

About two thirds of students showed harmoniously developed handwriting, 14% of them inconsistent, and 12% dysgraphic handwriting in the form of transcription. Distinctly dysgraphic handwriting was found in 6% of students. The average score in this test is 9.7, with the standard deviation of 5.3, indicating high variability of results.

No statistically significant correlations were found between the results in the Phonemic Discrimination Test and the Dysgraphic Handwriting Assessment Test (dictation, free topic, transcription), since Sig. > 0.05.

The Mann-Whitney U test found no statistically significant difference in the results of the Phonemic Discrimination Test between boys (Md = 39.50, N = 28) and girls (Md = 40.00, N = 22), U = 296.000, Z = -0.258, p = 0.797.

Table 4. Distribution of students on the Handwriting Dysgraphia Assessment Test in relation to gender and grade (form - transcription)

Transcription		Harm. dev. handwr.	Inconsist./ illeg. handwr.	Dysgr. handwr.	Dist. dysgr. handwr.	Total
Gender	boys	14	5	6	3	28
	girls	20	2	0	0	22
Grade	third	22	2	6	3	33
	fourth	12	5	0	0	17

Discussion

This research focused on examining the association between auditory discrimination of phonemes and the manifestation of dysgraphia in younger school children. The percentage of children with writing difficulties varies in range. In our research, the largest number of children in the third and fourth grade had writing abilities on average, while the smallest number of children demonstrated very superior writing abilities. 50 students of the third and fourth grade participated in the research, 56% of them were boys and 44% of them were girls.

Auditory discrimination is considered to be very important in the initial stages of writing and reading acquisition, while more complex processes (meaning, syntactic structure and context) begin to dominate later on. The tasks of auditory discrimination assess the ability to recognize the distinctive features of sounds, in isolation and in short words [13,14]. Our research showed that in auditory discrimination, as many as half of the tested students achieved the maximum score on the test, 8% of them achieved the score of 39, and every fourth the score of 38. About 15% of the students scored 37 or less points, with a minimum score of 32. No differences were observed in relation to gender, and fourth-graders showed significantly better results than third-graders in the test. However, according to Gligorović et al. (2011), the analysis of the relationship between the gender of the subjects and the achievement in speech comprehension assessment tasks showed that boys were significantly more successful than girls in the areas of auditory discrimination [15].

A study examining the link between phonological disorders and auditory discrimination showed that boys had a higher percentage of unsatisfactory results in the auditory discrimination test. Also, it was confirmed that the age of children affects the maturation of abilities necessary for sound differentiation [16,17].

In the Dysgraphic Handwriting Assessment Test, the children worked on three forms of writing: dictation, transcription and free composition on an agreed topic. All three forms included different modalities. Text dictation and transcription are based on auditory control (as well as physiologically healthy hearing), visual and graphomotor skill, phonemic awareness (the discrimination of voiced and unvoiced and acoustically similar phonemes), established linguistic basis as well as writing speed. Free composition on a given topic presupposes certain knowledge, which a child should demonstrate through a given topic. Difficulties occur in those who have not mastered the technique of writing, do not have sufficiently developed language structures, vocabulary and syntax. This test showed that two thirds of students had harmoniously developed handwriting, and the rest of them had difficulties with handwriting (12% of students had dysgraphia). Girls mostly had harmoniously developed handwriting, while more than half of the boys had dysgraphic handwriting. As for the grade, dysgraphia was more present in younger students, while it was absent in fourth grade students, which is understandable and shows that older students have reached a satisfactory level of skills through practice.

In comparison to other studies, we have observed somewhat higher frequency of dysgraphia in children of younger school age. It can be speculated that there is an increase of dysgraphia prevalence in population of this age, but follow-up studies are needed. [5,13,18].

It has also been proven that students with dysgraphic handwriting often show resistance toward writing and therefore, poorer results when it comes to mastering this skill [19].

The claim that children who exhibit difficulties in auditory discrimination of sounds have a higher number of writing errors proved to be incorrect in our research. No statistically significant associations were found between auditory discrimination and the occurrence of dysgraphia. What we noticed during the

research was that the most common type of writing errors, related to auditory discrimination, was the omission of graphemes. The study conducted by Duranović et al. (2017) on mistakes and creativity in writing included 59 students of the third and fourth grade of an elementary school in Tuzla. The most common type of writing errors in students of that age was the omission of graphemes [18]. The assumption that children with developmental dysgraphia have spelling problems in addition to handwriting problems is confirmed by a study showing that children with dysgraphic handwriting make significantly more mistakes at the level of words and sentences (phonological and lexical mistakes) compared to their peers of typical development. In addition to omitting graphemes in words, children with dysgraphic handwriting showed significantly more errors in copying already written content, as well as replacing already written content with other words and sentences [20,21].

Results show that errors in auditory discrimination and writing are more common in boys of younger school age. Studies conducted by Calasan et al. also show that writing errors are more dominant in boys. A study regarding the effect of writing disorders on school success in students with dysgraphia included 461 students of the third, fourth and fifth grades of three city schools on the territory of the Republic of Srpska. Results show the presence of dysgraphia in school-age children, significantly more common in boys than in girls [22]. Our results showed that girls generally had harmoniously developed handwriting, while more than half of the boys were in the categories of inconsistent handwriting or dysgraphia. Concerns regarding the existing differences between boys and girls are one of the main topics of the study of the impact of gender differences on student writing [23]. Encouraged by the results of the research, teachers' attitudes towards girls having a greater interest in writing and reading can lead to an unfavorable position of boys in the school grading system. This problem can be solved by adjusting the requirements of the teaching process as recommended by experts. Some of these recommendations stem from studies showing that boys are more successful at writing assignments if they are not required to write texts that are too long, as well as if the structure of the written assignment is clear [13].

The analysis of the obtained data showed that third grade students had dysgraphia in the form of dictation, transcription and writing on a free topic. In fourth grade students, there were no elements of dysgraphia in these writing tasks. From that we can conclude that age has a significant impact on the reduction of dysgraphic errors in students. In one of the studies concerning the frequency of mistakes in the use of morphological forms during writing in younger school age students, the authors characterized the period between the first and second grade as very important for acquiring knowledge about the written language. Namely, the second grade children had significantly fewer errors in writing morphological word forms compared to the first grade children. The author believes that this period may be a part of the normal language knowledge consolidation process, since a higher degree of fluency and productivity during writing was found in the second grade children [24].

Research results of some authors speak in favor of high correlation between the ability to read and write, which leads us to the conclusion that these two abilities have much in common. However, by thorough consideration it can be concluded that writing is far more demanding than reading. One of the reasons for this conclusion concerns the interpretation of the importance of orthographic rules necessary when writing. During reading, it is necessary to recognize the visual representation of words and connect them with meaning, but since the orthographic representation must be completely invoked from the mental lexicon, writing is a more complex function [25].

Conclusion

Based on the assessment of writing ability and auditory discrimination in children of younger school age, it was determined that there was no statistically significant correlation between auditory discrimination of sounds and manifestation of dysgraphic handwriting in all three forms of written expression (dictation, free topic, transcription).

The most common type of writing errors associated with auditory discrimination errors is the omission of graphemes.

Boys make more mistakes in auditory discrimination tasks and writing in all forms of written expression.

To our knowledge, there were no studies that compared the same variables in the Serbian-speaking area. The limitation of the study is that a small sample of students was included. Future research, in addition to a larger number of respondents, should also include younger students. Also, the presence of speech and language disorders, as well as learning disabilities, must be taken into account.

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Ethical approval. The Ethics Committee of the Faculty of Medicine, University of Novi Sad, approved the study and informed consent was obtained from all the individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Povezanost auditivne diskriminacije fonema srpskog jezika i disgrafije kod različitih formi pismenog izražavanja

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Uvod. Pisanje je najsloženija ljudska sposobnost i predstavlja najneposredniji oblik komunikacije. Auditivna diskriminacija je sposobnost razlikovanja glasova jezika. Nakon sedme godine teškoće u auditivnoj diskriminaciji, čak i sličnih glasova, smatraju se patološkom pojavom. Cilj istraživanja bio je da utvrdimo da li su poteškoće u auditivnoj diskriminaciji fonema povezane sa ispoljavanjem disgrafije kod dece mlađeg školskog uzrasta.

Metode. Istraživanje je sprovedeno u Osnovnoj školi „Vuk Karadžić" u Priboju, tokom 2020. godine uz prethodno dobijenu saglasnost direktora škole, kao i roditelja učenika. Uzorak istraživanja obuhvatio je pedesetoro dece trećeg i četvrtog razreda, uzrasta 9 i 10 godina. Za potrebe ovog istraživanja koristila su se dva testa, Test fonemske diskriminacije (Kostić Đ, Vladisavljević S, Popović M, 1983) i Test za procenu disgrafičnosti rukopisa (Ajuriaguerra, Auzias, 1971).

Rezultati. Nije ispoljena razlika u postignuću na testu auditivne diskriminacije između dečaka i devojčica. Polovina ispitivanih učenika postiže maksimalan skor na testu auditivne diskriminacije i prilično su ujednačeni u postignuću na testu auditivne diskriminacije fonema. Devojčice uglavnom imaju skladno razvijen rukopis, dok je više od polovine dečaka u kategorijama neskladan rukopis ili disgrafičan rukopis kada se radi o formi diktata, slobodnog sastava i prepisa. Nisu utvrđene statistički značajne povezanosti između rezultata na testu fonemske diskriminacije i testa za procenu disgrafičnosti rukopisa, $p > 0,05$.

Zaključak. Na osnovu procene sposobnosti pisanja i auditivne diskriminacije kod dece mlađeg školskog uzrasta utvrđeno je da ne postoji statistički značajna povezanost između auditivne diskriminacije glasova i ispoljavanja disgrafičnosti rukopisa u sve tri forme pisanog izražavanja (diktat, slobodna tema, prepisivanje).

Ključne reči: auditivna diskriminacija, učenici, pisanje, disgrafija

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Original article

Knowledge and frequency of contacts as factors in forming primary school children attitudes towards peers with developmental disabilities

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Summary

Introduction. Higher level of knowledge and frequent contacts with peers with disabilities can influence the emergence of more positive attitudes of students towards peers with disabilities. In regard to that, our aim was to test the importance of knowledge, contact frequency and other possible factors influencing attitudes of students toward disabled peers.

Methods. The study included 140 students of 4th and 5th grade of primary schools. The research was conducted in the period from December 2020 to March 2021 in two primary schools. The Chedoke McMaster scale was used to examine students' attitudes toward peers with disabilities, while Contact with Disabled Persons Scale and the Children's Knowledge about Handicapped Persons Scale were used to assess frequency of contact and knowledge about disabilities.

Results. Girls showed a significantly higher level (25.21±6.21) of frequency of contacts with students with disabilities compared to boys (19.66±7.30) (p=0.043) and higher level of knowledge (27.88±5.88) about disabilities compared to boys (25.50±4.69) (p=0.009). Respondents who attended school together with children with disabilities (31.07 ± 8.41) showed a significantly higher level of frequency of contacts with students with disabilities compared to respondents who did not attend school with peers with disabilities (13.72±6.32) (p=0.001).

Conclusion. Higher level of knowledge and frequent contacts with peers with disabilities does not have influence on the emergence of more positive attitudes of students towards peers with disabilities.

Key words: attitudes, primary school students, disability

Introduction

Developmental disabilities are defined as severe, chronic disabilities attributable to mental and/or physical impairments that are ''likely to continue indefinitely'', resulting in functional limitations in self-care, learning, receptive or expressive language, self-direction, capacity for economic self-sufficiency and independent living; manifested by the age 22 and requiring care, treatment

or other services of extended or lifelong duration [1]. World Health Organization estimates that more than a billion people live with some form of disability, or about 15% of the world's population [2]. The best possible building for an inclusive community is based on education. If children in schools are not taught to work and live in a community, to tolerate and appreciate differences related to origin, religion, culture, nationality, but also differences in cognitive, emotional, social and sensory-motor abilities, it will undoubtedly form a community that puts people with developmental disabilities at a disadvantage [3,4]. Children who have developmental disabilities already have a large number of challenges that have arisen as a result of their difficulties, such as health and social problems, and therefore society should avoid the possible obstacles in front of them in every possible way, including those of an educational nature. In order to avoid these obstacles, in recent years there has been a tendency to provide children with developmental disabilities, with the conditions to reach the maximum in their achievements and to inlude them in the regular educational system [3,4].

Inclusion implies the practice of including children with disabilities in the general education classroom with full involvement of all students in all aspects of schooling [5]. Given that inclusion is a very complex process influenced by many factors, special attention should be paid to the key participants of inclusion (teachers and students) and to their views on this issue, which should be positively directed [5–7]. Unfortunately, the access of children with developmental disabilities to regular schooling does not always guarantee a good outcome, full participation of inclusion members (teachers, pupils and students) or social acceptance. Although several studies have shown positive academic and social benefits of inclusive education [5,8,9], some others show that during inclusion, students with disabilities face a large number of social problems [10,11]. For example, students with developmental disabilities are significantly less likely than students without disabilities to report a sense of belonging to society, a sense of acceptance and security, or a feeling that other students are pleasant or kind to them, and significantly more likely to report interpersonal conflicts in school, loneliness and the existence of isolation [10].

It is known from literature that the attitudes of students of typical development towards peers with developmental disabilities range from predominantly negative to moderately positive [3]. It has been recognized that the main barrier to social inclusion in school is the existence of negative attitudes of peers, and these negative attitudes can often manifest themselves in the form of name-calling or harassment of students with developmental disabilities [12,13]. The importance of a positive attitude of peers towards children with developmental disabilities is shown by data from the literature, which states that a positive attitude is vital for the progress of students with developmental disabilities. However, in order for students with developmental disabilities to learn and progress to their full potential, it is necessary to find a socially acceptable and supportive environment [14–16].

Since it has already been identified that the emergence of negative attitudes is the beginning of the problem that children with developmental disabilities face during schooling, in order to assess attitudes towards people with disabilities, a large number of studies have been done to improve methods of assessment of attitudes. However, progress in identifying attitudes towards persons with developmental disabilities has been particularly advanced in terms of adult assessment (parents, teachers), but very few studies have worked to assess the knowledge and attitudes of children [3,4,17–19].

Although in our country there is a tendency towards inclusion in education, the literature lacks data on the attitudes of students towards the inclusion of their peers who have

developmental disabilities. There is a large amount of data in the literature on the attitudes of teachers and parents towards inclusion, but there is not much data on the attitudes, as well as frequency of contact and knowledge of children about their peers with developmental disabilities [20,21]. It is very important to understand the attitudes of the participants in inclusion and in which population of children negative attitudes occur more often, because only in this way it is possible to properly formulate and successfully implement inclusive education policy. This is the reason why this topic was chosen, and the main goal of the research was to examine whether there is a difference in attitudes, knowledge and frequency of contact with peers with developmental disabilities, between groups of respondents divided by gender, age and inclusion. Also, the objective of the research was to determine correlation between attitudes, frequency of contact and knowledge of students of typical development towards peers with developmental disabilities.

Methods

Prior to conducting the research, consents were collected from parents whose children participated in the research. A survey was conducted after all obtained consents. The study included 140 respondents, divided into two groups. The first group was consisted of 70 respondents from the typical population which attended classes together with children with developmental disabilities (group with inclusion). The other group was consisted of children from the typical population, which did not attend classes together with children with disabilities (group without inclusion). Students with disabilities attended classes according to a customized program from the first grade with their peers, and had a diagnosis of learning difficulties, as well as moderate intellectual disabilities. The study included approximately equal numbers of male and female respondents. Out of the total number of respondents, 71 (50.7%) were boys, while the remaining 69 (49.3%) were girls. Out of the total number of surveyed children, 30 (21.4%) were nine years old, the majority, 66 (47.1%) were ten years old, while 44 (31.4%) were eleven years old. Half of the respondents were the fourth grade students of primary school (50%), while the remaining 70 (50%) attended the fifth grade of primary school (Table 1).

Table 1. Respondent's socio-demographic characteristics

Characteristics	Number (%), or Mean ± SD
Gender	
Boys	71 (50.7)
Girls	69 (49.3)
Age	
Mean age, Y (SD)	23.1±2.8
9 years	30 (21.4)
10 years	66 (47.1)
11 years	44 (31.4)
Grade	
Fourth grade	70 (50.0)
Fifth grade	70 (50.0)

The research was conducted in two primary schools, "Hilmi ef. Saric" in Tarčin and in the primary school "9. maj" in Pazarić. Demographic data, gender, age, grade were collected through a specially compiled questionnaire, while achievement of students was collected from the grade book in collaboration with the class teachers involved in the research. The research was conducted in the period from December 2020 to March 2021.

Three scales were used in our research Chedoke-McMaster Attitudes Towards Children with Handicaps scale (CATCH) [22], Contact with Disabled Persons Scale (CDP) [23] and the Children's Knowledge about Handicapped Persons Scale (CKHPS) [24]. All three instruments have been translated into Serbian for the purposes of previous research, and we used that version of the instrument [25].

Attitudes of primary school students towards children with disabilities were assessed

by the CATCH scale. Since the research was aimed at assessing attitudes towards students with disabilities, the term "handicap" has been replaced by the term "developmental disabilities." This scale has been translated into Serbian by Talijan et al. [25] and that version was used. The CATCH scale is based on the three-component model of attitudes and encompasses the cognitive, affective, and behavioral domains. The scale includes 36 items with an equal number of positive and negative statements. The total score of the respondents was obtained by summing the items, dividing the sum by the number of particles and then multiplying by 10, while the score of separate domains was calculated by adding the number of points and then divided by the number of questions [26]. The higher score on the scale indicates more positive attitudes. Factor analysis of Rosenbaum et al. [22] showed that factors one and three included affective and behavioral items, and factor two cognitive items, the three-component model of attitudes was not confirmed, and authors suggested that a two-component model may be more appropriate. Therefore, in our research we used a two-component model of attitudes. The reliability of internal consistency for the scale as a whole is high (α = 0.909), as well as for the affective-behavioral component (α = 0.901), while this value is acceptable for the cognitive component (α = 0.705) [27].

In an original CDP scale the items are adapted to the experience of primary school children so that the scale will be focused on assessing the frequency of contact with children with disabilities. This scale consists of twenty items and the frequency of contact may be from never to very often. In order to assess psychometric characteristics, the authors of the scale conducted a study on a sample of 238 people. The internal consistency measured by the Cronbach alpha coefficient was 0.92 [23].

Knowledge of Persons with Disabilities was assessed using the CKHPS scale. The instrument includes 25 items that can be answered on a three-point scale: 0 (no), 1 (not sure) and 2 (yes). Scores can range from zero to 50, and a higher score indicates more knowledge about developmental disabilities. In order to assess the psychometric properties of the scale, Hazard (24) conducted a study on a sample of 411 students attending primary school. The results of the study showed that the reliability, estimated by the Spearman-Brown coefficient, is 0.53 [24].

The methods of descriptive and analytical statistics were used in the paper. Among the methods of descriptive statistics, measures of central tendency and measures of variability were used, namely: arithmetic mean with standard deviation and relative numbers for categorical variables. Distribution was normal and among the methods of analytical statistics Student's t-test and numerical one-factor analysis of variance (ANOVA) for bound samples were used to determine differences in mean values between two or more groups of respondents. Of the nonparametric tests, the chi-square test was used to assess the difference between the groups. For the correlation analysis, Pearson's correlation coefficient was used. The usual value of $p < 0.05$ was taken as the level of statistical significance of differences. Results were statistically analyzed in GraphPad Prism software (GraphPad, La Jolla, CA, USA) and SPSS software package version 21.0 (Statistical Package for Social Sciences SPSS 21.0 Inc, USA).

