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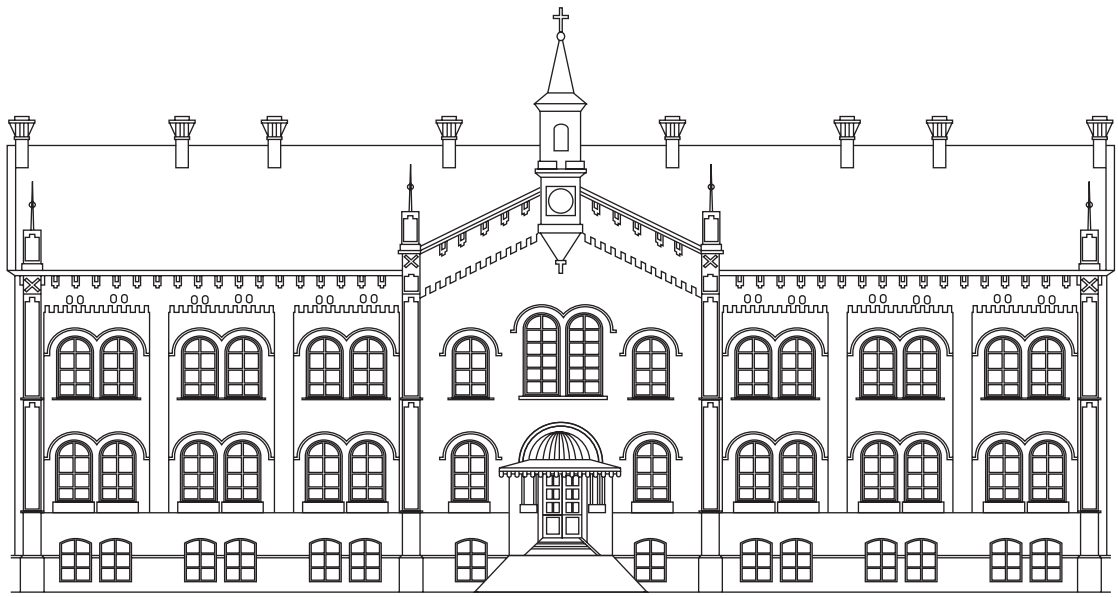

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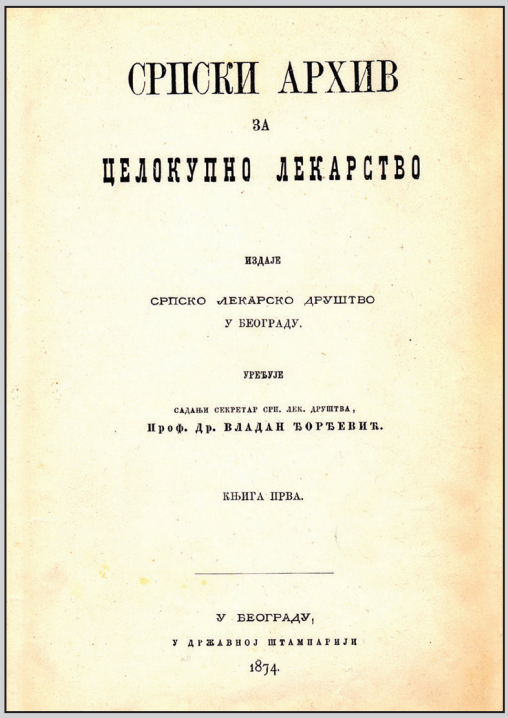

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ЗА

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1874.

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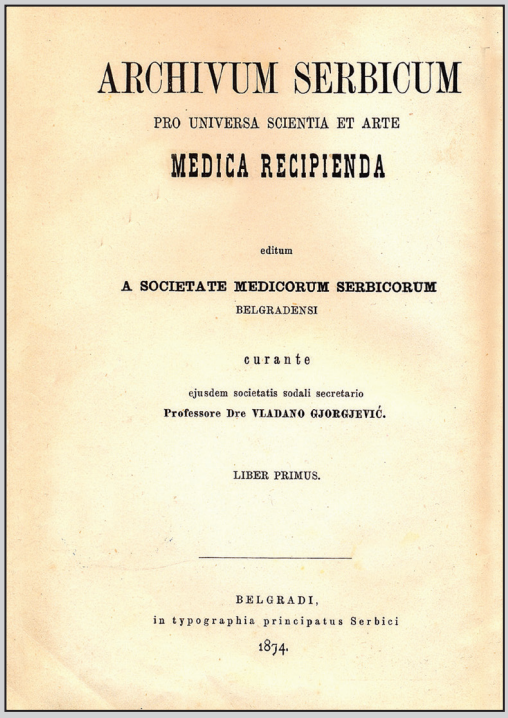

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1874.

The title page of the first journal volume in Latin

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ORIGINAL ARTICLES • ОРИГИНАЛНИ РАДОВИ

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Timing of clinical eruption of permanent teeth in children with molar incisor hypomineralization

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SUMMARY

Introduction/Objective Molar incisor hypomineralization (MIH) is a developmental defect and it has a multifactorial etiology; there could be variations in dental eruptions in the children with this condition. The aim of this study was the comparison of the clinical eruption status of the permanent teeth in children with MIH and patients without MIH.

Methods The study group comprised a total of 300 children (176 females and 124 males aged 6–12 years) who had been diagnosed with MIH but had no systemic disease. The control group comprised 300 age- and sex-matched children without MIH. In the study and control groups, the eruption of the permanent teeth (excluding third molars) was evaluated and compared. In addition, this comparison was performed separately for the males and females in the study and control groups. The independent samples t-test was used for statistical analysis.

Results No statistically significant difference was found between the mean age of the dental eruptions of the children with MIH and that of the children without mineralization disorders ($p > 0.05$). Regarding the mean age of the dental eruptions, the sex-matched comparison revealed no statistically significant difference between the study and control groups ($p > 0.05$).

Conclusion Although there was no statistically significant difference in the MIH group and the healthy control group regarding the mean age of the eruption of all teeth, a trend of accelerated dental development in the MIH group was observed.

Keywords: tooth eruption; molar incisor hypomineralization; developmental enamel defect

INTRODUCTION

Molar incisor hypomineralization (MIH) is defined as the hypomineralization of one or more permanent first molar. It is also sometimes associated with affected incisors. It is a developmental enamel defect without a definite etiology or general distribution. It is thought to be caused by ameloblasts that have been affected by local and systemic factors during the formation of enamel [1]. Studies have reported on eruption disorders in patients with developmental enamel defects other than MIH [2, 3]. The eruption is a dynamic process that begins at the initial positioning of the tooth in the alveolar bone and continues until the final positioning at which it is occluded by a dental antagonist. This is an important part of the developmental period [4]. MIH, which is thought to occur in relation to factors encountered in the development process, appears to be an important health problem. Because MIH is a developmental defect and it has a multifactorial etiology, there could be variations in dental eruptions in children with this condition. This study was planned after the literature survey identified the absence of evaluations of the effect of MIH on the timing of clinical eruption.

The purpose of this study was the comparison of the clinical eruption status of the permanent teeth of children with MIH and the patients without MIH. In addition, the timing of permanent tooth eruption in children with MIH was investigated.

METHODS

The study was approved by the Clinical Research Ethics Committee of Zonguldak Bulent Ecevit University (Protocol No.: 2017-75-09/08). To establish the study and control groups, 385 patients were examined and diagnosed with MIH. They had all applied for dental examinations at the Department of Pedodontics at Zonguldak Bülent Ecevit University between August 2017 and August 2018. In addition to the dental evaluation of all patients, medical anamneses were also taken. The study excluded patients with permanent teeth that were missing for reasons other than the extraction of the permanent first molars because of MIH (e.g., congenitally missing teeth or trauma). Other exclusions were children outside the age range of 6–12 years who exhibited localized pathology and severe malocclusion (e.g., marked skeletal mismatch or obstructed teeth), were already undergoing orthodontic treatment, presented with any systemic disease, and exhibited dental mineralization disorders other than MIH.

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The study group comprised a total of 300 children (176 females and 124 males aged 6–12 years) who had been diagnosed with MIH but had no systemic disease. The control group comprised 300 age- and sex-matched healthy children with no systemic disease or dental mineralization disorder.

Examination and assessment

In the present study, the permanent first molars and permanent incisors of children aged 6–12 years were examined for the presence of MIH. Halogen reflector lighting, a mirror, and a probe were used in the dental unit in accordance with the criteria established by Ghanim et al. [5]. The criteria include the presence or absence of demarcated opacities, destruction of enamel after eruption, atypical caries, and MIH-induced tooth extractions. The examination for MIH was conducted without drying the teeth. If necessary, cotton pellets were used to remove any residue on the teeth. The diagnosis of MIH was based on the detection of at least one affected permanent first molar.

The patients with only affected incisors were not diagnosed with MIH because these defects can be caused by local factors, such as trauma and caries [6, 7]. For the patients who lacked the teeth under examination because of extraction, the presence or absence of demarcated opacities in the other teeth was ascertained. If the other teeth had demarcated opacities, then the patient was considered to be affected by MIH. In this study, amelogenesis imperfecta (AI), dentinogenesis imperfecta, hypoplasia, diffuse opacities, white spot lesions, tetracycline-induced colorings, and fluorosis were considered to be the differential diagnosis.

In the study and control groups, permanent tooth eruption was evaluated in accordance with the criteria of Pahkala et al. [8]. According to these criteria, the eruption of each permanent tooth is evaluated on the basis of four codes:

Code 0: the tooth is not visible in the oral cavity;

Code 1: at least one tubercule of the tooth is visible in the oral cavity;

Code 2: the entire occlusal surface or mesiodistal width of the tooth is visible;

Code 3: the tooth is in occlusion.

In comparison of the eruption ages of the permanent teeth of the children in the study and control groups, tooth eruption was assumed to have occurred if any part of the crown was visible on the oral mucosa (Codes 1, 2, and 3). For each participant in the study and control groups, the eruption ages of teeth 11, 12, 13, 14, 15, 16, 17, 21, 22, 23, 24, 25, 26, 27, 31, 32, 33, 34, 35, 36, 37, 41, 42, 43, 44, 45, 46, and 47 were recorded. The mean age of dental eruption in the children in the study group was compared with that of the control group. In addition, this comparison was performed separately for the males and females in the study and control groups.

Statistical analysis

The data were analyzed with IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA). The independent samples t-test was used for the intergroup comparison of the ages. The results of the analysis of the quantitative data are presented as the mean ± standard deviation. For the qualitative data, they are presented as frequencies (percentages). The level of significance was set as $p < 0.05$.

RESULTS

The distribution of the mean ages of the patients in the study and control groups according to their sex is presented in Table 1.

Table 1. Distribution of the mean age of the patients and control group subjects in the study according to sex

Group	Females mean age ± SD	Males mean age ± SD	Total mean age ± SD
MIH	8.97 ± 0.14	8.7 ± 0.14	8.83 ± 0.1
Control	8.99 ± 0.14	8.96 ± 0.14	8.97 ± 0.1

MIH – molar incisor hypomineralization

Table 2. Mean age of permanent tooth eruption in patients and control group subjects

Tooth	MIH mean age ± SD	Control group mean age ± SD	Difference mean age ± SD	p
11	9.15 ± 0.1	9.36 ± 0.1	-0.21 ± 0.14	0.138
12	9.8 ± 0.1	9.89 ± 0.11	-0.09 ± 0.15	0.553
13	11.42 ± 0.16	11.37 ± 0.14	0.05 ± 0.21	0.797
14	10.51 ± 0.15	10.76 ± 0.14	-0.26 ± 0.21	0.233
15	11.01 ± 0.16	11.23 ± 0.14	-0.22 ± 0.22	0.314
16	8.98 ± 0.1	9.02 ± 0.1	-0.04 ± 0.14	0.759
17	11.89 ± 0.12	11.78 ± 0.13	0.11 ± 0.18	0.541
21	9.11 ± 0.1	9.35 ± 0.1	-0.25 ± 0.14	0.086
22	9.81 ± 0.1	9.88 ± 0.11	-0.07 ± 0.15	0.621
23	11.62 ± 0.11	11.41 ± 0.12	0.21 ± 0.16	0.195
24	10.56 ± 0.14	10.78 ± 0.12	-0.22 ± 0.19	0.245
25	11.13 ± 0.13	11.26 ± 0.14	-0.14 ± 0.19	0.480
26	8.96 ± 0.1	9.02 ± 0.1	-0.06 ± 0.14	0.652
27	11.89 ± 0.13	11.62 ± 0.12	0.27 ± 0.19	0.161
31	8.95 ± 0.1	9.04 ± 0.1	-0.09 ± 0.14	0.526
32	9.22 ± 0.1	9.41 ± 0.1	-0.19 ± 0.14	0.092
33	10.93 ± 0.14	11.21 ± 0.11	-0.28 ± 0.18	0.123
34	10.82 ± 0.15	10.98 ± 0.13	-0.15 ± 0.2	0.441
35	11.04 ± 0.15	11.23 ± 0.14	-0.18 ± 0.21	0.377
36	8.94 ± 0.1	9.02 ± 0.1	-0.08 ± 0.14	0.554
37	11.9 ± 0.11	11.68 ± 0.1	0.21 ± 0.16	0.179
41	8.97 ± 0.1	9.05 ± 0.1	-0.08 ± 0.14	0.561
42	9.21 ± 0.1	9.47 ± 0.1	-0.26 ± 0.15	0.075
43	10.83 ± 0.14	11.08 ± 0.12	-0.26 ± 0.19	0.178
44	10.74 ± 0.14	10.88 ± 0.14	-0.14 ± 0.2	0.475
45	11.22 ± 0.14	11.19 ± 0.14	0.02 ± 0.2	0.905
46	8.93 ± 0.1	9.02 ± 0.1	-0.09 ± 0.14	0.524
47	11.92 ± 0.1	11.75 ± 0.1	0.17 ± 0.15	0.243

MIH – molar incisor hypomineralization

The mean ages of the erupted permanent teeth (except for the third permanent molar) of the children in the study and control groups were compared. No statistically significant difference was found between the mean age of the dental eruptions of the children with MIH and that of

Table 3. Mean age of permanent tooth eruption in females in the patients and control group subjects

Tooth	MIH mean age ± SD	Control group mean age ± SD	Difference mean age ± SD	p
11	9.18 ± 0.14	9.46 ± 0.14	-0.28 ± 0.2	0.150
12	9.88 ± 0.14	9.94 ± 0.14	-0.06 ± 0.2	0.778
13	11.44 ± 0.22	11.29 ± 0.17	0.15 ± 0.27	0.583
14	10.48 ± 0.2	10.73 ± 0.18	-0.25 ± 0.27	0.344
15	11.01 ± 0.21	11.27 ± 0.15	-0.26 ± 0.25	0.307
16	9.07 ± 0.14	9.07 ± 0.14	0.01 ± 0.2	0.978
17	11.8 ± 0.17	11.68 ± 0.15	0.12 ± 0.24	0.610
21	9.14 ± 0.14	9.44 ± 0.14	-0.3 ± 0.2	0.129
22	9.86 ± 0.15	9.97 ± 0.14	-0.11 ± 0.2	0.574
23	11.65 ± 0.13	11.37 ± 0.15	0.28 ± 0.2	0.158
24	10.73 ± 0.18	10.89 ± 0.14	-0.16 ± 0.23	0.487
25	11.22 ± 0.16	11.21 ± 0.17	0.01 ± 0.24	0.977
26	9.04 ± 0.14	9.06 ± 0.14	-0.02 ± 0.19	0.900
27	11.81 ± 0.16	11.54 ± 0.15	0.27 ± 0.23	0.257
31	9.03 ± 0.14	9.1 ± 0.14	-0.06 ± 0.19	0.746
32	9.25 ± 0.14	9.62 ± 0.14	-0.38 ± 0.2	0.059
33	10.92 ± 0.17	11.18 ± 0.14	-0.26 ± 0.22	0.242
34	10.95 ± 0.17	11.02 ± 0.16	-0.07 ± 0.23	0.778
35	11.26 ± 0.17	11.11 ± 0.18	0.15 ± 0.25	0.552
36	9.01 ± 0.14	9.07 ± 0.14	-0.06 ± 0.19	0.752
37	11.83 ± 0.13	11.64 ± 0.12	0.19 ± 0.19	0.313
41	9.07 ± 0.14	9.11 ± 0.14	-0.04 ± 0.19	0.837
42	9.25 ± 0.14	9.57 ± 0.14	-0.32 ± 0.2	0.105
43	10.82 ± 0.17	11.03 ± 0.15	-0.21 ± 0.23	0.368
44	10.87 ± 0.18	10.96 ± 0.17	-0.09 ± 0.25	0.721
45	11.37 ± 0.16	11.31 ± 0.15	0.06 ± 0.22	0.775
46	9 ± 0.14	9.09 ± 0.14	-0.09 ± 0.2	0.645
47	11.88 ± 0.13	11.71 ± 0.13	0.17 ± 0.19	0.377

MIH – molar incisor hypomineralization

Table 4. Mean age of permanent tooth eruption in males in the patients and control group subjects

Tooth	MIH mean age ± SD	Control group mean age ± SD	Difference mean age ± SD	p
11	9.09 ± 0.15	9.21 ± 0.14	-0.12 ± 0.21	0.569
12	9.68 ± 0.14	9.8 ± 0.16	-0.12 ± 0.22	0.571
13	11.39 ± 0.19	11.63 ± 0.19	-0.24 ± 0.28	0.400
14	10.58 ± 0.24	10.86 ± 0.24	-0.28 ± 0.35	0.432
15	11.01 ± 0.26	11.09 ± 0.34	-0.07 ± 0.43	0.868
16	8.85 ± 0.14	8.95 ± 0.14	-0.11 ± 0.2	0.603
17	12.03 ± 0.12	12.16 ± 0.14	-0.13 ± 0.19	0.523
21	9.05 ± 0.15	9.23 ± 0.14	-0.17 ± 0.21	0.399
22	9.73 ± 0.14	9.73 ± 0.16	0 ± 0.22	1.000
23	11.54 ± 0.18	11.52 ± 0.2	0.02 ± 0.27	0.956
24	10.26 ± 0.22	10.53 ± 0.24	-0.27 ± 0.32	0.399
25	10.94 ± 0.23	11.43 ± 0.2	-0.5 ± 0.32	0.136
26	8.85 ± 0.14	8.96 ± 0.14	-0.12 ± 0.2	0.571
27	12.12 ± 0.15	11.88 ± 0.18	0.25 ± 0.25	0.356
31	8.84 ± 0.14	8.96 ± 0.14	-0.12 ± 0.2	0.542
32	9.1 ± 0.15	9.33 ± 0.15	-0.23 ± 0.21	0.275
33	10.94 ± 0.28	11.27 ± 0.16	-0.33 ± 0.32	0.302
34	10.59 ± 0.26	10.88 ± 0.25	-0.29 ± 0.37	0.429
35	10.88 ± 0.28	10.86 ± 0.16	-0.03 ± 0.37	0.941
36	8.85 ± 0.14	8.96 ± 0.14	-0.11 ± 0.2	0.577
37	12.12 ± 0.15	11.83 ± 0.16	0.29 ± 0.25	0.262
41	8.83 ± 0.14	8.96 ± 0.14	-0.13 ± 0.2	0.503
42	9.15 ± 0.15	9.32 ± 0.15	-0.17 ± 0.21	0.408
43	10.85 ± 0.25	11.23 ± 0.19	-0.38 ± 0.33	0.250
44	10.54 ± 0.2	10.74 ± 0.28	-0.19 ± 0.34	0.566
45	10.94 ± 0.24	10.9 ± 0.34	0.04 ± 0.41	0.923
46	8.85 ± 0.14	8.94 ± 0.14	-0.09 ± 0.2	0.655
47	12.03 ± 0.12	11.86 ± 0.16	0.18 ± 0.21	0.419

MIH – molar incisor hypomineralization

the children without mineralization disorders ($p > 0.05$; Table 2). Regarding the mean age of the dental eruptions, the sex-matched comparison revealed no statistically significant difference between the study and control groups ($p > 0.05$; Tables 3 and 4).

The data from these comparisons showed that the patients with MIH in the study group had a lower mean age of dental eruption than those in the control group. The statistically insignificant difference was $p > 0.05$.

DISCUSSION

It is important for dentists to understand the timing of the clinical eruptions of permanent teeth because children's dental development is a progressive and changing process that can be affected by a variety of factors [9, 10]. During mixed dentition, permanent tooth eruption occurs in chronological order, thereby providing an occlusal connection. A delayed, accelerated, or modified eruption sequence could be associated with malocclusions [4]. Potential deviations in the sequence or timing of the eruption may be related to complications, such as malocclusion, crowding, impaired oral hygiene, and periodontal diseases that require dental and orthodontic treatments [4, 11]. An understanding of the normal eruption process and the possible concomitant problems is important for the diagnosis and treatment plans for eruption disorders. The dental eruption is a process that can be affected by multiple factors; thus, deviations in the timing of eruption could indicate underlying local disorders or systemic diseases. Dentists should therefore consider the timing of eruption when designing dental treatment plans for children [9]. Knowledge about dental eruption is also important for determining children's growth and developmental levels. Dentists often use this information in the treatment of and surgical interventions in orthodontic patients. This information can also be used in forensic dentistry to predict a child's chronological age [12].

The close relationships among the components of dental germ development during odontogenesis have been highlighted [4]. The disorders that might occur during amelogenesis could affect dental development by causing developmental disorders or abnormalities in the various components of the developing teeth. For example, regional odontodysplasia is a rarely seen non-hereditary developmental anomaly that affects the dental ectodermal and

mesodermal layers. The shapes of the affected teeth are usually atypical, and the structures are hypoplastic and hypocalcified. In addition, the teeth could exhibit delayed eruption or impaction. It was reported that the eruption disorders observed in such patients could be the result of structural dental anomalies [13]. It is therefore conceivable that a disorder occurring during enamel formation, which is a part of dental development, could cause variations in the eruption times of children with MIH. In addition, the teeth affected by MIH are generally very sensitive and may undergo rapid enamel loss. In some cases, tooth extraction might be a preferred treatment option [14, 15]. Because of the clinical and pathological effects of MIH, the precise prediction of dental development and eruption is very important for the design of an appropriate treatment plan.

In the evaluation of an eruption, the visibility of any part of the dental crown in the oral mucosa is often used as a clinical marker for eruption [16]. In most clinical evaluations, tooth eruption is indicated if one of the tubercules or the incisal edge is visible on the oral mucosa [17, 18, 19]. A study by Moslemi et al. [19] in Iran in 2013 compared the timing of permanent tooth eruption in a patient group comprising 207 individuals, 96 males and 111 females aged 6–19 years, with cerebral palsy to that in an age- and sex-matched healthy control group. If the clinical examination revealed that any part of the crown was visible in the mouth, tooth eruption was assumed to have occurred. In 2017, Dashash and Al-Jazar [17] investigated the timing of permanent tooth eruption in 1211 children aged 5–13 years in Syria. In accordance with the criteria established by Pahkala et al. [8], they evaluated the eruption of permanent teeth in four stages. Tooth eruption was assumed to have occurred if any part of the tooth was visible on the oral mucosa. In Uganda in 2013, Kutesa et al. [20] investigated the correlation between the timing of permanent tooth eruption and children's heights and weights. The clinical examination of permanent tooth eruptions was performed, and the intraoral eruption stage of each permanent tooth was evaluated in accordance with the four codes established by the criteria of Pahkala et al. [8]. If any part of the dental crown was visible on the intraoral mucosa, tooth eruption was assumed to have occurred.

In the present study, the intraoral status of the teeth was evaluated on the basis of the criteria of Pahkala et al. [8]. The comparison of the eruption times of the permanent teeth of the children with MIH and the children who were healthy was performed on the basis of the aforementioned evaluation. There was no statistically significant difference in the MIH and healthy control groups regarding the mean age of the eruption of all teeth. However, most of the teeth of the patients with MIH were found to have erupted earlier than those of the control group. Tunç et al. [21] compared the development of the permanent teeth of 105 children aged 7–11 years with a diagnosis of severe MIH to that of an age- and sex-matched healthy control group. Dental development was evaluated through panoramic radiographs in accordance with the dental age estimation method of Demirjian et al. [22]. As was found in the present study, there was no statistically significant difference in dental development between the MIH group and the sex-matched healthy control group. The absence of a difference in dental development in the patients with MIH suggests that there may not be a difference in the eruption status. Thus, the present study supports previous findings. As is the case in the present study, Tunç et al. [21] did not find a statistically significant difference between the groups; however, they reported a trend of accelerated dental development in the males and females in the MIH group.

Seow [23] evaluated the correlation between AI, a developmental anomaly associated with eruption disorders, and permanent tooth development. The study comprised 23 patients (10 males and 13 females under the age of 16 years) diagnosed with AI and a control group of healthy age- and sex-matched individuals. The study reported that the AI cases exhibited faster dental development than the healthy individuals. The mean increase in the duration of dental development was approximately one year. Similar results were obtained for all the affected patients regardless of the type of AI. Vuorimies et al. [24] compared the timing of permanent tooth eruption in children diagnosed with osteogenesis imperfecta (OI), a disease associated with mineralization disorders, with that in the age- and sex-matched healthy control group. The patients with OI had a greater number of erupted teeth, faster eruption of the permanent teeth, and a more advanced dental age than the healthy controls. This was especially the case for the patients with OI type 1, which occurs concurrently with dentinogenesis imperfecta. Another study that evaluated the incidence of craniofacial and dental anomalies in children with OI reported a delay in the dental development of 21 percent of patients with OI type 3; however, 23 percent of patients with OI type 4 exhibited faster dental development [25].

In the present study, the trend in favor of an earlier eruption in children with MIH, albeit not statistically significant, can be attributed to the effects of the irregularity in enamel formation during the dental development process. A factor affecting the maturation phase of the enamel has been considered to be involved in the formation of MIH [15]. The enamel maturation stage is a slow progression throughout two-thirds of the entire enamel formation period [26]. An irregularity at this stage could affect dental development and thus eruption time. There was no statistically significant difference between the eruption times of the MIH and control groups. In addition, a comparative analysis of the results could not be performed because the literature review did not reveal any studies that had evaluated the eruption times of patients with MIH.

In most evaluations of eruption times, permanent tooth eruption was found to occur earlier in females than in males [8, 27]. Moslemi [18], who investigated the timing of permanent tooth eruption in 3744 children aged 4–15 years, found that the mean age of eruption was lower in females than in males. In Lithuania, Almonaitiene et al. [28] examined the timing of permanent tooth eruption in 3596 children aged 4–16 years. Eruption was found to have occurred significantly earlier in females than in males. The range was 1–10 months depending on the type of tooth. A similar study concluded that permanent tooth eruption

occurred earlier in females than in males. The eruption times ranged 4–6 months depending on the type of tooth [16]. A study of 1491 children (773 females and 718 males aged 5–15 years) by Bayrak et al. [29] in Turkey reported that the permanent teeth of females tended to erupt earlier than those of males. The reason has not yet been elucidated. However, the sex-related differences in the physical developmental stages and the earlier physical development and maturation of females are thought to contribute to the earlier occurrence of permanent dentition in females [30].

Given these differences, a sex-matched evaluation of the groups was performed. This obviated the confounding factor of sex in the comparisons of the timing of permanent tooth eruption in the MIH and healthy patients. The results for the two sex-matched groups were not statistically significant. The mean age of the eruption of a majority of the teeth was lower in the MIH group.

CONCLUSION

MIH, an important clinical problem with a recently increased incidence, is an issue that concerns not only dentists but all healthcare professionals. Because of the rapid and severe destruction of teeth affected by MIH, the treatment approach should be multidisciplinary. An accurate understanding of dental development and eruption times is important for predicting possible anomalies and applying appropriate treatment. In addition, eruption time in children is an important issue for both forensic medicine and medical doctors who follow the general health of the child. The review of the literature indicated a lack of studies on the clinical importance of MIH, which is currently a common disease, the eruption process, which is the most important part of dental development, and the time of the eruption in patients with MIH. Therefore, there is a need for further large-scale studies to evaluate the eruption times of various types of populations.

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Време ницања сталних зуба код деце са моларно-инцизивном хипоминерализацијом

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САЖЕТАК

Увод/Циљ Моларно-инцизивна хипоминерализација (МИХ) развојни је дефект глеђи са мултифакторијалном етиологијом, а код деце са овим стањем могу постојати разлике у времену ницања зуба.

Циљ овог истраживања био је да се упореди клинички статус времена ницања сталних зуба код деце са МИХ-ом у односу на децу без МИХ-а.

Методе Студијску групу чинило је укупно 300 деце (176 девојчица и 124 дечака узраста 6–12 година) којима је дијагностикован МИХ, али нису имали системску болест. Контролну групу чинило је 300 здраве деце подударне старости и пола. У студијској и контролној групи процењено је и упоређено време ницања сталних зуба (искључујући треће моларе), посебно за девојчице и дечаке. За статистичку анализу коришћен је *t*-тест независних узорака.

Резултати Није утврђена статистички значајна разлика између средње вредности времена ницања зуба код деце са МИХ-ом и деце без поремећаја минерализације ($p > 0,05$). Поређењем по полу, није откривена статистички значајна разлика у средњој вредности времена ницања зуба између испитиване и контролне групе ($p > 0,05$).

Закључак Иако није установљена статистички значајна разлика у средњој вредности у времену ницања сталних зуба код деце са МИХ-ом и контролне групе, приметан је бржи тренд развоја зуба код деце са МИХ-ом.

Кључне речи: ерупција зуба; моларно-инцизивна хипоминерализација; развојни дефект глеђи

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Changes in risk factors trends in coronary surgery over the past decade – a single-center validation

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SUMMARY

Introduction/Objective The risk factors in coronary patients indicated for surgery change during the years. The aim of this study was to analyze the trends of risk factors which enter into the composition of the European System for Cardiac Operative Risk Evaluation (EuroSCORE II).

Methods The research included 3996 patients who underwent coronary surgery from January 2012 to December 2020 at our clinic. For estimation of the risk factors and evaluation of the operative risk, the EuroSCORE II model was used. Kruskal–Wallis H test was used for testing differences of values of numerical variables between years. The calibration and the discriminative power of the EuroSCORE II were assessed by comparing the observed to the expected mortality ratio and by using area under the receiver operating characteristic curve (AUC).

Results Old age has shown a significant increasing trend ($p < 0.0005$), as well as diabetes mellitus on insulin therapy, before surgery ($p = 0.004$). The significant declining trend have shown: extracardiac arteriopathy ($p = 0.003$), critical preoperative condition ($p = 0.013$), preoperative NYHA Classes III or IV ($p < 0.0005$) and preoperative angina pectoris CCS Class IV ($p < 0.0005$). The mean value of the EuroSCORE II decreased from 1.73 to 1.53 ($p < 0.0005$). The observed mortality was 1.70% and the mean, predicted by the EuroSCORE II, was 1.75%. The O/E mortality ratio was 0.98; 95% confidence interval 0.95–1.03. The AUC was 0.825.

Conclusion Over the past decade the risk profile of patients for coronary surgery has changed. The mean value of the EuroSCORE II has a declining trend with a good predictive and discriminative power.

Keywords: cardiac surgery; risk factors; trends

INTRODUCTION

Risk stratification involves preoperative determination of operative risk based on number and severity of patients' risk factors. The essence of the model for outcome prediction and risk stratification in cardiac surgery is to single out risk factors that are important in relation to the outcome. A good calibration of the model exists if the difference between the expected outcome and the observed outcome is small, ideally zero. The discriminative power of the model is the ability to distinguish between low and high-risk groups in relation to the outcome.

European System for Cardiac Operative Risk Evaluation (EuroSCORE) was developed in the period 1995–1999 [1]. The model was initially additive [1].

In 2003, a logistics model was developed that proved to be better, especially in groups of patients with increased operative risk [2].

The application of EuroSCORE on other continents showed some differences in the risk profile of coronary patients indicated for surgical treatment. In Australia, the incidence of female sex, chronic obstructive pulmonary disease, extracardiac arteriopathy, reoperation, and emergency surgery was higher than in the European population [3]. In China, there were more patients with neurological deficits, while old age, chronic obstructive pulmonary disease, extracardiac arteriopathy, renal failure, unstable angina pectoris, recent myocardial infarction and left ventricular dysfunction were less common [4].

We analyzed the trends of all relevant risk factors of the additive EuroSCORE, from 2001 to the end of 2008. During the observed years, old age, extracardiac arteriopathy, recent myocardial infarction and emergency operation as risk factors significantly changed with an increasing trend, while chronic lung disease, neurological dysfunction and unstable pectoral angina had a decreasing trend [5]. The risk for isolated coronary artery bypass graft surgery (CABG) given by additive EuroSCORE increaseed over the years, but operative mortality decreased [6].

The EuroSCORE II, with new relevant risk factors, was defined and introduced into routine use in 2012. The initial results have shown better prediction than its original version. In our first study, from the beginning of 2012, the EuroSCORE II satisfactorily predicted hospital mortality and had solid discriminative power [7]. Recent results showed that the EuroSCORE II produces a valid risk prediction and outperforms the earlier models [8].

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The last two decades have seen a change in the risk profile of patient indicated for coronary surgery due to an increased number of percutaneous coronary interventions (PCI) with the implementation of various types of stents.

The aim of this study was to analyze the trends of risk factors in patients indicated for coronary surgery which make up the EuroSCORE II and to assess the predictive and discriminative power of the model.

METHODS

The research included 3996 consecutive patients who underwent isolated coronary surgery at Institute of Cardiovascular Diseases of Vojvodina, from January 2012 to December 2020 and was approved by the Ethics Committee of Institute of Cardiovascular Diseases of Vojvodina (No.: 1515-1/3). For each patient the EuroSCORE II was calculated prospectively, using the formulas available at the EuroSCORE website (www.euroscore.org). The postoperative mortality was considered as death from any cause within 30 days of the operation. Data were collected prospectively and analyzed retrospectively. Statistical analysis was performed using the IBM SPSS Statistics, Version 19.0 (IBM Corp., Armonk, NY, USA). The Kruskal–Wallis H test was used for testing differences of values of numerical variables between years. Relation between qualitative data was tested using the χ^2 test. Spearman's test was used to determine the strength and direction of association between two numerical variables. The calibration of the EuroSCORE II was assessed by comparing observed (O) to expected (E) postoperative mortality ratio and by Hosmer–Lemeshow (H-L) test. The discriminative power of the EuroSCORE II was examined using area under the receiver operating characteristic curve (AUC), where cut-off, sensitivity, and specificity were determined. The differences were considered significant if $p < 0.05$.

RESULTS

Total number of coronary patients operated in the period from 2012 until 2020 was 3996. During that time, it has come to significant changes in the frequency of some risk factors (Figures 1–6). Table 1 shows all the changes in the frequency of risk factors related to the patients who were operated on.

The average age of coronary patients who were operated on was 64.4 ± 1.17 (35–88) years. Over time, the patients get older. The observed years and the age of the patients were positively correlated ($r = 0.860$, $p = 0.003$), with an increasing trend (Figure 1).

There were 24.4% female patients. The frequency of female sex through the observed period was similar. The frequency of risk factors related to impaired kidney function, expressed by creatinine clearance < 85 ml/min, was 32.7%. It was significantly different through the observed period ($p < 0.0005$), but no trend was observed.

The observed years and frequency of extracardiac arteriopathy were related ($p = 0.003$). The years and average percentages of extracardiac arteriopathy in those years

Table 1. Risk factors from European System for Cardiac Operative Risk Evaluation (EuroSCORE) II in relation to time, significance, and trends

Risk factors	Total	2012	2013	2014	2015	2016	2017	2018	2019	2020	p	Trend
Age (years)	64.4 (35–88)	62.83	62.39	64.99	64.22	64.21	64.67	64.78	65.72	65.82	< 0.0005	↑
Female sex (%)	24.4	25.2	26.3	31.6	22.1	25.3	21.7	23.7	25.2	22.9	0.177	-
CC < 85 ml/min (%)	32.7	15	20.4	27.6	49.9	60.2	36.7	25.6	29.1	19.4	< 0.0005	-
Extracardiac arteriopathy (%)	15.3	19	20.9	11.6	15.5	15.7	14.1	13.2	12.2	14.6	0.003	↓
Poor mobility (%)	4.2	4.9	3.4	4.4	4.3	3.5	3.8	5.2	3.3	4.4	0.796	-
Previous cardiac surgery (%)	1.8	1.5	1.4	2.7	1.4	2.2	2.0	1.7	6	3.5	0.170	-
Chronic lung disease (%)	7.1	8.5	6.7	5.8	8.0	5.1	7.2	7.7	7.1	6.7	0.597	-
Critical preoperative state (%)	1.7	2.3	2	3.1	1.2	2.5	2.8	1.2	0.4	0.3	0.013	↓
Diabetes on insulin (%)	13	10.2	13.7	11.1	11.2	11.4	13.5	15.3	12.4	19.7	0.004	↑
NYHA III, IV (%)	16.9	27.7	32.4	25.8	13.3	13.1	10.4	9.1	12.8	18.1	< 0.0005	↓
AP-CCS Class IV (%)	6.6	9.1	7.8	8.4	6.7	13.9	9.8	1.9	1	0.3	< 0.0005	↓
LVEF < 50% (%)	36.3	32.3	36.6	33.8	38	35.3	41.4	33.2	38.4	38.4	0.064	-
Recent myocardial infarction (%)	25.9	27.1	26.3	21.8	26.6	29.6	25.9	26.5	19.5	28.6	0.022	-
Pulmonary hypertension (%)	22.6	18.6	19.63	16.9	27.6	30.6	27.1	18.9	20.3	19.4	< 0.0005	-
Urgent operation (%)	4.7	6.1	5	9.3	2.7	3.5	5.6	3.4	5.3	3.8	0.003	-

CC – creatinine clearance; NYHA – New York Heart Association; AP-CCS – Canadian Cardiovascular Society grading of angina pectoris; LVEF – left ventricular ejection fraction

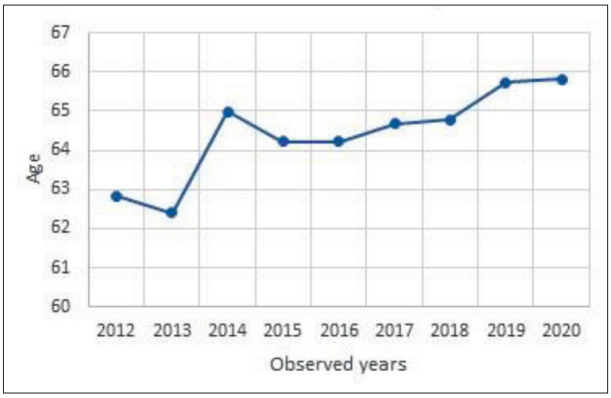

Figure 1. Trend of the mean age 2012–2020

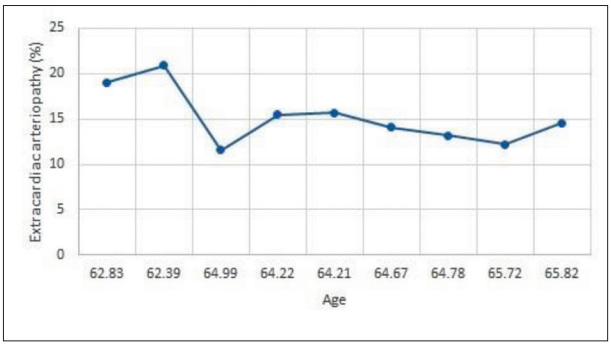

Figure 2. Frequency of extracardiac arteriopathy 2012–2020

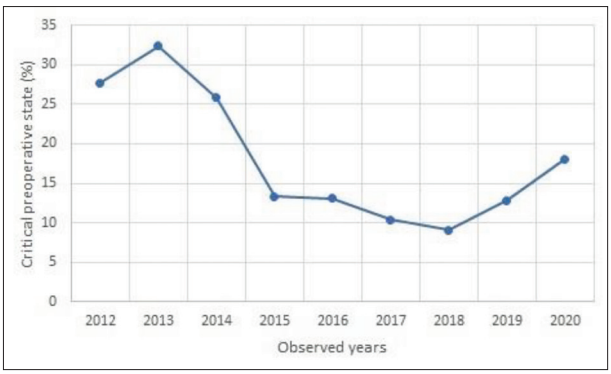

Figure 3. Average percentages of critical preoperative condition of patients 2012–2020

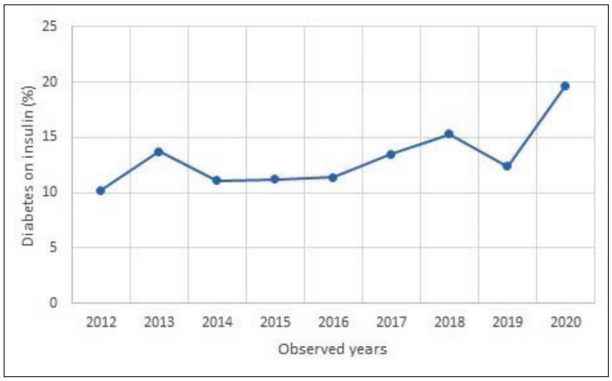

Figure 4. Percentage of patients with diabetes mellitus on insulin 2012–2020

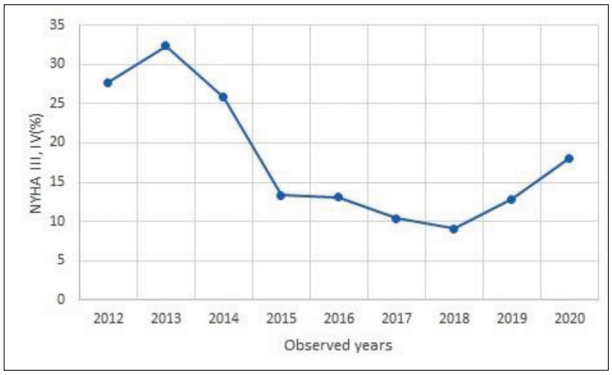

Figure 5. Frequency of patients in New York Heart Association (NYHA) Classes III or IV 2012–2020

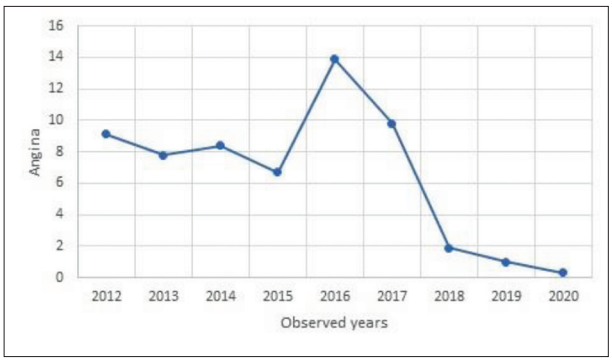

Figure 6. Average percentages of patients with angina pectoris Canadian Cardiovascular Society Class IV 2012–2020

were not correlated (r = -0.625, p = 0.072). This is close to statistical significance, which supports a negative correlation (Figure 2).

From 2012 until 2020 the percentage of patients with poor mobility or neurological deficit who were operated on was 4.2% and was similar during the observed period. It was the same with previous cardiac surgery and chronic lung disease, as risk factors.

The observed years and frequency of critical preoperative condition of patients were related (p = 0.013). The years and average percentages of critical preoperative condition were correlated (r = -0.669, p = 0.049), with a decreasing trend (Figure 3).

The percentage of patients who had diabetes mellitus on insulin therapy was 13%. The observed years and frequency of these patients were related (p = 0.040). The years and average percentages of patients who had diabetes on insulin therapy were correlated (r = 0.700, p = 0.036), with an increasing trend (Figure 4).

The observed years and frequency of patients in New York Heart Association (NYHA) Classes III or IV were related (p < 0.0005). The years and average percentages of patients in NYHA Classes III or IV were correlated (r = -0.720, p = 0.029), with a decreasing trend (Figure 5).

The observed years and the frequency of patients with angina pectoris Class IV, according to the Canadian Cardiovascular Society (CCS) were related (p < 0.0005). The total percentage of operated coronary patients with angina pectoris CCS Class IV was 6.6%. The years and average percentages of patients with angina pectoris CCS Class IV were close to statistical significance with

Table 2. Predictive power of the European System for Cardiac Operative Risk Evaluation (EuroSCORE) II in coronary surgery

	Number of patients	EuroSCORE II-expected mortality (%)	Observed mortality %	O/E mortality ratio (95% CI)	H-L test p-value
Coronary surgery	3996	1.75	1.7	0.98 (0.95–1.03)	< 0.0005

O – observed; E – expected: CI – confidence interval; H-L – Hosmer–Lemeshow

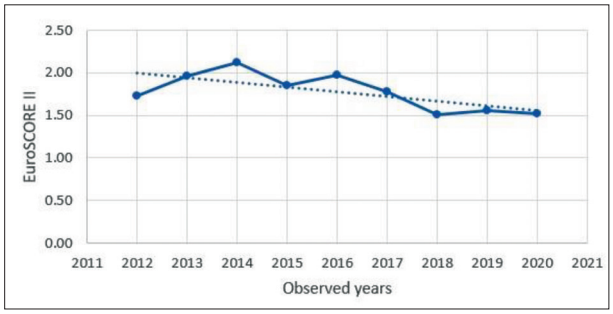

Figure 7. Trend of the mean value of the European System for Cardiac Operative Risk Evaluation (EuroSCORE) II 2012–2020

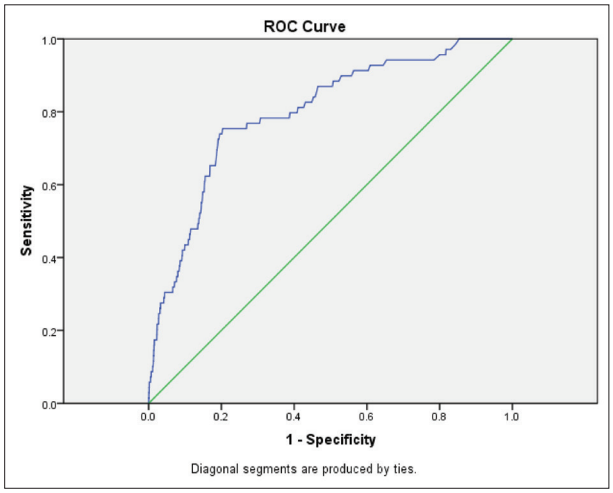

Figure 8. Discriminative power of the European System for Cardiac Operative Risk Evaluation (EuroSCORE) II;
ROC – receiver operating characteristic

a decreasing trend (Figure 6), but were not correlated (r = -0.653, p = 0.056).

