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Vojnosanitetski Pregled



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# VOJNOSANITETSKI PREGLED

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Međunarodni dan sreće se, na inicijativu Ujedinjenih nacija, obeležava 20. marta svake godine, počev od 2013. godine. Tema ovogodišnjeg dana sreće je „Budite pažljivi, budite zahvalni, budite ljubazni“. Sreća i mentalno i fizičko blagostanje su osnovno ljudsko pravo i univerzalni cilj. Ovaj dan podstiče pojedince da razmišljaju o sopstvenoj sreći i uoče segmente u kojima mogu napraviti kvalitativan pomak napred. Živimo u svetu koji se suočava sa velikim izazovima, u vremenu u kome je podrška dobrobiti ljudi ključ srećnijeg sveta za sve nas. Cenimo važnost sreće u životu i ponašajmo se u skladu sa tim, usrećimo sebe i druge ljude svojim delima!

The International Day of Happiness is celebrated on the initiative of the United Nations every year on March 20, starting from 2013. This year's theme for the Day of Happiness is "Be Mindful, Be Grateful, Be Kind". Happiness and mental and physical well-being are basic human rights and represent a universal goal. This day encourages individuals to reflect on their own happiness and spot areas where they can progress. We live in a world that faces great challenges, in a time where supporting people's welfare is the key to a happier world for all of us. We appreciate the importance of happiness in life and act accordingly, making ourselves and other people happy with our actions!



## The importance of direct genetic testing for determining female carriers of the mutation in dystrophinopathies

Značaj direktnog genetičkog testiranja za otkrivanje žena prenosioca mutacije kod distrofinopatija

Jasmina Maksić\*, Nela Maksimović†, Lukas Rasulić†‡, Olgica Milankov§||, Ana Marjanović¶, Dragana Cvetković\*\*, Vidosava Rakočević Stojanović†¶, Ivana Novaković†

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### Abstract

**Background/Aim.** Duchenne muscular dystrophy (MD) and Becker MD are caused by mutations in the gene for dystrophin (*DMD*). They are X chromosome-linked recessive diseases where males are affected, and females are healthy carriers of the mutation in most cases. It is estimated that 2/3 of mothers of Duchenne MD probands are carriers, while 1/3 of probands have *de novo* mutations. The aim of the study was to confirm the carrier status of female members of the families of Duchenne MD/Becker MD probands using direct genetic testing methods. **Methods.** The study included 38 females from 31 families of Duchenne MD/Becker MD probands with deletion/duplication in the *DMD* gene. Moreover, 4 cases of prenatal diagnosis of Duchenne MD/Becker MD were included. The methods of polymerase chain reaction - PCR and the multiplex ligation-dependent probe amplification - MLPA were applied for detecting deletions, i.e., deletion/duplication mutations in the *DMD* gene. **Results.** In the total of 31 Duchenne MD/Becker MD probands, 87.1% of deletions and 12.9% of duplications of one or

more exons in the *DMD* gene were detected. Of the 29 tested mothers, mutations were found in 17 of them (14 deletions and 3 duplications). Mutations were detected in 11 (57.9%) out of 19 mothers of probands with the Duchenne MD phenotype and 6 (60%) out of 10 mothers of Becker MD probands. Furthermore, 14 (56%) out of 25 mothers were carriers in probands with deletions, and 3 (75%) out of 4 mothers were carriers in probands with duplications. In the remaining 9 other female relatives of the patients, mutations were found in 4. In prenatal diagnosis, we identified a deletion in one male and one female fetus of one single mother who was confirmed as a carrier. **Conclusion.** The study showed that mothers were carriers in almost 60% of sporadic cases of Duchenne MD/Becker MD with deletions and duplications. In addition, the carrier frequency tended to be higher in mothers of the probands with duplications (75%) compared to mothers of probands with deletions (56%).

**Key words:** genes; genetic testing; muscular dystrophies; mutation; prenatal diagnosis; women.

### Apstrakt

**Uvod/Cilj.** Dišenova mišićna distrofija (MD) i Bekerova MD su uzrokovane mutacijama u genu za distrofin (*DMD*). To su recesivne bolesti vezane za X hromozom, od kojih obolevaju muškarci, a žene su uglavnom zdravi nosioci mutacije. Procenjeno je da su kod probanada obolelih od Dišenove MD 2/3 majki nosioci mutacije, dok 1/3 probanada ima *de novo* mutaciju. Cilj rada bio je da se potvrdi status nosioca mutacije kod ženskih članova porodica probanada obolelih od Dišenove MD/Bekerove MD prime-

nom metoda direktnog genetičkog testiranja. **Metode.** Studija je obuhvatila ukupno 38 žena iz 31 porodice probanada obolelih od Dišenove MD/Bekerove MD sa delecijom/duplikacijom u *DMD* genu. Takođe, u studiju su bila uključena i 4 slučaja Dišenove MD/Bekerove MD otkrivena prenatalnom dijagnostikom. Metoda lančane reakcije polimeraze (*polymerase chain reaction* – PCR) i metoda višestrukog umnožavanja vezanih proba (*multiplex ligation-dependent probe amplification* -MLPA) su korišćene za detekciju delecija, odnosno delecija/duplikacija mutacija u *DMD* genu. **Rezultati.** Kod ukupno 31 probanada obolelih od Dišenove

MD/Bekerove MD, utvrđeno je 87,1% mutacija tipa delecije i 12,9% mutacija tipa duplikacija jednog ili više egzona u *DMD* genu. Od 29 testiranih majki probanada, mutacije su nađene kod njih 17 (14 delecija i 3 duplikacije). Mutacije su detektovane kod 11 (57,9%) od 19 majki probanada sa fenotipom Dišenove MD i kod 6 (60%) od 10 majki probanada obolelih od Bekerove MD. Takođe, kod probanada sa delecijom, kod 14 (56%) od 25 majki je potvrđeno da su nosioci mutacije, a kod probanada sa duplikacijom, 3 (75%) od 4 majke su bile nosioci mutacije. Od ostalih 9 ženskih srodnika probanada obolelih od Dišenove MD/Bekerove MD, mutacije su nađene kod njih 4. Prenatalnom dijagnos-

tikom utvrđene su delecije kod jednog muškog i jednog ženskog fetusa iste majke koja je bila potvrđena kao nosilac mutacije. **Zaključak.** Istraživanje je pokazalo da su majke bile nosioci mutacija u skoro 60% izolovanih slučajeva obolelih od Dišenove MD/Bekerove MD sa delecijama i duplikacijama. Takođe, učestalost majki nosioca mutacije kod probanada sa duplikacijom (75%) se pokazala višom nego kod majki probanada sa delecijom (56%).

**Ključne reči:**  
**geni; genetičko testiranje; mišići, distrofija; mutacija; prenatalna dijagnoza; žene.**

## Introduction

Duchenne muscular dystrophy (MD) and Becker MD are diseases that result from mutations in the dystrophin gene (*DMD*). The gene is located on the short arm of the X chromosome (Xp21.1). Duchenne MD is the most severe form of dystrophinopathies because of the missing protein dystrophin. The incidence of Duchenne MD is 1 : 3,500 in live-born males, and it is characterized by early onset (approximately the second year of life), progressive weakness of skeletal and cardiac muscles, and a fatal outcome in the early twenties<sup>1</sup>. Becker MD occurs 10 times less often and represents a milder form of the disease that starts to manifest mostly around the age of 10, with variability in the clinical features – from practically asymptomatic forms to severe forms such as Duchenne MD.

The *DMD* gene is the largest human gene – it is 2.4 megabases in size and shows a high rate of spontaneous mutations<sup>2</sup>. The changes occurring in the *DMD* gene are deletions/duplications of one or more exons in 80% of patients, while mutations of less than one exon are present in 20% of patients (point mutations, microdeletions, microinsertions, and splice site mutations)<sup>3</sup>. The most common mutations in the *DMD* gene are deletions (65–75%) that show the specific distribution in the gene, appearing in the so-called “hot spots” of the exons 2–20 (the proximal part of the gene) and exons 45–55 (the distal part of the gene)<sup>4</sup>. Duplications are present in 5–15% of cases and can affect any part of the gene, more often the proximal part<sup>5</sup>. It has been estimated that two-thirds of Duchenne MD are familial cases, while one-third of the patients have *de novo* mutations<sup>6</sup>. Likewise, duplications and point mutations are more likely to be family cases<sup>7</sup>.

Duchenne MD and Becker MD are monogenic diseases, where males as hemizygous are affected, while females are generally phenotypically healthy heterozygous carriers of the mutation. According to the X-linked recessive inheritance model, the affected father passes the mutated allele to his daughters, who will be phenotypically healthy carriers of the disease. A woman, who is a heterozygous carrier of the mutation, has a 50% chance that her daughter will inherit a risky X-chromosome and become a carrier or a 50% chance that her sons will inherit that chromosome and become ill. In classical literature, based on family history, women are defined as obligatory, probable, and possible carriers of the mutation<sup>8</sup>.

According to the risk we have described, it is important to detect women who are phenotypically healthy carriers of the mutation using molecular genetic testing. The multiplex ligation-dependent probe amplification (MLPA) is a direct molecular genetic method that allows a quantitative analysis of all 79 exons of the *DMD* gene and the detection of deletions and duplications in the probands as well as the female carriers of the mutations<sup>9–12</sup>. In addition to direct gene analysis, in cases without deletion or duplication in the probands, an indirect method of gene analysis is applied, which is important in 20–30% of cases resulting in point mutations. The indirect analysis is based on linkage analysis and implies that it follows the inheritance of polymorphic DNA markers located within or near the *DMD* gene, indirectly determining whether a particular family member has inherited a mutation. However, indirect detection of mutations has its own limitations, which relate to the possibility of recombination within the gene, i.e., within the analyzed region, as well as on the limited information markers<sup>13–16</sup>.

The aim of this study was to confirm the carrier status of females in the families of Duchenne MD/Becker MD probands using direct genetic methods.

## Methods

### Study groups

The study group consisted of 38 female members (29 mothers and 9 other female relatives) from families of 19 Duchenne MD and 12 Becker MD probands with confirmed deletion/duplication in the *DMD* gene. All the cases of those affected were sporadic, except for one family with two affected sons (only one was analyzed in the study). In 25 cases, only the mothers of the patients were tested, and in 6 cases, the mothers and/or other female relatives of the patients were tested. In addition, 4 cases of prenatal diagnosis of Duchenne MD/Becker MD were included in the study.

The study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade. The study was carried out at the Clinic for Neurology, University Clinical Center of Serbia, Belgrade, and at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade. The second study group consisted of patients (probands) selected based on the following clinical parameters: the onset of the

disease, the clinical features, electromyography findings, and elevated levels of creatine phosphokinase.

The genomic DNA for analysis was isolated from the peripheral blood lymphocytes of the subjects using the isolation method according to standard procedure<sup>17</sup>. For prenatal diagnosis, the DNA was isolated from chorionic villus samples using a commercial kit (Qiagen DNA mini kit).

#### *Genetic analysis of the patients and their female relatives*

In the probands, we previously applied the polymerase chain reaction (PCR) and/or the multiplex ligation-dependent probe amplification (MLPA) for detecting deletions/duplications in the *DMD* gene<sup>18, 11</sup>. Likewise, the MLPA method was applied to detect deletions/duplications in female carriers. For prenatal diagnosis of Duchenne MD/Becker MD mutations, after determining the sex, the PCR method was used on one female and two male fetuses, while the MLPA method was used on one female fetus.

#### *MLPA method*

In the MLPA analysis, two complementary SALSA MLPA kits, P034 and P035 (MRC Holland, the Netherlands), were used, according to the protocol recommended by manufacturer<sup>19</sup>. The analysis was carried out using an ABI Thermal Cycler Verity and an ABI 3500 Genetic Analyzer, and the software was processed using the Coffalyser.Net program (MRC Holland).

#### *PCR analysis*

For the PCR analysis of 26 exons of the *DMD* gene, three sets of primers, A, B, and C, were used<sup>18</sup>. The PCR products were analyzed using 8% polyacrylamide gel and the nucleic acid electrophoresis standard procedure.

#### *Statistical analysis*

For statistical analysis, frequencies and percentages were used as descriptive statistics.

## **Results**

#### *Results of genetic analysis of Duchenne MD/Becker MD probands*

In 19 Duchenne MD and 12 Becker MD probands (total  $n = 31$ ) with major changes in the *DMD* gene, deletions were identified in 27 (87.1%) and duplications in 4 (12.9%) probands. The most frequent localization of dele-

tion/duplication was in the “hot spots” of the *DMD* gene, 22.6% in the proximal part of the gene (exons 2–20), and 61.3% in the distal part of the gene (exons 45–55), while 5 (16.1%) mutations were outside the predilection regions of the gene. In patients with the Duchenne MD phenotype, 15 deletions and 4 duplications were identified, while deletions were found in all patients with the Becker MD phenotype (Table 1).

#### *Results of genetic analysis of mothers of Duchenne MD/Becker MD probands*

Of the 29 tested mothers of 19 Duchenne MD and 10 Becker MD probands, mutations in the *DMD* gene were found in 17 (58.6%) out of 29 mothers. Deletions were detected in 14 (82.4%) out of 17 mothers, and duplications were detected in 3 (17.6%) out of 17 mothers. All the mutations found in mothers who were carriers were the same as in their affected sons, except in two cases where one mutation was bigger and the other smaller (Table 2).

Concerning the phenotype of the probands, mutations were found in 11 (57.9%) out of 19 mothers whose sons had a Duchenne MD phenotype and 6 (60%) out of 10 mothers whose sons had a Becker MD phenotype.

Concerning the type of mutation, in probands with deletions, mothers were carriers in 14 (56%) out of 25 cases, and in probands with duplications, mothers were carriers in 3 (75%) out of 4 cases.

#### *Results of genetic analysis in other female relatives*

Out of the remaining 9 female relatives of the Duchenne MD/Becker MD probands (5 sisters of 2 Duchenne MD and 1 Becker MD probands, 1 grandmother of a Duchenne MD proband, 1 niece whose uncle was a Becker MD proband, and 2 daughters whose father was a Becker MD proband), mutations were found in 4 [Table 2, case No. 5 (Duchenne MD) – grandson whose grandmother (mother’s line) had deletions in exons 49, 50; case No. 20 (Becker MD) – uncle had deletions in exons 45–47, his niece was a carrier of mutation; case No. 31 (Becker MD) – father had deletions in exon 13, 2 daughters were tested, and both were confirmed as carriers. The affected father had a third daughter who was not tested for carrier status].

Out of all the tested sisters of the probands, not one was confirmed as a carrier.

#### *Results of prenatal testing*

A prenatal diagnosis was performed on two of the three daughters whose father suffered from Becker MD (deletion of exon 13). A prenatal diagnosis was performed

**Table 1**

**Genetic analysis of Duchenne MD and Becker MD probands**

Phenotype	Deletion	Duplication	Total
Duchenne MD	15	4	19
Becker MD	12	/	12

**MD – muscular dystrophy.**

**Results are given as number of Duchenne MD or Becker MD probands.**

for the pregnancies of one daughter, previously tested and confirmed as a carrier (case No. 31, Table 2), and for the other daughter not previously tested on the carrier status. For the first daughter, in three pregnancies, the PCR method confirmed the deletion of exon 13 in one male fetus, while there was no deletion in the other male fetus. In the

third – female fetus, the MLPA method confirmed heterozygous deletion in exon 13 (carrier) (Figure 1).

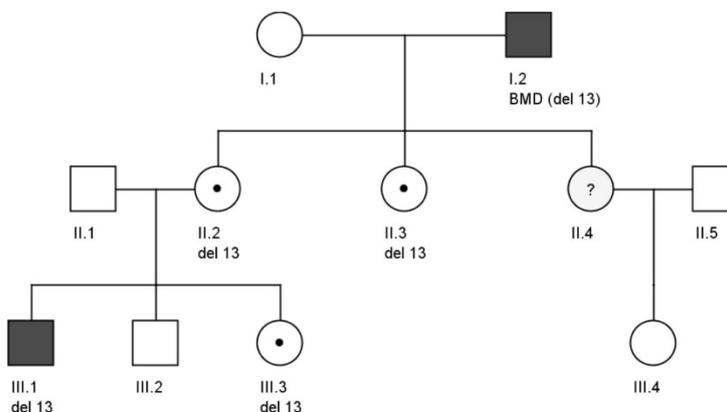
As for the second daughter, the PCR method did not confirm homozygous deletion in her female fetus, but it is still possible that the female fetus is a heterozygous carrier of the deletion.

Table 2

## Mutations found in Duchenne MD and Becker MD probands and their female relatives

Proband phenotype (case number)	Proband age (years)	Proband mutation type	Mother mutation type	Other female relatives mutation type
1- Duchenne MD	19	del 1	no del/dupl	
2- Duchenne MD	8	dupl 8-16	no del/dupl	
3- Duchenne MD	5	del 25-43	no del/dupl	
4- Duchenne MD	2	del 50	no del/dupl	
5- Duchenne MD	6	del 49,50	del 49,50	grandmother (del 49,50)
6- Duchenne MD	9	del 45-52	del 45-52	
7- Duchenne MD	12	del 1, DP427c	del 1, DP427c	
8- Duchenne MD	/	del 59	del 59	
9- Duchenne MD	7	del 35-52	del 35-52	
10- Duchenne MD	21	dupl 2	dupl 2	sister 1, 2 (no del/dupl)
11- Duchenne MD	16	del 45-50	no del/dupl	
12- Duchenne MD	6	del 46-50	no del/dupl	
13- Duchenne MD	6	del 44	no del/dupl	
14- Duchenne MD	2	del 33,34	del 33,34	
15- Duchenne MD	9	dupl 18-42 and 45-48	dupl 18-42 and 45-48	
16- Duchenne MD	7	dupl 52-62	dupl 52-62	
17- Duchenne MD	6	del 12-19	no del/dupl	
18- Duchenne MD	4	del 46-52	del 46-55	
19- Duchenne MD	9	del 3-15	del 3-15	sister (no del/dupl)
20- Becker MD	34	del 44-48	/	niece (del 45-47)
21- Becker MD	38	del 44-49	del 45-47	
22- Becker MD	34	del 48	del 48	
23- Becker MD	33	del 45-47	del 45-47	
24- Becker MD	24	del 45-49	no del/dupl	
25- Becker MD	22	del 45-49	no del/dupl	
26- Becker MD	30	del 45-48	del 45-48	
27- Becker MD	32	del 45-48	del 45-48	
28- Becker MD	14	del 9-12	no del/dupl	
29- Becker MD	24	del 12-43	no del/dupl	sister 1 and 2 (no del/dupl)
30- Becker MD	/	del 45-48	del 45-48	
31- Becker MD	/	del 13	/	daughter 1 and 2 (del 13)

MD –muscular dystrophy; del – deletion; dupl – duplication.



**Fig. 1 – Genealogy of case 31 – Becker muscular dystrophy (BMD) proband. Daughter II.2 is a confirmed carrier; she had a male fetus III.1 with del 13 and a female fetus III.3 with del 13. Daughter II.4 has not been tested for being a potential carrier; she had a female fetus without homozygous deletion. del – deletion.**

## Discussion

Our study included 19 Duchenne MD probands (mean age 8.5 years) and 12 Becker MD probands (mean age 28.5 years), where deletions or duplications were found in the *DMD* gene, using PCR and/or MLPA methods. The incidence of 87.1% of deletions and 12.9% of duplications, established in our group of probands, correspond to the data in literature<sup>3</sup>. The MLPA method revealed 3 larger deletions, 4 deletions outside of the “hot spots” in the gene, and 4 duplications in the probands, which confirms the effectiveness of the MLPA method in the detection of deletions/duplications in the *DMD* gene<sup>11, 20–22</sup>. The largest number of mutations was localized in the distal part of the gene (exons 45–55) in 19 out of 31 (61.3%) cases, which is the most common localization of deletions/duplications in sporadic Duchenne MD/Becker MD cases<sup>23</sup>.

In order to analyze the status of the carriers, a total of 38 female members from the proband families were tested. Of the 29 Duchenne MD/Becker MD proband mothers tested, a mutation of the *DMD* gene was found in 17 (58.6%) out of 29 mothers, similar to the results of other authors<sup>24</sup>. In 14 (82.4%) out of 17 mothers, we discovered deletion, and in 3 (17.6%) out of 17 mothers, we detected a duplication. Mutations found in mothers were the same as in their affected sons, except in two cases.

In sporadic Duchenne MD cases, it was estimated that 2/3 of the mothers were carriers of the mutation, 5–10% had gonadal mosaicism, while 25–30% had no mutation. In our study, in sporadic Duchenne MD cases, the mother was confirmed as a carrier in 11 (57.9%) out of 19 cases, a lower value than the estimated 2/3. Other authors also found this percentage to be lower than expected and that the detection of female carriers of the mutation, as well as the possibility of prenatal diagnosis, has led to a reduction in the number of children born from carrier mothers<sup>7, 25</sup>. In the sporadic Becker MD cases of our sample, 6 (60%) out of 10 mothers were carriers, which is lower than expected since the milder form of the disease in Becker MD patients allows them to have offspring and transfer the risky X chromosome to their daughters, who transfer it to the next generation. For instance, Lee et al.<sup>25</sup> found that the mothers of Becker MD probands were carriers in 89.5% of cases. In relation to the type of mutation in the probands, we established that mothers were carriers in 14 (56%) out of 25 cases for deletions and 3 (75%) out of 4 cases for duplications. This finding is in accordance with the abovementioned, i.e., the risk of a mother being a carrier is greater for certain types of mutations<sup>7, 26</sup>.

Of the other female relatives of the probands, mutations were present in 4 out of 9 cases. As there were known mutations in the probands, we could conclude whether they were carriers based on the MLPA findings in female relatives. However, in familial cases when the mutation cannot be identified, a mother who is an obligatory carrier has a 50% risk that her daughter will also be a carrier. A negative MLPA finding reduces the risk of the daughter being a carrier to 26.5%, and if the further analysis of gene sequencing is also negative, her risk of being a carrier is 3%<sup>27</sup>.

Furthermore, in our study, a prenatal diagnosis was performed on two of the three daughters whose father had Becker MD (Table 2, case No. 31). Two daughters were tested for carrier status and were positive, while one was not tested. A prenatal diagnosis in three pregnancies was carried out on one daughter (confirmed carrier) – a deletion was found in one male fetus and one female fetus (carrier), while one male fetus was healthy. In the case of the daughter who was not tested but also considered an obligatory carrier, homozygous deletion was not confirmed in the female fetus (Figure 1), which does not exclude the possibility of the fetus being a heterozygous carrier.

In some countries, it is recommended that testing for carrier status is performed when the female child reaches the age to decide on testing independently, which is in line with ethical principles. However, experience shows that in the Netherlands, for instance, 78% of girls over 16 years of age have not yet been tested, and the probable cause is that the average age of motherhood is about 28 years of age, so they are tested later<sup>28</sup>, while 1/3 of potential carriers are not tested at all<sup>29</sup>. It has been found that only 52.7% of women at risk of being a carrier of Duchenne MD/Becker MD gene mutations were tested for carrier status before conception<sup>30</sup>. It should be kept in mind that, in addition to the risk of bearing affected offspring, 10% of female carriers develop cardiomyopathy<sup>31, 32</sup>, so early detection of carrier status would allow adequate cardiac monitoring of these women, which is recommended by the age of 16<sup>33</sup>. In our environment, the practice is to let parents decide on testing the carriers, even prenatally. It is, therefore, of great importance to inform members of families affected by Duchenne MD/Becker MD about the nature of the disease, its inheritance, and possible risks, as well as about the method of testing and possible prevention.

In our study group, 12 (41.4%) out of 29 mothers were not confirmed as carriers, suggesting that mutation in the probands was new. It was mentioned earlier that 1/3 of mutations in the Duchenne MD probands were *de novo*. This occurrence is explained by an early fatal outcome in Duchenne MD patients, which leads to the elimination of 1/3 of mutations from the population, but this number is offset by the emergence of new mutations in the next generation<sup>2</sup>. It was found that *de novo* mutations are the most common deletions originating in oogenesis, while duplications and point mutations mainly occur due to events during spermatogenesis<sup>26</sup>. The problem is that *de novo* mutation in the *DMD* gene is clinically diagnosed only after the child experiences symptoms (about the second year at Duchenne MD, later at Becker MD) and at a molecular level, sometimes significantly later – as shown in our sample. It is particularly difficult when a second male child is born before the diagnosis of the older brother is confirmed.

Modern molecular genetic tests have enabled the reliable detection of carrier status. In familial cases of Duchenne MD/Becker MD, detection of the mutation in probands and testing for carrier status in female members in the family enables the determination of risk in the progeny and the provision of adequate genetic advice. In sporadic cases, when a

mother has one affected son, the risk of recurrence depends on whether she is a carrier of the mutation. If molecular analysis of the mother does not determine the presence of a mutation in somatic cells, the risk of recurrence is significantly reduced. However, when giving genetic advice, it should be noted that in 10–15% of cases, there may be gonadal mosaicism in the mother, and the smallest calculated risk is 4.3%<sup>34</sup>. When it comes to *de novo* mutations, they remain the biggest problem. The solution could be the introduction of screening for prenatal detection of mutations in the *DMD* gene in male fetuses, but there is still no consensus in the literature for this.

### Conclusion

In 31 Duchenne MD/Becker MD probands, we identified 87.1% of deletions and 12.9% of duplications of one or more exons. Mothers were confirmed as carriers in almost 60% of sporadic Duchenne MD/Becker MD cases with dele-

tions and duplications (mutations were found in 57.9% of mothers of Duchenne MD probands and 60% of mothers of Becker MD probands). In addition, the carrier frequency tended to be higher in mothers of the probands with duplications (75%) than in mothers of the probands with deletions (56%). Of the 9 other female relatives, mutations were found in 4. In the case of a mother confirmed as a carrier, deletion was detected in 2 out of 3 of her fetuses. These results point to the importance and need to determine mutations in probands, as well as the status of the carrier of the mutation of female members in families with Duchenne MD and Becker MD, which will allow individuals and other family members to receive adequate genetic advice.

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## Are the final-year medical students competent enough to tackle the immunization challenges in their practice?

Da li su studenti završne godine medicine dovoljno kompetentni da savladaju izazove imunizacije u svojoj praksi?

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### Abstract

**Background/Aim.** The competence of healthcare workers (HCWs) to advocate the importance of immunization to persons who are hesitant about vaccines is extremely important. The aim of this study was to evaluate the final-year medical students' competencies related to immunization challenges in their practice using their knowledge, attitudes, and study practices.

**Methods.** The cross-sectional study was conducted among 442 final-year students of the Faculty of Medicine, University of Belgrade, Serbia. The instrument used for data collection was an anonymous questionnaire consisting of 38 questions. Hierarchical multiple regression analysis was conducted to identify the predictive value of different factors in immunization knowledge among medical students. **Results.** The median total knowledge score with the interquartile range was  $7 \pm 3.00$  out of 13. There was a statistically significant positive correlation between the knowledge score and the grade point average (GPA) ( $r = 0.207$ ,  $p < 0.001$ ). Thirty-five percent of students felt moderately capable of conducting work regarding vaccination without supervi-

sion. Furthermore, 92.1% of students considered that additional training and information channels about immunization needed to be implemented in curricula for HCWs. The results of the hierarchical regression analysis showed that gender, age, GPA, study duration, self-confidence regarding immunization knowledge, the flu, and hepatitis B vaccination status explained a total of 36% of the variance in the immunization knowledge score. Less than two-thirds of students believed they had enough knowledge to reassure a person hesitant about immunization. **Conclusion.** The results of our study showed an average level of knowledge about immunization among final-year medical students. As less than two-thirds of students believe that they have enough knowledge to reassure a hesitant person, there is a need for a better understanding and improving the parts of the curricula of medical faculties regarding immunization.

**Key words:** attitude to health; health knowledge, attitudes, practice; students, medical; surveys and questionnaires; vaccination; vaccines.

### Apstrakt

**Uvod/Cilj.** Kompetentnost zdravstvenih radnika da objasne važnost imunizacije osobama koje se dvoume u vezi sa vakcinama izuzetno je važna. Cilj rada bio je da se procene kompetencije studenata završne godine medicine u vezi sa izazovima imunizacije u praksi koristeći njihovo znanje, stavove i praksu. **Metode.** Studija preseka sprovedena je među 442 studenta završne godine Medicinskog fakulteta Univerziteta u Beogradu. Za prikupljanje podataka korišćen je anonimni upitnik koji se sastojao od 38 pitanja. U cilju identifikacije prediktivne vrednosti različitih faktora koji utiču na znanje o imunizaciji među studentima medicine, sprovedena je hijerarhijska analiza multiple regresije. **Rezultati.** Medijana ukupnog skora znanja sa interkvartilnim opsegom bila je  $7 \pm 3,00$  od 13. Postojala je statistički značajna pozitivna korelacija između rezultata znanja i proseka ocena ( $r = 0,207$ ,  $p < 0,001$ ). Za obavljanje poslova u vezi sa vakcinacijom bez nadzora umejeno sposobnim osećalo se 35% studenata. Takođe, 92,1% studenata smatralo je da je u nastavnim planovima i programi-

ma za zdravstvene radnike potrebno uvođenje dodatne obuke i kanala informisanja o imunizaciji. Rezultati hijerarhijske regresione analize pokazali su da su pol, starost, prosek ocena, dužina studiranja, samopouzdanje u pogledu znanja o imunizaciji, vakcinalni status povezan sa gripom i hepatitisom B objašnjavali ukupno 36% varijanse u skor u znanja o imunizaciji. Manje od dve trećine studenata je verovalo da ima dovoljno znanja da uveri osobu koja okleva oko stava o imunizaciji. **Zaključak.** Rezultati našeg istraživanja pokazali su prosečan nivo znanja o imunizaciji među studentima završne godine medicine. S obzirom na to da je manje od dve trećine studenata verovalo da ima dovoljno znanja da uveri kolebljivu osobu, postoji potreba za boljim razumevanjem i unapređenjem delova nastavnih planova i programa medicinskih fakulteta koji se odnose na imunizaciju.

**Ključne reči:** stav prema zdravlju; zdravlje, znanje, stavovi, praksa; studenti medicine; ankete i upitnici; vakcinacija; vakcine.

## Introduction

At the moment when the world is amidst an unprecedented pandemic, the competence of healthcare workers (HCWs) to advocate the importance of immunization to persons who are hesitant about vaccines is more important than ever<sup>1</sup>. The vaccine coverage is not high enough to provide the herd immunity needed for preventing outbreaks of various vaccine-preventable diseases<sup>2-5</sup>. Multiple factors contributed to this, including the lack of adequate knowledge about immunization benefits and risks in the general population, lack of understanding of the severity of the diseases from which the vaccines protect, the activities of the anti-vaccination movement, and the spread of misinformation via the internet and social networks. All of that led to losing confidence in the importance, effectiveness, and safety of immunization<sup>6-9</sup>.

In 2012, the World Health Organization Regional Office for Europe developed the Tailoring Immunization Programs (TIP) approach to help countries achieve high and equitable vaccination uptake<sup>10</sup>. The TIP approach is based on three pillars, one of which is the theoretical model, which draws its messages from evidence gathered through behavioral science. This model sees capability, opportunity, and motivation as the key factors necessary to implement any (positive) health behavior. Capability, among other things, relates to the knowledge of HCWs. It emphasizes the need for HCWs to possess the necessary knowledge and education about vaccine safety, effectiveness and efficacy, contraindications, adverse events following immunization, potential risks related to vaccine-preventable diseases, national vaccination law, and regulations, vaccination coverage, as well as the skills to tailor their communication to different caregiver positions on vaccination<sup>10</sup>.

Studies that aim to examine the knowledge of HCWs and medical students on immunization offer insights into the gaps in knowledge about immunization that may exist among health professionals. There are indications that the senior medical students do not possess sufficient knowledge about vaccination because of the lack of competency-based curricula for vaccination at medical faculties<sup>11</sup>. Finding and addressing these gaps can be crucial in ensuring adequate vaccine coverage among this key target group, as well as among their patients, which is especially important in the light of the COVID-19 pandemic. Therefore, the aim of this study was to evaluate the final-year medical students' competencies related to immunization challenges in their practice using their knowledge, attitudes, and study practices.

## Methods

The cross-sectional study was conducted among sixth-year students of the Faculty of Medicine, University of Belgrade, between December 17 and December 23, 2019. All sixth-year students were included, of whom 442 filled in the questionnaire during their regular practical sessions (response rate 82.6%). The study was approved by the Institutional Review Board of the Faculty of Medicine, University

of Belgrade (No. 29/III-11, from March 28, 2016). All participants gave written consent to participate in the study.

The instrument used for data collection was an anonymous questionnaire consisting of 38 questions. The questionnaire was made based on the literature data<sup>12-14</sup>. The first part of the questionnaire contained demographic characteristics and the student's grade point average (GPA). The second part, the student's knowledge about immunization, was obtained through 13 questions regarding the immunization schedule in the Republic of Serbia (RS), herd immunity, the recent measles outbreak in the RS, elimination and eradication of infectious diseases, contraindications for vaccination, and adverse events following immunization.

The section aimed at investigating the attitudes of the participants on immunization contained 14 questions regarding the importance of vaccines for children, their safety and effectiveness, advocates of the anti-vaccination attitudes, the trust of the general public in the system of public health, and the ascertainment of the level in which the student's environment supports immunization.

The section of the questionnaire regarding the practices of immunization comprised six questions: students' vaccination status against hepatitis B infection and the flu, self-assessment of the capability of unsupervised work on immunization, and the arguments that the student would use in a real-life practical situation. Data on vaccination status against hepatitis B infection and the flu were based on students' self-report.

## Statistical analysis

Methods of descriptive statistics, correlation analysis, and the Mann-Whitney *U* test were used in the data analysis. Further, hierarchical multiple regression analysis was conducted to identify the predictive value of different factors in immunization knowledge among medical students. The dependent variable in this model was the knowledge score. The predictor variables were separated into four blocks. Sociodemographical variables (age and gender) were entered in the first block; GPA and study duration comprised the second block, followed by the attitude about self-confidence regarding immunization knowledge in the third block. Finally, the fourth block included vaccination status regarding the flu and hepatitis B. Keeping in mind that the whole study population was included in the hierarchical regression analysis, the ratio of valid cases ( $n = 442$ ) to the number of independent variables ( $n = 7$ ) was 63.1 to 1, which was equal to or greater than the minimum ratio.

## Results

Male students comprised 155 (35.1%) of the whole (442) sample. The mean age of the participants was  $24.8 \pm 1.6$  years, and the median GPA with the interquartile range was 8.6 (8.0–9.2). Concerning the knowledge about immunization, the vast majority of students were aware that the measles, mumps, rubella (MMR) vaccine does not cause autism (92.6% correct answers), that in the last measles out-

break in the RS, there were lethal outcomes (88.3% correct answers), while 86.7% of students knew what revaccination is (Table 1). One-fifth of students (19.9%) did not know that the vaccine against tuberculosis is included in the routine immunization schedule for children and adolescents in the RS. The question with the least number of correct answers was the one in which the students had to name 11 diseases for which vaccines are included in the routine immunization schedule for children and adolescents in the RS. The median total knowledge score was  $7 \pm 3.00$  (out of 13 points). A statistically significant correlation was observed between the knowledge score and students' GPA ( $r = 0.207, p < 0.001$ ).

Table 2 contains the students' answers to the questions regarding their attitudes toward immunization. A high percentage of students perceived that vaccines are important for children (96.8%), that vaccination needs to be mandatory (96.3%), that vaccines offer more benefits than risks (95.6%), and that they are effective (95.2%). Moreover, 92.1% of students consider that additional training and information channels about immunization need to be implemented in the curricula for HCWs. On the contrary, only 2.1% of students think that the general public has enough trust in the public health system, and 9.5% believe that the manufacturers of vaccines are concealing their adverse effects.

**Table 1****The knowledge of students regarding vaccination**

Statement	Correct answer, n (%)
The routine vaccination schedule for children and adolescents includes vaccines against the following diseases (multiple answers):	30 (6.8)
Against which diseases should the healthcare workers be immunized according to the Rulebook on immunization?	135 (30.5)
What is herd immunity?	268 (60.5)
What level of herd immunity is necessary to prevent an outbreak of measles?	262 (59.1)
How many cases of measles were there in the last outbreak in Serbia?	70 (15.8)
Were there any lethal cases in the last outbreak of measles in Serbia?	391 (88.3)
Is there still a need to vaccinate children against diseases that are eliminated or eradicated from these parts, such as diphtheria and poliomyelitis?	368 (83.1)
What is revaccination?	384 (86.7)
What are the general contraindications for immunization?	239 (54.0)
What are the most frequent adverse effects following immunization?	122 (27.5)
How often do severe adverse effects (such as encephalitis after administration of the MMR vaccine) occur?	35 (7.9)
Do you think that the MMR vaccine causes autism?	402 (92.6)
Do you think that preservatives, adjuvants, and stabilizers used in vaccines are toxic?	201 (46.2)

n – number of students; MMR – measles, mumps, rubella.

**Table 2****Attitudes of students regarding immunization**

Statement	Yes	No	Not sure	Do not know
Do you think vaccination needs to be mandatory for children and adolescents?	418 (96.3)	9 (2.1)	7 (1.6)	/
Do you think that more vaccines need to be mandatory for healthcare workers?	336 (78.5)	92 (21.5)	/	/
Do you consider vaccines important for children?	419 (96.8)	8 (1.8)	5 (1.2)	1 (0.2)
Do you think vaccines are safe?	380 (87.4)	7 (1.6)	45 (10.3)	3 (0.7)
Do you feel that you have enough knowledge to address the concerns of a person hesitant about vaccination?	274 (63.4)	29 (6.7)	115 (26.6)	14 (3.2)
Do you believe that vaccines are effective?	414 (95.2)	5 (1.1)	11 (2.5)	5 (1.1)
Do you believe that vaccines offer more benefits than risks?	413 (95.6)	6 (1.4)	11 (2.5)	2 (0.5)
Do you think the manufacturers of vaccines are concealing their adverse effects?	41 (9.5)	178 (41.2)	152 (35.2)	61 (13.8)
Do you feel that additional training and information channels about immunization need to be implemented for healthcare workers?	394 (92.1)	19 (4.4)	15 (3.5)	/
Does the general public have enough trust in the public health system?	9 (2.1)	316 (73.1)	107 (24.8)	/

All values are expressed as numbers (percentages) of students.

The practices of students related to immunization are shown in Table 3. The majority of participants stated that they feel informed to a great extent about immunization (53.0%), that their parents and friends support immunization completely (70.8% and 48.0%, respectively), or to a great degree (23.0% and 40.9%, respectively). Thirty-five percent of students stated that they feel moderately capable of conducting work regarding vaccination without supervision. Only 10 (2.3%) students have received their flu shot each year for the last 3 years, while 339 (78.1%) students had received the hepatitis B vaccine at some point in life.