Results

There was no statistically significant difference between the groups of respondents divided in relation to gender in the average values of the total score of attitudes, affective-behavioral domain or cognitive domain of attitudes towards students with disabilities. A statistically significant difference ($t=-2.315$; $p=0.043$) was observed between boys and girls in the average values of

the CDP scale which estimates the frequency of contacts with students with developmental disabilities. Girls (25.21±6.21) showed a significantly higher level of contact frequency with children with disabilities compared to boys (19.66±7.30). A statistically significant difference (t=-2.647; p=0.009) was observed between boys and girls in the average values of the CKHPS scale of children's knowledge about persons with disabilities. Girls (27.88±5.88) showed a higher level of knowledge about people or children with disabilities compared to boys (25.50±4.69) (Figure 1).

There was no statistically significant difference between the groups of children divided by age at 9, 10 and 11 years in the average values of attitudes towards children with disabilities, as well as the affective-behavioral or cognitive domain of attitudes, and no difference was observed in the frequency of contacts, nor knowledge about people with disabilities (Table 2).

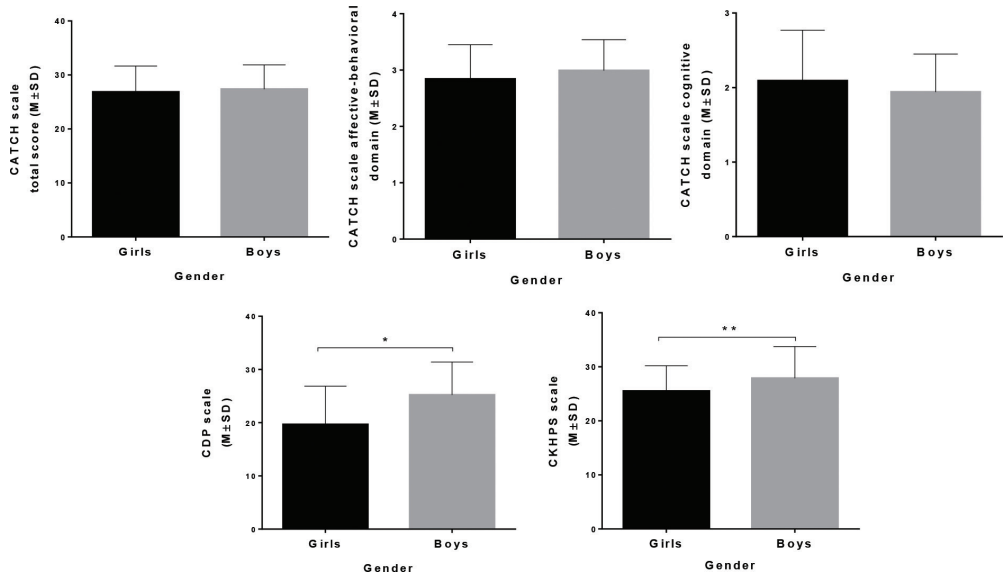

Figure 1. Mean values of attitudes, frequency of contact and knowledge between respondents divided by gender. CATCH - *Chedoke-McMaster Attitudes Towards Children with Handicaps scale; CDP - The Contact with Disabled Persons Scale; CKHPS - Children's Knowledge about Handicapped Persons Scale, M – mean, SD – standard deviation; *p<0.05; **p<0.001.*

Table 2. Attitudes, frequency of contacts and knowledge about people with disabilities in relation to the age of the respondents

Attitudes, frequency of contact and knowledge about developmental disabilities	Age Number (%)			F	p
	9 years	10 years	11 years		
CATCH (totale score)	26.18±4.89	27.35±4.35	27.32±4.99	0.719	0.489
Affective-behavioral domain	2.71±0.53	2.95±0.54	3.01±0.69	2.501	0.086
Cognitive domain	2.22±0.43	1.98±0.70	1.94±0.54	2.272	0.107
CDP (total score)	21.50±7.31	22.45±7.51	22.93±9.98	0.055	0.947
CKHPS (total score)	25.20±6.20	27.39±5.50	26.61±4.60	1.708	0.185

Neither statistically significant difference in the average values of the total score of attitudes towards students with disabilities, affective-behavioral or cognitive domain, nor knowledge about people or children with disabilities was observed between the groups of respondents divided in relation to inclusion. Between the groups of respondents divided into groups in relation to whether they attend classes together with students with disabilities or not, a statistically significant difference (t=6.386; p=0.001) was observed in the average values of the scale for assessing the frequency of contacts with children with developmental disabilities. Respondents who attend school together with children with disabilities (31.07±8.41) showed a significantly higher level of frequency in contacts with students with disabilities compared to respondents who do not attend school with peers with disabilities (13,72±6.32) (Figure 2).

Pearson's correlation coefficient showed the existence of a statistically significant positive and strong correlation between the overall score of attitudes towards children with disabilities with the affective-behavioral domain of attitudes (r=0.939; p<0.01), as well as moderately strong and positive correlations with cognitive attitude domain (r=0.432; p<0.01). A statistically significant, weak and positive correlation was also observed between the affective-behavioral and cognitive domains of attitudes (r=0.172; p<0.05). These results show that children who have more positive attitude towards children with disabilities, at the same time have higher level of affective-behavioral and cognitive attitudes. A statistically significant correlation between the frequency of contact and knowledge about persons with disabilities in relation to the attitude of the respondents towards students with disabilities was not observed (Table 3).

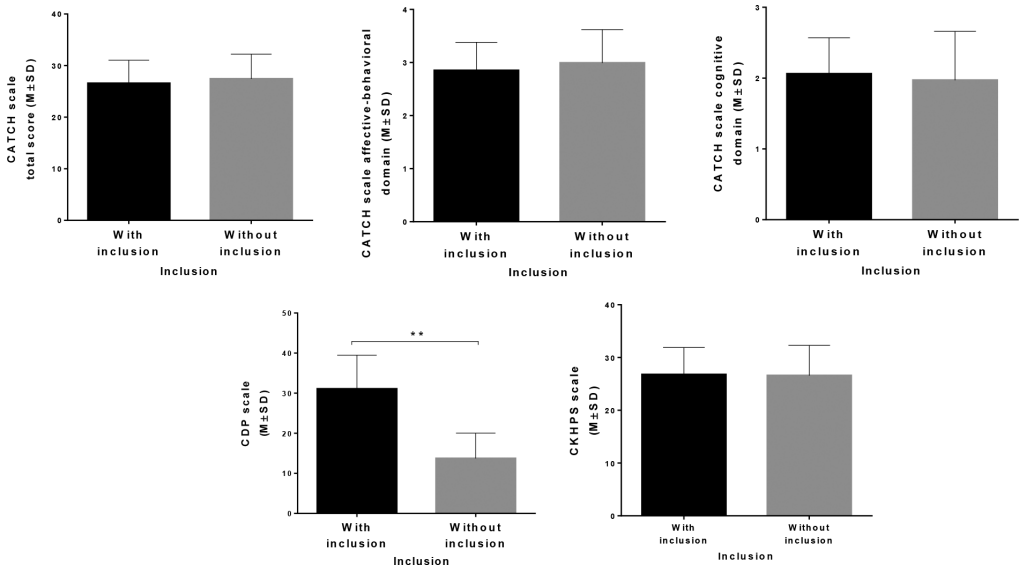

Figure 2. Mean values of attitudes, frequency of contact and knowledge between respondents divided by inclusion. CATCH - *Chedoke-McMaster Attitudes Towards Children with Handicaps scale; CDP - The Contact with Disabled Persons Scale; CKHPS - Children's Knowledge about Handicapped Persons Scale, M – mean, SD – standard deviation; *p<0.05; **p<0.001.*

Table 3. Correlation between the frequency of contact and knowledge about people with disabilities in relation to the attitude of respondents towards children with disabilities

	Attitudes (total score)	Affective--behavioral domain (attitudes)	Cognitive domain (attitudes)	Frequency of contact	Knowledge about people with disabilities
Attitudes (total score)					
Affective-behavioral domain (attitudes)	**0.939****				
Cognitive domain (attitudes)	**0.432****	**0.172***			
Frequency of contact	0.100	0.133	0.055		
Knowledge about people with disabilities	0.063	0.073	-0.148	0.020	

Pearson's correlation coefficient (r) was used, r values are shown in the table. * $p < 0,05$, ** $p < 0,01$

Discussion

In our study, we examined 140 fourth and fifth grade students. In relation to inclusion, we divided the respondents into two groups, a group of 70 respondents who do not attend classes together with students with disabilities and a group of 70 respondents who attend classes together with their peers with disabilities. In our research, neither statistically significant difference was observed between students who are in inclusion and those who are not in the overall score of attitudes, nor in the affective-behavioral or cognitive domain of attitudes. Our results are confirmed by the research of Dyson et al. [5] conducted on a sample of 77 children in Canada, which found that the attitudes of children with typical development, who had contact with children with developmental disabilities, were almost the same as those of children with typical development who had no contact with children with developmental disabilities [5]. Although most studies have shown conflicting results compared to ours and Dyson et al. [5], that children attending school with children with developmental disabilities have a significantly higher level of positive attitudes compared to children who do not attend inclusive classes and have never been in contact with a peer who has developmental disabilities [8,28]. A study conducted in Canada [28] on a sample of nearly 2,000 students showed a statistically significantly higher average value of the overall score of attitudes toward children with disabilities in children who are included, compared to children who are not. Also, the study of Nikolaraizi et al. [8] conducted in the United States of America (USA) and Greece has shown that children who are included accept children with developmental disabilities significantly faster and better than children who are not included [8].Although these results are encouraging, all mentioned studies indicate that children in inclusion have a positive attitude towards children with developmental disabilities, but they are not interested in socializing, playing, sitting together on a school bench or learning with peers with disabilities [8,28]. These results show that the existence of

a positive attitude of students does not mean their willingness to fully accept children with disabilities.

Most of the studies show that girls have a significantly more positive attitude towards peers who have developmental disabilities, which could be explained by the earlier development of empathy in relation to boys [18,19]. In our study, we neither observed a statistically significant difference between girls and boys in attitudes, nor between different groups of respondents divided by age. However, there are studies whose results collaborate with our results [29], where no differences in attitudes were observed between boys and girls.

Contact is defined as personal experience with members of a stigmatized group, and the effect of contacts on forming attitudes is the focus of a large number of studies that have shown that general personal contact with a person with a developmental disability has a positive effect on reducing negative attitudes. However, the relationship between contact and attitudes is much more complex, because making contact with a person with a developmental disability does not always result in the formation of positive attitudes [19,30]. Our results showed that there was no significant correlation between frequency of contact and attitudes of the respondents towards peers with developmental disabilities. Our results were confirmed in a study by Barr and Brachitta [31] who showed that a mere contact with people with disabilities was not necessarily correlated with positive attitudes and that better predictor of positive attitudes was the type of disability one had contact with [31]. However, our results showed that girls (25.21±6.21) had a significantly higher (p=0.043) level of contact frequency with students with developmental disabilities compared to boys (19.66±7.30). In our study we showed that contact with children with developmental disabilities was significantly (p=0.001) more frequent from respondents who attend school together with children with disabilities in comparison to respondents who do not attend school with peers with disabilities. However, between groups of children divided by age, we did not observe a significant difference in the average values of total score of CDP scale. These results showed that girls and included children were more willing to communicate and socialize with peers who have developmental disabilities compared to boys.

Acceptance of peers with developmental disabilities usually increases with age, most often because children gain more knowledge about the world around them as they grow. On the other hand, this knowledge does not always have to be positive, so it happens that children also learn negative cultural values and norms related to disabilities. According to a study by Hazzard et al. [24], conducted in the USA, on a sample of 367 students in which knowledge about people with disabilities was examined, no statistically significant difference was observed between boys and girls. However, the authors observed that older students (10 and 11 years of age) had significantly higher level of knowledge about people with disabilities compared to younger students (8 and 9 years). In contrast to this study, we did not notice a significant difference in the average values of the total score of CKHPS scale, neither in the score of CATCH and CDP scores between the groups of children aged 9, 10 and 11. However, the results of our study showed that girls (27.88±5.88) had a significantly higher level of knowledge about disabilities compared to boys (25.50±4.69) (p=0.009). The importance of acquiring knowledge about people with disabilities is shown in a study by Talijan et al. [25] in which a change in attitudes toward students with Down syndrome was examined using an imaginary contact program. The experimental group was educated by a program of imagining contact with children with Down syndrome for 6 weeks, while the control group

of the examined children was not educated in that period. The authors found that in the experimental group of children after education, the total score of attitudes (109.72±20.79) and knowledge (34.36±8.90) about persons with developmental disabilities was statistically significantly higher than the score of attitudes (73,52±23.57) ($p<0.001$) and knowledge (31.12±6.23) ($p=0.031$) before education by imaginary contact, while in the control group of examined students no statistically significant difference was observed, before and after the period without education.

One of the goals of the study by Hazzard et al. [24] was to examine whether knowledge had an impact on the attitudes of peers who have a disability. The study found that there was a statistically significant and positive correlation between knowledge and attitudes ($r=0.350$; $p<0.001$), which means that children who have more knowledge about people with disabilities have a more positive attitude and they accept these children better. Based on this data we assumed that children who have more knowledge about children with developmental disabilities also have more positive attitudes. However, our study did not find a statistically significant correlation between the knowledge or frequency of contacts in relation to the attitudes of respondents towards students with disabilities, which means that we found that higher frequency of contact and higher level of knowledge do not mean more positive attitude.

Conclusion

Our research has shown that girls have a significantly higher level of knowledge and frequency of contacts toward peers with developmental disabilities. Also, students who are included have more frequent contacts with peers with developmental disabilities than students who are not included, but difference in terms of attitudes and knowledge were not observed. The differences in attitudes, frequency of contact and knowledge between groups divided by age were not observed. Our findings suggest that frequency of contacts and knowledge of students about peers with disabilities does not impact the formation of more positive attitudes towards students with developmental disabilities.

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Ethical approval. The Ethics Committee of the Primary school "Hilmi ef. Šarić", Tarčin, approved the study and informed consent was obtained from all the individual respondents. The research was conducted according to the Declaration of Helsinki.

Conflicts of interest. The authors declare no conflict of interest.

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Znanje i učestalost kontakata kao faktora u formiranju stavova djece osnovnoškolskog uzrasta prema vršnjacima sa smetnjama u razvoju

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Uvod. Viši nivo znanja i česti kontakti sa vršnjacima sa smetnjama u razvoju mogu uticati na pojavu pozitivnijih stavova učenika prema vršnjacima sa smetnjama u razvoju. Cilj istraživanja je bio da se ispita važnost znanja, učestalosti kontakata i drugih faktora koji mogu uticati na stavove učenika tipične populacije prema vršnjacima sa smetnjama u razvoju.

Metode. Studija je obuhvatila 140 učenika 4. i 5. razreda osnovnih škola. Istraživanje je sprovedeno u periodu od decembra 2020. do marta 2021. godine, u dvije osnovne škole. Chedok McMaster skala korišćena je za ispitivanje stavova učenika prema vršnjacima sa smetnjom u razvoju, dok su skala za kontakt sa hendikepiranim osobama i skala o znanju djece o osobama sa smetnjama u razvoju korišćene za procjenu učestalosti kontakata i znanja o smetnjama u razvoju.

Rezultati. Djevojčice su pokazale značajno viši nivo (25,21±6,21) učestalosti u kontaktima sa učenicima sa smetnjom u razvoju u poređenju sa dječacima (19,66±7,30) (p=0,043) i viši nivo znanja (27,88 ± 5,88) o smetnjama u razvoju u poređenju sa dječacima (25,50±4,69) (p=0,009). Ispitanici koji školu pohađaju zajedno sa djecom sa smetnjama u razvoju (31,07 ± 8,41) pokazali su znatno veći nivo učestalosti kontakata sa učenicima sa smetnjama u razvoju u odnosu na ispitanike koji ne pohađaju školu sa vršnjacima sa smetnjama u razvoju (13,72±6,32) (p=0,001).

Zaključak. Naše istraživanje je pokazalo da djevojčice imaju viši nivo znanja i kontakata prema vršnjacima sa smetnjama u razvoju. Takođe, učenici koji su u inkluziji imaju češće kontakte sa vršnjacima sa smetnjama u razvoju od učenika koji nisu u inkluziji.

Ključne riječi: stavovi, učenici osnovne škole, smetnje u razvoju

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Original article

The knowledge of nurses about prevention of infections caused by the bacteria Clostridium difficile

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Summary

Introduction. Clostridium difficile is the leading cause of nosocomial diarrhea, associated with the use of antibiotics. The most common ways of transmitting the infection in hospitals are contaminated surfaces of the premises and the hands of medical staff.

Methods. The study involved 68 nurses/technicians employed at the University Hospital Foca in the departments of surgery and internal medicine. As a research instrument, we used a specially designed questionnaire, created by the authors for the purpose of this research.

Results. The research showed that 61.8% of respondents knew that hand washing with warm water and soap was considered the most effective prevention of the spread of infections, and 55.88% meant that they used chlorine-based preparations and hydrogen peroxide as the only effective disinfectant. Nurses with a work experience of less than 5 years showed better knowledge than other groups.

Conclusion. The knowledge of nurses about the prevention of C. difficile infection is not at a satisfactory level, which indicates the growing need for education of nurses.

Key words: Clostridium difficile, nurses' knowledge, nosocomial infections

Introduction

Clostridium difficile (C. difficile) is an anaerobic Gram-positive, sporogenic bacterium present in the soil and colon of the digestive tract of animals, healthy children and adults up to 5% of the microflora. It is excreted in the host's stool, and the infection is caused by spores present in the human digestive tract or by ingestion of spores and vegetative forms of the bacterium, contaminated food, water, and by dirty hands. Studies published so far indicate that C. difficile in most cases causes infections in hospitalized patients. The most common routes of transmitting the infection in hospitals are contaminated hospital surfaces and hands of medical staff [1].

Clostridium difficile infection (CDI) is acquired by ingestion of spores, usually transmitted from other patients, through the hands of medical staff and contaminated surfaces [2]. The spores of this bacterium are resistant to stomach acidity, so they can germinate into a vegetative form in the small intestine. Disruption of the normal intestinal flora, usually by exposure to antibiotics, allows C. difficile proliferation, causing a wide range of clinical manifestations that can range from asymptomatic colonization, diarrhea of varying severity to fulminant colitis, and even death [3]. Manifestations of CDI are abdominal pain and cramps, profuse diarrhea (mucous, dirty-green, watery stools with unpleasant odor) with fever and leukocytosis, and life-threatening complications such as pseudomembranous colitis, toxic megacolon and colon perforation [4]. CDI usually manifests a few days after taking antibiotics [1].

The topic of knowledge of health professionals about CDI has not been much covered in the professional literature, so there was not much data to compare the knowledge of nurses at the University Hospital in Foca. So far, there is no standardized questionnaire on nurses' knowledge of CDI.

Methods

The research was conducted as an observational cross-sectional study, in which 68 nurses and technicians participated, including interns (volunteers), at the University Hospital in Foca, employed in the departments of internal medicine and surgery. The study included nurses/ technicians who were at work on the days of the study and who gave their voluntary consent to participate in the study. The study was conducted in the period from June to October 2018.