The percentage of patients with left ventricular ejection fraction < 50% did not change significantly during the observed period (p = 0.064). The highest percentage was registered in 2017 (41.4%), 2019 (38.4%), and 2020 (38.4%). The percentage of coronary patients who had a recent myocardial infarction, pulmonary hypertension, or undergone emergency surgery and were operated on changed significantly (p = 0.022; p = 0.0005; p = 0.003; respectively), but without trends during the observed years.

The mean value of the EuroSCORE II and years were in negative correlation (r = 0.695, p = 0.038). The mean value of the EuroSCORE II decreased from 1.73 in year 2012 to 1.53 in 2020 (p < 0.0005). Trend of the mean value of the EuroSCORE II from 2012 to 2020 is shown in Table 2 and Figure 7.

The mean value of the EuroSCORE II for all 3996 coronary patients was 1.75, while the observed mortality was 1.7%. The difference was not statistically significant (p = 0.132). The predictive power of the EuroSCORE II in coronary surgery is shown in Table 2.

The EuroSCORE II showed a very good discriminative power in the period of nine years (AUC curve = 0.799, p < 0.0005). The cut-off value was 2.05, sensitivity 0.754, and specificity 0.797 (Figure 8).

DISCUSSION

The aim of this retrospective single centre study was to analyze the trends of risk factors that are part of the EuroSCORE II in patients indicated for coronary surgery and to assess the predictive and discriminative power of the model.

Two risk factors, old age and diabetes mellitus on insulin therapy before surgery, in the observed group showed a significant trend of increase during the observed period.

Four risk factors – extracardiac arteriopathy, critical preoperative condition, preoperative NYHA Classes III or IV, and preoperative angina pectoris CCS Class IV – showed a significant declining trend. The frequency of other risk factors during the last decade was similar, with no trends.

Mean value of the EuroSCORE II decreased from 1.73 in year 2012 to 1.53 in 2020 and the trend was declining. The predictive power was good and the discriminative power was very good.

The epidemiology of ischemic heart disease and comorbidities may be geographically different due to population differences, but also differences in prevention, diagnosis and therapy [9, 10, 11]. One of the first analysis of more than 11,700 coronary patients who were operated on, from six European countries, showed that the mean age was the lowest in the UK and the highest in France. Chronic lung disease was most common in Germany and Spain and the rarest in Finland and Italy. Chronic renal insufficiency was registered in 12.2% of patients in the UK, in 10.6% of patients in Spain, and in 3.4% of patients in Finland. Emergency myocardial revascularization was indicated in 2% of patients in Spain, in 3.9% in the UK, in 4% in Finland, in 4.3% in France, in 4.5% in Germany, and in 4.6% in Italy [12]. Nawata et al. [13] have compared Asian patients undergoing isolated CABG surgery between 2013 and 2016 in Japan and the United States. The patients in Japan were older (69 *vs.* 65 years) with a smaller body surface area (1.65 m^2 *vs.* 1.81 m^2) and body mass index (24 kg/m^2 *vs.* 26 kg/m^2). The prevalence of chronic lung disease (82% *vs.* 86%), and diabetes mellitus (54% *vs.* 60%) were similar.

The risk factors, as well as the overall level of expected operational risk, are not constant, unchanging values. They can change over time, even in the same population. In our earlier study, in the period 2001–2008, old age, extracardiac arteriopathy, recent myocardial infarction and emergency operation, as risk factors, significantly changed

with an increasing trend, while chronic lung disease, neurological dysfunction, and unstable pectoral angina had declining trend [6].

In the last decade only old age and the frequency of patients with diabetes on insulin therapy before surgery had an increasing trend. According to our findings, the mean age increased from 62.8 years in 2012 to 65.8 years in 2020, with a significant increasing trend. The number of PCI in our institution has rapidly increased, from 1946 in 2010 to 2777 in 2019. It is completely understandable that, with good prevention, improvements in medical therapy, and widespread use of PCI, coronary patients are coming to surgery older. Kindo et al. [14] have analyzed risk factors in two groups of patients, based on the years in which the operation was performed: Group A (2000–2003; 898 patients) and Group B (2009–2012; 1249 patients). They registered a significant increase in the prevalence of patients over 80 years of age (Group A = 4.3%; Group B = 6.8%; $p = 0.016$) and a significant increase of diabetes mellitus ($p < 0.0001$) over the years.

The prevalence of patients who were on insulin therapy before surgery in our study was 13%, for the whole observed period. The frequency has increased during the last nine years, from 10.2 % in 2012 to 19.7% in 2020. This is consistent with the data from the literature and may be explained by the fact that the onset of diabetes occurs in old age and may require several years to become clinically evident [9, 15].

In a recent study by Sharma et al. [16] the mean age of patients was found to be 58.87 years. The major comorbidities were hypertension in 88%, dyslipidemia in 69%, and type 2 diabetes mellitus in 51% of the patients [16].

Ziv-Baran et al. [17] compared changes in coronary patients' characteristics and outcomes during the first 15 years of the millennium. The period was divided into two sub-periods (2000–2008 and 2009–2014). Diabetes was more common in the later period ($p < 001$), while peripheral vascular disease and left main disease were more common in the earlier period [17].

The authors from Iran compared the risk factors of patients undergoing CABG surgery in 2010 and 2016. The frequency of diabetes mellitus increased in the second period (51.8% *vs*. 43.6%, $p = 0.025$), but the average age of patients significantly decreased (from 62.49 ± 8.05 to 58.09 ± 9.2) over time [18].

In a retrospective study Wang et al. [19] concluded that the diabetic patients had higher incidence of major adverse cerebral and cardiovascular events and mortality, after coronary surgery, compared with non-diabetic patients.

According to our results, the prevalence of coronary patients, who has extracardiac arteriopathy, for the whole observed period, was 15,3% and has a declining trend. The same trend was observed in the frequency of critical preoperative condition (the prevalence 1.7%), preoperative NYHA Classes III or IV (the prevalence 16.9%) and preoperative angina pectoris CCS Class IV (the prevalence 6.6%). Siregar et al. [20] presented the prevalence of the risk factors in 16 cardiothoracic centers in 46,883 consecutive cardiac surgery interventions in the Netherlands 2007–2009. The prevalences of the risk factors were as follows: extracardiac arteriopathy 12.2% (varied 9–16.5%), critical preoperative condition 4.7% (varied 2.2–8.7%), unstable angina pectoris 6.2% (varied 2.2–13.8%). Our declining trend of frequency of preoperative angina pectoris CCS Class IV could be explained by the fact that the majority of patients with this risk factor were referred to PCIs.

In a paper by Dinh et al. [21] from Australia, based on the research of 9372 patients in the 2001–2006 period, a considerable decline in the percentage of re-operations was registered (from 4.4% to 2.65%). It was explained by the improvement of the medical therapy and the increase of the number of PCIs.

According to Saeed et al. [22], South Asian and Middle-Eastern populations living in the West had significantly higher risk of diabetes and cardiovascular disease compared with native white Europeans. Effective and timely lifestyle intervention, education, physical activity, and diet can reduce the risk of diabetes.

Kindo et al. [14] found that the prevalence of extracardiac arteriopathy has significantly decreased regarding the two observed periods (group A = 19%; group B = 22.8%; $p = 0.035$), as well as prevalence of preoperative angina pectoris CCS Class IV (group A = 25.9%; group B = 9.7%; $p < 0.0001$). Contrary to our results, they showed a significant increase in the prevalence of preoperative NYHA Classes III or IV (group A = 8.6%; group B = 15.2%; $p < 0.0001$). The prevalence of recent myocardial infarction has significantly decreased (from 11.4% to 8.3%; $p = 0.019$), as well as the prevalence of reoperations (from 4.2% to 2.3%; $p = 0.012$) and ejection fraction under 50% (from 32.4% to 27.4%; $p = 0.005$). The four risk factors that have not changed significantly over time are female patients, chronic obstructive pulmonary disease, severe kidney failure, and urgent surgery.

In our earlier report the additive EuroSCORE has increased over the years and has overestimated mortality [6]. In this study, the declining trend of the EuroSCORE II was found, but the model proved to be a good predictor of mortality. Recent studies showed that the EuroSCORE II produces a valid risk prediction and outperforms the earlier additive and logistic models. Koszta et al. [23] concluded that the EuroSCORE II predicted better, compared to the initial models (O/E ratio: 0.75; the H-L test, $p = 0.5789$). Paparella et al. [24] presented an external validation of the EuroSCORE II, based on the results of 6293 coronary patients who were operated on. The discriminative power of EuroSCORE II was excellent (AUC 0.830) and the model proved to be a good predictor of hospital mortality.

In 2019, Nežić et al. [25] confirmed a good calibration of the EuroSCORE II in coronary surgery and an excellent discriminative power (AUC = 0.84). Recent studies, from different countries, are also in accordance with our results [26, 27, 28].

The limitation of our study is a relatively small sample size for precise analysis of the results. Furthermore, as it reflects a single-center experience, the results may not represent national and international practice and outcome. Further multicenter examinations with a larger number

of patients are necessary for more precise evaluation of the results.

CONCLUSION

Over the past decade, the risk profile of patients undergoing coronary surgery has changed. Old age and diabetes mellitus on insulin therapy before surgery have shown a significant increasing trend, while extracardiac arteriopathy, critical preoperative condition, preoperative NYHA Classes III or IV, and preoperative angina pectoris CCS Class IV have shown a significant declining trend. The mean value of the EuroSCORE II has a declining trend with a good predictive and discriminative power.

Conflict of interest: None declared.

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Промене у трендовима фактора ризика у коронарној хирургији у последњој деценији – потврда једног центра

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САЖЕТАК

Увод/Циљ Фактори ризика коронарних болесника индикованих за хируршко лечење временом се мењају.
Циљ ове студије био је да се анализирају трендови фактора ризика који улазе у састав Европског система за процену срчаног оперативног ризика (*EuroSCORE* II) и да се процене његова калибрација и дискриминативна моћ.

Методе Истраживањем је обухваћено 3996 оперисаних коронарних болесника у периоду од јануара 2012. године до децембра 2020. године на нашој клиници. За процену фактора ризика и евалуацију оперативног ризика коришћен је модел *EuroSCORE* II. Краскал–Волисов *H* тест коришћен је за тестирање разлике вредности нумеричких варијабли кроз године. Калибрација модела процењена је поређењем стварног и очекиваног морталитета, а дискриминативна моћ испитана је уз помоћ површине испод *AUC* криве.

Резултати Старост болесника имала је растући тренд ($p < 0,0005$), као и дијабетес мелитус на инсулинској терапији ($p = 0,004$). Значајан тренд опадања имали су: екстракардијална артериопатија ($p = 0,003$), критично преоперативно стање ($p = 0,013$), преоперативна *NYHA* класа III или IV ($p < 0,0005$) и преоперативна ангина пекторис *CCS* класе IV ($p < 0,0005$). Просечна вредност *EuroSCORE* II опала је са 1,73 на 1,53 ($p < 0,0005$). Стварни морталитет био је 1,70%, а очекивани 1,75%. Однос стварног и очекиваног морталитета био је 0,98; 95% интервал поверења 0,95–1,03. Површина испод *AUC* криве била је 0,825.

Закључак Профил ризика болесника за коронарну кардиохируршку интервенцију променио се у последњој деценији. Просечна вредност *EuroSCORE* II има тренд опадања, уз добру предиктивну и дискриминативну моћ.

Кључне речи: кардиохирургија; фактори ризика; трендови

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Predictive value of GATA3 and Ki-67 expression in biopsy and transurethral resection specimens in patients with urothelial carcinoma of the urinary bladder

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SUMMARY

Introduction/Objective Urothelial carcinoma is the most commonly diagnosed malignancy of urinary bladder in clinical and pathohistological practice where various prognostic factors play a significant role. One of the most important pathohistological prognostic factors is the intensity of immunohistochemical staining. Among various immunohistochemical markers that have been proven to influence disease progression and the patient's survival, role of Ki-67 and GATA3 in prediction of disease prognosis has not been completely clarified yet. The aim of this study was to determine the predictive value of GATA3 and Ki-67 mutual expression in urothelial carcinoma.

Methods Eighty patients were included in this study, out of which four groups were formed based on the pathological stage of urothelial carcinoma. After using preferred antibodies, their staining intensity was analyzed semiquantitatively.

Results Results showed that there was statistically significant correlation between the type of urothelial carcinoma, the pathological stage, and invasiveness and different grades of GATA3 expression, as well as statistically significant correlation between the type of urothelial carcinoma and the pathological stage and different grades of Ki-67 expression. The regression model showed low value of GATA3 and Ki-67 mutual expression. There was also statistical significance regarding the pathological stage and invasiveness of the tumor in survival analysis.

Conclusion Predictive value of GATA3 and Ki-67 mutual expression resulted as low from this study, but to our knowledge this was the first study to examine their predictive capability on biopsy and transurethral resection specimens.

Keywords: urothelial carcinoma; biopsy; transurethral resection; GATA3; Ki-67

INTRODUCTION

Urothelial carcinoma is the most commonly diagnosed malignancy of urinary bladder in clinical and pathohistological practice and it closely follows prostatic adenocarcinoma on the epidemiological malignancy scale of genitourinary system. Global data from 2020 showed 573,278 newly diagnosed urinary bladder carcinomas, which makes it the 10th one on the list of the most common malignancies in the general population [1]. In the 2006–2016 decade, annual frequency of urinary bladder carcinoma has declined by 1.3%, while mortality rate has not changed [2, 3]. The disease is more frequently diagnosed among males considering the differences in carcinogenic exposure between the sexes [4, 5]. Age represents a strong and independent risk factor as various demographic studies showed that patients older than 65 have 11-fold higher risk of getting this disease, unrelated to sex [6]. Considering the unchanged mortality rates during the last couple of years, the future burden of bladder cancer will fully depend on the newly formed diagnoses [3–6].

Pathohistological prognostic factors in bladder carcinoma include the histologic type, depth and tumor extension, stromal response and the intensity of inflammatory infiltrate, lymphovascular invasion, necrosis and disease stage, and intensity of immunohistochemical staining [7, 8]. Among various immunohistochemical markers that have been proven to influence disease progression and the patient's survival, Ki-67 and GATA3 have been chosen as the focus of the study, as their role in the disease prognosis has not been completely clarified yet [8, 9, 10].

The aim of this study was to determine the correlation between clinical and pathohistological parameters and urothelial carcinoma, as well as to determine the correlation between Ki-67 proliferation index and GATA3 expression with histological parameters of urothelial carcinoma and their predictive values.

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METHODS

During a one-year period (from October 1, 2020 to October 31, 2021), this retrospective–prospective study included pathohistological material from 80 patients who had been diagnosed with urinary bladder carcinoma or with a non-cancerous bladder lesion. The material for histological analysis was analyzed at the Center for Pathology and Histology of the University Clinical Center of Vojvodina. The study protocol was approved by the Ethics Committee of this institution (No. 00-400) and the Faculty of Medicine in Novi Sad (No. 01-39/299).

Study group

The cases of 80 patients who underwent biopsy or transurethral resection after clinical suspicion of bladder cancer existence were reviewed in this study, and an adequate tissue specimen was histologically analyzed. The exclusion criteria used in this study referred to patients with inadequate tissue specimens (not enough material suitable for immunohistochemical staining, remarkable exogenous tissue damage, and extensive necrosis present). All clinical data referring to demographic characteristics, type of procedure, pathohistological diagnosis, and stage were obtained from the patients' medical charts.

Urinary bladder tissue specimens for the histological analysis were fixed by 10% neutral formalin, then routinely paraffin-embedded. The specimens were cut at approximately 5 mm-intervals, sliced to 4 µm-thick sections, and stained with hematoxylin and eosin. By examining all tissue specimens, the following diagnoses were made and four numerically equal groups of 20 patients were formed:

- group I (control group) – tissue specimens where regular histological elements of bladder mucosa/wall as well as inflammation were present (in the form of *Cystitis cystica et glandularis, Cystitis polypoides et papillaris*), without any dysplastic epithelium changes or carcinoma *in situ* (CIS);
- group II – tissue specimens where papillary urothelial carcinoma, pTa stage was histologically confirmed and further divided into low grade and high grade (Figures 1A and 1B);
- group III – tissue specimens where infiltrating urothelial carcinoma pT1 stage was histologically confirmed and further divided into pT1m (microinvasive) and pT1e (extensive invasive) urothelial carcinoma (Figures 1C, 1D, and 1E);
- group IV – tissue specimens where infiltrating urothelial carcinoma, pT2 stage was histologically confirmed (Figure 1F).

The immunohistochemical analysis was performed on paraffine blocks with the biggest amount of preserved tissue and by using monoclonal antibody Ki-67 (clone MIB-1, DAKO, Glostrup, Denmark) and monoclonal GATA3 (clone L50-823, Cell Marque, Rocklin, CA, USA).

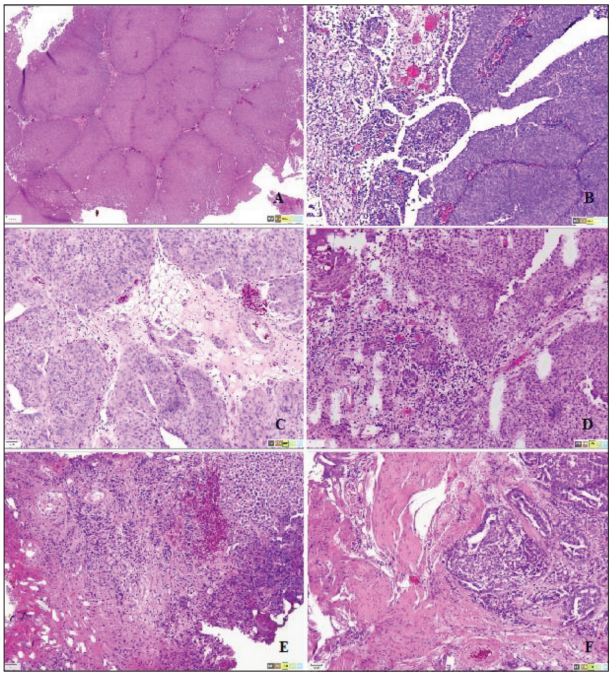

Figure 1. Microscopic appearance of urothelial carcinoma; (A) pTa, low grade, H&E, 2.5 ×; (B) pTa, high grade, H&E, 10 ×; (C) and (D) pT1, microinvasive, H&E, 10 ×; (E) pT1, extensive invasive, H&E, 10 ×; (F) pT2, H&E, 10 ×

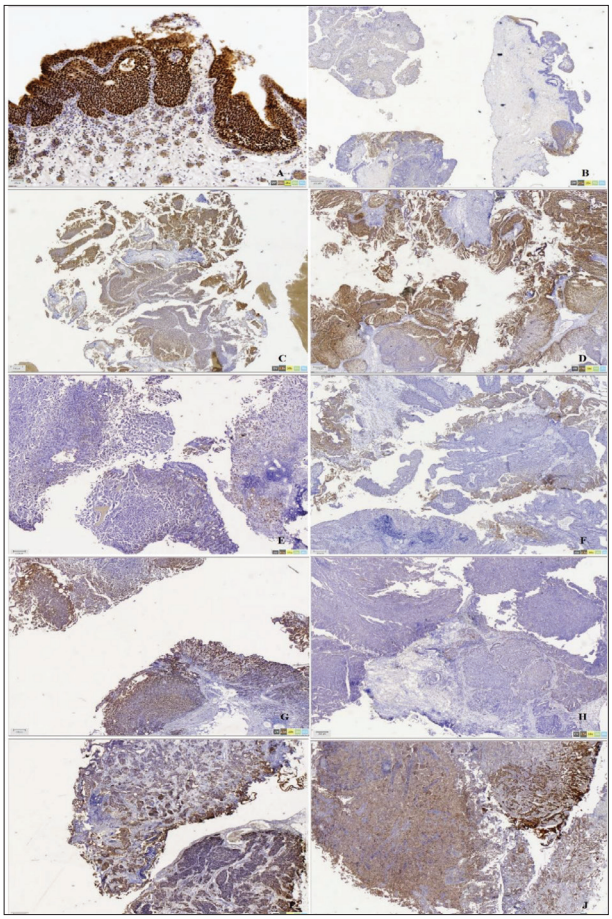

Figure 2. Micrographs showing GATA3 positivity in: (A) control group, 10 ×; (B), (C), and (D) pTa – grades I, II, III, 2.5 ×; (E), (F), and (G) pT1 – grades I, II, III, 2.5 ×; (H), (I), and (J) pT2 – grades I, II, III, 2.5 ×

Evaluation of Ki-67 and GATA3 immunohistochemical expression

The intensity of immunohistochemical staining was determined semiquantitatively, by analyzing areas where marker expression was most strongly presented. Ki-67 and GATA3 expression was evaluated as nuclear staining in tumor cells, while positive cytoplasmic staining was not considered important during the evaluation. Ki-67 proliferation index and GATA3 expression were defined as a percentage of positive tumor cells in regard to total number of tumor cells on histological section.

GATA3 positivity grading was defined as the following (Figure 2):

- grade 0 – no positivity;
- grade 1 – 1–10% positivity;
- grade 2 – 11–50% positivity;
- grade 3 – ≥ 51% positivity.

Ki-67 positivity grading was defined as the following (Figure 3):

- grade 0 – 0–10% positivity;
- grade 1 – 11–25% positivity;
- grade 2 – 26–50% positivity;
- grade 3 – ≥ 51% positivity.

Statistical analysis

The data were processed in the IBM SPSS Statistics, Version 23.0 (IBM Corp., Armonk, NY, USA). Data analysis methods used descriptive and inferential statistics. Numerical variables were presented by arithmetic mean and standard deviation, and the categorized variables through frequencies and percentages. Methods used to test statistical hypotheses were the χ^2 test and Fisher's exact test. The correlation between different parameters was determined with φ and Cramer's V correlation coefficients. Cumulative survival rates were calculated by the Kaplan–Meier method. All differences were considered significant for $p < 0.05$. The results are shown as tables (1–7).

RESULTS

Descriptive statistics and frequency of clinical and pathohistological characteristics

This study included 80 patients, 60 of which had a diagnosis of urothelial carcinoma, and 20 had a diagnosis of cystitis in different forms, without any elements of dysplasia, CIS, or carcinoma *per se*. The study included 54 men (67.5%) and 26 women (32.5%). Table 1 shows descriptive statistics of clinical characteristics and follow-up period of urothelial carcinoma and cystitis.

As patients with urothelial carcinoma were divided into three groups, 10 patients had a histological grade (HG) defined as low (16.7%), while 50 patients had high-grade urothelial carcinoma (83.3%). Twenty patients in each stage had pTa, pT1, and pT2 stages. Within patients with invasive pT1 and pT2 urothelial carcinoma, microinvasive pT1m stage was present in 11 patients (27.5%), while extensive invasive pT1e and invasive pT2 stage were present in 29 patients (72.5%).

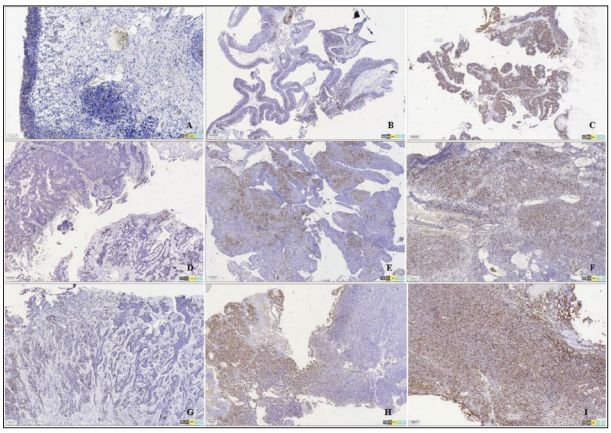

Figure 3. Micrographs showing Ki-67 positivity in: (A) control group, 10 ×; (B) and (C) pTa – grades I and III, 2.5 ×; (D), (E), and (F) pT1 – grades I, II, III, 2.5 ×; (G), (H), and (I) pT2 – grades I, II, III, 2.5 ×

Table 1. Descriptive statistics of clinical characteristics and follow-up period of urothelial carcinoma and cystitis

Age	Mean	68.87
	Median	69
	Standard deviation	10.13
	Minimum	30
	Maximum	88
Follow-up period (months)	Mean	12.71
	Median	12.5
	Standard deviation	7.37
	Minimum	1
	Maximum	27
Period until death outcome (months)	Mean	7.8
	Median	8
	Standard deviation	4.96
	Minimum	1
	Maximum	55

The correlation between urothelial carcinoma and cystitis with clinicopathological parameters

Table 2 shows summarized pathohistological parameters and their correlation with noninvasive and invasive urothelial carcinoma. As the results show, sex was not significantly associated with the presence of urothelial carcinoma, while significant associations were found between age groups, HG, and invasiveness with urothelial carcinoma.

In order to determine the predictive value of different age groups, multinomial logistic regression was used, in which regression model proved to be statistically significant (Table 3). In patients with noninvasive papillary urothelial carcinoma, age groups did not show any prediction value. On the other hand, age between 76 and 90 years was proven to be a good predictor for infiltrating urothelial carcinoma – patients of the aforementioned age had a

Table 2. Noninvasive and invasive urothelial carcinoma in different correlations with clinicopathological parameters

Parameters	Noninvasive papillary urothelial carcinoma (n/%)	Invasive (infiltrating) urothelial carcinoma (n/%)	Cystitis (n/%)	Total (n/%)	p	φ (p)
Sex						
Male	17 (21.3)	23 (28.7)	14 (17.5)	54 (67.5)	0.096*	
Female	3 (3.7)	17 (21.3)	6 (7.5)	26 (32.5)		
Age, years						
< 60	0 (0)	7 (8.8)	6 (7.5)	13 (16.3)	0.017**	
61–75	14 (17.5)	18 (22.5)	12 (15)	44 (55)		
76–90	6 (7.5)	15 (18.7)	2 (2.5)	23 (28.7)		
Histological grade						
Low grade	10 (16.7)	0 (0)		10 (16.7)	< 0.001***	0.632 (< 0.001)
High grade	10 (16.7)	40 (66.6)		50 (83.3)		
Invasiveness						
pT1m		11 (27.5)		11 (27.5)	< 0.001****	0.616 (< 0.001)
pT1e and pT2		29 (72.5)		29 (72.5)		

*χ^2 test = 4.672; **Fisher's test = 11.485; ***Fisher's test = 24.00; ****χ^2 test = 15.172

Table 3. Regression model for predictive values of different age groups

-2 Log	χ^2	p
16.834	14.269	0.006

Table 4. Age as a predictive factor for urothelial carcinoma of urinary bladder

Age groups	Noninvasive papillary urothelial carcinoma		Invasive (infiltrating) urothelial carcinoma	
	95% CI	p	95% CI	p
< 60	1 (reference group)		1 (reference group)	
61–75	1.5 (0.35–345.05)	0.17	0.78 (0.21–2.89)	0.70
76–90	2.57 (0.44–15.19)	0.29	**5 (0.96–25.94)**	**0.04**

five-fold greater chance of being diagnosed with infiltrating urothelial carcinoma than cystitis compared to patients younger than 60 years (Table 4).

The correlation between GATA3 and Ki-67 expression and histological parameters of urothelial carcinoma

Histological parameters of urothelial carcinoma and their association with classified positivity of GATA3 are presented in Table 5. The correlation between the type of urothelial carcinoma, pathological stage and invasiveness, and different grades of GATA3 expression was statistically significant, while the correlation between HG and GATA3 expression was not statistically significant. Table 6 shows histological parameters of urothelial carcinoma and their association with classified positivity of Ki-67. There was statistically important significance between type of urothelial carcinoma and pathological stage and different grades of Ki-67 expression, while correlation between HG, invasiveness and Ki-67 expression was not statistically significant.

Using the multinomial logistic regression, mutual predictive value of GATA3 and Ki-67 expression was examined, as well as separate predictive capability of these two markers. Analyzing mutual predictive capability, the regression model showed low values of GATA3 and Ki-67 mutual expression, thus separate expression of these markers had no statistically significant predictive values in urothelial carcinoma (Table 7).

Survival analysis

From the Kaplan–Meier plots, it can be concluded that the cumulative survival proportions varied between examined parameters. The cumulative survival proportion does not appear to differ remarkably considering HG, GATA3, and Ki-67 expression (Figures 4A, 4D, and 4E). It would appear that there was a statistical significance in regard to pathological stage (log rank (df = 2) = 8.327; p = 0.016, Figure 4B)

Table 5. The correlation between GATA3 expression and histological parameters of urothelial carcinoma

Histological parameters	Grade 0	Grade 1	Grade 2	Grade 3	Total	p	Cramer's V (p)
Urothelial carcinoma							
Noninvasive papillary	1 (1.3)	4 (5)	7 (8.8)	8 (10)	20 (25)	**< 0.001***	0.443 (< 0.001)
Invasive (infiltrating)	1 (1.2)	18 (22.5)	9 (11.3)	12 (15)	40 (50)		
Cystitis	0 (0)	0 (0)	0 (0)	20 (25)	20 (25)		
Pathological stage							
pTa	1 (1.7)	4 (6.7)	7 (11.7)	8 (13.3)	20 (33.3)	**< 0.001****	0.467 (< 0.001)
pT1	0 (0)	16 (26.7)	3 (5)	1 (1.7)	20 (33.3)		
pT2	1 (1.7)	2 (3.3)	6 (10)	11 (18.3)	20 (33.3)		
Histological grade							
Low grade	0 (0)	3 (5)	2 (3.4)	5 (8.3)	10 (16.7)	0.699***	
High grade	2 (3.3)	19 (31.7)	14 (23.3)	15 (25)	50 (83.3)		
Invasiveness							
pT1m	0 (0)	9 (22.5)	2 (5)	0 (0)	11 (27.5)	**0.008****	0.491 (0.012)
pT1e and pT2	1 (2.5)	9 (22.5)	7 (17.5)	12 (30)	29 (72.5)		

*Fisher's test = 32.347; **Fisher's test = 25.935; ***Fisher's test = 1.491; ****Fisher's test = 9.812

Table 6. The correlation between Ki-67 expression and histological parameters of urothelial carcinoma

Histological parameters	Grade 0	Grade 1	Grade 2	Grade 3	Total	p	Cramer's V (p)
Urothelial carcinoma							
Noninvasive papillary	14 (17.5)	5 (6.3)	0 (0)	1 (1.3)	20 (25)	< 0.001*	0.457 (< 0.001)
Invasive (infiltrating)	12 (15)	9 (11.2)	9 (11.3)	10 (12.5)	40 (50)		
Cystitis	20 (25)	0 (0)	0 (0)	0 (0)	20 (25)		
Pathological stage							
pTa	14 (23.4)	5 (8.3)	0 (0)	1 (1.7)	20 (33.4)	0.034**	0.325 (0.047)
pT1	6 (10)	5 (8.3)	4 (6.7)	5 (8.3)	20 (33.3)		
pT2	6 (10)	4 (6.7)	5 (8.3)	5 (8.3)	20 (33.3)		
Histological grade							
Low grade	7 (11.7)	3 (5)	0 (0)	0 (0)	10 (16.7)	0.099***	
High grade	19 (31.7)	11 (18.3)	9 (15)	11 (18.3)	50 (83.3)		
Invasiveness							
pT1m	0 (0)	9 (22.5)	2 (5)	0 (0)	11 (27.5)	0.275****	
pT1e and pT2	1 (2.5)	9 (22.5)	7 (17.5)	12 (30)	29 (72.5)		

*Fisher's test = 32.494; **Fisher's test = 13.08; ***Fisher's test = 5.515; ****Fisher's test = 4.121

Table 7. Regression model for predictive values of mutual GATA3 and Ki-67 expression

-2 Log	χ^2	p
5.589	0.587	0.344

and invasiveness of the tumor (Figure 4C). A log rank test was run to determine if there were differences in the survival distribution for these parameters and the results showed that patients with pT1m have better survival than patients with pT1e urothelial carcinoma [log rank (df = 1) = 2.989; p = 0.048, Figure 4C].

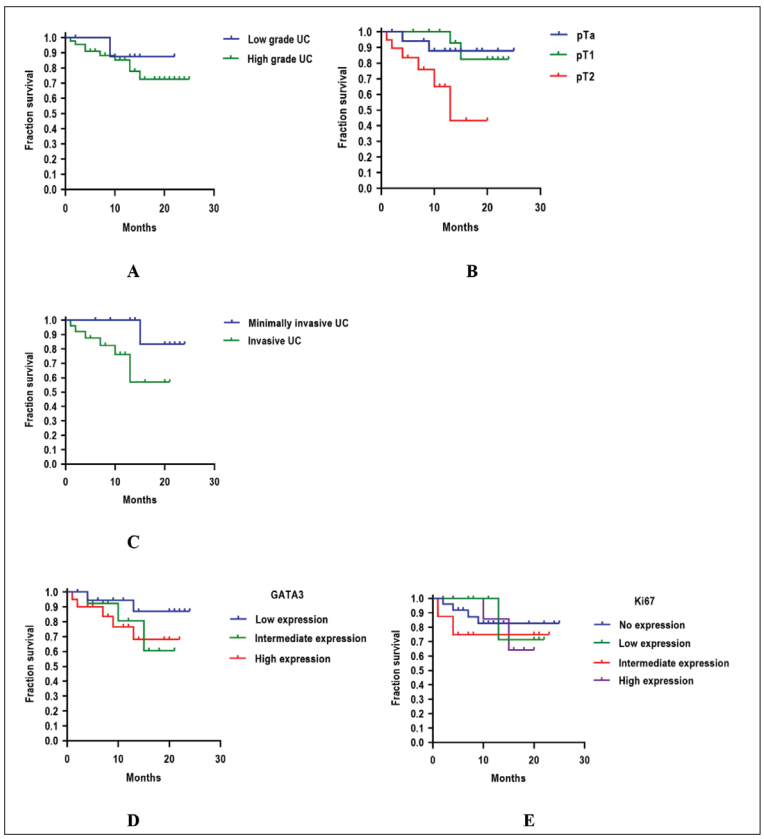

Figure 4. Cumulative overall survival curves stratified by: A) histological grade; B) pathological stage; C) invasiveness; D) GATA3 expression grades; E) Ki-67 expression grades

DISCUSSION

According to the International Agency for Research on Cancer and updated GLOBOCAN data from 2020, urinary bladder carcinoma is at the 10th place of the most commonly diagnosed carcinomas all over the world [1, 2]. This study included 40 men and 20 women with carcinoma, and male domination was compatible with data from larger studies, but with no statistical significance between the groups. Analyzing the sex influence on postoperative outcome from radical cystectomy, various authors showed that sex was an independent predictor; women had a two-fold greater risk of postoperative infection, extravesical extension, shorter disease-free period, and higher rate of relapse [6, 7]. In our study, patients in the 76–90 years age group had a five-fold greater chance of being diagnosed with infiltrating urothelial carcinoma compared to patients younger than 60 years. As a result of carcinogenic exposure, accumulation of somatic mutations and immune system changes, the general risk of urothelial carcinoma rises with age [11]. Autopsy studies frequently show many undetected malignancies, thus delayed detection reflects on lower cancer incidence, which is a result of less intense screening and diagnostic procedures [11, 12].

Numerous studies have also been dedicated to the analysis of the pT1 stage of urothelial carcinoma after radical cystectomy, its biological behavior and the ability for progression and relapse, as well as the possibilities for adequate and effective therapeutic modalities. According to these, the optimal solution for treating pT1 urothelial carcinoma was transurethral resection (TUR), and the most important predictors

are tumor size, multifocality, the presence of lymphovascular invasion, and concomitant CIS [13, 14]. Many studies have made their effort in order to stratify pT1 tumors according to the depth of invasion, and the results are highly variable. The possibility to substage pT1 varies 58–100%, and the percentage of accuracy is dependent on the number of specimens, sample quality, presence of muscularis mucosa, and the pathologist's level of experience [15, 16, 17]. According to one study, invasion depth 0.5–1.5 mm was defined as pT1, pT1b, and pT1c [15]. Based on this one and similar studies, pT1b and pT1c stages are significant predictors of disease progression [15, 16, 17]. pT1a/b/c substaging has not found constant and stable use in daily practice because it is associated with variable diagnostic accuracy 43–100% [16]. Other authors suggested a different pT1 substaging system, namely pT1m (microinvasive) and pT1e (extensive invasive). Studies showed that diagnostic accuracy of this substaging is higher and that patients with pT1e stage of urothelial carcinoma are at higher risk of disease progression [16, 17]. A study from 2018 is one of the rare studies which described a method for determining the depth of invasion within the pT1 stage, by biopsy or TUR specimens. Defining the linear extent of the urothelial carcinoma with optical micrometer and the cut-off value of 2.3 mm after regression analysis made this system 100% accurate for all included specimens, because the system did not require specific specimen orientation and did not depend on the presence of muscularis mucosa, histologic subtypes, lymphovascular invasion, and CIS [18].

There are different attitudes towards the correlation between GATA3 expression and the HG, as well as the pathologic stage. In the study by Agarwal et al. [19], statistically significant correlation between HG and GATA3 expression was present, as 100% of low-grade urothelial carcinoma showed intermediate to strong staining intensity, and more than half of pT2 urothelial carcinoma showed low intensity. Newer studies showed results similar to the study by Miyamoto et al., in which high-grade urothelial carcinoma was showing negative GATA3 expression [20]. On the other hand, Kamel et al. [21] suggested that GATA3 expression in non-muscle invasive urothelial carcinoma did not correlate with tumor stage, but was significantly downregulated in regard to tumor progression. In our study, the lowest intensity of GATA3 expression was associated with infiltrating pT1 stage of the disease. As study included smaller number of patients compared to the mentioned studies, the difference in results could be explained by the number and unpredictable behavior of the tumor in pT1 stage, which represents a therapeutic dilemma at the same time, because it is often difficult for a surgeon to decide whether to apply bacillus Calmette–Guerin immunotherapy or proceed with radical cystectomy, an aggressive and life-quality reducing surgery. Results from some other studies also show that GATA3 demonstrated significant correlation with oncological outcome, where higher expression was associated with longer disease-free period. GATA3-negative tumors had a tendency for an early relapse and the loss of GATA3 expression in invasive urothelial carcinoma increased the risk of death outcome, independent of age, morphology, and nodal status [20, 22]. GATA3 also represents a strong prognostic indicator for urothelial tumors of the upper urinary tract – a study by Inoue et al. [23] showed that GATA3-positive upper urinary tract tumors had a significantly lower risk of disease progression and cancer-specific mortality, in contrast to invasive bladder tumors.

Prognostic significance of Ki-67 reactivity in urothelial carcinoma has been analyzed through meta-analyses and cohort studies, but among patients who underwent radical cystectomy. High staining intensity was proved to be a predictor of significantly higher rate of cancer-specific mortality and shorter disease-free period [24, 25]. Critical values that would define high staining intensity were different between studies because of the non-existing standardized access, which contributes to the heterogeneity while interpreting Ki-67 expression. In most studies, 20%-value has served as a discriminator for poorer clinical outcome and shorter disease-free period [25]. In our study, Ki-67 expression within specimens of pTa urothelial carcinoma was absent or of low intensity. In the pT1 stage, Ki-67 expression varied from low to high intensity, as was the case with the pT2 stage. Similar results were found in the study by Ali Mohamed [26], but it should be mentioned that the study included twice as many patients. As Ki-67 is a cell cycle and proliferation regulative protein, it is probably capable of predicting tumor behavior based on histological grading and staging. Variable expression of Ki-67 in pT1 and pT2 urothelial carcinoma in our study could be explained by increasing bladder carcinoma's clinical, histological, and biological heterogeneity, where one marker is unlikely to predict precise prognosis.

Data showing mortality and five-year survival rate of patients with urothelial carcinoma are yet promising. Cumulative risk of death outcome from bladder carcinoma from birth to age of 74 years was 0.29% among men and 0.09% among women [1]. Data regarding five-year survival rate were obtained from the American Cancer Society for the 2010–2016 period. According to these, five-year survival rate depends on the stage. For example, five-year survival rate in CIS was 96%, 69% in patients with carcinoma confined to urinary bladder, and only 6% in patients with positive M descriptor (distant metastasis). With respect to all analyzed stages, patients' survival was brought down to noteworthy 77% [1, 2, 3].

CONCLUSION

In our study, cumulative probability of death outcome for microinvasive and extensive invasive pT1 urothelial carcinoma was proved to be statistically significant. Also, statistical significance was proved considering the pathological stage and invasiveness of the tumor. Even though this study found low predictive value of GATA3 and Ki-67 mutual expression, it was the first one to examine their predictive capability on biopsy and TUR specimens.

Conflicts of interest: None declared.

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Прогностички значај експресије *GATA3* и пролиферативног индекса *Ki*-67 у биоптичким узорцима и узорцима добијеним трансуретралном ресекцијом карцинома мокраћне бешике

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САЖЕТАК

Увод/Циљ Уротелни карцином је најчешће дијагностикована малигна неоплазма мокраћне бешике у клиничкој и патохистолошкој пракси, где различити прогностички фактори имају значајну улогу. Један од најзначајнијих патохистолошких прогностичких фактора је интензитет имунохистохемијског бојења. Међу различитим имунохистохемијским маркерима за које је доказано да имају утицај на прогресију болести и преживљавање болесника, заједничка улога *Ki-67* и *GATA3* у предикцији прогнозе болести није још потпуно разјашњена.

Циљ ове студије је дефинисање предиктивног значаја заједничке експресије *GATA*3 и *Ki*-67 у уротелном карциному.

Методе Осамдесет болесника је учествовало у студији, при чему су формиране четири групе на основу патолошког стадијума уротелног карцинома. Након употребе наведених антитела интензитет бојења је анализиран семиквантитативно.

Резултати Статистичка значајност је доказана између хистолошког типа, патолошког стадијума и инвазивности и различитих степена експресије *GATA*3, као и између хистолошког типа и патолошког стадијума и различитог степена експресије *Ki*-67. Регресиони модел је показао ниску предиктивну вредност заједничке експресије *GATA*3 и *Ki*-67. Такође је доказана статистичка значајност између патолошког стадијума и инвазивности тумора при анализи преживљавања.

Закључак Анализом предиктивног значаја заједничке експресије *GATA*3 и *Ki*-67 добијена је ниска вредност. Значај истраживања огледа се у његовој јединствености, што за циљ има испитивање успешности предикције наведених антитела у узорцима биопсије и трансуретралне ресекције уротелног карцинома.

Кључне речи: уротелни карцином; биопсија; трансуретрална ресекција; *GATA*3; *Ki*-67

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Clinicopathologic characteristics of cutaneous melanoma – a single-center retrospective study

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SUMMARY

Introduction/Objective Epidemiology of melanoma including the number of new cases and mortality have been established in most developed countries, but data on pathohistological features are mostly missing. The objective of the study was to investigate epidemiological, clinical, and pathohistological features of melanoma patients and compare the results with trends in other countries.

Methods Our sample comprised patients surgically treated for skin melanoma at the Hospital for Burns, Plastic and Reconstructive Surgery during the 2015–2017 period. Pathohistological, clinical, and demographic features of melanoma were studied.

Results The retrospective study comprised 201 patients (109 men and 92 women) aged 25–87 years. Melanoma was more common in men than in women (54.2% *vs*. 45.8%). Melanoma in male population most commonly presented on the trunk, while in females presentation on the trunk and lower extremities was almost equal. Superficial spreading melanoma was the most common type of melanoma (68.7%), without correlation to the sex. No correlation was observed in relation to the stage of the disease and the patient's sex ($p = 0.294$). A statistical difference was observed in relation to the type of melanoma and the Breslow classification ($p < 0.001$). Breslow's thickness correlated with neither age nor sex. In relation to tumor invasiveness, 12.4% of the lesions were classified as *in situ* lesions, while 87.6% of the lesions were invasive. The majority of patients were identified as stage pT1a.

Conclusion This study can help to identify patients at high risk for melanoma and contribute to optimize screening efforts in a defined target population.

Keywords: melanoma; Breslow; Serbian population; melanoma pathology; melanoma epidemiology

INTRODUCTION

Melanoma still represents a major diagnostic and therapeutic problem, with its rapid incidence rise worldwide [1]. The incidence of melanoma has been rising continuously over the last 50 years [2], with an approximate 3% annual rate according to age–period–cohort studies published so far [3]. The incidence and mortality rates vary by the regions, depending on the primary and secondary prevention strategies, early detection, and access to the latest treatment protocols [3].

Melanoma is among less commonly diagnosed skin tumors on the annual level (1–5%). According to the latest available data, 324,635 new cases were recorded in 2020, accounting for 1.7% of all newly diagnosed carcinoma on the global level, and with 57,043 related deaths its share in global carcinoma mortality is 0.6% [3]. In spite of development of new protocols and modalities for treatment and diagnostics, melanoma is still associated with the highest mortality rate of all skin tumors [1, 4]. In 2020, the age-standardized (for the world population) rate (ASR-W) was 3.4/100,000 on the global level, 18.9/100,000 in the Western Europe, and 35.8/100,000 in Australia and New Zealand as countries with the highest number of newly diagnosed cases [2]. In the same year, the standardized mortality rates were 0.56, 1.5, and 2.7/100,000, respectively [2]. It is more common in the 50+ years age population, with 59 years as the median age at diagnosis, but it is not uncommon among younger population either, particularly among women. Overall, melanoma is more common in men, but before the age of 50 it is more common in women [1]. It usually appears on the trunk, followed by the head and neck, lower and upper extremities, and finally the acral parts (hand, foot). Sex distribution shows that it is common on the trunk in men and on the lower extremities in women. In the USA, according to the SEER database, that keeps track of the five-year survival rates in patients diagnosed between 2011 and 2017, while reviewing cases of localized disease, the survival rate reaches 99.4%. In case of spreading to the lymph nodes, the percentage of the five-year survival is 68%, while in cases of distant metastases, the survival rate is only 29.8% [5]. In the light of the factual survival statistics, it is quite clear that early diagnosis of skin melanoma and easy access to clinical and dermoscopic examinations are very important factors in subsequent treatment. According to the National Cancer Registry of the Republic of Serbia, melanoma was the 12th most common

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malignant tumor in 2018, and 18th according to the number of fatal outcomes [6]. In 2018, the Dr. Milan Jovanović Batut Public Health Institute reported 709 new cases of melanoma, and 269 fatal outcomes. The ASR incidence was 10.6/100,000 in men and 8.6/100,000 in women [6], while the ASR mortality was 4.9/100,000 in men and 2.9/100,000 in women. The objective of the study was to investigate epidemiological, clinical and pathohistological features of melanoma patients and compare the results with trends in other countries.

METHODS

A retrospective study was conducted, with the data obtained from the medical records. Patients surgically treated for skin melanoma at the Hospital for Burns, Plastic and Reconstructive Surgery of the University Medical Center of Serbia (UKCS) in the period 2015–2017 were reviewed. The study was approved by the UKCS Ethics Committee (No: 602/1 Date: 30.12.2021).