The students believed that public figures (36.9%) and the internet and social media (36.2%) were the main means of propagating anti-vaccination attitudes (Figure 1).

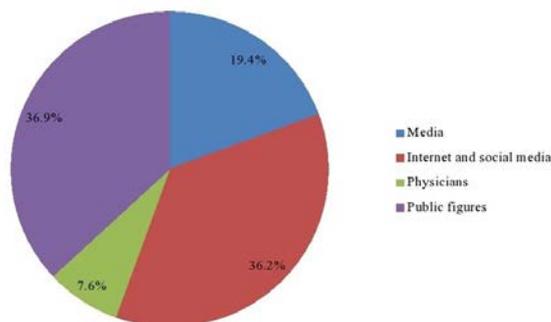
When asked about how they would respond to parents that are hesitant about vaccination, most of the students stated that they would explain that the benefits of vaccination greatly (84.4%) outweigh the risks. Many of the students would state that numerous research on large samples of children showed no link between immunization and autism (76.1%) and that the most frequent adverse effects following immunization are mild (68.4%) (Figure 2). Students singled

**Table 3**

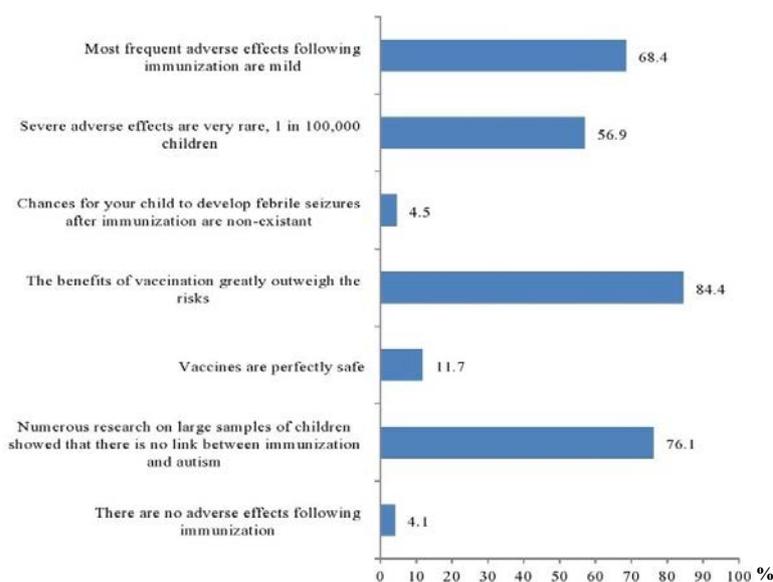
**Attitudes and practices of students regarding immunization**

Statement	Not at all	Not much	Moderately	To a great degree	Completely
In your opinion, how informed are you about immunization?	3 (0.7)	11 (2.5)	166 (38.2)	230 (53.0)	24 (5.5)
How supportive are your parents of immunization?	1 (0.2)	9 (2.1)	17 (3.9)	100 (23.0)	308 (70.8)
How supportive are your friends of immunization?	3 (0.7)	9 (2.1)	36 (8.3)	177 (40.9)	208 (48.0)
How capable do you feel to conduct work regarding vaccination and vaccines without the supervision of a more experienced physician?	73 (16.9)	123 (28.5)	151 (35.0)	67 (15.5)	18 (4.2)

All values are expressed as numbers (percentages) of students.



**Fig. 1 – The most important advocates of anti-vaccination attitudes.**



**Fig. 2 – The arguments that students would use to reassure parents who are hesitant about vaccinating their child.**

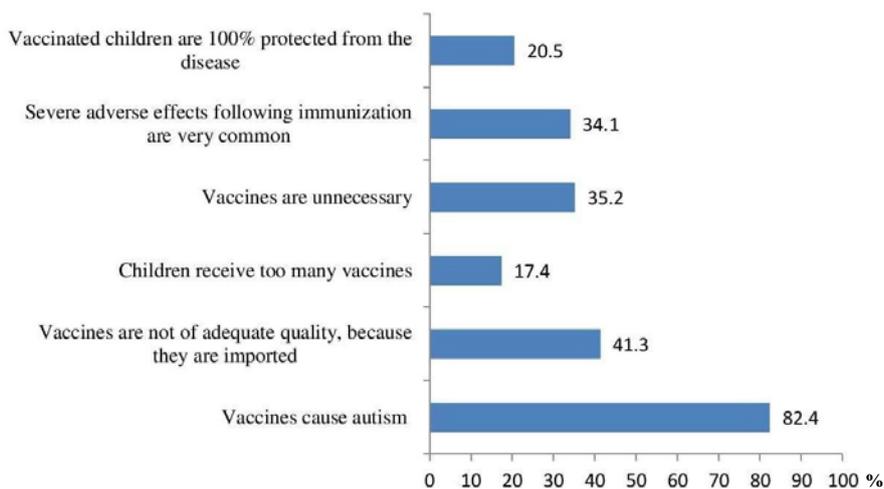
out the opinion that vaccines cause autism as the most important misconception about immunization in the general population (Figure 3).

The hierarchical regression analysis (Table 4) showed that the age and gender of medical students accounted for 17% of the variance of immunization knowledge score as a dependent variable ( $p < 0.01$ ) (model 1). When GPA and duration of studies were added, another 5% of the variance was explained ( $p < 0.05$ ). Adding the attitude regarding self-confidence about immunization knowledge (model 3) increased the proportion of variance explained for an additional 5% ( $p < 0.01$ ). Finally, the inclusion of the flu and hepatitis B vaccination status in the 4<sup>th</sup> model explained an additional 9% of the variance. It means that gender, age, GPA, study duration, self-confidence regarding immunization knowledge, the flu, and hepatitis B vaccination status explained a total of 36% of the variance in the immunization knowledge score.

**Discussion**

The problem of vaccine hesitancy is present not only in the general population but also among HCWs<sup>12-16</sup>. That is especially significant since HCWs are the primary source of information regarding immunization for parents, and the level of trust the patient has for his physician is deeply correlated with the final decision of the patient whether or not they will accept the vaccines<sup>17-19</sup>. HCWs who are hesitant about immunization are less successful in responding to parents' concerns regarding the immunization of their children<sup>13, 17</sup>.

The median knowledge score on immunization in our sample of final-year medical students was 7 out of 13 points. The mean knowledge score observed in a study among students in nursing, medical, and pharmacy programs at two universities in the USA was 3.34/5<sup>20</sup>. In our study, 60.5% of students knew the correct definition of herd immunity, while 59.1% knew what level of herd immunity is needed to pre-



**Fig. 3 – The most important misconceptions about immunization in the general population.**

**Table 4**

**Summary of hierarchical regression analysis of variables predicting immunization knowledge score**

Variable	Model 1			Model 2			Model 3			Model 4		
	B	SE (B)	$\beta$	B	SE (B)	$\beta$	B	SE (B)	$\beta$	B	SE (B)	$\beta$
Gender	0.36	0.19	0.09	0.33	0.19	0.08	0.33	0.19	0.09	0.19	0.19	0.05
Age	-0.16	0.06	<b>-0.14**</b>	-0.09	0.06	-0.08	-0.09	0.06	-0.08	-0.11	0.06	-0.10
GPA				0.40	0.14	<b>0.16**</b>	0.39	0.14	<b>0.15**</b>	0.31	0.13	<b>0.12*</b>
Study duration				0.001	0.001	0.001	0.001	0.001	0.005	0.00	0.001	0.014
Self-confidence regarding immunization knowledge							0.01	0.004	<b>0.15**</b>	0.01	0.004	<b>0.12*</b>
Flu vaccination										-0.60	0.30	<b>-0.09*</b>
Hepatitis B vaccination										-0.73	0.16	<b>-0.23**</b>
$R^2$		0.17			0.22			0.27			0.36	
F for change in $R^2$		<b>6.080*</b>			<b>4.351*</b>			<b>9.480*</b>			<b>13.811</b>	

GPA – grade point average; B – unstandardized beta coefficient; SE – standard error;  $\beta$  – standardized beta coefficient;  $R^2$  – coefficient of determination; F – F test of overall significance.

\* $p < 0.05$ ; \*\* $p < 0.01$ .

vent an outbreak of measles. Similar results were observed in a study conducted in a sample of university students in Poland, where 66.6% of students correctly responded to this question<sup>21</sup>. An interesting observation is that 4/5 of the students in our study knew that the vaccine against tuberculosis is included in the routine immunization schedule since this vaccine was included in the immunization schedule in the RS back in 1927. In the segment analyzing the practices related to immunization, the students stated that they consider themselves pretty informed about immunization (53.0%), and the largest percentage of students (35.0%) responded that they feel moderately capable of conducting work regarding vaccination and vaccines without supervision. Only 2.3% of students have received flu shots each year for the last three years. In contrast with our results, a study performed among healthcare students in Canada showed that 88.4% of students consider receiving annual flu shots important<sup>22</sup>. A Knowledge, Attitude, and Practice (KAP) study performed among medical college students in China has pointed out that the top 3 reasons among medical students for not getting a flu shot were poor knowledge of the vaccine, perceived lack of need for immunization due to good health, and worries about adverse reactions<sup>23</sup>.

The students who participated in the study believe that the most important promoters of anti-vaccination attitudes are public figures (36.9%) and the internet and social media (36.2%). The activities of the anti-vaccination movement are considered responsible for the rise in the number of parents who refuse to vaccinate their children in the last 20 years. The technological advances of modern society have facilitated the dissemination of these messages. Because of this, the importance of HCWs in providing the general public with the correct information and advice about immunization is greater than ever<sup>24</sup>.

The results of research conducted to assess the knowledge and attitudes about immunization among future HCWs have shown that the level of knowledge varies significantly depending on the curriculum<sup>25, 26</sup>. A project performed at the University of Munich, Germany, identified that not all relevant vaccination learning objectives were included in the curriculum at the University<sup>11</sup>. Implementing a competency-based curriculum on immunization can help the students acquire skills to communicate adequately about vaccination with the general public, and it can also make them capable of making decisions independently<sup>11</sup>. The principle of the competency-based curriculum is to integrate all of the important learning objectives about immunization into a sin-

gle teaching program, instead of students traditionally studying it superficially inside various subjects like microbiology, epidemiology, immunology, infectious diseases, pediatrics, public health, etc.

The importance of improving the knowledge, confidence, and communication tools of (future) HCWs has also been recognized by the European Joint Action on Vaccination. It emphasizes the need for educational activities in medical and paramedical curricula on vaccines and vaccination programs in Europe<sup>27</sup>. The benefits of such educational programs and activities have been shown in the populations of physicians as well, for instance, in a KAP study performed at the Mayo Clinic among physicians working in the internal medicine department<sup>28</sup>. This study, in which the participants were questioned prior to and after an education program on immunization, showed that the knowledge scores of the physicians, as well as their patients' influenza and whooping cough immunization rates, increased significantly after the educational program<sup>28</sup>.

Our study has several limitations. First, the cross-sectional design of the study makes it difficult to infer causality with high confidence. Another important issue is the self-reporting assessment of practices instead of using real-life situations; therefore, an information bias could be present.

## Conclusion

The results of our study have shown an average level of knowledge regarding immunization among final-year medical students of the University of Belgrade. It is alarming that barely two-thirds of students consider themselves competent enough to answer the concerns of a person who is a vaccine hesitator. That underlines the need for a better understanding and improving the parts of the curricula of medical faculties regarding immunization because it can offer insights into how knowledge, attitudes, and beliefs of students regarding immunization are formed and how to better train them for practice in immunization in their future work.

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## Abdominal injuries in road traffic accidents – an autopsy study

### Povrede trbuha u saobraćajnim nezgodama – autopsijska studija

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#### Abstract

**Background/Aim.** Traffic accident injuries are a major public health problem worldwide, with millions of people dying every year. Although the improvement of traffic safety is based on preventive action, it is necessary to conduct an analysis of victims in traffic in order to prevent future traffic accidents with the help of such findings. The aim of the study was to determine the manner of occurrence of abdominal injuries (AIs), gender differences, and the sitting position in the vehicle that affects the severity of AIs, as well as which AIs lead more often to fatalities in traffic accidents. **Methods.** Materials for the present study were collected from the medico-legal autopsies, showing AIs that occurred in road traffic accidents. The total number of people who died from sustained injuries or complications of the injuries after road traffic accidents was 525. **Results.** In the study sample, 38.3% of subjects sustained abdominal trauma. Men were more likely to have AIs, and passengers in the front seat and motorcycle drivers were identified as the most vulnerable category of these types of injuries. The liver was the most commonly injured abdominal organ. Liver destructions, liver lacerations, spleen lacerations, intestinal injuries, the simultaneous occurrence of head, chest, and AIs, or the simultaneous occurrence of head and AIs in a higher percentage led to death at the scene. **Conclusion.** The results of this study represent the basis for creating educational content for all traffic participants as well as the legislation in the field of traffic safety.

#### Key words:

abdominal injuries; accidents, traffic; autopsy; cause of death; risk assessment; sex factors.

#### Apstrakt

**Uvod/Cilj.** Povrede u saobraćajnim nezgodama, u kojima milioni ljudi umiru svake godine, predstavljaju veliki problem javnog zdravlja širom sveta. Iako se unapređenje bezbednosti saobraćaja zasniva na preventivnom delovanju, neophodno je sprovesti analizu nastradalih u saobraćaju, kako bi se uz pomoć tih rezultata sprečile buduće saobraćajne nezgode. Cilj rada bio je da se utvrde način nastanka povreda trbuha (PT), razlike prema polu i položaj sedenja u vozilu koji utiče na težinu PT, kao i vrste PT koje češće dovode do smrtnog ishoda u saobraćajnim nezgodama. **Metode.** Materijal za studiju prikupljen je iz rezultata sudskomedicinskih obdukcija, kojima su ustanovljene PT nastale u saobraćajnim nezgodama. Ukupan broj osoba umrlih od zadobijenih povreda ili komplikacija povreda nakon saobraćajnih nezgoda bio je 525. **Rezultati.** U analiziranom uzorku, PT imalo je 38,3% preminulih osoba. Muškarci su češće imali PT, a putnici na prednjem sedištu i vozači motocikala su bili najranjivija kategorija za nastanak tih vrsta povreda. Jetra je bila najčešće povređeni organ trbuha. Destrukcija i laceracija jetre, laceracija slezine, povrede creva, istovremene povrede glave, grudnog koša i PT ili istovremene povrede glave i PT doveli su u visokom procentu do momentalne smrti na mestu saobraćajne nezgode. **Zaključak.** Rezultati ove studije predstavljaju osnovu za kreiranje obrazovnih sadržaja za sve učesnike u saobraćaju, kao i kreiranje zakonske regulative u oblasti bezbednosti saobraćaja.

#### Ključne reči:

abdomen, povrede; udesi, saobraćajni; autopsija; smrt, uzrok; rizik, procena; pol, faktori.

#### Introduction

Along with industrialization and scientific and technological development in the recent century, trauma and its

complications have become an important issue, as it is one of the most prevalent causes of fatalities and morbidity worldwide<sup>1,2</sup>. Trauma is the most frequent cause of death in the first four decades of life, and it remains a major public health

problem in every country, regardless of the level of socioeconomic development (both in developing and developed countries) <sup>3-5</sup>.

The abdomen is the third most common injured region, and abdominal injuries (AIs) are the cause of considerable morbidity and mortality. The abdominal area is often exposed to injury due to its anatomical position, size it occupies, and minimal bone protection. Around 3/4 of cases of abdominal trauma result from road traffic accidents (RTA), while only 1/4 of cases result from falling from height and other causes <sup>6</sup>. The incidence of AIs in RTA has decreased because of road and car safety improvements, but they continue to represent life-threatening situations. The high number of AIs with severe consequences, even death in RTA, can be explained by an increased number of powerful motor vehicles as well as increased travel speeds <sup>7-9</sup>. When two objects collide, each of them has an amount of energy that is in a linear relationship with mass and exponential with velocity. As objects collide, energy gets transferred from one object to another depending on the direction, speed, position, and nature of the objects. That is why the relationship between velocity and mortality from road traffic collisions is exponential. AIs are usually associated with injuries of other body regions, so these injuries may be overlooked in patients with severe head, chest, and limb injuries or injuries of the pelvis <sup>10</sup>.

The aim of this study was to determine the frequency and characteristics of AIs due to RTA in order to better understand their significance and define preventive measures for the most vulnerable population categories.

## Methods

An epidemiological retrospective analytical autopsy study of AIs was conducted. Materials for the study were collected from the medico-legal autopsies, which presented AIs that occurred in RTA at the University Clinical Center of Kragujevac, Department of Forensic Medicine and Toxicology. The total number of studied deceased persons was 525 (of all 1,366 conducted autopsies performed on the territory of central Serbia, Kragujevac and the surrounding, in the period from 2001–2016). They died from sustained injuries or complications of the injuries after RTA. This study was conducted with the approval of the Ethics Committee of the University Clinical Center of Kragujevac (No. 01/13221).

Detailed information about the deceased was collected from different sources, including the request for a medico-legal autopsy obtained from competent courts or prosecutions, autopsy examination findings, and relevant clinical history found upon admission to the hospital and subsequently. Subjects were analyzed in terms of gender, age, and type of involvement in traffic accidents (pedestrians, motor vehicle drivers, front-seat passengers, rear-seat passengers, bicyclists, motorcyclists, and tractor drivers). AIs were classified into the following groups: liver injuries (lacerations, destructions, and subcapsular hematomas), spleen injuries (lacerations and destructions), intestinal injuries (intestinal contusions and intestinal rupture, mesentery hemorrhages and rup-

tures), kidney injuries (destructions, contusions, and lacerations), and urinary bladder injuries (hemorrhages of the bladder wall and ruptures). In addition, the analysis included the simultaneous occurrence of abdominal trauma and injuries of the chest or head. When considering the outliving period, the subjects were divided into two categories: subjects who died at the scene of the accident or on the way to the hospital and those who outlived their injuries for a certain period of time (6 hrs, 24 hrs, 72 hrs, 7 days, 14 days, 30 days).

## Data analysis

Statistical Package for Social Sciences – SPSS for Windows, Version 22 (SPSS Inc. Chicago, IL) was used for data processing. All numerical variables were tested with the Shapiro-Wilks and Kolmogorov-Smirnov tests for normal distribution. According to the data distribution, suitable descriptive statistics were employed (mean values with standard deviation). Student's *t*-test was used to estimate the differences between the variables that showed the parametric distribution. In variables that showed a nonparametric distribution, the Kruskal-Wallis test and Pearson's  $\chi^2$  test (with Yates correction) were applied. Binary logistic regression was chosen for analyzing the connection of dichotomous dependent variables (death on the spot vs. death after the outliving period) and observational independent variables (injuries of certain body parts). The results were presented as crude odds ratio (OR) with a 95% confidence interval (CI). After applying corrections for the influence of other independent and confounding variables, the acquired data were expressed as adjusted OR with a 95% CI. The *p*-value of 0.05 was considered significant.

## Results

### Demographic analysis

The study included subjects from 16 to 92 years old. The study excluded subjects younger than 14 years of age and those shorter than 150 cm. In the study sample, 201 (38.3%) subjects sustained abdominal trauma. Among all fatal RTA subjects with detected AIs, the males accounted for 3/4 of the subjects (152 males and 49 females). The average age of women was  $52 \pm 20.2$  years and average age of men was  $47.4 \pm 18.9$  years, but there was no statistically significant difference ( $t = -1.463$ ;  $p < 0.001$ ). Although there were slightly more RTA subjects with abdominal trauma in the 15–35 age group, the statistically significant difference in the number of participants per group was not found ( $\chi^2 = 3.981$ ;  $df = 3$ ;  $p = 0.264$ ). The distribution of RTA subjects with AIs by age range and gender is represented in Table 1.

RTA subjects with AIs (average age  $48.6 \pm 19.2$  years) were younger than other subjects without AIs (average age  $54.8 \pm 19.1$  years), which was statistically significant ( $t = 3.647$ ;  $p < 0.001$ ).

There was a statistically significant difference among the type of road users ( $\chi^2 = 194.423$ ;  $df = 6$ ;  $p < 0.001$ ). The

**Table 1****The distribution of road traffic accidents subjects with abdominal injuries by age range and gender**

Age range (years)	Men	Women	Total
15–35	47	10	57
36–50	37	10	47
51–65	34	12	46
> 65	34	17	51
Total	152	49	201

All values are expressed as numbers.

most vulnerable RTA subjects were front-seat passengers (39 out of 79, i.e., 49.4%). In terms of the frequency of AIs, motor vehicle drivers were in second place (44 out of 98, i.e., 45%). Among bicyclists, AI was present in the smallest number (5 out of 38, i.e., 13%) of RTA subjects.

*Most frequently injured organs*

Liver injuries were registered in 138 (26.3%) of 525 fatal RTA. The most common injuries were liver destruction (77, i.e., 14.7%) and liver lacerations presented in 58, i.e., 11%, fatal RTA. Subcapsular hematomas were found in only 3 subjects.

There was a statistically significant difference in the presence of liver injuries according to the type of participation in RTA ( $\chi^2 = 21.824$ ;  $df = 6$ ;  $p = 0.003$ ). The most common liver injury was among front-seat passengers (30 out of the total 79), compared to other subjects in RTA, which was statistically significant ( $\chi^2 = 5.867$ ;  $df = 1$ ;  $p = 0.015$ ). Among bicyclists, liver injury was present in the smallest number ( $\chi^2 = 8.211$ ;  $df = 1$ ;  $p = 0.004$ ), which was statistically significant compared to other participants (Table 2).

One in five RTA subjects (106, i.e., 20.2%) had spleen injuries. The spleen was most often destructed (85, i.e., 16%), while lacerations were much less present (21, i.e., 4%). According to the type of participation in RTA, a significant difference in the prevalence of spleen injuries was observed ( $\chi^2 = 18.388$ ;  $df = 6$ ;  $p = 0.010$ ), as presented in Table 2. The spleen injury was most common in front-seat passengers (25 out of the total 79 front-seat passengers, i.e., 31.6%), compared to other subjects in RTA, which is statistically significant ( $\chi^2 = 6.759$ ;  $df = 1$ ;  $p = 0.009$ ).

**Table 2****Frequency of liver, spleen, intestines, kidney, and urinary bladder injuries according to the type of participation in road traffic accidents (RTA)**

RTA subjects	Injuries							Total
	liver		spleen		intestines	kidney	urinary bladder	
	yes	no	yes	no	yes	yes	yes	
Pedestrians	51 (23.2)	169 (76.8)	33 (15.0)	187 (85.0)	37 (16.8)	12 (5.5)	12 (5.5)	220 (100)
Motor vehicle drivers	32 (32.7)	66 (67.3)	27 (27.6)	71 (72.4)	23 (23.5)	7 (7.1)	6 (6.1)	98 (100)
Front-seat passengers	30 (38.0)	49 (62.0)	25 (31.6)	54 (68.4)	18 (22.8)	8 (10.1)	6 (7.6)	79 (100)
Rear-seat passengers	4 (13.8)	25 (86.2)	3 (10.3)	26 (89.7)	3 (10.3)	3 (10.3)	1 (3.4)	29 (100)
Bicyclists	2 (5.3)	36 (94.7)	4 (10.5)	34 (89.5)	2 (5.3)	1 (2.6)	1 (2.6)	38 (100)
Motorcyclists	12 (30.8)	27 (69.2)	10 (25.6)	29 (74.4)	7 (18)	3 (7.7)	2 (5.1)	39 (100)
Tractor drivers	7 (31.8)	15 (68.2)	4 (18.2)	18 (61.8)	3 (13.6)	–	1 (4.5)	22 (100)
Total	138 (26.3)	387 (73.7)	106 (20.2)	419 (79.8)	93 (17.7)	34 (6.5)	29 (5.5)	525 (100)

All values are expressed as numbers (percentages).

Intestinal injuries were present in 93 (17.7%) RTA subjects. For easier analysis of intestinal injuries, we observed mesentery hemorrhages (58, i.e., 11%) and mesentery ruptures (31, i.e., 5.9%), while intestinal contusions and intestinal ruptures were present in significantly smaller numbers.

Kidney injuries were present in 34 (6.5%) RTA subjects. For easier analysis of kidney injury, we observed the following: destructions 20 (3.8%), contusions 9 (1.7%), and lacerations 5 (1%). Urinary bladder injuries were present in 29 (5.5%) RTA subjects. Hemorrhages of the bladder wall were present in 20 (3.8%), while bladder wall ruptures were present in only 9 (1.7%) cases. Depending on the type of fatal participants in RTA, injuries to the intestines, kidneys, and bladder did not show statistical significance (Table 2).

*Combined injuries*

According to our study, the liver was the most commonly injured abdominal organ, but liver injuries were often associated with injuries to other abdominal or extra-abdominal organs. The most commonly involved organ with liver was the spleen, 52% (72 of all 138 subjects with liver trauma), which is statistically significant ( $\chi^2 = 116.166$ ;  $df = 1$ ;  $p < 0.001$ ). Associated injuries of the liver and intestines were in second place with 39% (54 of all 138), and it was also statistically significant ( $\chi^2 = 57.930$ ;  $df = 1$ ;  $p < 0.001$ ). Associated injuries of the liver and kidney, 18% (25 of all 138), as well as liver and urinary bladder, 14% (19 of all 138), were less common but statistically significant, as presented in Table 3 with the results of the  $\chi^2$  test.

**Table 3**

Combined liver injuries and injuries of other abdominal organs in fatal road traffic accidents subjects							
Abdominal organs	Injuries	Liver		Total	$\chi^2$	df	p-value
		yes	no				
Spleen	yes	72 (13.7)	34 (6.5)	106 (20.2)	116.166	1	0.000
	no	66 (12.6)	353 (67.2)	419 (79.8)			
Kidney	yes	25 (4.8)	9 (1.7)	34 (6.5)	39.310	1	0.000
	no	113 (21.5)	378 (72.0)	491 (93.5)			
Urinary bladder	yes	19 (3.6)	10 (1.9)	29 (5.5)	22.286	1	0.000
	no	119 (22.7)	377 (71.8)	496 (94.5)			
Intestines	yes	54 (10.3)	39 (7.4)	93 (17.7)	57.930	1	0.000
	no	84 (16.0)	348 (66.3)	432 (82.3)			

All values are expressed as numbers (percentages).

It is important to note that about 1/3 of the subjects, 35.8% (188 out of 525), suffered from the concomitant chest and AIs, 21% (110 out of 525) sustained concomitant head and AIs, while the simultaneous occurrence of chest, head, and AIs was identified in 19.6% (103 out of 525) of instances ( $\chi^2 = 193.830$ ;  $df = 1$ ;  $p < 0.001$ ).

#### *Outliving after abdominal injuries*

In the group of fatal RTA participants who died at the scene, almost half of the subjects had some AI (Table 4).

In the group of fatal RTA subjects who outlived injuries for a certain period of time, 27.8% had an AI. RTA subjects with some AIs die more often at the scene of the accident,

which is statistically significant ( $\chi^2 = 18.934$ ;  $df = 1$ ;  $p < 0.001$ ). Details on outliving the RTA of subjects with AIs are shown in Table 5.

We also analyzed the prevalence of AIs in different RTA participants, and we observed a high prevalence of AIs in front-seat passengers and motor vehicle drivers who died at the scene of accidents (Table 6).

The existence of different AIs or concomitant injuries proved to be significantly connected with immediate deathly outcomes of RTA in two groups of subjects. After adjusting the results for gender and the occurrence of other AIs, only a few risk factors remained statistically significant to be associated with immediate deathly outcomes: the simultaneous occurrence of head, chest, and AIs; the simultaneous occur-

**Table 4**

#### Outliving abdominal injuries (AIs) after road traffic accidents

		A	B	Total
AIs	yes	136 (46.7)	65 (27.8)	201 (38.3)
	no	172 (53.3)	167 (72.2)	324 (61.7)
Total		291 (100)	234 (100)	525 (100)

All values are expressed as numbers (percentages) of subjects who died at the scene (A) and outlived the AIs (B).

**Table 5**

#### Details on outliving the road traffic accidents of the subjects with abdominal injuries (AIs)

Time	6 h	24 h	72 h	7 d	14 d	30 d	Total
AIs	29 (14.4)	12 (6)	2 (1)	10 (5)	6 (3)	6 (3)	65 (27.8)

All values are expressed as numbers (percentages) of subjects who outlived the AIs.

h – hours; d – days.

**Table 6**

#### Prevalence of abdominal injuries (AIs) in participants of different road traffic accidents (RTA) depending on outliving injuries

RTA subjects	Died at the scene	Outlived the AIs	Total
Pedestrians	48 (61.5)	30 (38.5)	78 (100)
Motor vehicle drivers	35 (79.5)	9 (20.5)	44 (100)
Front-seat passengers	29 (74.4)	10 (25.6)	39 (100)
Rear-seat passengers	5 (62.5)	3 (37.5)	8 (100)
Bicyclists	2 (40)	3 (60)	5 (100)
Motorcyclists	12 (70.6)	5 (29.4)	17 (100)
Tractor drivers	5 (50)	5 (50)	10 (100)
Total	136 (67.7)	65 (32.3)	201 (100)

All values are expressed as numbers (percentages).

rence of head and AIs; liver destructions, liver lacerations, spleen laceration, and intestinal injuries. The odds of occurrence (OO) of simultaneous abdominal and chest injuries in those who died instantly were approximately two times higher than in other subjects (OR 2.277; 95% CI 1.567–3.309;  $p < 0.001$ ), but the occurrence of simultaneous abdominal, chest, and head injuries were 3.6 times higher (OR 3.606; 95% CI 1.341–9.673;  $p = 0.002$ ).

Liver destructions were present in 61 (79.2%) RTA subjects who died at the scene of the accident ( $\chi^2 = 19.562$ ;  $df = 1$ ;  $p < 0.001$ ), and the OO of liver destructions in those who died at the scene are more than three times higher than in other subjects (OR 3.614; 95% CI 2.022–6.460;  $p < 0.001$ ). Similar results were present in the case of liver lacerations. Liver lacerations were present in 45 (77.6%) RTA subjects who died at the scene of the accident ( $\chi^2 = 11.969$ ;  $df = 1$ ;  $p = 0.002$ ), and the OO of liver lacerations in those who died at the scene are approximately three times higher than in other subjects (OR 3.110; 95% CI 1.634–5.917;  $p = 0.002$ ).

Of the 21 RTA subjects with a spleen laceration, as many as 19, i.e., 90.5%, died at the scene of accidents, which showed statistical significance ( $\chi^2 = 9.449$ ;  $df = 1$ ;  $p = 0.002$ ). The OO of spleen laceration in those who died at the scene are approximately eight times higher than in other subjects (OR 8.103; 95% CI 1.868–35.154;  $p = 0.005$ ).

Death at the scene of the accident rather than outliving the injuries occurs more often in the RTA subjects with intestinal injuries ( $\chi^2 = 6.344$ ;  $df = 1$ ;  $p = 0.012$ ), and the OO of intestinal injuries in those who died at the scene are approximately two times higher than in other subjects (OR 1.879; 95% CI 1.170–3.018;  $p = 0.009$ ).

## Discussion

According to the Law of the Republic of Serbia, performing a medico-legal autopsy is obligatory after RTA if there is a suspicion of a criminal offense. In addition to defining the exact cause of death and injury mechanism, one of the objectives of the autopsy is a detailed description of all external and internal injuries on the body; therefore, the autopsy report is an important document in further court proceedings and can serve scientific purposes because it contains a lot of medical facts. This study was conducted to research the pattern and type of AIs and to identify the risk of injuries to organs in the abdomen after RTA. Furthermore, the aim of this study was to estimate the frequency and characteristics of RTA AIs according to the age and gender of subjects, type of participants in traffic, and time distribution of death after injury (time from injury to death).

Of all fatal road accident participants, AIs were present in 38.3% of cases, which is slightly more than in other published studies. The main reason for that is the fact that our study was conducted on fatally injured RTA participants (autopsy study), where detailed autopsy methods note any minimal injury<sup>11–13</sup>. According to the results of many studies<sup>14–18</sup>, men are more commonly injured, and the ratio of men to women was about 3:1, and our results agree with that. The

fact that males are usually the breadwinners of the families makes them more mobile and thus more prone to traffic accidents. The most common age range of the RTA subjects was 15–35 years, which correlates with the study conducted by Subedi et al.<sup>17</sup>, and it can be explained by the fact that the participants of this age group are in the most active period of life, prone to proving and risky driving behavior<sup>6</sup>.

Observing the type of participation in RTA, every second front-seat passenger had an AI, which characterizes them as the most vulnerable RTA subjects when it comes to AIs (49.4%). Motor vehicle drivers were in second place among the injured RTA subjects with AIs (45%). In a study conducted on participants inside the vehicle, Daskal et al.<sup>19</sup> concluded that passengers inside the vehicle (front-seat or rear-seat passengers) were found to be at higher risk of serious AI than motor vehicle drivers, which correlates with the results of our study. Töro et al.<sup>18</sup> and Goniewicz et al.<sup>20</sup> point out that injuries of the abdominal organs are more common among protected participants in traffic accidents (participants inside the vehicle – motor vehicle drivers and front-seat passengers) compared to unprotected participants (pedestrians, bicyclists, and motorcyclists) with a frequency of about 20%. The higher incidence of AIs in vehicle participants can be explained by the mandatory use of a seat belt. In all types of motor vehicle crashes, seat belts are effective for preventing head injuries and death but increase AIs. Seat belts are the most common source of AI because when the restrained occupant is subjected to high-speed deceleration, abdominal compression occurs<sup>21–23</sup>. In contrast, Subedi et al.<sup>17</sup> point out that pedestrians and motorcyclists are RTA participants with a higher frequency of AIs and explain that the reason is a high-speed impact and lack of body restraints. According to our results, the smallest number of AIs was in bicyclists (13%), which also correlates with the results of the mentioned studies<sup>14–18, 20</sup>.

Every fourth RTA subject sustained a liver injury, so according to our results, it was the most commonly injured organ in the abdominal cavity. The high prevalence of liver injuries is explained by its size, fixation in the abdominal cavity, and the inelasticity of the tissue itself<sup>11, 24</sup>. Compression of the liver by the steering wheel causes the destruction of this organ. Several epidemiological studies have also reported the liver as the most commonly injured solid organ<sup>11, 13, 17, 25</sup>. There are studies in which the liver ranks second in frequency, just after the spleen<sup>12, 26</sup>. According to our results, the most common liver injury was destruction, which deviates from the literature data, according to which the most common liver injuries are lacerations<sup>11, 13, 27</sup>. In our study, the higher frequency of liver destruction can be explained by the high intensity of the force acting on the relatively weak and unprotected anterior abdominal wall during the RTA, resulting in severe injuries.

According to our results, the spleen is the most commonly injured organ after the liver, and it was found in every fifth RTA subject. Being struck by the intruded side door is the most common source of trauma in motor vehicle side collisions. Likewise, seat belt loading is a risk factor in some cases. Restrained drivers of small vehicles are subjected to

greater contact with the intruding vehicle. The side doors of heavier vehicles provide greater protection. However, unrestrained occupants sustained spleen injuries from instrument panel contact and the steering wheel. In autopsy studies after fatal traffic accidents, Tambuzzi et al.<sup>28</sup> and Reddy and al.<sup>13</sup> state that the spleen is second in frequency of injury after the liver, with a representation of about 18%, which is in correlation with the results of our study. Similar results have also been reported in other studies<sup>11, 25, 27, 29</sup>, but some studies in the literature describe the spleen as the most commonly injured organ in the abdominal cavity with a much higher frequency<sup>12, 26, 30, 31</sup>. As with the liver, the most common spleen injury was destruction, which also deviates from the literature data, according to which the most common spleen injuries are lacerations<sup>11, 13, 32</sup>.

With a similar frequency as spleen injuries of about 17%, intestinal injuries were present, primarily injuries in the form of mesentery hemorrhages. Intestinal injuries occur when a force acts on the anterior abdominal wall and presses them on the spinal column or due to the transmission of hydrostatic pressure through the fluid content in the intestines. There are studies in the literature in which intestinal injury was represented by 16%<sup>33</sup> and 17%<sup>29</sup>, similar to our results. Intestines, especially the small intestine, are the most damaged abdominal viscera in blunt abdominal trauma (19%), which reflects large energy transfers in traffic accidents in fatal cases<sup>34</sup>. Watts and Fakhry<sup>34</sup> have indicated that RTA subjects are 1.5 times more likely to present abdominal hollow viscera lesions than other blunt trauma mechanisms. Faduyile et al.<sup>11</sup> reported a slightly smaller incidence of intestinal injury, about 11%.

Injuries to the kidneys (7%) and urinary bladder (6%) were observed in a significantly smaller number of fatalities in traffic accidents. Kidney injuries are the rarest due to their anatomical position – they are protected in the space between the spine and ribs and are isolated by a layer of fatty tissue. The same is for the bladder, which is protected by the pelvic bones. Kidney injuries are significant because they are often accompanied by massive retroperitoneal bleeding that can be fatal. These findings are partially consistent with previous results<sup>13, 29, 35</sup>. In their study, Shetty et al.<sup>35</sup> pointed out that the kidney was the most commonly injured solid abdominal organ, with a frequency of about 23%.

According to our results, liver trauma usually occurs associated with other AIs. The most commonly involved organs were the spleen (52%), intestines (39%), kidney (18%), and urinary bladder (14%). Scollay et al.<sup>36</sup> established that almost 90% of liver trauma patients sustained associated chest, head, and orthopedic injuries, or other AIs, among which the most commonly involved were spleen (24%), kidney (21%), intestines (17%), and urinary bladder (4%). Liver injury was also associated with multiple regional injuries, as Subedi et al.<sup>17</sup> and Talving et al.<sup>37</sup> highlighted in their research. The most common associated extra- AIs included chest injury (36%), head injury (21%), and combined head and chest injury (20%). Associated injuries significantly raised mortality. The literature also emphasizes the im-

portance of associated injuries to the abdomen and other parts of the body, especially the chest<sup>11, 12, 27, 38</sup>.