A specially designed questionnaire, developed by the authors for the purposes of this research, was used to assess the knowledge of nurses/technicians about CDI prevention. The questionnaire contained 16 multiple-choice questions.

Computer processing of the data was performed using the Statistical Package for the Social Sciences (SPSS) version 20. Data are presented as percentages (%). The Chi-square test was used to determine knowledge about prevention in relation to work experience.

Results

The study involved 68 nurses and technicians, 39 from surgery and 29 from the internal medicine department. The total ratio of technicians and nurses was 19:49 or 27.9% of medical technicians and 72.1% of nurses.

The research involved nurses/technicians with different levels of education and different lengths of work experience. In the surgery department there were 29 nurses with high school, 1 nurse with higher school and 9 nurses with the university degree, while in the internal department there were 19 nurses with high school and 10 nurses with the university degree, there were no nurses with higher school.

There were 13 nurses with work experience of up to 5 years, 13 nurses with work experience of 5–10 years and 13 nurses with work experience of over 10 years, while the internal medicine department had 16 nurses with work experience of up to 5 years, 3 nurses with work experience of 5–10 years and 10 nurses with work experience over 10 years.

We have to mention that not all employees from these departments participated in the survey, so the data presented were obtained exclusively from survey participants.

During the survey, we found that 8.8% of respondents thought that Clostridium difficile causes urogenital infections, 7.4% thought that it causes wound infections, 5.9% thought that it causes respiratory infections, while 77.9% knew the correct answer or that Clostridium difficile causes infections of the gastrointestinal tract.

By processing the data, we have found that 10.3% of respondents think that Clostridium difficile infection is transmitted by droplets, 7.4% of them think that it is transmitted by blood, while 82.4% of respondents have an opinion that it is transmitted by fecal-oral route, which is the correct answer.

Among the respondents, 41 of 68 of them knew that all the listed antibiotics (cephalosporins, penicillins, fluoroquinolones and clindamycin) could be associated with the occurrence of CDI, which is 60.29% of the participants in the study. We received the most correct answers from the group of employees with work experience shorter than 5 years, which makes as many as 72.41% of respondents from this group. While 43.75% of employees with work experience from 5 to 10 years gave the correct answer, and the employees with work experience over 10 years, or 56.52% gave the correct answer.

In Table 1 we can see that only 36.8% of respondents know the correct answer, that the most successful preventive measure for the occurrence of CDI is the correct use of antibiotics. Also, 5.9% of respondents consider that the most successful preventive measure is the use of gloves during the examination of patients, hand washing 8.8%, preventive use of metronidazole 10.3%, and all of the given answers 38.2%.

By processing the data, we came to the conclusion that 10.3% of respondents thought that the most effective measure to prevent the spread of infection in hospital conditions was wearing gloves while working with patients, 7.4% believed that it was hand disinfection with alcohol-based disinfectants, 61.8% were aware that the most effective prevention of spreading CDI was washing hands with warm water and soap, while 20.6% thought that all of the offered answers were correct.

We also obtained data that 38 out of 68 subjects knew that the best disinfectant for the prevention of CDI was chlorine and hydrogen peroxide-based disinfectants, which is 55.88% of the participants. On this question, we received as many as 21 correct answers from employees whose work experience is shorter than 5 years, which makes 30.88% of all correct answers.

28 respondents or 41.2% knew that oral metronidazole was used as the first drug of choice.

Only 2.9% of respondents did not link the beneficial properties of probiotics during or after antibiotic therapy and do not recommend it to patients, 26.5% believed that it was sometimes recommended to give probiotics to patients during or after antibiotic therapy, while 70.6% of respondents believed that the use of probiotics was recommended with or after antibiotic therapy.

Table 1. Nurses' knowledge on the most successful CDI prevention measure

The most successful preventive measure	CDI Respondents
Proper use of antibiotics	36.8
Use of gloves during patient examination	5.9
Hand washing	8.8
Preventive use of metronidazole	10.3
All of the above	38.2

Discussion

Clostridium difficile is an anaerobic, sporogenic, gram-positive bacillus. It is the main cause of antibiotic-associated diarrhea, which is prevalent in hospital settings. Morbidity and mortality from CDI increased significantly due to the emergence of hypervirulent strains. Due to the poor clinical distinction between CDI and other causes of hospital-acquired diarrhea, a laboratory test for C. difficile is a very important intervention for the diagnosis of CDI [5]. Observing the

results of this research, we could notice that 77.9% of nurses and technicians knew what type of infection was caused by Clostridium difficile, or that they were infections of the gastrointestinal system. While as many as 82.4% were familiar with the route of CDI spread, or that it was a fecal-oral route of spread.

In 2012, Brady and colleagues published a paper on creating a standardized questionnaire to assess knowledge about CDI infection and prevention. These questionnaires were tested in control populations that included either a nurse - infection control specialist, or non-clinically trained individuals, and a cohort of medical staff. They found that all questionnaires studied significant discrimination between non-clinical and clinical populations and had similar levels of sensitivity and specificity in discrimination between these target populations. This study describes the development of a usable CDI knowledge assessment tool that can be used to assess knowledge levels, compare populations, and prepare targeted education [6].

Studies clearly indicate that certain antibiotics used for treatment are able to disrupt the intestinal microflora and lead to increased sensitivity of the patient, and thus allow the colonization of C. difficile. Broad-spectrum antibiotics disrupt the normal intestinal flora, so CDI is thought to be associated with antibiotic use [7].

Significant risk factors for patient-related CDI are antibiotic exposure, old age, and hospitalization [7]. Age > 65 increases the risk of CDI by 5 to 10 times compared with patients younger than 65. However, a significant share of CDI also occurs in the younger population. Age > 65 is a significant risk factor not only for CDI, but also for poor clinical outcome of the disease, due to comorbidities in the elderly [8,9]. Residents of nursing homes are at higher risk for CDI of the total population, but at lower risk than hospitalized patients (15%). This is mainly due to older age, comorbidities, more frequent hospitalizations and more frequent antibiotic therapies in this group compared to the non-institutionalized population. C. difficile is the most common cause of nosocomial diarrhea [10].

Today, the use of broad-spectrum antibiotics is extremely widespread and controlling the spread of C. difficile has become a huge task. The most common antibiotics often associated with CDI are cephalosporins, clindamycin, amoxicillin, and fluoroquinolones [11]. Almost every antibiotic is associated with the development of CDI, including drugs used to treat CDI: metronidazole and vancomycin [8]. Other well-defined risk factors for CDI include inflammatory bowel disease, gastrointestinal surgery, decreased immune response as a result of malignancies, transplantation, chronic kidney diseases, or the use of immunosuppressants [8,12]. Observing the results of the study, 60.29% of nurses and technicians knew that the above mentioned antibiotics could be associated with the occurrence of CDI. Other respondents thought that some of them could be associated with infection, which we cannot consider as a mistake since they are all associated with CDI. Even 30.38% of correct answers were from employees with a length of experience of less than 5 years.

The choice of antibiotic therapy should be adjusted to the severity of a disease. Common antibiotics used for milder forms of the disease are either oral metronidazole or vancomycin. Among them, metronidazole is usually recommended for the treatment of mild and moderate diseases, while oral vancomycin is generally not recommended [13,14,15]. New evidence suggests that vancomycin is superior to metronidazole and fidaxomicin is superior to vancomycin. The differences in efficacy between these antibiotics are not large, and the advantage of metronidazole is its far lower price compared to the other two antibiotics [16]. In our study, 41.2% of respondents knew that oral metronidazole was used as the first-choice antibiotic.

Hygienic hand washing with running water and soap is mandatory because alcohol-based hand sanitizers are not effective in neutralizing C. difficile spores. The patient's environment should be disinfected with chlorine preparations [17]. As the best way to prevent the spread of infection in hospital conditions, the World Health Organization advocates the use of soap with the use of alcohol-based preparations to limit the spread of spores [18], which was confirmed by 61.8% of respondents. However, 55.88% of respondents knew that chlorine and hydrogen peroxide-based preparations were recommended as the best means of disinfecting bedside tables, handles, bed rails, etc. Even 30.88% of respondents had a work experience of less than 5 years, which leads us to think that younger workers are more informed about modern research.

Thirty-one studies (8,672 participants) evaluated the efficacy of probiotics for preventing CDI among participants taking antibiotics. Their results suggest that when probiotics are given with antibiotics, the risk of developing CDI is reduced by an average of 60% [19], which was confirmed by 70.6% of respondents, believing that the use of probiotics during or after the use of antibiotics is recommended.

In a survey conducted in the United Kingdom in January 2007, we found that more than 50% of healthcare professionals correctly identified C. difficile as anaerobic bacillus. One-third of nurses were aware that 5% of adults carried C. difficile in their intestines. Among the respondents, 40% of healthcare workers were aware of the spectrum of diseases caused by C. difficile, while 26 (37%) nurses correctly identified various predisposing factors for acquiring CDI. Only 8% of medical staff was aware that antibiotic restriction was the simplest measure of CDI control. Only 28 (38%) nurses were aware that hand washing with soap and water was the most effective way to prevent CDI transmission. The results show that 70% of nurses correctly answered that oral metronidazole is a drug of choice for the treatment of CDI [20]. In our study, only 36.8% of respondents knew that the most successful preventive measure for the occurrence of CDI was the correct use of antibiotics, or limited use of antibiotics. Comparing other data, it can be said that the knowledge of our nurses is somewhat better than the results in the above given study.

Regardless of the mentioned results, we can consider that the knowledge of nurses about CDI prevention is not at a satisfactory level. The importance of CDI is indicated by a study of the prevalence of nosocomial infections, conducted in hospitals in eastern Herzegovina in August 2011, which shows that the prevalence of patients with nosocomial infections was 4.2%, of which Clostridium difficile is considered to be the cause of 40% or 1.68% of total infections [21]. At the time when the above study was done, the diagnosis was not well established, so there is an impression of low frequency. There are no recent data on the incidence of nosocomial infections at the University Hospital in Foca. Given that nurses' knowledge of CDI prevention is not at a satisfactory level, it is to be expected that the current incidence of these infections is higher.

The rate of C. difficile resistance to antimicrobial agents is growing rapidly worldwide. The CDI has become a major concern for the public health service. CDIs are unique due to increased incidence as well as the increased use of certain antibiotics [22].

Conclusion

Nurses' knowledge of CDI prevention is not satisfactory. Accordingly, it is necessary to define prevention measures and early detection of infected patients, and to prevent the spread of infection, in order to minimize this phenomenon. This indicates the growing need for education and information of nurses, both with high school and with the faculty. This problem could be solved through the

organization of seminars or online education for health professionals, training on methods of proper dressing, undressing and disposal of protective equipment used in the care of infected patients. Also, the introduction of training on disinfectants and methods of cleaning the ward, for staff involved in maintaining the hygiene of the ward, would help preventing the spread of infection in the ward, and thus the frequency of CDI.

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Ethical approval. The Ethics Committee of the University Hospital Foca approved the study and informed consent was obtained from all individual respondents.

Conflicts of interest. The authors declare no conflict of interest.

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Znanje medicinskih sestara o prevenciji infekcija izazvanih bakterijom Clostridium difficile

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Uvod. Clostridium difficile je vodeći uzročnik nozokomijalnih dijareja povezanih sa upotrebom antibiotika. Najčešći putevi prenosa infekcije u bolnicama su kontaminirane površine prostorija i ruke medicinskog osoblja. Cilj rada je bio da se ispita znanje medicinskih sestara o prevenciji infekcija izazvanih bakterijom Clostridium difficile.

Metode. U studiji je učestvovalo 68 medicinskih sestara/tehničara zaposlenih u Univerzitetskoj bolnici u Foči na odeljenjima hirurgije i interne medicine. Kao instrument istraživanja korišćen je posebno dizajniran upitnik, kreiran od strane autora u svrhu ovog istraživanja.

Rezultati. Istraživanjem smo došli do podatka da je 61,8% ispitanika znalo da se pod najefikasnijom prevencijom širenja infekcije smatra pranje ruku toplom vodom i sapunom, a 55,88% da se kao efektivno dezinfekciono sredstvo koriste preparati na bazi hlora i vodonik peroksida. Zaposleni sa radnim stažom kraćim od pet godina su pokazali bolje znanje od ostalih grupa.

Zaključak. Znanje medicinskih sestara o prevenciji infekcija izazvanih bakterijom Clostridium difficile nije na zadovoljavajućem nivou, što ukazuje na sve veću potrebu za edukacijom i informisanjem medicinskih sestara.

Ključne reči: Clostridium difficile, znanje medicinskih sestara, nozokomijalne infekcije

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Review

Late postoperative complications of arteriovenous fistula for hemodialysis

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Summary

The vascular approach is a prerequisite for performing hemodialysis, but their "weak points" are different and frequent complications. Modern guidlines recommend native arteriovenous fistula (AVF) as the first choice of vascular approach, because it is characterized by the longest survival and the least complications compared to other vascular approaches. All complications of AVF can be divided into intraoperative, early, and late postoperative. This paper presents the late postoperative complications of AVF, their frequency, causes, diagnosis and treatment.

The most important late postoperative complications are: stenosis, thrombosis, aneurysm or pseudoaneurysm formation, infection, hand edema, hematoma, ischemic steal syndrome, ischemic neuropathy, congestive heart failure. Large differences in the frequency of each complication in earlier studies can be explained by differences in surgical technique, localization of AVF, diagnostic methods, but, above all, differences between the presented groups of patients. It is described that the age of patients, sex, underlying disease, the presence of comorbid conditions and various metabolic and immune disorders characteristic of chronic renal failure, as well as the way of using and caring for AVF significantly affect the occurrence of AVF complications. One of the main predictors of AVF success and survival is the quality of the patients' blood vessels, and therefore careful examination of blood vessels before approaching AVF creation is of particular importance.

The creation, use and care of AVF is the task of the team of health professionals who take part in the treatment of these patients, and successful treatment requires their good cooperation, as well as cooperation with patients.

Key words: arteriovenous fistula, hemodialysis, late complications, risk factors

Introduction

The number of patients with end-stage renal disease requiring treatment with renal replacement methods is steadily increasing. According to the Renal Registry of Bosnia and Herzegovina, during the period from 2002 to 2018, the number of patients treated with kidney replacement methods increased from 1,616 to 2,703, and at the same time in the Republic of

Srpska the number of these patients doubled (536–1,082) [1]. The largest percentage of patients with end-stage renal disease is treated with hemodialysis worldwide, even in Bosnia and Herzegovina, where in 2018, 2,238 (83%) patients were treated with hemodialysis, and in 1,740 (78%) of them the vascular approach was arteriovenous fistula (AVF).

The vascular approach is a prerequisite for performing hemodialysis, and modern guidlines recommend native arteriovenous fistula (AVF) as the first choice of the vascular approach, because it is characterized by the longest survival and the least complications compared to other types of vascular approach [2,3]. However, vascular approaches are the "weak point" of hemodialysis because all vascular approaches, including AVF, are associated with various complications. A number of studies have shown that about 30% of hospitalizations of patients on hemodialysis are necessary for the formation and/or solving the complications of vascular approaches. The costs of vascular approaches account for 14–20% of the total costs of treating hemodialysis patients [4]. In addition, AVF complications are associated with increased morbidity and mortality, and prevention, early detection, and forehand and adequate treatment of complications can significantly reduce the number and duration of hospitalizations, other serious complications, and mortality [2,5,6].

As most patients with end-stage renal disease in our country are treated with hemodialysis, special attention should be paid to the formation of AVF, their care, and especially to the prevention and treatment of the complications. All complications of AVF can be divided into intraoperative and postoperative, and the latter can be early and late. This paper is presenting late postoperative complications of AVF, their frequency, causes, diagnosis, and treatment.

The most important late postoperative complications are: stenosis, thrombosis, inadequate flow through the fistula, aneurysm or pseudoaneurysm formation, infection, hand edema, hematoma, ischemic steal syndrome, ischemic neuropathy, congestive heart failure. In some of the examined groups not all of them are registered. Data on the prevalence of some late complications of AVF are very different, as illustrated by Table 1, which shows the data of several groups of authors. Aljuaid et al. [6] cite ischemic neuropathy (29.6%) and AVF aneurysms (25%) as the most common complications, and the last in several studies account for over 45% of all complications [8–10]. On the other hand, in a study by Greenberg et al. [11] AVF stenosis was most commonly registered (51.4%). The data of Schinstock et al. [12] who describe bleeding (33%) and AVF infection (26.8%) as the most common complications of AVF, are completely different. While bleeding, hematomas, and edema are not reported in most studies, there are studies in which the percentage of patients with these complications is not negligible [13,14]. In our prospective study, which included 250 patients, thrombosis and inadequate flow

Table 1. Incidence of late postoperative complications

Complication	In studies by other authors [4,6-14]	In the study by Z. Maksimović [15]
Stenosis	14-51.4	
Thrombosis	14.6-45	27.4
Aneurysm and pseudoaneurysm	3.8-60	1.2
Heart failure	12-17	
Ischemic neuropathy	1-29	
Infection	2-4.4 23.8	4.1
Bleeding	9.7-33	4.1
„Steal" syndrome	1.1-8	
Edema	1.5-3.7	23.3
Inadequate flow through AVF		24.7
Haematoma	8.4	16.4

AVF – arteriovenous fistula

through AVF are the most common complications [15]. Differences in the frequency of each complication can be explained by differences in surgical technique, AVF localization, diagnostic methods, but, above all, differences in the characteristics of patients and the quality of their blood vessels. It has been described that the age of patients, sex, underlying disease, comorbidities, but also the use and care of AVF significantly affect the occurrence of complications and survival of AVF [4,8,16].

Characteristics of late postoperative complications of arteriovenous fistula for hemodialysis

Stenosis. The basis of stenosis is the process of neointimal hyperplasia that occurs in the venous part of the AVF in response to endothelial cell injury. This injury can occur during surgery, AVF puncture, due to altered hemodynamic forces, effects of uremic toxins [17,18]. Damaged endothelial cells produce inflammatory mediators that activate platelet aggregation and attract leukocytes to the damaged site. At the same time, activated endothelial cells increase the expression of growth factors that stimulate the migration of smooth muscle cells from the medium into the intima, their proliferation and the deposition of the extracellular matrix. All this leads to the formation of neointimal hyperplasia, a fibromuscular thickening of the blood vessel wall [18,19]. The frequency of stenosis greatly varies in published studies, which is due among other things to the different diagnostic procedures used to diagnose it. In our prospective study conducted in the period from 2002 to 2010 there was no possibility to diagnose AVF stenosis, but it could be assumed that among patients with inadequate flow through AVF, a large percentage had stenosis, which was confirmed in 15 patients during reintervention [15]. Clinically significant stenosis should always be suspected when the flow through the fistula is reduced, but also when there are problems with puncture, prolonged post-hemodialysis bleeding, pain in the fistula, longer maintenance of hand swelling, increased venous pressure. Doppler ultrasound is used to diagnose stenosis, and peak systolic velocity greater than 400 cm/sec indicates the presence of stenosis. In addition, a flow drop of 20–25% per month is considered as an indicator of stenosis. The sensitivity of the ultrasound examination to detect significant stenosis ranges between 76–87%. Angiography is a reliable method for determining AVF stenosis, but it is a more expensive and invasive method and requires the use of contrast [4]. Treatment of stenosis includes balloon dilation, stent implantation, or surgical revision [19].

Thrombosis. Virchow in his concept of the pathogenesis of thrombosis has listed three basic pathogenetic factors: vessel wall injury, changes in blood flow, and blood disorders. Although established more than 150 years ago, this concept can still be applied today to venous thrombosis and even to AVF thrombosis [20].