The study comprised 201 patients (109 men and 92 women). The patients were divided into four age groups: < 40 years, 41–60 years, 61–80 years, and > 80 years. Tumor localization was divided into four regions: (a) head and neck, (b) trunk, (c) upper extremities, (d) lower extremities, (e) acral. The primary melanoma stage was determined according to the 2017 American Joint Committee on Cancer (AJCC) pT classification. Initially, narrow margin excisional biopsy with 1–3 mm margins was performed. The biopsy was interpreted by a pathologist experienced in melanocytic neoplasms. All patients had a confirmed diagnosis of cutaneous melanoma by histopathology, with all reported elements – histologic subtype: nodular melanoma (NM), superficial melanoma (SM), lentigo maligna (LM), acral, and others (desmoplastic and amelanotic); Breslow thickness (< 1 mm, 1.01–2 mm, 2.01–4 mm, > 4 mm), dermal mitotic rate per mm^2; present or absent ulceration and microsatellites, as well as the pT stage of the disease.

The study investigated clinical and histopathological features of melanoma and compared them between the sexes. The incidence of melanoma subtypes by the localization on the body was also investigated. Tumor thickness determined by Breslow classification was examined against patient age, melanoma subtype, stage of the disease, number of mitoses, presence of ulcerations and microsatellites.

Statistical analysis

The statistical software package SPSS for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA) was used. Parametric and nonparametric features were analyzed by using the χ^2 test, the Mann–Whitney test, the Kruskal–Wallis tests. Afterwards, the Kolmogorov–Smirnov normality test and Spearman's rank correlation coefficient were used.

P-values < 0.05 were considered statistically significant.

Ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the UKCS ethics committee (No.: 602/1 Date: 30.12.2021).

RESULTS

We evaluated a total of 201 tumor samples that were identified histologically as melanoma. All specimens were obtained from complete tumor excisions/excisional biopsy, which is the recommended excision technique if melanoma is suspected clinically.

Out of 201 confirmed melanoma samples, 25 were classified as *in situ* lesions (12.4%), defined by tumor growth confined to the epidermis with an intact basement membrane. The remaining 176 samples (87.6%) were identified as invasive melanoma. *In situ* tumors were almost equally common in both sexes. We assessed differences in the samples by sex (Table 1). The mean age of the patients was 61.47 ± 13.9 years and statistically significantly lower in women (63.39 ± 12.62 *vs.* 59.20 ± 15.03 [p = 0.036]). The majority of patients were in the 61–80 years old group. Patients under 40 years of age were more commonly women (6.4% of men, 15.2% of women), while men predominated among the elderly patients (7.3% of men and 4.3% of women).

Breslow's thickness did not differ between the sexes (p = 0.241) (Figures 1 and 2). The majority of patients (54.7%) had thin melanomas, less than 1-mm-thick tumors (49.6% of men and 60.9% of women). The incidence of 1.01–2 mm and 2.01–4-mm-thick tumors was almost identical in both groups (13.4% and 13.9%, respectively), with 1.01–2-mm-thick tumors slightly more common in men than in women. Breslow thickness above 4 mm was noted in 27 patients (17.9%), most of whom (59.3%) were 60–80 years of age, and slightly more frequent among male population. The patient age did not significantly correlate with tumor depth. On the other hand, a statistically significant difference was observed in relation to the type of melanoma and the Breslow classification (p < 0.001). SSM, LMM, and acral subtypes were mostly thin melanomas (79.1%, 17.3%, and 1.8%, respectively). In contrast, NM accounted for the majority of tumors thicker than 3 mm. Thin melanomas (< 1 mm) were almost equally distributed in the location of thorax and upper extremities, while thick melanomas (> 1 mm) were more frequent in the head and neck region and in the lower extremities (Figure 3).

No major differences between the sexes were detected in the histologic subtype. The most common subtype in both groups was SM, accounting for 68.7% of all tumors, followed by the nodular subtype. Melanoma localization by tumor type is shown in Table 2. The superficial type of melanoma was most often localized on the trunk (45.7%).

Table 1. Comparison of melanoma characteristics according to sex

Variable	Men n (%)	Women n (%)	Total n (%)	p
Age (years)				
≤ 40	7 (6.4)	14 (15.2)	21 (10.4)	0.123
41–60	27 (24.8)	27 (29.3)	54 (26.9)	
61–80	67 (61.5)	47 (51.1)	114 (56.7)	
> 80	8 (7.3)	4 (4.3)	12 (6)	
Total	109 (100)	92 (100)	201 (100)	
Melanoma localization				
Upper extremities	19 (17.4)	15 (16.3)	34 (16.9)	0.001
Head and neck	22 (20.2)	11 (12)	33 (16.4)	
Trunk	50 (45.9)	32 (34.8)	82 (40.8)	
Lower extremities	10 (9.2)	27 (29.3)	37 (18.4)	
Acral	8 (7.3)	7 (7.6)	15 (7.5)	
Total	109 (100)	92 (100)	201 (100)	
Histological type				
Superficial	70 (64.2)	68 (73.9)	138 (68.7)	0.232
Nodular	16 (14.7)	15 (16.3)	31 (15.4)	
Lentigo melanoma	15 (13.8)	6 (6.5)	21 (10.4)	
Acral	2 (1.8)	0 (0)	2 (1)	
Other	6 (5.5)	3 (3.3)	9 (4.5)	
Total	109 (100)	92 (100)	201 (100)	
Breslow thickness				
≤ 1	54 (49.6)	56 (60.9)	110 (54.7)	0.241
1.01–2	19 (17.4)	8 (8.7)	27 (13.4)	
2.01–4	16 (14.7)	12 (13)	28 (13.9)	
> 4	20 (18.3)	16 (17.4)	36 (17.9)	
Total	109 (100)	92 (100)	201 (100)	
pT stage				
Tis	12 (11)	13 (14.1)	25 (12.3)	0.370
T1a	32 (29.4)	35 (38.1)	67 (33.3)	
T1b	11 (10)	8 (8.7)	19 (9.4)	
T2a	14 (12.8)	4 (4.3)	18 (9)	
T2b	4 (3.7)	4 (4.3)	8 (4)	
T3a	8 (7.3)	8 (8.7)	16 (8)	
T3b	9 (8.3)	5 (5.5)	14 (7)	
T4a	10 (9.2)	8 (8.7)	18 (9)	
T4b	9 (8.3)	7 (7.6)	16 (8)	
Total	109 (100)	92 (100)	201 (100)	
Mitosis	55 (50.9)	40 (43.5)	95 (47.5)	0.293
Ulcerations	25 (22.9)	20 (21.7)	45 (22.4)	0.893
Microsatellites	7 (6.4)	1 (1.1)	8 (4)	0.073
Elevated S protein	17 (15.6)	14 (15.2)	31 (15.4)	0.941
Elevated LDH	42 (38.5)	36 (39.1)	78 (38.8)	0.931

LDH – lactate dehydrogenase

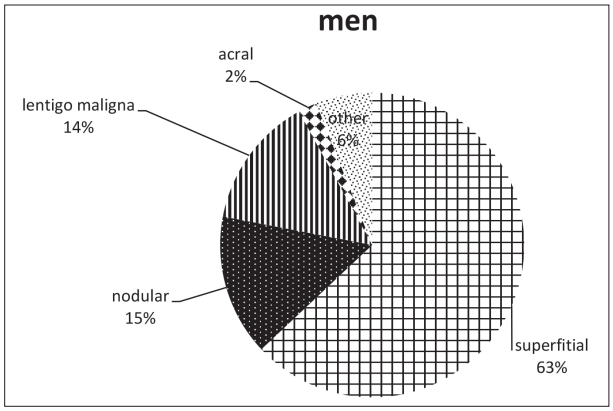

Figure 1. Distribution of melanoma subtype in male population

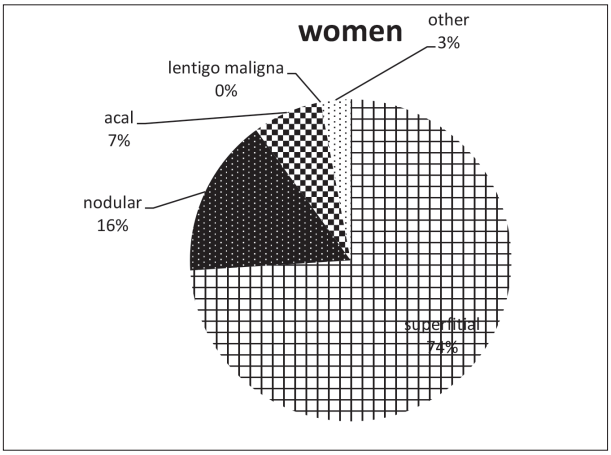

Figure 2. Distribution of melanoma subtype in female population

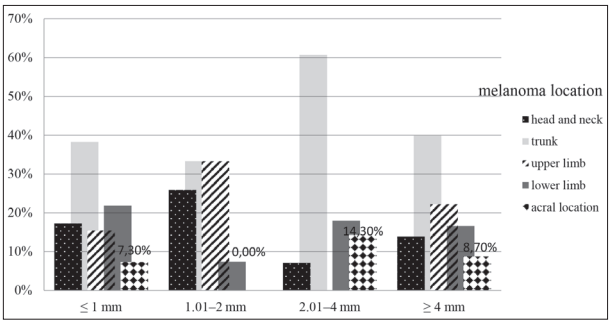

Figure 3. Distribution of melanoma thickness according to localization

Table 2. Comparison of melanoma location according to tumor type

Melanoma localization		Melanoma type					Total
		Superficial	Nodular	Lentigo melanoma	Acral	Other	
Upper extremities	n	25	7	3	0	0	35
	%	18.1%	22.6%	14.3%	0%	0%	17.4%
Head and neck	n	20	4	13	0	3	40
	%	14.5%	12.9%	61.9%	0%	33.3%	19.9%
Trunk	n	63	11	4	0	3	81
	%	45.7%	35.5%	19%	0%	33.3%	40.3%
Lower extremities	n	30	9	1	2	3	45
	%	21.7%	29%	4.8%	100%	33.3%	22.4%
Total	n	138	31	21	2	9	201
	%	100%	100%	100%	100%	100%	100%

The nodular type of tumor was also most commonly localized on the trunk (35.5% of cases). On the other hand, the most common type of tumor localized on the head and neck was lentigo melanoma, registered in 13 out of 21 patients (61.9%). The acral type of melanoma was registered only in two patients and it was localized on the lower extremities in both cases. Considering age distribution and melanoma subtype, superficial spreading, nodular and lentigo maligna were the most common subtypes of melanoma among the population 61–80 years of age (Figure 4). Also, in all locations of the body, melanoma was most frequently found in the age group above 61 years of age (Figure 5).

The study showed statistically significant difference in melanoma location between the sexes (p = 0.001).

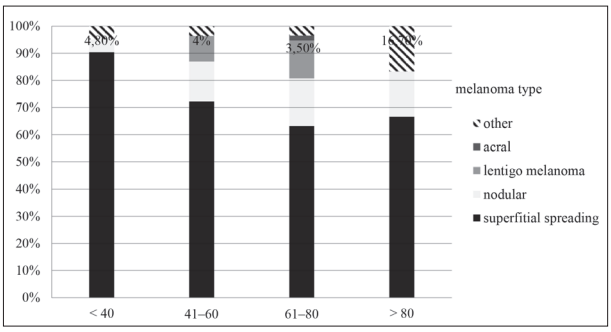

Figure 4. Distribution of type of melanoma according to age

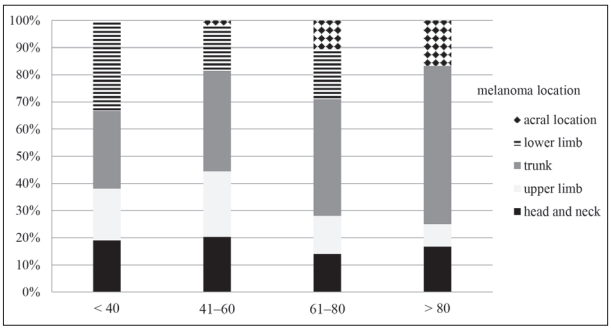

Figure 5. Distribution of melanoma location according to age

Melanoma was most often localized on the trunk 45.9%, followed by the head and neck 24.8% in male population, while in women, the incidence of melanoma was almost identical on the trunk and lower extremities (33.7% and 34.8%, respectively). No statistically significant difference was noted in the pT stage of the disease between the sexes (p = 0.370). Ulcerations were present in 22.4% of primary tumors. Mitoses and ulcerations were almost evenly distributed between the sexes with no significant difference (p = 0.293 and p = 0.893). Microsatellites were more often present in men than in women (p = 0.073), while distant metastases were registered in 2.5% of the patients, with no difference in frequency between the men and the women in our group of patients.

Table 3. Patient characteristics by the Breslow classification

Variable	Breslow				Total n (%)	p
	≤ 1 mm n (%)	1.01–2 mm n (%)	2.01–4 mm n (%)	> 4 mm n (%)		
Age (years)						
< 40	16 (14.5)	2 (7.4)	1 (3.6)	2 (5.6)	21 (10.4)	0.233
41–60	29 (26.4)	7 (25.9)	7 (25)	11 (30.6)	54 (26.9)	
61–80	58 (52.7)	16 (59.3)	20 (71.4)	20 (55.6)	114 (56.7)	
> 80	7 (6.4)	2 (7.4)	0 (0)	3 (8.3)	12 (6.30)	
Total	110 (100)	27 (100)	28 (100)	36 (100)	201 (100)	
Sex						
Men	54 (49.1)	19 (70.4)	16 (57.1)	20 (55.6)	109 (54.2)	0.241
Women	56 (50.9)	8 (29.6)	12 (42.9)	16 (44.4)	92 (45.8)	
Total	110 (100)	27 (100)	28 (100)	36 (100)	201 (100)	
Melanoma type						
Superficial	87 (79.1)	25 (92.6)	13 (36.1)	13 (36.1)	138 (68.7)	< 0.001
Nodular	0 (0)	2 (7.4)	8 (28.6)	21 (58.3)	31 (15.4)	
Lentigo melanoma	19 (17.3)	0 (0)	2 (7.1)	0 (0)	21 (10.4)	
Acral	2 (1.8)	0 (0)	0 (0)	0 (0)	2 (1)	
Other	2 (1.8)	0 (0)	5 (17.9)	2 (5.6)	9 (4.5)	
Total	110 (100)	27 (100)	28 (100)	36 (100)	201 (100)	
Mitosis	16 (14.5)	21 (17.7)	25 (89.3)	33 (94.3)	95 (47.5)	< 0.001
Ulcerations	3 (2.7)	8 (29.6)	13 (46.4)	21 (58.3)	45 (22.4)	< 0.001
Microsatellites	0 (0)	1 (3.7)	2 (7.1)	5 (13.9)	8 (4)	< 0.001

DISCUSSION

General epidemiological features of melanoma relating to the number of newly diagnosed cases and related mortality are mainly well established in many developed countries, but we miss the data on the histopathological features of melanoma [7]. The National Cancer Registry in Serbia keeps only the data on newly diagnosed cases and the number of related deaths, while important prognostic data, such as tumor thickness according to Breslow and the presence of ulcerations, are missing [6].

The results of our study have shown that most patients were over 60 years of age at the time of diagnosis establishing. Men were affected more frequently than women, but women were affected at an earlier age. In the age group under 40 years the number of women was twice the number of men. These results are in line with the latest published sex-specific trends published for populations all over Europe and USA indicating higher incidence among younger women, while men are affected mostly in their middle age [7–11]. These results coincide with the results published in the National Cancer Registry in the Republic of Serbia. This can be explained by different habits of sexes: men are less inclined to self-examine or present for an examination with a specialist. This probably contributes to detection of melanoma later and in more advanced stages [12, 13].

Localization of melanoma on the trunk may also be related to the later detection. The truncal melanomas, more common in men, are less visible and thus detected later. Melanomas in women are detected earlier so that they are thinner according to Breslow classification [14]. Men and women also differ in skin anatomy and physiology. The skin of men is thicker and richer in collagen and elastic fibers. It has less subcutaneous fat and different hair distribution, which responds differently to UV-induced skin trauma and is considered more photosensitive than the skin of women [15, 16]. The results of our study show greater prevalence of stage III I IV melanoma in men, which is in line with a study published by Behbahani et al. [14], suggesting the survival advantage of women, since simple surgical excision is the sufficient treatment method for thin melanoma. Analysis of the results of previous studies relating to anatomic localization and sex has shown that melanoma in men is most commonly localized on the trunk, while in women it usually affects the lower extremities [17].

However, recent studies show that this difference is fading out [18]. Our results also corroborate the trend showing that melanoma most commonly appears on the trunk (45.9%), and head and neck (24.8%) in men, while in women the incidence of melanoma on the trunk and lower extremities was almost identical (33.7% *vs*. 34.8%).

Our results show no statistical difference in the incidence of different types of melanomas between men and women. SM was the most common type of melanoma (68.7%), followed by NM (15.4%), LM (10.4%), and AM (1%), coinciding with the results published for the US and EU [19]. Comparisons of anatomic localizations of UV-exposed regions, as expected, showed LM presence most commonly on the head and neck regions (61.9%), while AM was present on the lower extremities only [9].

The most important prognostic factor was tumor thickness by Breslow, while penetration depth by Clark is still registered in HP reports although AJCC classification does not require it [20]. Most of our patients had melanoma less than 1 mm thick according to Breslow, while other groups were represented equally. Our study did not show statistically significant correlation of age and tumor thickness or sex and tumor thickness either.

The presence of ulcerations, mitotic activity and micro satellites is increased and is very much correlated with tumor thickness, as expected [20]. Numerous studies have shown that ulcerations, as a bad prognostic sign, are more common in men [18]. Our study, however, failed to identify any difference in the incidence of ulceration between the sexes.

CONCLUSION

Further investigations in the field of demographic and clinicopathological characteristics of melanoma are necessary to improve melanoma prevention, diagnosis, and treatment. Professional examination and self-examination should consider all parts of the body, including hidden localizations and particular attention should be paid in the population beyond 60 years. Understanding melanoma behavior and clearly identifying groups at risk in both sexes would allow us to create national programs of prevention and early detection of melanoma that could lead to better overall survival.

Conflict of interest: None declared.

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Клиничкопатолошке карактеристике кожног меланома – ретроспективна студија појединачног центра

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САЖЕТАК

Увод/Циљ Опште епидемиолошке карактеристике меланома, број новооболелих и морталитет, углавном су познате у већини развијених земаља, али оно што недостаје јесу подаци о хистопатолошким карактеристикама меланома. Циљ овог истраживања је било испитивање епидемиолошких, клиничких и патохистолошких карактеристика болесника и упоређивање резултата са трендовима у другим земљама.

Методе Наш узорак су чинили болесници оперисани због меланома коже на Клиници за опекотине, пластичну и реконструктивну хирургију у периоду од 2015. до 2017. године. Испитиване су хистопатолошке, клиничке и демографске карактеристике меланома.

Резултати Ова ретроспективна студија је обухватила 201 болесника (109 мушкараца и 92 жене), старости од 25 до 87 година. Мушкарци су оболевали чешће у односу на жене (54,2% према 45,8%). Меланом се код мушкараца најчешће јавља на трупу, док је код жена инциденца меланома на трупу и доњим екстремитетима идентична. Најчешћи тип био је меланом површног ширења (68,7%), без статистички значајне разлике међу половима. Није уочена корелација у односу на стадијум болести и пол болесника ($p = 0,294$). Статистички значајна разлика је уочена при поређењу дебљине меланома према класификацији по Бреслоу и типа меланома ($p < 0,001$). Није уочена разлика у дебљини меланома по Бреслоу међу половима и према старости болесника. Према инвазивности, 12,4% лезија су класификоване као *in situ* лезије, а 87,6% као инвазивне. Већина болесника је идентификована као стадијум *pT1a*.

Закључак Ова студија може олакшати идентификацију болесника са високим ризиком оболевања од кожног меланома и допринети оптимизацији скрининга у дефинисаној циљној популацији.

Кључне речи: меланом; Бреслоу (*Breslow*); популација Србије; патологија меланома; епидемиологија меланома

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

A clinical study of using a phentolamine alcohol wet dressing in the treatment of extravasation after a 20% fat emulsion intravenous infusion – a randomised trial

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SUMMARY

Introduction/Objective The aim of our paper was to investigate the clinical efficacy of using a phentolamine alcohol wet dressing to treat the extravasation of an intravenously administered infusion of milk fat. This study was designed as a randomized trial, and was done at the Hengshui people's Hospital, Hebei Province, China, from June 2019 to June 2020.

Methods In total, 300 patients were randomly divided into two groups. In the experimental group, the patients were treated using a phentolamine alcohol wet dressing, whereas in the control group, the patients were treated using a hydropathic compress with a 50% magnesium sulphate solution. The cure rate, healing time, and patient satisfaction of the two groups were compared and analyzed.

Results The cure rate of intravenous infusion extravasation was 92.67% (139/150) in the experimental group and 70.67% (106/150) in the control group ($p < 0.05$). In the experimental group, there were 66 patients whose cure time was less than 24 hours, 62 patients whose cure time was between 24 and 48 hours, and 22 patients whose cure time was over 48 hours. The cure time of the patients was significantly shorter in the experimental group than the control group. After treatment, in the experimental group, 67 patients were very satisfied, 52 patients were satisfied, 21 patients were generally satisfied, and 10 patients were dissatisfied; in the control group, 32 patients were very satisfied, 40 patients were satisfied, 56 patients were generally satisfied, and 22 patients were dissatisfied. The satisfaction of patients was significantly higher in the experimental group than in the control group.

Conclusion The effect of using a phentolamine alcohol wet dressing to treat the extravasation of an intravenous infusion of milk fat is significantly better than the effect of using a magnesium sulphate solution, and this type of dressing is worthy of clinical application.

Keywords: phentolamine alcohol wet dressing; magnesium sulphate solution; fat emulsion extravasation; cure rate; patient satisfaction

INTRODUCTION

Preface fat emulsion is an intravenously administered nutrition drug, and it is widely used in clinical practice [1, 2]. However, skin damage is common due to its high concentration and high permeability, and extravasation is a common complication that affects drug absorption [3, 4, 5], destroys the integrity of skin and tissue, and causes obvious pain [6, 7]. A detachable hydropathic compress is a new kind of wet compress method, which could effectively dilate blood vessels, improve blood circulation and reduce local pain.

Extravasation of intravenous infusion is relatively common in the clinic, and although the injury site is limited, the skin damage is severe. In recent years, the drug wet dressing method can partially improve the puncture effect because of its full drug action and strong absorbability. However, the traditional wet dressing method is complicated to operate and the steps are cumbersome. In addition, intravenous infusion puncture is affected by the physicochemical properties of the drug, which increases the risk of tissue damage. Phentolamine alcohol wet dressing can restore local blood supply and oxygen as soon as possible, improve microcirculation, reduce the occurrence of skin necrosis and improve the efficiency of wet dressing; reduce the risk of deterioration and failure of the drug during transportation and external environmental changes, prevent the volatilization of the drug and ensure the therapeutic effect [8, 9, 10]. At present, there are few clinical studies concerning the use of a phentolamine alcohol wet dressing to treat extravasation resulting from a milk fat intravenous infusion. Therefore, this study recruited 300 patients with extravasation following an intravenous infusion of milk fat at the Hengshui People's Hospital to investigate the clinical effect of using a hollow detachable hydropathic compress to treat the extravasation.

METHODS

Subjects

Between June 2019 and June 2020, patients with extravasation caused by the intravenous infusion of 20% fat emulsion were recruited for the

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study. The patients were divided into two groups. In the experimental group, patients were treated with a phentolamine alcohol wet dressing. In the control group, the patients were treated with a hydropathic compress with 50% magnesium sulphate solution. The cure rate, cure time, and patient satisfaction in the two groups were compared. All the participants signed informed consent forms, and the study was approved by the ethics committee of Hengshui People's Hospital (No: AF/SC-08/02.0).

Randomization and blinding

The randomization was conducted by an independent person who managed the randomization list, so the investigators and physicians involved in the trial had no access to this list. SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA) was used to generate a random allocation sequence, and 300 patients were randomly divided into a control group and a treatment group in a 1:1 ratio, with 150 patients in each group.

The experimental group was treated with a phentolamine alcohol wet dressing, and the control group was treated with a 50% magnesium sulphate solution. It was not possible to blind both the person performing the intervention and the subject during the intervention. However, it was designed to be evaluator-blinded in order to control for bias as much as possible. The participants were evaluated by researchers who had not performed the intervention or randomization.

Inclusion and exclusion criteria

Inclusion criteria: (1) patients with extravasation caused by the intravenous infusion of 20% fat emulsion, and (2) patients older than 18 years.

Exclusion criteria: (1) patients with extravasation caused by the intravenous infusion of other drugs, (2) patients with advanced malignant tumors, and (3) patients with incomplete case data.

Experimental methods

Control group: the gauze was half spread over a kidney basin, and 20 mL of 50% magnesium sulphate was poured on top, infiltrating the gauze. The gauze was then compressed onto the infusion extravasation and squeezed with tweezers until there was no excess water liquid. The site of the extravasation was covered, with care being taken to avoid the puncture point, twice a day, for 20 minutes each time.

Experimental group: the phentolamine alcohol wet dressing consisted of a non-woven fabric drug layer, a waterproof and breathable layer, and a protective layer. The drug layer was soaked with 1 mL phentolamine injection and 5 mL 75% alcohol. The protective layer was the largest and the drug layer the smallest.

The protective layer itself had three layers, comprising an inner film, a thermal insulation layer, and a packaging layer. The thermal insulation layer was between the inner film and the packaging layer, and the edge of the packaging layer was connected to the edge of the inner film by hot pressing. There was an anti-allergic medical tape inside the waterproof and breathable edge, and the phentolamine alcohol wet compress was sterilized using an ethylene oxide sterilizer before use. During use, the protective layer was placed on the affected area and fixed with the anti-allergic medical tape with care being taken to avoid the puncture point. The site of the extravasation was covered twice a day, for 20 minutes each time.

Main outcome measures

The main observation indicators of this study were sex, age, cure rate, healing time, patient satisfaction, and the numeric rating scale (NRS) score for pain, which was used before, in the middle, and at the end of the treatment. Treatment effect categories: wet dressing efficiency, patient satisfaction, patient pain score, and healing time of puncture site.

The treatment effect was observed after six hours of treatment in both groups. (1) The specific criteria for clinical efficacy were as follows: remarkable effect meant that local soft tissue swelling subsided, and redness, swelling, heat and pain completely disappeared; valid meant that local tissue swelling reduced, redness, swelling, pain significantly relieved, and no burning; and invalid meant that local tissue redness, swelling, heat and pain did not subside, skin color did not change, and even blisters and necrosis appeared. The total effective rate was remarkable effect + valid/ remarkable effect + valid + invalid × 100%. (2) The Digital Analog Self-Rating Scale worked as follows: a straight line was divided into 10 equal segments, and the degree of pain was evaluated from 0 to 10 points. A circle was drawn around the number describing the most severe pain in the previous 24-hour period. The total score was 10 points with mild pain scoring 1–3 points, moderate pain 4–6 points, severe pain 7–9 points, and extremely severe pain scoring 10 points. Thus, the lower the score, the milder the pain. (3) The cure time was measured as follows: the starting point was when the intervention measures were begun after the extravasation, and the endpoint was the complete disappearance of the clinical symptoms of intravenous infusion extravasation.

Statistical analysis

SPSS Statistics for Windows, Version 19.0, was used for statistical analysis. Continuous variables of normal distribution were expressed as mean ± standard deviation, and discontinuous variables were expressed as frequency [percentage (%)]. The t-test was used for the group comparisons, and the χ^2 test was used for countable data. A value of $p < 0.05$ was considered statistically significant.

Ethics approval statement

All the participants signed informed consent forms, and the study was approved by the ethics committee of the Hengshui People's Hospital (No: AF/SC-08/02.0).

RESULTS

General information

This study consisted of 155 males and 145 females, aged 18–81 years, who were randomly divided into an experimental group (n = 150) and a control group (n = 150). There were no significant differences in age, sex, weight, body mass index, treatment method, and type of disease between the two groups (both p > 0.05) (Table 1).

Table 1. Demographic characteristics

Index	Experimental group (n = 150)	Control group (n = 150)	p
Age (year, Mean ± SD)	37.1 ± 8.7	36.5 ± 6.8	0.174
Sex [male, n (%)]	71 (47%)	76 (50.6%)	0.094
Weight (kg)	76.8 ± 10.2	79.2 ± 11.1	0.105
Body mass index	23.1	21.7	0.099
Treatment method	0.063		
Surgical treatment	115	107	
Non-surgical treatment	35	43	
Type of disease	0.079		
Chronic intestinal obstruction	69	61	
Mesenteric ischemia	43	50	
Crohn's disease	18	16	
Perioperative nutrition supplement	20	23	

The cure rate of extravasation of an intravenous infusion

The cure rate of extravasation of an intravenous infusion was 98% (147/150) in the experimental group, and this was significantly higher than in the control group 86.7% (130/150) (p < 0.05) (Table 2).

Table 2. The cure rate of two groups

Variables	Experimental group	Control group	X^2/t	p
Cure rate	147 (98%)	130 (86.7%)	1.63124e^{-6}	< 0.05
Remarkable effect	66	59		
Effective	81	71		
Invalid	3	20		

The cure times

In the experimental group, there were 66 patients whose cure time was less than 24 hours, 62 patients whose cure time was 24–48 hours, and 22 patients whose cure time was more than 48 hours. In the control group, the cure time was less than 24 hours in 32 cases, 24–48 hours in 51 cases, and more than 48 hours in 67 cases. The cure time of patients in the experimental group was significantly shorter than it was in the control group (Table 3).

Table 3. The cure time of two groups

Group	< 24 h	24–48 h	> 48 h	Effective rate within 48 hours
Control group	66	62	22	128 (85.3%)
Experimental group	32	51	67	83 (55.3%)
X^2				6.07511e-6
p				< 0.05

Pain

The average NRS of the patients in the experimental group was 3.6 before treatment, 5.9 during treatment, and 7.1 after treatment. In the control group, the mean NRS was 1.1 before treatment, 4.1 during treatment, and 5.2 after treatment. No significant difference in the degree of pain was found between the two groups (Table 4).

Table 4. The numeric rating scale (NRS) score of two groups

Group	NRS score after hydropathic compress
Control group	1.8
Experimental group	4.2
t	7.2922
p	< 0.05

Patient satisfaction

In the experimental group, 67 patients were very satisfied after treatment, 52 patients were satisfied, 21 patients were generally satisfied, and 10 patients were dissatisfied. In the control group after treatment, 32 patients were very satisfied, 40 patients were satisfied, 56 patients were generally satisfied, and 22 patients were dissatisfied. The satisfaction of patients was higher in the experimental group than in the control group (Table 5).

Table 5. The satisfaction of two groups

Group	Very satisfied	Satisfied	Generally satisfied	Dissatisfied	Total satisfaction
Control group	67	52	21	10	140 (93.3%)
Experimental group	32	40	56	22	128 (85.3%)
X^2					0.024806937
p					< 0.05

DISCUSSION

The outcomes showed that the extravasation cure rate of intravenous infusion in the experimental group was significantly higher than that in the control group. The cure time of patients in the experimental group was significantly shorter than that in the control group. The satisfaction of patients increased in the experimental group when compared to the control group.

Phentolamine is a kind of alpha-receptor blocker, which can dilate blood vessels and improve blood circulation [11], while dopamine is a vasoconstrictor. An extravasation of

dopamine can lead to vasoconstriction of extravasated skin tissue and skin tissue necrosis [12, 13]. Phentolamine can antagonize the vasoconstriction of dopamine, dilating the blood vessels of local extravasated skin, improving blood circulation, and relieving pain, which is the only drug approved for vasopressin extravasation [14]. Although phentolamine is considered the standard for extravasation in current treatment protocols, it is often used with greater limitations [15].

Alcohol is a vasodilator, so it can dilate blood vessels in local tissue and improve blood circulation [16, 17]. A hydropathic compress of alcohol has an anesthetic effect on local tissues and nerves, thus reducing the pain suffered by patients. Furthermore, alcohol is a bacteriostatic drug, which can prevent local tissue infection and reduce local reaction [18].

It was found in this study that the hollow design was effective in preventing a skin infection, which can be caused by a hydropathic compress, at the puncture site, and the hollow design also increased the suction and viscosity around the site. The detachable design reduced the amount of labor associated with the daily moving of the wet compress, and the replacement was more convenient. Although the hydropathic compress position was relatively fixed, its effect was improved. The combination of these two methods effectively dilated blood vessels, improved local blood circulation, and reduced the contractile effect of dopamine on blood vessels and the degree of tissue damage. It also effectively reduced the skin pain at the exudation [19, 20], prevented local infection, reduced the healing time, and improved the satisfaction of patients.

Limitations: First, there was no blind method in this study. Second, there was only a small sample size, and so a further trial with a larger sample size is needed. Finally, the specific mechanism of a phentolamine alcohol wet dressing used to treat an extravasation of a milk fat intravenous infusion is still not clear, and, thus, further study is needed.

CONCLUSION

The effect of a phentolamine alcohol wet dressing in the treatment of an extravasation of an intravenous infusion of milk fat is significantly better than that of using a magnesium sulphate solution, and such a dressing is worthy of clinical application.

What this paper adds
What is already known on this subject

An extravasation of an intravenous infusion is a common complication and affects drug absorption. At present, there are few clinical studies concerning the treat extravasation resulting from a milk fat intravenous infusion.

What this study adds

The effect of a phentolamine alcohol wet dressing in the treatment of an extravasation of an intravenous infusion of milk fat is significantly better than that of using a magnesium sulphate solution.

The combination of a hollow design and a detachable design effectively dilated blood vessels and improved local blood circulation.

Clinical registration number: researchregistry6867
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Conflict of interest: None declared.

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Клиничка студија о коришћењу влажног завоја са фентоламин-алкохолом у лечењу екстравазације после интравенске инфузије са 20% масне емулзије – рандомизовано истраживање

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САЖЕТАК

Увод/Циљ Циљ рада је био да се испита клиничка ефикасност употребе влажног завоја од фентоламин-алкохола за лечење екстравазације интравенски примењене инфузије млечне масти. Ова студија је осмишљена као рандомизовано испитивање и рађена је у народној болници Хенгшуи, провинција Хебеј, Кина, од јуна 2019. до јуна 2020.

Методе Укупно 300 болесника је насумично подељено у две групе. У експерименталној групи болесници су лечени влажним завојем од фентоламин-алкохола, док су у контролној групи болесници лечени хидропатском облогом са 50% раствором магнезијум-сулфата. Упоређени су и анализирани стопа излечења, време излечења и задовољство болесника из две групе.

Резултати Стопа излечења екстравазације интравенском инфузијом била је 92,67% (139/150) у експерименталној групи и 70,67% (106/150) у контролној групи ($p < 0,05$). У експерименталној групи било је 66 болесника чије је време излечења било мање од 24 сата, 62 болесника чије је време излечења било између 24 и 48 сати и 22 болесника чије је време излечења било дуже од 48 сати. Време излечења болесника било је значајно краће у експерименталној групи него у контролној групи. Након лечења, у експерименталној групи 67 болесника је било веома задовољно, 52 болесника су била задовољна, 21 болесник је био генерално задовољан, а 10 болесника је било незадовољно; у контролној групи 32 болесника су била веома задовољна, 40 болесника је било задовољно, 56 болесника је било генерално задовољно, а 22 болесника су била незадовољна. Задовољство болесника је било значајно веће у експерименталној групи него у контролној групи.

Закључак Ефекат употребе фентоламин-алкохолног влажног завоја за лечење екстравазације интравенске инфузије млечне масти значајно је бољи од ефекта употребе раствора магнезијум-сулфата, а ова врста завоја је вредна клиничке примене.

Кључне речи: фентоламин-алкохолна влажна облога; раствор магнезијум-сулфата; екстравазација масне емулзије; стопа излечења; задовољство болесника

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Acute disseminated encephalomyelitis in children and adolescents – 20-year single-center experience in Serbia

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SUMMARY
Introduction/Objective Acute disseminated encephalomyelitis (ADEM) is the most common demyelinating disease of the central nervous system in pediatric patients. We aimed to evaluate the clinical profile of children with ADEM and to discern prognostic factors for disease outcome.
Methods A 20-year retrospective–prospective study was conducted in a cohort with the diagnosis of ADEM.
Results The study included 36 patients, with range of follow-up period of 6–120 months (median of 26 months). Prior infection was reported in 72.2% of the patients. In the clinical presentation of the disease, motor deficit was most common (81.1%), followed by ataxia (77.8%). More than a third of patients had back and limb pain or abdominal visceral pain, which highly correlated with MRI findings of myelitis. Abnormal brain CT findings were evident in 22.2% of the patients, and this was associated with higher Expanded Disability Status Scale (EDSS) and quicker progression of the disease. Median EDSS was 0 at the most recent follow-up visit, in all the patients. EDSS 0–2.5 was verified in 29 (80.6%) of the patients, while three (8.3%) patients scored 7–9.5 at the last visit. Two patients had a lethal outcome.
Conclusions ADEM is a serious disease in pediatric patients, but with a good prognosis, which is illustrated by the fact that 80.6% of our patients had a complete or almost complete recovery.
Keywords: encephalomyelitis; demyelination; children; adolescents; prognosis

INTRODUCTION

Acute disseminated encephalomyelitis (ADEM) is an immune-mediated inflammatory disorder of the central nervous system (CNS), characterized by disseminated demyelinating lesions, predominantly in the brain's white matter and the spinal cord. In absence of specific biomarkers, a diagnosis of ADEM is based on the clinical presentation and neuroradiological findings. In 2007, the International Pediatric Multiple Sclerosis Society Group (IPMSSG) published consensus criteria for demyelinating disorders of childhood, including ADEM, which was updated in 2013 [1, 2]. These criteria have significantly contributed to the accuracy of diagnosis and better management of demyelinating disorders in childhood. According to these criteria, ADEM is an acute or subacute disease characterized by signs of encephalopathy and multifocal neurological deficit. Magnetic resonance imaging (MRI) of the brain shows typically large, poorly demarcated white matter lesions, although involvement of the cortical gray matter is not uncommon [3, 4, 5]. The disease is usually monophasic; however, relapses are possible [5, 6, 7].

In 50–75% of ADEM patients, there is evidence of recent infection or immunization [8, 9, 10]. Most commonly, these are non-specific upper respiratory tract infections, which explains a higher incidence of the disease in winter and spring months. Inflammation is believed to be the result of a transient immune response to myelin or other autoantigens through a molecular mimicry mechanism or non-specific activation of autoreactive T cell clones. There is numerous evidence that ADEM is a T cell-mediated autoimmune disease. The yearly incidence of ADEM in children is estimated at 0.4–0.54 per 100,000 persons and is somewhat higher than in adults [3, 9].

The aims of our study were to evaluate 20-year experience from a single institution in Serbia in treating children and adolescents diagnosed with ADEM, and to examine relationships between clinical features, microbiology, neuroimaging, treatment, and outcomes of the disease.

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METHODS

This is a retrospective and prospective study of all patients diagnosed with ADEM at the Dr Vukan Čupić Mother and Child Health Care Institute of Serbia in Belgrade over a period of 20 years, from January 1999 to March 2020. This is a retrospective study in the period from 1999 to 2007 and a prospective one from 2008 to 2020, after the publication of Krupp's criteria in 2007 [1]. Clinical information was obtained from the inpatient medical records. Diagnosis of ADEM was based on the definition proposed by the IPMSSG [1, 2]. This definition was used to define the inclusion and exclusion criteria of our study.

The inclusion criteria were the following: 1) acute or subacute disease onset, 2) multifocal neurological disorder, 3) signs of encephalopathy, defined on the basis of at least one of the following two criteria: a) behavioral disorder, i.e. confusion, extreme irritability and/or b) disorder of consciousness (lethargy, somnolence, coma), 4) neuroimaging-verified areas of demyelination of the CNS. The exclusion criteria were the following: 1) previously registered lesions in the brain's white matter, 2) other neurological disorders, 3) congenital metabolic diseases, 4) infective or immunological diseases of the CNS, 5) absence of signs of encephalopathy, 6) presence of clinically isolated syndromes like optic neuritis, transverse myelitis, and brainstem encephalitis.

We retrospectively applied IPMSSG criteria to subjects treated for encephalitis and myelitis in our hospital from 1999 to 2007 in order to recruit ADEM cases. After the publishing of the IPMSSG criteria in 2007, we regularly use these criteria in our clinical practice for diagnosing ADEM.

The following demographics as well as clinical data were collected for each patient: sex, age, previous illnesses in personal history (including fevers with rashes and neurological diseases like febrile/afebrile epileptic seizures), preceding infection or vaccination within 2–30 days before clinical presentation of ADEM, interval between the previous infection/vaccination and the onset of ADEM, season, systemic and neurological symptoms and signs, clinical course and length of hospitalization. We evaluated consciousness disorder level according to the modified Glasgow Coma Scale (GCS) score [10]. We collected inflammation-related laboratory parameters (sedimentation, C-reactive protein, leukocyte count), cerebrospinal fluid (CSF) samples (cytological, biochemical, and bacteriological). An extensive work-up for bacterial and viral infections was performed. Bacteriological cultures and serological testing for numerous infectious agents in the serum and the spinal fluid, as well as by direct detection of DNA through the polymerase chain reaction for the herpes simplex virus (HSV) and the Epstein–Barr virus (EBV). Antibody titers were determined in the serum and the spinal fluid for the following agents: HSV1, HSV2, EBV, cytomegalovirus, varicella zoster virus, morbilli virus, rubella virus, *Mycoplasma pneumoniae*, *Borrelia burgdorferi* and seldom human immunodeficiency virus. The complement attachment reaction was used to test the possibility of an infection by the following viruses: influenza A and B, parainfluenza 3, adenoviruses, and lymphocytic choriomeningitis virus. A few patients were tested for serum anti-N-methyl D-aspartate (NMDA) receptor antibodies (anti-NMDAR) and anti-aquaporin-4 antibodies.

Computed tomography (CT) and MRI of the brain and the spinal cord were performed initially and during the follow-up of patients. Large, confluent or tumefactive MRI lesions > 2 cm were considered severe. Electroencephalogram (EEG), nerve conduction studies, visual evoked potentials and brainstem evoked response audiometry were analyzed. The Kurtzke Expanded Disability Status Scale (EDSS) was applied for assessment of the level of neurological damage during follow-up [11]. The main outcomes in the focus of our study were EDSS and patient survival.

Statistical analysis

Mann–Whitney test was used to compare the differences between two groups with non-parametric data. We used Pearson's χ^2 test in the form of contingency tables to analyze two attributive properties. Correlation of non-parametric variables was established by Spearman correlation method. To identify predictors of EDSS score at the last follow-up visit, we used the linear regression analysis, while logistic regression was used to assess risk factors for a fatal outcome. Independent variables were selected for regression analysis on the basis of previous knowledge of risk factors, assessment of potential other risk factors related to pediatric age, and also those variables that could affect the conclusions as confounding factors. In all analytical methods applied, the significance level was 0.05.

The study protocol was in accordance with the tenets of the Declaration of Helsinki and its later amendments. The study was approved by the Ethics Committee of our institution.

RESULTS

Thirty-six patients who met the inclusion criteria for ADEM were included in this study. Mean age of patients was 6.7 years (SD 3.58, median 5.7 years), ranging from six months to 14.2 years. The sex distribution was equal (1:1) among the patients older than 10 years, while in younger patients, male sex was more prevalent (1.5:1) ($p > 0.05$). Prior infection was reported for 26 patients (72.2%): respiratory illness in 14 (38.9%), non-specific febrile episode in five (13.9%), gastrointestinal infection in four patients, while rubella, varicella, and dental infection were reported in single patients. Previous acute infections of the upper respiratory tract and gastrointestinal tract were viral non-specific infections. There were no patients with recent vaccination. No trigger was identified in 10 patients (27.8%). The median period between the occurrence of the triggering event and the onset of ADEM was seven days (range 2–30 days). The shortest latency was found after non-specific respiratory illness (median seven days) and the longest after non-specific fever (15 days), ($p > 0.05$). The presence of the trigger was associated with significantly lower EDSS in

comparison with patients without an identifiable triggering factor for ADEM ($p < 0.05$). The presence of a trigger did not significantly affect survival (p 0.524). The majority of patients are diagnosed in winter (38.9%) and summer (25%) months, but the seasonal variation did not reach the point of statistical significance ($p > 0.05$). The overview and frequency of clinical manifestations of ADEM in the studied group are shown in Table 1.

Table 1. Initial signs and symptoms of acute disseminated encephalomyelitis

Signs and symptoms	Frequency n (%)
General signs and symptoms	
Fever	25 (69.4)
Headache	22 (61.1)
Vomiting	19 (52.8)
Pain (back, legs, abdomen)	14 (38.9)
Respiratory manifestations	9 (25)
Neurologic signs and symptoms	
Altered consciousness	36 (100)
Ataxia	28 (77.8)
Speech disturbance	27 (75)
Cranial neuropathy	19 (52.8)
Tetraparesis/tetraplegia	16 (44.4)
Hemiparesis/hemiplegia	8 (22.8)
Nystagmus	7 (19.4)
Paraparesis/paraplegia	5 (13.9)
Senzory neuropathy	5 (13.9)
Seizures	5 (13.9)
Extrapyramidal signs	4 (11.1)

Median time period from signs and symptoms' onset to the maximum of the clinical manifestations was 3.5 days (range 0–13). The rate of the disease progression did not significantly affect survival ($p > 0.05$). Moreover, there was no significant correlation between the period of disease progression and final EDSS ($p > 0.05$). The modified pediatric GCS ranged 3–14, with a median value of 11. Deep comma (GCS ≤ 8) developed in 30.6% of patients, moderate affection of consciousness (GCS 9–12) in 41.7%, and mild affection of consciousness (GCS ≥ 13) in 27.8%. There was no significant correlation between GCS and EDSS ($p > 0.05$). The length of hospital stay ranged 8–100 days, with a median of 28 days. Survival was not significantly affected by the length of hospitalization ($p = 0.284$). Additionally, the length of hospitalization showed no significant correlation with EDSS ($p = 0.493$).

CSF analysis was done in 34 patients. CSF cell count ranged 1–63 cells/ml with the median value of 6.5. Pleocytosis was found in 23 (67.6%) patients, with predominance of mononuclear cells. Glycorrhachia ranged 2.1–5.4 mmol/L with a mean of 3.81 mmol/L (SD 1.1). There were no patients with hypoglycorrhachia. CSF protein concentration ranged 137–780 g/L, with a median of 283.5 g/L. Elevated CSF proteins were found in 27.2% of patients. Spinal fluid cultures were sterile in all patients (100%). EDSS correlated significantly with CSF protein concentration (Spearman correlation coefficient = +0.39, $p = 0.02$) and negatively with CSF glucose concentration (Spearman correlation coefficient = -0.43, $p = 0.01$). OCB (oligoclonal bands) analysis in CSF and serum was done in 20 patients and was positive in four patients. One child had OCB only in CSF, while three patients had OCB in both CSF and serum.