According to the results of our study, almost half of the RTA subjects who died at the scene and 1/3 of those who outlived injuries for a certain period of time had some AI. Almost 80% of motor vehicle drivers and 74% of front-seat passengers with some AIs died at the scene of accidents, which is explained by the frequent occurrence of concomitant injuries, which in total represent severe bodily injuries incompatible with life. Ndiaye et al.<sup>39</sup> point out that more than three-quarters of motor vehicle drivers died immediately, which concurs with our results. One of the most important characteristics of injuries in traffic accidents is a massive action of a force which is the product of mass and acceleration absorbed by the body during a traffic accident. The injury occurs due to the absorption of the external force upon acceleration, deceleration, or impact, whereas the body tends to maintain its primary position and speed. As AIs increase in severity, other organ systems may become involved, so total mortality may result from the cumulation of all damaged organs (only abdominal or combinations of abdominal and other). The chances that participants who have died on the spot sustained simultaneous chest and AIs are more than two times higher, while the chances for the occurrence of simultaneous injuries to the head, chest, and abdomen are more than three times higher for RTA participants who died on the spot. Other authors have come to similar conclusions, according to which subjects with simultaneous head, chest, and AIs were most likely to die at the scene of the accident or during the first few hours following the accident<sup>38, 40–42</sup>. According to the obtained results, RTA participants who died on the spot more frequently have spleen lacerations (8 times higher), liver injuries (destructions 3.6 times or lacerations 2 times higher), and injuries of the intestines (2 times higher).

## Conclusion

Road traffic participants are not a uniform population; they are exposed to different kinds of hazards depending on conditions prevailing in that region, hence presenting different epidemiological findings. This study may help to take safety measures, implement strict traffic rules, and educate people. The results of our study reveal a clear difference between different road participants, the incidence of AIs, as well as the outcome after injury.

The most common AIs were present among men in the 15–35 age group. Among the type of road users, the most vulnerable RTA subjects were front-seat passengers and motor vehicle drivers.

The liver is the most commonly injured abdominal organ, and liver injuries are often associated with injuries to other abdominal or extra-abdominal organs. About 1/3 of the subjects suffered from the concomitant chest and AIs. RTA subjects with some AIs more often die at the scene of the accident. Liver destructions, liver lacerations, spleen lacerations, intestinal injuries, the simultaneous occurrence of

head, chest, and AIs, or the simultaneous occurrence of head and AIs in a higher percentage lead to death at the scene.

This information can provide decisive data for the correct choice between the various diagnostic and therapeutic options for early diagnosis of potentially fatal occult injuries. This type of research is pioneering research in the territory where it was conducted. The results of this study represent the basis for the creation of educational content for all traffic

participants, as well as the creation of legislation in the field of traffic safety. It is necessary to conduct such and similar research permanently in order to monitor the current state of suffering of traffic participants. Apart from the fact that such results serve to improve traffic safety, they also serve as a warning and encouragement that in the field of traffic safety, work must be done every day, as long as there are injured and killed traffic participants.

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## Femorodistal bypasses using venous “cuffs”

### Femorodistalni *bypass* korišćenjem venske „manžetne“

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#### Abstract

**Background/Aim.** Although distal venous patch and cuff techniques are recommended to improve the patency of bypass in the lower extremities, the advantage of these techniques remains unproven. Autovenous bypass graft remains the method of choice, but when there is no possibility of using the great saphenous vein, the venous cuff method may have an advantage. The aim of this study was to evaluate the results of femorodistal bypass procedures with a venous cuff in critical lower extremity ischemia. **Methods.** The study described the results of femorodistal bypass procedures after a follow-up period of 6 to 24 months. We followed 28 patients with distal composite anastomosis of femorodistal arterial reconstruction. Indications for surgical treatment were set on the basis of the clinical exam and morphological criteria based on multidetector computed tomographic angiography. Three types of distal graft-arterial anastomoses were applied: Miller’s cuff, Taylor’s patch, and St. Mary’s vein boot anastomosis. **Results.** After 6 months of surgery, all bypasses were passable; after one year, 2 (7%) patients had graft occlusion and above-knee amputation, and 8 (28%) patients died; after 24 months, 18 (64%) patients had primary bypass patency. **Conclusion.** Femorodistal bypass procedures using a synthetic graft and venous cuff have good long-term results. The optimal anastomosis type is St. Mary’s boot. A longer follow-up period and comparison with the autovenous bypass are necessary for a more detailed assessment of the final conclusion.

#### Key words:

computed tomography angiography; graft occlusion, vascular; ischemia; leg; multidetector computed tomography; vascular surgical procedures.

#### Apstrakt

**Uvod/Cilj.** Iako se distalni venski *patch* („zakrpa“) i *cuff* („manžetna“) tehnike preporučuju za poboljšanje prolaznosti *bypass*-a na donjim ekstremitetima, prednost tih tehnika ostaje nedokazana. Autovenski *bypass* graft ostaje metoda izbora, ali kada ne postoji mogućnost korišćenja velike potkožne vene, metode venske „manžetne“ mogu imati prednost. Cilj rada bio je da se procene rezultati femorodistalnih *bypass* procedura primenom venske „manžetne“ kod bolesnika sa kritičnom ishemijom donjih ekstremiteta. **Metode.** Prikazani su rezultati femorodistalnih *bypass* procedura nakon perioda praćenja od 6 do 24 meseca. Praćeno je ukupno 28 bolesnika sa distalnom kompozitnom anastomozom femorodistalne arterijske rekonstrukcije. Indikacije za hirurško lečenje postavljene su na osnovu kliničke slike i morfoloških kriterijuma, na osnovu nalaza multidetektorske kompjuterizovane tomografske angiografije. Primenjena su tri tipa distalnih graft-arterijskih anastomoza: *Miller’s cuff*, *Taylor’s patch* i *St. Mary’s vein boot* anastomoza. **Rezultati.** Nakon 6 meseci, svi bolesnici imali su prolazan *bypass*; nakon godinu dana, 2 (7%) bolesnika imalo je okluziju grafta i natkolenu amputaciju, a njih 8 (28%) imalo je smrtni ishod; nakon 24 meseca, 18 (64%) bolesnika imalo je primarnu prolaznost *bypass*-a. **Zaključak.** Femorodistalne *bypass* procedure korišćenjem sintetičkog grafta i venske „manžetne“, imaju dobre dugoročne rezultate. Optimalna anastomoza je ona po tipu *St. Mary’s boot*. Duži period praćenja i poređenje sa autovenskim *bypass*-om su neophodni za konačni zaključak.

#### Ključne reči:

angiografija, tomografska, kompjuterizovana; vaskularni graft, okluzija; ishemija; noga; tomografija, kompjuterizovana, multidetektorska; hirurgija, vaskularna, procedure.

#### Introduction

Femoropopliteal bypass procedures above or below the knee on the popliteal artery or the tibioperoneal trunk (fem-

orodistal bypass) are frequently necessary as “limb salvage” therapy. Those patients have critical limb ischemia because of an extensive occlusive arterial disease. Below-knee bypass becomes very often dysfunctional in the early postoperative

period because of technical reasons such as the choice of the artery, difficulties in creating anastomosis, and the choice of conduit. The most often used conduits for femorodistal reconstruction are autogenous vein, synthetic, or composite vein-synthetic grafts. The synthetic graft used for distal bypass revascularization in the early postoperative period becomes dysfunctional due to thrombosis<sup>1-4</sup>. In the second half of the 20<sup>th</sup> century, new techniques were described in below-knee femoropopliteal bypass surgery: Miller's cuff (1984), Taylor's patch (1992), and St. Mary's boot vein cuff (1997)<sup>5-7</sup>. Not long ago, the boat-form vein cuff technique appeared; it is easy to create and enables surgeons to adjust the anastomotic size and angle<sup>8</sup>. Vein cuff decreases intimal hyperplasia on end-to-side prosthetic-graft anastomosis<sup>9</sup>. Autovenous graft has better patency than polytetrafluoroethylene (PTFE) graft and a low degree of ischemic complication<sup>10</sup>. Distal vein cuff on composite femoropopliteal bypass leads to better long-term outcomes<sup>11</sup>. The saphenous graft should be a method of choice in femoropopliteal bypass surgery if the great saphenous vein (GSV) is available<sup>12</sup>. However, it may not be accessible in some cases, such as: if formerly used in the previous reconstruction or for coronary bypass; if the diameter of the GSV was small; previous thrombosis of the vein; previous stripping of the GSV. Therefore, the small saphenous vein, the superficial femoral vein, the basilic and cephalic vein, the part of the superficial femoral artery after endarterectomy, and the radial artery can be used for conduit. The interposition of vein tissue between the PTFE graft and artery reduces turbulence and also affects

the thrombogenicity-vein patch, which is a border between highly resistant arterial outflow and a little bit extended PTFE graft<sup>13,14</sup>. The aim of this study was to evaluate the results of femorodistal bypass grafting and vein cuff for critical lower extremity ischemia.

### Methods

This study presents early surgical morbidity and mortality results, patency, limb salvage, and survivals for femorodistal procedures performed from 2012 through 2014 at the Clinic for Vascular and Endovascular Surgery of the Military Medical Academy, Belgrade, Serbia. General characteristics of patients, demographic factors, and comorbidity are shown in Table 1.

Indications for surgical treatment were clinical and morphological features based on multidetector computed tomographic angiography (MDCTA) finding (Figure 1).

For all patients who had indications for below knee bypass treatment with composite distal anastomosis, popliteal bypass above the knee was created with composite anastomosis if the diameter of the popliteal artery was less than 4 mm.

Miller's cuff technique (Figure 2) uses a small part of the vein of 4–6 cm, with longitudinal incision and after excision of the valves. This vein patch was running suture to the edge of the arteriotomy (incision). The cuff was formed in that manner. PTFE graft was then running sutured to the edge of vein cuff<sup>6</sup>. The deficiency of this technique is the

**Table 1**

**General characteristics and comorbidities of patients with femoropopliteal reconstruction**

Parameter	Miller's cuff	Taylor's patch	St. Mary's vein boot	Synthetic femoropopliteal graft	Total
Man	5	3	18	31	57
Woman	/	/	2	16	18
Diabetes mellitus	1	/	9	19	29
Smoking	4	2	15	38	58
Arterial hypertension	5	3	19	42	67

All values are expressed as numbers (of patients).



**Fig. 1 – Multidetector computed tomographic angiography finding in a patient with an indication for femoropopliteal below-knee bypass.**

large anastomotic reservoir, turbulence blood flow, and “move” anastomosis.

Taylor’s patch technique (Figure 3) uses a part of the vein of 2–3 cm with longitudinal incision and after excision of the valve. The arteriotomy incision is also at a length of 2–3 cm. The part of the vein was sutured to the distal half of the arteriotomy incision. PTFE graft was directly sutured to the proximal half of the arteriotomy incision and the distal half of the PTFE graft to the edges of the vein cuff<sup>15</sup>.

Tyrrel and Wolf<sup>7</sup> improved their own technique – St. Mary’s vein boot (Figure 4). The suture technique is similar to Miller’s cuff technique, except the edges of the vein cuff were sutured to the proximal part of the arteriotomy incision, and, in that way, the anastomotic toe was formed. These authors forced this technique because, according to them, the previous techniques had deficiencies such as turbulence in blood flow, the low position of the anastomotic reservoir, and inadequate angle between graft and artery.

Surgical procedures were performed by 3 surgeons from the clinic, but in a standardized way so that there was no difference in surgical technique. The technique consisted of proximal latero-terminal (L-T) arterial graft anastomosis and distal composite anastomosis as previously described with previous popliteal artery endarterectomy.

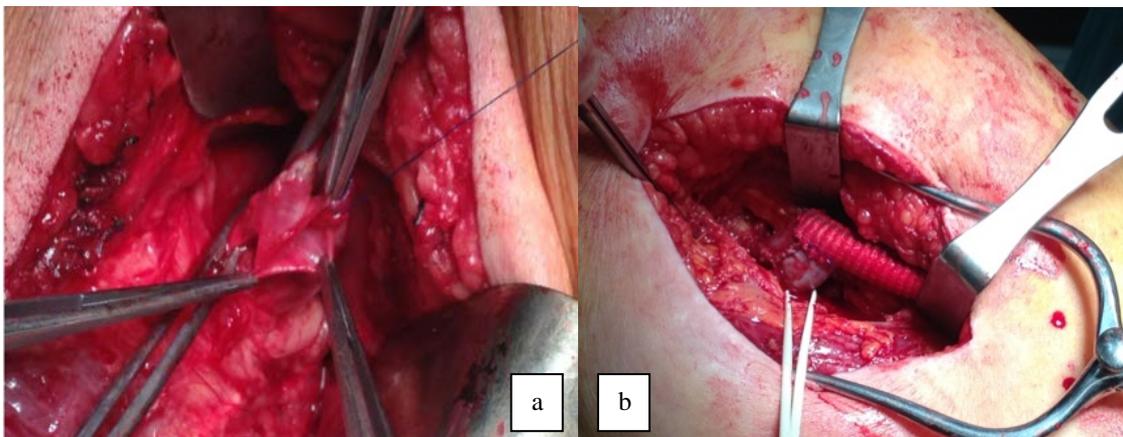
For the bypass patency, we used ultrasound duplex sonography, and all of the patients were monitored clinically for possible complications: graft thrombosis, infection, and amputation. Besides, general complications were followed: myocardial infarction, stroke, pulmonary embolism, and death.

## Results

In the period between 2012 to 2014, 75 patients were operated on due to femoropopliteal occlusive disease: 47 (63%) patients were treated with above-knee femoropopliteal synthetic graft bypass without vein cuff anastomosis, and 28 (37%) patients with vein cuff femorodistal reconstruction.

Out of the 28 followed patients with vein cuff distal anastomosis, 26 (93%) were male, and 2 (7%) were female. Femoropopliteal bypass above the knee was made on 10 (36%) patients, and femoropopliteal below the knee (or on tibioperoneal trunk) bypass was made on 18 (64%) patients. The indications for reconstructions were rest pain in 23 (82%) patients and tissue defect in 5 (18%) patients. We used PTFE graft on 10 (36%) patients, and on 18 (64%) patients, reconstruction was made with Dacron graft. Taylor’s patch was done on 3 (11%) patients, 5 (18%) had Miller’s cuff, and the remaining 20 (71%) patients had St. Mary’s boot reconstruction. Except for vascular reconstruction, no additional procedures, such as stenting or toe amputation, were necessary for our study.

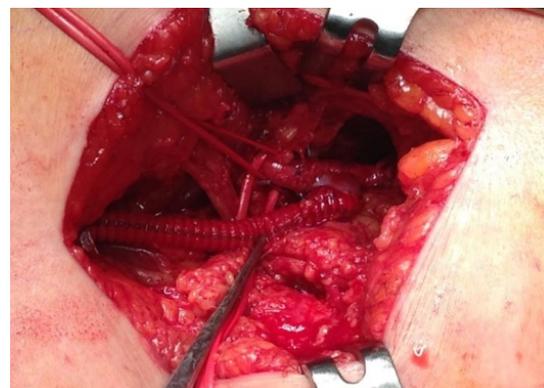
The period for evaluating primary patency was 6–24 months. The control examinations were 1, 3, 6, 12, and 24 months after surgical treatment. Through this period, we followed 28 patients. Eight (28%) of them had some general complications: 4 (14%) had a myocardial infarction, 3 (11%) had a stroke, and 1 (3%) had a pulmonary embolism 6 months after bypass surgery. Twenty (72%) patients had a real follow-up.



**Fig 2. – Miller’s-cuff technique: a) creation of anastomosis; b) anastomosis is completed.**



**Fig. 3 – Taylor’s patch technique: anastomosis is completed.**



**Fig. 4. – St. Mary’s vein boot technique: anastomosis is completed.**

After 6 months of follow-up, 20 (72%) patients had primary patency.

After one year of follow-up, 2 (7%) patients had an occlusion of bypass, and 18 (64%) had primary patency. One (20%) of the 5 patients with Miller's cuff and 1 (33%) of the 3 patients with Taylor's cuff had graft thrombosis, and amputation above the knee was indicated. Two (7%) more of the 28 followed up patients had amputation surgery.

After 24 months of follow-up, none of the new patients had occlusion of femorodistal bypass, and 18 (64%) had primary patency. During the follow-up period of 2 years, we did not observe infection as a complication.

## Discussion

According to the literature, the historical aspect of this method began in 1979 when Siegman<sup>5</sup> proposed a new technique – vein cuff. The aim of this technique is to lengthen the patency of distal bypass. Miller et al.<sup>6</sup> proposed a new variant of vein cuff to facilitate the anastomosis of a prosthetic graft to a small artery. Taylor et al.<sup>15</sup> reported results of vein cuff patency after one, three, and five years of evaluations. Tyrell and Wolf<sup>7</sup> reported results of distal bypass reconstruction with PTFE graft and Taylor's patch (74%) vs. Miller's cuff (47%), and very soon, they proposed their technique of venous cuff – St. Mary's boot.

The results of numerous studies prove that a technique with a composite PTFE-vein cuff graft should be useful for below-knee reconstruction when the GSV is not available. The disadvantage of this method is that it significantly extends the duration of the operation. In femoropopliteal bypass with a synthetic graft, the operation lasted on average 60–90 min, and in vein cuff femoropopliteal bypass, the duration of the operation was 120–150 min. Vein's tissue creates a "biological defensive zone" and reduces stimulation of factors responsible for neointimal hyperplasia<sup>16</sup>. During the follow-up period of 6–24 months, the most common reason for dysfunction of anastomosis was neointimal hyperplasia, and after 2 years of follow-up, it was the progression of atherosclerosis. Femorodistal bypass on tibioperoneal trunk with synthetic graft and vein patch for critical lower extremity ischemia has good long-term results comparable to those reported for bypasses without vein patch. Some authors have one-year results proving that the primary patency of below-knee bypasses with a vein patch is 62% and for synthetic

graft (PTFE) 52%. After two years of follow-up, bypasses with a vein patch have primary patency of 49%, but for synthetic grafting primary patency is 42%. Furthermore, lower limb amputation is reduced by 82% using a vein patch instead of PTFE grafting, which is at 62%<sup>14</sup>. Meta-analyses results reported that after five years of following patients with the below-knee bypass with synthetic graft, only 31% had primary patency<sup>17</sup>. After four years of follow-up, 49% of patients with below-knee venous bypass had patent grafts, and 12% with synthetic graft below-knee bypass had patent grafts. Moreover, lower limb amputation is more often necessary after using synthetic grafts<sup>18</sup>. The new studies are guiding the development and betterment of the quality of synthetic (PTFE) grafts by covering them with anticoagulant substances (Propaten®). For the first year of follow-up with these grafts for below-knee revascularization, primary patency was 80%<sup>19</sup>. Patients with below-knee bypasses need to continue the double antithrombotic therapy, but in some cases, anticoagulant therapy is necessary. In addition, following their blood pressure and lipid status is important<sup>20</sup>.

Given the small number of patients and the absence of a control group in this study, it is necessary to compare patients operated on with this method with patients operated on with an autovenous graft because many studies recommend saphenous graft as the method of choice<sup>21,22</sup>.

The disadvantages of this study, in addition to the above, are the retrospective design and the absence of a control group, and to some extent, the surgical technique, considering that there are possible differences in relation to the surgeons. Our results point to better primary patency in patients with below-knee bypass, created with synthetic graft and venous cuff.

## Conclusion

Femorodistal arterial bypass with synthetic graft and venous cuff in patients with critical limb ischemia has good long-term results. It is an optimal treatment when the venous conduit is not available in the appropriate length. The optimal venous cuff technique is St Mary's boot, with better results of primary patency of grafting. Using those techniques, the time of operation is prolonged, but the results are better. A longer monitoring period with a more detailed assessment and comparison with the autovenous bypass is necessary.

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# Sensorimotor rhythm neurofeedback training and auditory perception

## *Neurofeedback* trening senzomotornog ritma i auditivna percepcija

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### Abstract

**Background/Aim.** In everyday communication, people are exposed to a myriad of sounds that need to be sorted and relevant information extracted. The ability of a person to concentrate on certain sounds in a noisy background environment, perform selective attention, and focus their auditory attention is crucial for everyday functioning and communication. The aim of this study was to investigate the effect of the sensorimotor rhythm (SMR) (12–15 Hz) neurofeedback (NFB) training to improve auditory cognition measured by the achievements in the Quick speech-in-noise (QuickSIN) test, changes in the amplitudes and latencies of components of auditory evoked potentials (AEP) N100, N200, and P300 in the auditory oddball discrimination task, and changes in the spectral power of the SMR. **Methods.** The study included 16 healthy participants aged 25–40 years (8 males and 8 females). Each participant had 20 daily sessions of SMR NFB training. Auditory cognitive

functions and electrophysiological correlates of cognitive processing were recorded 5 times – before NFB, after 5, 10, and 20 sessions, and one month after the last session of NFB. **Results.** The results showed a statistically significant decrease in N200 and P300 latencies at frontal midline (Fz), central midline (Cz), and parietal midline (Pz) regions, an improvement on the QuickSIN test, and an increase in electroencephalogram SMR rhythm spectral power in the Cz region as a result of the NFB SMR training. No significant effect of the NFB training on the N100, N200, and P300 amplitudes on Fz, Cz, and Pz was found. **Conclusion.** The obtained results suggest that SMR NFB affects auditory perception in terms of shorter latencies of AEP and better performance on the QuickSIN test.

**Key words:** auditory perception; cognition; electroencephalography; event-related potentials, p300; evoked potentials, auditory; feedback, sensory.

### Apstrakt

**Uvod/Cilj.** U svakodnevnoj komunikaciji, ljudi su izloženi mnoštvu zvukova koje treba razvrstati i iz kojih treba izvući bitne informacije. Sposobnost osobe da se koncentriše na određene zvukove u bučnom okruženju, da selektivno i usredsređeno usmerava sluh je ključna za svakodnevno funkcionisanje i komunikaciju. Cilj studije bio je da se ispita efekat *neurofeedback* (NFB) treninga senzomotornog ritma (SMR) (12–15 Hz) na auditivnu percepciju koji se meri rezultatima postignutim na *Quick speech-in-noise* (QuickSIN) testu, promenama amplituda i latenci komponenti auditivnih evociranih potencijala (AEP) N100, N200 i P300 tokom zadatka auditivne diskriminacije i promenama spektralne snage SMR talasa. **Metode.** U studiju je bilo uključeno 16 zdravih ispitanika uzrasta od 25 do 40 godina (8 muškog i 8 ženskog pola). Svaki ispitanik imao je 20 svakodnevni SMR NFB treninga. Auditivne kognitivne funkcije i elektrofiziološke korelacije kognitivnih procesa snimane su 5

puta, i to pre primene NFB treninga, posle 5, 10, i 20 treninga i jedan mesec nakon poslednjeg treninga. **Rezultati.** Rezultati su pokazali statistički značajno smanjenje latenci N200 i P300 komponenti u regijama *frontal midline* (Fz), *central midline* (Cz) i *parietal midline* (Pz), bolje postignuće na QuickSIN testu kao i povećanje spektralne snage elektroencefalografije SMR ritma u Cz regiji kao rezultat NFB SMR treninga. Nije utvrđen statistički značajan efekat NFB treninga na N100, N200 i P300 amplitude u Fz, Cz i Pz regijama, niti na spektralnu snagu SMR talasa. **Zaključak.** Dobijeni rezultati ukazuju na potencijalni efekat SMR NFB treninga na poboljšanje procesa auditivne diskriminacije u smislu kraćih latenci komponenti AEP i boljeg postignuća na QuickSIN testu.

**Ključne reči:** percepcija, auditivna; saznanje; elektroencefalografija; potencijali povezani sa događajima, p300; evocirani potencijali, auditivni; povratna informacija, senzorna.

## Introduction

In everyday communication, people are exposed to a myriad of sounds that need to be sorted and relevant information extracted. The term “cocktail party effect” was first used by Cherry in 1953, which refers to focusing on one sound, often speech, while at the same time suppressing other unwanted sounds in a noisy background<sup>1</sup>. The cocktail party effect is an example of selective attention and illustrates the phenomenon of being able to focus auditory attention on a particular stimulus while filtering out a range of other stimuli<sup>2</sup>. The ability to concentrate on certain sounds in a multi-sound environment is crucial for daily functioning and communication.

The P300 event-related potential (ERP) can be considered a neurophysiological marker of auditory attention. P300 is an endogenous cognitive neuroelectric phenomenon that occurs under the influence of endogenous stimuli; it depends on the state of vigilance, concentration, and type of task the subject is required to perform. The ERP components are represented by a series of positive and negative waves (N100, P100, N200, P200, and P300) of different duration and amplitudes, the most significant of which is the P300.

Different treatments using different sub-disciplines of biomedical engineering are used to improve cognitive functions and thus improve the quality of life. Cognitive training with neurofeedback (NFB), as a form of operative conditioning, is being increasingly used in a healthy population with the aim of increasing performance. The use of NFB dates back to early experiments conducted by Kamiia in the 1960s and Serman in the 1970s, in which epileptic cats were trained to improve sensorimotor rhythm (SMR) of the brain, leading to less frequent epileptic seizures<sup>3</sup>. NFB, a form of biofeedback, is a therapeutic method based on monitoring the electroencephalogram (EEG) and providing feedback on the brain activity of subjects, which can be learned to regulate via operative conditioning<sup>4</sup>.

Using real-time NFB protocols allows the acquisition of control of localized brain activity. NFB allows the experimenter to noninvasively manipulate brain activity as an independent variable leading to specific behavioral changes<sup>5</sup>.

SMR waves training refers to cognitive function, better focus, and increased attention and concentration<sup>6</sup>. SMR waves (12–15 Hz) are beta waves that occur in the sensorimotor region of the brain regulated by the thalamocortical loop<sup>7</sup>. With the NFB SMR training, the subject trains to gain control in terms of increasing the amplitude of the SMR wave, resulting in increased attention and better focus. Literature data indicate that normal healthy individuals can learn to control and modify the components of their EEG activity and thus contribute to improving cognitive function.

The aim of this study was to investigate the effect of NFB SMR (12–15 Hz) training on auditory cognition measured by the following: the achievement of hearing speech in a noisy background, the Quick speech-in-noise (QuickSIN) test, changes in the amplitudes and latencies of event-related potentials (N100, N200, P300) in the auditory oddball dis-

crimination task, and changes in the spectral power of the SMR in healthy participants aged 25 to 40 years.

## Methods

### *Participants*

The study involved 16 healthy participants of both sexes (8 males and 8 females), 25 to 40 years old. The participants were recruited from the Institute for Experimental Phonetics and Speech Pathology and the Life Activities Advancement Center in Belgrade, Serbia, whose Laboratory for cognitive research conducted the experiments. The participants were without hearing or speech disorders, with no prior or current neurological or psychiatric illnesses (based on the participant’s verbal report). All participants were right-handed, according to the Edinburgh Handedness Inventory. Each participant gave their written informed consent before the experimental procedure. This study was approved by the local Ethics Committee (No. 22/19 from February 18, 2019) according to the Declaration of Helsinki.

### *Auditory event-related potentials recording*

The auditory event-related potentials (aERPs) were recorded using a Nihon Kohden Electroencephalograph (model EEG-4314F) and Neuroscan Acquire 4.0 software. To obtain the P300, an auditory “oddball” paradigm with two tones was used, with 80% non-target and 20% target stimuli. The participants had a task to react by pressing a control button with the right hand’s thumb each time they heard a tone that differed from the other mostly presented tones. A total of 80% of each presented tone had a frequency of 1,000 Hz, and 20% of tones were oddballs with a frequency of 2,000 Hz. The tones were randomly presented to the participants. The participants listened to the tones using earphones. Three Ag/Ag-Cl ring electrodes for aERPs registration were positioned according to the 10–20 International system for electrode placement at the frontal midline (Fz), central midline (Cz), and parietal midline (Pz) regions. The reference electrode was set to the ear lobes, and the ground electrode was on the forehead. The impedance was kept below 5k $\Omega$  with no more than 1k $\Omega$  difference between electrodes. The software has its own implemented tool for artifact rejection. Each recording section with more than 20% of rejected trials due to excessive artifacts was discarded and redone. Each participant underwent the experimental procedure in the morning hours (9–11 am). For each participant, averaged amplitude ( $\mu$ V) and latency (ms) of N100, N200, and P300 waves were obtained for each electrode (Fz, Cz, and Pz). The aERPs were recorded at the beginning (t1), after 5 (t2), 10 (t3), and 20 (t4) NFB SMR treatments, as well as one month after the last NFB SMR treatment (t5).

### *EEG recording and analysis*

EEG signal recording was performed on an EEG device Nihon Kohden (EEG – 1200K Neurofax) with a fixed cap

(Electrocap, model number 16755, International, Inc.) with Ag/AgCl surface electrodes filled with electro-conductive gel, which provides 19 EEG channels. The electrodes are positioned according to the 10/20 International Electrode Positioning System. During the experiment, participants were placed in a sitting position in a soundproof room. The task was to keep their eyes open and reduce movement as much as possible. EEG was recorded for 3 min during the “resting state” with no ongoing task. The participants were placed in a square-shaped cube made of white non-transparent curtains in order to eliminate visual stimuli that may have influenced the experimental tasks. The recording was done approximately around noon (12 am +/- 1 h). The EEG data were transposed into EEGLAB Software for Independent Component Analysis (ICA)-dependent artifact rejection. All artifacts, including body movement, eye blinks, eye movements, teeth clenching, or ECG artifacts, were removed from the EEG trace. From the 3 min resting state, we have selected six 10-s artifact-free periods: two segments from the first, second, and third minute of the recording. Those segments were averaged for each participant in further analysis.

We used a fast Fourier transform (FFT) to separate SMR rhythm (12–15Hz). Before computing FFT, each epoch was multiplied by an appropriate windowing function (Hanning window) in order to avoid border problems (leakage). Spectral power was calculated using in-house written MATLAB script (MathWorks, version 7) and EEGLAB software packages. For topographic spectral maps plotted in EEGLAB, we used all 19 electrodes.

#### *QuickSIN test*

The QuickSIN test is from the Hearing In Noise Test group of methods that uses test sentences mixed with defined doses of interfering noise. The attention of the respondents is crucial for the success of this test. With this method of measurement, speech communication in noisy environments is simulated. The starting hypothesis for measuring speech intelligibility in the presence of noise is that a person with normal hearing understands 50% of words if the signal/noise (S/N) ratio is 2 dB. The QuickSIN test uses sentences dosed with a certain level of noise of the “cocktail-party” type. The smallest test unit is a list with six test sentences. Each test sentence contains five key words (30 key words for one list). The level of interfering noise added to “clean” sentences depends on the position of the test sentence in the list. Sentences are played through speakers or headphones, and the respondent tries to recognize the key words. A comparison is made between what was heard and what was actually reproduced. For the needs of the QuickSIN method, 126 test sentences and 21 test lists were formed ( $21 \times 6 = 126$ )<sup>8</sup>. Due to the different levels of distracting noise in test sentences, not all sentences are of the same weight for perception. As the level of interfering noise increases, the intelligibility of key words decreases. The most difficult case is with the sixth test sentence, where the level of speech and disturbing noise is the same. For this sentence, it is predicted that a person with normal hearing

will correctly perceive one or two key words. If the respondent loses concentration on these sentences for a moment, he/she may not perceive any key words.

#### *Neurofeedback SMR protocol training*

The NFB SMR training was performed using BioTrace software for Nexus – 10B2015. The electrode was set to a Cz region (central midline – vertex region). The experimental task for participants was to perform an NFB SMR training by increasing the amplitude of SMR rhythm (12–15Hz). During the trials, the participant looks at the physiological responses on the screen in the form of pictures and video games. The information that comes from this process is feedback, which is reflected via changes in the image or sound of the video game used for training. The games are designed to let the participant advance in the game if he or she can bring the physiological function that is being rehearsed to the desired level. Each participant participated in 20 sessions of NFB SMR protocol training, three times a week for 28 min of effective recording: 2 min of resting state period (watching a blank computer screen) at the beginning, 4 training trials, each lasting 6 min, and 2 min of resting state at the end.

After 5 (t2), 10 (t3), and 20 (t4) NFB SMR training sessions, as well as one month after the last session (t5), participants were re-registered with aERPs using the same procedure as at the beginning.

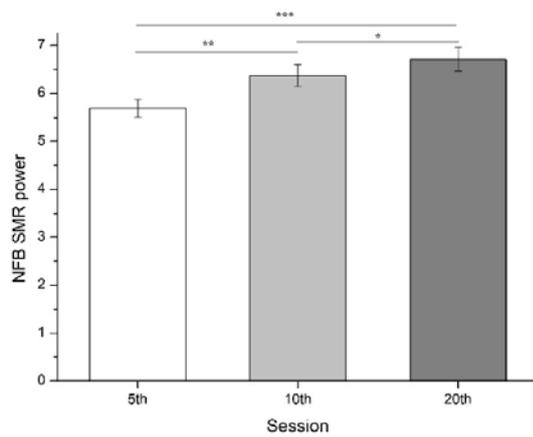
#### *Statistical analyses*

The sample size is small, and the data were with non-normal distribution. Hence, the comparisons of NFB SMR power, amplitudes and latencies of aERPs before and after NFB SMR training, and EEG SMR spectral power values were analyzed using nonparametric statistics – Kruskal-Wallis test for exploring the effect of time point (before NFB, after 5, 10, 20 sessions, and one month after the last training session) and Wilcoxon signed-rank test for post hoc multiple comparisons reporting Z score and *p*-value. The results of the QuickSIN test were tested using one-way ANOVA with the time point factor followed by Student’s *t*-test. In each comparison, a 95% confidence interval was used.

## **Results**

#### *NFB SMR power*

First, we analyzed the resting state NFB SMR power after 5, 10, and 20 NFB SMR training sessions (Figure 1). The Kruskal-Wallis test found a significant effect of session number on NFB SMR power in the Cz electrode location:  $H(47) = 3.478, p = 0.03$ . Post hoc Mann-Whitney *U* test found a statistically significant difference between NFB SMR power between the 5<sup>th</sup> and 10<sup>th</sup> session:  $Z = 2.327, p = 0.02$ , 10<sup>th</sup> and 20<sup>th</sup> session:  $Z = 1.965, p = 0.049$ , as well as 5<sup>th</sup> and 20<sup>th</sup> session:  $Z = 2.612, p = 0.009$ . The results showed a statistically significant linear increase in SMR power in the Cz region due to the application of the NFB SMR training protocol.



**Fig. 1 – Average neurofeedback (NFB) sensorimotor rhythm (SMR) power measured in the central midline (Cz) region after 5, 10, and 20 NFB SMR training protocols; \*  $p < 0.05$ ; \*\*  $p < 0.02$ ; \*\*\*  $p < 0.01$ , based on the Mann-Whitney  $U$  test.**

*Auditory event-related potentials*

The second task was to probe the effect of NFB SMR training protocol on aERPs amplitude and latency changes.

Using the Kruskal-Wallis test, we found no overall statistically significant effect of time point (number of NFB SMR training sessions) on aERP amplitudes of N100, N200,

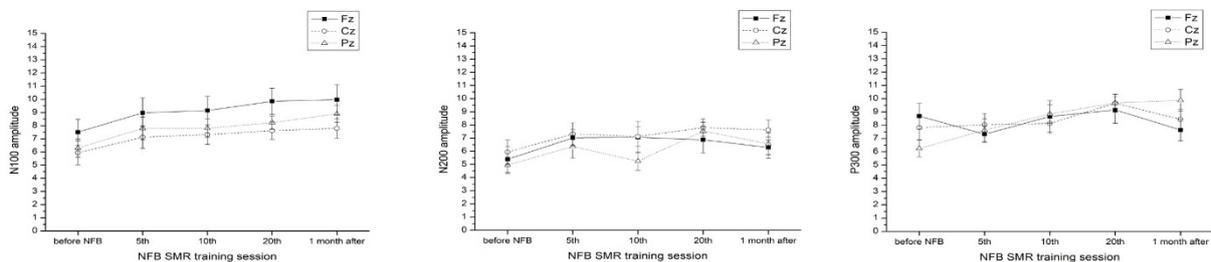
and P300. However, there is a trend of amplitude increase of the N100 wave when time point t1 (before NFB SMR training) is compared to time points t4 (after 20 NFB SMR training sessions) and t5 (one month after the 20<sup>th</sup> training session). In addition, for the P300 wave, there is a statistically significant difference for the Cz electrode location between t1 and t5 ( $Z = 2.327, p = 0.002$ ) with an almost statistically significant linear increase of amplitude in time (Figure 2).

The Kruskal-Wallis test found a statistically significant effect of time point (number of NFB SMR training sessions) on aERPs latency of N200:  $H(79) = 2.965, p = 0.018$ , and P300:  $H(79) = 3.889, p = 0.002$  (Figure 3).

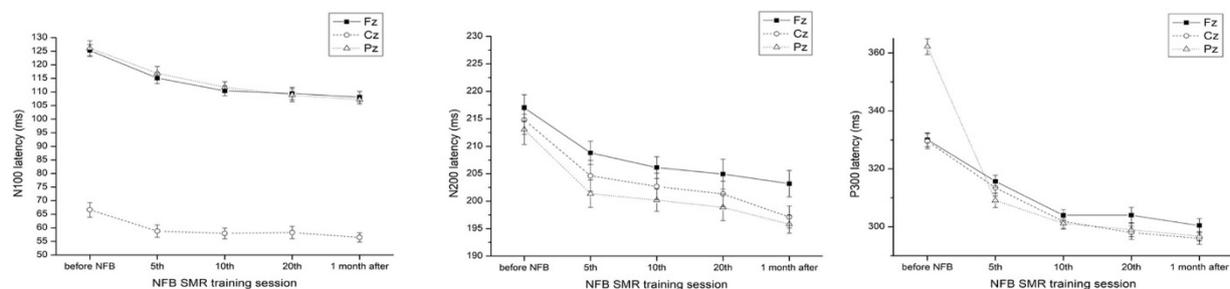
However, the post hoc Mann-Whitney  $U$  test found that the Cz electrode location had a linear decrease in latency for both N200 and P300 waves. For N200 wave, the following differences were present: t1 > t2:  $Z = 2.272, p = 0.023$ ; t1 > t3:  $Z = 2.330, p = 0.02$ ; t1 > t4:  $Z = 2.430, p = 0.015$ ; t1 > t5:  $Z = 2.992, p = 0.01$ . For P300 wave, the following differences were present: t1 > t2:  $Z = 2.561, p = 0.01$ ; t1 > t3:  $Z = 2.755, p = 0.006$ ; t1 > t4:  $Z = 3.517, p = 0.001$ ; t1 > t5:  $Z = 3.362, p = 0.001$ .