Damaged blood vessel wall and exposure of subendothelial structures to blood flow lead to platelet adhesion, which then secrete thromboxane A2 and adenosine diphosphate, stimulators of further adhesion. At the same time, "naked" collagen and released tissue thromboplastin start a coagulation cascade, and the key product of this cascade, thrombin and fibrinogen, form a thrombus [18].

In addition to neointimal hyperplasia, which is the main cause of stenosis and consequent thrombosis, many other disorders in chronic renal failure contribute to the development of thrombosis. While renal failure is characterized by decreased platelet function, hemodialysis patients are characterized by a prothrombotic condition. Chronic renal failure is accompanied by chronic inflammation, and CRP and other acute phase proteins are risk factors for AVF thrombosis. In addition, hyperhomocysteinemia, hypoalbuminemia, and hyperlipidemia also contribute to this prothrombotic

condition. Artificial membranes and systems through which blood flows during hemodialysis activate platelets and start the coagulation cascade [4,20].

Thrombosis is one of the main causes of the loss of AVF function resulting in missed dialysis sessions, hospitalization of patients, placement of temporary dialysis catheters, and accompanying complications. AVF thrombosis accounts for between 14.6 and 45% of complications observed in various studies [6–14], and in our group it is the most common complication and accounts for 27.4% of all late AVF complications [15]. The appearance of pain in the AVF area, palpation of the thrombus in the AVF and the loss of the "trill" above the AVF indicate thrombosis.

Various therapeutic methods for resolving thrombosis have been described, but it is of particular importance that treatment of thrombosis starts as early as possible. Today, various percutaneous methods of recanalization of vascular approaches for hemodialysis are used as alternatives to surgical thrombectomy, either using different thrombolytic drugs (urokinase, tissue plasminogen activator) alone or in combination with mechanical thrombectomy devices (Figure 1) [19,21,22]. One third of the patients in our series underwent thrombectomy, and those were patients who underwent surgery in the first 24 hours after the observed thrombosis, while in the others a new vascular approach had to be performed.

Aneurysms and pseudoaneurysms. An aneurysm is a pathological enlargement of the blood vessel wall that can occur on anastomoses or at the sites of a weakened venous wall due to repeated punctures and replacement of the blood vessel wall with fibrous tissue (Figures 2. and 3). Jankovic et al. [10] describe that there is a higher risk of developing aneurysm in patients with adult polycystic kidney disease, as well as in those who dialyze longer with high-flow membranes and high blood flow. The formation of aneurysms

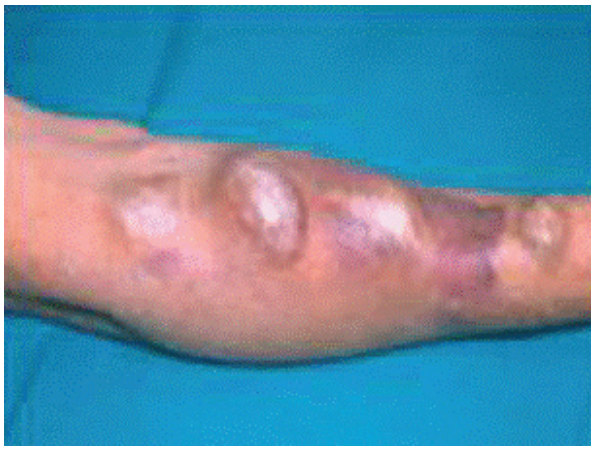

Figure 2. Aneurysms at puncture sites

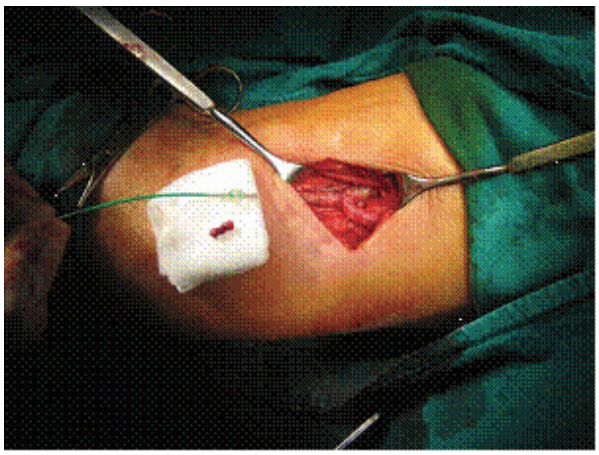

Figure 1. Thrombosis of the proximal radio-cephalic arteriovenous fistula. Thrombectomy with Fogarty catheter and new anastomosis

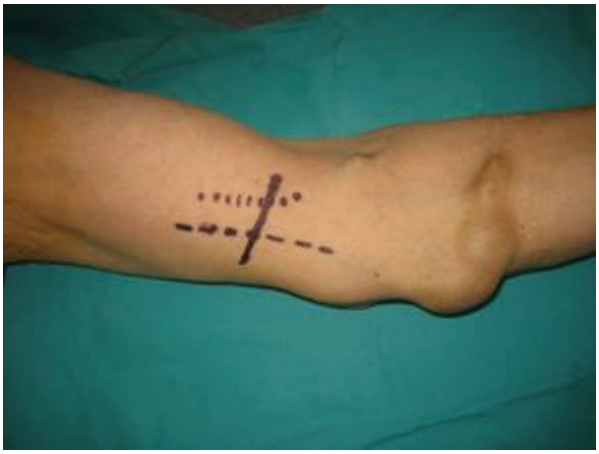

Figure 3. Several years duration dialysis via forearm AVF. Thrombosis of all aneurysmically dilated veins. Ultrasound confirmed the possibility of secondary AVF transposition of the basilic vein above the upper arm

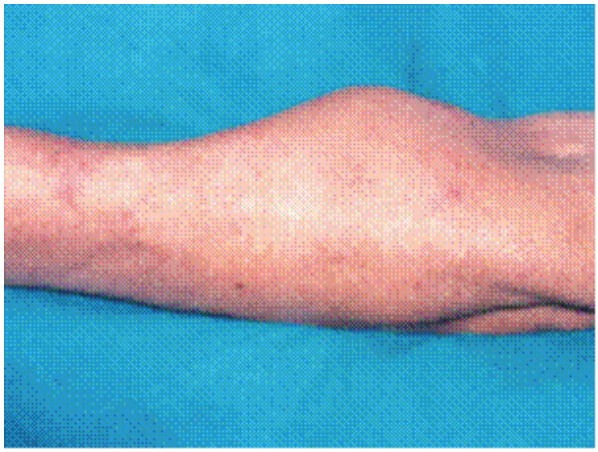

Figure 4. Pseudoaneurysm with chronic course

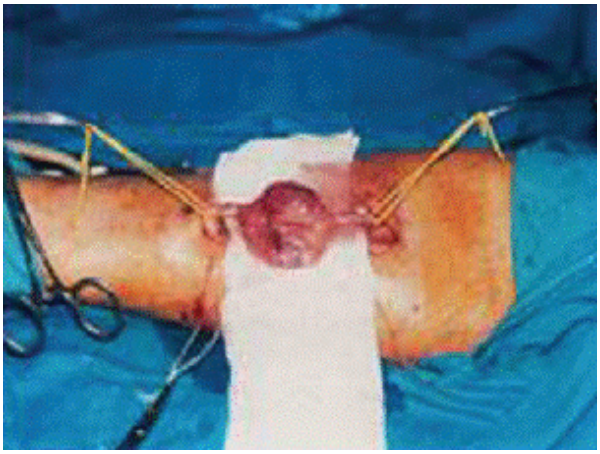

Figure 5. Surgical extirpation of the aneurysm with continued dialysis on the same arteriovenous fistula

and pseudoaneurysms can be prevented by regular controls of the vascular approach, the quality of the skin above it with the obligatory change of the puncture site, especially in patients with an increased risk of aneurysm formation [18,23].

In the studies published so far, there are huge differences in the frequency of AVF aneurysm. In some, aneurysm is the most common complication and accounts for about half of all AVF complications [8–10]. A slightly lower percentage of aneurysms are reported by Al-Thani et al. (32%) [24], while Greenberg et al. [11] describe an aneurysm as one of the rarest complications (3.8%). The occurrence of pseudo or true aneurysm was detected in our series in only three patients [15]. Such formations are more accurately diagnosed by color Doppler ultrasound examination, which can differentiate pseudoaneurysmal from aneurysmal enlargement, and on the basis of which a decision can be made about possible surgical correction (Figures 4. and 5).

Aneurysms and pseudoaneurysms can cause thromboembolism, local ischemia and skin necrosis, compression of surrounding nerves and paresthesia, fistula thrombosis, infection and sepsis, and can lead to rupture and severe bleeding during hemodialysis and also at home when it is dangerous for life [18,24]. The National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (K/DOQI) guide recommends that we do not use an aneurysmally altered part of AVF, to avoid it for puncture [3]. The aneurysm can be marked on the skin with ultrasound as a place to avoid puncture, and a usable part of the AVF can be marked on the skin with a felt-tip pen. In this way, the lifespan of the fistula is extended and reintervention is delayed, i.e. the formation of a new fistula at the proximal level. Surgery is indicated if an enlargement of the aneurysm is observed or if there is a pronounced stenosis of the AVF, in the case of a sudden increase in the aneurysm, the appearance of thinned skin, lack of puncture or rupture site [3,15,18]. One of our patients with pseudoaneurysm underwent surgery due to bleeding, while the other two avoided puncture in the pseudoaneurysm section.

Isolated AVF infection is a rare complication. Bylsma et al. [25] in an extensive meta-analysis that included 318 studies and 62,712 vascular approaches have shown that the risk of AVF infection is 4.1%, or 0.018 per 100 days of AVF.

Most often, AVF-related infection is only perivascular cellulitis, which is manifested by signs of inflammation (localized redness, swelling and pain) [26]. However, systemic bacteremia may develop, accompanied by malaise, fever, back pain, sometimes altered

state of consciousness, unexplained hypotension, leukocytosis. Infection can be associated with other complications of AVF such as aneurysms or hematomas and then it is a much more serious complication [7].

The most important measure to prevent AVF infection is to follow the rules of asepsis and antisepsis. If there are local signs of infection in the area around the AVF, you should take a swab, but be sure to look for hematogenous spread of the infection (blood culture, ultrasound examination of the heart valves). Infectious complications of AVF are resolved with systemic antibiotic therapy based on the results of swabs and blood culture, and, if necessary, some of the surgical procedures. Antibiotics are administered orally for two weeks if there is no bacteremia and fever, and according to modern guidelines in case of fever and bacteremia antibiotics are administered intravenously also for two weeks [22], although many authors still recommend antibiotic therapy in these cases for 4–6 weeks [7,15,18]. More extensive local signs of infection as well as larger hematomas or abscesses require classic surgical revision with postoperative drainage. AVF ligation is necessary only when it becomes a source of recurrent septic pulmonary embolism [22,26].

Heart failure. Although there is a traditional opinion that AVF has a negative effect on heart function, the real role of vascular approaches for hemodialysis in the development of heart failure is still unclear today. While some authors state that only AVF in which the flow exceeds 2,000 ml can lead to left ventricular hypertrophy and heart failure, others believe that AVF can cause heart failure only in patients with previous heart diseases [4,27–29]. However, even the most recent international guidlines do not provide a definition of high flow through vascular approaches or recommendations for fistula interventions that should be taken to prevent heart failure [3,22,28].

The mechanism by which AVF can lead to heart failure has not yet been fully explained. There is an opinion that there is a significant correlation between the flow through the fistula and the cardiac index (minute volume of the heart per unit of body surface area) and that the cardiac index increases immediately after the AVF formation. The AVF formation is thought to increase the minute volume by 15% and left ventricular end-diastolic pressure by 4% [3,22]. Basile et al. [29], however, prove that the relationship between flow through the AVF and cardiac index is complex and that the increase in the flow through the AVF does not follow a linear increase in cardiac index. They explain this by the existence of a functional reserve of the myocardium and the possibility of the heart adapting to the increase in flow through the AVF, which also prevents the occurrence of heart failure. However, this only applies to flow through the fistula between 950 and 2,200 ml/min, while blood flow through the vascular approach above this value is a significant predictor of heart failure with a sensitivity of 89% and a specificity of 100% [29]. Investigating the relationship between flow through the AVF and minute volume, Zamboli et al. [28] show that already flow through vascular approach ≥ 603 ml/min/m 2.7 is associated with an increased risk of heart failure, especially in patients who already have some echocardiographic signs of heart damage.

In patients with heart failure and high flow through the AVF, it is necessary to check the existence of other possible causes of heart failure before the intervention that would reduce the flow: anemia, hypertension, poorly assessed dry weight and consequent hypervolemia. Only when these problems are solved, and the signs of heart failure do not decrease, the correction of AVF is proposed. Several surgical techniques are used to reduce high blood flow through the AVF to treat heart failure: caliber reduction of the AVF anastomosis, graft interposition, placement of circumferential

polytetrafluoroethylene tape next to the anastomotic artery to reduce blood flow, or placement of a clip on the venous portion of the anastomosis to increase resistance as well as AVF ligation [27].

Limb ischemia induced by vascular approach. Limb ischemia, also known as blood theft syndrome or ischemic steal syndrome, occurs due to the retrograde flow of blood from an artery to a vein, which can cause ischemia distal to the anastomosis. There is an increased risk of developing this syndrome in patients with diabetes, people with atherosclerosis and peripheral vascular disease, the elderly and smokers [7]. Limb ischemia usually accounts for less than 10% of all AVF complications, and according to meta-analysis by Al-Jashi et al. [30], the incidence of this syndrome is 0.05/1000 patient days. The frequency is higher when A brachialis is used to create a vascular approach, and significantly less in radiocephalic AVF [18].

The clinical picture depends on the severity of the ischemia. Mild ischemia may be asymptomatic until the compensatory mechanisms of perfusion maintenance are exhausted, and then the patient complains of a feeling of coldness, pain, and the skin becomes pale or livid. Limb necrosis and ulceration occur in severe ischemia. Severe ischemia is an indication for closing the vascular approach, although reconstructive vascular operations are increasingly used [14, 18]. In a patient who developed hand ischemia after the formation of a brachiobasilic arteriovenous fistula (Figure 6), the pain stopped immediately after ligation of the fistula and the signs of ischemia soon disappeared.

Ischemic neuropathy is one of the rarer complications that accompany the AVF formation, which occurs as a consequence of ischemia. Most of the papers state that its frequency is between 1% and 10% [7], but a significantly higher percentage has also been described [6]. It is more common in patients with diabetes and already pronounced macroangiopathy, especially when the brachial artery is used to create AVF. Ischemic neuropathy of the upper extremities was first described by Bolton et al. [31], and the name "ischemic monomelic neuropathy" was introduced by Wilbourn [32], to point out that this is an isolated neuropathy due to reduced arterial blood supply of one extremity (from the Greek word melos - extremity) which should be distinguished from multiple neuropathy. There is also the opinion that ischemic neuropathy is a consequence of ischemic steal syndrome and only one of the clinical manifestations of that syndrome [33].

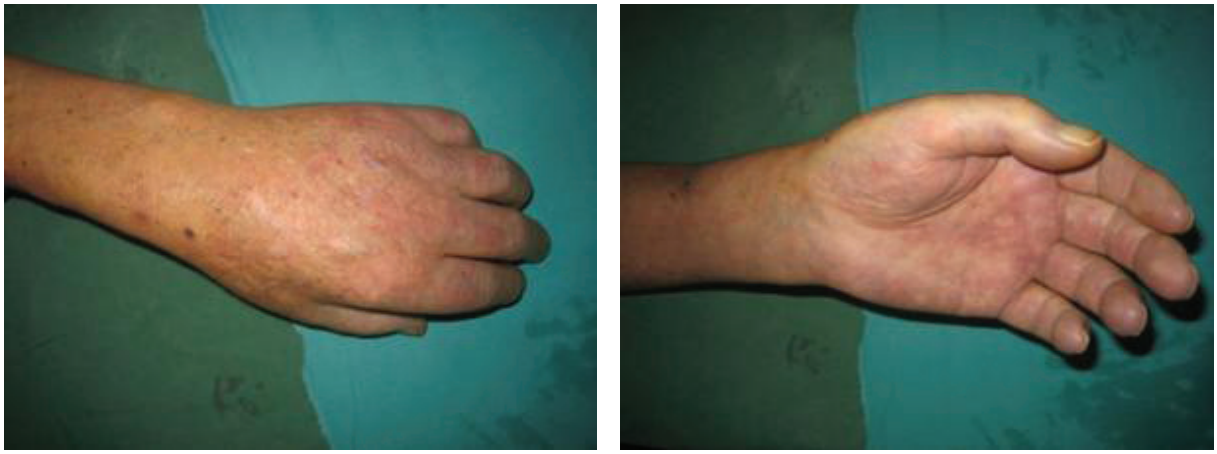

Figure 6. Ischemia of the hand occurring after the formation of a brachiobasilic arteriovenous fistula with transposition of the basilic vein

It is manifested by severe pain, paresthesias, and weakness of the arm. Neurological examination determines the weakness of the group of muscles innervated by n. medianus and sensory defect in the region of the nerve innervation. Although the diagnosis is usually made clinically, electromyography and measurement of nerve conduction velocity can be used to confirm the diagnosis [3,34].

Treatment of ischemic neuropathy aims to reduce blood flow to the vascular approach and increase limb perfusion distal to it. The AVF ligation, bandaging or angioplasty may be used for that purpose. Closure of functional AVF due to ischemic neuropathy should be carefully considered, as symptoms often persist or decrease only slightly after it [34]. However, it is thought that early closure of the vascular approach may lead to complete or partial recovery of the sensory and motor defect [33].

Other late complications of AVF, edema, bleeding, hematoma, are not mentioned in many papers. Edema is more common in brachiocephalic AVF and it is sometimes associated with wound infection. It is usually reduced by elevation of the arm, and it is very rarely necessary to ligate AVF due to edema [13]. Edema of the arms of our patients was mild and was treated conservatively in cooperation with specialists in internal and physical medicine [15]. Bleeding occurs more often as an early complication, and less often in the late postoperative period. It can occur due to overdose of heparin, damaged blood vessel wall during puncture, it can cause the formation of hematomas, and it rarely occurs due to rupture of the fistula [13,14]. Two of our patients with hematoma developed pseudoaneurysm, so one patient underwent reintervention due to bleeding. Most of our patients with hematoma were treated conservatively. More extensive hematomas can be resolved by ultrasound-guided aspiration, and less frequently classic surgical revision with postoperative drainage is required [15].

Risk factors

A number of factors are associated with the occurrence of AVF complications, and in the previous text, factors that may contribute to the occurrence of certain late postoperative complications have already been mentioned. Many of them act simultaneously and can jeopardize fistula function. As knowledge of risk factors for complications and AVF survival is very important for defining measures that can prevent complications, a large number of papers have been dedicated to their research.

Large differences in the frequency of complications in some studies also indicate differences in the frequency of some risk factors. In addition to demographic characteristics of patients, the occurrence of certain complications depends on the underlying kidney disease, the presence of comorbid conditions or various metabolic and immune disorders that occur in chronic renal failure and have already been mentioned in the pathogenesis of AVF stenosis and thrombosis [7,8,28]. Recently, Gardeezi et al. [35] have shown that low concentration of 25(OH)D and high concentration of FGF-23 and parathormone were associated with a higher risk for AVF reinterventions. Mineral metabolism disorder in chronic renal failure and its association with cardiovascular diseases are well known, but little is known about the impact of these disorders on AVF function [36,37].