Almost two-thirds (64.9%) of patients had diffusely slow EEG activity, while focal slow activity was found in 13.5%. There was no statistically significant difference of EDSS or GCS in regard to EEG findings ($p > 0.05$). Initial EEG features showed no significant association with later occurrence of epilepsy ($p > 0.05$).

Median time from disease onset to first CT scan was three days. Abnormal brain CT findings (oedema or hypodense lesions) were evident in 22.2% of patients. Abnormal brain CT scan was associated with higher EDSS (3.19 *vs*. 1.48).

Initial brain MRI scan was performed at median time of 10 days (range 2–98) after the symptoms' onset in 34 patients. Gadolinium enhancement was found in 36.1% of initial MRI scans with severe pathologic changes present in 61.1%. Patients with large and confluent brain lesions with mass effect did not have significantly different final EDSS when compared to patients with small brain lesions on MRI ($p > 0.05$). Furthermore, the presence of gadolinium enhancement was not found to affect EDSS ($p > 0.05$). Children with MRI lesions of spinal cord more commonly experienced pain (71.4%) when compared to children with normal spinal cord MRI (18.2%) (Pearson $\chi^2 = 10.21$, $p = 0.001$). Control MRI scans (median of 92 days after disease onset) showed regression of all changes in 83.3% of patients, stable findings in 8.3%, and worsening in further 8.3%. Overview of the localization of brain MRI lesions observed in studied patients is presented in Table 2.

Table 2. Localization of brain magnetic resonance imaging (MRI) lesions in patients with acute disseminated encephalomyelitis

Brain MRI changes	Frequency n (%)
Subcortical and deep white matter	24 (66.7)
Brainstem	20 (55.6)
Periventricular white matter	15 (41.7)
Basal ganglia	14 (38.9)
Spinal cord	14 (38.9)
Thalamus	12 (33.3)
Cerebellum	12 (33.3)
Cortical grey matter	8 (22.2)
Capsula externa	8 (22.2)

Antibacterial and antiviral treatments were administered until an infectious disease process was ruled out. Therapy for brain edema was universally administered. Most commonly used drug for the treatment of our patients was methylprednisolone in 80.6% of the cases, followed by dexamethasone in 13.9% and intravenous immunoglobulins in 25% of the patients. Therapeutic plasma exchange (TPE) was administered in two patients (5.6%). Corticosteroids were the first line of therapy in all the patients and initiated 1–45 days after the onset of the disease (median of eight days). There was no significant correlation between the length of treatment delay and final EDSS ($p > 0.05$).

The analysis of the complications of the disease shows that three children had urinary tract infection, two children had pneumonia, while sepsis, cardiopulmonary arrest, and thrombophlebitis of deep leg veins were found in single patients. Mechanical ventilation due to coma was used in six children over a period ranging 8–40 days (median of 21.8 days).

Outcomes

Only eight patients (22%) had normal neurological status at discharge, while 56% of the patients had completely normal findings at the most recent neurological exam. Median EDSS was found to be 0 at the most recent follow-up visit for surviving patients, after the range of follow-up period of 6–120 months (median of 26 months). EDSS in a range of 0–2.5 was verified in 29 (80.6%) patients, while three (8.3%) patients scored 7–9.5. Most recent neurological examination showed normal findings in 20 patients (55.6%) without any consequences and was abnormal in 14 patients. The frequency of different neurological sequelae is represented in Table 3. In our sample of subjects, ADEM caused two lethal outcomes. One patient died during the acute stage of the disease. In the second patient with lethal outcome, criteria for ADEM were initially present, but during the follow-up period the diagnosis of multiple sclerosis was established. We do not have more detailed data about the death of the patient with MS, because he died in another hospital.

Table 3. Frequency of neurological sequelae in acute disseminated encephalomyelitis patients at the most recent follow-up visit (median of follow-up period 26)

Neurologic signs and symptoms	Frequency n (%)
Cognitive impairment	8 (22.2)
Epilepsy	5 (13.9)
Hemiparesis/hemiplegia	5 (13.9)
Paraparesis/paraplegia	3 (8.3)
Tetraparesis/tetraplegia	3 (8.3)
Visual impairment	2 (5.6)
Hearing impairment	2 (5.6)

Patients treated before the publication of Krupp's criteria had significantly higher EDSS score in comparison to the group of patients treated after 2007 ($p = 0.003$). This finding was further supported by linear regression analysis showing that EDSS was significantly affected by the time period when the treatment was initiated. When binary regression was used to assess the impact or multitude of potential risk factors (demographic and clinical) to the lethal outcome of ADEM, not any factor was significantly affecting survival.

DISCUSSION

Our study is the first study aimed at evaluating pediatric patients with ADEM in the Serbian population. Our main goal was to assess the long-term outcomes of pediatric patients suffering from ADEM and prognostic factors for disease outcome.

The mean age at disease onset in our cohort was 6.7 years, which corresponds well to previously reported range (3.6–7 years) [12, 13, 14]. The sex ratio was equal in the cohort as a whole, while there was a predominance of males under the age of 10. A number of studies also showed an equal sex ratio among patients [13], but several authors reported male prevalence, especially at younger age [14, 15, 16], as in our study. Precipitating event over a four-week period before the onset of the clinical picture of ADEM was registered in 72.2% of the cases, similar to previous researches [14, 17]. There was no case with previous vaccination, unlike in some previous studies [3, 4]. Children with ADEM occurring after infection had a better long-term outcome compared to cryptogenic cases; in fact, analysis showed significantly lower final EDSS in the postinfectious group of patients (mean of 2.6) when compared to cryptogenic cases (mean of 1.4).

Tenembaum et al. [18] reported that neurological symptoms worsened after a mean period of 4.5 days in their group of 84 patients, while in our study that period averaged 3.5 days. Nishiyama et al. [19] presented the detailed clinical course of 24 pediatric ADEM patients in Japan and established that neurological progression typically took 4.1 ± 3.7 days with improvement onset on day 7 ± 4.5.

Each subject in our study had two or more general disease symptoms or signs, the most common being fever, headache, and vomiting, similar to other studies [13]. Motor deficit was most common (81.1%), which was also suggested by the majority of other published studies [10, 18, 20]. The initial presentation of the disease revealed that ataxia was found in a high percentage (77.8%) of the patients, but its presence did not affect EDSS significantly in the studied group. More than a third of our patients had back and limb pain or abdominal visceral pain, which was highly correlated with MRI findings of myelitis. The pain existed in 72% of our patients with MRI lesion in the spinal cord, while Barakat et al. [21] detected pain in 88% of 24 children with acute transverse myelitis. The pain in demyelinating disorders is the result of damage of the spinothalamic pathway or the dorsal column of the spinal cord. There is little research regarding pain in pediatric spinal cord demyelinating disease, in contrast to adult population. Barakat et al. [21] conducted research on pain and new quantitative MRI techniques (diffusion tensor imaging, magnetization transfer imaging) for spinal cord examination in children with demyelinating disease, which may be useful for monitoring the efficacy of management for myelitis in the future.

Interesting fact is that EDSS correlated significantly positively with CSF protein concentration and negatively with CSF glucose concentration, which could mean that higher degree of inflammation has adverse influence on disease outcome. OCB were positive in serum and CSF in three patients (15%), of 20 patients who were tested. One of them had OCB in CSF only and not in the serum. This patient developed MS later on. According to Dale et al. [20], out of their 35 patients, six (17%) had positive OCB

in CSF. Intrathecal oligoclonal bands were only present in 0–20% of the cases in other studies [3, 13]. It would be important to determine the biochemical markers in the CSF on the basis of which we could predict the outcome of the disease, in future research. We have not been able to determine the presence of anti-MOG antibodies in the cerebrospinal fluid before; however, it has been shown that MOG-Abs were identified in 33–66% of pediatric patients with ADEM [12, 22]. Rossor et al. [23] demonstrated that a higher relapse rate in children with MOG-Ab-associated ADEM, and a trend towards a greater risk of post-ADEM epilepsy. However, MOG-Ab alone are not sufficient to induce the disease. Proinflammatory cytokines are of great importance in the process of pathogenesis [24].

Hypodense CT lesions in the first days of the disease in our patients were associated with faster progression of the disease and poorer outcome. Brain edema was present on CT scan in one patient who died in the acute phase of the disease. Although we know that MRI is a much more sensitive method for detecting demyelinating lesions in ADEM [15], CT images can also be very important in the early diagnosis of brain edema. Distribution of demyelinating MRI lesions in our study was similar to other studies [13, 20, 25]. We failed to prove that there was a significant association between the extensiveness of lesions on brain MRI and the final outcome of the disease. Most recently repeated MR scans (median of 92 days after disease onset) in our study showed regression of all changes in 83.3% of patients, in accordance with data from other studies [10, 15].

Our first-choice treatment was i.v. administration of methylprednisolone in high doses for five days, followed by oral administration of prednisone in tapering doses. In case of failure to achieve significant clinical improvement early after starting corticosteroid therapy, intravenous immunoglobulins (IVIG) were used as the second treatment line. TPE was applied in two patients, with poor recovery from corticosteroids and IVIG and proved to be a very successful therapy. If there was no improvement within two weeks after IVIG administration, we decided to apply TPE. We applied five sessions of TPE on alternate days. There are no studies comparing the efficacy of corticosteroids, IVIG, and plasmapheresis.

In accordance with previous studies, our study asserts that children with ADEM have mostly favorable outcome [3, 15, 26]. Patients who developed epilepsy during the follow-up period did not have seizures in the acute phase of the disease. Five patients had acute attacks, but only one of them had subsequent attacks during the follow-up period. All patients with epilepsy after ADEM had large MRI lesions in acute stage of the disease, as depicted in Figure 1, showing brain MRI in a four-year-old boy.

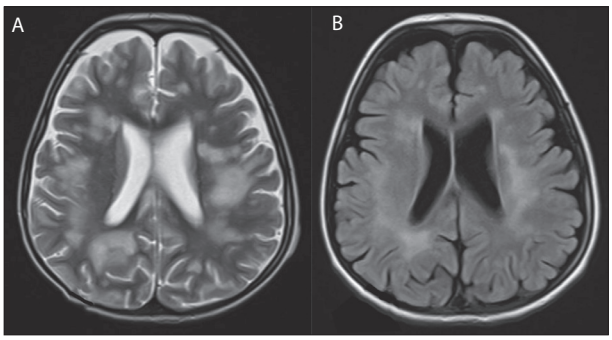

Figure 1. Acute disseminated encephalomyelitis in a four-year-old boy; A) T2W sequence of brain magnetic resonance imaging (MRI) scan shows large dissemianted hyperintense lesions; B) follow-up brain MRI obtained 15 months later shows a decrease and no appearance of new lesions

The main advantages of our study stem from long experience of treating children with ADEM. Our sample of children and adolescents was homogenous in terms of inclusion criteria. We also provide a realistic overview of clinical approach to pediatric patient with ADEM in a tertiary-level health care hospital in Serbia. This is the first decade-long study regarding pediatric ADEM in the region of the Balkans. The main disadvantage of our study is the variable length of patients' follow-up periods.

CONCLUSION

Our 20 years of experience have shown that ADEM is a serious disease in children, but with a good prognosis in the majority of patients, illustrated by 80.6% rate of complete or near-complete recovery, after a follow-up period ranging 6–120 months. Poor prognostic factors for disease outcome in terms of disability were the following: absence of previous infection as ADEM trigger, findings of brain edema or hypodense CT lesion in the first days of the disease, as well as higher values of protein in the CSF. Finding that EDSS was significantly better in ADEM patients treated after Krupp's criteria publication poses an important proof of their value in the clinical practice.

Conflict of interest: None declared.

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Акутни дисеминовани енцефаломијелитис код деце и адолесцената – двадесетогодишње искуство у једном центру у Србији

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САЖЕТАК

Увод/Циљ Акутни дисеминовани енцефаломијелитис (АДЕМ) најчешћа је демијелинизациона болест централног нервног система код педијатријских болесника. Циљ нашег рада је био да проценимо клиничке карактеристике деце са АДЕМ и да установимо прогностичке факторе за исход болести.

Методе Спроведена је двадесетогодишња ретроспективно-проспективна студија у кохорти болесника са дијагнозом АДЕМ.

Резултати Студија је обухватила 36 болесника, са периодом праћења 6–120 месеци (медијана 26 месеци). Претходна инфекција је пријављена код 72,2% болесника. У клиничкој презентацији болести моторни дефицит је био најчешћи (81,1%), а затим атаксија (77,8%). Више од трећине болесника је имало бол у леђима и екстремитетима или абдоминални висцерални бол, што је било у високој корелацији са МР налазима мијелитиса. Абнормални налази КТ мозга су описани код 22,2% болесника, што је било удружено са вишим скором на Проширеној скали степена онеспособљености (*Expanded Disability Status Scale, EDSS*) и бржим напредовањем болести. Медијана *EDSS* за целу кохорту на последњем контролном прегледу је износила 0. Код 29 (80,6%) болесника *EDSS* се кретао у опсегу 0–2,5, док су три (8,3%) болесника имала скор 7–9,5. Два болесника су имала летални исход.

Закључак Акутни дисеминовани енцефаломијелитис је озбиљна болест код педијатријских болесника, али са добром прогнозом, што илуструје податак да је 80,6% наших болесника имало потпуни или скоро потпуни опоравак.

Кључне речи: енцефаломијелитис; демијелинизација; деца; адолесценти; прогноза

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Cytokine gene polymorphisms of TNF, IFN-γ, and IL-12 as potential predictors in the onset of cervical disease in HR HPV-positive women with behavioral risk cofactors

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SUMMARY

Introduction/Objective The aim of this study was to investigate the distribution of genotypes and alleles of proinflammatory cytokines TNF, IFN-γ, and IL-12 and their effect on the development of a cervical illness and also to determine their associated influence with cofactors in HR HPV-positive women in Serbia.

Methods We have investigated 24 women and based on the cytological findings they were classified into four groups: PAP II, ASCUS, LSIL, and HSIL. Analysis of TNF, IL-12, and IFN-γ polymorphisms was performed using the real-time PCR TaqMan method. Statistical analysis was performed using parametric and non-parametric tests and correlation and multiple regression analysis.

Results Significantly higher frequency of high production-related TNF AA genotype was observed in severe dysplasia. The correlation between TNF gene polymorphism and cervical findings were highly significant. There was a moderate, significant correlation between low production IFN-γ AA genotype and earlier cervical infections. There was a significant correlation between the IL-12 polymorphism of the low production IL-12 AA genotype and cervical lesions.

Conclusion Results of this study show that HSIL is associated with significantly higher frequency of high production TNF AA genotype. It is known that polymorphisms of certain cytokine genes encoding proteins involved in Th1 and Th2 cellular responses may be associated with better or worse prognosis of cervical disease in women with persistent HR HPV infection. Therefore, they may be considered as biomarkers that may have a predictive role in the development of cervical cancer.

Keywords: cervical cancer; cofactors; gene polymorphism; TNF; IFN-γ; IL-12

INTRODUCTION

Cervical cancer is the fourth most frequent cancer in women with an estimated 604,000 new cases in 2020. Of the estimated 342,000 deaths from cervical cancer in 2020, about 90% of these occur in low- and middle-income countries. This frequency varies by geographical areas and ranges 17.2–55 per 100,000 women [1, 2, 3]. This tumor is highly correlated with infection by highly oncogenic types of human papilloma virus (high-risk HPV, HR HPV), which are the most common sexually transmitted pathogens [4, 5]. Since Harald zur Hausen proved the presence of highly oncogenic HPV types 16 and 18 DNA in cervical cancer cells in the early 1980s, it was clear that HPV infection is the key factor in its emergence [5, 6, 7]. In a majority of cases (> 80%), a spontaneous regression of changes occurs and the virus is eliminated within two years of initial infection. However, in a minority of cases persistent infection is established, from which 25% of infected women develop cervical intraepithelial neoplasia in the first degree (CIN I), with further progression (CIN II/III). Cervical cancer would develop in 10% of all patients and in 1% of HR HPV-positive women over a number of years [6, 8].

Recently, more attention has been dedicated to the role of genetic predisposition in the development of cervical cancer associated with various predisposing environmental cofactors. Genome-wide association studies have discovered a vast number of genes whose individual alleles are associated with a predisposition to develop cervical cancer. There are still contradictory data from different research teams, related to different ethnic populations and geographical areas, on polymorphisms in genes encoding proteins involved in the functioning of the Th1 and Th2 cellular response and their role in the pathogenesis of emerging HR HPV cervical cancer [9, 10]. Single nucleotide polymorphisms (SNPs) in genes encoding relevant proinflammatory cytokines, such as tumor necrosis factor (TNF), interferon-gamma (IFN-γ), and interleukin-12 (IL-12), are becoming highly significant genetic markers for assessing the risk of developing cervical cancer associated with HR HPV [11, 12].

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SNP at position -308 in the promoter region of the TNF gene (rs1800629) G/A, is highly correlated with CIN I associated with HR HPV. TNF plays an important role in various inflammatory diseases as one of the most important proinflammatory cytokines. It is considered that presence of A allele at the -308 TNF gene locus, which is a high secretory TNF phenotype, could have a strong influence on the development of HR HPV cervical cancer [13]. Studies show elevated TNF serum levels in women with CIN in comparison with healthy women. Also, AA genotype at the -308 locus of the TNF gene carries a higher risk for developing cervical tumors compared to GG and GA genotypes. A meta-analysis confirmed an increased risk of developing cervical cancer in the presence of the A allele, especially the AA genotype [14, 15].

IFN-γ is one of the most important Th1 immune modulators with antiviral and antitumor role. SNP at position +874 T/A (rs62559044) located in the first intron of the IFN-γ gene is associated with increased production of IFN-γ and effective defense against HPV infection, while its low level, which is present in cervical cancer, is associated with a poor prognosis [16, 17]. The DNA sequence containing the +874 T allele has a binding site for NF-kappa B-like transcription factor [18, 19]. Literature data shows that the presence of the T allele correlates with increased IFN-γ expression, thus reducing the cervical cancer risk, while a low secretory +874 IFN-γ AA genotype is associated with a high risk for developing HR HPV cervical tumors [7].

IL-12 is a heterodimer composed of two subunits – p35 (IL-12A) and p40 (IL-12B). Inheritance of functional SNP variants of this gene, which is associated with the destruction of cancer cells, leads to changes in expression, which also affects the function of other cytokines that are under its regulation. IL-12 stimulates the production of IFN-γ by signaling the molecular cascade, while inhibiting IL-4, which suppresses IFN-γ synthesis and stimulates the protective Th1 immune response. SNP at the +1188 A/C position in the 3'UTR region of the IL-12B gene (rs3212227) has been associated with a predisposition to cervical cancer in women with HPV HR. The AA genotype is also thought to be associated with the progression of HR HPV lesions in cervical cancer [20, 21].

The aim of this study was to investigate the distribution of genotypes and alleles of proinflammatory cytokines TNF, IFN-γ and IL-12 and their correlation with the grades of cervical illness. We also wanted to determine which genotypes possess a protective or favoring significance for the development of cervical cancer.

METHODS

From the patient registered with HR HPV positive cervical samples at the Institute of Microbiology and Immunology in Belgrade, Serbia, 24 patients were selected based on available colposcopic and cytological status determined during gynecological examination. Based on a Pap cytological findings and according to the Bethesda classification (2001), the patients were classified into four groups: women with normal cervical cytology or negative for intraepithelial lesion or malignancy (NILM) as control group; women whose cytology was defined as atypical cells of unknown origin (ASCUS); patients with low-grade squamous intraepithelial lesion (LSIL) or CIN I, corresponding to the slight changes in the cervical epithelium or koilocytosis (cells with perinuclear enlightenment indicative of HPV infection), and women with HSIL corresponding to moderate and/or severe cervical dysplasia (CIN II/III). The interview-based questionnaire was administered to all the participants involved in the study. It queried basic information about the patient, socio-demographic and behavioral data, patient's reproductive history, sexual habits, morbidity from other sexually transmitted diseases (STD), and other information of significance for HPV-related cervical disorders. To determine cytokine gene polymorphism, 5 ml of peripheral blood was taken from the patients using appropriate tubes (Becton Dickinson, New Jersey, USA) with anticoagulant ethylenediaminetetraacetic acid (EDTA), and has been transported within four hours to the Laboratory of Immunology of the Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, for further procedures.

Detection of TNF, IFN-γ, and IL-12B gene polymorphisms

DNA extraction

Genomic DNA was isolated from peripheral blood that had been sampled in tubes containing EDTA, using the Gene JET Whole Blood Genomic DNA Purification Mini Kit (Fermentas Thermo Fisher Scientific Inc., Vilnius, Lithuania) according to the manufacturer instructions.

SNP detection

Detection and analysis of the TNF –308 G/A (rs1800629) and IL12-B +1188 A/C (rs3212227) polymorphisms were performed using real-time PCR with commercial TaqMan probes (Applied Biosystems Inc., Foster City, CA, USA) and Maxima Probe qPCR Master Mix (Fermentas Thermo Fisher Scientific Inc.), according to the manufacturer instructions [22]. The IFN-γ +874 T/A (rs2430561) polymorphism was determined as previously described [23]. The thermal cycling conditions were 95°C for four minutes, followed by 40 cycles that were run for 15 seconds at 95°C, one minute at 55°C, and for 20 seconds at 68°C. Fluorescence readings were done at 68°C.

Written informed consent was obtained from all the women enrolled in the study. The protocol of the study was reviewed and approved by the Ethics Committee, Faculty of Medicine, University of Belgrade, decision number 29/XI-2.

Statistical analyses

Comparisons between genotype and allele frequencies in different populations were performed using the Pearson's χ^2 test, Fisher's exact test, or the Kruskal–Wallis test, followed by the Mann–Whitney U test, as appropriate. All

genotype frequencies were in Hardy–Weinberg equilibrium. Significance of differences was carried out at the probability level of $p < 0.05$.

RESULTS

Distribution of proinflammatory cytokine allele and genotype frequencies in cytological findings

The distribution of proinflammatory cytokine genotype frequencies in cytological findings is shown in Table 1. Statistical analysis of the significance of differences between the groups showed a significantly higher incidence of AA high secretory genotype of TNF cytokine (AA genotype, TNF gene, 75%, $p = 0.010$) only in cases of moderate and/or severe cervical dysplasia.

Table 1. Distribution of proinflammatory cytokine genotype frequencies in cytological findings

Genotype		Cytology results								p
		NILM		ASCUS		LSIL		HSIL		
		n	%	n	%	n	%	n	%	
TNF	GG	6	85.7	5	71.4	5	83.3	0	0	
	GA	1	14.3	2	28.6	0	0	1	25	0.010*
	AA•	0	0	0	0	1	16.7	3	75	
IFN-γ	**AA°**	1	14.3	4	57.1	3	50.0	0	0	
	AT	5	71.4	2	28.6	1	16.7	4	100	0.572
	TT	1	14.3	1	14.3	2	33.3	0	0	
IL-12	**AA°**	5	71.4	2	28.6	3	50	2	50	
	AC	2	28.6	5	71.4	2	33.3	2	50	0.513
	CC	0	0	0	0	1	16.7	0	0	

• – high production; ° – low production; LSIL – low-grade squamous intraepithelial lesion; HSIL – high-grade squamous intraepithelial lesions; ASCUS – atypical squamous cells of undetermined significance; NILM – negative for intraepithelial lesion or malignancy
*statistically significant – $p < 0.05$

In other groups of cytological findings (NILM, ASCUS, LSIL), allele distribution of the TNF gene did not differ significantly. The typical GG genotype dominance (72–86%) and an extremely rare occurrence of AA genotype was detected in all other groups.

In the analyzed sample, no significant difference was confirmed in the distribution of IFN-γ and IL-12 alleles in the control (NILM) and other groups ($p = 0.572$ and $p = 0.513$).

Low incidence of homozygous AA genotype of IFN-γ, which could, as hypothesized, pose an increased risk of cervical cancer, was slightly more common in ASCUS and LSIL groups (57.1% and 50%, respectively). The heterozygous AT genotype was predominant in the control group, but also in the group with HSIL findings (71.4% and 100%, respectively).

We got similar results for the distribution of IL-12 genotypes, such as that NILM (control group) had the highest frequency of homozygous AA genotype (71.4%), while the ASCUS group showed the same frequency of AC genotype of this gene (71.4%). High-risk low production AA genotype was detected in 50% of LSIL and HSIL cytological findings.

In order to achieve better overview of the distribution and influence of homozygous genotypes, we grouped them with respect to their heterozygous combinations and compared them according to the cytological findings in the control and other groups (Table 2).

Table 2. Distribution of proinflammatory cytokine genotype of high and low production according to the cytological findings in the control and other groups

Genotype		Control group and other results			
		NILM		ASCUS, LSIL, HSIL	
		n	%	n	%
TNF	GG + GA	7	35	0	0
	AA•	13	65	4	**100**
IFN-γ	**AA°**	10	62.5	7	**87.5**
	AT+TT	6	37.5	1	12.5
IL-12	**AA°**	10	**83.3**	7	58.3
	AC + CC	2	16.7	5	41.7

• – high production; ° – low production; LSIL – low-grade squamous intraepithelial lesion; HSIL – high-grade squamous intraepithelial lesions; ASCUS – atypical squamous cells of undetermined significance; NILM – negative for intraepithelial lesion or malignancy
*statistically significant – $p < 0.05$

Despite the high production TNF AA genotype being present in 65% in the control group of HR HPV-positive women without cytological changes, compared to all the other groups, statistical significance was not detected ($p = 0.160$).

The low production AA genotype of IFN-γ, which could potentially pose an increased risk of developing cervical cancer, was more prevalent in all other groups (87.5%) compared to the control group (65%) ($p = 0.204$).

The AA low secretory genotype of the IL-12 gene is mostly present in the control group (83.3%), compared to all others (58.3% – ASCUS, LSIL, HSIL), but statistically significant difference in this case was not proved ($p = 0.178$).

Previous analysis proved that, in the tested sample of proinflammatory cytokine gene polymorphisms, the mere presence of TNF AA genotype showed a statistically significant association with the progression of cervical dysplasia in HR HPV infection.

Correlation between cofactors and proinflammatory cytokines gene polymorphisms and their associated impact on the onset of cervical dysplasia

In the continuation of the statistical analysis, we applied the correlation and multiple regressions analyses to examine the correlations between polymorphisms and analyzed cofactors including environmental and behavioral cofactors such as high parity, oral contraceptives, tobacco smoking, infection with other STDs, early sexual intercourse, promiscuity, poor socio-economic conditions, dietary and nutritional factors, etc. Table 3 shows only the significant correlations of polymorphisms found in the studied group in relation to the tested cofactors.

TNF is related to cytological findings through a strong, significant and positive correlation coefficient. This means that a higher incidence of high production AA genotype occurs with less favorable cytological findings.

Table 3. Correlations of polymorphism with cytology results, previous infections, and cervical trauma

Cytology results, previous infections, and cervical trauma	p
TNF cytology results	
Spearman Correlation	0.592
Sig. (2-tailed)	0.002*
IFN-g	
Spearman Correlation	-0.472
Sig. (2-tailed)	0.020*
IL-12 cervical trauma	
Spearman Correlation	-0.444
Sig. (2-tailed)	0.030*

*statistically significant – $p < 0.05$

Table 4. High-grade squamous intraepithelial lesions prediction model based on proinflammatory cytokine polymorphisms

Prediction model HSIL	B	Std. error	t	p
(Constant)	0.229	0.172	1.333	0.198
TNF polymorphism	0.738	0.257	2.873	0.009*
IFN-g	0.007	0.201	0.034	0.973
IL-12 polymorphism	0.124	0.194	0.640	0.529

B – regression coefficient; HSIL – high-grade squamous intraepithelial lesions; *statistically significant – $p < 0.05$

Table 3 shows that there is a moderate but significant relationship between IFN-γ gene polymorphism and previously infections of the cervix. A negative sign of correlation (-0.472), indicates that low production of this cytokine is related to a greater number of previous cervical infections and vice versa.

The association between IL-12 production, represented by low production genotype, and the occurrence of cervical lesions is indicated by a negative and significant coefficient (-0.444.) This means that the high-risk low production AA genotype of the IL-12 gene is greatly correlated with the presence of cervical lesions.

The model for multiple regression analysis showed whether any of the polymorphisms examined may serve as potential genetic biomarker of susceptibility to cervical cancer in women with persistent HR HPV infection. In this regard, the occurrence of HSIL as a severe form of cervical dysplasia was taken as a dependent variable in relation to all the variables analyzed (Table 4). Through variance reduction, the step-by-step selection process identified only one significant predictor, namely the high production AA TNF genotype. This TNF gene polymorphism is significant in predicting the onset of HSIL in women with HR HPV infection.

DISCUSSION

In our previous study, a total of 541 women were processed and tested for the presence of HPV, out of which 105 were HPV-positive (19.4%) and 84 (15.5%) were HR HPV-positive [24]. The final investigated group included 84 women infected with HR HPV who were classified into four subgroups according to their cytological status of the cervix. The interview-based questionnaire which queried information of significance for HPV-related cervical disorders was administered to all the participants involved in the study. The cofactors found to be of significance in older age (46.7 ± 12.2 on average), body mass index > 25, lower educational level, long-term smoking (more than 20 years), previous genital infections and cervical interventions.

This study showed that, among the different groups of cytological findings, only the high production AA TNF genotype occurred with a significantly higher incidence in women with lesions of moderate to severe intensity. There was no significant difference between the other groups, both in genotypes determining TNF gene expression and in IFN-γ and IL-12 gene expressions. AA genotype prevailed in the lesions of moderate to severe intensity while in the other groups (PAP II, ASCUS, LSIL), the GG genotype was detected in 72–86% of findings. Correlation and multiple regression analysis showed that there was a highly significant, positive correlation coefficient between TNF and associated cytological findings. This means that the high production genotype of this cytokine was recorded in less favorable cytological findings.

We concluded that the presented sample of the analyzed cytokine gene polymorphisms provided indications of some of the presumed production trends that may have protective or better yet favoring significance for the occurrence of cervical cancer associated with HR HPV, but we were not able to prove the statistical significance in this sample size.

Previous analysis proved that, in the tested sample of proinflammatory cytokine gene polymorphisms, the mere presence of TNF AA genotype showed a statistically significant association with the progression of cervical dysplasia in HR HPV infection.

The association between SNP at position -308 and cervical cancer has been reported in many studies conducted among different races, where the presence of A allele has been associated with an increased risk of cervical cancer development [23].

In certain ethnic groups, SNP at position -308 has been shown to be associated with an increased risk of cervical cancer, but in some studies these results have not been confirmed [25].

Similar results were found in a study showing that A allele carriers were at higher risk for cervical cancer than individuals with both G alleles [26, 27].

In contrast, a study conducted in Africa showed that TNF gene polymorphism at position -308 had no effect on the development of cervical cancer [17].

Our results of the SNP gene study showed that there was no significant difference in allele distribution and IFN-γ production among patient groups with different cytological and colposcopic findings. Low IFN-γ production, determined by homozygous AA genotype, was found to be more common in the ASCUS and LSIL cervical findings, whereas the heterozygous AT genotype was more prevalent in the control and HSIL groups of patients.

Our study also established the existence of a significant relationship between IFN-γ gene polymorphism and earlier infections. A negative sign of correlation indicated that low

productions of this gene was related to a greater number of previous infections and vice versa.

The IFN-γ polymorphism at position +874 is associated with increased predisposition and progression of a number of diseases, including cervical cancer. The AA genotype (homozygous genotype) is responsible for low cytokine expression, since the DNA sequence containing T allele is a specific binding site for transcription factor NFκB, responsible for higher gene transcription and IFN-γ production. An *in vitro* study indicated that AA polymorphism is associated with a low AT polymorphism with a moderate and TT polymorphism with a high IFN-γ production [18]. These conclusions were also confirmed by a study by Zhou et al. [9], in which the IFN-γ AA genotype and the A allele were significantly more common in patients with CIN than healthy individuals.

The results of this study, as in the case of IFN-γ gene polymorphism, showed that there were no significant differences in IL-12 gene polymorphisms distribution and production among different groups of cytological findings. Low-production homozygous AA genotype had the highest incidence in the control group, whereas AC genotype was most common in the ASCUS group. The low-production AA genotype was detected in 50% of the LSIL and HSIL cytological findings.

This study showed that AA low-production genotype of the IL-12 is associated with the occurrence of cervical lesions by a strong, negative, and significant coefficient. This means that IL-12 low-production gene polymorphism correlates greatly with the presence of cervical lesions.

IL-12B polymorphism is associated with the development of diseases resulting from changes in IL-12 synthesis and secretion. A small number of studies have examined the correlation between IL-12B gene polymorphism and the development of cervical cancer. Studies conducted in Korea have shown that IL-12B with AC/CC genotypes (rs3212227) increases the risk of cervical cancer, but also that the difference is not statistically significant compared to women in whom this combination was not detected [28]. Similar results were reported in a study conducted in China. The IL-12B gene polymorphism (rs3212227) was not significantly correlated with an increased risk for the development of cervical pathological changes. A study conducted in China also suggested that IL12-B (rs3212227) AC/CC genotypes may, in some individuals, increase the risk of developing high-grade cervical lesions, especially if associated with a higher number of births [21].

By studying the IL-12 gene polymorphisms, we found that the AC genotype was not associated with the onset of cervical changes, whereas the AA genotype was significantly more common in patients with severe cervical dysplasia and cervical cancer. The AC genotype occurs more frequently in healthy individuals, indicating that the C allele has a protective role in the development of cervical lesions and their progression [20].

SNPs as genetic markers have received considerable attention from researchers in the past decade. Identifying SNPs in the gene's coding region that change the protein's amino acid sequence, which have been investigated in this study, in correlation with behavioral cofactors, can be helpful from a diagnostic point of view. According to the latest literature, statistical correlation also exists between polymorphisms present in the non-coding regions of the gene and enhanced risk of cervical cancer. But the effects of this polymorphisms residing in the non-coding regions (i.e., introns, promoters, 3′ and 5′ termini, etc.) have been investigated to a lesser extent as their exact mechanism of action is unknown and, therefore, demands additional attention [29].

CONCLUSION

It is known that polymorphisms of certain cytokine genes encoding proteins involved in Th1 and Th2 cellular responses may be associated with better or worse prognosis of cervical disease in women with persistent HR-HPV infection. Therefore, single nucleotide polymorphisms of TNF, IFN-γ and IL-12 genes may be considered as biomarkers that may have a predictive role in the development of cervical cancer, but further research would contribute to a better understanding of this subject. Also, combination of different gene polymorphism in one individual, a so-called gene profile, should be taken into consideration in future studies.

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Полиморфизми цитокинских гена *TNF*, *IFN*-гама и *IL*-12 као могући предиктори настанка цервикалне болести код жена позитивних на *HR HPV* са кофакторима ризичног понашања

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САЖЕТАК

Увод/Циљ Циљ ове студије је био да се испита дистрибуција генотипова и алела проинфламаторних цитокина *TNF*, *IFN*-гама и *IL*-12 и њихов утицај на настанак цервикалне болести, као и да се утврди њихов удружени утицај са кофакторима код *HR HPV* позитивних жена у Србији.

Методе Испитали смо 24 болеснице и на основу цитолошког налаза их поделили у четири групе: *PAP II*, *ASKUS*, *LSIL* и *HSIL*. Анализа полиморфизама *TNF*, *IFN*-гама и *IL*-12 извршена је методом *Real-time PCR TaqMan*. Статистичка анализа урађена је употребом параметарских и непараметарских тестова и корелационе и мултипле регресионе анализе.

Резултати Значајно већа учесталост високосекреторног генотипа *TNF AA* утврђена је у тежим облицима дисплазије. Позитивна корелација између високосекреторног полиморфизма *TNF* и цервикалних промена била је високо значајна. Утврђена је умерена, значајна корелација између нискосекреторног *IFN*-гама и ранијих цервикалних инфекција. Постоји значајна повезаност нискосекреторног генотипа *IL*-12 са раницама на грлићу материце.

Закључак Резултати ове студије показују да су *HSIL* промене у вези са значајно већом учесталошћу високосекреторног генотипа *TNF AA*. С обзиром на то да се полиморфизми одређених цитокинских гена који кодирају протеине укључене у *Th1* и *Th2* ћелијски одговор повезују са добром односно неповољном прогнозом цервикалне болести код жена са перзистентном *HR HPV* инфекцијом, могу се сматрати биомаркерима са предиктивном улогом у развоју цервикалног карцинома.

Кључне речи: цервикални карцином; кофактори; генски полиморфизми; *TNF*; *IFN*-гама; *IL*-12

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Prevalence of glaucoma in the city of Novi Sad

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SUMMARY

Introduction/Objective Our study aimed to estimate the prevalence of glaucoma and its subtypes in the population of Novi Sad, Vojvodina, Serbia, and provide the demographic and clinical analysis of glaucoma patients involved.

Methods Our study was designed as an observational, retrospective, cross-sectional, monocentric, including all the patients with the address of residence within the city of Novi Sad, with clinically diagnosed glaucoma, at least in one eye, treated at the University Eye Clinic, Clinical Centre of Vojvodina, Novi Sad. We analyzed the five-year prevalence of different types of glaucoma, together with the characteristics of visual field and risk factors in the form of coexisting diabetes mellitus and arterial hypertension.

Results Almost half of 3254 included patients (48.28%) were diagnosed with primary open-angle glaucoma (POAG), and its prevalence in the total population of Novi Sad was estimated to be 0.46%. The prevalence of other glaucoma types was as follows: primary angle-closure glaucoma (PACG) 0.17%, secondary glaucoma 0.09%, pseudoexfoliation glaucoma 0.09%, normal-tension glaucoma 0.13%, pigmentary glaucoma 0.01%, and juvenile glaucoma 0.01%. In the population above 40 years of age, the prevalence of all glaucoma cases was 1.9%, while the prevalence of POAG was 0.93%, and the prevalence of PACG was 0.35%.

Conclusion Our study represents the first attempt to address the epidemiological problems of glaucoma in our region in a comprehensive, evidence-based way. The prevalence of various glaucoma types and observed age-specific prevalence trends were lower than those published by other authors involving comparable populations, and we offered several potential explanations for this in our paper.

Keywords: prevalence of glaucoma; type of glaucoma; glaucoma epidemiology

INTRODUCTION

Glaucoma is progressive optic neuropathy accompanied by characteristic morphological changes of the optic nerve head and the nerve fiber layer of the retina. It manifests in visual field loss, usually asymptomatic in the early stages of the disease, leading to irreversible blindness if untreated. It represents the second leading cause of preventable blindness in the world. It has been estimated that 60.5 million people were suffering from glaucoma globally in 2010, with 6.7 million bilaterally blind. This number is expected to rise in the future, and it is estimated that 111.8 million people will have glaucoma in 2040 [1]. The main reason for this is considered to be a worldwide increase in the average life expectancy.

Based on its etiopathogenesis, glaucoma can broadly be divided into open-angle (OAG) and angle-closure (ACG) glaucoma, with further classification into primary, secondary, congenital (present on birth), and juvenile form (appearing between the ages of three and 40) [2].

Analysis of global glaucoma prevalence proved to be a complex undertaking, often leading to inconclusive and results that are difficult to compare. The situation improved after the introduction of the International Society of Geographical and Epidemiological Ophthalmology (ISGEO) classification of glaucoma for use in population-based surveys which more precisely defined diagnostic and classification criteria of different glaucoma types. However, many obstacles persist to this day [3].

Different subtypes of glaucoma have different racial and geographical prevalence. Africa and North America are found to have the highest prevalence of POAG (4% and 3.4%, respectively), while Oceania had the lowest (1.8%) [3]. The situation in China and India favors PACG, which accounts for 50–60% of all glaucoma cases [4].

According to the latest epidemiological studies and meta-analyses, the global prevalence of adult forms of glaucoma is estimated to be 4.79%, with POAG accounting for 3.05% and PACG for 0.5% of the cases, and these values seem to be increasing [5].

There are no available data regarding the number of people with glaucoma in Serbia. At the 2011 census, Serbia had 7,186,862 citizens and 3,849,267 people older than 40 years [6]. Extrapolating the prevalence data from the aforementioned studies, it has been estimated that around 100,000 people live with glaucoma in Serbia, with at least 75,000 POAG cases.

Our study aimed to estimate the prevalence of glaucoma and its subtypes in Novi Sad, Vojvodina, Serbia, with a population of 341,625 on the last census, and to provide with

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the demographic and clinical analysis of glaucoma patients involved.

METHODS

This observational, retrospective, cross-sectional, monocentric study included all the patients with clinically diagnosed glaucoma, at least in one eye, treated at the University Eye Clinic, Clinical Centre of Vojvodina, Novi Sad in the period from August 2007 to December 2012. The patients' data were populated from the electronic health records database used in the clinic. The dataset included 3254 patients, with the residence address within the city of Novi Sad, who met strict inclusion criteria of confirmed glaucoma diagnosis, regardless of age, sex, and type of glaucoma, excluding glaucoma suspects and patients with ocular hypertension. The onset of the disease was not necessarily in the period between January of 2007 and January of 2012, but a patient's first examination at the clinic was within that period. All the patients were examined by several experienced ophthalmologists working at the Glaucoma Department of the Eye Clinic.

We analyzed the period prevalence of different types of glaucoma, including POAG, PACG, secondary, juvenile, and congenital glaucoma, and the age and sex distribution of the patients. In addition, assessment of the cup/disk ratio of the optic disc using the 90D non-contact lens at slit lamp was done in every patient. Visual field testing was performed with Humphrey visual field analyzer (HFA; Carl Zeiss Meditec AG, Jena, Germany), C24-2 testing protocol, equipped with STATPAC. Mean deviation (MD) visual field index calculation has been considered an indicator of the stage of perimetric glaucoma disease. Finally, we analyzed the presence of two of the established glaucoma risk factors: diabetes mellitus and arterial hypertension.

Subjects' written consent was obtained in accordance with the Declaration of Helsinki, and the ethics committees of the Eye Clinic, Clinical Centre of Vojvodina, approved the study.

Data were statistically analyzed using SPSS, Version 16.0 (SPSS Inc., Chicago, IL, USA) using the following descriptive statistical methods: arithmetic mean, standard deviation, median, quartiles, frequencies, and percentages, with confidence interval (CI) set at 95%. The results are presented in the form of tables and graphs with comments.

All procedures performed in this study were in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written consent to analyze and publish all shown material was obtained from the patient, and the approval for the study was given by the Ethics Committee of Eye Clinic, Clinical Centre of Vojvodina.

RESULTS

There were 3254 patients enrolled in our study, the majority of them being females (1949 *vs.* 1305; 59.9% *vs.* 40.1%).

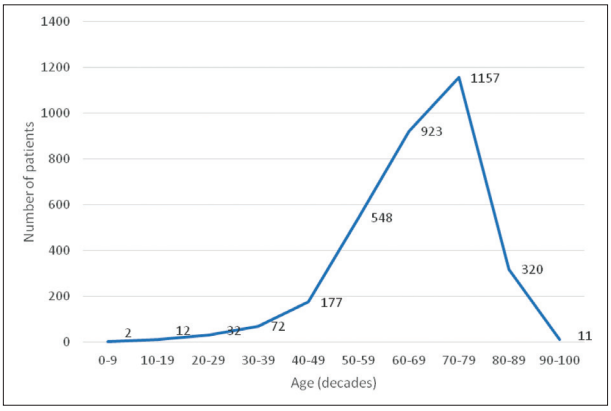

Figure 1. Age distribution of glaucoma patients

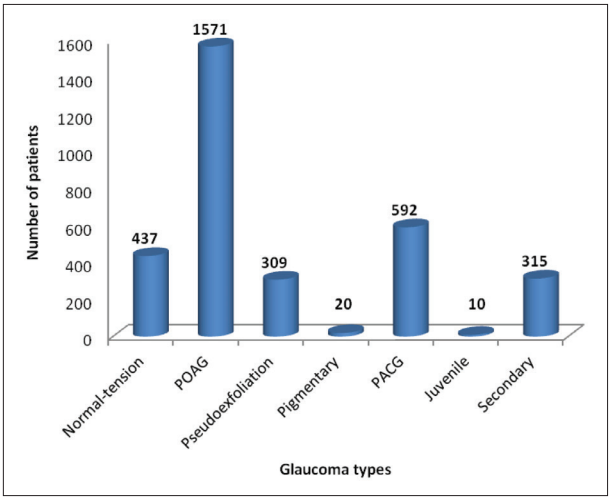

Figure 2. Distribution of glaucoma types;
POAG – primary angle-closure glaucoma; PACG – primary angle-closure glaucoma

The average age of the patients surveyed did not express normal distribution and was calculated using the median value (25th–75th percentile) at 68 (59–75) years. The youngest patient was eight years old, and the oldest was 100 years old. Age distribution of glaucoma patients shows a predominance of patients older than 40 years of age, with most cases during the eighth decade of life (Figure 1).

Among our patients, the most common glaucoma type was primary open-angle glaucoma (POAG), diagnosed in 1571 patients (48.28%). There were 592 (18.19%) patients with primary angle-closure glaucoma (PACG), while the secondary glaucoma was found in 315 (9.68%), pseudoexfoliation glaucoma in 309 (9.5%), normal-tension glaucoma in 437 (13.43%), pigmentary glaucoma in 20 (0.61%) patients, and 10 patients (0.31%) had juvenile glaucoma. There were no cases of congenital glaucoma in our group of patients (Figure 2).

According to the 2011 census, the population of the city of Novi Sad had a total of 341,625 citizens. There were 3254 glaucoma patients registered in our study, giving the total glaucoma prevalence of 0.96%. POAG had the highest prevalence of all glaucoma types (0.46%), followed by PACG (0.17%), and normal-tension glaucoma (0.13%).

There were 164,789 persons older than 40 years in the city of Novi Sad, making the total glaucoma prevalence

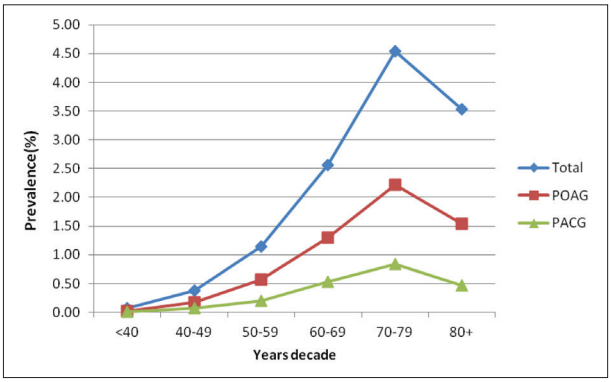

Figure 3. Age distribution of primary open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), and total glaucoma prevalence

for this age group 1.9%. The prevalence of patients in the group of 40–49 years was 0.38%, in the 50–59 years group the prevalence was 1.15%, for the group of 60–69 years the prevalence was 2.56%, for the age group of 70–79 years the prevalence was 4.54%, while the prevalence in the population above 80 years was 3.53% (Figure 3).