*QuickSIN test*

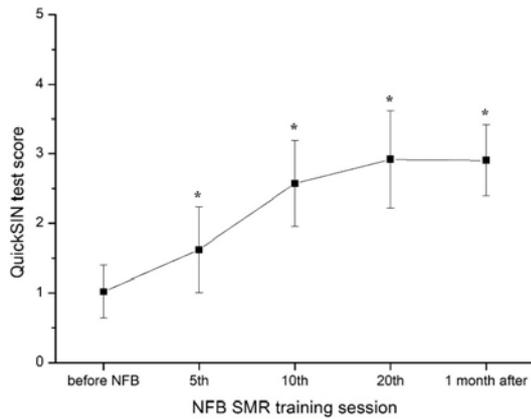
The obtained results showed a linear increase from t1 to t4 (Figure 4). The one-way ANOVA found a statistically significant effect of time point on the score:  $F(5, 1675) =$



**Fig. 2 – Average amplitude value ( $\mu\text{V}$ ) of N100 (left panel), N200 (middle panel), and P300 (right panel) waves at frontal midline (Fz), central midline – vertex (Cz), and parietal midline (Pz) electrode location at five time points: before neurofeedback (NFB) sensorimotor rhythm (SMR) training and after 5, 10, and 20 sessions, as well as one month after the last session.**



**Fig. 3 – Average auditory event-related potentials latency value (ms) of N100 (left panel), N200 (middle panel), and P300 (right panel) waves measured in frontal midline (Fz), central midline – vertex (Cz), and parietal midline (Pz) electrode location at five time points: before neurofeedback (NFB) sensorimotor rhythm (SMR) training protocol and after 5, 10, and 20 sessions, as well as one month after the last session.**

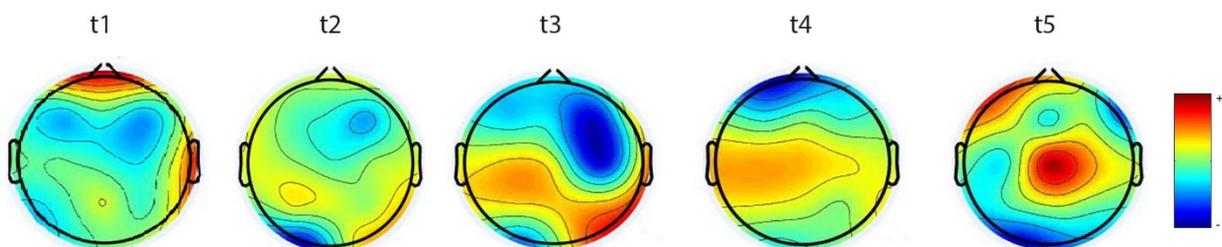


**Fig. 4 – The result of the Quick speech-in-noise (QuickSIN) test. The average number of perceived key words in the sixth test sentence, where the level of speech and disturbing noise is the same; \*statistically significant difference in test score compared to t1 – before neurofeedback (NFB) sensorimotor rhythm (SMR) training.**

5.248,  $p < 0.01$ . The post hoc Bonferroni correction found a difference between t1 and t2, t2 and t3, t1 and t3, t1 and t4, as well as t1 and t5. No differences were found between t3 and t4, nor between t4 and t5. The obtained results showed a better achievement on the QuickSIN test as a result of the NFB SMR training protocol.

#### EEG SMR spectral power

The final level of analysis was to probe the effect of NFB SMR training protocol on EEG SMR rhythm spectral power. The Kruskal-Wallis test found a significant effect of time points on the SMR spectral power in the Cz region:  $H(80) = 3.895$ ,  $p = 0.009$ . Post hoc Mann-Whitney  $U$  test showed that this effect was driven by the increase in SMR spectral power after training sessions compared to the initial t1 period (before NFB training). After 20 NFB SMR training sessions (t4), there was an increase in SMR spectral power over the entire sensorimotor region (C3, Cz, and C4 electrode location) (Figure 5). An interesting result was that even one month after the last NFB SMR training, there was still an increase in the Cz region.



**Fig. 5 – Averaged electroencephalogram sensorimotor rhythm (12–15Hz) spectral power [ $10 \times \log_{10} (\mu V^2/Hz)$ ] scalp distribution. t1 – before neurofeedback sensorimotor rhythm training protocol; t2 – after 5 training sessions; t3 – after 10 training sessions; t4 – after 20 training sessions; t5 – one month after the last training session.**

#### Discussion

This study explored the effect of NFB SMR training on auditory cognition measured by the achievement on the QuickSIN test, changes in the amplitudes and latencies of aERPs recorded at Fz, Cz, and Pz regions during standard auditory oddball discrimination task, and changes in the spectral power of the SMR measured by EEG.

An effect of NFB SMR training was found for aERP latencies. NFB SMR training caused a decrease in latencies of auditory ERP N200 and P300 waves. However, no differences in amplitudes were found (although there is a trend of increase in amplitude as a result of NFB SMR training). To generate the potential of P300, the oddball paradigm was used. It is the acoustic discrimination test, which uses two types of tone: high-frequency arrhythmic tone and low-frequency rhythmic tone. The difference between the two tones is in frequency and intensity<sup>9</sup>. The respondent is presented with two types of auditory stimuli – the first one being “rare” or “unexpected” arrhythmic tone, which represents the target stimulus and occurs in random order and differs in frequency from the second “standard” or “expected” tone. The participant is required to respond to the “unexpected” tone (by pressing a key) and ignore the “standard” tone, i.e., to recognize target stimuli in a series of stimuli that differ in their characteristics (volume, duration) and are less likely than the standard ones. The oddball experimental paradigm requires the attention and concentration of the respondents.

ERP waveforms are quantitatively described by amplitude level, latency length, and topographic distribution. The amplitude reflects the magnitude of neural activity and typically ranges from 1 to 30mV<sup>10</sup>. Latency represents the time interval, i.e., the period from the moment of stimulation, to the appearance of maximum amplitude, i.e., the peak of ERP, and ranges from several hundred ms.

Latency reflects the speed of processing sensory stimuli as a consequence of distinction from the other stimuli. Therefore, shorter latencies are considered to reflect more effective mental performance compared to longer latencies. In a study by Kober et al.<sup>11</sup> on healthy young adults after NFB SMR training, the experimental and not the control group showed an increase in the amplitudes of N100 and P300. We found a similar result regarding a sample of healthy individuals. In a study by Reichert et al.<sup>12</sup>, an in-

crease in N100 and P300 amplitude was observed in a post-stroke patient in an experimental group who had SMR training, whereas the control group showed no difference. This finding is partially in line with our study, which found a trend of amplitude increase of N100 wave when time point t1 is compared to time points t4 and t5. In addition, for the P300 wave, there is a statistically significant difference in the Cz electrode location between t1 and t5. The earliest wave in the sequence arises about 100 ms from stimulation and is designated as wave N100 due to the negative polarization. The negative wave (the N200 component) occurs 200 ms from stimulation and is associated with the process of sensory discrimination. The role of N200 is mainly focused on “cognitive controls,” a concept that encompasses monitoring and control of motor responses<sup>13</sup>.

The P300 wave has the longest latency and the highest amplitude registered above the central and parietal regions of the cerebral cortex. The time span of this P300 component by Coles and Rugg<sup>14</sup> can range from 250 ms to 900 ms, with an amplitude ranging from at least 5  $\mu$ V to the usual limit of 20  $\mu$ V. It is an endogenous response to an unknown task, i.e., response to target stimuli<sup>15</sup>. Extension of the P300 latency, which reflects the time of assessment and categorization of stimuli, indicates a slowdown in mental functions. The lack of attention causes a decrease in the P300 amplitude or the absence of a P300 wave.

A large number of studies of NFB training in a healthy population show that the SMR protocol is an effective method to improve cognitive performance in terms of increasing working memory, improving attention and perceptual ability, and reducing the time of reaction<sup>16–21</sup>.

Several studies by different authors have shown that the latency and/or amplitude values of P300 in normal adults are reproducible and stable without statistically significant differences in retesting state at different time intervals<sup>22–25</sup>. That is in line with our finding that P300 latency is stable even one month after the last training, which might point to a plastic change in the brain’s electric activity that can last for a longer period of time. In assessing the short-term and long-term effects of beta EEG-NFB in healthy subjects, Engelbregt et al.<sup>26</sup> found that frontal beta activity increased after 15 sessions of NFB and that these effects remained stable for at least 3 years. Regarding the changes in EEG, patients showed reduced strength of SMR after treatment, while NFB aimed to increase this frequency range. The observed effects in the EEG were specific for the narrow SMR frequency range of 12–15 Hz and were not found in the alpha and beta frequency bands, suggesting that the effects were specific for the frequency range. This finding suggests that NFB SMR leads to control over cer-

tain EEG frequencies but does not structurally regulate this EEG activity<sup>27</sup>.

In our study, we found an opposite result – an increase in EEG SMR spectral power as a result of NFB SMR training. Gadea et al.<sup>28</sup> showed in their study that healthy women were able to improve SMR rhythm after one training session with NFB, which was positively associated with performance improvement in the Dichotic Listening test that measures executive attention. NFB SMR training effects have been reported through the increment of the SMR on improvements in auditory attention and phonological awareness<sup>29, 30</sup>.

All respondents in the study had a better performance on the QuickSIN test with a linear trend of increasing the achievement from the beginning even to the one month after the last NFB SMR training session. The brain circuit of SMR has a thalamic-cortical origin. It is a bottom-up mechanism that reduces the interference of somatosensory information<sup>11</sup>. This inhibition, as a result of an increase in SMR, may lead to better integration of information processing in the cerebral cortex. Hence, the NFB SMR training might act within the inhibitory mechanism of the thalamic circuitry<sup>17, 31</sup>.

This finding indicates the potential usefulness of NFB SMR training as an operative conditioning paradigm by the SMR protocol of neuromodulatory therapy in improving auditory cognition. In addition to our study, other studies found that the standard NFB SMR training protocol in healthy individuals might be an efficient method for improving attention and perceptible abilities and reducing reaction times and errors by commission<sup>16</sup>.

## Conclusion

The limitation of our study was the small sample size. Hence, the generalization of the obtained results should be made with caution. In addition, the effect of NFB SMR training on other attention modalities (visual, for instance) was not assessed. That might be interesting and important for future research. However, the achieved long-lasting enhancement of selective auditory attention using NFB SMR training in our study might be a promising field of research towards its application not only in healthy individuals but also in neurotherapy in children with specific developmental disorders that affect auditory attention (hearing impairment, attention deficits, language disorders, etc.) as well as adults with neurological and/or cognitive impairments.

## Conflict of interest

The authors declare no conflict of interest.

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## Insulin resistance and serum metabolomics improvement in subjects with impaired glucose tolerance treated with *Hibiscus esculentus* L.

Smanjenje insulinske rezistencije i poboljšanje metabolomičkih osobina seruma kod osoba sa smanjenom tolerancijom na glukozu, tretiranih biljkom *Hibiscus esculentus* L.

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### Abstract

**Background/Aim.** Prediabetes (PD) refers to the condition in which the blood sugar level is higher than normal but has not reached the diagnostic criteria for diabetes mellitus (DM) yet. Impaired glucose tolerance (IGT) is a major prediabetic symptom since most patients with type 2 DM have progressed from the previous PD phase. The aim of the study was to observe the changes in serum metabolomics in patients with IGT treated with *Hibiscus esculentus* L. (*H. esculentus*) combined with the change of lifestyle. **Methods.** Sixty patients with IGT were divided into two groups. In one group, the subjects made a lifestyle change (LC group, simple diet control), and the other group of subjects made a lifestyle change combined with *H. esculentus* (LCH group) treatment with daily consumption of 20 g of dried *H. esculentus* fruit tea. The aim was to compare the blood glucose, homeostasis model assessment-estimated insulin resistance (HOMA-IR) index, and serum metabolomics after a 60-day clinical observation period. **Results.** There was no statistical significance in the glucose level between the two groups by the end of the observation period. The HOMA-IR index in the

LCH group was lower than in the LC group ( $1.7 \pm 1.1$  vs.  $2.4 \pm 1.2$ ,  $p = 0.030$ ). Serum metabolomics revealed that the levels of d-galactose, d-glucose, turanose, and uric acid in the LCH group were significantly lower than those in the LC group ( $16.7 \pm 3.9$  mmol/L vs.  $21.2 \pm 2.9$  mmol/L,  $101.5 \pm 40.2$  mmol/L vs.  $132.9 \pm 36.7$  mmol/L,  $1.8 \pm 1.6$  mmol/L vs.  $3.76 \pm 2.46$  mmol/L,  $44.56 \pm 15.7$   $\mu$ mol/L vs.  $67.8 \pm 23.5$   $\mu$ mol/L, respectively). The levels of lactic acid and conjugated linoleic acid in the LCH group were significantly higher than those in the LC group ( $3.3 \pm 0.5$  mmol/L vs.  $2.3 \pm 0.8$  mmol/L,  $6.9 \pm 6.1$  mmol/L vs.  $2.1 \pm 1.2$  mmol/L, respectively). **Conclusion.** *H. esculentus*, combined with a change of lifestyle, can reduce insulin resistance and the levels of multiple monosaccharides and blood uric acid in IGT patients. Regulation of the metabolism of lactic acid and conjugated linoleic acid may be the potential mechanism of how *H. esculentus* reduces insulin resistance.

**Key words:**  
glucose intolerance; insulin resistance; life style;  
metabolomics; plants, medicinal; prediabetic state;  
serum.

### Apstrakt

**Uvod/Cilj.** Predijabetes je stanje u kome je nivo šećera u krvi viši od normalnog, ali još nije dostigao dijagnostičke kriterijume za dijabetes melitus (DM). Smanjena tolerancija na glukozu (STG) je glavni predijabetesni simptom a kod većine obolelih od DM tipa 2 bolest se razvila kao posledica progresije predijabetesa. Cilj rada bio je da se odrede promene metabolomičkih osobina seruma kod osoba sa STG, koje su konzumirale čaj od biljke *Hibiscus esculentus* L. (*H. esculentus*), u kombinaciji sa promenom načina života. **Metode.** Ukupno 60 ispitanika sa STG podeljeno je na dve grupe. Jedna grupa ispitanika promenila je način života (jednostavna kontrola ishrane) – KI grupa, a u drugoj grupi bili su ispitanici koji su, pored toga što su promenili način života, konzumirali i čaj od

biljke *H. esculentus* (na dnevnom nivou 20 grama sušenog voćnog čaja) – KIH grupa. Cilj je bio da se uporede vrednosti glukoze u krvi, insulina, indeksa rezistencije na insulin (*homeostasis model assessment of insulin resistance* – HOMA-IR) i metabolomičkih osobina seruma između te dve grupe ispitanika nakon 60 dana kliničke opservacije. **Rezultati.** Nije bilo statistički značajne razlike u nivou glukoze u krvi između dve grupe po završetku 60-dnevnog perioda opservacije. Indeks HOMA-IR u grupi KIH bio je niži nego u KI grupi ( $1,7 \pm 1,1$  vs.  $2,4 \pm 1,2$ ,  $p = 0,030$ ). Rezultati ispitivanja metabolomičkih osobina seruma otkrili su da su nivoi d-galaktoze, d-glukoze, turanoze i mokraćne kiseline u KIH grupi bili značajno niži u poređenju sa KI grupom ( $16,7 \pm 3,9$  mmol/L vs.  $21,2 \pm 2,9$  mmol/L,  $101,5 \pm 40,2$  mmol/L vs.  $132,9 \pm 36,7$  mmol/L,  $1,8 \pm 1,6$  mmol/L vs.  $3,76 \pm 2,46$

mmol/L,  $44,56 \pm 15,7 \mu\text{mol/L}$  vs.  $67,8 \pm 23,5 \mu\text{mol/L}$ , redom). Nivoi mlečne kiseline i konjugovane linoleične kiseline u KIH grupi bili su značajno viši nego kod ispitanika iz KI grupe ( $3,3 \pm 0,5 \text{ mmol/L}$  vs.  $2,3 \pm 0,8 \text{ mmol/L}$ ,  $6,9 \pm 6,1 \text{ mmol/L}$  vs.  $2,1 \pm 1,2 \text{ mmol/L}$ , redom). **Zaključak.** Unos biljke *H. esculentus* u kombinaciji sa promenama načina života može umanjiti insulinsku rezistenciju i smanjiti nivo većeg broja monosaharida i mokraćne kiseline u krvi osoba sa

STG. Regulisanje metabolizma mlečne kiseline i konjugovane linoleične kiseline može biti potencijalni mehanizam koji objašnjava kako *H. esculentus* smanjuje insulinsku rezistenciju.

#### Ključne reči:

**glukoza, netolerancija; insulin, rezistencija; način života; metabolomika; biljke, lekovite; predijabetesno stanje; serum.**

## Introduction

The analysis report of the global diabetes mellitus (DM) prevalence survey by The International Diabetes Federation (IDF) showed that the number of global diabetic patients reached 415 million in 2015, and by 2040, the global number of diabetic adults is expected to reach 629 million<sup>1</sup>. Prediabetes (PD) refers to the state in which the blood sugar level is higher than normal but has not reached the diagnostic criteria for DM yet. Most patients with type 2 DM (T2DM) progress from the PD condition. Impaired glucose tolerance (IGT) is a major prediabetic symptom, and IDF reported that the global prevalence of IGT in 2017 reached 374 million<sup>1</sup>; in 2013, the survey in China also showed that the prevalence of IGT was as high as 15.5%<sup>2</sup>. Surveys have shown that 70% of people with PD can progress to T2DM<sup>3</sup>. Therefore, how to prevent or delay the progress of PD into DM is the focus of DM prevention. Appropriate lifestyle interventions can delay the progression of PD to D<sup>4-6</sup>. Diet regulation is an important way of changing lifestyle in the population with PD<sup>7</sup>. *Hibiscus esculentus* L. (*H. esculentus*) is a herbaceous plant also known as *Abelmoschus esculentus* L. of the Malvaceae family. Recent studies have shown that *H. esculentus* can help lower blood sugar and blood lipids and may become an early dietary choice for DM<sup>8</sup>. It has also been found that *H. esculentus* and its extracts can regulate dipeptidyl peptidase-4 (DPP-4), protect the islet  $\beta$  cells, and improve insulin sensitivity<sup>9,10</sup>. However, there are few clinical reports on *H. esculentus* regulating glucose metabolism in IGT patients, and the mechanism of *H. esculentus* regulating blood glucose still needs to be studied and clarified. Through comprehensive, real-time, and systematic contour analysis of metabolites, metabolomics research has made significant breakthroughs in recent years in screening DM biomarkers, analyzing metabolic pathways, and studying molecular pathways for pharmacological effects<sup>11</sup>. In this study, high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry analysis were applied to compare the changes in the glucose metabolism and serum metabolomics of IGT patients after simple lifestyle control or combined with *H. esculentus* tea consumption, aiming to explore the possible regulation pathways of blood glucose metabolism by *H. esculentus*.

## Methods

### General information

Inclusion criteria: a total of 60 IGT patients that were admitted to our hospital from May to November 2019 and

met the inclusion criteria were enrolled and divided into the LC group (subjects made a lifestyle change) and LCH group (subjects made a lifestyle change combined with *H. esculentus*) according to the random number expression method, with 30 cases in each group; the diagnosis was in compliance with the 1999 glucose tolerance abnormality diagnostic criteria (fasting blood glucose  $< 7 \text{ mmol/L}$ , blood glucose at 2-hr glucose load  $\geq 7.8 \text{ mmol/L}$ ,  $< 11.1 \text{ mmol/L}$ ) by World Health Organization (WHO)<sup>12</sup>; the patients were all  $< 75$  years old, including 32 males and 28 females; disease duration 0–12 months.

Exclusion criteria: patients with confirmed DM [type 1 DM (T1DM), T2DM, or special types of DM)], severe cardiac insufficiency (New York Heart Association cardiac function class III or higher), myocardial infarction, chronic liver disease or severe liver dysfunction (glutamyl aminotransferase  $\geq 3$  times of the normal upper limit), malignant tumors, tuberculosis, recent administration of nephrotoxic drugs, or cognitive abnormalities and estimated glomerular filtration rate (eGFR)  $< 60 \text{ mL/min}$ . Estimated GFR complies with the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine equation:

The equation for females –  $144 \times [\text{serum creatinine (sCR) / 0.7}]^{-0.329} \times 0.993^{\text{age}}$  (if sCR  $\leq 0.7 \text{ mg/dL}$ ) or  $144 \times (\text{sCR} / 0.7)^{-1.209} \times 0.993^{\text{age}}$  (if sCR  $> 0.7 \text{ mg/dL}$ ).

The equation for males –  $141 \times (\text{sCR} / 0.9)^{-0.411} \times 0.993^{\text{age}}$  (if sCR  $\leq 0.7 \text{ mg/dL}$ ) or  $141 \times (\text{sCR} / 0.9)^{-1.209} \times 0.993^{\text{age}}$  (if sCR  $> 0.7 \text{ mg/dL}$ ).

This study was conducted in accordance with the declaration of Helsinki, following the approval from the Ethics Committee of the Shanghai University of Traditional Chinese Medicine (Ethical Committee Approval number PTEC-R-2018-53-1, from October 25, 2018). Written informed consent was obtained from all participants.

There was no statistically significant difference in the basic information, such as age, gender, and course of the disease, between the two groups (Table 1,  $p > 0.05$ ).

The following steps were used for preparing fruit tea of dried *H. esculentus*: fresh *H. esculentus* (cultivated and harvested by Nanjing Youhewo Agricultural Co., Ltd.) was removed from the pedicel, entirely cleaned, and then the middle part was cut with a diameter of 15–20 mm into 5-mm thin slices with sterilized stainless steel knives, followed by 240 min of 60 °C air drying with the speed of 1 m/s (the vacuum drying oven: Type zKn4025, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.). The quality inspection was performed by the Comprehen-

**Table 1****Comparison of clinical data between groups**

Parameter	LC	LCH	F/ $\chi^2$	p-value
Age (years)	41.4 ± 5.9	40.8 ± 5.4	0.331	0.668
Male/female (n)	14/16	18/12	1.071	0.301
Course of disease (months)	7.3 ± 1.9	7.3 ± 1.6	2.4	0.943
Fasting blood glucose (mmol/L)	5.5 ± 1.0	5.6 ± 0.9	0.226	0.877
2-hr postprandial blood glucose (mmol/L)	8.9 ± 1.4	8.61.5	1.092	0.293
Glycated hemoglobin (%)	6.9 ± 1.8	6.9 ± 1.8	0.082	0.987
Fasting insulin (mmol/L)	10.4 ± 6.0	10.5 ± 5.8	0.032	0.962
2-hr postprandial insulin (mmol/L)	64.5 ± 22.3	65.0 ± 23.2	0.022	0.626
ALT (U/L)	24.3 ± 10.9	24.470 ± 10.2	0.153	0.961
Creatinine (μmol/L)	63.8 ± 18.4	64.5 ± 17.9	0.075	0.876

**ALT – alanine transaminase; LC – lifestyle change group; LCH – lifestyle change combined with *H. esculentus* treatment group; Results are given as mean ± standard deviation; F/ $\chi^2$  test is performed between the LC and LCH group.**

sive Testing and Inspection Center of Gaochun Dist., Nanjing, which obtained the following results: no metal or non-metallic foreign matter remaining, moisture content 3.4%, and lead/hexachlorocyclohexane/dichlorodiphenyltrichloroethane detection. Twenty g of the material was then sealed in polyethylene bags sterilized with an ozone concentration of  $\geq 20 \text{ mg} \times \text{m}^{-3}$  for 30 min<sup>13</sup>.

#### Groups and treatments

After 2 weeks of diet and exercise education for all the study subjects to cultivate their self-monitoring blood glucose habits to prevent the occurrence of hypoglycemia events or other adverse events, the study subjects started a 60-day clinical observation. The LC group continued changing their lifestyle, while the LCH group was given 20 grams of *H. esculentus* dried fruit tea daily (divided into 3 servings, brewed with 200 mL of warm water, to be consumed after breakfast, lunch, and dinner with chewed pulp) in addition to the lifestyle change. During that period, the study subjects were required to self-monitor the finger-terminal blood glucose before meals and at 9 pm twice a week.

#### Observation indices

After 60 days of observation, the serum fasting and 2-hr postprandial glucose, insulin, glycated hemoglobin, and lipids were compared between the two groups. Homeostasis model assessment-estimated insulin resistance (HOMA-IR) index (fasting blood glucose  $\times$  fasting insulin level/22.5) was calculated and compared, together with comparing the differences in the serum metabolomics between the two groups.

#### Specimen collection and processing

All the study subjects were fasting for more than 10 hrs before the blood sampling from the medial cubital vein in the morning. Then, 1.5 mL of the blood was collected into one coagulation tube with inert separation gel, one ethylenediamine tetraacetic acid-anticoagulation tube, and one heparin-anticoagulation tube, respectively. After standing for 30 min, the blood was centrifuged (3,500 rpm/15 min), and the super-

natant serum and plasma were collected and stored frozen at -70 °C. The enzymatic method was used to detect the blood glucose concentration, alanine transaminase (ALT), and sCR (Beckman Coulter Au Chemistry Systems, Suzhou, China); HPLC was used to detect the glycated hemoglobin (Arkray, Koka-shi, Japan); direct chemiluminescence was used to detect the serum insulin level (Simens Healthineers, Wuxi, China). Processing of metabolomics specimens: 400 μL of ice-cold methanol solution and 10 μL of internal standard (0.3 mg/mL, 2-chlorophenylalanine and undecanoic acid) were added to the 100 μL of the serum and mixed thoroughly, followed by 15 min centrifugation at 12,000 rpm at 4 °C. Then, 200 μL of concentrated and dried supernatant was transferred into an injection vial; 30 μL of methoxyppyridine solution (20 mg/mL) was added, shaken vigorously, and incubated at 37 °C for 1.5 hrs. Next, 30 μL of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), containing 1% chlorotrimethylsilane (TMCS) was added, incubated at 70 °C for 1 h, and then put to sit at room temperature. Reagents such as methanol were purchased from Sinopharm Group Co Ltd, Shanghai, China<sup>14,15</sup>.

#### Analysis of gas chromatography-mass spectrometry

Spectrometry was performed on a gas chromatography-mass spectrometer (Agilent, 7890A-7000, Palo Alto, California, US) and a vacuum concentrator (Labogene, MaxiVac Alpha, Copenhagen, Denmark). Chromatographic conditions were column HP-5 (30 m  $\times$  0.25 mm  $\times$  0.25 μm) and helium as the carrier gas. The heating processes were as follows: initial 80 °C for 2 min, increasing to 320 °C with the increasing speed of 10 °C/min, maintaining for 6 min. The inlet temperature was 280 °C. Mass spectrometry conditions were ionization mode EI, ion source temperature 230 °C, and temperature of the ion transmission line 150 °C. Scan mode was full scan, 50 ~ 600 m/z (mass-to-charge ratio).

#### Processing of metabolomics data

The experimental data were input into various (X) forms of chromatography-mass spectrometry (XCMS) data analysis platforms for extraction, peak alignment, retention time correction, etc., to finally obtain the original data. The

area of the original data was then normalized using Excel. The normalized data were then input into SIMCA-P14 software for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) for observing the natural distribution between samples and visually observing the possible existence of differences. The differential metabolites between the two groups were searched by the OPLS-DA method, and the variable importance in the projection (VIP) value (threshold value > 1) of the OPLS model was combined with the  $p$ -value (threshold value = 0.05) of the  $t$ -test to find the most differentially expressed metabolites. The identification of such metabolites used the National Institute of Standards and Technology (NIST) database.

#### Statistical analysis

SPSS 21.0 statistical software was used for processing. The measurement data were expressed as mean  $\pm$  standard deviation (SD). The changes in each observation index before and after treatment were tested by the  $t$ -test. All the statistical results were tested by the two-sided test, with  $p < 0.05$  being considered statistically significant.

## Results

#### Observation indices

In the LCH group, the fasting insulin level after treatment was  $7.4 \pm 4.2$  mmol/L, which decreased significantly compared with that in the LC group ( $9.8 \pm 4.4$  mmol/L,  $p = 0.035$ ). There were no significant differences in the fasting blood glucose, 2-hr postprandial blood glucose, blood lipids, and glycated hemoglobin between the two groups. In the LCH group, the HOMA-IR index after treatment was  $1.7 \pm 1.1$ , lower than that in the LC group ( $2.4 \pm 1.2$ ,  $p = 0.030$ ) (Table 2).

#### Analysis of gas chromatography-mass spectrometry metabolomics

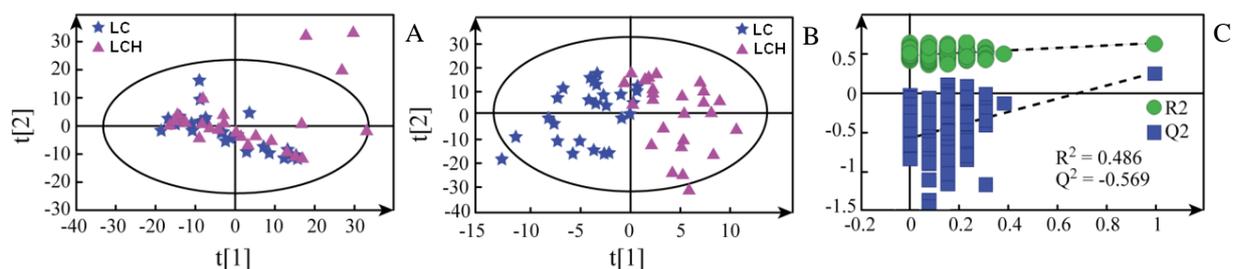
PCA analysis obtained the spatial distribution of serum in the two groups (Figure 1A). The samples in the LC and LCH groups did not show significant separation, and most of the serum samples in the two groups were distributed within the 95% confidence interval. To further verify the separation

**Table 2**

**Changes in serum metabolomics in the two groups after the 60-day clinical observation period**

Parameter	LC		LCH		$p$ -value	
	before	after	before	after	before	after
Fasting blood glucose (mmol/L)	$5.6 \pm 1.0$	$5.1 \pm 0.7$	$5.6 \pm 0.9$	$5.4 \pm 0.8$	0.877	0.125
2-h postprandial blood glucose (mmol/L)	$8.9 \pm 1.4$	$8.7 \pm 1.2$	$8.6 \pm 1.5$	$8.4 \pm 1.3$	0.293	0.261
Glycated hemoglobin (%)	$6.9 \pm 1.8$	$6.6 \pm 1.4$	$6.9 \pm 1.8$	$6.6 \pm 1.4$	0.987	0.989
Fasting insulin (mmol/L)	$10.5 \pm 6.0$	$7.4 \pm 4.2$	$10.5 \pm 5.8$	$9.8 \pm 4.4$	0.962	0.035*
2-hr postprandial insulin (mmol/L)	$64.4 \pm 22.3$	$58.7 \pm 17.6$	$65.0 \pm 23.2$	$60.1 \pm 15.4$	0.926	0.738
HOMA-IR	$2.6 \pm 1.7$	$2.4 \pm 1.2$	$2.6 \pm 1.6$	$1.7 \pm 1.1^\Delta$	0.976	0.030*
TC (mmol/L)	$4.6 \pm 1.6$	$4.3 \pm 1.6$	$4.6 \pm 1.6$	$4.2 \pm 1.4$	0.939	0.836
TG (mmol/L)	$2.4 \pm 1.0$	$1.9 \pm 0.8$	$2.4 \pm 1.1$	$1.9 \pm 0.8$	0.907	0.861
LDL (mmol/L)	$3.1 \pm 1.1$	$2.3 \pm 1.0$	$3.0 \pm 1.1$	$2.2 \pm 1.0$	0.867	0.723
HDL (mmol/L)	$1.8 \pm 1.3$	$1.9 \pm 1.2$	$1.8 \pm 1.3$	$1.9 \pm 1.1$	0.935	0.995
ALT (U/L)	$24.3 \pm 10.9$	$24.3 \pm 12.7$	$24.5 \pm 10.2$	$23.1 \pm 11.0$	0.961	0.697
Creatinine ( $\mu$ mol/L)	$63.8 \pm 18.4$	$62.4 \pm 20.3$	$64.5 \pm 17.9$	$62.9 \pm 20.5$	0.876	0.930
Uric acid ( $\mu$ mol/L)	$201.1 \pm 105.9$	$194.3 \pm 104.4$	$198.6 \pm 103.3$	$191.6 \pm 102.2$	0.929	0.922

LC – lifestyle change group; LCH – lifestyle change combined with *H. esculentus* treatment group; HOMA-IR – homeostasis model assessment-estimated insulin resistance index; TC – total cholesterol; TG – triglycerides; LDL – low-density lipoprotein; HDL – high-density lipoprotein; ALT – alanine transaminase; Results are given as mean  $\pm$  standard deviation;  $^\Delta p < 0.05$  – comparison with the data before treatment; \* $p < 0.05$  – comparison with the LC group.



**Fig. 1 – A) Principal component analysis between groups; B) Orthogonal partial least squares discriminant analysis between groups; C) Replacement verification model.**  
LC – lifestyle change group; LCH – lifestyle change combined with *H. esculentus* treatment group

Table 3

Differential serum metabolomics between the two groups				
Metabolites	LC	LCH	VIP	<i>p</i> -value
d-galactose (mmol/L)	21.2 ± 2.9	16.7 ± 3.9	1.57	0.004
d-glucose (mmol/L)	132.9 ± 36.7	101.5 ± 40.2	1.03	0.003
Turanose (mmol/L)	3.7 ± 2.4	1.8 ± 1.6	1.07	0.002
Lactic acid (mmol/L)	2.3 ± 0.8	3.3 ± 0.53	1.15	0.006
Uric acid (μmol/L)	67.8 ± 23.5	44.5 ± 15.7	1.17	0.008
CLA (mmol/L)	2.1 ± 1.2	6.9 ± 6.1	1.08	0.0003

LC – lifestyle change group; LCH – lifestyle change combined with *H. esculentus* treatment group; VIP – variable importance in the projection; CLA – conjugated linoleic acid.

Results are given as mean ± standard deviation.

between the two groups, OPLS-DA analysis was performed on the two groups' sera (Figure 1B). The results showed that the samples of the two groups significantly separated, with interpretability  $R^2X = 0.895$ ,  $R^2Y = 0.738$ , and predictability  $Q^2 = 0.651$ , indicating that the fit and prediction ability of the OPLS-DA data model can better explain the differences between the two groups. The replacement test was used to verify the model, and after 200 times of replacement tests, the intercept was  $R^2 = 0.486$  and  $Q^2 = -0.569$  (Figure 1C), indicating that the model had good reliability.

The VIP value of differential metabolites can reflect the importance of each variable in establishing the model. In this study,  $VIP \geq 1$  and a two-sided *t*-test ( $p < 0.05$ ) were used to screen and identify the major differential metabolites in the two groups. The results showed that the plasma lactate level in the LCH group was  $3.3 \pm 0.5$  mmol/L, higher than that in the LC group ( $2.3 \pm 0.8$  mmol/L,  $p = 0.006$ ). In the LCH group, the levels of serum uric acid, plasma d-galactose, d-glucose, and turanose were  $44.5 \pm 15.7$  μmol/L,  $16.7 \pm 3.9$  mmol/L,  $101.5 \pm 40.2$  mmol/L,  $1.8 \pm 1.6$  mmol/L, respectively, all significantly lower than those in the LC group ( $67.8 \pm 23.5$  μmol/L,  $21.2 \pm 2.9$  mmol/L,  $132.9 \pm 36.7$  mmol/L,  $3.7 \pm 2.4$  mmol/L, respectively, with  $p = 0.008$ ,  $0.004$ ,  $0.003$ ,  $0.002$ , respectively). The plasma-conjugated linoleic acid (CLA) level in the LCH group was  $6.9 \pm 6.1$  mmol/L, which was significantly higher than that in the LC group ( $2.1 \pm 1.2$  mmol/L,  $p = 0.003$ ) (Table 3).

## Discussion

The purpose of this study was to compare whether *H. esculentus* combined with lifestyle change can improve glucose metabolism abnormalities in IGT patients more efficiently than just simple lifestyle changes and analyze possible metabolic pathways of such regulatory effects. The results showed that the fasting insulin level and HOMA-IR of patients in the LCH group were significantly lower than those in the LC group, suggesting that the patients had reduced insulin resistance. Insulin resistance is the basic pathological mechanism throughout the development of T2DM, which also causes other metabolic disorders, such as islet β-cell secretion defects, insulin resistance in peripheral tissues, increased liver glucose output, lipid metabolism disorders, weakened insulinotropic effect, and hyperglycemia. Insulin resistance also participates in the whole occurrence and de-

velopment process of T2DM and its complications. Because of that, early improvement of insulin resistance may help delay the onset and progression to T2DM<sup>16</sup>. Our results suggest that *H. esculentus* combined with lifestyle change can significantly improve insulin resistance in IGT patients and may help delay their transition to type T2DM. The hypoglycemic effect of *H. esculentus* has been observed in several animal studies. For instance, Khatun et al.<sup>17</sup> reported that *H. esculentus* extract can reduce blood glucose by reducing glucose intestinal absorption in rats. Previous studies reported that *H. esculentus* extract significantly reduced the fasting blood glucose and glycated hemoglobin, and improved the liver function damage in diabetic rats, the super-dose of which is not toxic to rats<sup>18</sup>. Certain *in vitro* tests have confirmed and explored the specific molecular mechanism of *H. esculentus* in improving insulin resistance. Other studies have confirmed that *H. esculentus* polysaccharides can improve insulin sensitivity in mice, and the effect may depend on the regulation of the liver X receptor and the expressions of peroxisome proliferators-activated receptor (PPAR) and its target genes<sup>19, 20</sup>. Our results show that *H. esculentus* combined with lifestyle significantly improves insulin resistance in IGT patients more than lifestyle management alone and validate previous scholars' conclusions that *H. esculentus* can improve insulin resistance at a clinical level. Since IGT patients had no significant changes in clinical DM when their plasma glucose changed, our study results did not observe significant reductions in the levels of glucose and glycated hemoglobin in IGT patients before and after treatment. However, *H. esculentus* can improve insulin resistance in patients, implying it assists in long-term glucose metabolism regulation and DM prevention in IGT patients. Thereby prolonging the observation period may provide more clinical evidence on the regulation of glucose metabolism by *H. esculentus*.

We analyzed the differential metabolites between the two groups through metabolomics to explore possible metabolic pathways involved in regulating glucose metabolism and insulin action. The results showed that the plasma d-galactose, d-glucose, and turanose were significantly lower in the LCH group than in the LC group, suggesting that monosaccharide molecules in the LCH group were significantly reduced. These monosaccharide molecules can be interchanged with glucose in peripheral blood, which is an important factor leading to elevated plasma glucose and insulin

resistance. Our results show that the monosaccharide molecular metabolites of the patients in the LCH group are significantly reduced, which provides theoretical support for our hypothesis of the clinical application of *H. esculentus* to regulate blood sugar and suggests that the monosaccharide metabolism pathway may be the main metabolic pathway involved in the improvement of insulin resistance by *H. esculentus*.