One of the main predictors of AVF success and survival is the quality of patients' blood vessels [6,15] and therefore the importance of careful examination of blood vessels before approaching AVF creation is emphasized. In recent years, more precise preoperative procedures have been introduced to assess the quality of blood vessels. The KDOQI guidelines provide clear recommendations on when and how to perform vessel mapping before approaching the creation of a vascular approach [3]. Donfrid et al. [38] based on their extensive experience, point out that in the preoperative

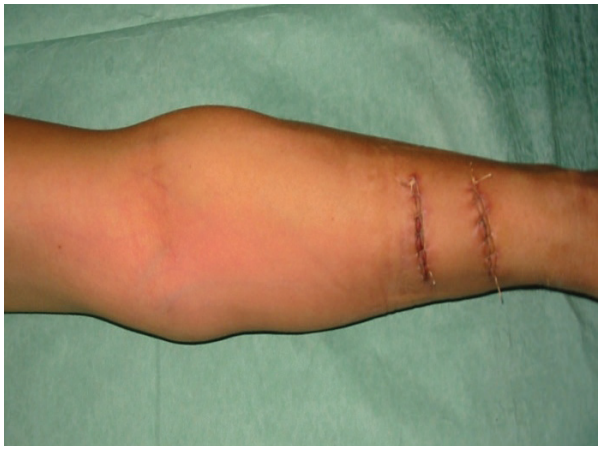

Figure 7. Without ultrasound diagnostics, AVF operation failed twice in one day

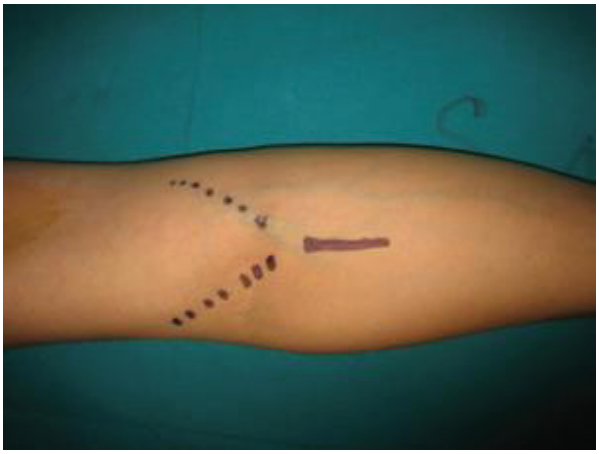

Figure 8. Color Doppler showed possible AVF of the cubital region after which a successful AVF was done

diagnosis, a vascular approach requires an exhaustive anamnesis and clinical examination, and that vitium artis is not to perform an ultrasound examination (Color Doppler sonography) of blood vessels in both arms (Figure 7. and 8). A well-planned AVF and performed on a prepared patient using all the necessary diagnostics with a well-educated vascular surgeon are a guarantee of a successful creation of a vascular approach. It is a big mistake that the first vascular approach is performed by a poorly-educated surgeon. The failure of the first approach is an introduction to later difficult-to-resolve complications (Figure 9) [38].

Of great importance for the occurrence of complications of AVF is the way of their use, and especially it is necessary to avoid premature puncture of AVF after surgery, repeated punctures in the same place, excessive compression. Examining the factors associated with the loss of AVF patency, we have previously shown that hypotension, diabetes, anemia, previous venous cannulation used for AVF, artery and vein quality, use of AVF for hemodialysis less than 45 days after its creation, number of intraoperative and postoperative complications, are significant independent risk factors for loss of AVF patency [16]. In addition to all these factors, we and many other authors emphasize the importance of surgical technique and only strict adherence to all recommended details of AVF surgery can ensure the optimal result of the procedure [6,7,16,38–40].

Most of chronic kidney diseases are progressive and end in end-stage renal disease. Therefore, we should draw the attention of all those who treat patients with chronic kidney diseases, as well as of the patients themselves, that keeping blood vessels from the initial stages of the disease is extremely important for the later successful creation of AVF and its functioning.

Conclusion

Functional AVF is a prerequisite for successful hemodialysis, but frequent complications of AVF are still one of the most common causes of hospitalization of patients treated with hemodialysis. Knowledge of the etiopathogenesis and all risk factors for the occurrence of AVF complications enables their prevention, early detection and timely and adequate treatment. AVF creation, usage and care are a continuous task of a team of health professionals (nephrologists, surgeons, radiologists, nurses, etc.) who need to achieve good cooperation with patients, which only enables successful treatment.

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Kasne postoperativne komplikacije arteriovenske fistule za hemodijalizu

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Vaskularni pristup je preduslov za izvođenje hemodijalize, ali su različite i česte komplikacije njihova "slaba tačka". Savremeni vodiči preporučuju nativnu arteriovensku fistulu (AVF) kao prvi izbor vaskularnog pristupa, jer je odlikuje najduže preživljavanje i najmanje komplikacija u odnosu na druge vaskularne pristupe. Sve komplikacije AVF mogu se podijeliti na intraoperativne, rane i kasne postoperativne. U ovom radu prikazane su kasne postoperativne komplikacije AVF, njihova učestalost, uzroci, dijagnostika i liječenje.

Najvažnije kasne postoperativne komplikacije su: stenoza, tromboza, formiranje aneurizme ili pseudoaneurizme, infekcija, edem ruke, hematom, ishemijski steal sindrom, ishemijska neuropatija, kongestivna srčana slabost. Velike razlike u učestalosti pojedinih komplikacija u dosadašnjim studijama mogu se objasniti razlikama u hirurškoj tehnici, lokalizaciji AVF, metodama dijagnostike ali, prije svega, razlikama između prikazanih grupa bolesnika. Opisano je da starost bolesnika, pol, osnovna bolest, prisustvo komorbidnih stanja i različitih metaboličkih i imunskih poremećaja karakterističnih za hroničnu insuficijenciju bubrega, kao i način korišćenja i njege AVF značajno utiču na pojavu komplikacija AVF. Jedan od glavnih prediktora uspjeha i preživljavanja AVF jeste kvalitet krvnih sudova bolesnika i zato je pažljivo ispitivanje krvnih sudova prije pristupa kreiranju AVF od posebnog značaja.

Kreiranje, korišćenje i njega AVF je zadatak tima zdravstvenih radnika koji učestvuju u liječenju ovih bolesnika, a za uspješno liječenje potrebna je njihova dobra saradnja, kao i saradnja sa bolesnicima.

Ključne riječi: arteriovenska fistula, hemodijaliza, kasne komplikacije, faktori rizika

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Review

The function of autophagy as a fundamental process of preserving cell homeostasis

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Summary

Autophagy is a dynamic process, conserved in all eukaryotes. It is responsible for the degradation of cytoplasmic content. Autophagy is crucial in cell survival and cell death. It plays a significant role in the cell response to stress, nutrient deficiencies, embryonic development, tumor suppression, response to pathogens and aging. The process of autophagy is also involved in the pathology of human diseases, such as cancer, diabetes, cardiomyopathy, and neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Autophagy is a mechanism that involves degradation of cells, proteins, damaged organelles and pathogens through the lysosomal mechanisms, thus autophagy supports cell survival during starvation, hypoxia and metabolic stress. However, if extensive and/or excessive, autophagy can promote apoptosis (type I) or function as an alternative cell-death pathway, called autophagic cell death (type II). Autophagy can either promote cancer cell death, or serve as a survival mechanism against apoptosis or necrosis induced by various anticancer treatments. Given the contradictory role of autophagy during tumor initiation and progression, the use of autophagy in therapy depends on the context and must be approached individually.

Key words: autophagy, cell survival, cell death, tumor

Introduction

Autophagy (Greek meaning 'self-eating') is a dynamic process, conserved in all eukaryotes. It is responsible for the degradation of cytoplasmic content. It plays a significant role in the cell response to stress, nutrient deficiencies, embryonic development, tumor suppression, response to pathogens and aging. The process of autophagy is also involved in the pathology of human diseases, such as cancer, diabetes, cardiomyopathy, and neurodegenerative diseases such as Alzheimer's and Parkinson's disease.

There are three types of autophagy: chaperone-mediated autophagy, microautophagy, and macroautophagy. Chaperone-mediated autophagy is present in higher eukaryotes, where chaperones bind to a specific protein leading to its unwinding and allowing it to pass through the lysosome membrane [1]. In microautophagy, the cytoplasmic content reaches the lysosome or vacuole (in plants) by invagination. However, autophagy mainly refers to the third type, macroautophagy (hereinafter autophagy), which is also the best studied [2].

The morphological characteristic of autophagy is *de novo* formation of autophagosomes, vesicles with a double membrane containing cytosol and captured organelles. The outer membrane fuses with the lysosome or vacuole by releasing the vesicle with the inner membrane into the lumen where the entire contents are degraded [3].

The autophagy has gained a lot of interests since 3rd October 2016, when Yoshinori Ohsumi was awarded the Nobel Prize in Physiology or Medicine for discoveries of mechanisms of autophagy. Approval of its beneficial effects in various diseases led to the wide autophagy fasting promotion. However, contradictory data about the role of the autophagy in cancer initiation and progression emphasizes the importance of individual and more careful approach in autophagy applications. The best evidence for the importance and popularity of autophagy research are publications. Since then, number of publication investigating autophagy rapidly increase (2016 – 5482; 2017 – 6509; 2018 – 7198; 2019 – 8154; 2020 – 9158). Only, from the beginning of 2021, 965 publications were published [4].

Autophagy function

Cell survival

During starvation, in lack of nutrients, which lasts for hours, autophagy produces the necessary amino acids, which are further used in several different ways. Within the first day of starvation, glucose is produced in the liver by gluconeogenesis, via lactate and amino acids. Alanine is delivered to the liver from peripheral tissues, primarily muscles, and is converted to glucose via the glucose-alanine cycle. Amino acids can further be used as an energy source in the Krebs cycle. In the third way, amino acids are used for intensive synthesis of proteins necessary for the adaptation of cells to starvation conditions. The greatest mobilization of nutrients through autophagy occurs during remodulating development, as the body's response to conditions (spore production in yeast, multicellular association in *Dictyostelium discoideum*, insect metamorphosis). It should be emphasized that the production of amino acids by autophagy is an acute or urgent response, and can support cell survival for a short time. Little is known about the role of autophagy in chronic starvation.

One of the most important roles of autophagy is the removal of cytoplasmic contents. Accumulation of abnormal proteins and deformed organelles in hepatocytes, karyomiocytes, and neurons lacking autophagy can lead to neurodegeneration or tumorgenesis. Accumulation of autophagosomes has been observed in Alzheimer's disease, polyglutamine recurrent disease (Huntington's disease, fragile X chromosome mental retardation, bulbar muscular atrophy, myotonic dystrophy type 1) and Parkinson's disease.

Autophagy can also serve as a transport system from the cytoplasm to lysosomes/endosomes or vacuoles. The best example is the Cvt pathway in yeast. Autophagy is also used to present endogenous class II major histocompatibility complex (MHC) antigens, which are recognized by CD4 + T cells. Autophagy is involved in the removal of pathogens (xenophagy). Influenza antigen binds to LC3 and is incorporated into autophagosome and is represented by MHC class II molecules. Dendritic cells, on the other hand, use the autophagy pathway to recognize viral single-stranded

RNA. Some of the toll-like receptors, the basic molecules of innate immunity, are located on plasma membranes, others on endosomes such as TLR7, which recognizes viral single-stranded RNAs and triggers an immune response, or secretion of inflammatory cytokines transported by autophagy.

Packaging of cell contents into autogaphosomes, but without degradation, is an important function of autophagy in cell survival. Autophagy can be caused by several events, including stress ER. Autophagy is thought to protect the cell by packaging ER in autophagosome. When yeast cells are treated with DTT or tunicamycin, autophagosomes with parts of the ER appear in the cytoplasm and still do not fuse with the vacuole. In this way, the dissemination of toxins in the cell is prevented. Although autophagy is involved in the immune system, it has been observed that some pathogens (*Legionella pneumophila, Coxiella burneti, Brucella abortus and Porphyromonas gingivalis*) use autophagosomes to protect against degradation, forming intracellular niches for survival and replication. The exact mechanism of this process is unknown. A similar example was observed in mouse hepatitis virus and picornavirus replication in vacuoles of the origin of autophagosome [5].

Autophagic cell death

Based on morphological criteria, there are three types of cell death, type I (apoptosis), type II (autophagy) and type III (necrosis) [6]. Autophagic cell death refers to a form of cell death that is morphologically different from apoptosis and is the result of excessive autophagy, respectively the presence of autophagosomes in a dying cell [7]. It is commonly described during embryogenesis, in order to maintain homeostasis, in diseased tissues, and in cell lines treated with chemotherapeutics or other cytostatics. In apoptosis (type I cell death), the cytoskeleton collapses in early stages, and the organelles remain preserved until late stages of apoptosis. In autophagy (type II cell death), there is degradation of organelles in early stages, but preservation of the cytoskeleton until later stages. Cell death by apoptosis is regulated by cascade reactions of caspases and DNA fragmentation, while in autophagy these processes occur late or not at all. Unlike necrosis, there is no inflammatory response [8].

However, an increased number of autophagy markers, such as the number of autophagosomes, does not necessarily mean an increased autophagic flux, but may be the result of blocking the maturation of the autophagosome (lysosome fusion), and if autophagic flux in a dying cell is indeed increased, it may be an attempt to save cells from death. Therefore, the true significance of autophagic cell death would be cell death by autophagy, not cell death in parallel with autophagy [9].

Shen and Codogno (2011) [10] proposed a new definition of autophagic cell death, according to which autophagic cell death is a form of necrosis in which autophagy serves as a mechanism of cell death and must meet the following criteria:

- Cell death without apoptosis mechanisms, such as caspase activation;
- Increased autophagic flux is not just the autophagy marker;
- Suppression of autophagy by pharmacological inhibitors and genetic blocking eliminates cell death [9].
- On the other hand, autophagy can serve as a mechanism of cell death by promoting apoptosis, respectively autophagic cell death due to excessive cell degradation.

Autophagy and apoptosis

Autophagy and apoptosis occur when cells are under stress. Under certain circumstances, these are two independent processes, but in others, the activation of autophagy inhibits

apoptosis or autophagy occurs before apoptosis. Regulators of apoptosis, members of the Bcl-2 family, caspase 8, and FADD can regulate autophagy, and proteins involved in autophagy such as Atg5, beclin 1, and Atg4D play a role in apoptosis, suggesting the overlapping of these two processes. Normally, autophagy precedes apoptosis and maintain cell homeostasis, but is often described as a type II cell death known as autophagic cell death, thus acting as a guardian or executor of apoptosis depending on the environment.

The best example of the molecular overlap between the regulation of apoptosis and autophagy is the interaction of the autophagy protein beclin 1 and the anti-apoptotic protein Bcl-2. Binding of these two proteins inhibits both processes at the basal level. Under normal conditions, Bcl-2 inhibits beclin 1, and during stress they separate and further stimulate autophagy [10]. Nutrient deficiency activates JNK 1 (C-Jun N-terminal protein kinase 1) which phosphorylates the Bcl-2 regulatory loop and then terminates the interaction with beclin 1. Released beclin 1 triggers autophagy. Phosphorylated Bcl-2 interacts with Bax maintaining the integrity of the mitochondrial membrane preventing apoptosis. However, prolonged starvation causes JNK1-mediated Bcl-2 hyper-phosphorylation, leading to Bax dissociation and caspase-3-mediated apoptosis activation [11].

As in apoptosis, caspases are involved in the regulation of autophagy [12,13]. Caspases cleave beclin 1, Atg5 and p62 which inhibit autophagy. Caspase 8 inhibits autophagy by cleaving Atg3. In the absence of caspase 8 cleavage, excessive autophagy occurs in T cells [14]. Caspase 9, involved in the intrinsic apoptosis pathway, induces autophagy by increasing LC3 lipidation through interaction with Atg7 [12,15]. Caspase 3 cleaves beclin 1 to form two fragments of 37 and 35 kDa with C-terminus and N-terminus. Beclin 1 with N-terminus is localized on the nucleus, and beclin 1 with C-terminus on the mitochondria, leading to the release of cytochrome c and apoptosis [16].

The role of autophagy in tumors

In normal cells and tissues, autophagy plays a complex role. Lack of autophagy is thought to contribute to many diseases, including neurodegenerative diseases and aging. Previous work has shown that it suppresses initiation, but promotes tumor progression [17].

Autophagy as a tumor suppressive mechanism

Autophagy has an anti-inflammatory role [18], removes inflammasomes (responsible for the secretion of inflammatory interleukin-1β and intreleukin-18), damaged mitochondria [19]. It is associated with inhibition of a number of pro-inflammatory signals [20]. Lack of autophagy causes oxidative stress and genomic instability, an inflammatory microenvironment, favorable conditions for malignant transformation and progression. Chronic inflammation, as a result of lack of autophagy due to mutation of Thr300Ala on *ATG16L1* gene, is present in Crohn's disease. Patients with Crohn's disease have an increased risk of developing cancer in areas of inflammation because the cells are more susceptible to intracellular bacterial infection, which leads to chronic inflammation, tissue damage and an increased risk of tumorgenesis [21].
Mutation of *AMBRA1*, *Atg5* and *Atg7* gene in mice leads to the development of benign neoplasms [22,23].
An increase in oxidative stress activates nuclear factor, erythroid-2-like 2 (NRF2), which can stimulate tumor growth [24]. Loss of autophagy in the liver is toxic, causing chronic hepatocyte death and inflammation processes leading to the development of liver tumors [25]. P62 deficiency reduces toxicity and tumorigenesis caused by lack of autophagy.

Increased expression of *p62* promotes oxidative stress and tumor growth. It is not known how p62 contributes to tumorigenesis, a given protein is involved in several oncogenic signaling pathways through which it can affect, such as NRF2, mTOR and NF-kB [26].

It is involved in maintaining metabolic homeostasis, removing non-functional mitochondria involved in tumor cell metabolism [20].

Autophagy suppresses tumorigenesis by preventing viral and bacterial infections. Cancer-causing pathogens, such as hepatitis B virus, human herpes virus, human papillomavirus types 16 and 18, Epstein-Barr virus, *Helicobacter pylori*, *Streptococcus bovis*, *Salmonella enterica*, and *Chlamydia pneumoniae*, after infection, activate xenophagy, which removes pathogens and triggers a specific immune response [18].

These facts indicate that autophagy, by preserving homeostasis, helps prevent malignant transformation.

The role of autophagy in tumor promotion

Autophagy plays a contradictory role in tumors. Although it suppresses tumor promotion and certain aspects of tumorigenesis, autophagy contributes to the survival of established tumors in response to stress. Tumor cells depend on autophagy significantly more than normal cells. Due to increased nutrient consumption and increased proliferation rates, tumor cells suffer from metabolic stress and hypoxia, and increased basal autophagy protects them from apoptosis and necrosis [27]. It also helps in the late stages of tumor progression, dissemination and metastasis. Separation of cells from the extracellular matrix induces autophagy in epithelial cells, which protects them from cell death called anoikis, and allows metastasis [28]. Autophagy helps metastasis and secretion of cytokines, such as the proinvasive cytokine IL6, which is necessary for invasion [29]. Autophagy protein degradation in stellate cells helps the metabolism of tumor cells by secreting amino acids, primarily alanine, whose carbon tumor cells are used for the Krebs cycle. And other carbon sources such as glucose are used to synthesize serine, which in many types of tumor cells is used to produce nucleotides [30]. Inhibition of autophagy blocks cell migration and invasion in vitro and reduces metastasis in vivo, as shown by research on GEMM (genetically engineered mouse model) for hereditary breast cancer [31]. Defects in the autophagy signaling pathway usually limit the proliferation, invasion, and metastasis of malignant cells [20]. In K-ras lung and pancreatic cancers, loss of autophagy directs tumor progression to benign neoplasm, through increased p53 activity. In GEMM lung cancer, the *Atg7* deletion redirects the pathological path from carcinoma to benign oncocytomas, or tumors that accumulate damaged mitochondria [22]. Since damaged mitochondria are the main substrate of autophagy, this points to the fact that benign tumors actually have defects in autophagy [32].