The prevalence of POAG among populations older than 40 years was 0.93%, while the prevalence of PACG in the same population was 0.35%. POAG showed a higher prevalence throughout all age groups, comparing to PACG. As with total glaucoma cases, the highest prevalence of both POAG and PACG is found among the population in the eighth decade of life (2.22% and 0.83%, respectively), with the decrease among the patients over the age of 80 (Table 2).

Table 1. Prevalence of glaucoma types

Glaucoma type	Prevalence % (95% CI)
Normal-tension	0.13 (0.1159–0.1399)
POAG	0.46 (0.4371–0.4825)
Pseudoexfoliation	0.09 (0.0803–0.1005)
Pigmentary	0.01 (0.0032–0.0084)
PACG	0.17 (0.1593–0.1872)
Juvenile	0.01 (0.0011–0.0047)
Secondary	0.09 (0.0821–0.1023)

POAG – primary open-angle glaucoma; PACG – primary angle-closure glaucoma

Table 2. Age distribution of open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), and total glaucoma prevalence

Years of life	POAG prevalence %	PACG prevalence %	Total glaucoma prevalence % (95% CI)
< 40	0.02	0.01	0.07 (0.0546–0.0787)
40–49	0.18	0.08	0.38 (0.3274–0.4403)
50–59	0.56	0.2	1.15 (1.0528–1.244)
60–69	1.3	0.53	2.56 (2.394–2.721)
70–79	2.22	0.83	4.54 (4.28–4.791)
80+	1.54	0.47	3.53 (3.158–3.9055)

Taking into consideration all types of open-angle glaucoma (POAG, normal-tension, and pigmentary glaucoma), there were 2028 cases recorded in our study. This makes the collective prevalence of these types of glaucoma at 0.59% in the general population and 1.23% if we calculate only for the population above the age of 40 years.

Automated perimetry using Humphrey visual field analyzer (HFA) (C24-2 testing protocol) showed a mean MD index value of -6.51dB (± 8.4 dB).

Of all the risk factors for the development of glaucoma, this study included two: diabetes mellitus and arterial hypertension. Diabetes was found in 76 (2.32%) patients, while the arterial hypertension was present in 86 (2.62%) patients.

DISCUSSION

Our study aimed at estimating the prevalence of glaucoma for the population of the city of Novi Sad for the 2007–2012 period. We have analyzed the records of 3254 patients, of the median age of 68 (59–75) years, with confirmed glaucoma diagnosis, treated at the Glaucoma Department of the Eye Clinic of the Clinical Centre of Vojvodina. Our clinic is the only tertiary ophthalmological center in our region, and most glaucoma patients are diagnosed and treated within its Glaucoma Department.

Sex predilection for glaucoma is still a debated topic in the literature. It was considered that women are more prone to suffer from glaucoma during their lifetime, as several population-based studies indicated in the past [7, 8]. This was especially true with PACG in people of Asian descent [3]. However, available data seem to show that males have a higher chance of being POAG patients. In the final multivariable model, Khachatryan et al [9] found male sex to be more associated with POAG risk (OR, 1.64; 95% CI, 1.44–1.87; $p < 0.001$), after adjusting for age, systemic hypertension, diabetes, and BMI.

There was a higher number of female patients (59.9%) in our group. This might reflect sex distribution among the general population of Novi Sad, with 52.54% of the population being women and due to the women's longer average life expectancy at birth (78.4 *vs*. 72.9 years) [6, 10]. Another potential explanation might be the phenomenon of sex-related differences in health behavior and the fact that women generally tend to visit physicians earlier and more often than men, that might lead to the larger pool of undetected male glaucoma patients in the general population [11].

The prevalence of glaucoma and its types shows significant regional variations, determined by the racial characteristics of the population. The latest pooled prevalence data, for the people over the age of 40, estimate it to be between 2.4% (95% CI 2 ~ 2.8%) globally, and between 2.23% and 2.93% for the people of European ancestry [5].

The population of the city of Novi Sad and Serbia is mainly Caucasian, of European origin [12]. The prevalence data found in our study was considerably lower than the ones in other sources. It was found to be 0.96%, including patients of all age groups, and 1.9% involving patients older than 40.

POAG is the most common type of glaucoma globally. Its overall prevalence is estimated 2.2–3.05%, being most prevalent in the people of the black, African ancestry, reaching 5.4%, and least common in the people of Asian

ancestry [13]. The prevalence for the white population is estimated to be within the 1.29–2.93% range [5, 14]. The results of our study show a prevalence of 0.46% for the general population and 0.93% in the population older than 40 years, which is lower than published data found by other researchers.

The situation with PACG regarding its geographical distribution is quite the opposite. Its prevalence is the lowest among the people of white and black race ranging 0.03–0.85% [5, 15]. On the other hand, Asian people have higher values reaching up to 1.2%, while the prevalence among the people of European ancestry is 0.42–0.69% [5, 13, 16]. The prevalence of this type of glaucoma in our survey was 0.17% for all age groups and 0.35% for patients above 40 years of age and was more similar to the results of earlier studies.

Numerous studies have shown that age-specific prevalence for both POAG and PACG increases with age [15, 17, 18]. Our research findings agree with most of the other studies, although with lover prevalence across all age groups. In addition, we found the maximum prevalence for both POAG and PACG among patients in the eighth decade of life, declining in later life. Similar results are present in the study by Kreft et al. [17], who conclude that the reason for this might be in the increased risk of additional health care risks in the form of more severe comorbidities or because of the mortality selection process, a tendency that fitter persons with a generally lower risk of morbidities, among them POAG and its risk factors, reach the highest ages.

Other glaucoma types have also shown the tendency of racial/geographical distribution, with variable prevalence globally. Normal-tension glaucoma is defined as open-angle glaucoma with IOP values within the physiological range for the given population (mean value of IOP +/- 2SD). It has been found in up to 30% of all POAG cases, depending on the selected cut-off IOP value [1, 19]. Our research found its prevalence to be 0.13%, which makes 22% of OAG prevalence (59%), the result comparable with the available data from the literature.

Pseudoexfoliation glaucoma also exhibits regional variations comprising 20% to more than 50% of open-angle glaucoma cases [20, 21]. We analyzed pseudoexfoliation glaucoma as a separate clinical entity and found its prevalence to be 0.09% in all age groups. We are not aware of any other study presenting these results in a similar, comparable fashion.

A secondary glaucoma is a diverse group of glaucoma diseases, occurring together with another underlying eye disease (lens-related, aphakic, pseudophakic, neovascular, traumatic, uveitic). According to published studies, it has been known to represent 1–11% of all glaucoma cases [4, 22]. Our data establish its prevalence at 0.09%, with 315 patients suffering from it.

The reason for the prevalence values obtained in our study to be at the lower end of the reported range might be in the fact that although most glaucoma patients in our region are treated at our clinic, some of them are registered at secondary levels of ophthalmology health care in community health care centers or several privately run clinics. Incorporating the data from these centers in our analysis should be the goal of future studies.

ISGEO defined diagnostic and classification criteria for glaucoma in population-based surveys. Among other criteria, it specified the "gold standard" of glaucomatous visual field loss using the threshold test strategy with the 24-2 test pattern of the Zeiss-Humphrey field analyzer II as a referent device [23]. We followed these recommendations in our survey and found the mean MD index value of -6.51dB (± 8.40dB) for all the glaucoma patients.

Multiple risk factors were associated with glaucoma and its subtypes. POAG link with diabetes mellitus was established by numerous authors [17, 24]. In the latest meta-analysis of seven prospective studies, Zhao and Chen [25] found diabetes mellitus to be associated with a significantly increased risk of glaucoma. The prevalence of secondary (neovascular) glaucoma was also linked with diabetes, although there has been a marked decrease in it in recent years, after the introduction of anti-VEGF therapy[26]. In our study, we report diabetes comorbidity in 2.32% of all glaucoma patients. Arterial hypertension is another risk factor associated with an increased risk of POAG development [27]. Our survey found it to be present in 2.62% of all the included patients.

CONCLUSION

Our monocentric, cross-sectional, retrospective, observational study represents the first attempt to address the epidemiological problems of glaucoma in our region in a comprehensive, evidence-based way. Its results demonstrated the sex and age distribution of glaucoma patients treated at our clinic for the follow-up period of five years. We estimated the period prevalence of various glaucoma types and observed age-specific prevalence trends in our population. The prevalence values found were compared with the ones published by other authors, involving an equivalent population. It is evident that our total and individual glaucoma type prevalence rates are lower than previously published, and we tried to give a credible explanation for that. Apart from that, we have also analyzed the average level of visual field defect and the presence of two glaucoma risk factors (diabetes mellitus and arterial hypertension) in our patients.

Conflict of interest: None declared.

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Преваленца глаукома на територији града Новог Сада

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САЖЕТАК

Увод/Циљ Циљ нашег истраживања био је да се процени преваленца глаукома, као и његових типова у популацији града Новог Сада и да се изврши анализа демографских и клиничких карактеристика болесника који болују од глаукома.

Методе Наша студија је осмишљена као опсервациона, ретроспективна, моноцентрична студија пресека, која је обухватила све болеснике са пребивалиштем на територији града Новог Сада, са клинички постављеном дијагнозом глаукома бар на једном оку, а који су лечени на Клиници за очне болести Клиничког центра Војводине. Испитивали смо петогодишњу преваленцу различитих типова глаукома, заједно са особеностима налаза видног поља и присуством шећерне болести и артеријске хипертензије као факторима ризика.

Резултати Код скоро половине од 3254 укључена болесника (48,28%) постављена је дијагноза глаукома отвореног угла и процењена преваленца овог обољења у становништву Новог Сада износи 0,46%. Преваленца осталих типова глаукома била је следећа: примарни глауком затвореног угла 0,17%, секундарни глауком 0,09%, псеудоексфолијативни глауком 0,09%, нормотензивни глауком 0,13%, пигментни глауком 0,01% и јувенилни глауком 0,01%. Код становништва старијег од 40 година преваленца свих глаукома износила је 1,9%, док је преваленца глаукома отвореног угла била 0,93%, а преваленца глаукома затвореног угла 0,35%.

Закључак Наша студија представља први покушај епидемиолошке анализе питања глаукома у нашем региону на свеобухватан, на доказима засновани начин. Процењена је преваленца различитих типова глаукома и уочени су њени трендови у зависности од узраста становништва. Добијене вредности преваленце ниже су од оних објављених од стране других аутора у популацијама сличним нашој и у нашем раду смо пружили неколико могућих објашњења за ту чињеницу.

Кључне речи: преваленца глаукома; тип глаукома; епидемиологија глаукома

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Impact of aesthetic rhinoplasty on the respiratory function of the nose in patients with a straight nasal septum

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SUMMARY

Introduction/Objective Aesthetically, the nose adds special signature to a person's look. This results in many nasal pyramid surgeries, either functional or aesthetic. The problem arises in aesthetic surgeries. Patients often tend to present their dissatisfaction with the appearance of the nose as a breathing difficulty, as they often lack the support of the environment in the decision to undergo cosmetic surgery. The objective of the paper was to examine, using subjective assessment and objective measurements, the change in the nasal respiratory function in patients who undergo aesthetic nose surgeries, despite having a straight nasal septum before the surgery.

Methods The study was conducted as a prospective, cross-sectional one, and involved 32 patients of both genders. Before and at six months after surgery all patients underwent subjective nasal breathing assessment using visual analog scale (grade 0–10) as well as objective nasal respiratory function assessment using rhinomanometry and acoustic rhinometry after anemization.

Results There were seven male and 25 female patients, age ranging 18–27 years. Objective measurements after surgery showed that the nasal cavity volume, minimum cross-sectional area, as well as the airflow through the nose reduced, while resistance to the nasal airflow increased, but with no statistical significance. The subjective assessment of nasal breathing statistically significantly improved after the surgery.

Conclusion The subjective assessment of nasal breathing postoperatively is not a relevant indicator of the objective state of the nasal respiratory function in patients after aesthetic rhinoplasty.

Keywords: aesthetic rhinoplasty; rhinomanometry; acoustic rhinometry; nasal airflow; subjective nasal breathing sensation

INTRODUCTION

Nose represents the central structure that dominates the whole face. Aesthetically, it adds special signature to a person's look and contributes to the expressiveness and beauty. For this reason, numerous nose deformities impact the person's look itself. This results in many nasal pyramid surgeries, either functional or aesthetic. Functional rhinoplasty implies a nasal pyramid surgery in cases when it is not possible to establish a good respiratory function by correction of the nasal septum only. Aesthetic surgeries are those that are performed at the request of the patient due to dissatisfaction with the appearance of the nose, without the respiratory function being compromised by the shape of the nose itself.

Primary functions of the nose are respiratory and protective ones. Protective function reflects in cleaning the air, moistening it and warming, i.e., conditioning it for lower airways. The aim of each functional rhinoplasty surgery is to establish a good nasal respiratory function. The problems arise in aesthetic surgeries. Patients often tend to present their dissatisfaction with the appearance of the nose as a breathing difficulty, as they often lack the support of the environment in the decision to undergo cosmetic surgery.

The second problem is the readiness of the surgeon not to give in to the patient's tendency to have the smallest nose possible, and this way to lead to a serious violation of the nasal respiratory function.

The success of the surgery in rhinoplasty/septorhinoplasty is measured through many aspects: the patient's satisfaction with his/her look, subjective quality of nasal breathing, but also the satisfaction of the surgeon himself/herself with the results of the surgery. There are many questionnaires for the analysis of surgical results [1–7].

It has been proven, without a doubt, that after the surgery of a deformed nasal pyramid, with the mechanical barriers for airflow through the nasal cavity present before the surgery, a good respiratory function was established. By using objective tests, such as rhinomanometry, acoustic rhinometry, and measuring of the peak nasal inspiratory flow, one obtains statistically significant improvements in the nasal respiratory function in these patients. Still, the question remains what happens with the nasal respiratory function in patients who have good respiratory function, and undergo nasal surgeries purely for aesthetic reasons.

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The objective of the paper was to examine the changes in the nasal respiratory function, using subjective assessment and objective measurements, in patients who undergo aesthetic nasal surgeries but had a straight nasal septum prior to the surgery.

METHODS

The study was conducted as a prospective and cross-sectional one, and involved 32 patients of both genders who were operated on at the ENT department of a tertiary level institution. None of the patients had a deviated septum or other nasal pathology or risk factors, such as allergy, that could affect nasal breathing. All participants had an undisturbed nasal respiratory function prior to surgery. The condition for participating in the study was a nasal septum without deviation and dissatisfaction with the nasal pyramid appearance. Before they were included in the study, all the patients had undergone an ENT examination, subjective nasal breathing self-assessment, as well as an objective nasal respiratory function assessment. In order to accurately evaluate the condition of the nasal septum, beside anterior rhinoscopy, the nasal endoscopy using rigid Storz 4 mm optics at an angle of 30°, was performed. The subjective nasal breathing sensation was self-assessed by the patient using the visual analogue scale (grade 0–10), where 0 stands for minimal and 10 for maximal nasal airflow rate. Objective assessment of the nasal respiratory function was carried out by using rhinomanometry and acoustic rhinometry. The nose volume was measured for the first 5 cm from the nasal openings. Such distance was taken into consideration in order to better establish the effects of the nose volume changes because they model the nasal pyramid, making it smaller. In this case, the Rhinoscan SRE 2000 (Interacoustics A/S, Middelfart, Denmark) device was used. Before the objective assessment of the nasal respiratory function, all the patients underwent nasal anemization. The anemization was performed in accordance with the 2005 Consensus by using 0.1% oxymetazoline spray, 50 μg in each half of the nose; the process is repeated in five minutes with one dose in each half of the nose. After the application, it was necessary to wait for 15–30 minutes and then perform the assessment [8, 9]. The anemization was performed in order to exclude the mucous component during the assessment.

All the patients underwent an aesthetic rhinoplasty procedure with the correction of the size of the nose, in order to adjust the nose to the anthropometric measurements of the face. A low-to-low lateral nasal osteotomy with cartilaginous grafts was used for this purpose.

Six months after the surgical procedure, all the patients underwent subjective and objective assessments of the nasal respiratory function, the same way it was done before the surgery. Digital and print face photos (front and profile view) were taken before and after surgery as well.

Since these are dependent sets of results, the analysis of the effect of the surgery had to be carried out by the factorial analysis, and not by comparing parametric and non-parametric properties of assessed variables. The analysis of the change in values was carried out by performing Duncan's test. The significance level values of $p < 0.05$ were considered statistically significant.

The study was carried out according to the principles of the Helsinki Declaration and it was approved by a local ethics committee (protocol number 6/00-29).

RESULTS

There were seven male (21.87%) and 25 female (78.13%) patients. The age of the patients ranged 18–27 years; the average age was 23.79 years. Age and gender distribution of study participants is shown in Figure 1.

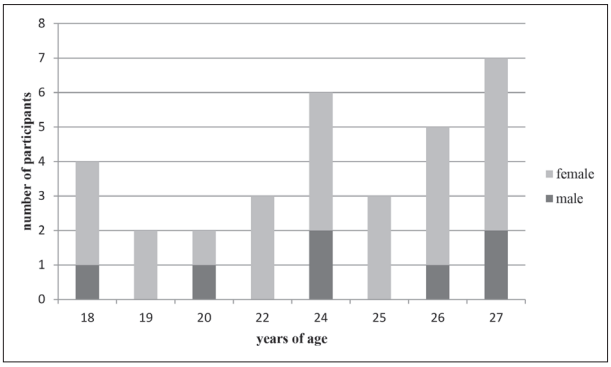

Figure 1. Age and gender distribution of the participants

A statistically significant difference in the assessment of nasal respiratory function before and after surgery was obtained only in the subjective assessment of nasal respiratory function, which proved to be significantly better after surgery in both the left ($p = 0.00072$) and the right half of the nose ($p = 0.00015$). Although all objective parameters of nasal respiratory function show postoperative degree of deterioration in relation to preoperative values, this difference was not statistically significant in any of the parameters, as shown in Table 1.

Table 1. Results and comparison of mean values of subjective and objective parameters in nasal respiratory function before and after the surgery

Parameters	Before surgery	After surgery	p
Resistance on the left side (Pa/cm³/s)	0.5408	0.5687	0.6794
Resistance on the right side (Pa/cm³/s)	0.5112	0.5445	0.6127
Total resistance (Pa/cm³/s)	0.2524	0.2737	0.4034
Airflow on the left side (L/min)	306.30	284.89	0.5357
Airflow on the right side (L/min)	319.40	278.65	0.2281
Volume on the left side (cm³)	6.7075	6.0042	0.1243
Volume on the right side (cm³)	6.2883	6.0233	0.5528
Minimum cross-sectional area on the left side (cm²)	0.4675	0.4192	0.1121
Minimum cross-sectional area on the right side (cm²)	0.4583	0.4200	0.1797
Subjective breathing sensation on the left side (VAS)	6.4348	9.1467	**0.00072**
Subjective breathing sensation on the right side (VAS)	6.3478	9.3333	**0.00015**

VAS – Visual Analogue Scale (grade 0–10)

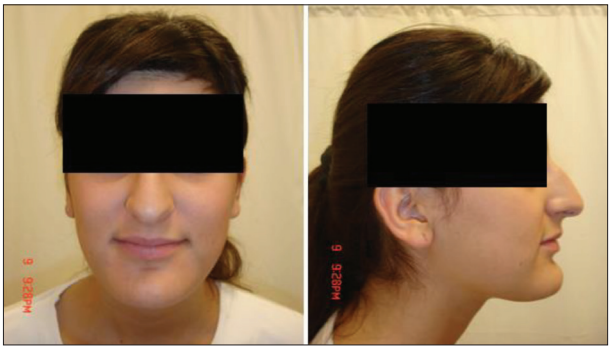

Figure 2. Frontal and profile face image before the surgery

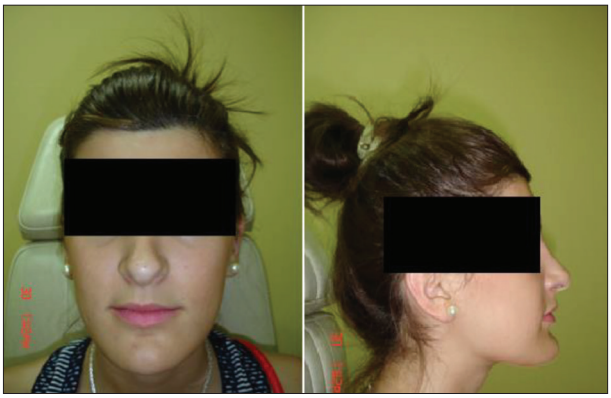

Figure 3. Frontal and profile face image after the surgery

Examples of the appearance of the nasal pyramid in frontal and profile view images, pre and postoperatively, are shown in Figures 2 and 3.

DISCUSSION

The nose, besides its aesthetic significance, providing its functions, has a remarkable impact on the quality of life. If the breathing and protective functions of the nose are impaired, patient breathes through the mouth, which results in constant sore throat and consequent damage of the lower airways.

It is well known that nasal respiratory function improves after functional rhinoplasty, but the surgeons need to be aware that nasal septal surgery affects nasal anthropometry and can consequently cause aesthetic complications [10].

The question remains what happens with the nasal respiratory function in patients who have preserved respiratory function, and undergo nasal surgeries for cosmetic reasons only. The presented study aimed to answer this question.

Certain authors found that aesthetic rhinoplasty does not impede the nasal respiratory function, at least not with proved statistical certainty [11, 12].

Unlike them, there is an opinion that when the low-to-low osteotomy is used, the change in the internal nasal valve results in impaired nasal breathing [13]. However, Kamburoglu et al. [14] could not prove the occurrence of nasal breathing disturbance after the reduction rhinoplasty procedures with statistical certainty.

Okland et al. [15], according to their retrospective study, with patient-centered questionnaires used, indicate that reductive rhinoplasty is not associated with a greater risk of breathing obstruction when performed with modern airway preservation techniques, but report the initial increase in obstructive symptoms only on the first postoperative visit due to perioperative swelling.

Based on their research, McKiernan et al. [16] concluded that there is some benefit after rhinoplasty, particularly in patients who also have a cosmetic reason for the surgery.

Pfaff et al. [17], in a systematic review and meta-analysis of 25 studies, found that functional and aesthetic nasal operations appear to significantly improve olfaction as well, which is directly correlated with improvement in nasal airflow and the quality of life.

By analyzing the results of our study, it could be stated that all the objective parameters for the nasal respiratory function after the surgery worsened in comparison to those before the procedure. Such differences are insignificant, but still present. There was an increase in both nasal halves individually and in the overall nasal airflow resistance, which indicates the narrowing of the respiratory space inside the nose. The reduction of the nose volume in the observed range also indicates reduction of the respiratory space. During the surgery, the size of the nasal pyramid reduces, and this results in the reduction of the nasal volume and the minimal cross-sectional area.

Pousti et al. [18] stated that the nasal cross-sectional area is the main factor which determines the nasal respiratory function, and that the nasal airflow proportionally reduces with the minimal cross-sectional area.

In our material, the minimum cross-sectional area reduced on average by 0.0483 cm^2 on the left, and by 0.0383 cm^2 on the right side. The reduction of the minimum cross-sectional area is the main reason for the increase in both individual and total nasal airflow resistance, but at the same time it is the cause of the airflow reduction.

Savović et al. [19] stated that rhinomanometry significantly correlates with the subjective score of the nasal respiratory function and that it can be used as the objective indicator in daily clinical practice. The same authors also found that the acoustic rhinometry does not correlate with the subjective score of the nasal breathing and that it has more significance in research than in clinical practice [19]. Unlike this research, Skouras et al. found that the acoustic rhinometry is good in detecting and monitoring the effects of impaired breathing, in particular in plastic surgeries of nose and nasal septum [20].

Although the objective respiratory parameters indicated the reduction of the nasal airflow and an increase in nasal airflow resistance, though not with a significant difference, the finding with regards to subjective nasal breathing indicated significant improvement of the respiratory function. After the surgery, all the patients rated their nasal breathing as significantly better. Such difference is statistically highly significant. The explanation for such occurrence may go in three directions. The first one is that the reduction of a bigger nasal pyramid and nasal volume result in better stimulation of olfactory receptors,

and therefore the persons have better sensation of the airflow in the nose. The second reason for such result might be the fact that these persons were primarily operated on for aesthetic reasons, and that only the fulfilment of their aesthetic requirements and satisfaction with the outcome leads to exaggerated results when scoring the nasal respiratory function after the surgery. The third reason could be the fact that these persons presented their nasal respiratory function before the surgery worse than it really was, in order to justify their aspiration to undergo a nose surgery as soon as possible.

Although there is no significant difference in the reduction of nasal respiratory functions following surgery in patients who had preserved nasal respiratory function before the surgery, it is important to point out that these patients need to be monitored continuously. This was also highlighted by Constantinides et al. [21], who emphasised that after aesthetic nose surgeries the patients may have asymptomatic nasal breathing difficulties for a long time. As the same authors underline, these patients do not have good correlation between the subjective score and objective nasal breathing findings.

CONCLUSION

Nasal pyramid reduction after aesthetic rhinoplasty, in patients with a nasal septum without deviation preoperatively, results in statistically insignificant reduction of the nasal airflow as well as an increase in nasal airflow resistance, as a direct consequence of the reduction of the minimal cross-sectional area as well as the volume of the nose itself.

Subjective self-assessment of nasal breathing is not a relevant indicator of the nasal respiratory function in patients who undergo aesthetic rhinoplasty.

Conflict of interest: None declared.

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Утицај естетске ринопластике на дисајну функцију носа код пацијената са правом носном преградом

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САЖЕТАК

Увод/Циљ Естетски, нос даје печат изгледу особе. Ово је разлог честих операција носне пирамиде, како функционалних, тако и естетских. Проблем се јавља код естетских операција. Пацијенти су често спремни да свој проблем са изгледом носа приказују као проблем отежаног дисања, будући да околина често нема разумевања за њихову жељу за естетском операцијом.

Циљ рада је био да се, коришћењем субјективне процене и објективних мерења, испита промена у дисајној функцији носа код пацијената који се подвргавају естетској операцији носа а имају праву носну преграду пре операције.

Методе Истраживање је спроведено као проспективно, укрштено, и обухватило је 32 пацијента оба пола. Пре и шест месеци после операције свим пацијентима је дисајна функција носа процењивана субјективно коришћењем визуелне аналогне скале (оцена 0–10) и објективно помоћу риноманометрије и акустичке ринометрије уз претходну анемизацију.

Резултати Студија је укључила седам пацијената мушког и 25 женског пола, старосне доби од 18 до 27 година. Објективна мерења после хирургије показала су смањење запремине носа, минималне површине попречног пресека, као и протока ваздуха кроз нос, док се отпор протоку ваздуха кроз нос повећао, али без присутне статистичке значајности. Субјективни осећај дисања на нос се статистички знатно поправио после операције.

Закључак Субјективни осећај дисања на нос није релевантан показатељ објективног стања дисајне функције носа код пацијената после естетске ринопластике.

Кључне речи: естетска ринопластика; риноманометрија; акустичка ринометрија; проток ваздуха кроз нос; субјективни осећај дисања на нос

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Motivation and job satisfaction among hospital nurses in Bulgaria – a cross-sectional study

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SUMMARY

Introduction/Objective The competitive healthcare market is focused on quality health services and the search for effective methods to improve the quality of these services represents a continuing challenge for healthcare managers. It has been found that satisfaction with the work of nurses directly affects the quality of care and patient satisfaction. The aim of this study was to examine the factors influencing the motivation and job satisfaction of hospital nurses.

Methods A cross-sectional study in private and public hospitals of Plovdiv region (second largest region in Bulgaria), established as training and teaching bases for nurses was conducted. The sample comprised of nurses employed in internal wards, surgical wards, intensive care units, and neurology ward settings.

Results Most of the participants 55 (67.1%) are working in public hospitals and 27 (32.9%) in private ones. All of the respondents were women with a mean age of 50.57 ± 11.6 years. Only 40.3% of nurses were satisfied with their job and employees in public hospitals were generally more dissatisfied, although both sectors have identified "payment" and "working conditions" as being an important source of dissatisfaction.

Conclusion Motivation and job satisfaction studies play an important role in providing appropriate medical care in the healthcare sector. The results of this study demonstrate the importance of good job salary, good working conditions, and co-workers' support for job satisfaction.

Keywords: cross-sectional study; job motivation; job satisfaction; nurses; healthcare

INTRODUCTION

Job satisfaction is one of the most actively and extensively studied topics in the field of organizational behavior. Theorists' and researchers' interests on that topic dates back to the beginning of the last century as a part of attempts to understand the human behavior in working conditions [1]. The competitive healthcare market is focused on quality health services and the search for effective methods for improving the quality of these services represents a continuing challenge for healthcare managers. Many researchers are looking for a link between job satisfaction and job performance [2]. The interest in the motivation and satisfaction of employees arises exactly from the need to identify those subjective factors that explain the differences in behavior and performance of employees. Locke defines job satisfaction as a pleasant or positive emotional state resulting from the evaluation of someone's work or work experience [3]. It can also be described as a phenomenon which is a result of complex interactions between work experience, organizational environment, and personal factors. This phenomenon is inextricably linked to work motivation, understood as "a reason that makes workers act in a certain way, striving to achieve personal and organizational goals" [4]. Neither job satisfaction nor motivation are directly visible, but both are critical to the retention and performance of health professionals [5].

In the field of healthcare, health services and the nursing profession, satisfaction is one of the key determinants of job performance [6]. Performance is studied from different points of view. Some studies examine the impact of organizational characteristics (such as staff shortages, lack of equipment, etc.) on satisfaction [7]. Other researchers have assessed the impact of management and organizational situation factors on satisfaction [8]. Satisfaction is important and influences job performance and attitudes towards leaving the workplace [9]. Factors such as poor working conditions, ethical problems, or stress in the workplace lead to dissatisfaction and low labor productivity [10, 11, 12]. Studies show that job satisfaction directly affects the quality of health care and patient satisfaction [13]. The results of studies found that high levels of nurse burnout are associated with lower patient satisfaction [14].

Nurses' satisfaction contributes directly to patients' satisfaction with nursing care, which is an important performance indicator of the quality of care [15, 16]. Therefore, work motivation and satisfaction are factors, influencing the quality of health services, efficiency and commitment to the health organization and they directly affect the cost of healthcare

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[6, 13]. The constant investigation of the most important factors influencing the motivation and job satisfaction of healthcare professionals is one of the ways to retain staff and increase the efficiency of health organizations.

The aim of this study was to examine the factors influencing the motivation and job satisfaction of hospital nurses in the Plovdiv region, Republic of Bulgaria.

METHODS

A cross-sectional study in private and public hospitals of the Plovdiv region (the second largest region in Bulgaria), established as training and teaching bases for nurses, was conducted. Data was collected for the November 2019 – February 2020 period. The sample comprised nurses employed in internal wards, surgical wards, intensive care units, and neurology ward settings. It was an anonymous self-administered questionnaire survey on a voluntary basis. It contained a permission form, purpose of the research with brief description and guidelines on how to fill out the questionnaire. Data collection was performed using a questionnaire, designed by the authors of this study, and based on the theory by Herzberg et al. [4]. The study protocol was approved by the Ethics Committee of the Medical University – Plovdiv. Confidentiality and anonymity were assured to the participants, and they were informed of the right of withdrawal from the study.

All study participants received information on the study objectives and recruitment process. Three University hospitals in which nurses are trained in the Plovdiv region were selected at random. The inclusion criteria for this study required nurses to be: (a) full-time employees and (b) being hospital staff for at least six months. The sample size was obtained using a formula for cross-sectional studies [17]:

$$N = \frac{[Z^2 \times P \times (1-P)]}{D^2}$$

where, N = number of participants; Z = SD at 95% CI (1.960); D = amount of error we will tolerate = ± 5% P = 11.7% – obtained from a pilot study [18].

$$N = \frac{[1.960^2 \times 0.117 \times 0.883]}{0.05^2}$$

We used the modified Kish and Leslie equation to calculate available sample size [17]:

$$n = \frac{N}{1 + \frac{(N-1)}{K}}$$

where n is the sample size and K is the estimated overall population of the study population (approximately 1000 nurses). To cater for non-correspondents, 10% of the sample size was added to the calculated sample size. Therefore, the actual total sample size was 150 respondents. A total of 95 questionnaires were completed and returned by the respondents, which represented 63.3% of the targeted nurses. Eighty-two valid questionnaires were included in the final analysis.

The questionnaire consisted of two parts. The reliability coefficient measured by Cronbach α was equal to 0.849. The first part of the questionnaire included sociodemographic characteristics and data related to the workplace. The second part of the tool contained 22 questions for self-assessment of work motivation and satisfaction. A five-point Likert scale was used to rate from 1 – "not important at all" / "does not satisfy me at all" to 5 – "very important" / "very satisfying." Higher results correspond to higher work motivation and satisfaction with a specific factor. Five-point Likert scale was used to respond to all questions (1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, and 5 = strongly agree).

The data was processed with the SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA). The basic descriptive parameters were calculated (mean, standard deviation, number, and percentage). Univariate ANOVA was used to test the differences in the motivating factors according to age, position, and other characteristics. Verification of the hypotheses was conducted with $p < 0.05$.

RESULTS

Most of the participants 55 (67.1%) work in public hospitals and 27 (32.9%) in private ones. All of the respondents were women with a mean age of 50.57 ± 11.6 years. Older nurses are the majority in Bulgarian hospitals. Thirty-seven of the participants (45.1%) in the study have a bachelor's degree and only 17.1% (14 participants) have a master's degree. The highest number of all nurses having work experience between 21–30 years is 37 (45.1%), which implies significant experience in practicing the nursing profession, and only 6.1% have less than five years of experience. Most participants are married [63 (76.8%)]. The distribution of the sample, according to demographic and work-related variables, is shown in Table 1.

Table 2 provides the mean, the standard deviation, 95% confidence interval, and overall ranks of the 11 motivational factors for both hospital types. The ranking is based on the mean of the factors. The lowest mean value has the highest rank because the respondents were asked to rank with a most effective motivator, then with a second most effective motivator, and so on. For nurses working in both public and private hospitals, "payment" is the first most important motivational factor, followed by "job security." These factors also receive the lowest mean value (3 and 4.44, respectively). The factor with least motivational importance is "responsibility at work" (Table 2).

Satisfaction from various investigated factors in the workplace are given in Table 3. Every other nurse is satisfied or very satisfied with her supervisor (n = 46, 56.1%); 12.2% of nurses working in private hospitals are dissatisfied with their job. Among persons working in public hospitals, this percentage reaches 28%. Most of the respondents are

Table 1. Socio-demographic and workplace characteristics of the respondents (n = 82)

Characteristic	Value	n	%	p
Age group	< 30	6	7.3	0.001
	31–40	9	11	
	41–50	26	31.7	
	51–60	23	28	
	over 60	18	22	
Marital status	lives alone	13	15.9	0.411
	married	63	76.8	
	unmarried, divorced, widowed	6	7.3	
Educational level	college	31	37.8	0.005
	bachelor	37	45.1	
	master	14	17.1	
Rank	head nurse	15	18.3	< 0.001
	nurse	63	76.8	
	nurse manager	4	4.9	
Form of ownership at the hospital	private	27	32.9	0.002
	public (state/municipal)	55	67.1	
Work experience (years)	< 5	5	6.1	< 0.001
	5–10	6	7.3	
	11–20	18	22	
	21–30	37	45.1	
	> 30	16	19.5	
Departments	internal medicine department	44	53.7	< 0.001
	surgical department	26	31.7	
	intensive care unit	3	3.7	
	neurology department	9	11	

satisfied with the "working hours" (n = 37, 45.1%) and with their "relationships with colleagues" (n = 33, 40.3%). On the other hand, almost three-quarters of nurses are dissatisfied or very dissatisfied with the "salary they receive" (n = 54, 65.8%) and with the "working conditions" (n = 52, 63.4%).

Married nurses have lower job satisfaction than unmarried ones ($p < 0.05$). Nurses with a college degree (mean rank = 52.79) report a higher level of job satisfaction than those with a master's degree (mean rank = 44.62) or a bachelor's degree (mean rank = 32.68). There is a significant difference in the overall job satisfaction of the respondents depending on the educational level ($\chi^2 = 8.59$, $p = 0.014$). However, nurses with a master's degree work more often in private hospitals (n = 23, 28%) and those with a bachelor's degree in public hospitals (n = 14, 17%). Nurses working in private hospitals reported a higher overall level of job satisfaction (mean rank = 44.22) than those working in the public sector (mean rank = 40.16) ($\chi^2 = 20.85$, $p < 0.001$).

DISCUSSION

Work motivation is a proven factor which influence nurses' intention to pursue the profession and job satisfaction [18, 19]. The practice of the nursing profession is influenced by the complex effect of various environmental factors – socio-economic, legal, political, demographic, cultural, as well as specific factors for the profession. The shortage of nurses is a challenge not only in Bulgaria but is a critical issue in many countries [6, 20, 21]. Nowadays, in Bulgaria, a number of nurses are leaving their profession to join other non-nursing fields or emigrate abroad [22]. In our country, shortage of hospital nurses is associated with factors such as increased migration of professional nurses, decreased number of nurse graduates, and ageing workforce [23, 24, 25]. Job satisfaction of healthcare professionals is a very important factor of the quality of health care and has a great impact on achieving good performance and efficiency in healthcare organization. Many studies have focused on job satisfaction through nurses because of its proven impact on patient safety and health outcomes [11, 25]. Therefore, this study set out to examine the nurses' motivation and job satisfaction and factors associated with it in public and private hospitals in the region of Plovdiv.

As in a number of researches that suggested that, generally, nurses' job satisfaction is usually moderate or low, our findings showed that only about 40.3% of nurses were satisfied with their jobs, which is an extremely low level [6, 14]. Overall, nurses in public hospitals were generally more dissatisfied than those working in private ones, although

Table 2. Ranking of motivators: a comparison between hospitals (public and private)

No.	Motivators	Mean		SD		95% confidence interval		Rank	
		public	private	public	private	public	private	public	private
1	opportunity for advancement and promotion	6.42	5.11	3.56	3.08	5.46–7.38	3.89–6.33	6	3
2	payment/salary	3.09	3.22	3.01	2.85	2.28–3.9	2.1–4.35	1	1
3	job security	4.44	4.11	2.35	1.52	3.8–5.07	2.33–1.53	2	2
4	working conditions	4.64	5.22	2.35	3.07	4–5.27	4.01–6.44	3	5
5	interesting job	4.71	5.19	2.52	3.56	4.03–5.39	3.58–6.59	4	4
6	working time / flexible working hours	5.98	6.67	2.69	3.34	5.24–6.69	5.35–7.99	5	7
7	additional benefits	6.93	5.37	2.48	2.86	6.26–7.6	4.24–6.5	8	6
8	co-worker relationship, teamwork	6.71	7.3	2.8	2.95	5.95–7.47	6.13–8.46	7	8
9	social position and prestige	7.22	8.07	3.03	2.29	6.4–8.04	7.17–8.98	9	10
10	job responsibility	8.44	8.3	2.34	1.77	7.8–9.07	7.6–9	11	11
11	supervisor support	8	7.44	2.7	2.97	7.27–8.73	6.27–8.62	10	9

Table 3. Frequency and percentage of each item in the job satisfaction

No.	Items	Very dissatisfied	Dissatisfied	Neither satisfied nor dissatisfied	Satisfied	Very satisfied
		n (%)	n (%)	n (%)	n (%)	n (%)
1	opportunity for advancement and promotion	19 (23.2)	14 (17.1)	29 (35.4)	12 (14.6)	8 (9.8)
2	payment/salary	33 (40.2)	21 (25.6)	20 (24.4)	4 (4.9)	4 (4.9)
3	job security	7 (8.5)	12 (14.6)	33 (40.2)	18 (22)	12 (14.6)
4	working conditions	29 (35.4)	23 (28)	14 (17.1)	10 (12.2)	6 (7.3)
5	interesting job	17 (20.7)	16 (19.5)	37 (45.1)	10 (12.2)	2 (2.4)
6	working time/ flexible working hours	4 (4.9)	10 (12.2)	31 (37.8)	16 (19.5)	21 (25.6)
7	additional benefits	10 (12.2)	8 (9.8)	37 (45.1)	12 (14.6)	15 (18.3)
8	co-worker relationship, teamwork	6 (7.3)	6 (7.3)	37 (45.1)	18 (22)	15 (18.3)
9	social position and prestige	14 (17.1)	14 (17.1)	27 (32.9)	18 (22)	9 (11)
10	job responsibility	17 (20.7)	10 (12.2)	30 (36.6)	16 (19.5)	9 (11)
11	supervisor support	10 (12.2)	8 (9.8)	18 (22)	28 (34.1)	18 (22)

both sectors have identified "payment" and "working conditions" as important sources of dissatisfaction. According to Nantsupawat et al. [25], job dissatisfaction is a major cause of high nurse turnover, as well as increased absences. Another reason is the low nursing occupational prestige. These are demotivating factors that cause young people in Bulgaria to study nursing or to practice this humane profession.

The nurses in this study were females, most of them over the age of 50, married and with a bachelor's degree. Masum et al. [26] revealed that married nurses have higher job satisfaction than unmarried ones, but we found the opposite results. The finding of the present study revealed that nurses with a master's degree are most dissatisfied with their work, followed by nurses with a bachelor's degree and those with a college degree. The cause could be that more educated staff have higher expectations, which healthcare organization are often unable to fulfill. Also, master's degree nurses were less satisfied with their occupation, probably because the nurse salary structure in our country does not differentiate between groups.

The research results show that hospital nurses are dissatisfied with their job salary (65.8%), working conditions (63.4%), and their social position and prestige (40.2%). The respondents were least satisfied with "payment/salary." The main motivator in both studied groups is "payment/salary," but this factor is associated with the greatest dissatisfaction. Two-thirds of the study participants were least satisfied with their salary, which is consistent with studies in other countries [6, 14, 26–29]. Although the salary of nurses in Bulgaria has started to increase in recent years, this fact suggests that Bulgarian nurses may not be satisfied with the level of income they receive now. A low satisfaction score and high significance score of the factors show that there is a prospect to improve job satisfaction by increasing payment. However, satisfaction with material status may not be related to the amount of remuneration received as much as to the perception of remuneration being fair.

In our study, important factor influencing dissatisfaction is "working conditions." Poor working conditions and limited resources reduce job satisfaction and motivation among nurses, which can affect service quality and distress [6]. It has been found that less favorable working conditions such as heavy workload, lack of staff and extended working hours are negatively associated with job satisfaction. Physical working conditions, such as noisy environment, and poor living conditions can also reduce job satisfaction. Grujičić et al. [28] in a survey performed in healthcare centers in three districts of Vojvodina (Serbia) show that healthcare workers are more satisfied with a positive work environment.

Among the eleven items of job satisfaction, nurses collectively expressed high level of satisfaction with supervisor support (56.1%) and flexible working hours (45.1%). The results from this study are following the results of Masum et al. [26] and Labrague et al. [30], who identified "supervisor support" as an important factor of nurses' job satisfaction. In our study, "relationships with colleagues" and "supervisor support" predict a nurse's job satisfaction positively. This is consistent with studies done in hospitals in Slovenia and Serbia, where nurse-physician relationships had impact on nurse satisfaction [27, 29]. The collaboration between a doctor and a nurse is linked to positive job satisfaction [31]. When there are good work relationships, there is a sense of recognition and feeling of respect between them, which further brings satisfaction at work.

Our study had some limitations. In this study, the sample size was relatively small and limited to nurses who were employed in private and public hospitals which are training and teaching bases in the Plovdiv region, Bulgaria. As it is a cross-sectional study, inferences on the cause–effect relationships could not be made. The information gained from participants is based upon their subjective perceptions.

CONCLUSION

Motivation and job satisfaction studies play an important role in providing appropriate medical care in the healthcare sector. Low job motivation and job satisfaction have been found to reduce the quality of healthcare, to worsen patient satisfaction, and ultimately to increase healthcare costs. The findings of this study indicate that nurses with good job salary, good working conditions and co-worker support

tend to be more satisfied with their job. These findings can be used by hospital managers, professional associations, and health policy makers to develop strategies to increase income in this occupational group, improve working conditions, and safety. This will increase motivation and job satisfaction in hospitals and reduce the nurses' intention to leave the profession.

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Радна мотивација и задовољство болничких медицинских сестара у Бугарској – студија пресека

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САЖЕТАК

Увод/Циљ Конкурентно тржиште здравствене заштите оријентисано је на квалитетне здравствене услуге, а потрага за ефикасним методама за побољшање квалитета ових услуга стални је изазов за менаџере у здравству. Утврђено је да задовољство радом медицинских сестара директно утиче на квалитет неге и задовољство болесника.

Циљ ове студије био је да испита факторе који утичу на мотивацију и задовољство послом медицинских сестара.

Методе Спроведена је студија пресека у приватним и јавним болницама регије Пловдив (други по величини регион у Бугарској), које су базе за обуку и наставу за медицинске сестре. Узорак је обухватио медицинске сестре које раде на интерним одељењима, хируршким одељењима, јединицама интензивне неге и неуролошким одељењима.

Резултати Већина учесника, 55 (67,1%), ради у јавним болницама, а 27 (32,9%) у приватним болницама. Сви испитаници су жене просечне старости 50,57 ± 11,6 година. Само 40,3% медицинских сестара је задовољно својим радом, а особље јавних болница обично је незадовољније, иако оба сектора наводе плату и услове рада као важне изворе незадовољства.

Закључак Студије о мотивацији и задовољству послом играју важну улогу у пружању одговарајуће медицинске заштите у здравственом сектору. Резултати ове студије показују значај добре плате на радном месту, добрих услова рада и подршке колега за задовољство послом.

Кључне речи: студија пресека; мотивација за посао; задовољство послом; медицинске сестре; здравствена заштита

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Microanatomical characteristics of arterial vascularization of the anterior cruciate ligament

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SUMMARY

Introduction/Objective The aim of this study was to examine the immunohistochemical features of the vascularization of the anterior cruciate ligament (ACL), as well as the quantification of capillaries within the three segments of the ACL; proximal, middle and distal. The quantification and metric characteristics of mast cells of the ACL are the second goal of this research.