Our metabolomics analysis also showed that the blood lactic acid level in the LCH group was significantly higher than that in the LC group, suggesting that the lactic acid metabolic pathway may be involved in the metabolic mechanism of *H. esculentus* regulating insulin action. The role of circulating lactic acid in glucose metabolism is mainly manifested in regulating liver glucose output. Lactic acid is the final product of glycolysis and is also the main raw material of liver gluconeogenesis, which can increase liver glucose output and maintain fasting blood glucose stability. In the state of insulin resistance, the inhibitory effect of insulin on the activation pathway of liver gluconeogenesis key enzymes weakens, leading to the abnormal activation of the gluconeogenesis pathway in liver cells; therefore, the liver glucose output continues to be activated when the blood glucose increases, resulting in blood glucose elevation. The lactic acid in the liver is taken up by hepatocytes through the lactic acid cycle, causing lactic acid to participate in liver gluconeogenesis and blood sugar increase. The increase in the lactic acid level by the hypoglycemic agent metformin may be related to inhibiting the uptake and utilization of lactic acid by liver cells, thereby reducing the liver gluconeogenesis precursors and inhibiting the liver glucose output<sup>21, 22</sup>. Our study observed that the insulin resistance in the LCH group improved while the lactate level increased, suggesting that *H. esculentus* may be involved in regulating the lactic acid cycle and liver glucose output, thereby reducing insulin resistance. Nevertheless, the specific mechanism of *H. esculentus* regulating gluconeogenesis and lactic acid metabolism still needs further studies. There was no statistical significance in liver enzymes and serum creatinine levels between the two groups, suggesting that the increased lactic acid level in the LCH group did not cause liver or kidney function damage. However, the effect of *H. esculentus* on lactic acid metabolism needs to be observed for a longer period of time for further evaluation.

Our study found that the blood uric acid level in the LCH group was significantly lower than that in the LC group, suggesting that inhibition of uric acid overproduction may be involved in the improvement of insulin resistance metabolic pathways by *H. esculentus*. Recent studies have confirmed that elevated uric acid is directly involved in the development of lipid metabolism disorders and DM, as well as their complications<sup>23</sup>. Studies have found that prediabetic patients with hyperuricemia have higher insulin levels, suggesting that increased blood uric acid worsens insulin resistance and accelerates the progression of type T2DM<sup>24</sup>. In addition, persistent hyperuricemia can aggravate islet  $\beta$ -cell damage<sup>25</sup>. Zhu et al.<sup>26</sup> and other studies have found that hyperuricemia inhibits the activities of insulin signaling

pathway protein kinase B (PKB) and insulin receptor substrate (IRS) and increases insulin resistance through oxidative stress, thus leading to insulin resistance in mice. Our results support the findings of other scholars. However, the molecular mechanism of *H. esculentus*' regulation of the uric acid metabolic pathway needs further studies. We have used mass spectrometry to detect the main components of dried *H. esculentus* fruit tea, which showed polysaccharides and flavonoids, etc. We also used the water extraction method to extract the *H. esculentus* polysaccharides and applied them in high-fat diet-induced insulin-resistant mice, and found that they can reduce pyruvate-induced blood sugar increase and decrease the expression of the key enzymes of hepatic gluconeogenesis [phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphate (G6P)], suggesting that *H. esculentus* may have a regulatory effect on hepatic gluconeogenesis. The main components of *H. esculentus* are quercetin and isoquercetin. Our *in vitro* experiments have confirmed that they can reduce the expression of PEPCK and G6P in hepatocytes and activate adenosine 5-monophosphate-activated protein kinase (AMPK) and its phosphorylation simultaneously, which have similar effects to metformin. This study is a clinical observation of *H. esculentus* on glucose metabolism in the IGT population. The results suggest that *H. esculentus* can improve insulin resistance and increase blood lactate levels, which supports the results of *in vitro* studies and suggests that *H. esculentus* may be involved in regulating hepatic gluconeogenesis, thereby reducing hepatic glucose output and reducing blood sugar<sup>27</sup>.

Metabolomics analysis also showed that the CLA level in the LCH group was significantly higher than that in the LC group. CLA is an isomer of linoleic acid and is widely found in ruminant meat and dairy products<sup>28, 29</sup>. Recent studies have found that it has the effects of improving immunity, confronting cancer, atherosclerosis, and oxidation, reducing blood sugar and lipids, and improving insulin sensitivity<sup>30, 31</sup>. Its effect on glycolipid metabolism may be related to activating PPAR $\alpha$ , down-regulating CD36, and promoting acetyl-CoA carboxylase (ACC) phosphorylation<sup>32, 33</sup>. Studies have shown that CLA can act as a ligand of the nuclear transcription factor PPAR- $\gamma$  to increase the expression of adiponectin mRNA, play a role in glucose metabolism organs such as the liver and skeletal muscles, and improve insulin resistance<sup>34</sup>. Recent studies have confirmed that CLA promotes white adipocyte differentiation by activating PPAR- $\gamma$ , which increases insulin-sensitive small adipocytes, thereby increasing insulin sensitivity<sup>34</sup>. Our research shows that *H. esculentus* combined with lifestyle change increases the CLA level, which is consistent with previous results on CLA regulating glucose metabolism; therefore, this study can provide a clinical basis for *H. esculentus* in ameliorating insulin resistance and preventing the progression of IGT.

## Conclusion

This study found that *H. esculentus* combined with lifestyle change can improve insulin resistance in patients with glucose tolerance. Metabolomics analysis showed that the

contents of various monosaccharides such as galactose and serum uric acid decreased, while the levels of serum lactic acid and CLA increased in the LCH group, suggesting the involvement of the two elements in *H. esculentus*' improving insulin sensitivity in IGT patients. In addition, our findings imply *H. esculentus* regulating glucose metabolism may be related to inhibiting the liver glucose output by reducing liver lactic acid circulation. However, this study has a small number of study subjects and a short observation cycle. In the future, more prediabetic patients need to be included for long-term clinical observations to verify the mechanism of *H. esculentus* in improving early-stage abnormal glucose metabolism. In addition, metabolomics analysis only suggested the effects of *H. esculentus* on glucose metabolism-related products, more in-depth research is needed in the future to explore the molecular mechanism of *H. esculentus* in im-

proving early-stage metabolic abnormalities and insulin resistance so as to provide a more theoretical basis and research directions for *H. esculentus*' antidiabetic effects.

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### Conflicts of interest

The authors declare no conflict of interest.

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## Dental and skeletal changes occurring after orthodontic-surgical treatment of mandibular prognathism

Dentalne i skeletne promene nastale posle ortodontsko-hirurškog lečenja mandibularnog prognatizma

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### Abstract

**Background/Aim.** Mandibular prognathism (MPG) is a severe form of facial and jaw deformity. This kind of anomaly usually requires combined orthodontic-surgical treatment. The aim of this study was to determine the changes in the craniofacial complex after orthodontic-surgical treatment of patients with MPG by analyzing the cephalometric parameters on teleradiography images before and after treatment. **Methods.** The study included a sample of 40 subjects (mean age  $24.1 \pm 4.1$  years) who underwent orthodontic-surgical treatment of MPG. Vertical and sagittal parameters which characterize MPG were measured on profile teleradiography images before and 12 months after treatment. Based on the analysis of cephalometric parameters on preoperative and postoperative teleradiography images, dental and skeletal changes that occurred after treatment were determined. **Results.** After the end of the treat-

ment, most of the parameter values that characterize MPG were significantly reduced. A drop in values was noted in the following parameters: SNB, SNPg, NS/SpP, NS/MP, SpP/MP, NSAr, ArGoMe, Bjork polygon, NMe, NSna, SnaMe, SSnp, I/SpP. There was a statistically significant increase in the values of the following parameters SNA, ANB, GoArNS, SGo and i/MP. No significant changes in values were recorded on the OP/NS and SARGo parameters. **Conclusion.** Orthodontic-surgical treatment of MPG leads to changes in the bone and dental structures of the craniofacial system. As a result of such treatment, there is a functional improvement and an improvement in the appearance of the face.

**Key words:** cephalometry; malocclusion, angle class III; mandible; orthognathic surgical procedures; prognathism; treatment outcome.

### Apstrakt

**Uvod/Cilj.** Mandibularni prognatizam (MPG) predstavlja težak oblik deformiteta lica i vilice. Ova vrsta anomalije najčešće zahteva kombinovano ortodontsko-hirurško lečenje. Cilj ove studije bio je da se utvrde promene na kraniofacijalnom kompleksu nakon ortodontsko-hirurškog lečenja osoba sa MPG analizom kefalometrijskih parametara na teleradiografskim snimcima pre i posle lečenja. **Metode.** U istraživanje je uključeno 40 osoba (prosečne starosti  $24,1 \pm 4,1$  godine) kod kojih je sprovedeno ortodontsko-hirurško lečenje MPG. Vertikalni i sagitalni parametri koji karakterišu MPG mereni su na profilnim teleradiografskim snimcima pre lečenja i 12 meseci posle lečenja. Na osnovu analize kefalometrijskih parametara na preoperativnim i postoperativnim teleradiografskim snimcima utvrđene su dentalne i skeletne promene nastale nakon lečenja. **Rezultati.** Nakon

završenog lečenja značajno su smanjene vrednosti većine parametara koje odlikuju MPG. Zabeležen je pad vrednosti sledećih parametara: SNB, SNPg, NS/SpP, NS/MP, SpP/MP, NSAr, ArGoMe, Bjork polygon, NMe, NSna, SnaMe, SSnp, I/SpP. Došlo je do statistički značajnog povećanja vrednosti sledećih parametara SNA, ANB, GoArNS, SGo, i/MP. Nisu zabeležene statistički značajne promene vrednosti parametara OP/NS i SARGo. **Zaključak.** Ortodontsko-hirurško lečenje MPG dovodi do promena na koštanim i dentalnim strukturama kraniofacijalnog sistema. Kao rezultat takvog lečenja dolazi do funkcionalnog poboljšanja i do poboljšanja izgleda lica.

**Ključne reči:** kefalometrija; malokluzija, klase III; mandibula; hirurgija, ortognatska, procedure; prognatizam; lečenje, ishod.

## Introduction

Mandibular prognathism (MPG) is a severe form of facial and jaw deformity, a hereditary developmental type dominated by disturbed sagittal and vertical intermaxillary relations caused by excessive growth and development of the lower jaw in relation to the upper jaw and cranial base<sup>1</sup>.

MPG, one of the most complex and unattractive orthodontic irregularities, is manifested by inadequate size, shape, and position of the lower and often the upper jaw. During growth and development, there is a growing discrepancy in the size of the upper and lower jaw. This deformity often manifests only at puberty and reaches full expression with the end of growth<sup>1</sup>.

The frequency of MPG varies depending on the observed population. The highest incidence was observed in the Asian population (about 15%) and the lowest in the Caucasian population (1%)<sup>2</sup>. Recent research has shown an occurrence range of 2% to 6% for the European population, while the most common occurrence of MPG is in the Chinese population<sup>3</sup>.

Patients with MPG have a specific facial appearance, occlusal relationships, and a specific craniofacial skeletal structure. Specific characteristics of MPG are the increased total anterior facial height, especially the anterior lower facial height, decreased dimensions of posterior lower facial height and length of the posterior cranial base, increased values of angles defining the anteroposterior and vertical ratio of upper and lower jaw to the anterior cranial base [lower jaw prognathism angle (SNB), gonial angle (ArGoMe), Bjork polygon, mandibular plane (MP) angle relative to the anterior cranial base (NS/MP), ratio of the upper and lower jaw in the vertical plane (SpP/MP)], and negative values of the inter-jaws angle (ANB). MPG is accompanied by specific occlusion: compensatory protrusion of the upper, pronounced retrusion of the lower incisors, large interincisal angle, the large angle between the occlusal and MP, negative incisal step, and often present frontal open bite.

Due to the changed anatomical relationships in such patients, all oral functions and facial appearance are significantly impaired, endangering the psychosocial status of the patient, which has a negative impact on the quality of life.

Treatment of MPG is possible by growth modification during growth and development, orthodontic treatment (dent-alveolar compensation – camouflage), and orthodontic-surgical treatment<sup>4</sup>.

The largest number of patients with MPG achieve satisfactory functional and aesthetic results with combined orthodontic-surgical treatment. Surgery is performed after the facial and jaw bones have completed the growth and development phase in order to prevent the negative impact of post-treatment growth on the achieved results<sup>1</sup>.

Before the operation, the patient goes through the phase of pre-surgical orthodontic preparation to correct the irregularities of both dental arches, which makes it easier for the surgeon to perform a stable reposition of the bone segments. Orthognathic surgical treatment involves surgical repositioning of the upper and lower jaws, which achieves the correct

ratio of bone structures to the base of the skull<sup>5</sup>. The combination of bilateral sagittal split osteotomy and Le Fort osteotomy are the most common surgical procedures used to treat MPG<sup>6</sup>. Surgery on both jaws in the treatment of MPG achieves many advantages, such as better functional results, significantly reduced recurrence rate, significant harmonization of facial dimensions, balanced facial proportions in the sagittal and vertical directions, and greater stability of post-operative results<sup>7, 8</sup>. The surgery is followed by a phase of post-surgical orthodontic treatment, which performs the final movements of the teeth and thus completes the entire therapy<sup>5</sup>.

Numerous X-ray cephalometric parameters can confirm MPG. MPG is characterized by negative values of ANB and increased values of angles that determine the anteroposterior and vertical relationship of both jaws to the anterior cranial base: SNB, ArGoMe, Bjork polygon, NS/MP, face profil angle (NAPg).

Analyses of cephalometric parameters on profile telero-diography images of the head are necessary for the diagnosis of MPG, in the planning of appropriate orthodontic and surgical treatment, as well as in the assessment and evaluation of treatment outcomes.

After orthodontic-surgical treatment of MPG, skeletal and soft tissue changes of the craniofacial complex occur<sup>6, 9–11</sup>.

Orthodontic-surgical treatment of MPG leads to the establishment of craniofacial balance, changes in facial appearance, improvement of oral functions, and patient's quality of life.

The aim of this study was to determine the changes in the craniofacial complex after orthodontic-surgical treatment of patients with MPG by analyzing the cephalometric parameters on telero-diography images before and after treatment.

## Methods

The patients were treated at the Department of Orthopedics of the Jaws, the Dental Clinic, and the Clinic for Maxillofacial Surgery of the Military Medical Academy in Belgrade, Serbia. All activities and procedures applied in this study were approved by the Ethics Committee of the Military Medical Academy in Belgrade (from 25 December, 2018) and informed consent was obtained from the patients.

A total of 40 patients, 19 (47.5%) males and 21 (52.5%) females, participated in this study. The age of the patients ranged from 19 to 34 years, and the mean value  $\pm$  standard deviation (SD) was  $24.1 \pm 4.1$  years. All patients were diagnosed with MPG and underwent orthodontic-surgical treatment of this deformity.

The patients in the study were divided into two groups. The first group included patients who underwent surgery on one jaw (monomaxillary group), and the second group consisted of those who underwent surgery on both jaws (bimaxillary group). Out of the total number of patients, 16 (40%) were in the monomaxillary group, while 24 (60%) were in the bimaxillary group.

All patients underwent preoperative orthodontic treatment with the same protocol to achieve adequate and stable postoperative occlusion. After the orthodontic preparation, surgical repositioning of the jaws (mono or bimaxillary type) with rigid fixation was performed. In the area of the lower jaw, a standard sagittal step osteotomy was performed, while in the area of the upper jaw, a Le Fort osteotomy of the middle face was performed. The research included the analysis of cephalometric parameters on telerradiography images before and after orthodontic-surgical treatment.

The following patients were excluded from the study: patients with cleft lip and palate and all other craniofacial deformities; patients with a history of facial trauma or some orthognathic surgery; patients with temporomandibular joint diseases; patients with facial asymmetries, etc.

Angular and linear parameters describing dental and skeletal changes were measured on telerradiography images of 40 patients with MPG before orthodontic preparation (T1) and twelve months after surgical treatment of MPG (T2). Figure 1 shows the cephalometric points and planes used in the analysis of telerradiography images.

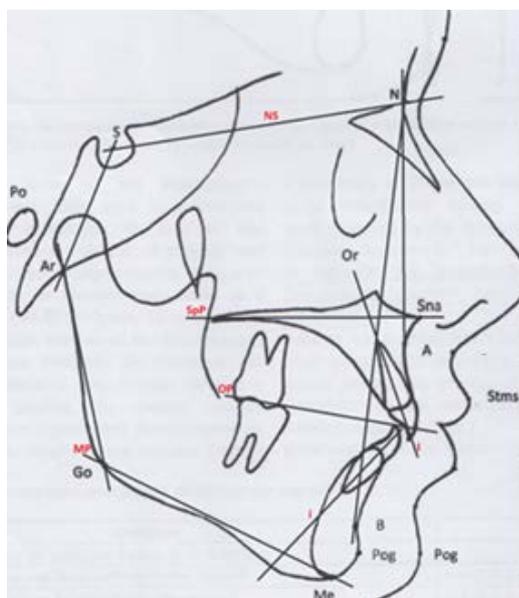
#### *Analysis of the facial skeleton in the sagittal direction*

Parameters of the facial skeleton in the sagittal direction include the following: upper jaw prognathism angle (SNA), SNB, ANB, lower jaw prognathism angle/facial angle (SNP<sub>g</sub>), and inclination of the ramus to the cranial base angle (GoArNS).

The first group of parameters in this study includes the analysis of the facial skeleton in the sagittal direction. These are angular measurements (unit of measure is degree) of bone structures that indicate the size and manner of movement of the lower and upper jaws during surgery.

#### *Analysis of the facial skeleton in the vertical direction*

Parameters of the facial skeleton in the vertical direction include the following: palatal plane angle relative to the anterior cranial base (NS/SpP), NS/MP, SpP/MP, occlusal plane angle relative to the anterior cranial base (OP/NS), saddle angle (NSAr), articular angle (SArGo), ArGoMe (for all angular measurements the unit of measurement is degree),



**Fig. 1 – Cephalometric parameters.**

**N** – nasion (the most anterior point on the frontonasal suture); **S** – sella (the center of the *sella turcica*); **A** – subspinale point (the deepest midline point between the anterior nasal spine and prosthion); **B** – supramentale point (the deepest point in the bony outline between the infradentale and the pogonion); **Me** – menton (the lowest point on the bony outline of the mandibular symphysis); **Or** – orbitale (the lowest point on the infraorbital margin); **Go** – gonion (the most lateral external point at the junction of the horizontal and ascending rami of the mandible); **Po** – porion (the uppermost point of the bony external auditory meatus); **Pog** – pogonion (the most anterior point of the bony chin); **Stms** – most anterior contour point of upper lip; **OP** – occlusal plane (the denture plane bisecting the posterior occlusion of the permanent molars and premolars and extending anteriorly bisecting the overbite); **NS** – nasion-sella plane (the anterior cranial base formed by projecting a plane from the sella-nasion line); **SpP** – palatal plane (formed by connecting anterior nasal spine to posterior nasal spine, used to measure the vertical tilt of maxilla); **Sna** – anterior nasal spine (the most prominent point of the upper jaw); **Ar** – articulare (a point at the junction of the posterior border of the ramus and the inferior border of the posterior cranial base – occipital bone); **MP** – mandibular plane (the tangent drawn to the inferior border of the mandible); **I** – inclination of the upper incisors

total front face height (NMe), front upper face height (NSna), front lower face height (SnaMe), total rear face height (SGo), and rear upper face height (SSnp) (for all linear measurements, the unit of measurement is millimeter).

#### *Incisor position analysis*

Incisor position analysis assessed the inclination of the upper incisors relative to the palatal plane (I/SpP) and inclination of the lower incisors relative to the MP (i/MP) parameters (unit of measure is degree).

#### *Statistical analysis*

The Kolmogorov-Smirnov test was used to examine the layout of a statistical series.

Pearson's Chi-squared test ( $\chi^2$ ) was used to test the relationship between the two qualitative variables. Differences in numerical variables were examined using One-Factor Analysis of Variance (ANOVA) and/or t-test for large independent samples.

To examine the relationship between the two continuous variables, Pearson's correlation coefficient was used as a parametric test, and Spearman's correlation coefficient as a

nonparametric substitution. Differences in the values of numerical variables measured in several time intervals were tested by the Repeated measure ANOVA test.

Statistical significance was defined at the level of probability of the null hypothesis of  $p < 0.05$ . Statistical processing and analysis were done in the computer program SPSS V24 (Statistical Package for the Social Sciences), and graphical and tabular presentations in the software package Microsoft Office (Excel and Word).

## Results

The analysis of cephalometric parameters on teleradiography images before and 12 months after the end of treatment revealed the changes in the measured parameters (Table 1). After treatment, statistically significantly higher values were recorded on the parameters SNA, ANB, GoArNS, SGo, SSnp, and i/MP. While the parameters SNB, SNPg, NS/SpP, NS/MP, SpP/MP, NSAr, ArGoMe, Bjork polygon, NMe, NSna, SnaMe, and I/SpP showed statistically significantly lower values after treatment. The value of OP/NS and SArGo parameters did not change significantly 12 months after orthodontic-surgical treatment of MPG.

**Table 1**

**Differences in measured parameters in all patients before (T1) and 12 months after (T2) orthodontic-surgical treatment of mandibular prognathism**

	Parameters	T1	T2	<i>p</i>
Sagittal direction	SNA	79.4 ± 5.0	81.2 ± 4.5	<b>0.012</b>
	SNB	8.9 ± 4.8	81.5 ± 4.2	<b>0.000</b>
	ANB	-5.6 ± 2.6	-0.2 ± 2.3	<b>0.000</b>
	SNPg	87.2 ± 4.1	84.3 ± 3.6	<b>0.000</b>
	GoArNS	82.2 ± 5.1	83.9 ± 7.7	0.159
	NS/SpP	8.0 ± 2.5	6.8 ± 2.4	<b>0.036</b>
	NS/MP	33.9 ± 4.8	31.1 ± 4.6	<b>0.002</b>
	SpP/MP	28.8 ± 4.3	25.4 ± 3.8	<b>0.000</b>
Vertical direction	OP/NS	14.69 ± 4.39	13.5 ± 4.02	0.114
	NSAr	121.6 ± 3.9	121.2 ± 3.9	0.383
	SArGo	141.1 ± 3.0	140.9 ± 3.0	0.779
	ArGoMe	133.9 ± 4.9	130.8 ± 4.9	<b>0.015</b>
	Bjork	396.6 ± 4.5	392.9 ± 4.2	<b>0.000</b>
	NMe	99.6 ± 15.2	90.1 ± 11.5	<b>0.004</b>
	NSna	42.0 ± 5.7	37.8 ± 5.4	<b>0.001</b>
	SnaMe	57.7 ± 9.9	52.4 ± 6.9	<b>0.020</b>
Incisors position analysis	SGo	57.6 ± 6.8	65.1 ± 10.3	<b>0.000</b>
	SSnp	33.9 ± 6.9	35.3 ± 6.0	0.432
	I/SpP	116.7 ± 4.0	113.1 ± 5.4	<b>0.003</b>
	i/MP	77.2 ± 6.3	81.1 ± 6.2	<b>0.001</b>

SNA – upper jaw prognathism angle; SNB – lower jaw prognathism angle; ANB inter – jaws angle; SNPg – lower jaw prognathism angle or facial angle; GoArNS – inclination of the ramus to the cranial base angle; NS/SpP – palatal plane angle relative to the anterior cranial base; NS/MP – mandibular plane angle relative to the anterior cranial base; SpP/MP – ratio of the upper and lower jaw in the vertical plane; OP/NS – occlusal plane angle relative to the anterior cranial base; NSAr – saddle angle; SArGo – articular angle; ArGoMe – gonial angle; Bjork – Bjork polygon angles; NMe – total front face height; NSna – front upper face height; SnaMe – front lower face height; SGo – total rear face height; SSnp – rear upper face height; I/SpP – inclination of the upper incisors relative to the palatal plane; i/MP – inclination of the lower incisors relative to the mandibular plane.

Results are presented as arithmetic mean ± standard deviation; bolded values are statistically significant; the *t*-test of repeated measurements was applied.

*Differences between measured and reference values of cephalometric analysis parameters*

Table 2 shows the differences between the reference and measured values of the parameters before and after orthodontic-surgical treatment.

Apart from the SArGo parameter, the values of all other parameters measured before treatment differ statistically significantly in relation to the reference values. After the completion of the treatment, the analysis established that the parameters SNA, SNB, NS/MP, OP/NS, and I/SpP are in the reference values, while in the other parameters, there is a statistically significant difference concerning the reference values. Despite the deviations, their values are close to the reference values in relation to the condition before treatment, which leads to the harmonization of skeletal and dental structures, improvement of oral function, and the appearance of the patient's face.

*Differences in cephalometric analysis depending on the type of surgical procedure*

Based on the analysis of cephalometric parameters conducted before and after treatment, statistically significant differences were found between patients of the monomaxillary and bimaxillary groups in the period before and after treatment.

Before treatment, patients from the bimaxillary group had higher values of the following parameters: NS/MP, SpP/MP, ArGoMe, NMe (NSna, SnaMe), and SGo. The exception is the I/SpP parameter, which showed higher values in patients from the monomaxillary group before treatment.

The established differences in the values of the mentioned parameters before treatment were essential for choosing the operative procedure. Considering that both in the monomaxillary and bimaxillary groups, the values of the measured parameters after the treatment approached the reference values, this proved to be justified (Table 2).

In patients with a more pronounced form of MPG, both in the sagittal and vertical directions, better functional and

aesthetic results are achieved by surgical intervention on both jaws.

After completing the treatment, all patients, regardless of the type of surgery, had significantly lower measured values of parameters. The difference measured on the parameters before the treatment remained even after treatment, with their values approaching the reference values. The parameters NS/MP, NMe, NSna, and SnaMe are statistically significantly higher after treatment in patients from the bimaxillary group, except for the parameter I/SpP, which is higher after treatment in patients from the monomaxillary group.

*Differences between linear and angular parameters before (T1) and 12 months after orthodontic-surgical treatment (T2) of patients from the monomaxillary group and patients from the bimaxillary group*

In patients from the monomaxillary group, in whom sagittal split osteotomy of the ramus was performed by analysis of cephalometric parameters before orthodontic-surgical treatment, larger deviations of parameters in the sagittal direction compared to reference values were observed. The vertical parameters also deviated from the reference values, but to a much lesser extent compared to the patients from the bimaxillary group. After the completion of orthodontic-surgical treatment, the reference values of the parameters SNA, SNB, NS/MP, OP/NS, and I/SpP were achieved. Other parameters have been improved but not completely corrected. At the end of the treatment, the parameters in both groups of patients were close to the reference values, which speaks in favor of a well-chosen method of treatment (Table 3). Significantly larger deviations before treatment in both sagittal and especially vertical parameters in relation to reference values were noted in patients from the bimaxillary group, in contrast to patients from the monomaxillary group (Table 4). These deviations speak in favor of a more severe form of deformity, but the orthodontic-surgical treatment succeeded in at least ameliorating, if not completely correcting, even these bigger declinations.

**Table 2**

**Differences between reference and measured values of cephalometric analysis parameters before (T1) and 12 months after (T2) orthodontic-surgical treatment of mandibular prognathism**

Parameters	Reference values	Measured values		<i>t</i>	<i>p</i>	Measured values		<i>t</i>	<i>p</i>
		T1				T2			
		mean	SD			mean	SD		
SNA	82	79.0	5.1	-3.696	<b>0.001</b>	81.8	4.9	-0.191	0.849
SNB	80	85.3	5.7	5.873	<b>0.000</b>	81.3	5.2	1.605	0.116
ANB	2	-6.3	3.2	-16.143	<b>0.000</b>	0.5	2.3	-4.059	<b>0.000</b>
NS/SpP	12	7.9	3.3	-7.903	<b>0.000</b>	6.7	2.8	-12.087	<b>0.000</b>
NS/MP	32	37.2	8.0	4.120	<b>0.000</b>	33.8	7.1	1.634	0.110
OP/NS	14	16.0	6.2	2.105	<b>0.042</b>	15.0	5.6	1.156	0.255
NSAr	123	120.9	5.1	-2.588	<b>0.013</b>	120.4	5.4	-3.080	<b>0.004</b>
SArGo	143	141.8	4.0	-1.879	0.068	141.2	3.8	-3.030	<b>0.004</b>
ArGoMe	130	135.9	5.1	7.386	<b>0.000</b>	131.9	4.8	2.463	<b>0.018</b>
Bjork	396	398.7	6.3	2.719	<b>0.010</b>	393.4	5.4	-3.069	<b>0.004</b>
I/SpP	110	112.8	6.9	2.556	<b>0.015</b>	110.3	6.1	0.309	0.759
i/MP	90	75.4	8.6	-10.788	<b>0.000</b>	80.0	8.1	-7.831	<b>0.000</b>

For abbreviations see under Table 1; *t* – *t*-test for one sample; bolded values are statistically significant.

Table 3

Differences between patients from the monomaxillary group and patients from the bimaxillary group in the cephalometric analysis of telerradiography images before (T1) and 12 months after (T2) orthodontic-surgical treatment of mandibular prognathism

Parameters	T1		<i>p</i>	T2		<i>p</i>
	monomaxillary	bimaxillary		monomaxillary	bimaxillary	
SNA	79.4 ± 5.0	78.8 ± 5.3	0.711	81.2 ± 4.5	82.2 ± 5.3	0.539
SNB	84.9 ± 4.8	85.5 ± 6.3	0.748	81.5 ± 4.2	81.2 ± 5.9	0.865
ANB	-5.6 ± 2.6	-6.8 ± 3.6	0.247	-0.2 ± 2.3	1.0 ± 2.2	0.081
SNPg	87.2 ± 4.1	87.3 ± 6.1	0.981	84.3 ± 3.6	82.9 ± 5.9	0.42
GoArNS	82.2 ± 5.1	79.5 ± 7.5	0.209	83.9 ± 7.7	83.8 ± 9.1	0.964
NS/SpP	8 ± 2.5	7.9 ± 3.7	0.907	6.8 ± 2.4	6.6 ± 3.1	0.892
NS/MP	33.9 ± 4.8	39.5 ± 8.9	<b>0.029</b>	31.1 ± 4.6	35.7 ± 7.9	<b>0.042</b>
SpP/MP	28.8 ± 4.3	32.8 ± 6.7	<b>0.044</b>	25.4 ± 3.8	28.8 ± 6.4	0.063
OP/NS	14.7 ± 4.4	16.9 ± 7.0	0.259	13.5 ± 4.0	16.0 ± 6.3	0.163
NSAr	121.6 ± 3.9	120.5 ± 5.7	0.523	121.2 ± 3.9	119.8 ± 6.3	0.434
SArGo	141.1 ± 3.0	142.2 ± 4.6	0.395	140.9 ± 3.0	141.4 ± 4.3	0.69
ArGoMe	133.9 ± 4.9	137.4 ± 4.8	<b>0.032</b>	130.8 ± 4.9	132.6 ± 4.8	0.26
Bjork	396.6 ± 4.5	400.1 ± 6.9	0.1	392.9 ± 4.2	393.8 ± 6.1	0.619
NMe	99.6 ± 15.2	113.5 ± 19.5	<b>0.021</b>	90.1 ± 11.5	104.0 ± 18.2	<b>0.01</b>
NSna	42 ± 5.7	47.6 ± 8.1	<b>0.022</b>	37.8 ± 5.4	43.6 ± 7.8	<b>0.013</b>
SnaMe	57.7 ± 9.9	65.5 ± 11.9	<b>0.037</b>	52.4 ± 6.9	60.5 ± 11.2	<b>0.014</b>
SGo	57.6 ± 6.8	65.1 ± 11.9	<b>0.029</b>	65.1 ± 10.3	70.8 ± 12.6	0.143
SSnp	33.9 ± 6.9	36.2 ± 6.4	0.082	35.31 ± 6.0	39.1 ± 6.8	0.296
I/SpP	116.7 ± 4.0	110.2 ± 7.2	<b>0.002</b>	113.1 ± 5.4	108.5 ± 5.9	<b>0.018</b>
i/MP	77.2 ± 6.3	74.4 ± 9.7	0.259	81.1 ± 6.2	79.3 ± 9.2	0.504

For abbreviations see under Table 1.

Results are presented as arithmetic mean ± standard deviation; bolded values are statistically significant; the *t*-test of repeated measurements was applied.

Table 4

Differences in the cephalometric analysis of telerradiography images before (T1) and 12 months after (T2) orthodontic-surgical treatment of mandibular prognathism within the monomaxillary and bimaxillary groups

Parameters	Monomaxillary group			Bimaxillary group			
	T1	T2	<i>p</i>	T1	T2	<i>p</i>	
Sagittal direction	SNA	79.4 ± 5.03	81.2 ± 4.5	<b>0.012</b>	78.8 ± 5.3	82.2 ± 5.32	<b>0.000</b>
	SNB	84.9 ± 4.8	81.5 ± 4.2	<b>0.000</b>	85.5 ± 6.3	81.2 ± 5.9	<b>0.000</b>
	ANB	-5.6 ± 2.6	-0.2 ± 2.3	<b>0.000</b>	-6.8 ± 3.6	1.0 ± 2.2	<b>0.000</b>
	SNPg	87.2 ± 4.1	84.3 ± 3.6	<b>0.000</b>	87.3 ± 6.1	82.9 ± 5.9	<b>0.000</b>
	GoArNS	82.2 ± 5.1	83.9 ± 7.7	0.159	79.5 ± 7.5	83.8 ± 9.1	<b>0.005</b>
	NS/SpP	8 ± 2.5	6.8 ± 2.4	<b>0.036</b>	7.9 ± 3.7	6.6 ± 3.1	<b>0.029</b>
	NS/MP	33.9 ± 4.8	31.1 ± 4.6	<b>0.002</b>	39.5 ± 8.9	35.7 ± 7.9	<b>0.000</b>
Vertical direction	SpP/MP	28.8 ± 4.3	25.4 ± 3.8	<b>0.000</b>	32.8 ± 6.7	28.8 ± 6.4	<b>0.000</b>
	OP/NS	14.7 ± 4.4	13.5 ± 4.0	0.114	16.9 ± 7.0	16.0 ± 6.3	0.223
	NSAr	121.6 ± 3.9	121.2 ± 3.9	0.383	120.5 ± 5.7	119.8 ± 6.3	0.064
	SArGo	141.1 ± 3.0	140.9 ± 3.0	0.779	142.2 ± 4.6	141.4 ± 4.3	0.085
	ArGoMe	133.9 ± 4.9	130.8 ± 4.9	<b>0.015</b>	137.4 ± 4.8	132.6 ± 4.8	<b>0.000</b>
	Bjork	396.6 ± 4.5	392.9 ± 4.2	<b>0.000</b>	400.1 ± 6.9	393.8 ± 6.1	<b>0.000</b>
	NMe	99.6 ± 15.2	90.1 ± 11.5	<b>0.004</b>	113.5 ± 19.5	104.0 ± 18.2	<b>0.000</b>
	NSna	42 ± 5.7	37.8 ± 5.4	<b>0.001</b>	47.6 ± 8.1	43.6 ± 7.8	<b>0.000</b>
	SnaMe	57.7 ± 9.9	52.4 ± 6.9	<b>0.020</b>	65.5 ± 11.9	60.5 ± 11.2	<b>0.000</b>
	SGo	57.6 ± 6.8	65.1 ± 10.3	<b>0.000</b>	65.1 ± 11.9	70.8 ± 12.6	<b>0.000</b>
Incisors position analysis	SSnp	33.9 ± 6.9	35.31 ± 6.0	0.432	36.2 ± 6.4	39.1 ± 6.8	<b>0.001</b>
	I/SpP	116.7 ± 4.0	113.1 ± 5.4	<b>0.003</b>	110.2 ± 7.2	108.5 ± 5.9	0.065
	i/MP	77.2 ± 6.3	81.1 ± 6.2	<b>0.001</b>	74.4 ± 9.7	79.3 ± 9.2	<b>0.000</b>

For abbreviations see under Table 1.

Results are presented as arithmetic mean ± standard deviation; bolded values are statistically significant; the *t*-test of repeated measurements was applied.

## Discussion

Facial and jaw deformities are irregularities in the shape, size, and structure of the jaws that lead to significant facial asymmetry, mutilation, and functional problems<sup>12</sup>. MPG, as the most common deformity of the face and jaws, causes not only aesthetic problems in patients but also has psychosocial consequences associated with impaired masticatory and speech functions<sup>13,14</sup>.

The most common motive for patients to decide to correct this deformity is to improve dentofacial aesthetics<sup>15</sup>, while for some patients, the primary goal is to improve masticatory function<sup>16</sup>. Therefore, many kinds of research were focused on determining the changes in the bone and soft tissue structures of the face after the completion of orthodontic-surgical treatment of patients with MPG.

Combined orthodontic-surgical treatment is the best possible treatment for MPG<sup>13,17</sup>.

Treatment of MPG aims to correct dental anomalies, improve facial appearance, and harmonize a patient's facial profile<sup>10</sup>. This type of treatment establishes functional occlusion, facial and dental symmetry, improvement of all oral functions<sup>6,18,19</sup>, reduction of psychological and social problems of patients<sup>20</sup>, as well as improvement of self-confidence and quality of life of patients in general<sup>21</sup>.

Recognition of the aesthetic problem of deformity and correct prediction of post-treatment changes in soft and hard tissues is an important part of the diagnosis and planning of treatment of MPG with orthodontic surgery therapy<sup>11,22</sup>.

The results of our study showed, apart from the SARGo parameter, a significant difference in the measured parameters in all patients with MPG before orthodontic-surgical treatment in relation to the reference values. After the treatment, the analysis showed that the parameters SNA, SNB, NS/MP, OP/NS, and I/SpP are within the reference values, while significant differences remained in other parameters in relation to the reference values. Despite the remaining deviations from the reference values, they were significantly corrected in relation to the condition before treatment, which leads to the harmonization of skeletal and dental structures, improvement of oral function, and appearance of the patient's face. The selection of the appropriate type of surgical procedure in orthognathic surgery is based on the type and degree of manifestation of dentofacial deformity.

As part of orthodontic-surgical treatment, the bimaxillary surgery is performed most frequently because this method offers a higher probability of achieving sufficiently good anteroposterior and vertical intermaxillary relations<sup>7</sup>. Studies have shown that the bimaxillary surgery gives much more stable results during longer follow-up after treatment than in patients who underwent the monomaxillary surgery<sup>23</sup>. The monomaxillary surgery has its own indications; these are primarily patients with MPG in whom the vertical dimensions of the face are not increased, as shown by our study<sup>24</sup>.

As facial appearance is one of the main motives for patients to undergo this type of treatment, a large number of studies have focused on predicting changes in facial appearance due to changes in hard tissues after orthodontic-surgical

treatment of MPG. These predictions have become an integral part of therapy planning. Our study showed that the analysis of cephalometric parameters is still a good enough standard for assessing and predicting the outcome of orthodontic-surgical treatment. The results of our study, as well as the Rustemeyer and Martin<sup>25,26</sup> studies, showed that both monomaxillary and bimaxillary jaws reposition favorably affect changes in bone structures, leading to improved facial convexity and approximation to aesthetic norms if primary differential diagnosis of MPG and selection of treatment types were correct.