The importance of autophagy in the treatment of tumors

Pharmacological inhibitors such as 3-methyladenine (3-MA), baphilomycin A1 (BafA) and chloroquine (CQ) are used in clinical studies to inhibit autophagy. 3-MA is a class III PI3K inhibitor, Vps34, and inhibits autophagy in the early stages of autophagosome isolation membrane formation. BafA prevents the function of lysosomes, and blocks the late phase of autophagy, the degradation of autophagosomes. CQ as a weak base increases the lysosomal pH value, also blocking the degradation of the contents. Although these inhibitors are effective, none of them are specific for autophagy, they also participate in other cellular processes such as endocytosis, intracellular transport and lysosome production. Therefore, the use of inhibitors, which aim at

specific molecules of the autophagy signaling pathway, is suitable for studying the therapeutic effect of inhibiting autophagy.

So far, the use of autophagy inhibitors together with other chemotherapeutics has been shown to be effective in eliminating tumor cells. Imatinib (Gleevaec), a tyrosine kinase inhibitor that inhibits BCR/ABL, is conventionally used to treat chronic myeloid leukemia (CML). However, its use leads to CML stem cell resistance and relapse in patients. The combined use of imatinib with CQ significantly leads to the death of tumor cells in as many as three different stages of the disease, in newly diagnosed patients, in patients who use only imatinib and in patients with imatinib resistance. Similar results were achieved with RNA interference of the ATG5 and ATG7 genes, directly linking the inhibition of autophagy with tumor cell death. Studies of the combined use of CQ with chemotherapeutics, performed on other tumor models, have shown that inhibition of autophagy causes p53-mediated apoptosis and tumor regression [33].

Also, recent studies have shown that Δ9-tetrahydrocannabinol (THC) causes autophagy in glioma cells, which indirectly causes their death by promoting apoptosis. THC causes endoplasmic reticulum stress and inhibits the AKT/mTOR pathway. Inhibition of autophagy or apoptosis prevents cell death, which indicates the interdependence of the two processes [34]. Coordination of autophagy and apoptosis has been shown to be necessary for efficient melanoma cell death in in *vitro* and *in vivo* studies. Inhibition of autophagy with BafA, CQ, or ATG5 deletion or treatment with caspase inhibitors suppresses cell death suggesting that induction of autophagy is necessary for later activation of apoptosis [35].

Conclusion

Autophagy is associated with many human diseases, including neurological, autoimmune, infectious diseases, metabolic disorders, and cancer. However, the largest number of clinical trials related to autophagy are, clinical trials of cancer, which involve the inhibition of autophagy. Studies have shown that autophagy plays a key role in suppressing tumor initiation, but also that it helps tumor cell progression and metastasis. Therefore, autophagy both promotes and inhibits tumor growth, and the role of autophagy in cancer therapy depends on the context and should not be generalized. On the other hand, autophagy has a protective role in normal tissues and its deficiency results in neurodegenerative and metabolic disorders, i.e. it is toxic to normal tissues.

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Funkcija autofagije kao osnovnog procesa očuvanja ćelijske homeostaze

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Autofagija je dinamičan proces, očuvan kod svih eukariota. Odgovorna je za razgradnju citoplazmatskog sadržaja. Autofagija je presudna u ćelijskom preživljavanju i ćelijskoj smrti. Ima značajnu ulogu u reakciji ćelije na stres, nedostatku nutrijenata, embrionalnom razvoju, suzbijanju tumora, odgovoru na patogene i starenje. Proces autofagije takođe je uključen u patologiju humanih bolesti, poput tumora, dijabetesa, kardiomiopatije i neurodegenerativnih bolesti poput Alzheimerove i Parkinsonove bolesti. Autofagija je mehanizam koji uključuje degradaciju ćelija, proteina, oštećenih organela i patogena putem lizozomskih mehanizama, pa tako autofagija omogućava preživljavanje ćelije tokom gladi, hipoksije i metaboličkog stresa. Međutim, ako je opsežna i/ili prekomjerna, autofagija može promovisati apoptozu (tip I) ili funkcionisati kao alternativni put ćelijske smrti, koji se naziva autofagna ćelijska smrt (tip II). Autofagija može promovisati smrt ćelija karcinoma ili služiti kao mehanizam preživljavanja protiv apoptoze ili nekroze izazvane raznim tretmanima protiv tumora. S obzirom na kontradiktornu ulogu autofagije tokom inicijacije i progresije tumora, upotreba autofagije u terapiji zavisi od konteksta i mora joj se pristupiti individualno**.**

Ključne riječi: autofagija, ćelijsko preživljavanje, ćelijska smrt, tumor

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Review

Puberphonia: from classic to modern approach

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Summary

Voice is a significant component of communication that allows us to express information and emotions, so it is the foundation of verbal communication. Maturation of the body involves dilation of the larynx and lower positioning of the larynx in the neck, resulting multiple changes in voice quality. The rapid changes in the human larynx during puberty are more evident in males. Such changes can result in voice mutation – puberphonia. Puberphonia, also called mutational dysphonia or mutational falsetto, is the failure of a natural decrease in fundamental frequency or pitch. We can also defined puberphonia as persistent adolescent voice even after puberty in the absence of organic cause. This functional voice disorder can have multiple consequences on the personality and quality of life of an individual that often encounters problems that include psychological, emotional, social, and professional difficulties. This article aims to review the relevant and accessible literature on puberphonia in a comprehensive concise manner, highlighting the etiology, prevalence, clinical manifestation, consequences on quality of life, as well as evolution of the approach and attitude to its treatment.

Key words: voice mutation, puberty, puberphonia

Introduction

During ontogenetic development, a human goes through two very distinct biological phases of his development, which visibly affect his physical and mental state. In the first phase is the biological ascent, puberty, or the age of significant changes in the child's body and a major turning point in boys and girls, and the second is the biological decline, or menopause. Both phases significantly affect an individual's voice. Numerous literature in the field of speech therapy, acoustics, phonetics, phoniatrics, psychology, psychiatry, vocal pedagogy, as well as individuals' own experiences testify to the importance of voice and speech in human communication [1]. Voice is a multidimensional whole that allows us to express information and emotions, so it is the foundation of verbal communication [2]. It is a product of the synergy of several systems: respiratory, phonatory, resonant and articulatory systems. We are often not sufficiently aware of the importance of the voice and the information it gives us. By listening

to someone's voice, we can get information about the speaker's physical characteristics and also more subtle information, such as temperament, intention, emotion, or mood [3]. As we mentioned earlier, the period of puberty is a period of intense changes in an individual's voice. Puberty occurs and lasts very individually. In some it occurs earlier, in some later, and that depends on several factors, such as environment, diet and genetics. In our area, the maturation of girls occurs between the ages of 12 and 15, and in boys between the ages of 13 and 16. The duration of puberty, and therefore the mutation of voice, cannot be precisely determined. Voice mutation or so-called puberphonia is a change in the vocal apparatus due to the growth of the larynx and its muscles, and the enlargement of the vocal cords. It is a transitional period of changing the voice of a child into the voice of an adult. If not properly treated or given importance, this functional voice disorder can have multiple consequences on the personality and quality of life of an individual, so we believe that it is important to review relevant and available literature, make a review of knowledge about this voice disorder, which will be useful to both, clinicians and scientists.

Definition, etiology and prevalence of puberphonia

Mutation of the voice during puberphonia is a functional disorder which is manifested as the voice with the children's characteristics after puberty. The appearance of voice in a higher level or in the voice register is the main symptom that lasts even after puberty. The voice of patients is continuous and weak for a long time, thin, breathable, hoarse, feminized and immature. Other symptoms are pitch interruptions, inadequate resonance, and shallow breathing [4,5]. Other terms used to describe this condition include puberphonia, adolescent transitional dysphonia, persistent falsetto, incomplete mutation, and mutational falsetto [6].

The etiology of this voice disorder has not been fully elucidated. However, it is considered that the main cause is the influence of testosterone and growth hormone with incompatible growth of the larynx, especially the vocal cords. A recent study shows that there are aberrant hormonal changes in people with puberphonia, expressed through higher values of GPER-1, 17β-HSD, as well as cAMP compared to the control group [7]. During puberty, developmental mutations are observed in both males and females. However, this change is more obvious in boys compared to girls. During this period, the larynx descends, and its dimensions in the sagittal and transverse planes increase. In men, the angle of the thyroid cartilage decreases to 90°, the length of the vocal cords increases, and the dimension of the epiglottis decreases. Changes that occur during puberty in all organs responsible for phonation include an increase in respiratory capacity, because the length and extent of the breast also increase [8]. The neck increases in length and width, which leads to a relative lowering of the larynx and a consequent expansion of the vocal tract, which increases the resonatory system. The growth of the paranasal sinuses also changes the quality of the voice. The vibrational source, the vocal cords, is therefore only one of several parts of the vocal apparatus that change size during puberty, but the fundamental frequency of the voice is directly related to the vibration of the vocal cords [9]. In addition to these causes, the etiological factors include emotional stress, delayed development of secondary sexual characteristics, psychogenic causes and excessive maternal protection [10].

Voice frequency in men falls by approximately one octave. The voice remains in high intonation, but occasionally it breaks and there is a mixing of the chest register and the head register. The vocal register is a psychoacoustic

term and the term "register" was used to perceptually describe different registers of voice quality contained in certain tone ranges. The three main modes of phonation are classified as laryngeal modes or registers. These are chest, middle and head registers. These three registers usually correspond to frequency ranges: low, medium, and high [11]. The typical fundamental frequency of an adult male is between 85–180 Hz, and of an adult female around 165–255 Hz [12]. The characteristic of puberphonia is that a boy continues to use higher tonality that strains the laryngeal muscles. Therefore, the voices of girls and boys that show similarities before puberty, after this period differ significantly in gender and voice quality in terms of specific, low frequencies in men [8,13]. There are three phases in the mutation period (9–16 years). In the permutation phase, the first symptoms of mutational voice change appear at the age of 9 to 10 years. The main mutational phase is characterized by a rough, hoarse voice quality and a successive decrease in pitch over a relatively short period of time; it can take several months. The postmutation phase is the last phase and involves the stabilization of the male voice; it is characterized by lowering the volume range and stabilizing the pitch. Most adolescents complete the mutation phase at the age of 17.

Although mutational falsetto is a temporary voice, in cases when it is not treated, it can turn into a chronic voice disorder [14]. Especially, if resonance is interrupted during vocal performance and if voice control is reduced, it can negatively affect men in early adolescence in a psychosocial sense [15]. Vocal therapy in this case, as with all other voice problems, helps the individual to regain a healthy voice in accordance with their age and gender. Applied vocal therapy increases the voice quality of young people and adults, helping them to control vocal performances. The prevalence of this voice disorder has not been clearly established, there is a lack of epidemiological studies that deal with this issue. However, research done in India finds that the prevalence is 1 per 900,000 inhabitants [16]. Also, some studies find that of all voice disorders 2–3% are mutational falsetto, or puberphonia [17]. Other studies that address this problem always refer to this frequency data, as no more comprehensive study has been done to examine the prevalence of this voice disorder.

Puberphonia and quality of life

The human voice is an individual product of the interaction of a complex physiological function that reveals uniqueness, because individuals can be recognized by voice [18,19]. The personal characteristics of the voice heard by the person-listener can shape the flow of communication [20]. When vocal characteristics deviate from gender, age and culturology in the background of the expected norm, the sound of the voice can attract more attention than the messages themselves. Therefore, it is not surprising that individuals with a voice disorder can be identified by their voice disorder instead of the whole personality [21,22]. In the same way, the detrimental consequences of voice impairment include the risk of losing social role [23]. Typical psychologically accompanying problems of chronic illness in general, as well as in persistent voice disorders, include depression, anxiety, and tension [24]. Voice production is a critical component of social interactions. It is therefore clear that as a consequence of voice disorders, detrimental effects on the quality of life of people are imposed [23]. The impact of voice disorders varies from person to person. Occupation, environment, family members, and complete personality are all variables that can affect the way a voice disorder affects a particular person [25]. People with puberphonia often encounter problems that include psychological, emotional, social, and professional difficulties [26]. In recent

decades, more and more attention has been focused on health-related quality of life, and research conducted by Wilson et al stressed the importance of including quality of life measures in voice assessment [27]. That is how the instrument, the so-called The Voice Handicap Index (VHI) was developed and validated by Jacobson et al. [28]. It was initially developed to meet the patient's requirements in terms of treatment outcomes with an emphasis on the patient's physical, emotional and functional changes during treatment. The first version of this instrument had 85 items, and then it was reduced to 30 items as VHI-30, which is also the most popular scale used in clinical practice, as well as in research [29]. Each VHI subscale tends to score 40, giving a total of 120. A VHI score of 0 to 30 represents low score indicating that there is a minimal handicap associated with voice disorder. A score of 31 to 60 indicates a moderate handicap as a result of a voice disorder. Then, a VHI score of 60 to 120 represents a significant and severe handicap due to voice problems and is often seen in patients with vocal cord paralysis or vocal cord scar. Many researchers reported that VHI is a useful tool for monitoring treatments for a wide range of voice disorders [30,31]. VHI is also used to assess the effect of voice disorders on a patient's daily life [28]. Also, the overall VHI score is taken as the change between the VHI score before and after the intervention, and the scores on the VHI subscales may be important for assessing treatment options and outcomes. In addition to the impact on voice, this disorder also has an impact on the social and psychological levels.

The effects of puberphonia in men include: physical/mental health; destroys their self-respect; leads to low self-esteem, self-doubt, anxiety and depression; worrying behavior; obsessive thoughts about yourself; eating disorders such as anorexia and overeating; late marriage or inability to form a partnership; loneliness or leaving home; suicidal tendencies [32]. Several studies have also revealed the social aspect of the problem of puberphonia [32,33,34], and researchers with their personal treatment experience have concluded that many other psychosocial problems such as employment and marital issues arise in society. Therefore, the researchers are motivated to find all the consequences of the voice mutation as well as the appropriate treatment model. The most striking consequences of puberphonia are functional communication and social participation. Namely, critical aspects of communication skills are influenced by puberphonia, so adolescents with this functional disorder are teased and bullied more than their peers. Adolescents may not find this voice stigmatizing, however, they prefer not to talk about it with other people. However, in addition to the above, puberphonia can have a broad psychosocial influence. This is especially true for adolescents who may face additional physical, emotional changes as they enter the adult world. A recent study [32] reports the findings of a survey investigating the social and communication impacts of puberphonia. The following messages emerged from the mentioned research, which should be recognized by the public and medical professionals: 1. Puberphonia is a very common problem; 2. It is necessary to take advantage of the available treatment; 3. It is not a hormonal disease or a physiological disease; 4. People with puberphonia are of orderly intelligence; 5. Inadequate parenting is not the etiology of puberphonia; 6. This voice disorder is curable.

Diagnosis and possible treatments of puberphonia

Quality assessment of the voice is very important, because based on it, the vocal therapist designs a treatment plan and program, and implements adequate methods and techniques that include the ultimate care for the voice of each patient [35]. The diagnosis of

puberphonia relies on auditory-perceptual characteristics, acoustic analysis, laryngovideostroboscopy findings, psychological assessment, tests to exclude hypogonadism, assessment of secondary sexual characteristics, Gutzmann pressure test (external downward pressure on the thyroid cartilage will cause normal voice), as well as clinical examination of the larynx. The diagnostic profile of an individual with puberphonia is relatively simple. Typically, the patients are male after puberty, with relatively normal physical development, including secondary sexual characteristics. The pitch of these patients is significantly higher than expected and similar to the pitch of females. They also rarely have a height that goes through the chest register. Patients lack knowledge of their own potential for a male voice in the chest register and are therefore unaware of how to choose and maintain such a voice [9]. There is a lack of data in the literature on the results of vocal therapy in the case of puberphonia. Recently, there has been a dramatic change in the approach to treating this condition. While older concepts were almost synonymous with vocal therapy, recent trends are prone to surgical correction. Watkinson [36] actually says that surgery is contraindicated in the treatment of puberphonia. Nevertheless, surgical correction of puberphonia is now successfully performed, which has been confirmed through many studies and case reports around the world. In order to decide on a therapeutic modality, we must first define the goals of puberphonia treatment, and regardless of the therapy we apply, the goals must be the same: 1. The patient should be taught to perform phonation in the lower register; 2. The patient should be taught to make full use of phonatory and respiratory muscles; 3. The patient must be convinced that the new, lower voice must be used instead of the old, higher one.

Different therapeutic models are available, mainly categorized into three groups: vocal therapy, digital laryngeal manipulation and surgery. Before undergoing vocal therapy, patients need to be properly consulted on the basics of voice anatomy, human growth, and voice production. This will help alleviate the patient's anxiety before the same therapy. Vocal therapy is a behavioral method to change the way the voice is produced. Therapeutic techniques used in vocal therapy can even improve wound healing after vocal cord injury. Vocal therapy is an effective and appropriate method of treatment either as the only therapy for voice disorder or in combination with other treatment modalities (e.g. surgery, medications). It is considered to be the primary method of treatment of puberphonia [37]. Vocal therapy is traditionally divided into two categories, direct and indirect vocal therapy. A program of vocal therapies that combine direct and indirect approaches is considered to give the best results. Historically, indirect vocal therapy has involved vocal rest. If inadequate or traumatic speech is the cause of voice problems, in most cases the voice temporarily is improved after vocal rest. However, dysphonia usually returns after continued use of the voice. Therefore, vocal rest alone cannot solve the long-term voice problem. Indirect vocal therapy also consists of educating the patient about the dangers of vocal abuse and vocal hygiene. In contrast to indirect, direct vocal therapy involves changing the patient's speech technique in an attempt to increase vocal efficiency and improve voice quality. Vocal therapy usually requires 1–2 sessions once a week for approximately 6–8 weeks. Exceptions exist, including speech therapy before phonosurgery, which is usually limited to a few sessions before surgery and approximately 1–2 weeks after surgery. The techniques of direct vocal therapy focused on puberphonia are as follows: Cough: The patient learns to cough by pressing Adam's apple. This maneuver shortens the length of the vocal cords where it reduces its vibrating tone. The patient is advised to do this exercise at home. This will allow the patient to get

used to the lower fundamental frequency of the voice. The next technique is masking the range of the voice - this procedure is known to improve the quality of the voice. The use of auditory masking is used to produce a reflex response. Speaking in a noisy background has been found to have deep effects on how an individual speaks. It can change the quality of an individual's speech. This procedure also makes the voice clearer and louder. An instrument known as a facilitator is used for this purpose. Masking is in the range between 100–8000 Hz. Another technique under the guise of direct vocal therapy is a glottal attack before a vowel - The vowel is a very important sound in speech. It is also easily amenable to therapy/change. Glottal attack involves bringing both vocal cords closer. The patient is asked to breathe in and create air pressure in the subglottic area. This causes increased muscle tension in the laryngeal area. The vowel is pronounced as the air is breathed out. This procedure allows a patient to settle down to his basic fundamental frequency of voice. Also, there are techniques for relaxing the laryngeal musculature: Yawn technique, M warm-up, Visipitch, chewing technique, Boone's technique, Mcfarlane and Boone's Boom swallowing technique [10]. However, there is a lot of controversy about the usefulness of these techniques for relaxing the laryngeal muscles and their effectiveness. Some have even been found to cause damage to the vocal cords [38]. In addition to vocal therapy, there is also laryngeal manipulation as a method of treating puberphonia. This method was first described by Dr Sudhakar Vaidia in his study [34]. Patients were examined under local anesthesia using a Macintosh intubation laryngoscope. The long blade of the laryngoscope was placed in the vallecula and the patient was asked to speak the long "EEE". The pressure in the vallecula stretched the vocal cords. With additional external pressure on the thyroid cartilage, the quality of the voice improved, in the sense that the patient's voice immediately improved from a child's high tone to a lower adult male voice. Digital laryngeal manipulation means that the thyroid cartilage is compressed and the patient is asked to speak. The patient is advised to repeat this procedure at home. Also, injecting botulinum toxin into the cricothyroid muscle could also be helpful. In the same study, it was found that ideally 15 units of Botulinum toxin should be injected in each direction. We can conclude that a conservative approach to the treatment of puberphonia includes speech therapist and otolaryngologist, and that a motivated patient and a dedicated team of therapists usually succeed in treatment in most patients. When this approach to treatment does not work, a surgical option remains in those rare patients. Namely, when all these conservative methods fail, surgery must be the option. The first case of surgical treatment of puberphonia was reported by Pau and Murthi [39].