Methods In total, 30 human ACL of 30 persons, obtained during routine autopsy, were examined under the microscope, following immunohistochemical reactions against CD34 of blood vessels and MastTrip of mast cells.

Results The middle genicular artery close to the ACL gave off branches for the supply of ligament itself. Each field of mm^2 contained an average number of 1113.84 (959–1240), microvessels in ACL proximal third, an average number of 1145.43 (924–1310) microvessels in ACL middle third, and an average number of 1134.55 (889–1451) microvessels in ACL distal third. An average number of mast cells of the ACL was 3.8 per mm^2. In the peripheral synovial zone of the ACL, we counted 12.6 mast cells per mm^2. An average area value of the mast cells was 124.7 μm^2, and an average value of shorter and longer axis of the mast cells was 11.2 × 15.0 μm.

Conclusion There was no statistically significant differences between the average numbers of intraligamentous microvessels of the ACL thirds ($p > 0.05$), confirming and supporting our hypothesis of uniform distribution of blood supply within the ACL.

Keywords: anterior cruciate ligament; intraligamentous microvessels; mast cells; immunohistochemistry

INTRODUCTION

The anterior cruciate ligament (ACL) is an intracapsular but extrasynovial ligament. The cruciate ligaments are covered by a fold of synovial membrane which incompletely divides the joint in the sagittal plane. The histological structure shows that the intercellular matrix of cruciate ligaments consists of parallel bundles of collagen fibers, separated by thin reticular fibers. Ligaments belong to the group of hypocellular tissues, and the cells that can be found in the structure of ligaments have the characteristics of fibroblasts [1]. ACL injuries are associated with various risk elements related to the extrinsic and intrinsic factors. Intrinsic factors of ACL tear include morphology of intercondylar notch of femur and proximal plateau of tibia, and the distribution of blood supply within the ACL [2].

The popliteal artery (PA) is the main blood vessel that supplies the knee joint, and its injury is common in the knee luxation and fractures of the lower extremities' bones. After originating from the PA, the middle genicular artery (MGA) pierces through the posterior part of articular capsule and enters the area of the intercondylar fossa, where it divides by providing numerous branches to both cruciate ligaments and menisci [3]. With its terminal branches, the MGA participates in the vascularization of cruciate ligaments, branching out into small blood vessels that form a periligamentous network within the synovial sheath surrounding the ligaments. From the synovial membrane the capillaries originate and penetrate the ligaments at right angles, where they are longitudinally oriented forming intraligamentous vascular network. The distal part of the ACL is also supplied by branches of the PA: inferior lateral genicular arteries (ILGA) and inferior medial genicular arteries (IMGA) [4].

The network of blood vessels surrounding the ACL is significantly more developed comparing with the intraligamentous area, whereas the distal part of the ligament is the least vascularized. The reason for this difference can be found in the specific histological structure of

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the distal part of an ACL ligament, i.e., there is a zone of fibrocartilaginous tissue, which results in a certain degree of compression of blood vessels, consequently leading to weaker vascularization of this part [4, 5]. The mast cells (MCs) are present in different tissues close to blood vessels, containing different granules with bioactive molecules (histamine, cytokines, heparin, and tryptase) [6].

Immunohistochemical methods of staining enable precise identification and analysis of the capillary network of three parts; proximal, middle and distal segment of the ACL, as well as the quantification and metric characteristics of MCs of the ACL.

METHODS

Immunohistochemical studies were performed on 30 isolated ACL specimens obtained from cadavers of persons of both sexes (18 males and 12 females) aged 36–72 years (mean age 58.6 years) during autopsies at the Institute of Pathology of the Medical Faculty of Belgrade, with the approval of the Institute of Pathology. The material for histochemical and immunohistochemical staining methods was prepared in a standard manner at the Institute of Histology and Embryology and the Institute of Pathology of the Faculty of Medicine in Belgrade. The ACL samples were fixed in 4% neutral buffered formaldehyde solution for 24 hours, in a volume 20 times greater than the volume of the immersed tissue. After the fixation was completed, the samples were divided into three thirds (proximal, middle and distal) knowing that the lower part of the ligament was marked with the blue thread. They are then further prepared by a routine procedure, which includes dehydration, impregnation, and molding in paraffin/paraplast (Bio-Plast plus, Bio-Optica, Milan, Italy). Each sample of 15 ligaments embedded in paraffin was cut transversely, and 15 ligaments were cut longitudinally, serially, on a microtome (RM 2255, Leica Microsystems GmbH, Frankfurt, Germany) until the ligament was completely cut and the order of sections was carefully marked. Tissue sections 4–5 μm thick were mounted on special high-adhesion glass slides (SuperFrost Plus, Dako, Glostrup, Denmark), dried for 60 minutes in a thermostat at 56°C and then stained. The sections underwent immunostaining by incubation with two primary antibodies, CD34 (antibody Dako Denmark A/S, M 7165, dilution 1:25), and anti-mast cell tryptase (Dako Denmark A/S, M 7052, dilution 1: 100) according to the staining protocol.

During the staining process of ligament slices, negative controls were represented by tissue samples to which a non-immune serum was applied instead of primary antibodies. The intensity of the immune response was determined semiquantitatively by two independent researchers as strongly positive (+++), moderate (++), weak (+) or negative (-). The number of microvessels identified by immunostaining was counted manually in the software system "Leica Interactive Measurements" (Leica Microsystems GmbH, Frankfurt, Germany), on 10 randomly selected fields of view of each section at × 400 magnification (field size 341.7 μm × 250 μm), then the number for the area of 1 mm^2 was subsequently calculated. The number of immunostained MCs was counted with a similar procedure applied.

The analyses of descriptive statistics that we used were arithmetic mean values with standard deviations, and minimum and maximum values. We used the student's t-test for independent samples and one-way analysis of variance (ANOVA) followed by Bonferroni's corrective. The probability level of $p < 0.05$ was considered as a statistically significant difference. All statistical analyses were performed using the statistical program SPSS (SPSS for Windows, release 17.0, SPSS Inc., Chicago, IL, USA). The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade, Belgrade, Serbia (No. 1322/V-10, Date 20-05-2021).

RESULTS

Our research has shown that the MGA, branch of the PA, in the close vicinity of the ACL sent more small arteries for the vascularization of the ligament itself. The distal part of the ACL received branches from the ILGA and IMGA, also originating from the PA (Figure 1). Precise measurements gave diameters of these branches, from 0.022 mm to 0.049 mm (in average 0.032 mm). Upon separation from the PA, ILGA, and IMGA branched and formed a fine synovial arterial network on the ACL surface from which originated thin penetrating vessels for ligament tissue. Studying the ligament slices, we noticed small arteries, arterioles, precapillaries, capillaries, venules and small veins. Only the capillaries reached the deepest parts of the ligament. The MGA also sent penetrating nutrient branches to the femoral attachment of ACL, and surrounding bony tissue of the lateral femoral condyle. The ILGA and IMGA branches

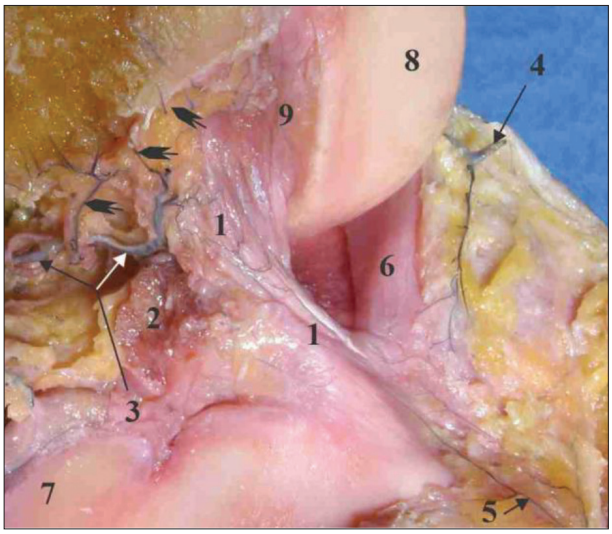

Figure 1. 1 – the anterior cruciate ligament; and 2 – the posterior cruciate ligament are supplied from the 3 – middle genicular artery, 4 – the inferior lateral genicular artery and 5 – the inferior medial genicular artery; note the femoral nutrient branches of the middle genicular artery (arrows), 6 – the lateral meniscus, 7 – the medial meniscus, 8 – the lateral condyle of femur, and 9 – the intercondylar fossa (medial view, dissection of knee specimen injected with India ink)

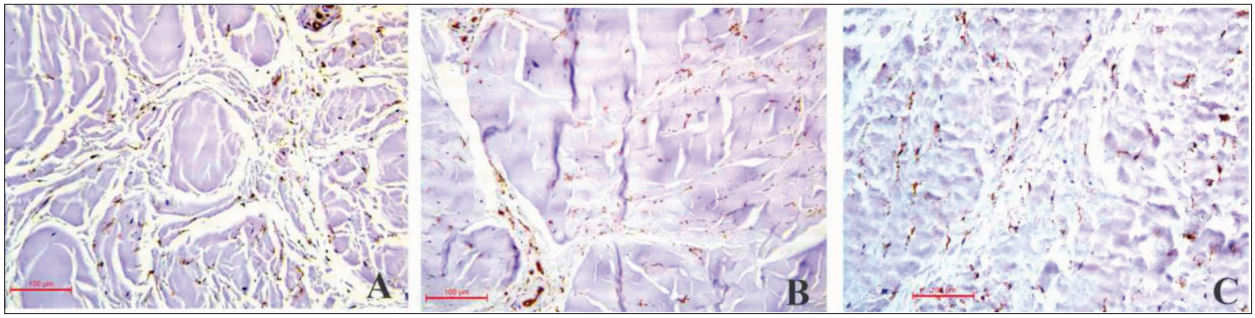

Figure 2. Intraligamentous microvessels in the transverse sections of A – proximal part; B – middle part; C – distal parts of the anterior cruciate ligament (CD34 immunostaining)

Table 1. Vascular density in three parts of anterior cruciate ligament: proximal, middle and distal third; mast cell density in synovial and intraligamentous tissues; and metric characteristics of tryptase positive mast cells

Anterior cruciate ligament	Proximal third	Middle third	Distal third
N° of microvessels/ mm²: min-max (M)	959–1240 (1113.84)	924–1310 (1145.43)	889–1451 (1134.55)
Tissue	**Synovial tissue**		**Intraligamentous tissue**
N° of mast cells/mm²: min–max (M)	6–18 (12.6)		0–7 (3.8)
	Average shorter and longer, and mean diameter (μm): min–max (M ± SD)		**Area value (μm²): min–max (M ± SD)**
Metric characteristics of tryptase positive mast cells	11.2 × 15 (13.13 ± 0.9)		91.5–155.8 (124.7 ± 16.2)

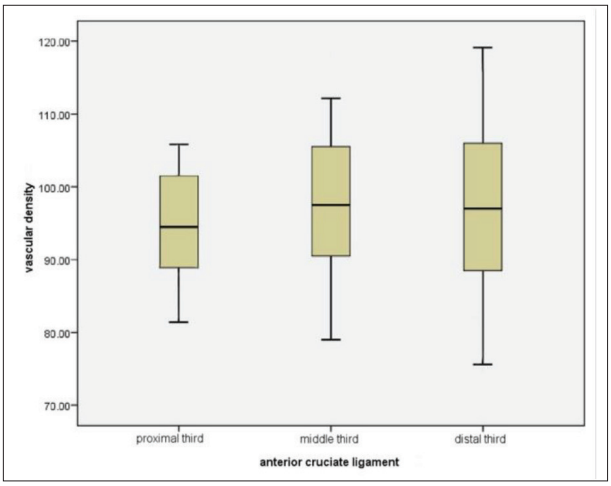

Figure 3. Distribution of number of microvessels in proximal, middle and distal third of anterior cruciate ligament

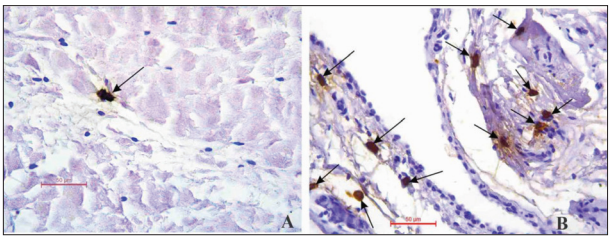

Figure 4. Tryptase-positive mast cells (arrows) in A – intraligamentous tissues; B – synovial tissues of the anterior cruciate ligament (anti-mast cell tryptase immunostaining)

supplied the tibial attachment of ACL, and the upper bony surfaces of the lateral and medial tibial condyles.

Dividing the longitudinal central axis of the ACL into thirds enabled us to analyze the partial vascularization of the ACL. The average microvessel intraligamentous density of the proximal third of the ACL counted in microscopic fields was 95.2 (82–106), and recalculated for the area of 1 mm² of ACL tissue was 1113.84 (959–1240) (Figure 2 and Table 1).

The intraligamentous vascular network of the middle third of the ACL, showed the average number of blood vessels per mm² of ACL tissue was 1145.43 (924–1310) (Figure 2 and Table 1).

The intraligamentous density of CD34 positive microvessels of the distal third of the ACL, the average number of blood vessels per mm² of ACL tissue was 1134.55 (889–1451) (Figure 2 and Table 1).

Testing the significance of the difference in the number of ACL microvessels, based on analysis of variance (ANOVA), by comparison on three thirds of the ACL, proximal, middle and distal, it was observed that all values are not statistically significant, $p > 0.05$, i.e., there was no statistically significant difference in the number of microvessels with regard to thirds of the ACL. Blood vessels are uniformly distributed regardless of the observed part of the ACL (Figure 3).

An average number of immunostained MCs of the ACL was 3.8 (0–7) per mm². In the peripheral synovial zone of the ACL, reach in blood vessels, we counted 12.6 (6–18) MCs per mm² (Figure 4). An average area value of the MCs was 124.7 ± 16.2 μm² (91.5–155.8 μm²), and an average value of shorter and longer axis of the MCs was 11.2 × 15 μm (on average 13.13 ± 0.91 μm) (Table 1).

DISCUSSION

Our analysis of the ACL specimens related to their periligamentous vascularization are in agreement with already published results on this topic. The ACL receives arterial blood from MGA, ILGA and IMGA [1, 3]. The arteries originating from the MGA branch in the form of a network in the synovial tissue that surrounds the ligament. From the synovial tissue, small arteries deep into the ACL

and continue as longitudinally oriented intraligamentous vessels. Blood vessels are also found in the interfascicular connective tissue, between the longitudinal bundles of fibers, where they are protected from the forces of ligament stretching.

The ILGA and IMGA, branches of the PA, supply the bony parts and the skin above the lateral tibial condyle, and at the level of the upper border of the medial tibial condyle respectively [7, 8]. Developing and planning the perforating flaps in this region may be initiated including these arteries [9]. The ILGA and IMGA are also responsible for the blood supply to the distal part of the ACL, what was evident in our study. The surgeons should acquire a profound anatomical knowledge of the respective arteries and the extent of the perfused area in order to preserve the distal attachment of the ACL.

The blood supply to the ligament itself is much smaller than the vascular network of the synovial membrane. The network of periligamentous vessels within the synovial membrane extends the entire length of the ligament. The authors also describe these vessels as tortuous blood vessels, which allow them to withstand the demands of complex ligament movements [4, 5]. Thus, the synovial arterial network is very dense, and extends the entire length of the ligament, while in the ACL itself the capillary network is not so intense. In the ligament itself, capillaries follow the bundles of connective fibers. Our findings in the ACL arterial supply agree with this statement. These intraligamentous blood vessels of the ACL could rupture as a result of minor trauma creating an intraligamentous hematoma followed by knee pain and limitation of motion [10].

ACL injuries are among most frequent disabling injuries associated with athletic or other activities (e.g., different accidents). Most ACL injuries occur during sports like skiing, pivoting (e.g., soccer) or non-pivoting sports (e.g., running), that involve dynamic movements such as jumping, pivoting, and cutting. The ACL reconstruction procedure is currently the most common surgical technique for ACL tears. The procedure includes direct arthroscopy, preparation of graft material (e.g., bone-patellar tendon-bone graft), drilling a canal, and fixation of femoral and tibial sides of graft with titanium screws [11]. Both, ACL reconstruction, and non-surgical treatment (e.g., rehabilitation) are accepted procedures with the potential to restore acceptable knee joint function, offering good quality of life. The patients with a surgical procedure of ACL reconstruction report better knee function compared with the non-surgical group [12]. In the other study, most patients who had unsuccessful rehabilitation therapy after ACL injury reported instability, not optimal knee function, and pain during activity. They required and underwent a delayed ACL reconstruction [13]. Increased vascularity and remodeling of human ACL graft in the first year after surgery indicate its insufficient mechanical properties, and need for an extended rehabilitation program [14].

Complications of the ACL reconstruction have been described, including the vascular injuries. The surgeons should be aware about the possible injury to geniculate arteries [15, 16]. Vascular injury or avulsion of the MGA can also occur during posterior cruciate ligament (PCL) reconstruction during open procedure with a posterior approach [17]. According to our investigation reported avascular necrosis of the medial femoral condyle, as a complication of ACL or PCL surgery, is the result of not preserved femoral nutrient branches of the MGA.

In case of a femoral avulsion tear, primary ACL repair in carefully selected patients possess apparent advantages over ACL reconstruction. Natural healing of the repaired proximal ACL tear is additionally achieved by using a high strength suture tape for the stabilization and protection of ligament from excessive elongation. Restoration of native ACL maintains its structural anatomical characteristics, proprioceptive and biomechanical functions. ACL primary repair is safe less invasive procedure, avoids graft-related complications, and faster rehabilitation is possible [18, 19]. Preserved periligamentous synovial sheath should be confirmed before the repair because of the mesenchymal stem cells which are evident in the tissue surrounding the blood vessels [20]. For revascularization, it is necessary that the site of graft attachment in case of its reconstruction is covered with a synovial membrane. The remnant tissue promotes formation of a fibrin-platelet scaffold, revascularization and ligamentization of ACL [21]. Primary repair promotes cell proliferation in the tendon-bone transitional zone and ligament portions, reduces osteoarthritis-like pathological changes, and maintains blood vessels within the ACL. Histological immunohistochemical research using CD34, the most sensitive endothelial marker of blood vessels, confirmed that the microvessels have a significant effect on tissue healing during the primary repair [18]. Our research performing CD34 immunostaining reported a fine synovial arterial network on the ACL surface from which originated thin vessels for the fibrous tissue of the ligament itself, and penetrating vessels for its femoral attachment and surrounding femoral bony tissue. The precursor cells from the surrounding tissue could invade the area and start fibroblast proliferation and collagen production within the repaired tissue as a basis for biological repair.

Studies using the injection technique have shown uneven vessel density in parts of the ACL. One group of authors claims that the vascularization of the proximal and distal parts of ligaments is rich, while between the vascular network of the central and distal part there is a completely avascular area. The coincidence of a poor vascularity and the occurrence of fibrocartilage in the ACL may be caused by the compression in area of the ligament related to the anterior border of the intercondylar fossa when the knee is in full extension. The proximal portion of the ACL has better vascularity. The MGA gives rise to ligamentous branches proximally, and courses distally along the dorsal aspect of the ACL [1, 3, 4, 5]. According to some authors, the poor healing potential of the ACL is a consequence of the weaker vascularization of the lower central parts of the ligament. According to the other study the distal portion of the ACL have many blood vessels and vascular-derived stem cells than in the middle third of the ligament [22]. One recent study quantified relative perfusion

of proximal, middle, and distal thirds of ACL injected with contrast using magnetic resonance imaging scanner. They demonstrated greatest mean relative perfusion within the proximal ACL region, followed by the middle third, and the least relative perfusion in the distal part [23].

To contrast previous studies, the present research has clearly shown the uniformity of vascularization of all parts, the three thirds of the ACL. The difference between the results of our research and the previous studies is probably in the method used in this study. Injection techniques can give unexpectedly unreliable results depending on the condition of the blood vessels and the quality of the injection material. Many of the earlier studies have been done on an animal model, which often cannot be extrapolated to humans, especially due to the different anatomy of the structures being compared. Because of the small number of analyzed cases in a number of papers the reported results are not relevant. In our opinion the immunohistochemical results are more relevant for analyzing an average ACL vascularization. Analyzing the position of extraligamentous synovial blood vessels approaching the ACL it was evident the existence of greater vascular network close to the proximal attachment of the ligament. We already stated that penetrating vessels from the synovium supply the femoral attachment of the ACL, and surrounding femoral bony tissue, but the intraligamentous vascular density was uniformly distributed through the ACL.

The MCs are present close to blood vessels in all connective tissues. Activation and degranulation of MCs comprise different granules with bioactive molecules (histamine, cytokines, heparin, and tryptase) into the local tissues. MCs can cause endothelial activation, vasodilatation and plasma extravasation, and contribute to neurogenic inflammation [24]. Activated MCs participate in tissue remodeling, by activating connective tissue cells and angiogenesis, by releasing histamine and the activation of other cells (macrophages and platelets) [6, 25]. Our research demonstrated very modest presence of MCs in the tissue of ACL, an average of 3.8 cells per mm^2. The peripheral synovial tissue of the ACL, reach in blood vessels, contained 12.6 MCs in average per mm^2. Our observations suggests that the periligamentous synovial sheath of the ACL is reach in blood vessels, nerves and MCs, and has a great potential to promote ligament healing.

The limitations of our study could be related to the missing subgroup matching for the gender and age comparison. We formed only one group of the available ACL specimens in order to establish precise definitions on the variations of intraligamentous microvessels of these structures. Obviously higher values for length and cross-sectional area of the male comparing to the female ACL, and more prominent ability of the male relating to the female ACL to alter its morphology and mechanical properties during intense loading (e.g., a sport training), may suggest sex-based differences in ACL structure and microvascularization. The ACL is composed of fibrous tissue exposed to the various risk elements related to the extrinsic and intrinsic factors. Age related complex changes in microcirculation activate microvascular inflammatory processes and result in dysfunction. It is possible that ACL vascular density in older may differ from that of young persons. Future investigation should improve understanding of potential gender and age-related differences in ACL microvascular supply.

CONCLUSION

Comparing the three thirds of ACL, proximal, middle and distal, it was observed that all values were not statistically significant, $p > 0.05$; i.e., there is no statistically significant difference in the number of microvessels per third of the ACL. Blood vessels are uniformly distributed regardless of the observed part of the ACL. The highest presence of blood vessels and MCs was noticed in the peripheral synovial tissue of the ACL.

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Микроанатомске карактеристике артеријске васкуларизације предње укрштене везе

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САЖЕТАК

Увод/Циљ Циљ студије је био да се проуче имунохистохемијске карактеристике васкуларизације предње укрштене везе (ПУВ), као и квантификација капиларних судова у проксималном, средњем и дисталном сегменту ПУВ. Други циљ овог истраживања била је квантификација и одређивање мерних карактеристика мастоцита ПУВ.

Методе Тридесет хуманих ПУВ пореклом од 30 особа, добијених рутинском обдукцијом, проучавани су под микроскопом после имунохистохемијских реакција на ендотелни маркер *CD*34 крвних судова и мастоцитну триптазу присутних мастоцита.

Резултати Средња артерија колена (*a. media genus*) у непосредној близини ПУВ даје више малих артерија намењених васкуларизацији самог лигамента. Просечан број капилара на квадратном милиметру површине поља препарата проксималне трећине ПУВ износио је 1113,84 (959–1240), средње трећине 1145,43 (924–1310), док је код дисталне трећине ПУВ износио 1134,55 (889–1451). Просечан број мастоцита по квадратном милиметру препарата ПУВ износио је 3,8. У периферној синовијалној зони ПУВ постојало је просечно 12,6 мастоцита по квадратном милиметру. Просечна површина триптаза позитивних мастоцита била је 124,7 μm^2. Просечна вредност краћег и дужег пречника ћелија била је 11,2 × 15,0 μm.

Закључак Поређење три трећине ПУВ, проксималне, средње и дисталне, показало је да вредности капиларне густине у вези нису статистички значајне, $p > 0,05$; односно не постоји статистички значајна разлика у броју микросудова по трећинама ПУВ. Крвни судови се униформно распоређују без обзира на посматрани део ПУВ.

Кључне речи: предња укрштена веза; интралигаментозни микросудови; мастоцити; имунохистохемија

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Pulmonary and central nervous system aspergillosis in a patient with COVID-19 infection

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SUMMARY

Introduction Patients with COVID-19 infection are vulnerable to a variety of serious complications, including invasive fungal infections such as aspergillosis. Because pulmonary aspergillosis is difficult to confirm with perfect confidence, it has been classified as "proven," "probable," and "possible." We present a patient with COVID-19 infection in whom a "probable" pulmonary aspergillosis was complicated by hematogenous spread into brain with formation of multiple abscesses.

Case outline A 67-year-old female was diagnosed with COVID-19 infection using polymerase chain reaction (PCR) from a nasopharyngeal swab. The patient had never been vaccinated before. Despite standard therapy and noninvasive oxygen support, the patient's health deteriorated one month following the onset of the disease, with chest discomfort, cough, and hemoptysis. Thoracic computed tomography (CT) revealed bilateral infiltrative lesions with varying diameters of cavities, primarily in the left lung, as well as modest effusions in both pleural spaces. Aspergillus hyphae were isolated from tracheobronchial aspirates. Despite therapy with Amphotericin B, which was only available antifungal medication at the time, the patient fell into a coma. A CT scan of the skull revealed several infiltrative lesions inside the brain, some with cavities suggestive to metastatic abscesses, most likely of fungal etiology (Aspergillus) as a result of hematogenous spread of pulmonary aspergillosis. Despite therapy and all other precautions, the patient died. The autopsy was not carried out.

Conclusion In addition to other complications, COVID-19 patients may develop pulmonary aspergillosis, which can be fatal because of the possibility of hematogenous spread to the brain.

Keywords: COVID-19; invasive pulmonary aspergillosis; brain

INTRODUCTION

Fungal infections are one of the problems that people with COVID-19 infection are prone to. As a result of respiratory alveolar damage brought on by COVID-19 infection, these tissues are more vulnerable to microbial infections and fungal invasions [1]. COVID-19-associated pulmonary aspergillosis (CAPA) is an invasive fungal complication, which emerged in intensive care units even in previously immunocompetent patients which were on mechanical ventilation [2, 3]. CAPA may also appear in severely ill COVID-19-infected patients receiving noninvasive ventilation in intensive care units, especially in those who have concomitant conditions and are receiving protracted immunomodulatory medication therapy [4].

CASE REPORT

An unvaccinated against COVID-19 67-year-old female presented on September 15, 2021 with cough, chest pain, and high fever. A nasopharyngeal swab was positive for COVID-19 by polymerase chain reaction (PCR). She was treated on the outpatient basis with antibiotics (cephixime, levofloxacine), fraxiparine – low molecular weight heparin (LMWH) and antiviral drug – favipiravir. As the patient`s condition did not improve, she was admitted to the General hospital in Kraljevo four days later. On examination dyspnea dominated. She was under oxygen mask and O_2 saturation was normal (98%). Chest radiography showed bilateral basal pneumonia. Otherwise, she was submitted to angioplasty of right coronary artery due to myocardial infarction in 2009 and she has put on permanent treatment with aspirin (100 mg/day), angiotensin-converting enzyme inhibitors and nitrates medication. Echocardiography showed reduced ejection fraction of 50%. At admission laboratory data were as follow: white blood cells (WBC) 10.36×10^9/l, neutrophils 88.4% (9.16×10^9/l), lymphocytes 8.2% (0.850×10^9/l), hemoglobin (Hb) 141 g/l, platelets 124×10^9/l. Except moderately elevated glycaemia, biochemical analyses were within normal ranges (Table 1). D-dimer 1.54 mg/l, (C-reactive protein) CRP 17.1 mg/l, Fe 4.2 μmol/l, ferritin 110 μg/ml, troponin 6.0 pg/ml. Fourth day after admission to hospital the patient suffered more severe chest pain and dyspnea so she had to be transferred to pulmonary unit. Control laboratory data worsened (Table 1). She was developing covid cytokine storm. The dose of corticosteroids

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Table 1. Laboratory results

Date	15.09.2021	19.09.2021	23.09.2021	1.10.2021	7.10.2021	15.10.2021	26.10.2021	29.10.2021
White blood cell count × 10^9/l	8.59	10.36	14.31	7.6	11.44	7.28	6.02	12.29
Hemoglobin g/l	140	141	135	152	115	104	93	105
Neutrophil count % (absolute count × 10^9/l)	75.6% (6.49)	88.4 (9.16)	94.9 (13.58)	87.1 (6.61)	91.1 (10.43)	94.7 (6.89)	95.9 (5.77)	98 (12.059
Lymphocyte count % (absolute count × 10^9/l)	17% (1.46)	8.2 (0.85)	3.5% (0.5)	9.5 (0.72)	6.5 (0.74)	3.4 (0.25)	2.8 (0.17)	1.2 (0.15)
Platelet count × 10^9/l	134	127	161	202	132	107	99	73
Blood urea nitrogen (BUN mmol/l)	6.90	7.60	6.53	12.40	10.92	9.05	11.47	20.5
Creatinine mg/l	81	80	58	68	64	89	71	137
Glycaemia mmol/l		10.44	13.56	13.93	16.35	31.19	6.97	8.44
C reactive protein mg/l	2.3	17.1	187.2	14.7	2.5	10.8	172.7	263.5
Ferritin ng/l		110	474	451	786	494	1494	/
D-dimer mg/l FEU	2.77	1.54	0.57	1.85		0.78	/	/
Lactate dehydrogenase IU/l	410	510	504	4.54	505	473	688	1126
FIBRINOGEN		3.1			1.9	2.4	5.2	8.3

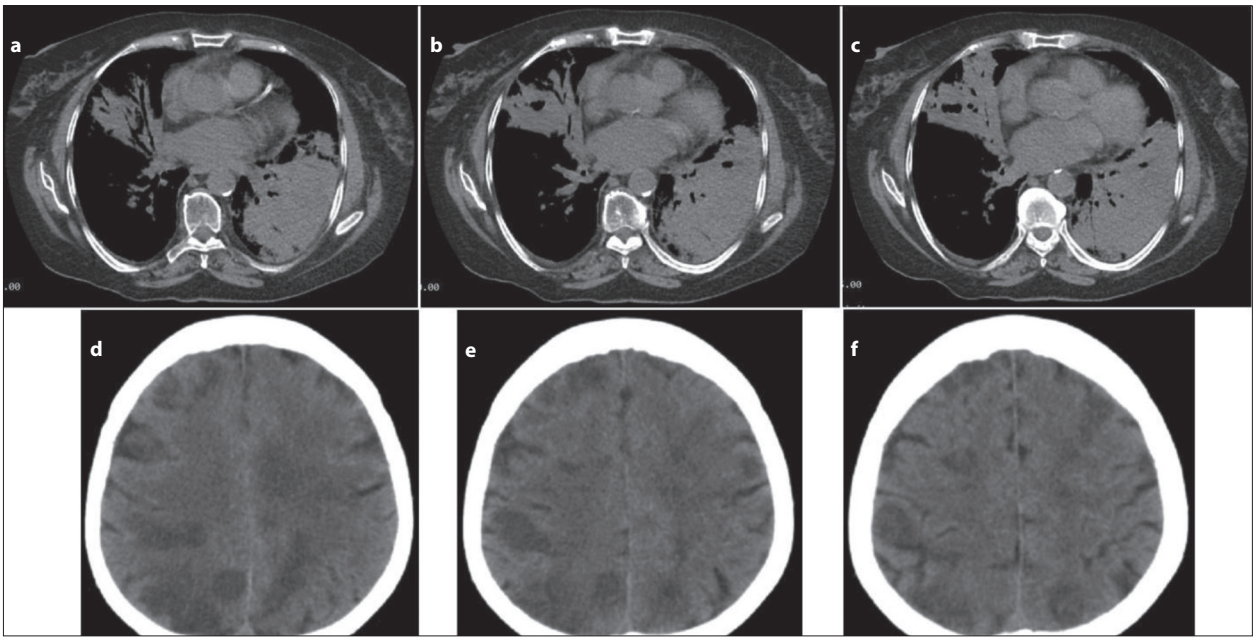

Figure 1. a, b, c – thoracic computed tomography scan showed generalized, partially necrotized inflammatory consolidation of distal part of left lower lobe with signs of numerous cavities of different size, on the right lung with partially necrotized inflammatory infiltrates with signs of cavities within; d, e, f – computed tomography scan of head showing multiple inflammatory lesions through the brain some of which forming abscesses up to 34 mm in diameter

was increased up to 240 mg of methylprednisolone. She was also treated with broad spectrum antibiotics, LMWH, fluconazole and put on permanent noninvasive oxygen therapy, and diabetes mellitus (of which she was not previously aware of) was regulated by infusion pump of insulin. Corticosteroid therapy was not interrupted during the whole hospitalization, but its dose was reduced during periods of the improvement. The patient suffered *Clostridium difficile* enterocolitis which was at first unsuccessfully treated with metronidazole orally, afterwards vancomycin was introduced in doses of 125 mg every six hours, and the patient responded well to treatment. However, soon after, general condition deteriorated developing hemoptysis in the middle of October. Laboratory data showed low number of lymphocytes 0.25/l × 10^9/l, CRP was 10.8 mg/l, Hb dropped to 104 gr/l.

Chest CT scan was performed on October 20, 2021 showing bilateral moderate pleural effusions, interstitial focal consolidations and restrictive segmental atelectasis, complicated by cavitary zone of consolidation in lower left pulmonary lobe with significant reduction of parenchyma. The next day condition further deteriorated. During the night between October 29 and 30, the patient became soporose, and was transferred to intensive care unit. The patient's family disagreed with starting the patient on mechanical ventilation, so she stayed on noninvasive ventilation. Laboratory data also deteriorated, especially factors of inflammation CRP 310.5 mg/l, troponin 8.35 ng/l, WBC 9.59 × 10^9/l, neutrophils 97.8% (9.38 × 10^9/l), lymphocytes 1.4% (0.13 × 10^9/l), Hb 112 g/l, platelets 76 × 10^9/l. Control thoracic CT scan performed on October 26, 2021 showing generalized, partially necrotizing inflammatory

consolidation of distal part of the lower left lobe with signs of numerous cavities of different size, on the right lung with partially necrotized inflammatory infiltrates with signs of cavities (Figure 1a, b, and c). From tracheobronchial aspirate branching hyphae of aspergillus were isolated. Amphotericin B was given as the only drug that was available, colistin, meropenem, corticosteroids and LMWH. As the patient became comatose, endocranial CT scan was performed showing multiple inflammatory lesions throughout brain. Some of the inflammatory focuses in brain were radiographically classified as abscesses up to 34 mm in diameter (Figure 1d, e, and f). The patient died on November 1, 2021. Autopsy was not performed.

This study was done in accordance with the institutional committee on ethics.

DISCUSSION

It is well known that immunodeficient patients with hematological malignancies such as acute leukemia patients, patients with hematopoietic stem cells transplantation are at highest risk for invasive fungal infections such as aspergillosis, candidiasis and mucormycosis [5, 6]. CAPA may even occur in immunocompetent patients, most frequently in those in intensive care units who are on mechanical ventilation, usually 10–14 days after establishing the diagnosis. Less frequently it may occur in patients who are not on mechanical ventilation particularly during cytokine storm, acute respiratory distress syndrome (ARDS) or in those who are on prolongued immunomodulatory drugs. The most frequent cause of fungal infection is *Aspergillus fumigatus* [7]. According to different authors the incidence of CAPA ranges between 3.8% and 33.3% of patients, depending on geographic area, while the overall incidence was 7.6% for proven, probable or possible CAPA [2, 8].

A low number of lymphocytes and severe lung damages caused by SARS-CoV-2 virus are risk factors for developing invasive fungal infection [1]. The further risk factors for development of fungal infection are an administration of immunomodulatory agents, especially prolonged use of corticosteroids in severe COVID-19 patients [2, 8] as well as significant comorbidities such as previous pulmonary disease, renal failure, diabetes mellitus, liver failure, malignancy etc.

The European Confederation of Medical Mycology/The International Society for Human and Animal Mycology expert group established criteria for diagnosis and classification of CAPA, based on clinical factors, chest imaging and microbiological findings of *Aspergillus* presence, according which a pulmonary aspergillosis may be defined as "proven", "probable" and "possible", depending on the reliability of diagnostic procedure that have been performed [3]. The group advised diagnostic bronchoscopy in patients with high clinical suspicion for fungal infection in order to collect secret from bronchi for evaluation (PCR, galactomannan test and cultures). However, it turned out that in real practice a bronchoscopy was not frequently performed in the intensive care units because it generates aerosols which is considerable risk for spreading COVID-19 disease to health care workers [5]. CAPA galactomannan test in serum is usually negative, while in bronchial secret is positive. In our patient there were no technical possibilities to perform either galactomannan test in serum, or in tracheobronchial secret. It is also possible that hemoculture could be positive if taken in time of hematogenic dissemination of fungal infection to the central nervous system. CAPA is an invasive fungal complication that may develop in immunocompetent patients who are in intensive care units and receiving invasive ventilation, but it occurs less frequently in patients receiving noninvasive ventilation, particularly if they have comorbid conditions, have experienced ARDS, cytokine storm, or have received prolonged treatment with high doses of corticosteroids or other immunomodulatory medications (tocilizumab).

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Аспергилоза плућа и централног нервног система код болесника са инфекцијом ковидом 19

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САЖЕТАК

Увод Болесници са инфекцијом ковидом 19 подложни су разним врстама компликација, па и инвазивним гљивичним инфекцијама, као што је аспергилоза. Аспергилоза се код њих не може са сигурношћу лако доказати, тако да се њена дијагноза квалификује као „доказана", „вероватна" и „могућа". Приказујемо болесницу са инфекцијом ковидом 19 код које је „вероватна" аспергилоза плућа метастазирала у мозак доводећи до мултиплих апсцеса мозга.

Приказ болесника Код жене старе 67 година дијагностикована је инфекција ковидом 19 ланчаном реакцијом умножавања (*PCR*) из назофарингеалног бриса. Иначе болесница није била вакцинисана. Упркос стандардном лечењу и неинвазивној оксигенотерапији, стање болеснице се погоршало месец дана после почетка болести са појавом болова у грудима, кашљем и хемоптизијама. Компјутеризована томографија грудног коша показала је инфилтрате у оба плућна крила са формирањем кавитета разне величине у њима и мање изливе у обе плеуралне дупље. Из трахеобронхијалног аспирата изоловане су хифе аспергилуса. Упркос лечењу амфотерицином Б, који је једино био доступан, болесница је развила коматозно стање, а компјутеризована томографија мозга је показала мултипле инфилтративне лезије са местимичним формирањем кавитета који су одговарали метастатским апсцесима, вероватно гљивичне етиологије (*Aspergillus*), настале хематогеном дисеминацијом из плућа. Упркос свим терапијским мерама, дошло је до леталног исхода. Сходно препорукама, аутопсија није рађена.

Закључак Међу бројним компликацијама, оболели од ковида 19 могу развити аспергилозу плућа, која због могућности хематогеног ширења на мозак може довести до леталног исхода.

Кључне речи: ковид 19; инвазивна аспергилоза плућа; мозак

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Intrathecal baclofen therapy and COVID-19 infection – report of three cases

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SUMMARY

Introduction Patients with severe spasticity are effectively treated with intrathecal baclofen therapy (ITB), but because of their invalidity, in case of infection, prognosis is poor.

Case outline We present three cases (two men and one woman) of patients treated with baclofen intrathecal therapy due to spasticity of all four extremities who underwent SARS-CoV2 virus infection. Two of them have multiple sclerosis, and one has trauma of the cervical segment of the spinal cord. In all three patients, the clinical presentation of COVID-19 infection occurred within six months of implantation of the pump for ITB. They were successfully treated in hospital with same dose of the drug and without exacerbation of neurological status. Barthel index (BI) and modified Rankin score were same before and after COVID-19 infection. In two cases BI was 20, and in one 69; and modified Rankin score (mRS) was 3 in one case, and 5 in two cases.

Conclusion Patients with severe spasticity who require intrathecal baclofen therapy can be safely treated regardless of the pandemic.

Keywords: intrathecal therapy; baclofen; spasticity; COVID-19

INTRODUCTION

Spasticity is a consequential symptom of several neurologic conditions that result in central paresis. It has been described in stroke, multiple sclerosis (MS), brain trauma, and in children with cerebral palsy and can heavily affect quality of patients' life [1]. Treatment of spasticity is based on medication and rehabilitation in order to improve their daily activities. They are usually treated with oral administration of baclofen (GABA-B agonist), tizanidine (alpha-2 adrenergic agonist) or tolperisone (sodium and calcium channel blocker at the level of brain stem) [2, 3]. When oral therapy is insufficient or the patient does not tolerate the drug side effects, intrathecal therapy may be considered. If testing for intrathecal baclofen therapy (ITB) is successful, these patients can be effectively treated [4].

Recent Corona virus pandemic caused major turbulence in the majority of services, including health care. Patients with ITB are prone to poor prognosis in case of infection [5].

We present three cases of patients treated with ITB due to spasticity of all four extremities who have been infected with COVID-19. Degree of neurological disability was measured with several scales: spasticity by the Ashworth scale, performance in activities of daily living by the Modified Barthel Index (BI), level of pain by the visual analogue scale (VAS) and level of function by the Modified Rankin scale (mRS) [6, 7, 8].

CASE REPORT

Case 1

A 60-year-old male patient, previously diagnosed with C7-Th1 discus hernia, was admitted to our hospital due to the spinal cord trauma at the C7-Th1 level in November 2019. Lower extremity plegia and high-grade palsy of the upper extremities on both sides were stated during examination. Four days after the trauma a massive pulmonary embolism occurred, limiting possible therapy for next two months. Also, spastic type of paralysis (Ashworth grade III) has developed. Patient's therapy was continued on the physical medicine ward (BI 2, mRS 5). Since maximum dosage of tizanidine therapy gave no results considering spasmolysis (Ashworth grade III, pain level 9 on VAS scale), it was decided that the patient should be tested for intrathecal Baclofen application (positive test on 100 μg bolus for four hours) and Synchromed II pump (Medtronic Inc., Dublin, Ireland) was implanted in September 2020. After the operation, tizanidine therapy was canceled, and daily dosage of Baclofen was gradually risen during physical therapy to 800 μg in 24-hour intrathecal continuous infusion. Significant reduction of pain (VAS 3) and extremities spasticity (more notably on upper extremities) were achieved, BI was 12 and mRS 5.

Two months after the implantation of ITB system, nasopharyngeal swab was taken and

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PCR test on SARS-CoV-2 was performed, due to high fever (39°C), drop of lymphocytes in leukocytic formula, as well as an occurrence of breathing difficulties. The test came back positive. Chest radiography showed transparency reduction ("ground glass") on both sides. COVID-19 therapy protocol gave subjective improvement in the first days, but on the 18th day of COVID infection, worsening of the patient state was observed. His oxygen saturation has decreased, followed by anuria and deterioration in consciousness which progressed to somnolence. In the following period, lymphocytes continued to drop and there was sudden rise of all inflammatory factors, so the patient was admitted to the Intensive Care Unit (ICU), where he was intubated and started on mechanical ventilation. During his stay at the ICU, spasticity worsened to preoperative level.

One week later, after stabilization of patient's state of consciousness and oxygen saturation recovery to 96%, he was transferred back to the neurosurgical ward and his physical therapy was resumed. One month after the SARS-CoV-2 infection he was vaccinated with the first dose of vaccine. Patient was active in a wheelchair (upper extremities palsy 4/5, lower extremities spastic plegia, on all extremities Ashworth grade II, BI 19, mRS 5) and because of sphincter disfunction, permanent urinary catheter was applied. Baclofen therapy was not discontinued and it was resumed in unchanged dosage (800 μg in 24-hour infusion).

Case 2

Our second patient was 49-year-old female, diagnosed with MS. Her first symptoms occurred in 2010 as sense of tingling in her feet which progressed to lower legs and later occurred in her hands. Her walk was unstable with maximal distance estimated 1 km. Her illness was primarily progressive and she began to use a walking device in 2017. By 2018 she was coerced to use the wheelchair and her condition worsened with leg stiffness and hands tremor. During 2020, she had occasional episodes of generalized fatigue, that could last 12 hours during the day and resolve spontaneously.

In neurological status, she had severe spastic quadriparesis, which was more prominent in lower extremities (Ashworth grade III+ on lower and grade III on upper extremities, BI 12, mRS 5). Because of painful spasticity (VAS 5), she was tested for intrathecal baclofen application which reduced spasticity and pain after 100 μg bolus for 3.5-hour period.

The patient was operated on in May 2021, when Synchromed II pump (Medtronic PLT) was implanted and programmed for 200 μg/24-hour administration. Dose was gradually increased to 320 μg/24-hour during her hospitalization at the clinic for neurosurgery, and in the end reached 500 μg/24-hour during her stay at the clinic for physical rehabilitation (BI 20, mRS 5). During this period, she was not vaccinated against SARS-CoV-2.

Four months after ITB implantation, her body temperature rose to 38°C, oxygen saturation was 75% and interstitial pneumonia pattern was observed on radiography. She was hospitalized and treated according to National standards for COVID-19 infection. Two weeks later, she was discharged in good general and unchanged neurological condition (BI 20, mRS 5). Her daily Baclofen dosage remained the same during her hospitalization. There was no discontinued of ITB therapy during infection.

Case 3

The third patient was 42-year-old man with MS. He was treated since 2009 for primarily progressive form of the disease, and by 2020 he started to use wheelchair (BI 51, mRS4). Because of prominent spasticity (Ashworth grade III) and oral spasmolytic therapy intolerance, he was tested for ITB positive in April 2021 after 50 μg of baclofen bolus. Synchromed II pump (Medtronic PLT) was implanted in May 2021 and dosage was set at 100 μg/24 hrs. After the operation spasticity was reduced and initial rehabilitation treatment was prescribed (BI 69, mRS 3). He was vaccinated with two doses of SARS-CoV-2 vaccine.

Four months after, he was hospitalized in COVID hospital due to confirmed infection (body temperature 39°C, positive PCR). During this time oxygen saturation did not drop below 95% and chest radiography did not show any signs of inflammation. After one week he was discharged from the hospital for house care.

After the end of quarantine period, he was admitted to the hospital for regular follow-up. His neurological status remained unchanged (severe spastic palsy of the lower extremities – Ashworth grade II, BI 69, mRS 3), so his daily dosage was same, without any discontinued during infection.

Written consent for publication of this article has been obtained by the patients.

DISCUSSION

Intrathecal drug delivery systems are widely used as an option for treatment of severe spasticity and intractable pain in last four decades [9, 10, 11]. It is the most effective treatment in situation when conservative methods have proved insufficient or intolerable [12, 13]. This treatment is expensive at first, but two years after implantation the costs of the device equate with alternative therapy [14–17].