In our study, we obtained objective data on the type of MPG based on the results of the analysis of cephalometric parameters. Based on those results the future treatment plan was built and treatment results monitored.

By analyzing the parameters of the sagittal direction (SNA, SNB, ANB, SNPg, and GoArNS), we established how much and in which way the jaws were moved during the orthodontic-surgical treatment. SNA angle values were statistically significantly higher after treatment compared to pre-treatment values. The increase in the value of the SNA angle is due to the forward movement of the upper jaw during the operation. The SNB angle was statistically significantly reduced after treatment due to the backward movement of the lower jaw during treatment. As a direct consequence of changes in the values of the SNA and SNB angles, there is a change in the value of the ANB angle, which represents their difference and shows statistically significant increased values after treatment. Increasing the SNA angle and decreasing the SNB angle led to the normalization of the anteroposterior relationship between the lower and upper jaw, as well as the relationship of the lower jaw to the anterior cranial base.

There was a decrease in the SNPg parameter after treatment compared to preoperative measurement as a consequence of lower back jaw movement, while the GoArNS parameter was significantly higher after orthodontic-surgical treatment for the same reasons.

The results of our study for this group of parameters are in line with the findings of Aydil et al.<sup>9</sup>, who also did not find a statistically significant difference at the end of the treatment between patients with monomaxillary or bimaxillary surgery.

By analyzing the parameters of the vertical direction (NS/SpP, NS/MP, SpP/MP, OP/NS, NSAr, SARGo, ArGoMe, NMe, NSna, SGo, and SSnp) before treatment, a significant deviation from the reference values was observed in patients from the bimaxillary group in relation to patients from the monomaxillary group. Increased vertical parameters were the reason for the decision on the necessity of bimaxillary surgery<sup>24</sup>. After the completion of the treatment, the approximation of the reference values was noticed in both the monomaxillary and bimaxillary groups of patients, which only confirms that a correct decision on the type of surgical procedure was made.

The NS/SpP parameter, which represents the ratio of the upper jaw base to the cranial base in the vertical plane, shows a slight decrease compared to preoperative measure-

ment. That means that during the orthodontic-surgical treatment of MPG, there was a slight rotation of the upper jaw in the cranial direction. Since the values of this parameter after treatment in patients from the bimaxillary group are lower than in patients from the monomaxillary group, it means that during the operation, the upper jaw, in addition to being moved forward, is also rotated cranially.

The NS/MP parameter, which represents the ratio of the mandibular base to the cranial base in the vertical plane, is statistically significantly lower after treatment in patients from the monomaxillary and bimaxillary groups. Changes in this parameter occur as a result of a change in the position of the lower jaw (back and up) during surgery on the lower jaw.

The values of the parameter SpP/MP are significantly lower after the treatment due to the rotation of the central fragment of the lower jaw cranially. The base of the upper jaw was also slightly changed in the vertical plane (rotated cranially), which confirms the values of the NS/SpP angle. Reduction of NS/MP angle and SpP/MP angle after treatment indicate that the operation normalized the ratio of the lower jaw in the vertical direction, which significantly reduced the total anterior height of the patient's face.

The NSAr and SARGo parameters indicate changes in the position of the articular head of the lower jaw. The values of the NSAr parameter are slightly lower after treatment but without statistical significance, while the SARGo parameter does not show statistically significant changes after treatment. These results show that there are no significant changes in the anteroposterior position of the articular process of the lower jaw during the surgical procedure of the MPG treatment. The findings for this group of parameters are consistent with the findings of Jacobson et al.<sup>27</sup> and Becker et al.<sup>28</sup>. The ArGoMe angle decreased significantly after treatment due to backward jaw movement. These results indicate one of the basic goals of surgery, which is to reduce the length of the body and the angle of the lower jaw.

Twelve months after the orthodontic-surgical treatment, the sum of the angles that make up the Bjork polygon shows a significant drop in value. The decrease in the value of the ArGoMe angle and the entire Bjork polygon after treatment is a consequence of the anterior rotation of the proximal segment of the mandible during bilateral sagittal osteotomy of the ramus of the mandible. In this way, the skeletal ratio of the jaws in the sagittal direction and the ratio of the anterior and posterior height of the face were corrected, and so was the overall profile of the face in these patients.

After the completed treatment, the total anterior facial height NMe and the lower anterior facial height SnaMe were significantly reduced, while the values of the posterior facial height SGo were increased compared to preoperative measurement.

The values of the angles I/SpP and i/MP, which represent the ratio of the axes of the upper and lower central incisors to the base of the upper and lower jaws, respectively, changed statistically significantly. The value of the angle I/SpP is significantly lower 12 months after the end of treatment compared to preoperative measurement, while the angle i/MP recorded a significant increase after the end of the

treatment. This result shows that orthodontic-surgical treatment greatly affects the inclination of the upper and lower incisors in relation to the basic planes of the face. More precisely, this is the result of adequate preoperative orthodontic positioning of the upper and lower incisors (retrusion of the upper and protrusion of the lower). Appropriate orthodontic preparation creates the possibility for adequate surgical correction and repositioning of bone segments, which achieves stable functional and aesthetic results.

Based on our results, the inclination of the lower incisors in relation to the basic plane of the lower jaw MP was increased, which reduced their retro inclinations typical of MPG. The ratio of the lower incisors to the basic plane of the lower jaw, even after treatment, deviates from the biometric norm and is typical for MPG. That means that a certain degree of retrusion of the lower incisors is maintained even after orthodontic-surgical treatment.

Johnston et al.<sup>7</sup> observed that, after treatment, lower incisors remain in retrusion below normal values in 46% of patients. This incomplete decompensation is associated with possible extractions of teeth in the lower jaw during the pre-surgical orthodontic phase, incomplete orthodontic treatment phase, inadequate labial bone for incisor displacement, the resistance of the lower lip muscles to mandibular incisor displacement as well as values of cephalometric parameters before treatment: high values of SNA angle, greater inverse overlap, and greater retrusion of the lower incisors.

All changes that occur on bone and dental structures as a result of orthodontic-surgical treatment lead to changes in the surrounding facial structures (nose, zygomatic bones, infraorbital areas, and chin), which significantly affects the appearance of the patient's face<sup>6, 9-11</sup>. The bimaxillary surgery has a greater impact on the vertical relationships of the upper and lower face, nose, lower jaw, and chin<sup>29</sup>. In connection with these bone changes – a decrease in SNB and ANB angles and an increase in SNA angle values – the profile and appearance of the patient's face improved due to backward movement of the chin and lower lip protrusion, thus increasing facial convexity.

Baherimoghaddam et al.<sup>30</sup> analyzed telerradiography images before and after bimaxillary surgery in patients with MPG and found a decrease in SNB angle and an increase in SNA and ANB angles as in our study. Moreover, in this study, a significant difference was noted between the lower anterior height of the face before and after treatment. Due to changes in the above parameters, as well as consequent changes in the mentolabial angle, facial convexity angle, and upper lip protrusion, the basic features of MPG have been significantly corrected.

Marsan et al.<sup>10</sup> confirmed that the bimaxillary surgery in people with MPG improves both the vertical and horizontal proportions of the face and corrects the concave profile of the face. Due to the increase in the SNA angle and the decrease in the SNB angle, there was a decrease in the convexity angle of the face as well as the protrusion of the upper lip. The values of the ANB angle and incisal step were improved, and the soft tissue profile of the face was significantly changed. That study, as well as ours, confirmed that the repositioning of the upper and

lower jaw during the bimaxillary surgery has a significant effect in both horizontal and vertical directions, leading to increased nasolabial angle, decreased mentolabial angle, improved lip posture, and tooth appearance, which leads to improved orthognathic facial profile in patients with MPG.

In contrast to our study, Downarowicz et al.<sup>6</sup> analyzed telerradiography images of patients at the beginning of orthodontic treatment, immediately before surgery, and 3 to 6 months after the end of treatment. Their results also showed the normalization of cephalometric parameters. Similar to our results, the SNB angle was significantly reduced after the treatment, the SNA angle was significantly increased, while the ARGoMe angle was reduced compared to the initial condition, and the total height of the face was reduced, although the lower part of the face is still more dominant than the middle part. After surgery, both the occlusion and the patient's appearance improved significantly.

Chew<sup>31</sup> investigated the changes in hard tissues after orthodontic-surgical treatment of patients with MPG. They performed the analysis of cephalometric parameters on telerradiography images immediately before the operation and 6 months after the end of treatment. The results showed there was a change in the cephalometric parameters after the end of the treatment, such as the values of the ANB angle and the overlap of the incisors. At the beginning of the study, all patients had a concave profile of the face with a protruded lower lip. Bimaxillary surgery has improved facial convexity, nasolabial angle, and protrusion of the upper and lower lip according to appropriate aesthetic norms.

Bone tissue changes after orthodontic-surgical treatment of MPG were examined by Aydil et al.<sup>9</sup>. They analyzed telerradiography images before treatment and 1.5 years after completion of the treatment. After treatment, the upper incisors were protruded, and the lower incisors were retruded.

Similar to our study, the study by Johnston et al.<sup>7</sup> confirmed that patients who underwent planned bimaxillary surgery had higher negative values of ANB angle before treatment, as well as lower values of SNA angle, while patients who underwent planned monomaxillary surgery had higher initial values of the SNB angle. After the end of the treatment, the values of the SNB and ANB angles were reached approximately close to the reference values in patients with bimaxillary surgery, while in patients with monomaxillary surgery, deviation from the reference values is still present.

The results of our study showed that after orthodontic-surgical treatment, a large number of linear and angular parameters typical of MPG changed. After the end of treat-

ment, the total posterior and posterior upper facial height was increased, and the total anterior and anterior lower facial height was reduced. That improved the relationship between the total anterior and posterior facial height and led to the harmonization of the facial profile after the completion of treatment. As a consequence of orthodontic-surgical treatment, there were significant changes in the values of the angles SNA, SNB, and ANB. The angles of SNA and ANB were increased, while the angle of SNB was reduced after the end of treatment. Most of the vertical components of MPG have been reduced. Significant reduction of NS/MP, ArGoMe, and Bjork polygon angles improved the positions of the upper and lower jaws towards the anterior cranial base and the interrelationship of the jaws in the vertical direction. That resulted in a reduction in total anterior facial height, especially lower anterior facial height, and harmonization of occlusion. After the surgery, there were significant changes in the size of the SpP/MP angle, which represents the relationship between the upper and lower jaws in the vertical plane.

Orthodontic-surgical treatment has significantly improved dental and bone parameters, established proper occlusal relationships, and improved facial appearance in patients with MPG.

## Conclusion

After the orthodontic-surgical treatment MPG, dental and skeletal changes occur on the craniofacial skeleton, which significantly affects the appearance of the patient's face.

The results of our study showed that after the end of the treatment, there were no statistically significant differences between patients who underwent surgery of the monomaxillary type and patients who underwent surgery of the bimaxillary type, although, before treatment, there were significant differences between these two groups of patients. Patients who underwent bimaxillary surgery had significant deviations in parameters in both the vertical and sagittal directions before the surgery.

After the end of the treatment, regardless of the initial differences, all the measured parameters were brought closer to the reference values, which speaks in favor of the adequately chosen treatment method and surgical technique.

This paper showed that the correct treatment method, the correct choice of surgical technique, and orthodontic therapy in patients with different forms of MPG achieve similar results close to the reference values.

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# The Serbian version of the Brunnsviken brief quality of life scale: reliability, validity, and psychometric features among the population of high school students

Srpska verzija kratke skale *Brunnsviken* o kvalitetu života: pouzdanost, validnost i psihometrijske karakteristike srednjoškolaca

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## Abstract

**Background/Aim.** Measuring the quality of life (QoL) is vital in daily clinical practice because it shows significant information in addition to symptoms. There are a large number of scales for assessing the QoL. The Brunnsviken Brief Quality of Life scale (BBQ) measures importance-adjusted satisfaction across six life areas. A validation study of the BBQ scale showed good questionnaire features (high concurrent and convergent validity, internal and test-retest reliability, and sensitivity to change). Therefore, the BBQ scale is excellent for use in psychiatric patients to measure outcomes, as well as for everyday screening. **Methods.** This prospective cross-sectional study was performed among the final-year students in five high schools in Kragujevac, Serbia. Students completed the BBQ questionnaire. Statistical analysis was performed using a standard statistical method for scale validation and standardization. **Results.** Our study showed that the BBQ scale had high internal consistency (Cronbach's alpha = 0.687). That result showed that the scale had good reliability in our study. The average BBQ score was  $69.63 \pm 16.70$  (male:  $69.66 \pm 18.46$ ; female:  $69.83 \pm 15.78$ ,  $p = 0.944$ ). **Conclusion.** The Serbian version of BBQ satisfies all the criteria of successful validation. Therefore, this scale can be helpful in assessing the QoL in the healthy youth population in Serbia.

**Key words:** quality of life; serbia; students; surveys and questionnaires.

## Apstrakt

**Uvod/Cilj.** Merenje kvaliteta života (KŽ) je veoma važno u svakodnevnoj kliničkoj praksi, jer pored simptoma pruža značajne informacije. Postoji veliki broj skala za procenu KŽ. Kratka *Brunnsviken* skala o KŽ (BKŽ) meri zadovoljstvo u šest životnih oblasti. Validaciona studija BKŽ skale je pokazala dobre karakteristike upitnika (visoku konkurentnu i konvergentnu validnost, visoku internu pouzdanost i pouzdanost ponovnog testiranja i osetljivost na promene). Zbog toga je upitnik BKŽ odličan za upotrebu kod psihijatrijskih bolesnika za merenje ishoda lečenja, kao i za svakodnevni „skrining“. **Metode.** Prospektivna studija preseka sprovedena je među učenicima završnih razreda pet srednjih škola u Kragujevcu, Srbija. Učenici su popunili upitnik BKŽ. Statistička analiza izvršena je korišćenjem standardne statističke metode za validaciju i standardizaciju skale. **Rezultati.** Naša studija je pokazala da je skala BKŽ imala visoku internu konzistentnost (Cronbach-ov alfa = 0,687). Takav rezultat je pokazao dobru pouzdanost skale u našoj studiji. Prosečan skor BKŽ bio je  $69,63 \pm 16,70$  (muškarci:  $69,66 \pm 18,46$ ; žene:  $69,83 \pm 15,78$ ,  $p = 0,944$ ). **Zaključak.** Srpska verzija BKŽ zadovoljava sve kriterijume uspešne validacije. Zbog toga, ta skala može biti korisna za procenu kvaliteta života populacije zdravih mladih osoba u Srbiji.

**Ključne reči:** kvalitet života; srbija; studenti; ankete i upitnici.

## Introduction

Quality of life (QoL) measures provide information beyond what is conveyed by symptom measures, making them meaningful complements in daily clinical practice<sup>1</sup>. The defini-

tion of the term “quality of life” is essential. However, authors are often unable to define precisely what they mean by the QoL<sup>2</sup>. On the other hand, subjects differ from each other according to what they deem important for a quality life<sup>3</sup>. Moreover, there is an overlap between the QoL and life satisfaction<sup>4</sup>.

There are a large number of scales for assessing the QoL; more than 150 instruments for measuring the QoL were published back in 1994<sup>2</sup>. Today, there are numerous valid and reliable self-rating scales for the assessment of subjective QoL [for instance, RAND-36, Assessment of QoL (AQoL-4D), EuroQol (EQ-5D), Healthy Days core questions (CDC HRQoL-4), Patient-Reported Outcomes Measurement Information System – Global Health Scale (PROMIS), Quality of life scale (QoL scale), Medical Outcomes Study Short-Form 36 (SF-36), etc.]<sup>5-15</sup>. Considering that these previously enumerated scales measure the lack of symptoms, these scales are inappropriate for use in healthy subjects. Moreover, for measuring the QoL, these scales are valid and reliable, but several limitations affect their wide application<sup>1</sup>. A good scale for clinical practice should be brief, have a simple scoring procedure, and have easy and free accessibility. However, all of these scales lack some of these characteristics.

The Brunnsvikien Brief QoL (BBQ) measures importance-adjusted satisfaction across six life areas<sup>1</sup>. The original validation study of the BBQ scale showed good questionnaire features (high concurrent and convergent validity, internal and test-retest reliability, and sensitivity to change). Therefore, the BBQ scale is excellent for use in psychiatric patients for measuring outcomes, as well as for everyday screening<sup>1</sup>. The BBQ is freely accessible for non-commercial use in at least 30 different languages (<http://www.bbqscale.com>)<sup>16</sup>. Lindner et al.<sup>1</sup> showed that this scale for measuring the QoL is a valid and reliable instrument. Moreover, this scale was shown to be sensitive to differences between subjects with clinical symptoms and subjects without them, while it was not sensitive to gender or age.

The aim of the study was to evaluate the reliability, validity, and factor structure of the BBQ scale among the population of high school students in Serbia.

## Methods

The study was conducted at five High Schools in Kragujevac (Serbia) among 225 subjects. Data for this study were collected during the 6 months, from October 2020 to February 2021. Approval for this study was signed by the School Principals, after which they informed school psychologists, who distributed questionnaires in paper form to students. All of the respondents were adults, so parental approval was not required. Data were collected from this non-clinical sample anonymously.

The BBQ scale was first translated into Serbian (by a native Serbian speaker), and then it was translated again from Serbian into English (by a native English speaker), using the procedure of forward-backward translation. This procedure comprises the following steps: initial translation, translation synthesis, back translation, committee review (in this case, two psychiatrists) and pretesting, and drafting a final version of the scale. After that, the original English version (Appendix 1) of the questionnaire and the English version obtained after the translation from Serbian into English

were compared, and these two versions were identical<sup>17</sup>. The Expert Committee drafted the final Serbian versions of the BBQ scale (Appendix 2), which was pretested on 10 students.

BBQ questionnaire was used for measuring QoL<sup>1</sup>. The BBQ scale has 12 items which measure six life areas: Leisure (1<sup>st</sup> and 2<sup>nd</sup> items), View on life (3<sup>rd</sup> and 4<sup>th</sup> items), Creativity (5<sup>th</sup> and 6<sup>th</sup> items), Learning (7<sup>th</sup> and 8<sup>th</sup> items), Friends and Friendship (9<sup>th</sup> and 10<sup>th</sup> items), and View on self (11<sup>th</sup> and 12<sup>th</sup> items)<sup>1,18</sup>. Each pair of items for each of the six life areas makes a question using satisfaction and importance. Items are scored using a five-step Likert rating scale (from 0 – Strongly disagree to 4 – Strongly agree). The score is computed by multiplying the Satisfaction and Importance items for each of the six life areas. The total BBQ score represents the sum of the six products for Satisfaction and Importance items for each of the six life areas. Possible values of the score are from 0 to 96.

In order to assess the test-retest reliability of the questionnaire, a test was performed in a 2-week interval on the same 24 respondents, 10 male and 14 female. The average age in this group was 17.05 [standard deviation (SD) 0.23].

All respondents also answered the World Health Organization Quality-of-Life Scale (WHOQOL-BREF). The WHOQOL-BREF is a 26-item instrument consisting of four domains: physical health, psychological health, social relationships, and environmental health; it also contains QoL and general health items. Each item of the WHOQOL-BREF is scored from 1 to 5 on a response scale, which is stipulated as a five-point ordinal scale<sup>19,20</sup>. The scores are then transformed linearly to a 0-100 scale.

The statistical analysis was performed using the program IBM SPSS Statistics 26.0 (IBM, USA, 2019). Continuous variables are shown as mean  $\pm$  SD. Questionnaire reliability analysis was performed by determining Cronbach's alpha ( $\alpha$ ). Mutual correlations of questions were analyzed with the help of a correlation matrix (inter-item Pearson's correlations). The Kaiser-Meyer-Olkin Measure of Sampling Adequacy and Bartlett's test were performed to examine the suitability of the results for factor analysis. Factors were extracted after orthogonal rotation using the varimax method with the criterion for the number of the extracted components, eigenvalue  $> 1$ . The factor loading of 0.3 or greater was considered<sup>17</sup>.

The study was approved by the Ethics Committee of the Faculty of Medical Sciences in Kragujevac (Approval No. 01-6228, from July 30, 2020). Data were collected anonymously.

## Results

The study was conducted among 225 subjects (70 males and 155 females). The average age in the whole group was 17.82 (SD 0.41).

The average BBQ score was  $69.63 \pm 16.70$ . According to gender, there were no significant differences by BBQ between males and females (male:  $69.66 \pm 18.46$ ; female:  $69.83 \pm 15.78$ ; independent samples *t*-test,  $p = 0.944$ ).

The BBQ total score followed an approximately normal distribution. The BBQ scale was found to have high internal consistency with Cronbach’s  $\alpha = 0.687$ , which means that the reliability of the scale is good.

Six item pairs of BBQ were subjected to principal components analysis (PCA). Prior to conducting the analysis, the suitability of the data for factor analysis was assessed. Examination of the correlation matrix revealed many correlation coefficients greater than 0.3 (Table 1). Correlations between the item pairs for each of the six life areas’ original items also showed a good correlation in all life areas (from 0.284 to 0.640). The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy value of 0.763 indicated the adequacy of the sample, while the value of Bartlett’s Test of Sphericity  $< 0.001$  was highly statistically significant, which altogether indicated the factorability of the correlation matrix. After that, a factor analysis was conducted to determine how many phenomena are measured by the BBQ questionnaire.

The analysis of the main components revealed the presence of two components with characteristic values over 1, which explains a total of 58.10% of the variance (I 41.05%; II 17.05%). The scree plot revealed the existence of a clear breakpoint behind the two components (Figure 1). Based on Katel’s criteria, it was decided to keep both components.

The component matrix revealed the existence of a simple structure, with two components having quite high weights (I component in the range of 0.480–0.810, II component in the range of 0.323–0.596). To make these components easier to interpret, a varimax rotation was performed. The rotated solution revealed the existence of a simple structure, with two components having quite high weights (I component in the range of 0.543–0.791, II component in the range of 0.312–0.764) (Table 2). Finally, the first component includes item questions View of self, Leisure time, View on life, and Learning. The second component includes questions about Friends, Friendship, and Creativity. Name suggestions of the first and second components are “view of one’s own life” and “contact with the environment”, respectively.

Finally, if any item is removed in this two-component model, the internal reliability will not change significantly; Cronbach’s  $\alpha$  ranges from 0.578 to 0.686.

The total BBQ scores in the 2-week interval on the same 24 subjects had a strong positive correlation (Pearson’s  $r = 0.989, p < 0.001$ ) (Figure 2A), suggesting satisfactory test-retest reliability.

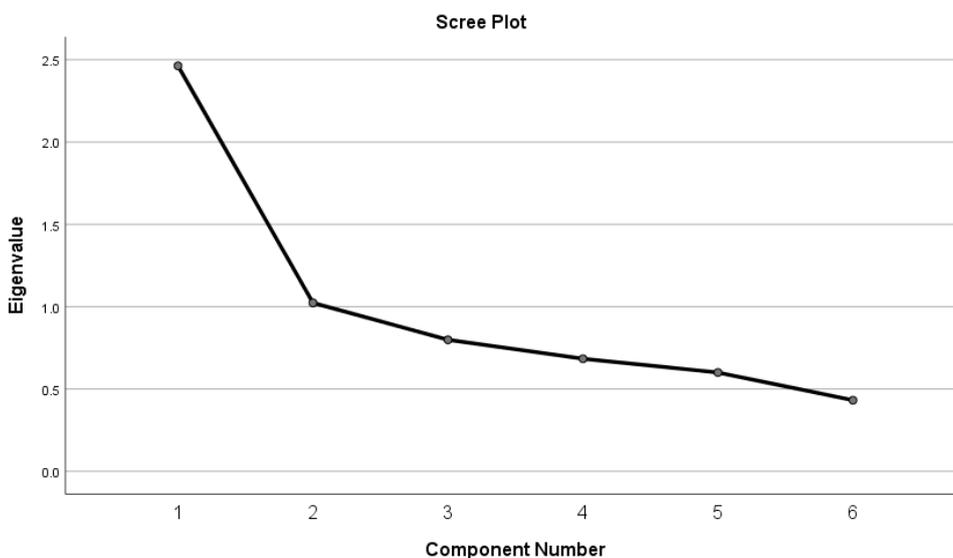
Regression analysis between the BBQ and the WHOQOL-BREF scale showed a strong positive correlation (Pearson’s  $r = 0.640, p < 0.001$ ) (Figure 2B). The average QoL measured by the WHOQOL-BREF scale was  $98.51 \pm 12.70$ .

**Table 1**

**Correlation matrix for Brunnsvikien Brief Quality of Life scale**

Items	Mean $\pm$ SD	Leisure time	View on life	Creativity	Learning	Friends and friendship	View of self	Item-pair
Leisure time	11.04 $\pm$ 4.39	1.000						0.450
View on life	12.50 $\pm$ 4.41	0.343	1.000					0.450
Creativity	10.58 $\pm$ 4.72	0.116	0.378	1.000				0.444
Learning	9.30 $\pm$ 4.81	0.218	0.370	0.263	1.000			0.612
Friends and friendship	13.24 $\pm$ 4.01	0.106	0.321	0.266	0.157	1.000		0.284
View of self	12.98 $\pm$ 4.35	0.393	0.529	0.225	0.357	0.181	1.000	0.640

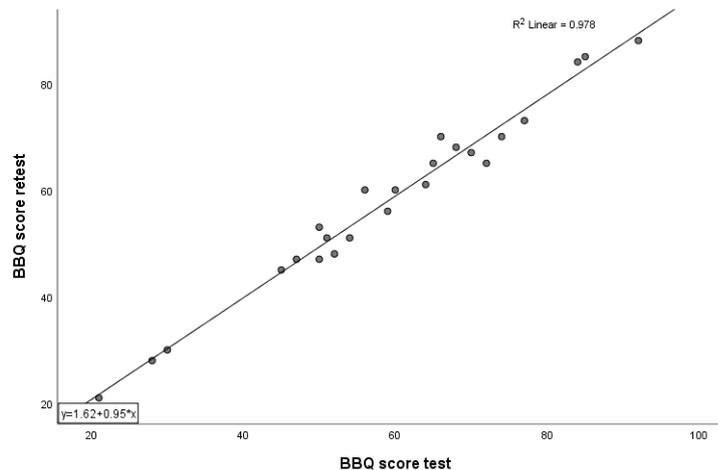
SD – standard deviation



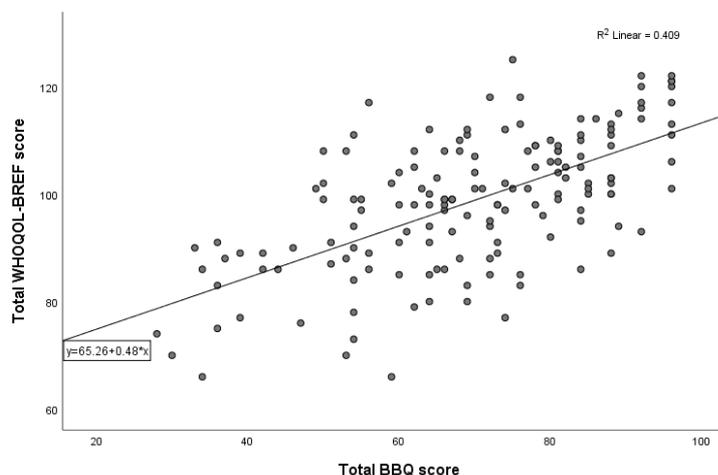
**Fig. 1 – Scree plot diagram of factor analysis**

**Table 2****Component matrix for Brunnsviken Brief Quality of Life scale after Varimax rotation with Kaiser normalization**

Variable	Pattern matrix	
	1	2
View of self	0.791	
Leisure time	0.777	
View on life	0.648	0.486
Learning	0.543	0.312
Friends and Friendship		0.764
Creativity		0.744

**Extraction method: principal component analysis.**

A)



B)

**Fig. 2 – A) The total Brunnsviken Brief Quality of Life (BBQ) scale score: test vs. retest; B) Regression results between both scales for measuring the quality of life: the BBQ questionnaire and the World Health Organization Quality-of-Life Scale (WHOQOL-BREF).**

## Discussion

Self-assessment of health status and QoL are an integral part of population health studies<sup>21</sup>. The assessment of QoL is complex and can be based on a different set of indicators, which may differ between countries. Therefore, the choice of the questionnaire, as well as translation and validation, represents an adequate and the most important step in the QoL as-

essment. Since there are numerous scales for measuring QoL, health professionals are often in a situation to choose one of the many questionnaires for measuring QoL<sup>22</sup>. A health professional with a good understanding of the disease and/or the research requirements can select a questionnaire for measuring QoL by carefully examining the scale items and judging to what extent the set of items matches the research requirements.

The respondent's education level is very important in the cases of self-administered questionnaires because understanding the questions and the response rates significantly influence the results of the questionnaires. Completing the questionnaire without interviewers significantly increases the probability of misunderstanding and missing responses, as well as anxiety and uncertainty experienced by the respondents<sup>23,24</sup>. The face-to-face questionnaires are the best option for these people<sup>25-27</sup>. There are also biased respondents to distort responses in a favorable direction to avoid negative answers in the case of self-administered questionnaires.

Assessing the QoL of young healthy people has long been a topical issue. In their previous study, Chen et al.<sup>28</sup> showed the development and psychometric assessment of the Young Adult Quality of Life (YAQOL) instrument, which measures the QoL of young adults aged 18 to 25. The YAQOL instrument has 14 multi-item scales. This instrument assesses physical health and aspects of psychological well-being, social relationships, role function, and environmental context. The average reliability coefficient for this instrument was 0.73. Twelve out of all 14 YAQOL scales distinguished young adults with personality disorders from the subjects without personality disorders. A negative relationship was also shown between the YAQOL scale scores and psychiatric disorder symptoms. Therefore, the YAQOL instrument reliability and utility were shown as strong measures of QoL in young adults.

Measuring the QoL is essential in daily clinical practice because it shows significant information in addition to symptoms. The BBQ self-rating scale of QoL has all the good characteristics that a scale should have – it is brief, easy to use with a simple scoring procedure, and has free accessibility<sup>1</sup>. The first validation study of the BBQ scale showed good questionnaire features – that the scale is a valid and reliable measure of QoL. Furthermore, this scale appeared to be sensitive to the difference between subjects with clinical symptoms and subjects without them. Therefore, the BBQ scale is excellent for use in psychiatric patients for measuring outcomes, as well as subjects without psychiatric symptoms. The BBQ has shown high internal consistency, high test-retest reliability, and satisfactory classification accuracy when comparing clinical patients with healthy controls<sup>1</sup>.

In our study, the BBQ showed good internal reliability. The Cronbach's  $\alpha$  as a measure of internal consistency in our study was 0.687. This value may appear as merely unsatisfactory in light of the often-reported threshold of 0.70<sup>29</sup>. However, this interpretation guideline is well argued to be inappropriately applied in cases such as the BBQ because scales with few items (such as the BBQ) tend to have lower  $\alpha$ . In this case, inter-item correlations, preferably between 0.20 and 0.40, are considered more appropriate measures of scale reliability<sup>30</sup>. Six item pairs of the BBQ during the analysis of the main components revealed the presence of two components which explains a total of 58.10% of the variance. The average BBQ score was  $69.63 \pm 16.70$ . According to gender, there is no significant difference between males and females by BBQ. Our factor analysis showed that the first component includes item questions about the View of

self, Leisure time, View on life, and Learning, while the second component includes questions about Friends and Friendship and Creativity. We suggested the name "view of one's own life" for the first component and "contact with the environment" for the second component. The first component refers to the characteristics of the individual's personality against which one perceives their own life and assesses its quality. On the other hand, the second component portrays a person's relationship with the environment, i.e., describes how a person experiences themselves through the eyes of the environment (meaning how a person thinks he/she is judged by other people or what role he/she plays in daily life situations). However, in the original article, the parallel analysis scree plot for the BBQ revealed a marked, consistent drop in eigenvalues following the first factor and a large difference *vis-à-vis* the resampling-derived eigenvalue only for the first factor, suggesting a satisfactory and interpretable unifactorial solution<sup>1</sup>. Because of that, the BBQ was designed to measure a single latent factor corresponding to overall subjective QoL. Similar observations have been shown in our paper, so a one-factor solution could also be applied here.

In our study, similar to the first validation study of BBQ<sup>1</sup>, men and women did not differ in BBQ scores, suggesting that the BBQ was not biased toward any sex group. Moreover, the BBQ in our study showed good reliability in terms of internal. In our study, regression analysis between the new validation BBQ scale and the previously validated WHOQOL-BREF scale showed a strong positive correlation, similar to the first study with this scale<sup>1</sup>. That shows that the newly translated QoL measurement scale, the BBQ scale, measures QoL in the same way as the previously validated and widely used scale, the WHOQOL-BREF scale.

The BBQ questionnaire can be applied in clinical practice. The BBQ was already used in the randomized controlled clinical trial as a secondary outcome measure after an intervention – internet-delivered extinction therapy for worry<sup>31</sup>. The study investigated an effect of a newly developed internet-based extinction therapy protocol in reducing worry in a sample of high-worrier subjects. The study showed a very large reduction of worry symptoms in a sample of high-worrier subjects compared to a control group. On the other hand, there was also a moderate effect on QoL. In the new study, these authors showed that internet-delivered extinction therapy was superior to waiting-list in reducing cognitive avoidance, intolerance of uncertainty, depressive symptoms, and also increased quality of life<sup>32</sup>.

In addition, BBQ was applied in a four-week randomized controlled pilot study for measuring QoL to test the effectiveness of the intervention "Boost & Balance online course" (combination of positive psychology, yoga, and mindfulness practice for five min per weekday) on measures of ill-being, well-being, and mindfulness<sup>33</sup>. Intervention in this study showed a significant decrease in aspects of psychological ill-being and a significant increase in aspects of psychological well-being, as well as the level of mindfulness in a group of office workers (mean of BBQ was pre-intervention 61.14 vs. post-intervention 64.73), in comparison to a control group assigned to a waitlist condition (mean of BBQ was pre-intervention 66.95 vs. post-intervention

64.53). The BBQ was used in several other studies, and it showed satisfactory results in all of them<sup>34–38</sup>.

Previous findings show that QoL instruments can help health professionals make informed decisions about disease management<sup>9</sup>. Furthermore, the instruments for assessing QoL are valuable tools for monitoring health-related QoL.

#### Limitations of the study

Our Serbian validation study of BBQ did not include a clinical sample. Future studies should investigate whether the BBQ is as valid and reliable when used in clinical samples.

#### Conclusion

This study showed nearly identical results as the first validation study of BBQ. The Serbian version of BBQ satisfies all the criteria of successful validation. Therefore, this scale will help assess the QoL in the healthy Serbian youth population. We hope that the BBQ scale for measuring the QoL may help in the everyday screening of mental disorders among young patients. In the end, the BBQ self-rating scale of the QoL has all the good characteristics that a scale should have – it is brief, easy to use, with a simple scoring procedure, and free accessibility.

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## Appendix 1

## Original Brunnsviken Brief Quality of life (BBQ) scale in English

## Brunnsviken Brief Quality of life scale (BBQ)

The following 12 questions are about how you experience your quality of life. It covers six areas, how satisfied you are with these, and how important they are to you. Circle the number that best reflects your experience.

	Do not agree at all				Agree completely	
1	I am satisfied with my <b>leisure time</b> : I have the opportunity to do what I want in order to relax and enjoy myself.	0	1	2	3	4
2	My <b>leisure time</b> is important for my quality of life.	0	1	2	3	4
3	I am satisfied with how I <b>view my life</b> : I know what means a lot to me, what I believe in, and what I want to do with my life.	0	1	2	3	4
4	How I <b>view my life</b> is important for my quality of life.	0	1	2	3	4
5	I am satisfied with opportunities to be <b>creative</b> : to get to use my imagination in my everyday life, in a hobby, on the job, or in my studies.	0	1	2	3	4
6	Being able to be <b>creative</b> is important for my quality of life	0	1	2	3	4
7	I am satisfied with my <b>learning</b> : I have the opportunity and desire to learn new, exciting things and skills that interest me.	0	1	2	3	4
8	<b>Learning</b> is important for my quality of life	0	1	2	3	4
9	I am satisfied with <b>friends and friendship</b> : I have friends that I associate with and who support me (as many friends as I want and need).	0	1	2	3	4
10	<b>Friends and friendship</b> are important for my quality of life	0	1	2	3	4
11	I am satisfied with <b>myself as a person</b> : I like and respect myself.	0	1	2	3	4
12	My satisfaction with <b>myself as a person</b> is important for my quality of life	0	1	2	3	4

The BBQ may be used freely and without costs by researchers and clinicians. For more information, visit [www.bbqscale.com](http://www.bbqscale.com)

## Appendix 2

## Translation of the Brunnsvikien Brief Quality of Life (BBQ) scale to Serbian

## Kratka skala Brunnsvikien za procenu kvaliteta života (BKŽ)

Pred vama se nalazi dvanaest tvrdnji koje se tiču načina na koji doživljavate kvalitet svog života. Skala uključuje šest aspekata kvaliteta života, a vaš zadatak je da procenite u kojoj meri ste njima zadovoljni i koliko su oni važni za vas. Za svaku tvrdnju zaokružite broj koji najbolje odražava vaš stepen slaganja sa njom.

Od 0 – uopšte se ne slažem, do 4 – u potpunosti se slažem.

1	Zadovoljan/na sam svojim slobodnim vremenom: jer mogu da radim kako bi se opustio i uživao.	0	1	2	3	4
2	Moje slobodno vreme je važno za kvalitet mog života.	0	1	2	3	4
3	Zadovoljan/na sam time kako gledam na život: znam šta mi mnogo znači, u šta verujem i šta želim od života.	0	1	2	3	4
4	Način na koji doživljam svoj život važan je za kvalitet mog života.	0	1	2	3	4
5	Zadovoljan/na sam svojim mogućnostima da koristim maštu u svakodnevnom životu, kada se bavim nekim hobijem ili kada sam u školi.	0	1	2	3	4
6	Mogućnost da budem kreativan/a je važna za kvalitet mog života.	0	1	2	3	4
7	Zadovoljan/na sam svojim učenjem: imam mogućnost i želju da naučim nešto novo i uzbudljivo, kao i da usvojim veštine koje me interesuju.	0	1	2	3	4
8	Učenje je važno za kvalitet mog života.	0	1	2	3	4
9	Zadovoljan/na sam svojim prijateljima i prijateljstvima: imam prijatelje sa kojima se družim i koji me podržavaju (onoliko prijatelja koliko želim da imam i koliko mi je potrebno).	0	1	2	3	4
10	Prijatelji i prijateljstva su važni za kvalitet mog života.	0	1	2	3	4
11	Zadovoljan/na sam sobom kao osobom: volim i poštujem sebe.	0	1	2	3	4
12	Za kvalitet mog života važno je da sam zadovoljan/na sobom kao osobom.	0	1	2	3	4

BKŽ mogu koristiti slobodno istraživači i kliničari bez ograničenja i naknade. Za dodatne informacije, posetite veb-sajt [www.bbqscale.com](http://www.bbqscale.com).