Conclusion

Voice is an essential component of individual expression and as a connection with others. As a basic part of one's inner being, the voice is highly influenced by thoughts and self-awareness [40]. The functionality and dysfunction of the voice must be understood within a psychosocial context. Voice characteristics appropriate to age, gender, and cultural background are a critical aspect of functional voice use and consequently successful communication. Future directions in research include replication of existing research and comparisons of various puberphonia treatment techniques to determine efficacy in terms of method/technique and duration of therapy. Also, additional directions include examining participants who belong to other groups, such as individuals with additional communication difficulties. The combination of clinical outcomes with empirical evidence from controlled scientific studies may be most acceptable through a joint effort between clinical environments and

institutional research. Such combined resources can improve knowledge of functional voice dynamics and psychosocial issues, as well as facilitate the establishment of reliable differential diagnostic procedures and the development of treatment approaches.

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Puberfonija: od klasičnog do savremenog pristupa

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Glas je značajna komponenta komunikacije koja nam omogućava prenošenje informacija i izražavanje osjećanja, stoga se posmatra kao temelj verbalne komunikacije. Sazrijevanje tijela obuhvata širenje grkljana i niže pozicioniranje grkljana u vratu, što rezultira višestrukim promjenama u kvalitetu glasa. Brze promjene na ljudskom grkljanu tokom puberteta očiglednije su kod muškaraca. Takve promjene za posljedicu mogu imati mutaciju glasa - puberfoniju. Puberfonija, koja se naziva i mutaciona disfonija ili mutacioni falset je neuspjeh prirodnog smanjenja osnovne frekvencije ili visine tona. Puberfoniju možemo definisati i kao perzistentan adolescentni glas i nakon puberteta u odsustvu organskog uzroka. Ovaj funkcionalni poremećaj glasa može imati višestruke posljedice na ličnost i kvalitet života pojedinca, koji često nailazi na probleme psihološke, emocionalne, socijalne i profesionalne prirode. Ovaj rad ima za cilj da pregleda relevantnu i dostupnu literaturu na temu puberfonije na sveobuhvatan, sažet način, ističući etiologiju, prevalenciju, kliničku manifestaciju, posljedice na kvalitet života osoba sa puberfonijom, kao i evoluciju pristupa i odnosa prema njegovom liječenju.

Ključne riječi: mutacija glasa, pubertet, puberfonija

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Review

Vitamin D and atherosclerosis

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Summary

Cardiovascular diseases rank first on the mortality list globally or 31%. The basic measure of prevention in accordance with the recommendations of the World Health Organization is a change in risk lifestyle in terms of diet, physical activity, tobacco and alcohol consumption. Vitamin D was previously recognized as a regulator of calcium and phosphorus ratio, bone remodeling or the main controller of skeletal pathophysiology. However, vitamin D enjoys great interest in clinical and epidemiological research in terms of its possible impact on reducing the risk of cardiovascular diseases. Among other things, vitamin D deficiency is associated with an increased risk of endothelial dysfunction. Although the deficiency has been identified as a risk marker for cardiovascular diseases, the mechanism of action of vitamin D on the path from endothelial dysfunction to cardiovascular diseases has not been fully revealed. The findings in this segment of activity of vitamin D would be significant in terms of reducing morbidity and mortality from cardiovascular diseases.

Key words: vitamin D, endothelial dysfunction, atherosclerosis, cardiovascular diseases, mortality

Introduction

Vitamin D in the body is not dependent exclusively on nutritional sources, which somewhat refutes its name "vitamin", because the way it originates in the body, "hormone" would be more appropriate name. According to its chemical structure, it belongs to the group of steroids [1]. Whether it originates from nutritional food sources of plant (ergocalciferol, D2) or animal origin (cholecalciferol, D3) or is formed under the reaction of ultraviolet B (UVB) rays on the

skin from 7-dehydrocholesterol (D3), in order to become active, double hydroxylation is necessary [1]. The first hydroxylation of vitamin D takes place in the liver through the enzyme D3-25, hydroxylase. The second hydroxylation takes place via the enzyme D3-1a-hydroxylase (primary kidney cells). After double hydroxylation, the active form of vitamin D or calcitriol was obtained. There is another active form of vitamin D that is formed by dihydroxylation in the kidneys (positions 24 and 25), known as 24,25-dihydroxycholecalciferol [1,2]. Vitamin D (D represents D_2, or D_3, or both) that is ingested is incorporated into chylomicrons, which are absorbed into the lymphatic system, enter the venous blood and interact with its nuclear vitamin D receptor in the small intestine, kidneys, and other tissues [2]. It should be noted that D2 and D3 are used for food fortification and in vitamin D supplements [2].

Epidemiological data

Clinical and epidemiological studies have linked vitamin D deficiency to non-skeletal disorders. Based on epidemiological studies a wide range of non-skeletal diseases have been linked to vitamin D such as cardiovascular, malignant and immune diseases, then diabetes mellitus and multiple sclerosis [3,4,5].

The role of vitamin D in CVD received an epilogue at a time when scientists came up with statistics that the prevalence of ischemic heart disease and hypertension is higher in countries that have fewer sunny days a year. Similar studies have shown that the incidence of cardiovascular adverse events is more common in winter [6].

Also, in survey from 2013 to 2015 based on measuring the concentration of 25(OH) D in 1,078 patients and basic information, scientists concluded that prevalence of CVD was 59.28% in the vitamin D deficient group [7]. Researchers of Framingham study have proved that concentrations of 25(OH)D <15 ng/ml were associated with 60% increased risk of a future cardiovascular event. The highest risk was evident in individuals with hypertension [8]. A study in Finland that included 6,219 men and women without cardiovascular diseases has proved that the largest quantile of 25(OH)D had 24% lower risk for CVD [9].

In the Vindicate study which included 229 patients (179 men) with chronic HF due to LVSD and vitamin D deficiency (cholecalciferol <50 nmol/l [<20 ng/ml]) participants were allocated to 1 year of vitamin D3 supplementation (4,000 IU [100 μg] daily) or matching non-calcium-based placebo [10]. Authors have concluded that the one year of 100 μg daily vitamin D3 supplementation does not improve 6-min walk distance but has beneficial effects on LV structure and function in patients on contemporary optimal medical therapy. Further studies are necessary to determine whether these translate to improvements in outcomes [10].

Houghton and Lew suggested that there was an increased risk for hypercalcemia related to over supplementation with vitamin D. They presented the hypervitaminosis D-induced hypercalcaemia in an individual taking 50,000 IU of vitamin D supplement daily for several months due to vitamin D deficiency. Over supplementation with vitamin D caused hypercalcemia (>2.6 mmol/L) with reported mild chronic abdominal pain with constipation, nausea, vomiting, hypertension and tachycardia at the first (of seven) hospital admission. Due to persistent symptomatic hypercalcemia (liposolubility of vitamin D), patient required six more admissions. Authors suggested that the increased awareness of vitamin D deficiency and accessibility of vitamin D supplements may increase frequency of hypervitaminosis D-induced hypercalcemia [11].

The ARIC study included four cities in the United States of America and was conducted as a prospective cohort study with over 15,000 respondents for a period of 33 years of

follow-up. Namely, the design of the study is such that the subjects were visited five times during the stated period, and that the variables necessary for the study were collected at that time. Serum samples were collected during the second visit and stored at – 70° C, analyzed by liquid chromatography for the presence of 25(OH)D during 2012 and 2013. After statistical analysis, the researchers concluded that lower HDL concentrations and higher TGL/HDL – C ratio were recorded in participants with a deficiency of 25(OH)D (<20 ng/ml) compared to participants with an optimal concentration of 25(OH)D (≥30 ng/ml) with CI of 95% [12]. A study conducted in the United States indicated that 83.5% of patients (n = 314) admitted to hospital for acute myocardial infarction had a low serum concentration of 25(OH)D (25(OH)D <75 nmol/l) [13]. The risk of myocardial infarction and stroke is significantly higher in people with vitamin D deficiency showed the European Prospective Investigation into Cancer and Nutrition (EPIC) [14].

In population-based cohort study - Roterdam study, authors found an association between vitamin D and prevalent stroke. Out of 9,680 participants, 339 had a history of stroke at baseline. Serum 25-hydroxyvitamin D concentration was associated with prevalent stroke (OR: 1.31; 95% CI, 1.14-1.51). Only severe vitamin D deficiency was associated with incident stroke (hazard ratio, 1.25; 95% CI, 1.05-1.50) [15].

During a clinical study in Poland involving 266 men and 144 women aged 65.4 ± 10.8, the researchers concluded that coronary artery lesions detected by coronary angiography were more common in patients with lower concentrations of 25(OH)D. Also, they unequivocally proved that patients with acute coronary syndrome had a significantly lower concentration of 25(OH)D compared to patients with stable coronary disease [16].

A retrospective study conducted from 2004 to 2015, including patients with chest pain and patients who underwent angiography. The results indicated that patients with normal concentration of 25(OH)D had coronary arteries without damage compared to a patient with low concentration (20 – 29.9 ng/ml), or lack (> 20 ng/ml) of 25(OH)D (OR: 7, 95% CI: 5.2 – 9.5, p <0.0001). Also, patients with low concentration or lack of vitamin D metabolites show signs of obstructive coronary artery disease in 62% of cases (n = 624, OR: 2.9, 95% CI: 2.3 – 3.7, p <0.0001), or 25% without signs of coronary artery obstruction (n = 249, OR: 1.5, 95% CI: 1.1 – 2, p = 0.02) [17].

The role of vitamin D in atherogenesis

Morbidity and mortality from CVD have a great socio-economic impact and represent the greatest burden on health systems globally [18]. The role of vitamin D in CVD is not clear at this point. Recent decades of research have shown that vitamin D is associated with atherogenesis. The discovery of vitamin D receptors in other organs (gastrointestinal tract, parathyroid glands, thyroid glands, pituitary gland, central nervous system, immune system, respiratory system, connective tissue and myocardium) reveals possible roles of vitamin D [3,4,5]. The most important are:

- increase in the concentration of nitric oxide in the endothelium,
- inhibition of platelet and leukocyte aggregation and adhesion,
- reduction of oxidative stress in the endothelium,
- influence on muscle tone of blood vessels,
- reduction in the release of vasoconstrictor substances,
- inhibits the release of proinflammatory cytokines,
- modulation of the immune response,
- inhibition of proliferation and migration of smooth muscle cells of blood vessels.

Endothelium is the largest regulator of

vascular homeostasis, which is reflected in the effects of vasoconstriction, vasodilation, smooth muscle tissue proliferation, thrombogenesis, inflammation and fibrinolysis [19]. If vascular endothelial dysfunction occurs, atherosclerosis develops. Vascular endothelial cells show the expression of vitamin D receptors (VDRs), which may play a protective role in the preservation of endothelium through various mechanisms of action. Also, VDR in endothelial cells expresses the enzyme 1a-hydroxylase, which consequently hydroxylates circulating 25(OH)D to the active form of vitamin D (calcitriol) [20]. The next step in the action of vitamin D on the endothelium is to regulate the production and release of nitric oxide (NO) through direct transcription and consequent vasodilation. If there is a decrease in the serum concentration of vitamin D, the consequence is a lower bioavailability of NO in the endothelium. In addition to the vasodilatory effect, NO plays a significant role in reducing platelet and leukocyte aggregation/adhesion. Vitamin D has been shown to have a protective role on the endothelium. Namely, vitamin D successfully prevents the accumulation of superoxide in endothelial cells. An observational study (n = 957) indicated that a low serum concentration of 25(OH)D was associated with an increased degree of inflammation, i.e. the concentration of IL-6 and CRP increased with a decrease in the concentration of the anti-inflammatory cytokine IL-10 [21]. Also, the low level of 25(OH)D in the circulation indicated a connection with the lipid profile of patients with diabetes, obesity and hypertension [22]. Namely, vitamin D limits the formation of foam cells and improves the transport of HDL, which indicates a possible protective mechanism [23].

There is evidence that vitamin D affects vascular tone depending on blood pressure. It is assumed that the mechanism of action is the reduction of endothelium-dependent contractions. The influx of Ca ions into endothelial cells causes the activation of the enzyme Ca-dependent phospholipase A2 and then starts the reaction of conversion of phospholipids from the cell membrane into arachidonic acid metabolites - endothelial vasoconstriction factors. This reaction is catalyzed by cyclooxygenase (COX) enzymes or the conversion of arachidonic acid into endoperoxides, which are converted into prostaglandins and thromboxane A2 under the action of synthase enzymes. It is thought that in this reaction, vitamin D may lead to a reduction in the release of endothelial vasoconstriction factors by preventing an increase in concentration of Ca in endothelial cells [24]. Also, research has shown that vitamin D reduces the expression of the enzyme COX and increases the expression of 15-hydroprostaglandin dehydrogenase, which ultimately results in reduced COX secretion and inactivation of prostaglandin [25].

In the process of atherosclerosis, in addition to the endothelium, the cells of the smooth muscle of the blood vessels also play an important role. Namely, these cells participate in the production of the extracellular matrix, which represents the beginning of atherogenesis [26]. Smooth muscle cells also possess VDR, which expresses the enzyme 1a-hydroxylase. Vitamin D has an antiproliferative effect on smooth muscle cells by inhibiting the enzyme endothelium-dependent DNA synthase and cell proliferation, then the effect on elastogenesis and immunomodulation [27]. Physiological doses of vitamin D inhibit the release of proinflammatory cytokines, adhesion molecules and the proliferation of smooth muscle cells of the blood vessels, which results in the suppression of calcification of the intima and media of blood vessels [27].

If endothelial damage occurs, it triggers a series of immune reactions that lead to atherosclerosis. Inflammation also activates T helper lymphocytes, macrophages and other immune response cells, while endothelial lesions lead to cholesterol infiltration. T helper

lymphocytes are primarily responsible for the proatherogenic response during endothelial inflammation by the production of cytokines (interleukin 2, interferon gamma, TNF alpha and beta, etc.) that cause macrophage activation. At the same time, T helper lymphocytes secrete interleukins that affect the production of antibodies by B lymphocytes, i.e. the immune response to inflammation [28].

Although the role of T helper lymphocytes in atherogenesis is unclear, there is evidence to suggest that their function is antiatherogenic due to the secretion of interferon gamma and certain cytokines [4]. There is evidence that vitamin D inhibits the proliferation of T lymphocytes and thus reduces the expression of interferon gamma and interlukin 2, which play a major role in the proatherogenic response. There is also evidence to suggest that vitamin D affects a stronger T helper lymphocyte response and has a direct effect on B lymphocyte regulation and antibody production. The result is an atheroprotective effect with stronger protection and reduced immune response to the inflammatory process [29,30]. However, it should be noted that the effects of vitamin D on B lymphocytes as an indirect consequence of its effect on T lymphocytes are mentioned earlier in the manuscript.

Studies have shown that vitamin D affects the reduction of the process of taking up oxidized LDL by monocytes and macrophages, which results in a reduction in the formation of foam cells. Vitamin D has a similar effect immediately, acting on TNFa, which also prevents the migration of monocytes and transformation into foam cells [31]. Also, vitamin D reduces the expression of adhesion molecules (vascular adhesion molecule 1 and matrix metalloproteinases 1) which prevent platelet aggregation [32].

Vitamin D also shows an indirect atheroprotective effect by acting on pancreatic β cells, serum lipoproteins and the renin-angiotensin-aldosterone system (RAAS). Studies have shown that there is VDR on pancreatic β cells and that it has a function in the normal insulin secretion when vitamin D is bound to VDR. There are indications that the serum concentration of vitamin D is directly linked to the pancreatic β cells, causing better control of the serum concentration of glucose. Researchers searched the EMBASE, PUBMED and COCHRAINE databases, with the keywords glycosylated hemoglobin, serum vitamin D and blood sugar, and concluded that supplementation with reduced serum concentrations of vitamin D had a direct effect on reducing the markers of insulin resistance [33]. RAAS is the largest regulator of blood pressure in the body. The components RAAS, angiotensin II and aldosterone are associated with the pathogenesis of atherosclerosis. Also, studies indicate that vitamin D is a potent endocrine suppressor of RAAS [34]. In vitro models have shown that vitamin D suppresses renin and angiotensin gene expression, while clinical studies have found that serum concentration of vitamin D is inversely associated with plasma renin activity [35].

The study, which included 80 patients divided into 4 groups depending on the number of affected coronary arteries, concluded that the concentration of vitamin D, TGF-β1 and IL-35 was negatively correlated with the severity of coronary artery disease [36].

The study that included genetic variants of VDVP (n= 1080) indicated that there was no association with severity of coronary disease. Also, from the same study, it was concluded that the concentration of 25(OH)D is a predictor of coronary lesions during angiography [37].

Researchers examining the association between coronary heart disease, diabetes and vitamin D status (n = 1859, of which 34.5% with diabetes) concluded that lower vitamin D concentrations in patients with diabetes were independently associated with increased prevalence and the severity of coronary artery disease [38]. Recent studies due to COVID-19 pandemia showed that decreased serum concentracion of 25(OH)D was inversely associated

with increased serum concentracion od IL-6 and CRP, and as a conclusion increased risk of heart failure, diabetes and pneumonia [39].

Recommendations for vitamin D intake

Vitamin D deficiency in children has the greatest impact on the skeleton and leads to rickets and in adults to osteomalacia and osteoporosis. Since the production of vitamin D in the body depends on UVB radiation, residents who live at a greater distance from the equator are at higher risk of developing vitamin D deficiency. Also, people with darker skin absorb less UVB radiation, which leads to reduced vitamin D synthesis. Daily exposure to sunlight at a dose of one third of the minimal erythema dose is considered sufficient to produce the required amounts of vitamin D [40]. The World Health Organization states in its document on UV radiation and vitamin D that it is necessary to sunbathe the peripheral parts of the body for at least 5 to 15 minutes, two to three times a week during the summer months in order to maintain the concentration of vitamin D [41].