As with any therapeutic modality, complications are possible. ITB is often placed in patients who are already in medically difficult state and complex to treat and as such, have higher risk of illness at baseline. Therefore, these devices require close supervision and time-sensitive management by a specially trained medical professionals [18]. Puck-Faes et al. [11] has found that patients with a spinal origin of spasticity, lower mRS and higher BI have a higher risk to sustain complications. Complications can be divided in four categories: drug-related, pump-related, catheter-related, and infections [9, 19]. Infections, as complication, have been reported in 5–26% of cases and were more common among traumatic spinal cord injuries [9]. They may appear at any time, after implantation or after revision [11, 20]. Long-term aftercare with baclofen pump refill was proved to be safe, with an infection rate of 0.6 % per puncture for each refill [4]. In our experience with ITB,

we have not observed any infection related to surgery or refill of the pump to date. Also, no drug, pump or catheter related complication were detected.

COVID-19 outbreak led to significant changes in almost all aspects of everyday life, affecting healthcare practice as well. It poses a great risk for patients on ITB due to increased occurrence of infection, as well as the possibility of missing the pump supplementation with the drug. In some cases, this can be life-threatening [21].

Two out of the three presented patients had severe disability with mRS score of 5 and with BI less than 20, while the third patient had moderate disability with mRS score 3 and BI 69, which is in accordance with experience of Puck-Faes et al. [11]. The patient with spinal trauma had the most severe disability among the presented cases. Presented patients did not had ITB system-related infection, but like all those who have suffered a severe disability, there are higher risks.

Based on our first case in November 2020, we have decided that any patient with ITB and COVID-19 infection, should be treated in hospital conditions in order to avoid complications and aggravation of their already difficult state. Since the effect of spasmolytic therapy was satisfactory, the daily dose of baclofen was not changed. Our opinion is that COVID-19 infection does not require a change in ITB therapy protocol, as well as the refill of the ITB system. It could not be remotely managed according to telemedicine services [22].

During COVID-19 pandemic, special attention should be paid to patients in more severe condition, referring to their mobility. The availability of health service is necessary, both in order to supplement the system and, given their altered immunity, to treat them adequately in case of infections. Patients with severe spasticity who require ITB can be safely treated regardless of the pandemic, as it is a safe method that significantly reduces spasticity and alleviates the degree of disability.

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Интратекална терапија баклофеном и инфекција ковидом 19 – приказ три болесника

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САЖЕТАК

Увод Болесници са тешким спастицитетом ефикасно се лече интратекалном терапијом баклофеном. Због њихове инвалидности, у случају инфекције прогноза је лоша.

Приказ болесника Представљамо три болесника (два мушкарца и једну жену) лечена интратекалном терапијом баклофеном због израженог спастицитета сва четири екстремитета који су прележали инфекцију вирусом *SARS-CoV2*. Два болесника имају мултиплу склерозу, а један трауму цервикалног сегмента кичмене мождине. Сва три болесника развила су клиничку слику инфекције ковидом 19 унутар шест месеци од уградње пумпе за интратекалну терапију баклофеном. Лечени су у болничким условима са непромењеном дозом лека, без погоршања неуролошког статуса. Бартелов индекс и модификовани Ранкин скор били су исти пре и после инфекције ковидом 19. У два случаја Бартелов индекс је био 20, а у једном 69, а модификовани Ранкин скор био је 3 у једном случају и 5 у два случаја.

Закључак Болесници са тешким степеном спастицитета, код којих је неопходна интратекална терапија баклофеном, могу се безбедно лечити без обзира на пандемију ковида 19.

Кључне речи: интратекална терапија; баклофен; спастицитет; ковид 19

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

The use of adipose-derived stem cells, platelet-rich and platelet-poor plasma in the maxillary cyst treatment

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SUMMARY

Introduction The case report describes the effect of combination therapy using adipose-derived stem cells (ADSC), platelet-rich plasma (PRP) and platelet-poor plasma (PPP) in the treatment of a maxillary cyst.

Case outline A maxillary cyst between the central incisors was identified in a healthy 54-year-old male patient during a routine dental check-up. Following thorough clinical and radiographic examinations, the treatment plan was presented and explained to the patient and written informed consent was obtained. Initially, the conservative periodontal treatment was performed. Afterwards, the adipose tissue was collected from the patient's belly fat and ADSC, PRP, and PPP were obtained, following the Institution's surgical and laboratory protocols. The maxillary cyst was then surgically removed and ADSC, PRP, PPP, and resorptive collagenous membrane were placed on the surgical site. Three-year-follow up radiographs showed significantly reduced radiolucency and bone regeneration around apexes of central incisors. Clinically, there were no signs of inflammation or pain.

Conclusion The positive outcome of the case presented in this report could be considered as a promising way to treat large bone defects using ADSC, PRP, and PPP.

Keywords: stem cells; platelet-rich plasma; platelet-poor plasma; maxillary cyst

INTRODUCTION

Commonly seen cysts of the anterior region of the maxilla are radicular cysts and nasopalatine duct cysts (NPDC). Radicular cysts are inflammatory odontogenic cysts developed around the apices of the teeth with infected or necrotic pulp. Clinically, the lesion may be asymptomatic and discovered by accident on a routine dental radiograph. Their incidence is higher between the third and the sixth decade of life with male predominance [1]. NPDC or incisive canal cyst is the most common non-odontogenic developmental cysts which arises from embryogenic remnants of nasopalatine duct. Patients with this lesion may also be asymptomatic, but many will manifest with one or more symptoms [2]. According to previous studies, prevalence of NPDC is significantly higher in male patients [3]. Both mentioned cysts can grow to significant dimensions and their surgical enucleation can leave large defect in the alveolar bone.

Postnatal stem cells, such as dental pulp stem cells or adipose-derived stem cells (ADSCs), play a significant role in tissue repair and regeneration [4]. The ADSCs are multipotent stem cells, which can be easily obtained in large quantities during simple surgical procedure [5]. According to several studies, ADSCs can differentiate into many different cell types and have great potential for bone regeneration, which has been demonstrated in different in vitro experimental studies [6, 7]. The platelet-rich plasma (PRP), as a bioactive scaffold, took a significant place in regenerative dentistry. Because of its variety of growth factors, PRP influences bone remodeling and wound healing [8].

The present case report describes the effect of combination therapy using ADSCs, PRP and platelet-poor plasma (PPP) in the treatment of a maxillary cyst.

CASE REPORT

A healthy 54-year-old male came to the Simed Zobozdravstvo Clinic, in Ljubljana, Slovenia for a routine dental check-up in September 2017. He had no subjective problems, and the medical history and family history were non-contributory. Retroalveolar radiography (Figure 1a), made in September 2017, showed a cystic lesion that extends below the nasal spin and is associated with the incisive canal. A cone-beam computed tomography (CBCT) of the intercanine segment of the maxilla was done in December 2017 (Figure 2) showed a radiolucent, clearly demarcated lesion located along the incisive canal and labially from it, extending from the nasal floor to the edge of the alveolar ridge between the upper central incisors. The dimensions of cyst measured on CBCT were 11.63 mm × 13.42 mm × 14.82 mm (Figure 2). The apexes of both upper central incisors

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were projected into the lesion; however, they were both surrounded by bone. The buccal lamella above the cyst was intact, while palatal lamella appeared to be affected. Periodontal involvement of all intercanine teeth was severe, and the bone deficit along tooth 21 extended completely marginally. The orthopantomogram image previously made in 2013, brought by the patient, showed that cyst between the upper middle incisors was already visible but in a smaller dimension (approximately 10 × 6 mm).

In the beginning of 2018, the patient was addressed by another dentist to three different oral and maxillofacial surgeons for an examination and consultation due to a lesion that was discovered on a routine examination on an orthopantomogram image. In their reports it was stated that during extraoral examination no obvious abnormality was detected. The mouth opening was not blocked, the occlusion was stable and correct, and the lips were palpable without pathological resistance. A clinical examination revealed that both upper and lower dental arches were provided with fixed partial dentures. Also, generalized periodontal disease with periodontal pockets was present. In the region of the upper central incisors the cyst was not palpated, and when pressed from the vestibular side, no secretion from the gums was observed. When probing, the gums immediately bled, and the increased probing depth of 8 millimeters was present in both incisors distally. All three oral and maxillofacial surgeons believed it was necessary to surgically remove the cyst and to extract both upper middle incisors. The periodontal treatment was also advised.

The patient returned to the Simed Zobozdravstvo Clinic, Ljubljana, Slovenia where complex periodontal-surgical treatments were performed, upon written informed consent has been obtained from the patient. Based on the clinical and radiographical appearance, it was supposed that the lesion was a nasopalatine cyst, which was later confirmed by the histopathological analysis. First, in March 2018 the conservative periodontal treatment was performed. Then, in June 2018, following the institution's protocols, the patient was prepared for the liposuction by injecting local anesthetics and collagenase, prepared according to the prescription (Lekarna, Ljubljana), in the subcutaneous tissues around the patient's waist. Half an hour later, the adipose tissue from the patient was collected from the belly fat, following a standard liposuction protocol, using a 100 ml syringe. After obtaining the lipoaspirate, heparin was added to prevent coagulation and 40 minutes of centrifugation was performed using predetermined programs (Centrifuge, Domel Holding, Železniki, Slovenia). Then, three fractions were aspirated into three syringes: ADSCs approx. 1.5 ml, PRP approx. 2 ml and PPP approx. 2 ml. Before application of the fractions in the surgical field, calcium was added to all three syringes to start coagulation and block heparin. The mentioned autotransplantation protocol has been approved by the Agency for Medicinal Products and Medical Devices of the Republic of Slovenia, that granted the Donor Center status to Clinic Simed Zobozdravstvo (EU Register Simed SI100053). The nasopalatine cyst was then surgically removed under local anesthesia. Incision and nasolabial flap were made. The lesion was located along the incisive canal and extended from the nasal floor to the edge of the alveolar ridge between the upper central incisors. The apexes of both upper middle incisors were surrounded by bone, but also projected into the lesion. Careful enucleation of the cyst was performed without extraction of adjacent teeth, and on the surgical site ADSCs and PRP were applied and covered with collagenous resorptive membrane Evolution (OsteoBiol®, Torino, Italy) and PPP.

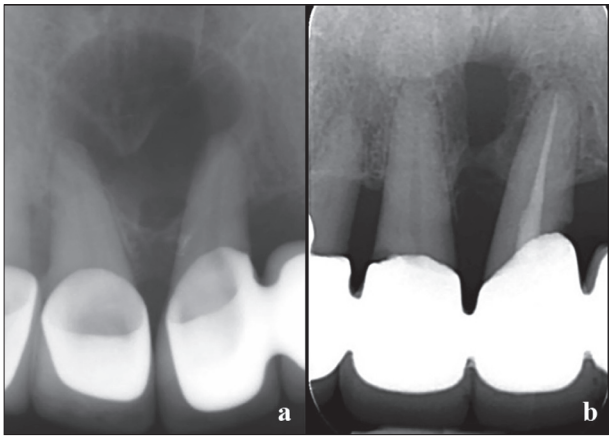

Figure 1. a – preoperative retroalveolar radiograph showing a cystic lesion between roots of the central incisors; b – the 3-year follow-up retroalveolar radiograph showing reduced radiolucency; 900 pixels wide (300 PPI)

The sutures were removed after seven days, and the surgical wound was in the healing process without complications. In September 2018 the dental check-up was conducted, tooth 21 was endodontically treated. After almost three years, in April 2021, a follow-up retroalveolar radiograph (Figure 1b) and CBCT (Figure 3) showed significantly reduced radiolucency (5.44 mm × 8.4 mm × 9.84 mm) and bone regeneration around both apexes were visible. Also, clinically there were no signs of inflammation or pain, and periodontal condition of the teeth was acceptable.

DISCUSSION

Over the past decades, the regenerative dentistry represents a progressing field of dentistry, that also involves stem cell technology. The adipose tissue is most used as a stem cells source for tissue engineering, because of high concentration of adult stem cells [9]. According to an earlier study, the ADSCs may be applied in the management of alveolar bone defects, specifically in periodontal disease, and, as stated by the authors, further verification through human clinical trials would be required [10]. Experiences from other research found this application acceptable [9].

Based on a previous study, biomaterials represent significant component in tissue engineering because of their constant stem cells supply with nutrients, and also acting like a biological barrier that protects cells from immune attacks [11]. It is considered that PRP may have a considerable role in tissue regeneration as a suitable biomaterial scaffold for ADSCs, because of the high levels of growth

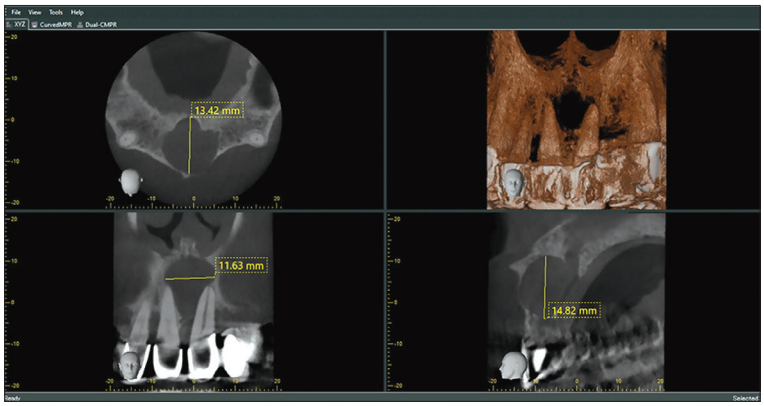

Figure 2. Cone-beam computed tomography showing a radiolucent clearly demarcated lesion located in the intercanine segment of the maxilla; 900 pixels wide (300 PPI)

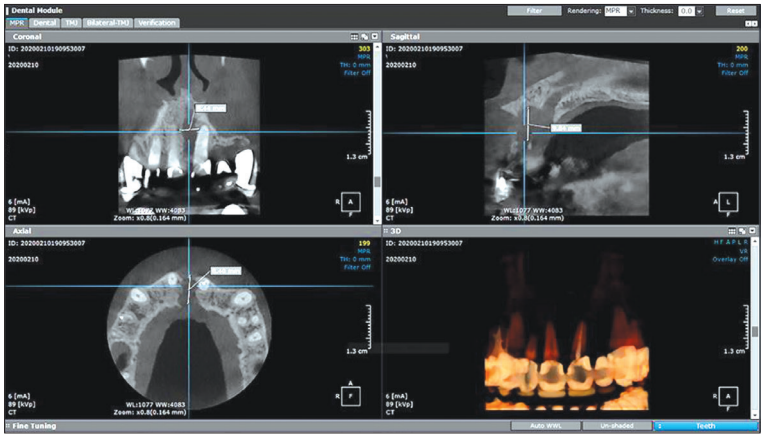

Figure 3. The 3-year follow-up cone-beam computed tomography showing reduced size of the radiolucency; 900 pixels wide (300 PPI)

factors and secretory proteins [12]. Minimal risk of infectious disease transmission, immunologic reactions and rejection are associated with PRP preparation from autologous blood [13]. According to Aly LAA et al [14], the use of PRP in bone surgery can be explained by the local release of various growth factors, from alpha granules, involved in reparative process of osteogenesis. They include platelet derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and endothelial growth factor (EGF) [14]. PDGF contributes in many specific activities such as angiogenesis and macrophage activation, collagen synthesis and enhance the proliferation of bone cells; IGF-I stimulates protein synthesis and improves bone formation because of the osteoblasts and their proliferation and differentiation; TGF-β shows the ability to induce deposition of bone matrix, to promote the production of extracellular matrix, to enhance the proliferative activity of fibroblasts, and also may inhibit osteoclast formation and bone resorption; VEGF is important for vasculogenesis and angiogenesis and EGF leads to DNA synthesis causing an increased expression of certain genes by binding to the epidermal growth factor receptors (EGFR) [15].

Platelets activation in PRP is one of the crucial elements which will induce the release of multiple growth factors from α-granules by thrombin or calcium [16]. Previous studies have often reported usage of platelets concentrates in oral and maxillofacial surgery due to the sudden release of platelets as a result of mixing PRP with calcium chloride and thrombin [17]. PRP growth factors, upon release from platelets, bind to receptors that mesenchymal stem cells express on surface of target cells in site of application and initiate a signaling pathway that can inhibit or stimulate cell differentiation and proliferation [12, 18]. PPP is another platelet-derived fraction which can have a significant role in bone regeneration. Study carried out by Hatakeyama et al. [19] implied that PPP gel is stronger than PRP gel because of the slightly higher concentration of fibrinogen and that PPP can stimulate bone formation due to the presence of a fibrin network that allows the space making for bone regeneration. Both PRP and PPP promote wound healing and their similar biological responses indicate that they may conduct positive effect on gingival repair [20].

Due to significant regulatory role of PRP and its ability to affect bone remodeling and healing, some authors believe that the use of PRP together with ADSCs could increase adhesion, migration, proliferation, and differentiation of stem cells, and thus enhance their effectiveness in bone formation and mineralization [21]. Other studies, which have used combination of ADSCs and PRP on experimental animal models, reported differentiation into alveolar bone, cement and periodontal-like structures at the site of the implementation [12]. In vitro investigation in rats, carried out by Huang and Wang [22], reported excellent cell compatibility and proliferation, as well as the influence of PRP growth factors on increased osteogenic cell differentiation. They indicated that growth factors from PRP are adequate approach for bone tissue engineering. Some studies suggested that ADSCs can express synergistic effect with PRP in bone defects therapy [14] and that human PRP demonstrate higher concentration of TGF-β1, PDGFAA, -AB, and -BB than animal PRP [23]. Although there are divided opinions regarding the use of PRP in bone regeneration, some in vivo studies from other research field have shown a positive PRP effect in stimulating the healing process of bone defects [24]. Also, advanced, second generation of platelet concentrates, platelet-rich fibrin, proved to have beneficial effect in the treatment of patients with chronic periodontitis when used in combination with conservative periodontal therapy [25].

In this case report, the PRP fraction was used as a source of growth factors, which encourages stem cells to proliferate and differentiate faster. Even though PRP contains PPP, a separate fraction of PPP was used as a surgical dressing. After the application of stem cells and PRP in the operative field after cyst enucleation, PPP served as a

natural glue for the stabilization and strengthening of the collagenous membrane. Also, after suturing the wound, the PPP fraction served as a bandage that covers the wound, allowing for faster clot formation and better protection of the wound against the infection. The positive outcome of the case presented in this report could be considered a promising way to treat large bone defects.

Conflict of Interest: None declared.

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Употреба матичних ћелија масног ткива, плазме богате тромбоцитима и плазме сиромашне тромбоцитима у лечењу максиларне цисте

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САЖЕТАК

Увод Приказ болесника описује ефекат комбиноване терапије коришћењем матичних ћелија добијених из масног ткива, плазме богате тромбоцитима и плазме сиромашне тромбоцитима у лечењу максиларне цисте.

Приказ болесника Код здравог 54-годишњег мушкарца је током рутинског стоматолошког прегледа случајно установљена максиларна циста између средишњих секутића. После детаљних клиничких и радиографских прегледа болеснику је представљен и објашњен план лечења и добијен је писмени информисани пристанак за лечење. Прво је спроведена конзервативна пародонтолошка терапија. После тога је сакупљено масно ткиво из сала на стомаку болесника и из њега су добијене матичне ћелије масног ткива, плазма богата тромбоцитима и плазма сиромашна тромбоцитима, према хируршким и лабораторијским протоколима Института. Максиларна циста је затим хируршки уклоњена и на оперативно поље су апликоване матичне ћелије из масног ткива, плазма богата тромбоцитима и плазма сиромашна тромбоцитима, као и ресорптивна колагенска мембрана. На контролном рендгенском снимку после три године уочено је значајно мање расветљење и регенерација кости око врхова корена горњих средишњих секутића. Клинички нису били присутни знаци запаљења или бола.

Закључак Позитиван исход приказаног случаја може се сматрати обећавајућим начином лечења великих дефеката виличних костију употребом матичних ћелија из масног ткива, плазме богате тромбоцитима и плазме сиромашне тромбоцитима.

Кључне речи: матичне ћелије; плазма богата тромбоцитима; плазма сиромашна тромбоцитима; максиларна циста

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

^{18}F-fluorodeoxyglucose positron emission tomography / computed tomography in primary Ewing sarcoma of the lung

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SUMMARY

Introduction Ewing sarcoma is rare in medical practice, and evaluating positron emission tomography / computed tomography (PET/CT) imaging of soft tissue Ewing sarcoma is a challenge. Primary Ewing sarcoma of the lung is an infrequent diagnosis.

Case outline A 22-year-old female patient was sent for PET/CT examination to the Center for Nuclear Medicine with Positron Emission Tomography, of the University Medical Center of Serbia, with a referral diagnosis of primary Ewing sarcoma of the right lung. In parallel to tumor visualization, the PET/CT imaging showed a radiological entity named "kissing sign," due to an enlarged beaver tail liver.

Conclusion According to the concept of functional mimicry and tissue specificity of molecular markers, a better understanding of the molecular mechanisms of soft tissue Ewing sarcoma is the challenge. These observations can be the platform for further investigation of new therapeutic regimens.

Keywords: Ewing sarcoma of the lung; ^{18}F-FDG PET/CT; kissing sign of the liver and spleen; the beaver tail liver; Tartrate-resistant acid phosphatase

INTRODUCTION

Ewing sarcoma belongs to the group of primitive neuroectodermal tumors originating from a neuroendocrine cell in bone or soft tissue [1]. As reported by Haas et al. [1], this kind of tumor often occurs in patients under 25 years of age, but has the congenital presentation of extraosseous Ewing sarcoma, although it is exceedingly rare. Multiple loci of soft tissue Ewing sarcomas develop at multiple localization: lungs [2–6], liver [7], and extremely rarely, primary Ewing sarcoma occurs in female genital organs, in the uterus [8].

According to the positron emission tomography / computed tomography (PET/CT) imaging, this study described an extremely rare visualization of tumor mass of Ewing sarcoma clinically confirmed as primarily localized in the right lung.

CASE REPORT

The present study refers to a 22-year-old female patient admitted for PET/CT examination at the Center for Nuclear Medicine with Positron Emission Tomography, University Medical Center of Serbia, diagnosed with Ewing sarcoma of the right lung three years ago. Upper right lobectomy and chemotherapy were performed. There are multiple focal partially confluent secondary tissue changes in the right hemithorax.

Before the diagnostic examination, the patient signed her informal consent for the ^{18}F-FDG PET/CT study.

After the patient's fasting six hours before the PET/CT study, and the median cubital vein cannulation, injection dose of 225 MBq ^{18}F-FDG was applied, followed by a 85-minute data acquisition. ^{18}F-FDG PET/CT examination on a 64-slice hybrid PET/CT scanner (Biograph; Siemens Medical Solutions USA, Inc., Malvern, PA, USA). A three-dimensional PET scan (three minutes per bed position acquisitions) and low-dose non-enhanced CT scan was acquired from the base of the skull to the mid-thigh. Multidetector CT was acquired with 120 kV and with automatic, real-time dose modulation amperage (CareDose4D [Siemens Healthare GmbH, Erlangen, Germany], with the baseline being 45 mA) (slice thickness of 5 mm, the pitch of 1.5, and a rotation time of 0.5 seconds). CT, PET (attenuation-corrected), and combined PET/CT images were displayed for analysis on a single Multimodality Workplace (Siemens Healthcare GmbH).

The PET/CT study shows the condition after upper right lobectomy, elevated anterior part right hemidiaphragm (Figures 1, 2B). In the middle and lower lobe of the right lung, multiple single and fused, nodular, and soft tissue changes of variable size are observed, partly continuously with the mediastinal, costal, and diaphragmatic pleura, which inhomogeneous intensively uptake ^{18}F-FDG (SUVmax 11) with zones of absent accumulation corresponding

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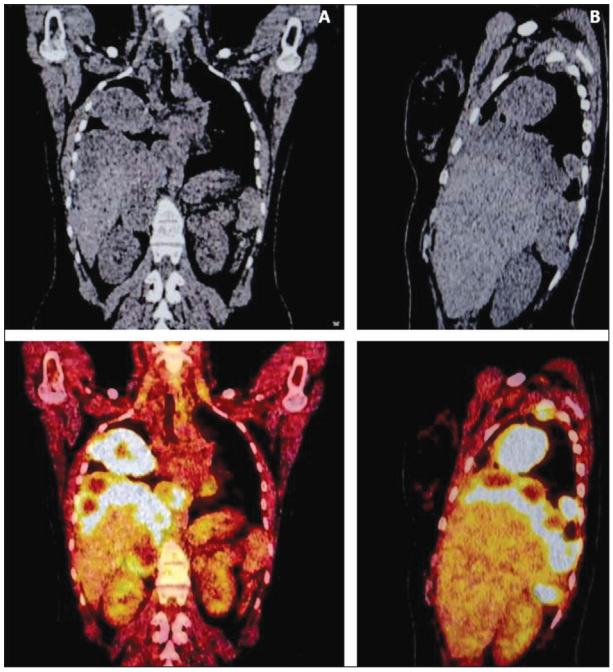

Figure 1. A – coronal; B – sagittal computed tomography, fused positron emission tomography/computed tomography images of elevated anterior part right hemidiaphragm after upper-right lobectomy

to [18]F-FDG zones of necrosis or hemorrhage (Figures 1, 2, and 3). There is an enhanced [18]F-FDG accumulation in activated brown adipose tissue in supraclavicular regions (Figure 2A).

Liver enlarged diameter of 13 × 22 × 22 cm (Figures 1B, and 3B) contacting the left lobe of the liver and spleen, a radiological finding known as the "kissing sign" of the liver and spleen has appeared (Figure 3), which is due to morphological variation of the beaver tail liver.

DISCUSSION

Dharmalingam et al. [3], state that "Primary Ewing sarcoma of the lung is anecdotally rare, with few cases reported in the literature" until 2020. The "Ewing family of tumors" includes other tissue types, such as soft tissue origin classified as extraosseous Ewing sarcoma or primitive neuroendocrine origin [2]. Because of the rarity of extraosseous Ewing sarcomas, the therapeutic approach is the same as for osseous ones [7].

The patient in this study showed no signs of reducing tumor mass despite chemotherapy. The present study indicates the radiological finding of the "kissing sign," which may exist because of the morphological variety of the left liver lobe. The enlarged liver in this patient showed the morphological type of beaver tail liver. Radiological finding known as the "kissing sign" of the liver and spleen in this specific case is a consequence of contact of the left lobe of the beaver tail liver and spleen.

Ewing sarcomas, both bone and soft-tissue varieties, are aggressive neoplasms. In one-quarter of patients, there are clinically evident metastases at presentation. The primary therapeutic approach for Ewing sarcoma is systemic

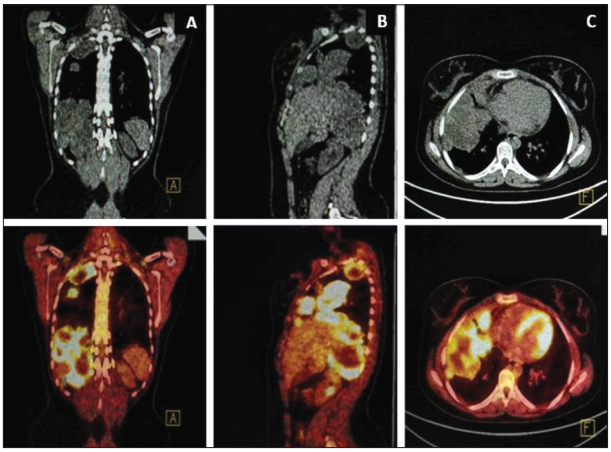

Figure 2. A – coronal; B – sagittal; C – axial computed tomography, fused positron emission tomography/computed tomography images (mediastinal window) of multiple single and fused nodular and soft tissue changes of variable size in the middle and lower lobe of the right lung, partly continuously with the mediastinal, costal and diaphragmatic pleura, which bind [18]F-fluorodeoxyglucose inhomogeneous intensely, with zones of absent [18]F-fluorodeoxyglucose accumulation corresponding to zones of necrosis or hemorrhage

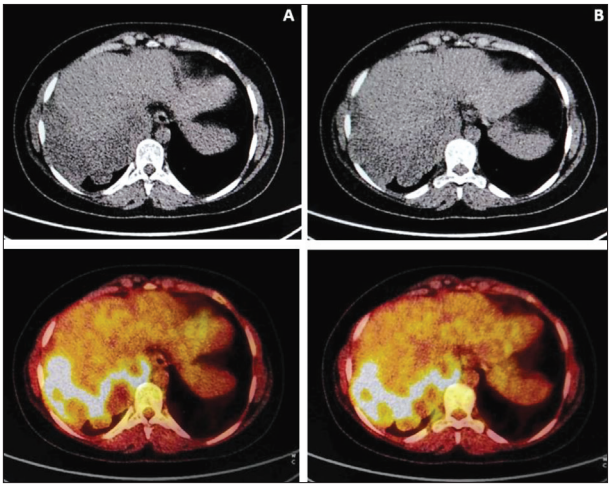

Figure 3. A, B – axial computed tomography, fused positron emission tomography/computed tomography images "kissing sign" liver and spleen

treatment [9]. Relapse is systemic mainly, followed by combined and local relapse. The five-year post-relapse survival rate is 15–25% [9].

Understanding the molecular biology of these tumors and novel treatment approaches are the challenges.

The facts of Ewing sarcomas as a systemic illness evoke the thesis on functional mimicry and tissue specificity of the acid phosphatase family of enzymes. Acid phosphatases are a family of enzymes with different structural, catalytic, and immunological properties, tissue distribution, and subcellular location [10]. There are five isoenzymes of acid phosphatase. The tartaric acid inhibits isoenzymes 1–4, but isoenzyme 5 is resistant to the inhibition [11]. The functional relationship between the lysosomal acid phosphatase (tartrate sensitive) and tartrate-resistant acid phosphatase is unknown.

Isoenzyme 5 is called the tartrate-resistant acid phosphatases (TRAP). Human TRAP is a member of a

widely-distributed and structurally highly-conserved group of iron-containing proteins [12]. The alveolar macrophages and osteoclasts exert TRAP enzyme activity [13]. TRAPs are expressed in bone-resorbing osteoclasts (5b form), alveolar macrophages (5a form), or can be markers of inflammation [14]. The functional importance of TRAP is the involvement in bone resorption and iron homeostasis (transport, metabolism) [15]. The TRAP exerts a homology to uteroferrin, a purple acid phosphatase family [16].

According to GeneCards®: The Human Gene Database (https://www.genecards.org), tartrate-resistant acid phosphatase has marked expression in bone, stomach, colon, liver, kidney, lung, pancreas, and prostate. The same organs/tissues, including bone and lung, are referred to in the scientific literature as loci for primary Ewing sarcoma of soft tissue [7, 17–21]. As TRAP has a promotive effect on cancer cell elongation, proliferation, migration, and invasion [22], we can propose that TRAP inhibitors may be possible therapeutic agents. According to Boorsma et al. [23], the Au(III) compound AubipyOMe is the most potent inhibitor of TRAP activity. The formula of Au(III) compound AubipyOMe can be a step forward for Fullerene-based delivery systems in the body as a new therapeutic approach for (extra)osseous Ewing sarcomas [24].

Although Ewing sarcoma of soft tissues, including lungs, is rare, the unfavorable outcome is a challenge in understanding the molecular mechanisms of the pathogenesis of soft tissue Ewing sarcoma. There is an urgent need to test high technology and nanomaterials as potential therapeutic tools in this type of cancer.

The present study showed a rare clinical condition related to primary Ewing sarcoma of the lung using ^{18}F-FDG PET-CT. This method is convenient in staging, restaging, and assessing therapy response in patients with Ewing sarcoma. As a diagnostic (pretreatment) tool, ^{18}F-FDG PET-CT of patients with Ewing sarcoma improves the detection of metastases compared to conventional imaging [25]. According to Hack et al. [26], PET/CT metrics in soft tissue and bone-originated sarcomas could not be the same. The maximum standardized uptake value is a better prognostic factor for soft tissue Ewing sarcomas, but tumor volume, rather than FDG PET activity, is more informative in evaluating bone Ewing sarcoma [26].

Conflict of interest: None to declare.

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[18]Ф-флуородеоксиглукозна позитронска емисиона томографија / компјутеризована томографија у примарном Јуинговом саркому плућа

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САЖЕТАК

Увод Јуингов сарком није чест у клиничкој пракси. Углавном захвата скелетне структуре, док је изузетно ретка појава Јуинговог саркома са примарном локализацијом у меким ткивима. Појава примарног Јуинговог саркома плућа је реткост и представља дијагностички и терапијски изазов.

Приказ болесника Болесница (22 године старости) са дијагнозом примарног Јуинговог саркома десног плућног крила упућена је на испитивање позитронском емисионом томографијом / компјутеризованом томографијом у Центар за нуклеарну медицину са позитронском емисионом томографијом Универзитетског клиничког центра Србије. Извршена је лобектомија горњег десног плућног лобуса две године раније и примењена је хемиотерапија. [18]Ф-флуородеоксиглукозна позитронска емисиона томографија је приказала мултипле фокалне, делимично конфлуентне секундарне ткивне депозите у десном хемитораксу. Уочен је радиолошки феномен „знака пољупца" слезине и увећане јетре са анатомском варијацијом левог лобуса јетре која се описује као феномен дабровог репа.

Закључак Пратећи до сада објављене публикације које се односе на ванскелетну примарну локализацију Јуинговог саркома плућа, приметно је да се овај тумор јавља углавном у ванкоштаним ткивима у којима је изражена експресија киселе фосфатазе резистентне на тартарат. Сходно концепту функционалне мимикрије и ткивне специфичности, ова сазнања чине платформу за истраживање нових дијагностичких и терапијских приступа код Јуинговог саркома нескелетног порекла.

Кључне речи: Јуингов сарком плућа; [18]Ф-флуородеоксиглукозна позитронска емисиона томографија / компјутеризована томографија; знак пољупца слезине и јетре; знак дабровог репа; кисела фосфатаза резистентна на тартарат

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Bilateral atypical femoral fracture related to bisphosphonate therapy

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SUMMARY

Introduction Although bisphosphonates represent the most commonly prescribed antiresorptive therapy for the treatment of osteoporosis and fracture prevention, paradoxically, their continuous use in some patients can lead to an atypical femoral fracture. This type of fracture is characterized by specific features regarding clinical presentation, mechanism of injury and radiological manifestations. The objective of this article was to present a case of a bilateral femoral fracture associated with bisphosphonate usage.

Case outline A 70-year-old female patient was admitted to the emergency department with a severe, throbbing pain in both thighs after a ground-level fall. Radiographs of both femurs verified bilateral complete fracture localized in the diaphyseal region. Prior to hospitalization, she had continuously been taking ibandronate for three years. The patient underwent percutaneous osteosynthesis with intramedullary nailing with interlocking screws of both femurs. Six weeks after the surgical treatment, radiographic findings indicated the presence of early signs of healing on both femurs. At the four-month follow-up, the complete union of the bone fragments was achieved and she managed to walk without any pain and walking aids.

Conclusion Physicians still face doubts regarding optimal duration of bisphosphonate therapy. Although atypical femoral fracture represents a rare adverse event related to bisphosphonate use, all patients receiving this antiresorptive treatment who are complaining of new-onset unexplained pain in the thighs for more than two weeks should be completely diagnostically evaluated in term of atypical femoral fracture. Patients with confirmed atypical femoral fracture should undergo surgical treatment.

Keywords: osteoporosis; antiresorptive treatment; intramedullary nailing

INTRODUCTION

Considering the steady trend of prolongation of the average life expectancy, a decrease in bone density, osteoporosis, continues to be a health concern for the growing elderly population [1]. Although the available therapeutic modalities have shown to be effective in treating this skeletal disorder, the rare adverse event associated with antiresorptive therapy is an atypical femoral fracture (AFF) [2]. AFF represents a stress fracture following minimal or no trauma with a fracture line being localized distal to the lesser trochanter and proximal to the supracondylar flare of the thigh bone [3]. Despite the fact that bisphosphonates are usually the treatment of choice for patients suffering from osteoporosis, AFF develops more commonly in patients on bisphosphonate therapy. Additionally, prolonged treatment duration with this type of medications increases risk of fracture [4]. It has been hypothesized that their use could potentially lead to the severe suppression of bone turnover, which eventually causes accumulation of microdamage and consequent change in bone structure [5].

In this article, we report a case of a bilateral femoral fracture affiliated with bisphosphonate usage.

CASE REPORT

A 70-year-old female patient was presented to the emergency department with severe, throbbing pain in both thighs after a ground-level fall. The patient gave a history of preexisting dull aching pain over both thighs that lasted four months before admission to the hospital. She was diagnosed with osteoporosis three years prior to hospitalization. The diagnosis was established after a mechanical provocation which led to a vertebral fracture at the level of L2–L3. She was continuously taking ibandronate tablets in the monthly dose of 150 mg for three years. Apart from bisphosphonate therapy she was on a thyroid hormone replacement therapy due to a total thyroidectomy. Her medical history was positive for iatrogenic hypothyroidism. The patient was a smoker and her body mass index (BMI) was 22 kg/m^2.

Physical examination revealed presence of a bilateral deformity as well as tenderness to palpation over both thigh regions. Active and passive motion of hip and knee joint were limited due to the pain. On admission, lower extremities were neurovascularly intact.

Radiographic examination verified the presence of a bilateral complete fracture localized at the level of the diaphysis. Regarding the

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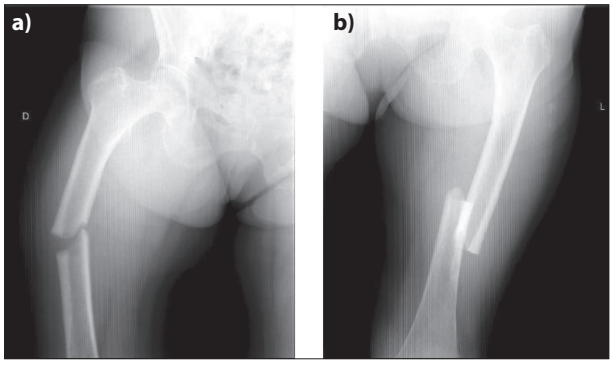

Figure 1. Radiographs showing complete fracture at the diaphysis on the right (a) and left (b) femur

Table 1. Laboratory and DXA assessment

Parameters	Value
Intact parathyroid hormone (pg/mL)	66.5
25(OH)D total (nmol/L)	84
tP1NP (ng/mL)	262.1
CrossLaps (pg/mL)	561
DXA scan	
T score L1-L4	-3.3
BMD (g/cm^2)	0.787

tP1NP – total procollagen 1 amino-terminal propeptide;
DXA – bone density test

fracture configuration, fracture lines were initially transverse with an oblique segment at the medial aspect of both femurs, without comminution (Figure 1.). The results obtained by analysis of the complete blood count as well as the biochemical panel were within the reference range.

When the diagnosis of an AFF was confirmed, antiresorptive treatment was stopped and patient underwent percutaneous osteosynthesis with intramedullary nailing with interlocking screws of both femurs.

Intraoperative and postoperative course was uneventful. After discontinuation of bisphosphonate, laboratory and Bone Density Test (DXA) assessment were carried out and the results are presented in the Table 1. She was commenced on vitamin D and calcium supplement therapy. The patient was discharged home on the seventh postoperative day in a good general health condition.

Six weeks after surgery, radiographic findings indicated the presence of early signs of healing on both femurs (Figure 2 a) and b)) and the patient was allowed to walk with a walker bearing her weight. At four months follow-up, the complete union of the bone segments was achieved and she managed to walk without any pain and walking aids (Figure 2 c) and d)). On further medical checkup, six months postoperatively, she did not report any ailment and the mobility of the hip joints reached a satisfactory level of recovery. Differences in quality of life before and after the surgery were measured by Short Form Health Survey (SF-36) and scores are shown in Table 2.

One year after the operation, due to the high risk of fracture as well as presence of osteoporosis on lumbar vertebrae revealed in DXA scan, alendronate was introduced to therapy.

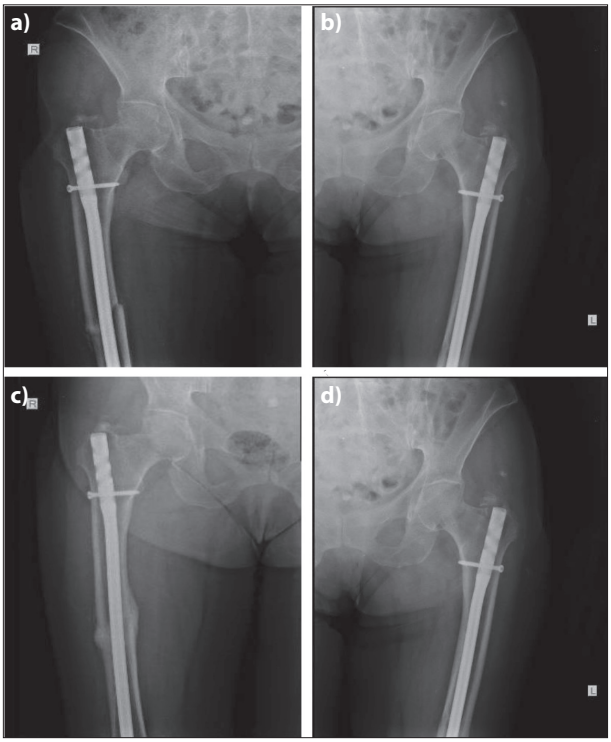

Figure 2. Radiographs showing the presence of early signs of healing six weeks after surgery [(a) and (b)] and complete union on both femurs four months postoperatively [(c) and (d)]

Table 2. Short Form Health Survey (SF-36) scores

Short Form Health Survey (SF-36)	Before	After
Physical functioning (%)	15	65
Role limitations due to physical health (%)	0	50
Role limitations due to emotional problems (%)	33.3	100
Energy/fatigue (%)	40	70
Emotional well-being (%)	56	80
Social functioning (%)	50	75
Pain (%)	22.5	77.5
General health (%)	35	55
Health change (%)	0	75

DISCUSSION

In clinical practice, the most commonly used group of medicines for the treatment of osteoporosis and fracture prevention are bisphosphonates. Although generally well tolerated and effective, paradoxically, bisphosphonate therapy in some patients can cause AFF. They interfere with bone remodeling processes leading to changes in the mechanical and regenerative properties of bone [6]. Management of osteoporosis with bisphosphonates is followed by high relative risk of developing AFF, ranging from 2.1 to 128, while the absolute risk expressed numerically ranges from 3.2 to 50 cases per 100,000 person per year. Prolongation of therapy correlates with a significantly increased risk which was observed to be 100 cases per 100 000 person per year [3]. The mechanisms of AFF's development have still not been completely understood. It is assumed that bisphosphonates are responsible for the irregular and increased collagen cross-linking, which leads

to the formation of focal zones of dense and brittle bone, mostly in the subtrochanteric region, which is sensitive to the high tensile and compressive forces. Low bone turnover in these zones causes microdamage accumulation which subsequently progresses to fracture [7].

There are still no clear guidelines regarding the optimal duration of a bisphosphonate therapy. Prolonged retention at the bone surface can be explained by their high affinity for hydroxyapatite [8]. Although current guidelines suggest that after five years of treatment with bisphosphonates patient should undergo reevaluation and therapy should be stopped, study conducted by Lo et al. [9] demonstrated that patients receiving this therapy are at higher risk for AFF even after three years of continual antiresorptive treatment.

In 2010, the Task Force of the American Society for Bone and Mineral Research (ASBMR) defined the major and minor criteria following diagnosis of AFF, which were afterwards revised and published in 2014. In the case of AFF, localization is essential for diagnosis and the fracture line is placed in the diaphysis region somewhere distal to the lesser trochanter to just proximal to the supracondylar flare. To diagnose AFF presence of four major features out of five is required, that may or may not be accompanied with minor features. Major features are: association with no or minimal trauma, no or minimal comminution, involvement of the lateral cortex in both, complete and incomplete fracture, though complete fracture might be associated with a medial spike, transverse fracture line with the potential oblique orientation medially, localized periosteal or endosteal thickening at the lateral cortex ("beaking" or "flaring"). Minor features include generalized cortical thickening of the femoral shaft, bilaterality, prodromal symptoms (pain in the groin or thigh) and prolongation of healing time [3]. The fractures in this report were consistent with all of the major and with a few of the minor features. Considering previously mentioned minor features, our patient complained of pain in both thighs prior to the fall and she experienced bilateral femoral fracture. Therefore, diagnosis of a bisphosphonate induced AFF was made.

Koh et al. [10] demonstrated that duration of bisphosphonate therapy for more than five years without pause period, prolonged use of glucocorticoids and a higher BMI increase the risk of AFF. Findings of another research article were consistent with previously mentioned predisposing factors, although authors reported that Asian race also has an additional impact on the increase of the fracture incidence [11]. Rheumatoid arthritis, increased anterior and lateral femoral curvatures and thicker lateral femoral cortex of diaphysis were recognized as a possible contributor for development of an AFF as well [12]. None of the listed risk factors were present in our patient.

Several factors determine the therapeutic approach: type of a fracture (complete or incomplete), presence of symptoms and the general condition of the patient. A conservative treatment may be the option for asymptomatic patients with incomplete AFF. Indication for intramedullary nail fixation is complete fracture or incomplete fracture associated with pain [13]. Other criteria that should be considered in patients with incomplete fracture are the extent and depth of the fracture line, presence of symptoms, and patient's choice [2]. Endochondral ossification is a process responsible for the union of bone fragments in patients with complete fractures. The bone remodeling, as a stage of endochondral ossification, is essential for union of the stress fracture segments. Bisphosphonates inhibit remodeling through suppression of the osteoclast mediated bone resorption. Intramedullary reconstruction full-length nails provide regenerative processes to take place inside fracture crack [13].

According to a study conducted by Kim et al. [14], after the operation, patients achieved radiological union between three to 10 months with an average of 5.5 months. Kang et al. [15] demonstrated that observed healing time differed according to the duration of therapy. Fracture union was achieved in 4.8 ± 2.5 months in patients who taken bisphosphonates for less than three years, but the time required for healing was 9.3 ± 3.7 months in those who taken antiresorptive therapy for more than three years. In our patient the time required for healing was four months.

An observational follow-up study was conducted in order to investigate the incidence of new fragility fractures following an AFF. Results demonstrated that in a group of patients who sustained an AFF incidence of fractures was high. The majority of reported fractures were located on vertebrae, hip, humerus or forearm. In patients with high risk of fragility fractures bisphosphonates or denosumab might be continued after the completion of healing process in surgically managed bilateral AFFs [16].