## Nickel-titanium files in endodontics: development, improvement, and modifications of nickel-titanium alloy

Nikl-titanijumski instrumenti u endodonciji: razvoj, usavršavanje i modifikacije nikl-titanijumskih legura

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### Key words:

dental alloys; endodontics; mechanical phenomena; nickel; root canal preparation; surface properties; titanium.

### Ključne reči:

legure, stomatološke; endodoncija; fenomeni, mehanički; nikl; zub, lečenje korenskog kanala; površina, svojstva; titanijum.

### Introduction

Numerous innovations in endodontic instrumentation were introduced in the early 1990s, and the concept of developing flexible endodontic files was significantly improved using nickel-titanium (NiTi) alloy instead of long-used stainless steel. The introduction of “smart” NiTi alloy is one of the biggest evolutionary shifts in endodontics because it has significantly improved the quality and efficiency of canal processing, with a significant reduction in complications and errors<sup>1-4</sup>.

Civian et al.<sup>5</sup> were the first to suggest the use of NiTi alloys for the production of endodontic files in 1975, and Walia et al.<sup>6</sup> were the first to introduce hand-held NiTi endodontic files made of treated orthodontic wire. The first NiTi rotary files with a 0.002 cone (introduced in 1992) were designed by Dr. John McSpadden<sup>1</sup>.

Complications during instrumentation related to the inherent stiffness of steel files have been significantly reduced thanks to the specific characteristics of the innovative NiTi alloy. Superelasticity (SE) and shape memory (SM) are the results of the phase transformation of the NiTi alloy, i.e., solid phase changes and transition from austenitic (parent phase) to martensitic phase<sup>7</sup>.

Numerous sets of rotary NiTi files have been introduced during the last few decades (there are currently more than 160 of them), and new ones appear every day<sup>1</sup>. The development of machine files is chronologically classified into

five generations, with a tendency to improve their individual performance and a maximum reduction of possible shortcomings<sup>8,9</sup>.

The focus of the endodontic dental industry was initially aimed at increasing the cutting efficiency of the files, i.e., reducing complications (fractures), during canal treatment. Modern generations primarily include different designs of the working part of the file related to cross-section, fixed or variable conicity, number and appearance of blades, and arrangement of blades along the active part, i.e., the presence or absence of radial surfaces<sup>1,7,8</sup>.

However, despite different design solutions, the occurrence of deformations and sudden fractures during instrumentation was not excluded, so technological solutions related to the modification and transformation of the basic NiTi alloy followed. In addition to changes related to surface and heat treatments of NiTi alloys, the number of files required for instrumentation has been reduced, and a new concept of activating machine files has been introduced to prevent the deformation and breakage of files<sup>8,10-13</sup>. It has been confirmed that different production processes, design solutions, but also the physical properties of the alloy, affect the clinical performance of machine files, i.e., their efficiency and safety during instrumentation<sup>8</sup>. The focus was, therefore, shifted towards the application of various technologies of heat and mechanical treatment of NiTi alloy in order to optimize the microstructure and thus increase the flexibility of endodontic files<sup>11,14</sup>.

Modern technological processes of the development of machine files are based on the knowledge that the mechanical properties of equiatomic NiTi alloy depend on the phase transformation caused by stress and the fact that these microstructural changes can be controlled by thermomechanical treatments<sup>14</sup>. It has also been confirmed that the metallurgical characteristics of NiTi alloy (composition, microstructure, and phase constitution) significantly affect the basic performance of files<sup>11, 15, 16</sup>.

The aim of this paper was to present the development, improvement, and modification of NiTi alloy and indicate how the effects of different thermomechanical alloy treatments and specific surface and heat treatments of files affect the improvement of clinical performance and safer and more efficient root canal cleaning and shaping.

### Crystallographic structure of NiTi alloys

The equiatomic NiTi alloy consists of approximately 56 percent by weight (wt%) of Ni and 44 wt% of Ti. The difference in weight percentages is due to the different molecular weights of these two elements (Ni 58.69 g/mol and Ti 47.87 g/mol)<sup>17</sup>. The specifics of alloys are the direct and strong bonds between electrons, which are responsible for their exceptional characteristics, such as SE and SM effect (SME). The NiTi alloy has three different phase microstructures that are temperature dependent: austenite, martensite, and a transient, R-phase. The austenitic phase gives a strong and hard form, and the martensitic and R-phase a softer form of the alloy that is easily deformed<sup>18</sup>. All three phases of transformation affect the mechanical characteristics of the alloy, and the change of phase in the solid state is responsible for the elastic properties, i.e., the martensitic transformation induced mainly by stress or temperature reduction<sup>1</sup>. After elastic or pseudoplastic deformation, the alloy undergoes a thermoelastic transformation from the austenitic to the martensitic phase. Quite a small force is enough to cause the alloy to bend at this stage. The whole structure recovers after the cessation of stress, returning to the austenitic phase, where it takes on its original shape (stress-induced thermoelastic transformation). The martensitic phase is generated by the stress of the material in the austenitic state and allows high stresses<sup>17</sup>. Atoms in the martensitic transformation are rearranged into a new, more stable crystal structure without changing the chemical composition of the matrix but with a macroscopic change in the shape of the material. This phase transformation occurs by transitioning the high-temperature, spatially centered cubic or tesserall austenitic, parent phase, into a rhombic or monoclinic martensitic phase. The martensitic transformation occurs due to shear forces and most often passes through the middle tetragonal phase (R-phase), where the martensitic areas have the same crystal structure but a different spatial orientation. By heating the material in the martensitic phase, a reversible transformation occurs, and martensite returns to the austenitic phase<sup>17</sup>.

A specific property of SM is the ability of the alloy to completely regain its original shape when heated above the temperature of transformation of martensite into austenite

(temperature varies depending on the chemical composition of the alloy). Among various metal alloys that show SE and SM, NiTi has the best biocompatibility and exceptional corrosion resistance due to the existence of a surface layer of Ti oxide<sup>19</sup>. Although only one manufacturer (Dentsply, Maillefer Instruments SA, Ballaigues, Switzerland) has published the absolute composition of the conventional alloy (56 wt% of Ni and 44 wt% of Ti) and the detailed technological process of producing rotary files, it is considered the best ratio that provides superelastic properties<sup>20</sup>.

The dominance of the desired properties of the files (superelastic or more pronounced SME) is enabled by the variation of the composition of the NiTi alloy. Increasing the proportion of Ni or its replacement by trace elements, e.g., cobalt (less than 2% by weight), results in a reduced phase transformation temperature, while an increase in temperature (e.g., annealing) also increases the phase transformation temperature<sup>7</sup>. The temperature transformation of NiTi alloys depends on the volume ratio of Ni and Ti and can range from -50 °C to +100 °C<sup>7</sup>. It has been confirmed that even a change of 0.1% in the composition of the alloy can lead to a change in the transformation temperature of 10 °C, which can subsequently affect its mechanical characteristics<sup>21</sup>. Otsuka and Ren<sup>19</sup> found that increasing the Ni content leads to a drastic decrease in the transformation temperature, so the focus of the development of new NiTi files is directed toward Ni-rich alloys.

### Thermo-mechanical treatments of NiTi alloy

Thermo-mechanical treatment is a metallurgical process that involves combining mechanical (compression or forging, rolling, drawing, etc.) and heat treatment (water quenching, heating, and cooling) methods of alloy processing in a single process<sup>1, 20-22</sup>. Thermo-mechanical treatments can be applied during the production process of the alloy and files, i.e., subsequent treatment of finished files.

Heat treatment of alloy is a modern approach to the regulation of increasing resistance to cyclic fatigue of endodontic files. This process consists of heating the material to a certain temperature and cooling it after a certain time under controlled conditions<sup>14, 20</sup>. It has been confirmed that temperature, i.e., the heating time and the cooling rate during alloy production, affect the SE and SM of NiTi files<sup>1</sup>.

New endodontic files with superior mechanical properties have been developed by the thermo-mechanical treatment that goes through the processes of aging, annealing, and recrystallization of the alloy<sup>1</sup>. Heat treatment leads to four different reactions: a) changes in chemical composition, b) recovery of defects, c) reduction of defects by recrystallization, and d) transformations of the structural phase<sup>1</sup>.

Aging gives the alloy greater mechanical strength and implies even heating of the alloy to about 500 °C and then rapid cooling (usually in water) because this prevents the deposition of alloying elements. Hardened parts are removed by annealing (heating to 300 °C–500 °C), and the alloy is then slowly cooled. Recrystallization implies a cold deformed structure with a changed set of grains, which reduces the hardness of the alloy and increases its forgeability<sup>1</sup>.

Heat treatment maintains the crystallographic structure of the alloy and provides files with greater flexibility and fracture resistance<sup>23,24</sup>. NiTi endodontic files mainly contain an austenite phase (conventional NiTi, M-wire, R-phase) or a martensitic phase [controlled memory (CM) wire and gold and blue heat-treated NiTi alloys]<sup>23,25</sup>. Superelastic alloys with a stable martensitic phase, which shows a lower modulus of elasticity (30–40 GPa) compared to austenitic (80–90 GPa), have also been developed by special heat treatment, while this modulus in the R-phase is lower than in martensite<sup>25</sup>. The mechanical properties of the alloy also depend on the ambient temperature: if the temperature is above the austenite finish (austenitic state), the alloy becomes hard, with superior superelastic properties; if the temperature is below the martensite finish (martensite state), it becomes soft and ductile and deforms easily with SME. The martensitic phase provides superior resistance to cyclic fatigue compared to austenitic due to the reorientation of the double phase structure<sup>24,26,27</sup>.

### Thermal preparation of NiTi alloy

Conventional NiTi wires are formed by cold drawing, resulting in a microstructure containing martensite residues in the austenitic matrix. Internal stresses can be released, and the disadvantages of rearrangement of the crystal lattice can be reduced by heat treatment of such an alloy (450 °C–550 °C)<sup>1,28,29</sup>.

### M-wire

The M-wire, characterized by an austenitic phase with a small proportion of stable R- and martensitic phase<sup>11,14</sup>, has contributed to the development of superelastic characteristics of NiTi endodontic files. This wire was introduced (2007) by Sports Wire LLC (Langley, OK, USA) and Dentsply Tulsa (Dental Specialties, Tulsa, OK, USA) and proposed for use in endodontics<sup>24–26</sup>.

The M-wire is composed of Nitinol 508 (Ni 55.8%), which is subjected to specific heat treatments at different temperatures. This type of alloy contains areas in the deformed martensitic phase, premartensitic R-phase, but also in austenitic phase. The finishing of the wire in the austenitic phase ranges from 45 °C to 50 °C, maintaining a pseudoelastic state<sup>20</sup>. Unlike the austenitic form, where the alloy is resistant and hard, the M-wire in the martensitic phase becomes softer, more flexible, and easily deformed, with a significantly lower possibility of stress fracture<sup>11</sup>. Martensitic phase transformation also has excellent resistance to fatigue due to its ability to absorb energy<sup>20</sup>.

Compared to files produced from conventional NiTi alloys, files made of M-wire have higher resistance to cyclic fatigue and improved mechanical properties<sup>14,24,26</sup>.

Files made of M-wire are ProFile GTKS, ProFile Vortex, ProTaper Next, Path Files (Dentsply Sirona, York, PA, USA), and WaveOne (Dentsply Maillefer, Ballaigues, Switzerland) and Reciproc (VDW, Munich, Germany). De Vasconcelos et al.<sup>30</sup> found that increased alloy flexibility and

increased fatigue resistance (martensitic phase) also provide a better arrangement of the crystal structure. Gao et al.<sup>31</sup> explained the higher resistance to cyclic fatigue of files made of M-wire by the lower appearance of cracks due to the better crystal orientation of the martensitic phase.

### R-wire

During the production process, a conventional NiTi alloy in the austenitic phase is transformed into a rhombohedral crystal structure, with the formation of the middle R-phase (the form between austenite and martensite)<sup>11,19</sup>.

It is formed during the direct transformation of martensite into austenite (due to heating) or during the inverse transformation from austenite to martensite (by cooling) and shows a lower modulus of elasticity compared to the martensite and austenitic phases. Files made of R-phase alloy have a complete austenitic form at ambient and body temperature and allow greater flexibility and increased resistance to cyclic fatigue<sup>14,20</sup>. In contrast, Park et al.<sup>32</sup> have observed that this production process does not affect the increased resistance to torsional fractures of files.

R-wire endodontic files include Twisted and K3 systems. SironEndo (Orange, CA, USA) developed the Twisted File system in 2008 and the Twisted File Adaptive in 2013 using the production process that involved transforming raw austenitic NiTi wire by thermal process into R-phase, twisting the wire and then conditioning its surface<sup>14,25</sup>.

Ghabbani's profilometric studies confirm the lower occurrence of surface deformations after instrumentation on the Twisted File system compared to M-wire files (ProTaper Next, Dentsply Maillefer, Ballaigues, Switzerland)<sup>33</sup>.

SironEndo developed K3KSF (SironEndo Orange, CA, USA) in 2011, which is a system that takes advantage of R-phase technology and represents an advanced version of the files, as it is subjected to a specific heat treatment<sup>14</sup>. These files are made by the micro-milling process and then subjected to a special heat treatment that significantly improves flexibility and strength, with modification of the crystal structure of the alloy<sup>14</sup>. Heat treatment can change the temperature of the phase transformation by repairing the crystal lattice defects and reducing the internal deformation energy. K3KSF files are a new evolutionary variant of the K3 system (created in 2011), and the R-wire provides them with better mechanical properties compared to files produced by a traditional process<sup>1,34</sup>. Unlike Twisted File and Twisted File Adaptive, produced by plastic deformation, the innovative way of production involves twisting metal wire and its heat treatment by recrystallization and provides the Twisted File system with greater elasticity and resistance to cyclic fatigue<sup>25</sup>. The protected production process ensures greater integrity of the crystal lattice structure of the metal by twisting and significantly increases the resistance of the files to breakage<sup>1</sup>. Increased flexibility in R-phase files allows better centering along the canal and a lower possibility of transport compared to conventional NiTi systems, while their resistance to cyclic fatigue is similar to files made of M-wire<sup>25,35</sup>.

### Alloy with controlled memory wire

The NiTi wire with CM-wire was introduced in 2010 and represents the first thermomechanically treated alloy that does not possess superelastic properties, neither at room nor at body temperature<sup>25,36</sup>. This wire is made of Nitinol SE508 alloy, with a lower Ni mass percentage (52%), compared to conventional SE alloys (54%–57%)<sup>1,14</sup>. The protected thermal process of CM-alloy formation involves heating and cooling in order to obtain a stable martensite phase at body temperature and thus better mechanical properties (increased flexibility, reduced SM, and increased transformation temperature)<sup>1,25,33</sup>.

Recent studies have shown that the formation temperature of the austenitic phase in files made of CM alloy is about 47 °C when the composite of martensitic phase, R-phase, and austenitic phase is achieved at room temperature<sup>1,25,37</sup>. This alloy allows the files to be bent before application into the canal, which significantly increases their resistance to cyclic fatigue. Files made of CM-wire are returned to their original state after sterilization and can be reused until the moment of irreversible deformation when they should be discarded<sup>1,17,25,37</sup>. These files show increased flexibility and better centering along the canal<sup>1,17,25</sup>.

Endodontic files made of CM alloy (CM-wire) include Hyflex CM, Typhoon Infinite Flex, and Pro Design systems. Coltene/Whaledent (Cuyahoga Falls, OH) introduced Hyflex CM files made of this alloy in 2011. These files show greater resistance to cyclic fatigue and are subjected to plastic deformation during use but return to their initial state after autoclaving<sup>1,15,17</sup>. Shen et al.<sup>38</sup> pointed out that HyFlex CM files show three to eight times higher resistance to cyclic fatigue compared to files made of conventional NiTi wires. Despite their lower tensile strength (1094 MPa vs. 1415 MPa in conventional files), CM files are more resistant to deformation (58.4%–84.7%) compared to the conventional ones (16.7%–27.5%), which indicates their superior flexible properties<sup>1</sup>. The transport rate of HyFlex CM files is similar to other super-elastic NiTi files, but canal correction is significantly reduced compared to Revo-S (Micro-Mega, Besançon, France), ProTaper Next (Dentsply Sirona Endodontics, Ballaigues, Switzerland), and Reciproc files (VDW, Munich, Germany)<sup>9,23</sup>.

Typhoon Infinite Flex files (Clinician's Choice Dental Products, New Milford, CT, USA) were created in 2011; they show 150% higher resistance to cyclic fatigue than files made of M-wire and 390% compared to those of conventional NiTi alloy<sup>1</sup>.

ProDesign R and ProDesign Logic systems (Easy Dental Equipment, Belo Horizonte, MG, Brazil) were created in 2014; they have an S-shaped cross-section, an inactive tip, and variable spiral angles with two blades. These systems are designed with a single file concept and differ only in the way the files are activated (ProDesign Logic is used in full rotation, while ProDesign R is designed for reciprocal activation)<sup>1</sup>.

### Max-wire

FKG (FKG Dentaire, La-Chaux-de-Fonds, Switzerland) developed a special NiTi alloy Max-wire (Martensite-Austenite Electropolishing-Flex, FKG) for the production of XP systems (XP-endo Shaper, XP-endo Finisher, XP-endo Retreatment) in 2015<sup>1</sup>. The transition from martensitic to austenitic phase occurs during the processing of the alloy at temperatures equal to or higher than 35 °C. These files are relatively straight at room temperature (M-phase, martensitic state), but due to the exposure to intracanal temperature and phase transformation, they change into another, bent form (A-phase, austenitic state). The transition from the martensite phase to the austenite phase occurs naturally at body temperature between 32 °C and 37 °C (austenite temperature around 35 °C).

XP-endo Shaper with a diameter of #30 size and a variable cone (0.01–0.04) and XP-endo Finisher with a diameter of #25 size and a conicity of 0.00 are, with eccentric rotation, able to adapt to the morphology of the root canal system, expanding or contracting as they progress along the canal<sup>1,39,40</sup>.

Leoni et al.<sup>41</sup> pointed to more efficient cleaning of the XP-endo Finisher dentinal walls compared to passive ultrasonic irrigation, while Keskin et al.<sup>42</sup> confirmed that XP-endo Finisher removes calcium hydroxide residues from the canal more effectively in combination with passive ultrasonic irrigation. Živković et al.<sup>39,40</sup> confirmed efficient cleaning of the apical part of the canal using XP-endo Finisher files.

### T-wire

The new martensitic alloy heat treatment system is known as T-wire technology, which, according to the manufacturer, significantly increases the resistance of files to cyclic fatigue (up to 40%)<sup>1</sup>.

Endodontic files made of NiTi alloy (T-wire) are One Endo and Exo Endo, or TwoShape system. The design of these files was created by combining two or more different cones on the same file. The files have a specific tip design, which provides more efficient processing of narrower canals, lower possibility of transport, and blockage of canals, i.e., lower need for mandatory initial passability. The ONE Endo file is used for the initial extension and the EXO Endo for the final canal formatting<sup>1</sup>.

The Two Shape system (MicroMega, France) is made of T-wire by enhancing One Shape austenitic files but with a new asymmetric design that improves cutting efficiency and detritus evacuation. Uslu et al.<sup>37</sup> have found that T-wire technology is responsible for increasing cyclic fatigue resistance by 40% compared to One Shape files.

### C-wire

Heat-treated NiTi alloy is the basis for making the One Curve system (Micro-Mega, Besançon, France) with a single file in full rotation, which was created by different heat treatment processes of C-wire. The file has a CME, increased flexibility, and greater resistance to cyclic fatigue<sup>43</sup>. The

One Shape system (Micro-Mega, Besancon, France) showed 2.4 times higher resistance to cyclic fatigue compared to the previous generation<sup>43</sup>. The cross-section varies along the blade of the file, which improves the centering in the apical third of the canal and provides more efficient debridement<sup>43,44</sup>.

### Specific heat treatments of finished files

After making endodontic files, heat treatments can correct the defects that have occurred during the processing of the wire and affect the modification of the crystal structure of the alloy. Thermocycling of NiTi alloy allows martensitic transformation in two phases instead of one<sup>23</sup>. Furthermore, the phase transformation of austenite into martensite takes place first, after additional heat treatment due to the formation of the R-phase and deposition of Ti<sub>3</sub>Ni<sub>4</sub> particles; additional cooling of the alloy is necessary to form the martensite phase<sup>1,23</sup>.

### Blue-wire

Dentsply Tulsa Dental (Tulsa, OK, USA) introduced ProFile Vortex Blue in 2011, the first endodontic file with a recognizable blue color<sup>25</sup>.

Vortex Blue is a rotating system made of M-wire with increased fatigue resistance, better cutting efficiency, flexibility, and better centering along the canal<sup>14,20</sup>. The files were created by a new, protected wire treatment, which involves a two-stage transformation of the alloy at the austenitic phase temperature (38 °C). The recognizable blue color originates from the surface layer of Ti oxide (thickness 60 nm–80 nm), which, in addition to better cutting efficiency, also affects the increased resistance to wear<sup>14,20</sup>. Goo et al.<sup>45</sup> tested the influence of alloy type (stainless steel, conventional SE NiTi, M-wire, and Vortex Blue) on cyclic file fatigue in an artificially formed stainless steel canal and confirmed the best characteristics of Vortex Blue sets in terms of flexibility and resistance to fracture. The protected production process of Vortex Blue files reduces the SME compared to standard NiTi files but allows the default shape to be maintained during canal instrumentation<sup>45</sup>.

The Sequence Rotary and X1 Blue File systems (MK Life, Porto Alegre, RS, Brazil) were created in 2017. The systems include files created by multiple heat treatments of CM-wire (alternating heating and cooling), where a bluish hue that originates from a thin Ti oxide layer (60 nm–80 nm) is formed<sup>1</sup>.

The new generation of Reciproc Blue (VDW, Munich, Germany) files was created in 2016. This system combines the simplicity of the original RECIPROC concept of a single file with the application of reciprocal movements that result in greater safety in primary or re-endodontic treatment. Innovative heat treatment provides greater flexibility and easier and safer file progress in the canal<sup>10,46</sup>. The characteristic blue color, which leads to modifications of the molecular structure and an increase in flexibility and resistance to cyclic fatigue, is formed by heat treatment of the CM-wire<sup>10,46,47</sup>.

### Gold-wire

The unique heat treatment before and after file creation led to the development of the WaveOne Gold (Dentsply Sirona) system in 2011. In this process, the conventional alloy is first subjected to a heat treatment process (410 °C–440 °C, under a constant load of 3 kg–15 kg), and the finished file is then subjected to another thermal process (120 °C–260 °C). This technological process results in a surface gold color of the file (Ti oxide layer, 100 nm–140 nm), which significantly improves the flexibility and file resistance<sup>20</sup>.

The ProTaper Gold (Dentsply Sirona) system was created by subsequent heat treatment of files at a temperature of 370 °C–510 °C in 2013<sup>1</sup>. ProTaper Gold is similar to the ProTaper Universal system (size, convex triangular cross-section, and a progressive cone), but features a gold color which affects increased flexibility and resistance to cyclic fatigue, and ensures better file centering<sup>1,14,48</sup>. The golden color of the files originates from the layer of Ti oxide (thickness of 100 nm–140 nm); it affects the weaker effect of SM, so slight deformations are often noticeable in new, unpacked files<sup>20</sup>. This property allows files to retain the bent shape even after removal from the bent canal after processing<sup>14,20</sup>.

AF Blue (Fanta Dental CO., Ltd, Shanghai, China), the new NiTi file system, is made of specially heat-treated AF-wire (AFTM-H) with excellent mechanical properties (strength), flawless surface finish, higher resistance to cyclic fatigue, and good cutting efficiency. The extreme flexibility of these files prevents the occurrence of transport, and the variable cross-section ensures minimal radial contact and reduces the possibility of screwing<sup>44</sup>.

S-One (Fanta Dental CO., Ltd., Shanghai, China) is a heat-treated file used in the technique of single file in full rotation. The file has an unusual design with a tip diameter of #25 size and a 6% of a constant cone, i.e., an S-shaped cross-section<sup>24,26</sup>.

### Treatment by electric discharge machining of NiTi alloy

Electric discharge machining (EDM), or EDM technology, is a non-contact thermal erosion process used in the production of electrically conductive materials and involves controlled electrical discharge in the presence of insulating fluid.

Coltene introduced the Hyflex EDM system (Coltene/Whaledent, Cuyahoga Falls, OH) in 2016, which is made of NiTi CM 495 alloy and produced by spark technology<sup>37</sup>. The surface of the metal (NiTi alloy) is “melted” by this process, with partial evaporation leaving an eroded surface. The file is exposed to heat before or after ultrasonic cleaning (between 300 °C and 600 °C for 10 min to 5 hrs), which improves cyclic fatigue resistance by more than 700% at room or body temperature. Analysis of the surface structure of Hyflex CM and Hyflex EDM files, before and after processing of strongly bent canals, indicated fewer surface defects in Hyflex EDM systems<sup>37</sup>. These systems have different cross sections on the working part and provide greater

cutting efficiency, facilitate penetration, and reduce the risk of fracture<sup>20,37</sup>.

Sterilization allows these files to be recovered and restored to their original form. However, smaller files are usually permanently deformed, so special care should be taken when reusing them<sup>37</sup>.

Neoniti (Neolix, Chatres-La-Forêt, France), a new NiTi system with a single file in full rotation, is made of a special alloy subjected to subsequent heat treatment. The rectangular cross-sectional design and specific heat treatment significantly increase the flexibility and effect of SM. Treatment of the working part of the file by the EDM creates a rough surface and enhances the abrasive properties, activating the cutting efficiency<sup>44</sup>.

### Specific surface treatments after making files

Improvement in the physical and mechanical properties of files can be achieved not only by heat treatments after machining but also by specific surface treatments of finished files (ionic implementation, nitriding, cryogenic treatment, electropolishing)<sup>1,14</sup>.

Ion implantation of endodontic instruments in plasma was introduced in the late 1980s<sup>20</sup>. A highly negative pulsating voltage is applied to the plasma-submerged file in the vacuum chamber. In this way, ions are extracted from the plasma and penetrated on the surface of the file. Studies have shown the success of this procedure by incorporating argon, boron, and nitrogen<sup>1,14,20</sup>. Ionic implantation improves surface characteristics without affecting the superelastic properties of the alloy. Gavini et al.<sup>1</sup> have shown that ionic nitrogen implantation improves resistance to cyclic fatigue and that this treatment also leads to increased cutting efficiency, i.e., improved wear resistance.

Due to the higher affinity of Ti for binding to oxygen, prolonged exposure of the alloy to moderate temperature leads to the formation of a surface oxide film of TiO<sub>2</sub>, which is formed slowly<sup>20</sup>. The surface porous oxide film, which increases the stability of the surface layers of the alloy and protects against corrosion, is formed by coating endodontic files with a flexible protective layer of TiO<sub>2</sub> using the immersion sol-gel method<sup>14,49</sup>.

Titanium nitride, which consists of a thin outer layer of TiN and a thicker inner layer of Ti<sub>2</sub>Ni<sup>14,20</sup>, is formed by the method of thermal nitriding on the surface of files. Nitriding is performed at 250 °C (at 300 °C, the superelastic character of the file is lost), and the TiN layer on the files significantly increases the corrosion resistance in contact with 5.25% NaOCl<sup>14,25</sup>.

The deep dry cryogenic process increases resistance to corrosion and wear and improves the strength and microhardness of treated metals<sup>20</sup>. This method involves suspending the metal through a super-cooled bath with liquid nitrogen (-196 °C) and then gradually warming it to room temperature. The mechanism of action is twofold because the transformation of martensite into austenite is more complete after cryogenic treatment, and the deposition of smaller carbide particles within the crystal structure has also been observed<sup>14,20</sup>. Cryogenic file treatments affect significantly

higher microhardness and higher cutting efficiency without affecting wear resistance, with a significant improvement in the cyclic fatigue resistance of NiTi rotary files<sup>14</sup>.

Electropolishing is an electrochemical process for surface finishing of NiTi files and was first applied by FKG (La Chaux-de-Fonds, Switzerland) in 1999<sup>1,20</sup>. This procedure leads to the removal of surface irregularities (by dissolving metal ions) by immersing the files in an electrolyte bath (concentrated solution of high-viscosity sulfuric or phosphoric acid) at a controlled temperature. The passage of current causes the oxidation of the metal and its dissolution in the electrolyte, and a hydrogen release reaction takes place at the cathode. The formation of a thin passive layer and the dissolution of the surface leads to the removal of surface irregularities, which increases the resistance to cyclic fatigue and torsional loading of files and provides greater resistance to corrosion<sup>1,17,24,26</sup>. Examples of these files are RaCe systems (FKG, La Chaux-de-Fonds, Switzerland), available in several variations and clinical sets (RaCe, IRaCe, BioRaCe, Series ISO 10, Scout RaCe, BT RaCe). Such a design provides better cutting, reduces the possibility of screwing inside the canal, and provides better apical penetration of files<sup>1,17</sup>.

EndoSequence files (Brasseler, Savannah, GA, USA), created in 2009, were also subjected to electrochemical polishing and immersed in an ionic solution through which an electric current passes in order to remove all surface irregularities that occurred during the production process.

The results indicated greater resistance to corrosive defects in files with an electropolished surface and less prevalence of production defects on the work surface<sup>17,22,34</sup>. However, some authors state that file design has a more significant effect on file resistance to cyclic fatigue than the finishing itself<sup>1,49</sup>.

### Conclusion

Owing to the thermomechanical treatments of the alloy, the latest technological advances have enabled the development of new NiTi instruments (M-wire, files with CM, CM-wire, R-phase wires, Max-, T-, and C-wires), which with different start kinetics and the design features of the working part show improved flexibility and greater resistance to cyclic fatigue compared to traditional superelastic NiTi systems.

Conventional NiTi alloy usually contains an austenitic phase, and thermomechanically treated alloy contains different amounts of R-phase and martensite. Thermally modified alloy enables the production of more flexible rotary files with greater resistance to defects and fractures.

Improvement in the physical and mechanical properties of NiTi rotary files can be achieved by heat treatments after machine file making (Blue-wire, Gold-wire, EDM) or by specific surface treatment of files (ionic implementation, electropolishing, nitriding, cryogenic treatment).

Files with a dominant austenitic phase are superelastic and more suitable for processing straight or slightly bent canals, and such an alloy compensates for the reduced torsional resistance of the files to form the initial patency. Martensitic form files are more flexible and are characterized by in-

creased resistance to cyclic fatigue and lower torque, and are suitable for processing bent and complex canal systems.

Files made of heat-treated CM-wire show greater flexibility and a weaker SME. Therefore, they can be bent before application into the canal and thus more efficiently clean and shape bent canals.

The dental practitioner is the most important factor for the success of the endodontic intervention. In addition to essential skills, they must know the complex anatomy of the canal and the characteristics of the file design, choose the right set of files and adapt the instrumentation technique to each individual case.

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## Moyamoya syndrome in Schimke immuno-osseous dysplasia

### Mojamoja sindrom u Šimkeovoj imuno-osealnoj displaziji

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#### Abstract

**Introduction.** Schimke immuno-osseous dysplasia (SIOD) is a rare autosomal recessive multisystem disorder associated with biallelic mutations of the *SMARCAL1* gene. Vascular central nervous system complications in the form of Moyamoya syndrome (MMS) have been reported as a comorbidity in nearly half of the patients clinically presenting with severe migraine-like headaches, transient ischemic attacks (TIA), and ischemic or hemorrhagic infarctions. We present an illustrative case of an infantile form of SIOD with MMS, with a review of the latest diagnostic possibilities, as well as current diagnostic and therapeutic dilemmas in managing SIOD. **Case report.** We present a female patient with the infantile form of SIOD. The proband was born small for gestational age in the 34th gestation week with characteristic dysmorphic features. Genetic testing found a biallelic, nonsense mutation c.2542G>T in the *SMARCAL1* gene. The patient presented early with TIA, seizures, and recurrent ischemic strokes. Magnetic resonance imaging (MRI) confirmed the pres-

ence of progressive brain atrophy with bilateral occlusion/stenosis of middle cerebral artery and anterior cerebral artery and a smoke-like collateral vessel appearance consistent with the MMS. At the age of 5 years and 9 months, the patient developed a high fever and cough with unknown cause, with a low erythrocyte and white blood cell count during four weeks, with a poor therapeutic response to antibiotics, transfusion of red blood cells, and granulocyte growth factor. She later died. **Conclusion.** Patients with SIOD may present progressive cerebral vascular changes and clinical neurologic deterioration early in the course of the disease. In such patients, early diagnosis and preventive revascularization surgery are of paramount importance. In diagnosing MMS, MRI angiography can be an appropriate substitute for standard invasive cerebral angiography.

#### Key words:

cerebrovascular disorders; diagnosis; magnetic resonance imaging; moyamoya disease; mutation; neurologic manifestation.

#### Apstrakt

**Uvod.** Šimkeova imuno-osealna displazija (SIOD) je autozomno recesivno multisistemsko oboljenje, povezano sa bialelskim mutacijama gena *SMARCAL1*. Vaskularne komplikacije u centralnom nervnom sistemu u formi Mojamoja sindroma (MMS) javljaju se kao komorbiditet kod gotovo polovine bolesnika, manifestujući se klinički jakim glavoboljama nalik na migrenozne, tranzitornim ishemijskim atacima (TIA) i ishemijskim ili hemoragijskim infarktima. Prikazujemo ilustrativan slučaj infantilnog oblika SIOD sa MMS, sa pregledom najnovijih dijagnostičkih mogućnosti i trenutno najvažnijih dijagnostičko-terapijskih dilema vezanih za SIOD. **Prikaz bolesnika.** Prikazujemo bolesnicu sa infantilnim oblikom SIOD-e, koja je rođena

sa težinom manjom od referentne za gestacijsku starost, u 34. gestacijskoj nedelji, sa manifestovanim karakterističnim dismorfizmima. Genetsko testiranje je otkrilo istovetnu *nonsense* mutaciju c.2542G>T na oba alela gena *SMARCAL1*. Bolesnica je imala više epizoda TIA, konvulzija i ishemijskih moždanih udara. Ispitivanja magnetskom rezonancom (MRI) pokazala su progresivnu atrofiju mozga sa bilateralnom okluzijom/stenozom srednje i prednje cerebralne arterije i razvoj kolateralnih krvnih sudova pod slikom „duvanskog dima“ karakteristično za MMS. U uzrastu od 5 godina i 9 meseci, bolesnica je dobila visoku temperaturu i kašalj, nepoznatog uzroka, praćene niskim brojem eritrocita i leukocita tokom 4 nedelje i slabim odgovorom na terapiju antibioticima, transfuziju eritrocita i faktor stimulacije rasta granulocita. Nakon ove epizode

bolesnica je preminula. **Zaključak.** Oboleli od SIOD-e mogu razviti progresivne promene krvnih sudova mozga i kliničke manifestacije neurološkog propadanja u ranoj fazi bolesti. Kod takvih pacijenata od najvećeg značaja je rano postavljanje dijagnoze i preventivna hirurška revaskularizacija. U dijagnozi MMS, magnetna angiografija

može biti adekvatna zamena standardnoj invanzivnoj angiografiji mozga.

**Ključne reči:**  
**cerebrovaskularni poremećaji; dijagnoza; Moyamoya bolest; mutacija; magnetska rezonanca, snimanje.**

## Introduction

Schimke immuno-osseous dysplasia (SIOD) is an autosomal recessive multisystem disorder associated with mutations of the *SMARCAL1* (SWI/SNF2 related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1) gene<sup>1</sup>. *SMARCAL1* encodes a chromatin remodeling enzyme with multiple roles in DNA restructuring<sup>2</sup>. Mutations of this gene are essentially affecting cellular proliferation and differentiation<sup>3,4</sup>. Disruption of chromatin remodeling is a predisposing factor for nonatherosclerotic occlusive cerebrovascular disease observed in such patients<sup>5</sup>. Vascular central nervous system complications in the form of Moyamoya syndrome (MMS) have been reported as a comorbidity in nearly half of the cases clinically presenting with severe migraine-like headaches, transient ischemic attacks (TIA), and ischemic or hemorrhagic infarctions<sup>3,6,7</sup>. Moyamoya disease (MMD) is a rare progressive bilateral stenooclusive arteriopathy of unknown origin. The disease involves the distal end of both internal carotid arteries and their branches, clinically presenting with recurrent strokes in children<sup>8</sup>.

SIOD is hallmarked by growth failure, skeletal dysplasia, steroid-resistant nephrotic syndrome, renal failure, immunodeficiency, and neurologic and pulmonary abnormalities. Patients can be generally divided into two groups – those with a juvenile or early-onset form of SIOD have a much more severe disease course, while patients with later onset have more favorable outcomes, surviving into adulthood<sup>2,3,6,7</sup>.

Stajić et al.<sup>9</sup> should be credited with leading the way in managing and treating SIOD patients in Serbia, and, to the best of our knowledge, they described and published the first case of SIOD from Serbia. Boerkoel et al.<sup>10</sup> were the first to show cerebral vascular abnormalities on MRA in two unrelated female SIOD patients. Both patients had cerebral vascular changes, first officially named MMD (“puff of smoke”) by Suzuki and Takaku<sup>11</sup> in 1969. As already pointed out, when Moyamoya appears in the form of an isolated condition, it is named MMD, but when it is associated with certain known comorbid genetic conditions, such as SIOD, neurofibromatosis I, sickle cell anemia, or Down syndrome, it is named MMS. In either case, the hallmark of Moyamoya is progressive occlusion of major cerebral arteries<sup>12,13</sup>.

## Case report

The presented female patient was born by an emergency Cesarean section in the 34th gestational week, 44 cm long and weighing 1,280 g. Diagnosis of intrauterine growth retardation (IUGR) was made prenatally. She was the firstborn child of

healthy, nonconsanguineous Serbian parents. During the first 23 months of life, characteristic dysmorphic features became evident: disproportionate short stature (below the 3rd percentile for age and gender), spondyloepiphyseal dysplasia (flattened vertebral bodies and dysplastic acetabular fossae), a short neck, triangular face, broad nasal tip, sparse and dry hair, multiple hyperpigmented macules, and absent dentition. Growth hormone secretion and thyroid function tests were normal. The immunophenotype of peripheral blood lymphocytes demonstrated a strikingly low number of lymphocytes, particularly of T cells, with a normal CD4/CD8 ratio and a slightly increased number of natural killer (NK) cells. Immunoglobulin levels were normal. Her neurologic development was appropriate for her age. The patient’s clinical and laboratory findings suggested the diagnosis of SIOD, which was confirmed by genetic testing demonstrating that she had a biallelic, nonsense mutation c.2542G>T in the *SMARCAL1* gene.