The National Institutes of Health of the USA has published a Recommended Dietary Allowance (RDA) for vitamin D for a healthy population in accordance with age, good bone health and maintenance of calcium homeostasis. From the moment of birth to the first year, it is necessary to take 400 IU or 10 μg, from the first year of age to 70 years, as well as during pregnancy/lactation 600 IU or 15 μg. People over the age of 71 have higher vitamin D needs of 800 IU or 20 μg [42].

For determination of vitamin D status in clinical research and public health 25-hydroxycholecalciferol (25(OH)D) is used. By itself, 25(OH)D has a long half-life and is suitable for assessing the status of vitamin D in the blood. Measuring the concentration of 25(OH)D integrates exposure to nutritional sources of vitamin D as well as UV radiation or supplementation from a few months back [43].

After extensive research, experts from the Institute of Medicine established reference value of 25(OH)D in the blood (Table 1).

Determination of serum concentration of 25(OH)D is usually carried out by liquid chromatography. Depending on the laboratory method, which may be different even from the usual one, falsely high or falsely low serum concentration of 25(OH)D values can be obtained.

Table 1. Serum concentration 25(OH)D and health

Health status	nmol/L	ng/mL
Associated with vitamin D deficiency, leads to rickets or osteomalacia	<30	<12
It is considered inadequate for maintaining the bone health of healthy individuals	30-49	12-19
It is considered adequate for maintaining the bone health of healthy individuals	≥50	≥20
Evidence pints to potential side effects especially concentrations greater than 150 nmol/L or 60 ng/mL	>125	>50

* Retrieved from the Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC: National Academy Press, 2010

Vitamin D supplementation and cardiovascular risk

Vitamin D deficiency is common, especially in developed countries, where the prevalence of vitamin D deficiency is from one third to one half of the adult population [4]. Also, in developed countries, there is a high prevalence of CVD and hypertension. Vitamin D deficiency is easily corrected by oral supplementation,

which would greatly contribute to the possibility of simple and cheap intervention with the aim of reducing cardiovascular risk. Experimental evidence of an association between vitamin D metabolism and cardiovascular physiology suggests a causal relationship. However, animal models cannot reliably represent nutritional vitamin D deficiency in humans. Most existing clinical trials are designed to investigate the primary effect of supplementation on the skeleton, while the secondary effect is on the cardiovascular system.

One of the largest clinical studies is the Women's Health Initiative (WHI) with a randomized sample of > 36,000 women, with 400 IU vitamin D supplementation and 1,000 mg daily calcium carbonate supplementation. Researchers have shown that there is no evidence that active supplementation with vitamin D and calcium carbonate leads to a reduction in the risk of coronary events or stroke (HR1.04, 95% CI 0.92 – 1.18) [44]. Also, by meta-analysis of randomized clinical trials, researchers indicated that vitamin D supplementation was not associated with a reduced risk of acute cardiovascular events (myocardial infarction, stroke), cardiovascular mortality. [45]. However, a large number of observational studies have shown that there is a significant bond between low serum concentration of vitamin D and cardiovascular events [46]. Sistematic review and meta-analysis based on the effects of vitamin D supplements on cardiovascular risk factors demonstrated that vitamin D supplementation (> 4,000 IU) improved serum concentrations of 25(OH)D, significantly lowered blood pressure, serum PTH, hs-CRP, TC, LDL, and TG and increased HDL. Vitamin D supplementation also appears to improve arterial stiffness [47].

Conclusion

From the time of the discovery of vitamin D until today, clinical and epidemiological studies have confirmed that vitamin D has a wide range of roles in the body in addition to those recently known roles in Ca and P homeostasis. Also, researches link vitamin D to CVD, which are at the very top of the morbidity and mortality list. Clinical evidence suggests that vitamin D participates in the process of atherogenesis acting anti-atherogenic, and epidemiological data speak in favor of this discovery because people with vitamin D deficiency have higher risk for CVD.

Namely, the scientists processed the data and came to the conclusion that the prevalence of CVD is higher in countries with fewer sunny days a year, and that the incidence of cardiovascular events is more frequent in winter when there are fewer sunny days. Based on such data, world authorities have given recommendations for daily intake of vitamin D as well as the recommended time of exposure to UVB rays. Since clinical trials were designed to primarily investigate the effect of vitamin D on the skeleton and secondarily on CVS, it is necessary to conduct larger number of clinical trials to investigate the role of vitamin D on the cardiovascular system.

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Vitamin D i ateroskleroza

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Kardiovaskularne bolesti zauzimaju prvo mesto na listi mortaliteta globalno, odnosno 31%. Osnovna mera prevencije u skladu sa preporukama Svetske zdravstvene organizacije je promena rizičnih ponašanja u pogledu ishrane, fizičke aktivnosti, konzumacije duvana i alkohola. Vitamin D je ranije prepoznat kao regulator odnosa kalcijuma i fosfora, remodeliranje kosti, odnosno glavni kontrolor skeletne patofiziologije. Međutim, vitamin D uživa veliko interesovanje u kliničkim i epidemiološkim istraživanjima u pogledu njegovog mogućeg uticaja na smanjenje rizika od kardiovaskularnih bolesti. Između ostalog, deficit vitamina D se dovodi u vezu sa povećanim rizikom od endotelne disfunkcije. Iako je deficit identifikovan kao marker rizika za kardiovaskularne bolesti, mehanizam delovanja vitamina D na putu od endotelne disfunkcije do kardiovaskularnih bolesti nije u potpunosti otkriven. Otkrića u ovom segmentu delovanja vitamina D bi bila značajna u pogledu smanjenja morbiditeta i mortaliteta od kardiovaskularnih bolesti.

Ključne reči: vitamin D, endotelna disfunkcija, ateroskleroza, kardiovaskularne bolesti, mortalitet

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Case reports

A rare thyroid disorder mimicking mitochondrial disease

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Summary

Introduction. Patients affected with Allan-Herndon-Dudley syndrome (AHDS) have a deficiency of monocarboxylate transporter 8 (MCT8), a protein primarily responsible for the transport of triiodothyronine (T3) into the brain. This X-linked disorder affects almost exclusively males with clinical presentation encompassing developmental delay, axial hypotonia, dystonia, poor head control, quadriplegia and absence of speech.

Case reports. Patient 1 is a male child referred to a hospital investigation at 11 months due to severe developmental delay and elevated blood ammonia level (163 mcmol/L). Hypotonia and dystonic movements were noted at admission, with facial dysmorphic features. Laboratory findings revealed increased blood lactate (17.2 mmol/L), alanine (533 mcmol/L) and ammonia (391 mcmol/L) concentrations. Serum creatine-kinase levels showed substantial increase over the course of hospitalization up to 6,855 IU/L. Clinical exome sequencing detected a novel hemizygous frameshift insertion c.1456insC in gene SLC16A2, predicted to cause loss of normal protein function either through protein truncation or nonsense-mediated mRNA decay. Segregation genetic testing of the family members revealed that mother, maternal uncle and maternal grandmother carry the same mutation in SLC16A2. The boy`s mother experienced learning difficulties through childhood while maternal uncle is severely affected by AHDS. Patient 2 is a boy referred to clinical geneticist due to severe psychomotor delay of unknown etiology. Moderate serum lactate elevation was the only laboratory abnormality during initial investigations. Diagnosis of AHDS was established by clinical exome sequencing, and subsequent hormonal evaluation revealed increased triiodothyronine (T3) level which corresponds well to genetic diagnosis.

Conclusion. Presence of lactic acidosis and/or hyperammonemia in children with severe developmental delay is not specific for inborn disorders of energy production, such as mitochondrial disease. Clinicians should consider thyroid hormones profiling in cases of unexplained severe developmental delay in male children, especially if associated with axial hypotonia and dystonic movements.

Key words: Allan-Herndon-Dudley syndrome, hyperammonemia, lactic acidosis

Introduction

Seventy years after the first clinical description of Allan-Herndon-Dudley syndrome (AHDS), it was found that mutations in SLC16A2 are the genetic basis of this rare disease [1]. Patients affected with AHDS have a deficiency of monocarboxylate transporter 8 (MCT8), a protein primarily responsible for the transport of triiodothyronine (T3) into the brain. This X-linked disorder affects almost exclusively males and its incidence could be underestimated due to absence of pathognomonic clinical features. With the onset of genome-based diagnostic approach, substantial number of new cases has been reported in recent years, from different ethnic and geographic backgrounds [2–4]. Clinical presentation of AHDS is variable but in most cases encompasses severe developmental delay, axial hypotonia, dystonia, poor head control, quadriplegia and absence of speech [5,6]. Brain MRI findings usually reveal delayed myelination [7]. Recently, treatment options for AHDS have emerged with the use of T3 analogues such as tiratricol (Triac) [8].

Pattern of hormonal status in affected children includes elevated 3,3′,5-triiodothyronine (T3), high or normal thyroid-stimulating hormone (TSH) and low or normal tetraiodothyronine (T4) (5). However, there have been reports of AHDS patients with normal thyroid status [9] or with unusual laboratory findings, e.g. elevated serum lactate and/or ammonia [9,10].

Small number of female carriers of mutations in MCT8 have presented with manifestations related to the AHDS [10]. Clinical spectrum in female carriers ranges from asymptomatic to mild mental retardation, and in some cases to more complete clinical and hormonal profile of AHDS [11]. Herein, we describe two boys with distinct presentation of AHDS.

Case reports

Patient 1 is a male child born at term from the first uneventful pregnancy of non-consanguineous parents with birth weight of 3,500 grams, birth length of 51 cm and normal head circumference (35 cm). Apgar score was 10 at first minute. Hypotonia was already noted in neonatal period, but the boy was not referred to hospital investigations until 11 months. The reasons for referral were a severe developmental delay and elevated blood ammonia level (163 mcmol/L) registered during an outpatient neurological evaluation. Hypotonia and dystonic movements were noted at admission. Face was noted as wide, with epicanthal folds and micrognathia and normal head circumference (P25-50). Bilateral cryptorchidism was verified. Laboratory findings revealed high blood lactate (17.2 mmol/L) and ammonia (391 mcmol/L) concentrations. Arterial blood pH was 7.22, with base excess at -12.4 mmol/L. Level of creatine-kinase in serum showed substantial increase over the early course of hospitalization, from 819 IU/L at admission to 6855 IU/L on the second day. Amino acid analysis in plasma revealed increase of alanine (533 mcmol/L) while urine organic acid profile as well as plasma total carnitine, free carnitine and acylcarnitine profile were normal. MRI of the brain showed delayed myelination with thin corpus callosum and overall reduction of supratentorial white matter. Acidosis was corrected and thiamine and carnitine were introduced in maintenance dosage, due to suspicion of inborn error of energy metabolism. Overall condition of the child remained stable despite laboratory abnormalities, and he was discharged after 20 days. Ammonia level was reduced to 64 mcmol/L at discharge. Clinical exome sequencing was performed at 21 months and detected a novel hemizygous frameshift insertion c.1456insC in gene SLC16A2. The mutation was predicted to cause loss of normal protein function either through protein truncation or

nonsense-mediated mRNA decay. After acquiring genetic diagnosis, thyroid hormone profile was performed for the first time and revealed elevated T3 and free T3 plasma levels, high TSH and low free T4 concentration: TSH 9.71 mIU/L (ref. 0.27–4.2), fT4 6.87 pmol/L (ref. 12.0–22.0), fT3 15.74 pmol/L (ref. 2.92–7.53), T3 5.68 pmol/L (ref. 0.6–3.9)

Segregation genetic testing of the family members revealed that mother, maternal uncle and maternal grandmother carry the same mutation in SLC16A2. The boy`s mother had learning difficulties through childhood and adolescence. Maternal uncle is affected with severe mental retardation and quadriplegia. Maternal grandmother does not have any clinical manifestations relevant to AHDS. X-inactivation study was performed in female carriers in this family and it revealed similar pattern of skewed inactivation of X chromosome in both, favouring chromosome without SLC16A2 mutations (90:10).

During the second year of life, severe developmental delay, absence of head control and dystonic movements were persevering. Blood ammonia level was measured at follow up visits every three months, and it showed non-consistent findings, ranging from 49 mcmol/L to 377 mcmol/L, without signs of acute encephalopathy. At two years of age, treatment with L-thyroxine was introduced due to consistently elevated TSH level, but without apparent clinical effect.

Patient 2 is a boy referred to clinical geneticist due to severe psychomotor delay of unknown etiology at 18 months. Clinical exam revealed abundance of involuntary dystonic movements. Moderate serum lactate elevation (5.1 mmol/L) was the only laboratory abnormality during initial investigations. Diagnosis of AHDS was established by clinical exome sequencing when novel variant c.45G>A in SLC16A2 gene was found. Subsequent hormonal evaluation revealed increased T3 level and normal TSH which corresponds well to the established genetic diagnosis. Delay of psychomotor development is severe with inability to speak or sit independently at the age of 2.5, while serum lactate remains mildly to moderately elevated during follow-up. At this age, delayed myelination was the most prominent finding of the performed brain MRI. Triac was recently initiated at the age of 3 and discrete motor improvement was observed over the weeks of therapy.

Discussion

Severe developmental delay in a hypotonic child with elevated blood lactate and/or ammonia, as observed in our patients, was highly suggestive of mitochondrial disease. Only after the exome sequencing, the correct diagnosis of AHDS was established. Hyperlactatemia and hyperammonemia have already been anecdotally reported in patients with AHDS [10,11]. Herzovich et al. attributed these findings to peripheral hyperthyreotic effects, speculating that high catabolic rate in skeletal muscles of AHDS patient results in such abnormalities: high lactic acid was explained by the enhanced glycolysis/glycogenolysis, and high ammonia in plasma by accelerated purine nucleotide catabolism [12]. Significant hyperlactatemia was also noted in case report by Langley et al., but ammonia level was normal in this AHDS patient [10]. Elevated serum concentration of CK was verified in patient 1 as unique finding for AHDS so far. However, several authors reported hyperammonemia and elevated serum hyperCKemia in patients with hypothyroidism [13]. In a study by Turkish authors, CK levels have been shown to negatively correlate with TSH levels in both overt and subclinical hypothyroidism [14]. Therefore, the transitory presence of hyperCKemia in our patient 1 could not be explained by thyroid disorder.

The impact of thyroid hormones on mitochondrial metabolism has been extensively studied [15]. The role of T3 as the inducer

of mitochondrial biogenesis and regulator of mitochondrial gene expression is recognized [16]. In recent paper, Zimmerman et al. reported a significant reduction of respiratory chain complex I in oncocytic cells of patients with hypothyroidism [17]. These authors propose possible role of complex I deficiency in immune pathogenesis of hypothyroidism. Reduced presence of respiratory chain complex I in muscle cells of AHDS patient in our report, casts a new light onto the relationship of thyroid disorders and mitochondrial metabolism. There are, however, scarce reports of mitochondrial disturbance in the presence of thyrotoxicosis, such as massive enlargement of these organelles and activation of mitochondrial apoptotic pathway [18]. These processes could underlie some of the observed similarities between AHDS and mitochondriopathies.

There are reports of AHDS patients previously considered to have other genetic diseases. Vaurs-Barrière et al. showed that a significant portion of patients with initial diagnosis of Pelizaeus-Merzbacher were found to actually suffer of AHDS. A number of AHDS cases was genetically diagnosed with the use of next generation sequencing tools [3,19]. Methodology of NGS proves valuable in population of patients with severe developmental delay of unclear etiology. However, use of much cheaper and quicker thyroid hormone profiling could direct the genetic analysis toward a single gene analysis for the confirmation of AHDS.

Neuroimaging is one of the key diagnostic tools when dealing with children with profound developmental delay associated with movement disorder, as seen in AHDS. Inadequate myelination and brain atrophy have been recognized as the imaging features of this particular disorder, but they are not universally present [6]. In a proportion of patients central white matter myelination shows gradual improvement over time [20,21]. Studies have revealed that MCT8 deficiency, diminished thyroid stimulation of oligodendrocytes leading to hypomyelination in critically important period of early life [22]. Drawing from our own experience with two patients, brain MRI can be a helpful tool in establishing suspicion of AHDS. Also, follow-up of the myelination status can be important part of treatment response assessment.

Conclusion

Presence of lactic acidosis and/or hyperammonemia in male children with severe developmental delay is not specific for inborn disorders of energy production, such as mitochondrial disease. Clinicians should consider thyroid hormones profile in cases of unexplained severe developmental delay in male children, especially if associated with axial hypotonia, dystonic movements and MRI findings of hypomyelination, even in presence of atypical laboratory findings.

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Redak poremećaj tireoidne funkcije sa ispoljavanjem sličnim mitohondropatiji

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Uvod. Oboleli od Allan-Herndon-Dudleyevog sindroma (AHDS) imaju nedostatak monokarboksilatnog transportnog proteina 8 (MCT8) koji omogućava ulazak trijodtironina (T3) u centralni nervni sistem. Ovaj X-vezani poremećaj uslovljen mutacijama u SLC16A2 genu pogađa gotovo isključivo dečake i klinički se ispoljava usporenim psihomotornim razvojem, hipotonijom osovine tela, distonijom, kvadriplegijom i odsustvom razvoja govora.

Prikaz slučajeva. Pacijent 1 je muško dete upućeno na ispitivanje u uzrastu od 11 meseci zbog usporenog psihomotornog razvoja i povišene koncentracije amonijaka u krvi (163 mcmol/L). Na prijemu se uočavaju hipotonija, distonični pokreti i diskretna dismorfija lica. U laboratorijskim nalazima iz krvi dobijaju se povišene koncentracije laktata (17,2 mmol/L), alanina (533 mcmol/L), amonijaka (391 mcmol/L) i serumske kreatin-kinaze (postepeni porast do 6855 IU/L). Kliničko egzomsko sekvencioniranje pokazalo je postojanje nove hemizigotne frameshift insercije c.1456insC u genu SLC16A2. Segregaciona analiza sprovedena kod članova porodice je pokazala da majka, ujak i baba po majci nose istu mutaciju u SLC16A2. Dečakova majka je imala određenih poteškoća u učenju tokom detinjstva, dok ujak ima kliničku sliku AHDS. Pacijent 2 je dečak upućen kliničkom genetičaru zbog teškog psihomotornog zastoja. U inicijalnim analizama se registruje umereno povišena koncetracija laktata u krvi. Dijagnoza AHDS je postavljena na osnovu kliničkog sekvencioniranja egzoma, a dalja dijagnostička obrada je pokazala povišene vrednosti T3 u krvi što odgovara ovoj dijagnozi.

Zaključak. Prisustvo laktične acidoze i/ili hiperamonijemije kod dece sa teškim psihomotornim zaostajanjem ne susreće se samo kod urođenih poremećaja energetskog metabolizma, odnosno mitohondropatija. Ispitivanje statusa tireoidnih hormona u slučaju psihomotornog zastoja nepoznate etiologije kod dece muškog pola ima značaja, pogotovo ako su prisutni aksijalna hipotonija i distonični pokreti.

Ključne reči: Allan-Herndon-Dudley sindrom (AHDS), hiperamonijemija, laktična acidoza

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Dijelovi rada: naslovna strana, kratak sadržaj sa ključnim riječima, tekst rada, literatura, prilozi (*tabele, grafikoni, slike*).

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Knjiga:

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

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Disertacija:

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Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

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Book:

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

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