The first neurologic symptoms appeared at the age of 25 months, with a brief transient aphasic episode. The patient’s electroencephalogram (EEG) showed a bilateral parietal-occipital slow delta dysfunction, and a diagnosis of TIA was made. Four months later, she had a different type of attack manifested by tremors in the right hand and leg lasting under 5 min, followed by a similar left-sided attack about a month later. Based on a repeated EEG, a diagnosis of partial epilepsy was made, and carbamazepine therapy was initiated.

Her first magnetic resonance imaging (MRI), done when she was 2.5 years old, demonstrated brain atrophy, ischemic leukoencephalopathy, and subcortical laminar cerebral necrosis (Figure 1A). Three-dimensional time of flight (3D TOF) (magnetic resonance angiogram – MRA) showed an absence of flow in the right middle cerebral artery (MCA) (Figure 1B). She had two more aphasic episodes, and after the last one, she was left with a permanent inability to speak at the age of 32 months. Due to her low body weight and poor general condition, she was not accepted for revascularization surgery. Tests for congenital and acquired thrombophilia were negative. Antithrombotic therapy with acetylsalicylic acid (Aspirin®) was initiated after her first MRA; however, since her neurological condition deteriorated, her treatment continued with combined antithrombotic and anticoagulation drugs (Aspirin®, dipyridamole, and warfarin) bearing in mind her international normalized ratio (INR) which had to be kept below 3.

MRI and MRA were repeated 14 months after the first MRI examination, showing more pronounced brain atrophy and bilateral chronic subdural hematomas (Figure 1C). Cerebral angiography demonstrated bilateral occlusion/stenosis of MCA and anterior cerebral artery (ACA) with collateral

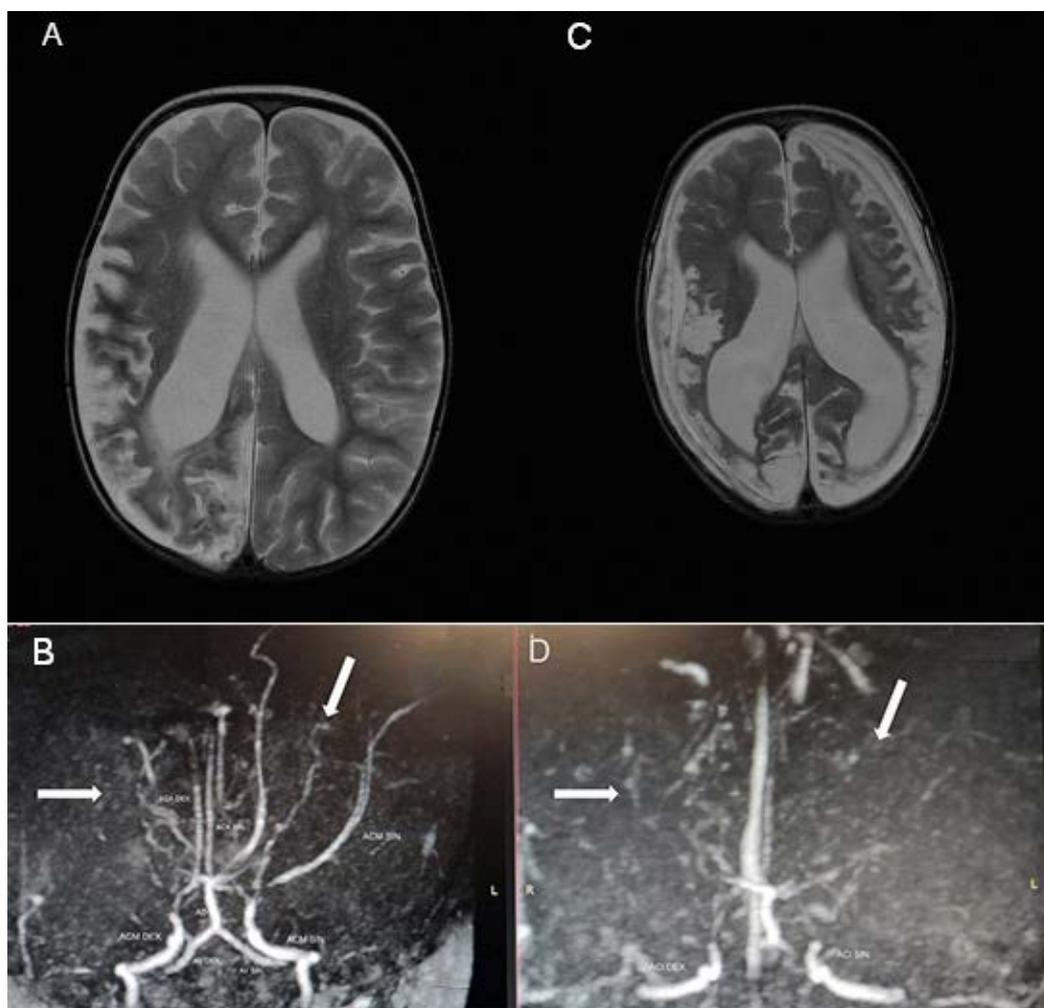
circulation consistent with MMS (Figure 1D). Extracranial vessels ultrasound was not performed, but MRI did show the presence of extracranial collateral circulation. Because of the presence of subdural hematomas, Aspirin® and warfarin were withdrawn, and dipyridamole was continued. She subsequently had several more attacks, usually left-sided. Spastic left-sided weakness gradually developed and remained present on repeated physical examinations. Over time, contractures in her lower extremities affected her everyday activities until her ability to walk was lost completely.

Significant proteinuria was first observed when she was 30 months old (5 months after her first aphasia attack), and hypercholesterolemia was noticed a month later. Our patient never had high blood pressure. The signs of renal failure never appeared, and her urea and creatinine were constantly within normal limits. Chronic anemia developed when she was 5 years old. The white blood cell count (WBC) was

within the normal range except during her infrequent acute infections, with the absolute lymphocyte count slightly below the normal range. At the age of 5 years and 9 months, she developed a high fever and cough, with a low erythrocyte and WBC count. The fever with a low WBC count, marked by a very low neutrophil count, continued for the next four weeks. She had a poor therapeutic response to antibiotics, transfusion of red blood cells, and granulocyte growth factor. The cause of the fever was never determined and, during this episode, she died.

### Discussion

SIOD is a rare multisystem autosomal recessive disorder. Its prevalence is unknown, but in the United States, it is estimated at between 1 : 1,000,000 and 1 : 3,000,000<sup>14</sup>. SIOD is mainly characterized by disproportionate short stature



**Fig. 1 – A. Right temporal and partial occipital lobe atrophy with increased signal intensity in the cortex, and the dilatation of the ventricular system; B. Absence of contrast flow in the right middle cerebral artery (MCA) with the bilateral abnormal collateral flow and bilateral shadows (arrows) on the same scan level at the basal ganglia region consistent with magnetic resonance imaging Moyamoya stage 2; C. Bilateral chronic subdural hematomas with sediment in the posterior portions of hematomas on the left side and more pronounced both brain atrophy and ventricular dilatation; D. Absent flow in both MCAs and flow reduction in both anterior cerebral arteries with the significantly less prominent collateral flow (arrows) consistent with advanced magnetic resonance imaging Moyamoya stage 3.**

due to spondyloepiphyseal dysplasia, T-cell immunodeficiency, and progressive steroid-resistant nephropathy<sup>3, 6, 7</sup>. Although this combination is unique to SIOD, a definitive diagnosis was made only after molecular genetic testing, which confirmed the presence of the biallelic pathogenic mutation in *SMARCAL1* gene. However, there are significant phenotype-to-genotype variations suggesting that SIOD is modified by factors such as environment, epigenetics, and oligogenic inheritance<sup>7</sup>. Moreover, since only 50%–60% of individuals with typical SIOD features have detectable *SMARCAL1* gene mutations, it is proposed that mutations of unidentified genes can also produce the same phenotype<sup>15</sup>.

Our patient presented with all the typical characteristics of the infantile form of SIOD. Physical features such as IUGR and short stature are present in 70% and 99% of affected individuals, respectively<sup>7</sup>. Lymphopenia caused by T-cell deficiency, as in the presented case, affects about 80% of SIOD patients and is consistent with reduced production of T cells by the thymus<sup>7, 16</sup>. Absent expression of interleukin 7R $\alpha$  is blamed for the reduction of the total T cell count<sup>17</sup>. Bertulli et al.<sup>18</sup> recently described a familial SIOD case with defective expression of interleukin 7R $\alpha$  and alterations of the NK cells. Bone marrow failure occurs in up to one-third of SIOD patients, and it could explain the deficiencies of all blood count lineages observed in our patient at the onset and during her final febrile illness<sup>3, 7</sup>. Infection is the primary cause of death of such patients (23%), while stroke (13%) and renal failure (11%) are less represented<sup>7</sup>.

Even though our patient developed steroid-resistant nephrotic syndrome during the course of the disease, signs of renal failure never appeared, and renal disease did not show clinical progression. Treatment attempts with angiotensin-converting enzyme inhibitors and prednisolone were unsuccessful, as described in most publications<sup>7, 14</sup>. Unfortunately, a renal biopsy was never performed, so we could not assess the probable presence and extent of focal glomerular sclerosis.

MMD and MMS is a rare progressive bilateral stenotic arteriopathy clinically presenting with recurrent strokes in children<sup>8</sup>. As stenosis progresses, a compensatory mesh of small collateral vessels develops, presenting on catheter angiography in the form of a cloudy, smoke-like appearance named “moya-moya”, which is the Japanese term for a “puff of smoke”. According to the results from the International Pediatric Stroke Study, approximately one-third to one-half of SIOD has a MMS. Nevertheless, SIOD is a rare cause of MMS, while sickle cell anemia, Down syndrome, and neurofibromatosis I are much more frequent<sup>8</sup>.

MMD is responsible for about 8% of arterial strokes in children<sup>8</sup>. Ninety percent of children with MMD had an ischemic stroke, 7.5% presented with TIA, while hemorrhagic stroke was the dominant form of presentation in only 2.5%<sup>8</sup>. Among the children with arterial ischemic stroke, hemiparesis was the most common presenting sign (79%), speech difficulties were observed in 49%, while headache and seizures were reported in 47% and 30%, respectively<sup>8</sup>.

TIA was the first mode of presentation in our patient who initially had a brief aphasic episode. Rafay et al.<sup>19</sup> state that in children with MMS, TIA is often associated with hy-

perventilation suggesting hypoperfusion rather than thrombotic vasooclusion as a prominent mechanism. Some other authors do not make a clinical distinction between stroke/TIA<sup>9</sup>. After her first aphasia attack, the girl rapidly and completely recovered, suggesting cerebral hypoperfusion as an underlying mechanism.

Nonetheless, within the next 5 months, her neurological status rapidly deteriorated with progressive mental decline, behavioral changes, more profound speech difficulties, and partial epilepsy, clinically strongly suggesting the presence of ischemic strokes. It is not possible to assess the exact time of appearance of infarctions since the first brain MRI done 5 months after the appearance of the neurological symptoms already demonstrated the presence of brain atrophy, ischemic leukoencephalopathy, and subcortical laminar cerebral necrosis (Figure 1A). Such findings are consistent with both fresh and old ischemic strokes. Middle and proximal anterior cerebral arteries (ACA) are known to be the most frequently involved arterial territories affected by ischemic infarctions in pediatric MMD<sup>19</sup>. During the next 14 months, her clinical condition further deteriorated, and repeated MRI showed complete obstruction of both MCA and ACA with a mesh of tiny collateral network confirming the MMS cerebral angiopathy within the SIOD.

The exact mechanism of arterial stenosis in MMD is yet to be fully elucidated. The theory of cerebral atherosclerosis was supported by vascular changes observed on *post-mortem* examinations which showed focal intimal lipid deposits, focal myointimal proliferation, macrophage invasion, foam cells, fibrous transformation, and calcium deposits<sup>7</sup>. Hypertension and hyperlipidemia caused by nephrotic syndrome, present in almost all SIOD patients, were also believed to accentuate thromboembolic atherosclerosis with the progression of arterial stenosis<sup>20</sup>. However, more than 15 years ago, it was demonstrated that successful renal transplantation in SIOD does not stop the progression of cerebral vascular stenosis, suggesting that renal disease alone is not the main reason for the progression of cerebral angiopathy<sup>14, 17</sup>.

Efforts should be made in early diagnosing such subgroups of patients in order to enable them for early preventive revascularization surgery<sup>9</sup>.

Antiplatelet agents are recommended in Japanese 2012 guidelines for the treatment of ischemic MMD<sup>13</sup>. The J-ASPECT study demonstrated that prehospital use of Aspirin® in patients with ischemic MMD in Japan was associated with a better functional status on hospital admission<sup>21</sup>. The combination of two antiplatelet drugs (Aspirin® and dipyridamole as the most common) seems to be more effective than a single antiplatelet drug in preventing early stroke recurrence<sup>22</sup>.

Most pediatric MMD patients were treated with Aspirin® alone, but 20–50% were on anticoagulation therapy<sup>8, 19</sup>. Initially, our patient was treated with Aspirin® alone. As the neurological disease progressed, dipyridamole was added, but it did not affect the appearance of her recurrent ischemic attacks. She was refused revascularization surgery due to her low body weight and poor general condition. Since there were no other treatment options, off label use of combined antiplatelet and

anticoagulation therapy was attempted bearing in mind to keep her INR below 3. That was based on adult data which suggested that adding warfarin to Aspirin® reduces the risk of thromboembolic stroke by one-quarter with a non-significant increase in intracranial bleeding and no difference in deaths<sup>22</sup>. A repeated MRI showed the presence of bilateral subdural hematomas, which obviously was the adverse effect of combined antiplatelet and anticoagulation therapy. On the other hand, such therapy showed no effect on reducing the rate of ischemic strokes, pointing toward the concept that atherosclerotic thrombosis is not the basic mechanism of progressive cerebral arterial stenosis in MMD patients.

### Conclusion

One of the major observations that emerge from this case report is that mild neurological symptoms appeared very

early in the course of the disease before any other system was clinically affected. Nearly half of SIOD patients have neurologic symptomatology. In such a case, the course of the disease and the final outcome of the patient we presented would, at least partly, be avoided. Finally, with advances in genetic diagnostics, a step forward toward genetic therapy for MMD is reasonably expected.

### Informed consent statement

Consent was obtained from the patient's mother for the publication of this report and any accompanying images.

### Conflict of interest

The authors declare no conflict of interest.

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# Giant intrapericardial lipoma: clinical and forensic implications

## Veliki intraperikardni lipom: kliničke i forenzičke implikacije

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### Abstract

**Introduction.** Even though lipomas are the most common benign tumors, they are rarely found in the pericardial cavity. Although histopathologically benign, they can cause life-threatening complications by rapid growth and may therefore be clinically considered malignant. **Case report.** We present an 80-year-old female who was injured during a syncopal episode when falling from a standing height and suffered bodily injuries for which she was hospitalized. In the further course of her short-term hospital treatment, death occurred, and the cause of death was marked as “unknown”. At the autopsy, a dilated and tense pericardium filling up a large part of the chest cavity was noted. A well-encapsulated soft tissue mass, 20 × 18 × 3 cm in size, weighing 820 g, was visualized in the pericardial cavity. Histopathological examination revealed that the mass was a lipoma and showed acute myocardial necrosis; therefore, it was assumed that the cause of death was probably due to the compression of lipoma on coronary arteries. **Conclusion.** Even though intrapericardial lipomas are benign tumors, they can cause life-threatening complications and sudden cardiac death. There are numerous diagnostic methods capable of detecting intrapericardial lipomas, and with timely treatment, the patient can be cured.

### Key words:

autopsy; cause of death; diagnosis; lipoma; myocardial infarction; pericardium; syncope.

### Apstrakt

**Uvod.** Lipomi su najčešći benigni tumori, ali su retko lokalizovani u perikardnoj šupljini. Mada benigni po svojim patohistološkim karakteristikama, zbog ubrzanog rasta i posledičnih komplikacija koje mogu ugroziti život, mogu se klinički smatrati malignim. **Prikaz bolesnika.** Prikazana je 80-godišnja žena, koja je nakon epizode sinkope, pri padu sa sopstvene visine, zadobila telesne povrede zbog kojih je hospitalizovana. U daljem toku kratkotrajnog bolničkog lečenja nastupio je smrtni ishod, a uzrok smrti označen je kao „nepoznat”. Obdukcijom je utvrđen uvećan i napet perikard, koji je ispunjavao značajan deo grudne duplje. Dobro inkapsulirana tkivna masa dimenzija 20 × 18 × 3 cm, težine 820 g, uočena je intraperikardno. Histopatološkom analizom pokazano je da je uočena promena lipom, a na srčanom mišiću uočeni su znaci akutne nekroze miokarda, na osnovu čega je pretpostavljeno da je infarkt miokarda najverovatnije nastao kao posledica pritiska lipoma na koronarne arterije. **Zaključak.** Intraperikardni lipomi mogu biti maligni po lokalizaciji i dovesti do iznenadne srčane smrti. Postoje različite dijagnostičke metode pomoću kojih je moguće otkrivanje intraperikardnih lipoma, a pravovremenom intervencijom bolesnik može biti izlečen.

### Ključne reči:

autopsija; smrt; uzrok; dijagnoza; lipom; infarkt miokarda; perikard; sinkopa.

### Introduction

Primary pericardial tumors are rare, with an estimated prevalence of 0.00–0.007%<sup>1</sup>. These tumors can be benign (teratoma, fibroma, angioma, lipoma) or malignant (mesothelioma, sarcoma)<sup>2</sup>. Lipomas are the most common benign tumors usually seen in the subcutaneous tissue but may also be deep-seated. They are infrequently seen in the thoracic

cavity and even less frequently in the pericardial cavity<sup>3,4</sup>. Cardiac lipomas can originate from the subendocardium, subpericardium, or myocardium<sup>5</sup>. Lipomas usually grow slowly, and patients may remain asymptomatic for many years. Therefore, most intrapericardial lipomas are detected as accidental findings during an autopsy<sup>6</sup>. Lipomas can be diagnosed using noninvasive imaging methods such as echocardiography, computed tomography, or magnetic resonance.

If diagnosed, lipomas are usually in the advanced stage of development and present as extremely large masses that, if not asymptomatic, cause symptoms including effort angina, fatigue, atrial arrhythmias (by compressing the coronary arteries), and dyspnea (by tamponade)<sup>7,8</sup>.

Herein, we present a rare case of cardiac death caused by compression of intrapericardial lipoma on coronary arteries.

### Case report

We present an 80-year-old female who was injured when falling from a standing height after experiencing a syncope episode. On admission to the secondary care hospital, she presented with dizziness, breathlessness, and chest pain. She had a history of hypertension. The main finding on physical examination was systolic ejection murmur grade III/VI, while the rest of the examination was unremarkable. The electrocardiogram (ECG) revealed sinus rhythm, 90 beats per minute, and left ventricle hypertrophy with secondary repolarization abnormalities. Focus transthoracic echocardiogram (TTE) showed concentric left ventricle hypertrophy, nonsevere aortic stenosis, and preserved ejection fraction (65%), with no revealed pericardial mass or effusion.

On the second day after admission, she suddenly deteriorated. Initial ECG showed atrioventricular dissociation that

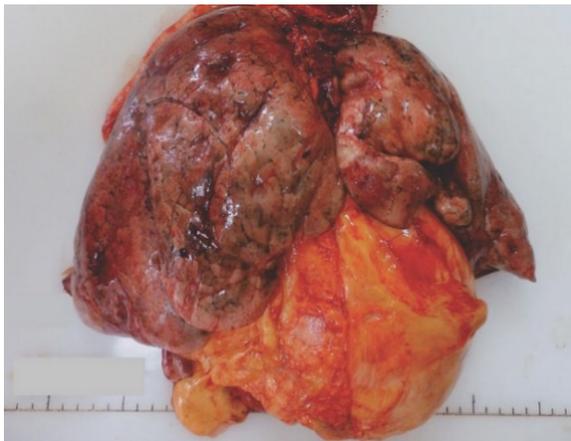
progressed to cardiopulmonary arrest. Following unsuccessful resuscitation attempts, she was pronounced dead.

The patient was of average osteomuscular build, with a body mass index of 24 kg/m<sup>2</sup>. During the autopsy, an external examination revealed multiple bruises, excoriations, and superficial lacerations on the left side of the face. During the internal examination of the thorax, a dilated pericardium filling up a large part of the chest cavity was noted (Figure 1).

A well-encapsulated soft tissue mass, 20 × 18 × 3 cm in size, weighing 820 g was observed in the pericardial cavity (Figure 2).

The mass was free in the pericardial sack, located behind the left auricle, and connected with a vascular peduncle to the left atrium (Figure 3).

The heart itself weighed 460 g. The left ventricular wall was 16 mm thick, and the aortic valve was stenotic, with a ring circumference of 6 cm. An ischemic area, 2 × 2 cm in size, was noted in the interventricular septum (IVS), and it was observed that the anterolateral papillary muscle (ALPM) was pale, with hemorrhages. The three major coronary arteries had numerous atherosclerotic plaques that were calcified in some parts. The level of their stenosis was approximately 60%. Other macroscopic findings were unremarkable. Histopathological examination revealed that the tumor mass was a lipoma composed of mature fat cells (Figure 4).



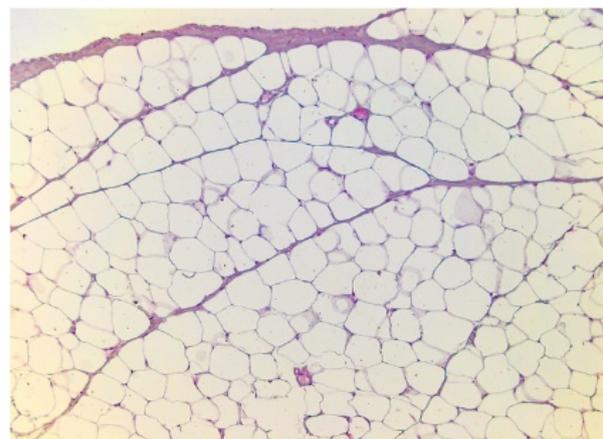
**Fig. 1 – Dilated pericardium filling up a large part of the chest cavity.**



**Fig. 2 – Well-encapsulated soft tissue mass in the pericardium.**



**Fig. 3 – Free tumor in the pericardial sack, connected to the left atrium, just behind the auricula by a vascular peduncle.**



**Fig. 4 – Histological appearance of the intrapericardial lipoma: mature fat cells (hematoxylin-eosin, ×40).**

The heart tissue showed acute ischemia. Therefore, we concluded that the cause of death was myocardial infarction (MI), probably due to the compression of lipoma on coronary arteries.

### Discussion

In our opinion, the most important autopsy findings were macroscopic and microscopic findings on the heart and pericardium. The examination of the pericardium showed a well-encapsulated lipoma located behind the left auricle and connected with a vascular peduncle to the left atrium. Furthermore, the aortic stenosis, as well as the stenosis of coronary arteries, were observed, but individually, they were not severe enough to cause the death of the patient. Therefore, it was assumed that the cause of death was MI, probably due to the compression of lipoma on coronary arteries. This hypothesis is encouraged by the fact that an ischemic area in the IVS, as well as ischemia of the ALPM, were noted. It is well-known that the left anterior descending artery (LAD), through its branches, provides blood supply for a major part of the left ventricular myocardium, as well as the anterior and mid thirds of the IVS<sup>9</sup>. Its first diagonal branch provides blood for ALPM<sup>10</sup>, while the anterior part of the IVS obtains blood from the septal branches of the LAD<sup>9</sup>. Bearing in mind the anatomic location of the mentioned blood vessels, in addition to the location of the lipoma, there is a strong possibility that the compression of these arteries caused by the tumor mass leads to the ischemia of the IVS and ALPM.

On the other hand, the question arises whether the pressure of the intrapericardial lipoma was enough to completely stop the blood flow through the coronary arteries and cause myocardial necrosis. We speculate that, in this case, aortic stenosis was a significant contributing factor. As previously mentioned, aortic stenosis was probably not severe enough to individually cause myocardial ischemia. Nevertheless, patients with aortic stenosis are susceptible to myocardial ischemia due to increased metabolic demands of the hypertrophic myocardium, which, in case of decreased blood supply, could have been contributing factor for myocardial ischemia<sup>11,12</sup>. Although the ischemic zone was not extensive, we hypothesize that the alteration of metabolism in cardiac myocytes caused by the ischemia leads to malignant ventricular arrhythmia and sudden cardiac death.

MI with nonobstructive coronary arteries (MINOCA) is an uncommon but well-documented phenomenon, and one of the potential causes includes external compression of the coronary arteries. However, reports of extrinsic compression

of epicardial coronary arteries are uncommon. Gue et al.<sup>13</sup> presented a case of a 44-year-old patient with MINOCA due to compression of coronary arteries caused by the enlarged mediastinal lymph nodes in Hodgkin lymphoma. Aggarwala et al.<sup>14</sup> showed the case of a 71-year-old female patient who presented with findings suggestive of an acute MI due to extrinsic cardiac mass encasing the left circumflex and right coronary arteries (RCA), which caused compression and spasticity of the RCA. However, there are no reports in the literature focusing on cases with similar clinical presentation in patients with intrapericardial lipoma.

Although being the first-choice method in identifying intrapericardial masses, the diagnostic value of echocardiography has its shortcomings. Transesophageal echocardiogram (TEE) can provide more accurate imaging than TTE, but either TTE or TEE is insufficient to distinguish pericardial adipose tissue from lipoma. Therefore, additional computed tomography or magnetic resonance imaging is often needed since they are able to give a more comprehensive view of the structure and its origin<sup>15,16</sup>. In this case, even though the lipoma was very large, it was neglected with the TTE exam. The possible explanation might be the lack of time for a detailed examination since the TTE examination was performed in the emergency room shortly after the syncopal episode. However, some case reports describe a successful diagnosis of cardiac lipoma using TTE<sup>17</sup>. Therefore, bearing in mind its low price and availability, TTE should be the first-line examination for suspect cardiac lipoma.

Neoplasms, either primary cardiac tumors such as pericardial lipoma or metastatic disease, are a rare cause of extrinsic compression of coronary arteries; therefore, there is a paucity of papers focusing on their clinical effects<sup>14</sup>. That is why they are often neglected by clinicians. However, as we have shown, they can lead to a fatal outcome; thus, considering pericardial tumors in patients presenting with chest pain and no sign of coronary artery disease is essential for establishing the diagnosis and initiating appropriate treatment.

### Conclusion

Even though intrapericardial tumors are often histopathologically benign, they can cause life-threatening complications and sudden cardiac death. However, these tumors are seldom considered and often overlooked and mistreated. There are numerous diagnostic methods capable of revealing them; therefore, careful examination of the patient is always required because, with adequate diagnosis and timely treatment, the majority of these patients can be cured.

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## Isolated fracture of the lesser tuberosity of the humerus – a rare injury that requires surgical treatment

Izolovani prelom malog tuberkuluma humerusa – retka povreda koja zahteva operativno lečenje

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### Abstract

**Introduction.** An isolated fracture of the small tuberosity is a very rare injury that can often be overlooked. That injury is significant due to the function of the *subscapularis* muscle, which loses its attachment by separating the small tuberosity. **Case report.** A 45-year-old male, former athlete, suffered a left shoulder injury while falling downhill. Although the clinical picture was not convincing, a computed tomography scan showed a fracture. Open repositioning and osteosynthesis were performed. After rehabilitation, the patient regained a full active range of motion in the shoulder joint three months after the operation. We present the mechanism of injury, clinical picture, necessary diagnostics, and technique of surgical treatment of isolated fracture lesser tuberosity of the humerus. The results of other authors whose series are also quite small were analyzed. **Conclusion.** It was concluded that an isolated fracture of the small tuberosity requires surgical treatment to preserve a good range of motion in the shoulder joint.

**Key words:** diagnosis; fractures, avulsion; humeral fractures; orthopedic procedures; tomography, x-ray, computed.

### Apstrakt

**Uvod.** Izolovani prelom malog tuberkuluma je veoma retka povreda, koja se često može prevideti. Takva povreda je značajna zbog funkcije podlopatičnog mišića, koji odvajanjem malog tuberkuluma gubi svoj pripoj. **Prikaz bolesnika.** Muškarac, bivši sportista, star 45 godina, zadobio je povredu levog ramena pri padu na nizbrdici. Mada klinička slika nije bila ubedljiva, snimak kompjuterizovanom tomografijom pokazao je postojanje preloma. Učinjena je otvorena repozicija i osteosinteza. Posle sprovedene rehabilitacije, tri meseca posle operacije, pacijent je vratio pun aktivan obim pokreta u zglobu ramena. Prikazujemo mehanizam povrede, kliničku sliku, neophodnu dijagnostiku i tehniku hirurškog lečenja pacijenata sa izolovanim prelomom malog tuberkuluma humerusa. Analizirani su rezultati drugih autora, čije su serije analiziranih slučajeva takođe jako male. **Zaključak.** Izolovani prelom malog tuberkuluma zahteva hirurško lečenje kako bi se očuvao dobar obim pokreta u ramenom zglobu.

**Ključne reči:** dijagnoza; prelomi, avulziona; humerus, prelomi; ortopedske procedure; tomografija, kompjuterizovana, rendgenska.

### Introduction

Isolated lesser tuberosity fracture is an extremely rare injury. In the series used as the basis for the Müller AO Classification (AO classification), these fractures represented only 2 of the 730 analyzed cases<sup>1</sup>. In their work, Robinson et al.<sup>2</sup> estimate that this injury occurs in 0.46 cases of humeral injuries per 100,000. As with the greater tuberosity fracture, which occurs due to anterior shoulder dislocation, when it

comes to lesser tuberosity fractures, posterior dislocation should be suspected<sup>3</sup>. Lesser tuberosity fracture is often associated with severe trauma, which includes multiple proximal humerus fractures. In order to be isolated, nondisplaced fractures of the anatomical or surgical neck of the humerus should be excluded.

Anatomically lesser tuberosity is significant due to the subscapularis muscle attachment, which represents the dynamic front stabilizer of the joint and provides its internal rotation.

These fractures are often associated with posterior shoulder dislocation; after its reduction, the fracture of the lesser tuberosity can be overlooked. The clinical picture in the acute phase is also not convincing: less pain in the front of the shoulder, lesser restriction of movement, and minimal swelling and bruising. The anatomical position of the small tubercle is such that its fracture is often not seen on regular X-rays<sup>4</sup>. Therefore, it is considered that the percentage of overlooked fractures is very high. Vezeridis et al.<sup>5</sup> demonstrated that 75% of the initial radiographs were interpreted as normal, with the diagnosis identified only on subsequent magnetic resonance imaging (MRI). Axial X-rays help detect the level of displacement, although today, the supreme method for diagnosis is a computed tomography (CT) scan. If the fracture is not diagnosed and treated adequately, there is an impairment of shoulder function with limited internal rotation.

### Case report

A 45-year-old male, a former athlete, suffered a left shoulder injury while falling downhill. It has been four days since the injury; the patient complained of pain in the anterior aspect of the shoulder joint. According to the patient, his arm was abducted and in external rotation when he fell. A

clinical examination showed local pain on palpation as well as a hematoma in the anterior aspect of the shoulder joint and on the left side of the chest. He actively performed all shoulder movements except total internal rotation. An anteroposterior radiography of the shoulder was performed, on which a larger ossification was seen in the *supraspinatus* tendon, and a fracture of the small tubercle was suspected (Figure 1). Therefore, a CT diagnosis was made, which confirmed the fracture, with separation and caudal displacement of the small tubercle (Figure 2).

Surgery was performed through a 5 cm-long deltopectoral incision, which was sufficient to access the fracture site and the dislocated fragment. The lesser tuberosity was pulled by the force of the subscapularis muscle medially and distally. In order not to damage the bone fragment during repositioning, the tendon of the subscapularis muscle was sutured with the Krackow stitch technique, and, together with the fragment, it was pulled toward the anatomical site. The lesser tuberosity was again placed in the anatomical position and fixed with a 4.0 mm titanium cannulated screw, and one Arthrex PushLock® SP 4.5 mm suture anchor was used for tendon fixation in the humeral head. Calcification in the supraspinatus tendon was identified and removed, while the defect was closed side-to-side with an Arthrex FiberWire® 2-0 suture (Figure 3).



**Fig. 1 – X-ray radiograph of the shoulder (anterior/posterior view) shows a large ossification in the *supraspinatus* tendon and a suspected fracture of the small tubercle.**



**Fig. 2 – Computed tomography image of the shoulder shows the fracture with separation and caudal displacement of the small tubercle.**



**Fig. 3 – Intraoperative X-ray finding shows fracture-related defect closed side-to-side with an Arthrex FiberWire® 2-0 suture.**

The patient wore Desault's bandage immobilization for the next 3 weeks while elbow movements were allowed. After that, shoulder passive motion was started (shoulder continuous passive motion – CPM machine) for the next 2 weeks. A program of exercises to strengthen the rotator cuff muscles followed. The patient returned to the full active range of motion after 3 months.

## Discussion

Isolated lesser tuberosity fracture is an extremely rare injury. Therefore, the experience in the treatment is very limited. Rare works in the literature are mostly reduced to case reports<sup>6-20</sup>. The lesser tuberosity is protected from direct injuries due to its small size and its position on the medial side of the humerus. Most authors believe that the main mechanism of injury is excessive muscle pull<sup>7, 8</sup>. When the subscapular muscle forcefully contracts to resist abduction and external rotation of the shoulder, the resultant strong traction force avulses the lesser tuberosity. Less probable mechanisms, such as stress fractures and fractures due to extreme internal rotation and shoulder extension (back-reach position), as well as fractures due to epileptic seizures and during electroconvulsive therapy for psychiatric disorders, have also been described in the literature<sup>10, 11, 21</sup>. Symptoms specific to acute cases include pain in the anterior aspect of the shoulder that worsens with external rotation and limits the internal rotation of the joint. On plain radiographs, these fractures may be misdiagnosed as calcific tendinitis of the rotator cuff or osseous Bankart lesions<sup>12</sup>. An AP radiograph in maximal internal rotation sometimes does not provide a good projection of the lesser tuberosity. Large, displaced fractures can be seen on standard AP radiographs, while smaller fragments need the axillary view in order to reveal the degree of their displacement. An axillary view is sometimes not easy to do due to pain. A supreme method in diagnosis today is CT, and it is most often necessary in diagnosing this injury. Although MRI is not necessary, it allows the evaluation of the entire rotator cuff and better visualization of a minimally displaced fragment<sup>13, 20, 22</sup>.

Although the literature describes cases of nonsurgical treatment with a satisfactory result<sup>23</sup>, our opinion is that surgical treatment must be considered in dislocated fractures, primarily due to the active role of the subscapularis muscle in the internal rotation of the shoulder joint. If such an injury

is neglected, the patient will certainly not have the full range of motion in the shoulder, primarily the internal rotation. It is clinically manifested by positive tests for damage to the subscapularis (lift-off or belly-press test). The stability of the joint should not be forgotten, as cases of instability resulting from the lack of a dynamic role of the subscapularis muscle have also been described<sup>14</sup>. Medial dislocation of torn fragments reduces the coracohumeral distance, which can restrict movements, create coracoid impingement, and cause pain<sup>15</sup>.

Most of the acute cases that have been reported in the literature were treated by open reduction and internal fixation, resulting in an excellent clinical outcome (Table 1).

For acute nondisplaced fractures of the lesser tuberosity, nonoperative treatment can provide satisfactory results. It is necessary to be careful as a contraction of the subscapularis muscle can lead to retraction of the fractured tuberosity, which requires operative treatment<sup>8</sup>. Operative treatment is recommended for patients with displacement greater than 5 mm, fragment angulation greater than 45°, limited joint mobility, significant clinical weakness, or continued pain<sup>16</sup>.

Research has shown that pediatric patients have excellent outcomes after lesser tuberosity fractures and may benefit more from surgery in comparison to nonoperative treatment. While the outcomes of adults are also acceptable, the fact that one-third of patients do not regain the full range of motion without surgery implies the advantage of surgical treatment<sup>17</sup>.

The surgical approach to treatment is anterolateral-deltopectoral. A long head of the bicep should be identified to make sure that it is not medially displaced into the joint. There is a possibility of dislocation of the biceps tendon in fractures of the lesser tuberosity, which affects sulcus bicipitalis. In the case of an acute fracture of a small tubercle, the subscapularis is freely mobile, and if the bone fragment is large, it should be repositioned and fixed with two 4.0 cannulated screws. If the fragment is small and soft, the fixation is performed with strong non-absorbable sutures and anchors, using a similar technique used in the reconstruction of the subscapularis tear. In old cases, the subscapularis becomes shorter, and the bone fragment is retracted medially, so there is a need for its release and mobilization of muscle and tendons. Repairing such a condition is usually difficult due to scars, retraction of the bone fragment, and muscular fatty degeneration. The result may be slightly less external rotation after reconstruction.

**Table 1**

### Characteristics of the patients that have been reported about the topic in the literature

Authors	Number of pts.	Age-gender	Duration	Method of treatment	Outcome
Kanso and Bricout <sup>4</sup>	1	31-f	acute	ORIF	excellent
Tosun and Kesemenli <sup>6</sup>	2	19-m, 33-f	acute	ORIF	excellent
Ogawa and Takahashi <sup>12</sup>	10	12-m, 56-m, 68-f, 28-m, 17-m, 50-f, 13-m, 31-m, 15-m, 11-m	6 acute 4 chronic	ORIF in 5 pts. (3 acute, 2 chronic) 5 non-operative (3 acute, 2 chronic)	6 excellent 4 very good
Paschal et al. <sup>16</sup>	2	14-f, 15-m	chronic	ORIF	1 excellent, 1 good
Cheng et al. <sup>19</sup>	1	15-m	chronic	ORIF	excellent
Levine et al. <sup>20</sup>	1	14-m	acute	ORIF	excellent

pts – patients; m – male; f – female; ORIF – open reduction and internal fixation.

Rehabilitation after this operation is individualized and similar to the rehabilitation after surgical treatment of a torn rotator cuff<sup>18</sup>.

### Conclusion

Isolated lesser tuberosity fracture is a rare injury that most often requires surgical treatment. Additional diag-

nostic is needed because the fracture can easily be overlooked. This injury affects the function of the rotator cuff. Separation and poor healing of the lesser tuberosity reduce the role of the subscapularis. The prerequisite for a good functional outcome of isolated lesser tuberosity fractures is anatomical reposition and stable fixation. Only then can we expect a good range of motion of the shoulder joint.

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