In conclusion, physicians still face doubts regarding optimal duration of bisphosphonate therapy. Although AFF represents a rare adverse event related to bisphosphonate use, all patients receiving this antiresorptive treatment who are complaining of new-onset unexplained pain in the thighs for more than two weeks should be completely diagnostically evaluated in term of AFF. Patients with confirmed AFF should undergo surgical treatment.

Conflict of interest: None declared.

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Обострани атипични прелом фемура повезан са бисфосфонатном терапијом

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САЖЕТАК

Увод Иако бисфосфонати представљају најчешће прописивану антирезорптивну терапију за лечење остеопорозе и превенцију прелома, парадоксално, њихова континуирана примена код неких болесника може да доведе до атипичног прелома фемура. Ову врсту прелома одликују специфичне карактеристике у погледу клиничке презентације, механизма повређивања и радиолошких манифестација.

Циљ овог рада је био да се прикаже случај обостраног прелома фемура који је удружен са применом бисфосфоната.

Приказ болесника Седамдесетогодишња болесница примљена је на одељење ургентне медицине због снажног, пулсирајућег бола у обе бутине који је настао после пада на истом нивоу. Радиограмом оба фемура верификован је обострани прелом фемура локализован у регији дијафизе. Она је три године пре хоспитализације у континуитету узимала ибандронат. Болесница је подвргнута перкутаној остеосинтези са интрамедуларним клином и закључавајућим шрафовима. Шест недеља после оперативног захвата радиографски налаз је указао на присуство раних знакова зарастања на оба фемура. На контролном прегледу после четири месеца коштани ломци су комплетно срасли и болесница је могла да хода без појаве бола и помагала.

Закључак Лекари и даље имају недоумице везане за оптимално трајање терапије бисфосфонатима. Иако атипични прелом бутне кости представља редак нежељени догађај који је удружен са применом бисфосфоната, сви болесници на овој антирезорптивној терапији и који се жале на новонастали бол у бутинама неразјашњене етиологије, а који траје дуже од две недеље, треба да буду потпуно дијагностички обрађени у правцу атипичног прелома фемура. Болесници са потврђеним атипичним преломом бутне кости треба да се подвргну оперативном лечењу.

Кључне речи: остеопороза; антирезорптивна терапија; интрамедуларна фиксација

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Chronic lymphocytic leukemia diagnosed during pregnancy – case report and review of literature

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SUMMARY

Introduction B-cell chronic lymphocytic leukemia (CLL) can be easily overlooked in pregnancy, particularly in cases with inadequate antenatal care. We report a case of pregnant woman diagnosed with CLL and evaluate this patient with cases in literature.

Case report An asymptomatic 35-year-old woman presented with slightly elevated absolute lymphocyte count at antenatal monitoring in her second pregnancy. Further hematological investigations disclosed CLL with monoallelic deletion of chromosome 13q14. She was monitored during throughout the pregnancy, being asymptomatic and without treatment, and delivered a healthy child at term with no complications. After almost four years of follow up patient is without any signs of disease progression and her absolute lymphocyte counts remained on predelivery levels.

This is the first published case of CLL diagnosed during pregnancy in Serbia. Rare similar cases published so far have been discussed, especially in terms of disease course, long-term prognosis and available therapeutic modalities.

Conclusion Due to the fact that nowadays many women are delaying childbearing in middle age it can be expected that cancer diagnose could be more often found in pregnant women in the future. In a view of the complex nature of such condition, a multidisciplinary approach for diagnosing and treating of pregnant women is highly recommended.

Keywords: chronic lymphocytic leukemia; pregnancy; treatment; outcome

INTRODUCTION

Approximately 1 in 1000 pregnancies is complicated by any cancer diagnosis, whereby hematological malignancies comprising 11.5–18% of these cases [1, 2, 3]. Among hematological malignancies the most common are Hodgkin's disease, non-Hodgkin's lymphoma and acute myeloid leukemia, accounting for 6%, 4.7%, and 3.2% cases, respectively [1]. The most common reason of induced abortion among pregnancy induced malignancies is hematologic disease (21%) [3]. Although it is the most common adult leukemia, B-cell chronic lymphocytic leukemia (CLL) has been reported in pregnancy in less than 10 cases [4–13]. The median age of CLL is 70 years and approximately 2% of patients are aged < 45 years [14, 15]. Considering the male:female ratio of 2:1, extremely small proportion of patients with CLL are expected to be females of potentially child-bearing age [14, 15]. In such circumstances, CLL can be easily overlooked in pregnancy, particularly in cases with inadequate antenatal care (rural areas, health habits, low social milieu, etc.).

We describe the case of CLL occurring in pregnancy in a Serbian patient, diagnosed three months before delivery. The review of literature data was the basis of discussing some important issues related to diagnosis and management of CLL in women of childbearing age and in pregnancy.

CASE REPORT

We retrospectively analyzed 2020 (1315 males and 705 females, M/F ratio 1.86) consecutive untreated patients with *de novo* CLL referred for diagnosis in the Laboratory for Immunophenotyping and Flow Cytometry, Diagnostic Department, Clinic of Hematology, University Clinical Center of Serbia, in the time period from January 2003 until June 2020. Diagnosis was established according to the standard criteria [16, 17]. Among these 2020 patients with CLL, 320 patients were younger than 55 years (15.8%), including 9.8% (198/2020) male and 6% (122/2020) female, M/F ratio 1.62. Only one of these "younger" CLL patients was a pregnant female and her clinical characteristics, labor outcome and course of the malignant disease are described in the text bellow.

A 35-year-old Caucasian woman in her second pregnancy (first delivery three years prior, delivered via Caesarean section), was first seen by a gynecologist in April 2017 on her first prenatal visit at eight-weeks' gestation. According to Article 16 of the Law on Patients' Rights, written consent was obtained for performing

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invasive diagnostic and therapeutic procedures, as the Approval of the Committee on ethics for the case publishing. Complete blood count (CBC) showed elevated white blood cell count (WBC) of 14.8 × 10^9/l with lymphocyte predomination (65% WBC). Other routine antepartum laboratory results were otherwise within normal range together with hemostasis parameters. She was without symptoms and denied experiencing any weakness, fever, fatigue, or swelling. Since absolute lymphocytosis persisted during next few gynecological checkups, she was referred to a hematologist in August 2017. Physical examination did not reveal any lymphadenopathy or hepatosplenomegaly. Abdominal ultrasonography was normal. Obstetric ultrasonography showed normal fetal anatomy and normal fetal circulation. CBC showed elevated WBC count of 36.4 × 10^9/l accounting for 71% of lymphocytes in differential. Diagnostic immunophenotyping by multicolor flow cytometry of native peripheral blood specimen (September 2017), showed accumulation of atypical monoclonal mature B-cells ($CD19^+CD5^+/SSC^{low}$ cells → 57% WBC → 20.8 × 10^9/l) with specific immunophenotype: $CD19^+$, $CD20^{+low}$, $CD21^{+low}$, $CD22^{+low}$, $CD23^{+intermediate}$, $CD5^{+intermediate}$, $CD79b^{+low}$, $FMC7^{+low}$, $CD43^{+low}$, $CD27^{+low}$, $CD200^{+high}$, $mIgkappa^{+low}$; CLL Score = 4 points). According to these findings, a diagnosis of CLL/variant $FMC7^+$ was made. Expression of prognostic markers, CD38 and CD49d, was not detected.

The patient was clinically doing well, and mutual decision of gynecologist, perinatologist, and hematologist was to carefully monitor pregnancy. In November 2017, the patient was delivered by elective Caesarean section at 39 weeks' gestation. She gave birth to a healthy female infant, weighing 2400 grams with Apgar score of 10. Histopathologic examinations of the chorion and umbilical cord were normal. She had an uneventful postpartum course and regular lactation (lasting for six months). CBC were checked up on six-week periods during pregnancy and lactation and after that, two times a year for almost four-year follow-up period (Figure 1). Whole body computed tomography scan was done in March 2018, and did not reveal significant lymphadenopathy or organomegaly. G-banding and fluorescent in situ hybridization for chromosome 12, 13q14.3 deletion, 17p13 deletion and 11q22 deletion was performed and showed a 13q14.3 deletion in 80% of cells. The patient remained without symptoms and in good clinical condition. On her last follow-up (January, 2021) CBC showed elevated WBC of 33 × 10^9/l with 80% of lymphocytes in differential count.

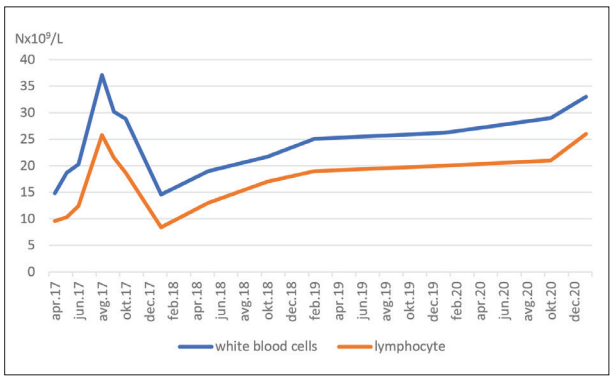

Figure 1. Changes in leukocyte count and absolute lymphocyte count during four-year follow-up of the patient

DISCUSSION

The WBC count begins to increase in early pregnancy, mainly due to an absolute increase in neutrophil numbers, but absolute lymphocyte count (ALC) (normal range 1–4 × 10^9/l) and ratio of B and T cells is essentially unchanged [18]. This increase in the WBC count peaks during the second and third trimester, with return to normal female values within the first week post-delivery. Therefore, any increase in lymphocyte count, especially if it is persistent and/or progressive, requires a detailed differential diagnostic approach.

In a large prospective study of 1744 women of reproductive age (i.e., 15 years and older) with non-Hodgkin's lymphoma, including 198 women with CLL, Adami et al. [19] concluded that the hormonal and immunological changes associated with pregnancy had little or no effect on the development of CLL. Indeed, significant changes in lymphocyte count were not noticed during careful monitoring of our patient during pregnancy, lactation and regular follow-up during four-year period. Histopathologic examination of the chorion and umbilical cord of our patient were normal. There were no evidence regarding transplacental transmission of CLL to the fetus, although two cases of placental invasion have been reported in literature [4, 5].

According to the actual guidelines [16, 17], a watch-and-wait approach is advised in early stages of CLL due to the indolent nature of the disease. Since there are no existing guidelines for CLL in pregnancy, closer monitoring throughout pregnancy is obligatory to look for potential complications, particularly increased susceptibility to infection or occurrence of autoimmune cytopenias. Indications for specific treatment include a very high ALC, followed by symptoms or risk of leukostasis or antenatal complications. Leukostasis is rarely seen in CLL, and patients can be asymptomatic with an ALC as high as 500 × 10^9/l [20]. A high ALC should not necessarily be a trigger for treatment, unless there is placental insufficiency, intrauterine growth restriction or other antenatal complications that may be CLL-related. If there is a significantly ALC raise, cytoreduction could be achieved with leukapheresis. There is only one case in the literature where this has been performed successfully for CLL during pregnancy [7].

In the matter of chemotherapy in pregnancy, the main parameters that influence the choice of treatment are gestational term, type and stage of cancer, the possibility of transplacental transfer and risk of teratogenicity of the drug, but also the patient's opinion on the continuation of the pregnancy if the disease is diagnosed at an early term [21]. The fetus is most vulnerable to drug-related teratogenicity during the first trimester, and consequently chemotherapeutic agents, especially genotoxic drugs in combination, should be avoided. Chemotherapy could

be considered in the second and third trimester if there is a rapidly progressive disease or local compressive disease [22], and fludarabine-cyclophosphamide-rituximab would be the combination of choice. Single-agent monoclonal antibodies (e.g., rituximab), in the setting of CLL in pregnancy, are potentially attractive first-line treatment because of their low teratogenic risk. García et al. [13] reported the only case of rituximab treatment in pregnancy with successful outcome. The safety of novel agents [23] in the treatment of CLL (B-cell receptor pathway inhibitors) in pregnancy is not yet determined and while their efficacy and low side effect profile may be promising, they should therefore be avoided until more data is available. It may be preferable to deliver the infant prior to cytotoxic treatment, particularly during the third trimester. If delivery is planned during the courses of chemotherapy, delivery should be timed 3–4 weeks after treatment to avoid myelosuppression and infectious risk in both the mother and newborn. Also, very important question in this case is the assessment of risk factors for development of venous thromboembolism in pregnancy and the puerperium. Royal College for Obstetricians and Gynecologists classifies pregnant women with cancer as intermediate risk patients and recommends considering of antenatal prophylaxis with low molecular weight heparin [24]. This approach and decision for antenatal prophylaxis requires postnatal prophylaxis for at least six weeks. In our opinion, the decision of ante- and postnatal prophylaxis in group of pregnant women with CLL and other hematological malignancy have to be analyzed individually.

Since the concurrence of cancer and pregnancy is a relatively rare medical problem, single institutional or regional initiatives are not able to provide sufficient information on the safety of cancer treatment during the pregnancy for both mother and fetus [2]. Currently, many women are delaying childbearing in the period 30–49 years, the age group with higher cancer incidence. Due to that it can be expected that cancer will be diagnosed more often in pregnant women in the future [25]. We highly recommend a multidisciplinary approach for diagnosing and treating pregnant women due to the complex nature of such a condition.

Conflicts of interest: None declared.

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Хронична лимфоцитна леукемија дијагностикована током трудноће – приказ болесника и преглед литературе

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САЖЕТАК

Увод Б-ћелијска хронична лимфоцитна леукемија може се лако превидети у трудноћи, посебно у случајевима неадекватне антенаталне неге. Приказујемо случај болеснице којој је током трудноће постављена дијагноза хроничне лимфоцитне леукемије и упоређујемо га са досад објављеним случајевима у светској литератури.

Приказ болесника Почетком трећег месеца антенаталног праћења код 35-годишње труднице у другој трудноћи уочен је благо повишен апсолутни број лимфоцита. Хематолошким испитивањем постављена је дијагноза хроничне лимфоцитне леукемије са моноалелном делецијом хромозома 13*q*14. Болесница је током трудноће била без симптома и родила је, без икаквих компликација, здраво дете у термину. После скоро четири године праћења болесница је била без икаквих знакова напредовања болести, а њен апсолутни број лимфоцита остао је на нивоу пре порођаја.

Ово је први приказани случај Б-ћелијске хроничне лимфоцитне леукемије дијагностиковане током трудноће у Србији. Дискутовано је о ретким, досад објављеним сличним случајевима, посебно у погледу тока болести, дугорочне прогнозе и доступних терапијских модалитета.

Закључак У данашње време велики број жена одлаже порођај за средње животно доба, па се чешће може очекивати и дијагноза малигне болести током трудноће. Због комплексности природе оваквог стања препоручује се мултидисциплинарни приступ у постављању дијагнозе и лечењу таквих трудница.

Кључне речи: хронична лимфоцитна леукемија; трудноћа; лечење; исход

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REVIEW ARTICLE / ПРЕГЛЕД ЛИТЕРАТУРЕ

The role of the blood–brain barrier in psychiatric disorders

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SUMMARY
The blood–brain barrier (BBB) is formed by continuous, closely connected endothelial cells, enveloped in the basal lamina, pericytes, and foot extensions of astrocytes. BBB has a vital role in brain metabolism and protects the brain parenchyma from harmful agents present in the systemic circulation. Damage to the BBB and an increase in its permeability have an important role in many neurodegenerative diseases. This paper aims to review the literature on the impact of the BBB damage on psychiatric illness, a largely neglected and under-researched area. Links between BBB impairment and specific neuropsychiatric disorders are described including schizophrenia, affective disorders, dementias with behavioral disorders, and alcohol use disorder, with comparison to typical hereditary small vessel diseases affecting the BBB such as cerebral autosomal dominant arteriopathy with subcortical infarction and leukoencephalopathy and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes. The authors critically summarize possible pathogenic mechanisms linking BBB damage and these common disorders.
Keywords: blood–brain barrier; schizophrenia; affective disorders; alcohol use disorder; dementia; hereditary small vessel disease

INTRODUCTION

Current research has shown that various neurological diseases occur due to the structural and/or functional disorder of the blood–brain barrier (BBB) [1]. Most frequently studied entities include multiple sclerosis, infectious or inflammatory brain diseases, certain vascular as well as degenerative disease such as Alzheimer's (AD), Parkinson's disease, and amyotrophic lateral sclerosis [2]. The pathogenesis of these conditions includes damage to the occludent endothelial junction and subsequently increased BBB permeability, expression of a procoagulant endothelial phenotype, as well as the production of free radicals and other mediators of inflammation [3]. Changes in the structure of the BBB can be transient and mild corresponding to the opening of the tight junctions, but also chronic with permanent damage to the BBB leading to alterations in protein and enzyme transport. The consequent activation of microglia and infiltration of the brain parenchyma with plasma proteins and immune cells induce the disruption of the central nervous system (CNS) homeostasis and damage to the surrounding brain tissue [1].

Interestingly, data in the literature on the impact of the BBB damage on psychiatric illnesses are mostly lacking. One intriguing contemporary hypothesis states that the BBB dysfunction and subsequent increase in its permeability may be the basis for the development of certain psychiatric disorders [4]. Previous research on experimental models indicated that the development of psychiatric conditions as well as the co-existence or overlap between certain neurological and psychiatric diseases may be explained by BBB disruption, which may widen the therapeutic approach [4]. Therefore, our paper aims to review possible pathogenetic mechanisms linking BBB disruption to the main psychiatric conditions.

THE STRUCTURE OF THE BBB

The BBB is a very complex cellular system consisting of endothelial cells, pericytes, perivascular microglia, astrocytes, and continuous basal laminae made of pericytes and foot extensions of astrocytes [2]. These components configure a very restrictive wall named BBB as its main role is to maintain a safe extracellular environment for CNS neurons [2]. The impermeability of the BBB is mostly achieved through the specific structure of the endothelium [3]. The BBB protects the brain from toxins in the blood and controls the transport of glucose and other nutrients to the CNS, but also manages the removal of various metabolites from the brain tissue.

The vascular wall lining the brain capillaries is characteristically formed by uninterrupted endothelial cells surrounded by branching

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cytoplasmatic extension of astrocytes and pericytes [3]. The endothelium of the BBB is continuous, and endothelial cells form an inner sheath of capillaries. These cells secrete numerous vasoactive substances that have a role in the regulation of the vascular tone [1]. Nitric monoxide (NO) is one of the most important vasodilators secreted by these cells, and its deficiency is an important indicator of endothelial dysfunction [5]. Endothelial cells are interconnected, predominantly by occluding (tight) junctions, the most important components of BBB, as well as with adherent compounds that provide mechanical stability [3]. Occluding compounds form a selective barrier that regulates and restricts the transport of certain metabolites, ions, macromolecules, toxic substances, pathogenic microorganisms, erythrocytes, and leukocytes from blood to the brain tissue [3]. Various nutrients including glucose and amino acids enter the brain tissue through the transporters, while the introduction of large molecules such as insulin, leptin, transferrin, and low-density lipoprotein is carried out by receptor endocytosis [2]. Recent research has shown that claudins (CLDNs), a family of 24 members, are the main structural and functional elements of the occluding compounds [4]. Specifically, claudin-5 (CLDN5) is the most expressed occluding compound in the BBB, and its suppression leads to BBB damage and the emergence of a schizophrenia-like phenotype in experimental animals (mice) [4, 6]. In addition, important integral proteins of occluding compounds are CLDN-12 [2]. The transmembrane claudin and occluding proteins are linked by the Zonula Occludens-1 (ZO-1) protein to the actin filaments of the endothelial cell cytoskeleton, making ZO-1 essential for maintaining the integrity and functioning of the occluding compound [2]. The function of the occluding compound is regulated by a number of factors, including vascular endothelial growth factor, G-proteins, tyrosine kinases, Ca++, cAMP, proteases, TNF- α (tumor necrosis factor-α) [7].

The characteristic feature of pericytes is contractile, branched cytoplasmatic extensions surrounding endothelial cells. They express receptors for vasoactive mediators, such as catecholamines, endothelin-1, and angiotensin II [1]. All these molecules play a vital role in regulating cerebral blood flow through capillaries [1]. The pericytes are critical in the maintenance, stability, and selectivity of the BBB. The most important factor for the functioning and homeostasis of pericytes and BBB itself, is the platelet-derived growth factor [3]. The role of pericytes have been evidenced in the cerebral autosomal dominant arteriopathy with subcortical infarction and leukoencephalopathy (CADASIL), AD, Huntington's disease and amyotrophic lateral sclerosis [3].

The foot extensions of astrocytes are interconnected by junctions that communicate with each other (Gap Junctions; nexus) and form a perivascular glial membrane that envelops the outer surface of the brain capillaries [2]. Astrocytes regulate endothelial vasodynamic, proliferative and phenotypic activities as well as their capacity for the formation of occluding compounds within the BBB [1]. Astrocytes synthesize factors such as glial neurotrophic factor, TGF-β-growth basal fibroblast growth factor, and angiopoietin-1, all acting as stimulants for the endothelial cells [8]. They also contain aquaporins (AQP), and water transport channels, with AQP4 being specific for astrocyte foot extensions [9]. Changes in the BBB stability, as well as changes in the secretion of antioxidative compounds of glutathione and superoxide dismutase, are implied in the pathogenesis of many neurodegenerative diseases [2].

Perivascular microglia is positioned between the endothelial basal lamina and cytoplasmatic extensions of astrocytes, making an interconnected web of brain macrophages [10]. These macrophages have significant phagocytic activity, by which they internalize proteins and other substances, thus reducing the detritus from the brain parenchyma. Moreover, these cells can secrete specific cytokines enhancing the inflammatory response [11]. Perivascular microglia have a significant role in the presentation of antigens in the CNS. This has been demonstrated in experimental allergic encephalomyelitis and studied in animal models of multiple sclerosis [12]. Microglial cells can quickly reduce inflammation and eliminate infectious agents before they damage brain tissue [11].

In recent years several studies identified BBB dysfunction and its increased permeability as potentially important factor in the development of psychiatric disorders [4, 13, 14]. Numerous control mechanisms can become disrupted due to infiltration of brain parenchyma with neurotoxic substances, resulting in inflammation and oxidative stress. Our previous work has shown that oxidative stress has a significant role in the development and progression of psychiatric disorders, including major depressive disorder (MDD) and bipolar affective disorder (BAD) [15, 16]. In addition to affective disorders, current literature recognizes the role of BBB dysfunction in schizophrenia, dementia, and addictive disorders such as alcohol abuse.

BBB AND SCHIZOPHRENIA

Schizophrenia is a heterogeneous clinical syndrome characterized by a constellation of symptoms divided into positive, negative, and cognitive, often severely impacting individual functionality [17]. The pathogenesis of schizophrenia is still poorly understood, and the variability of its clinical manifestations indicates several distinct pathophysiological processes in play [17]. The roles of neurotransmission disturbances, immune response, and low-grade inflammation ("low-grade encephalitis") have been all implied [18, 19]. Recent data indicate that the oxidative stress associated with neuroinflammation and neurovascular endotheliopathy can lead to impairment of BBB integrity and decreased cerebral perfusion and disruption of neuronal homeostasis in patients with schizophrenia [13, 19]. The damage to occluding compounds between endothelial cells of continuous BBB capillaries (i.e., decreased CLDN5 expression in the hippocampus) has been demonstrated in schizophrenia [20]. Decreased levels of mRNA in charge of the synthesis of occluding compounds such as CLDN5, CLDN12, and ZO-1 appear to be associated with schizophrenia [20]. In addition, 5-HT

regulates the functioning of the BBB by increasing the expression of CLDN5, potentially playing an important role in the pathogenesis of schizophrenia. [6].

The plasma levels of astrocyte protein S100B are elevated in schizophrenia, making 100B protein levels a putative treatment marker particularly in patients with predominantly negative symptoms [14, 20]. Since S100B is a calcium-binding protein, specifically found in the astrocytes, its elevation indicates astrocyte activation with increased permeability of the BBB, which may lead to neurodegeneration [21]. Moreover, S100B synthesis induces oxidative stress, potentially resulting in the promotion of NO synthesis and an increase of pro-inflammatory cytokines which has a detrimental effect on neuronal integrity [1, 20]. There are also data indicating beneficial effects of psychotropic medications by reducing the oxidative stress in the CNS [22].

BBB ROLE IN AFFECTIVE DISORDERS

There is an increasing body of evidence linking major affective disorders (MDD and BAD) to BBB dysfunction [23–26]. Both functional and structural abnormalities are heterogenous and widespread in schizophrenia [27]. Similarly to schizophrenia, elevated values of S100B have been observed in the serum of patients with affective disorders, and are also associated with the severity of symptoms in depression [28]. Moreover, the elevation of S100B is a predictor of a successful response to antidepressant therapy in MDD [29]. In addition, chronic social stress can have an effect on CLDN5 levels, potentially affecting the BBB integrity [26]. The most intense passage of proinflammatory cytokines through the dysfunctional BBB has been observed in the region of nucleus accumbens, which is a brain region important for mood regulation [29].

Recent data indicate an association between mRNA levels for CLDN5, CLDN12, and ZO-1 and the onset and duration of MDD and BAD [4]. It has been shown that BAD patients with impaired BBB integrity have a more pronounced clinical expression of the disease and its progression [4, 30]. Both acute or chronic BBB damage increases the passage of proinflammatory cytokines such as IL-1, IL-6, TNF-α, and reactive oxygen species (ROS) [15]. This triggers the activation of microglial cells with a central role in the pathway of neuroinflammation, and also promotes local oligodendrocyte and myelin sheath damage, compromising neuronal network integrity [15, 31]. Positron emission tomography imaging revealed increased microglial activity and neuroinflammation in the hippocampus of patients with affective disorders [4]. In addition, oligodendrocyte density is decreased in BAD which indicates potential instability of the neural circuitry [31].

BBB STATUS IN DEMENTIA

Frequently, patients with dementia present with additional psychiatric symptoms such as depression, agitation, anxiety, apathy, or psychosis [32, 33]. The brain endothelium becomes progressively dysfunctional in aging, with corresponding alterations in the BBB [34]. However, in the two most common types of dementia, AD and vascular dementia, the critical role of BBB dysfunction has been documented in the last years [2, 3]. The data is especially robust concerning pericyte degeneration [3]. It has been shown that an influx of immune cells (CD4+ and CD8 T cells, dendritic cells, B cells) via disturbed BBB, lead to damage of multiple efflux transporters (adenosine triphosphate dependent pumps that remove harmful agents out of the brain tissue), and accumulation of molecules prone to aggregation, such as amyloid beta [1, 35]. In AD in particular, amyloid beta as the main component of amyloid plaques is a key pathological feature of the disease [35]. High levels of amyloid beta and oligopeptides lead to activation of microglial cells and astrocytes, resulting in increased production of toxic molecules, and consequent synaptic and neuronal damage [1]. Microglia activated in relation to amyloid beta plaques leads to the activation of astrocytes which results in the release of cytokines such as IL-1, TNF-β, TNF-α, TGF-β, NGF, bNGF, and ROS [1]. In addition, the amyloid beta has been shown to stimulate the nuclear kappaB transcription factor (NF- κB) which in turn also induces the transcription of TNF-α, IL-1, IL-6, MCP-1 (monocyte chemotaxis-1 protein), and NO synthase [34]. Activation and migration of immune cells, as well as the release of cytokines, damage the integrity of the BBB [1]. The dysfunction of BBB is reflected in the impaired functioning of the amyloid beta transporter whose role is to transport these peptides from brain tissue to the blood, via BBB [1, 36]. Dysfunction of the amyloid beta transporter could be the precipitant in the cascade of events leading to the damage of neurons, synapses, and glial cells [36].

The pathophysiology of the subcortical small-vessel disease, the most frequent type of vascular cognitive impairment, is also characterized by endothelial damage and BBB dysfunction [32, 33, 37, 38]. Cerebral hypoxia due to vascular damage leads to increased levels of inflammatory molecules causing apoptosis [34]. The inflammatory molecules such as IL-1, IL-6, MMPs (MMP-2, MMP-9), TNFα, and TLR4 (toll-like receptor 4) cause demyelination and damage to the axons and oligodendrocytes associated with the white matter lesions [33, 37]. Besides risk factors, the clinical presentation including cognitive decline and behavioral changes, AD and vascular dementia also share several aspects of brain pathology, such as increased oxidative stress, disturbed amyloid beta clearance, and BBB disruption [33, 36].

BBB IN ALCOHOL USE DISORDER

Cognitive decline is common in patients with alcohol use disorder [38]. Largely, an association between alcohol use and the risk for dementia has been demonstrated in studies with robust methodology [39]. Particularly vulnerable might be the elderly and patients with documented neuroinflammatory conditions. Although the pathophysiological

pathways of neurodegeneration associated with alcohol use are not sufficiently explored, it is clear that dietary deficiencies, such as depletion of vitamin B1 and B12, in combination with alcohol metabolites (such as acetaldehyde) have a neurotoxic effect [40, 41, 42]. In addition, chronic alcohol abuse leads to increased BBB permeability via the inhibition of protein expression such as ZO-1, occludins, VE-cadherins, AQP4), thus stimulating neuroinflammation and oxidative stress, most intensely in the hippocampal region [41]. In vitro studies demonstrated a detrimental effect of alcohol on tight junctions, endoplasmic reticulum, and an increase in ROS production [41, 42].

BBB STATUS IN HEREDITARY CEREBRAL SMALL VESSEL DISEASE

The BBB likely has an important role in the pathogenesis of the CADASIL, the most frequent inherited form of vascular dementia [43]. This clinical phenotype is characterized by four basic manifestations: strokes, migraine with aura, psychiatric symptoms, and cognitive decline that progresses to subcortical vascular dementia [44]. Symptomatology of CADASIL can start with neuropsychiatric symptoms, sometimes delaying the diagnosis, with depression being the most frequent psychiatric manifestation [44, 45]. The main pathological characteristics of CADASIL are damage to smooth muscle cells of small cerebral arteries and arterioles, as well as deposition of granular osmiophilic material in the vascular wall, due to mutation in the Notch3 gene on chromosome 19p13 [46, 47]. Recent data suggest a significant role for BBB in this syndrome, with pronounced destruction of pericytes in the vascular wall of the brain, increasing the permeability of the BBB [48].

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome is a rare hereditary progressive systemic disease with encephalopathy as a leading clinical feature [45, 49]. Although the most prominent clinical manifestations of the MELAS are neurological, comprising migraine, stroke-like episodes, seizures, and cognitive decline, depressed mood, anxiety, and behavioral changes are often neglected but important clinical features [44, 45, 49]. At the core of this syndrome are 29 specific point mutations documented so far, occurring at the level of various mitochondrial DNA (mtDNA) [45]. The key pathological process is a disorder in the synthesis of intramitochondrial proteins including respiratory chain enzymes and leading to reduced adenosine triphosphate synthesis [49]. Endothelial dysfunction is also evidenced in the pathogenesis of stroke-like episodes since there is a lack of vasodilatation in these patients due to reduction of NO synthesis by the endothelium [49]. There is also the metabolic hypothesis based on a generalized mitochondrial cytopathy, but the neuro-vascular hypothesis merging all potential pathological pathways appears most comprehensive [49].

CONCLUSION

There is evidence of involvement of all elements of the BBB in major psychiatric disorders as well as neurological conditions with behavioral changes. The role of astrocytes seems important since damage to the BBB leads to astrocyte activation and altered gene expression, resulting in disturbances in the extracellular environment of neurons. Activation of astrocytes also increases cytokine synthesis and secretion, thus contributing to local inflammation of brain tissue. Therefore, the "astrocytic hypothesis" of schizophrenia and depression, as well as the "low-grade encephalitis" hypothesis of schizophrenia remain highly relevant today. Importantly, emerging infectious agents, such as SARS-CoV-2 virus infection, might add additional knowledge to the inflammatory processes adding to psychiatric conditions and cognitive disturbances. As taking into account the BBB hypothesis for the development of certain psychiatric disorders seems justified, we advocate for the development of precise molecular markers which would enable clinicians to measure the presence and the degree of BBB damage, as well as markers of brain parenchyma inflammation connected to BBB dysfunction in these entities. Increasing knowledge of these pathogenetic mechanisms may open new frontiers in the therapeutic approach to these chronic and burdening conditions.

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Улога крвно–можданe баријере у психијатријским обољењима

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САЖЕТАК

Крвно–можданa баријера (КМБ) састоји се од континуираних, тесно спојених ендотелних ћелија омотаних базалном ламином, перицитима и стопаластим продужецима астроцита. КМБ има виталну функцију у можданом метаболизму и штити мождани паренхим од штетних фактора присутних у системској циркулацији. Показано је да оштећење КМБ и повећање њене пропустљивости имају значајну улогу у многим неуродегенеративним обољењима.

Циљ овога рада је преглед литературе о значају оштећења КМБ код психијатријских обољења, у великој мери занемареној и недовољно истраженој области. Повезаност између поремећаја КМБ и неуропсихијатријских поремећаја посебно је анализирана за схизофренију, афективне поремећаје, деменције са поремећајима понашања, поремећаје повезане са употребом алкохола, са посебним осврtом на наследне болести малих крвних судова мозга са оштећењем КМБ, као што су церебрална аутозомно доминантна артериопатија са супкортикалним инфарктима и леукоенцефалопатијом и митохондријска енцефаломиопатија са лактатном ацидозом и епизодама налик можданом удару. Аутори критички сумирају могуће патогенетске механизме који повезују оштећења КМБ са овим честим обољењима.

Кључне речи: крвно–можданa баријера; схизофренија; афективни поремећаји; поремећај употребе алкохола; деменција; наследне болести малих крвних судова мозга

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HISTORY OF MEDICINE / ИСТОРИЈА МЕДИЦИНЕ

Has something changed about chronic cocaine abuse over time? An instructive example from the forensic collection

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SUMMARY
Introduction Herein we present an illustrative case from the Forensic Museum collection made by Professor Milovan Milovanović (1884–1948). Museum specimen No. 465 represents a jar containing three glass syringes and two small bottles of 10–20 ml, sealed with corks, found in the pockets of the deceased whose autopsy was performed in 1929.
Case outline It was a 30-year-old male, found dead in a tavern shed, a former medical student, lieutenant, and Russian émigré who came to Belgrade, Serbia in 1921 following the commanding general of the anti-Bolshevik White Army, Pyotr Nikolayevich Wrangel. He was an alcoholic, a drug user, and a member of the so-called Russian cocaine quartet gang. In the autopsy report, Professor Milovanović described a textbook example of a drug user: extremely malnourished body, skin covered with scabs, multiple „purulent abscesses" and „livid infiltrations," and attenuated nasal septum with mucosa covered with scabs. Internal autopsy findings included fatty liver, pneumonia, and anemia of all internal organs. At the time, the whole brain, tissue of internal organs, and contents of the stomach and intestines were used for the analysis ("the Stas–Otto method for extraction of alkaloids"). Qualitative analyses showed "the presence of cocaine" in all the examined organs, and the analysis of the content from the "cloudy, colorless liquid" found in the dark bottle showed that it contained "0.0113 g of morphinum hydrochloricum."
Conclusion Contemporary analyses of the material from one of the syringes and the transparent glass bottle performed 90 years later showed the presence of cocaine, morphine, and codeine, confirming that the cause of death was drug-related.
Keywords: autopsy; museum collection; 1920s; cocaine; morphine; toxicology

INTRODUCTION

In the early 1920s, Professor Milovan Milovanović (1884–1948) officially founded the Forensic Museum of the Institute of Forensic Medicine. Its collection contains exhibits of medicolegal and forensic importance related to actual cases from the Professor's daily practice. This is a story about one such object and a related case.

CASE REPORT

Museum specimen No. 465

The preserved label attached to the jar, bearing text written in Cyrillic, refers to forensic case No. 331 with the autopsy performed by Professor Milovanović on September 27, 1929 (Figure 1a). The specimen contains three glass syringes and two small bottles of 10–20 ml, one made of transparent glass and the other of dark glass, sealed with corks (Figures 1b and 1c).

Case history, autopsy and toxicological findings and the cause of death

From the autopsy report (Figure 2), but even more from the paper published by Professor Milovanović [1], we learn information about the deceased: a 30-year-old male who was found dead in a tavern shed, on the outskirts of the city. As a lieutenant, he came to Belgrade in 1921 with General Pyotr Nikolayevich Wrangel (1878–1928) (commanding general of the anti-Bolshevik White Army in Southern Russia, later one of the most prominent exiled white émigrés in the Kingdom of Yugoslavia). He was a failed medical student, an alcoholic, and a cocaine and morphine user, as well as a member of the so-called Russian cocaine quartet, a gang that operated in Belgrade between the First and the Second World War. He used about three grams of cocaine per day, injecting it more than 60 times per day subcutaneously. He eventually ended his life as a thief and beggar. During the investigation, in the deceased's coat pockets, the police found three glass syringes, needles, and two small bottles: one bottle was transparent, with "a minimal amount of white powder," and the other one dark with "a small amount of cloudy, colorless liquid."

During the external examination, Professor Milovanović noticed that the deceased was "extremely malnourished and neglected ... his skin covered with lice." The nasal septum was "attenuated," and "its mucosa was covered with scabs." The skin of the front side of the body, and even more pronounced on the left arm,

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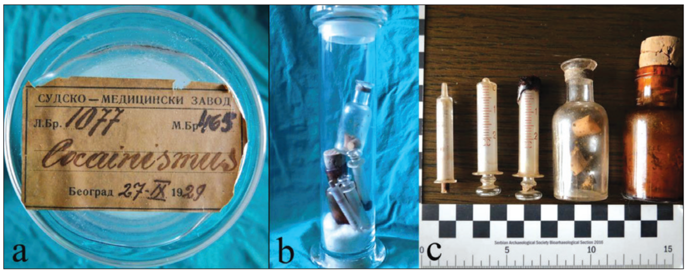
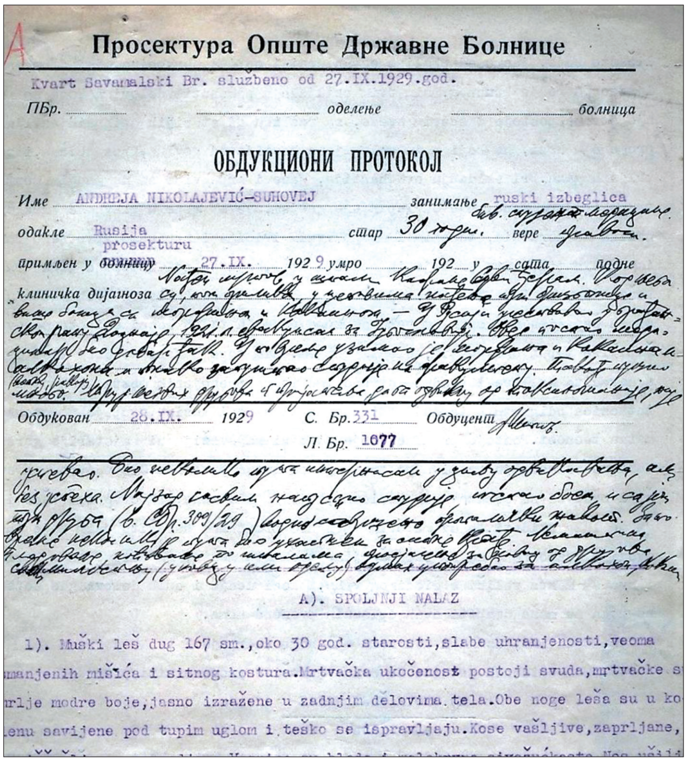


Figure 1. Museum specimen No. 465, related to forensic case No 331, from 1929; a: label attached to the museum specimen; b: jar with three glass syringes and two small bottles, one made of transparent and the other of dark glass, sealed with corks; c: closer aspect of the syringes and bottles



Просектура Опште Државне Болнице

Kvart Savamalski Br. službeno od 27.IX.1929.god.

ПБр. .... одељење .... болница

ОБДУКЦИОНИ ПРОТОКОЛ

Име ANDREJA NIKOLAJEVIĆ-SUHOVEJ занимање ruski izbeglica

одакле Rusija стар 30 год. вере

примљен у prosekturu 27.IX. 1929 умро 192 у сата подне

клиничка дијагноза

Обдукован 28.IX. 1929 С. Бр. 331 Обдуцент

Л. Бр. 1077

A). SPOLJNJI NALAZ

1). Muški leš dug 167 sm.,oko 30 god. starosti,slabe uhranjenosti,veoma ...anjenih mišića i sitnog kostura.Mrtvačka ukočenost postoji svuda,mrtvačke s... ...rlje modre boje,jasno izražene u zadnjim delovima tela.Obe noge leša su u ko... ...enu savijene pod tupim uglom i teško se ispravljaju.Kose vašljive,zaprljane,

Figure 2. Autopsy records from September 27, 1929, forensic case No. 331; autopsy was performed by Professor Milovan Milovanović

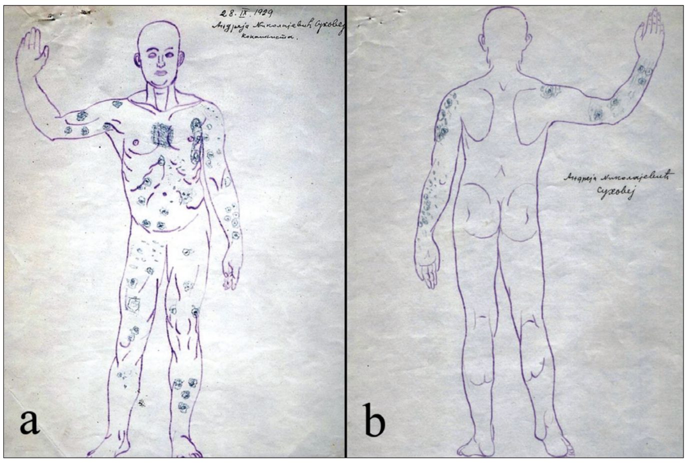


Figure 3. Two autopsy diagrams of the a: anterior and b: posterior view of the body, with numerous prominent skin lesions caused by repeated cocaine injections in "the typical regions"

was covered with multiple "purulent abscesses" and "livid infiltrations," focally spotted with scabs. Professor Milovanović illustrated all of these changes in the body diagrams (Figure 3), which were saved and preserved in the autopsy report. Internal autopsy findings included fatty liver, pneumonia, and anemia of all internal organs.

The toxicological analysis was performed in the State Chemical Laboratory: its original report is still attached to the autopsy report (Figure 4). The whole brain (weighing 1420 g), the tissue of other internal organs (parts of the lungs, heart, spleen, liver, and kidneys – weighing 1580 g in total), the stomach and intestines with their contents, and "the contents" of the dark bottle found in the coat pocket of the deceased were analyzed using "the Stas–Otto method for extraction of alkaloids." Qualitative analyses showed "the presence of cocaine" in all the examined organs. The analysis of the "cloudy, colorless liquid" found in the dark bottle showed that it contained "0.0113 g of morphinum hydrochloricum."

Professor Milovanović concluded that the cause of death was pneumonia "facilitated by malnutrition and reduced resistance to disease ... due to chronic purulent skin infection ... which were all caused by cocaine and morphine abuse," i.e., formally, this was pronounced a drug-related death.

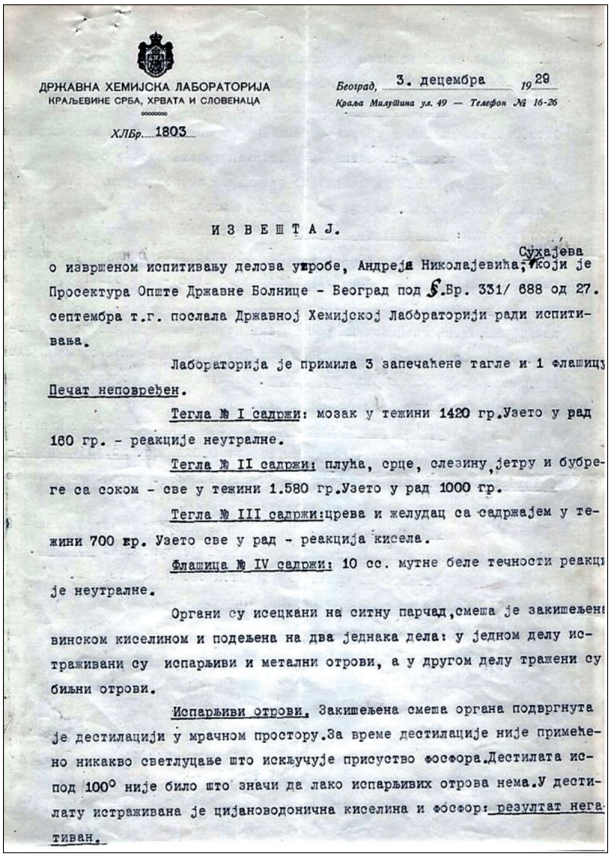
This article presents a historical case and, therefore, ethical approval was neither necessary nor obtained.

DISCUSSION

According to Professor Milovanović, no one in the domestic population in Serbia used cocaine in the first decade after the First World War [1]. Cocaine was introduced to our people by the Russians, the so-called white émigrés. At one point in 1920, there were about 40,000 Russian refugees in the Kingdom of Yugoslavia, mostly belonging to the middle or upper class, landowners, persons of liberal professions, merchants, industrialists, and army officers to the most considerable extent: all of whom escaped the Bolshevik Revolution [2]. According to the census in 1931, about 16,000 Russian refugees were in Belgrade. In addition to opera singers, ballet dancers, and various artists, the habit of cocaine use also came with the refugees from Russia.

The Drug Law passed in December 1931 in the Kingdom of Yugoslavia defined the term "controlled psychoactive substances," specifying which of them were illegal and including among them "cocaine, its salts and all chemical substances with more than 0.1% of cocaine" [3].

Professor Milovanović published his paper about pathoanatomical changes in chronic cocaine abusers in a domestic medical journal *Medicinski*



ДРЖАВНА ХЕМИЈСКА ЛАБОРАТОРИЈА
КРАЉЕВИНЕ СРБА, ХРВАТА И СЛОВЕНАЦА

Београд, 3. децембра 1929
Краља Милутина ул. 49 — Телефон № 16-26

ХЛБр. 1803

И З В Е Ш Т А Ј.

о извршеном испитивању делова узгробе, Андреја Николајевића, Сужајева који је Просектура Опште Државне Болнице - Београд под Ѕ.Бр. 331/ 688 од 27. септембра т.г. послала Државној Хемијској Лабораторији ради испитивања.

Лабораторија је примила 3 запечаћене тегле и 1 флашицу Печат неповређен.

Тегла № I садржи: мозак у тежини 1420 гр.Узето у рад 160 гр. - реакције неутралне.

Тегла № II садржи: плућа, срце, слезину, јетру и бубреге са соком - све у тежини 1.580 гр.Узето у рад 1000 гр.

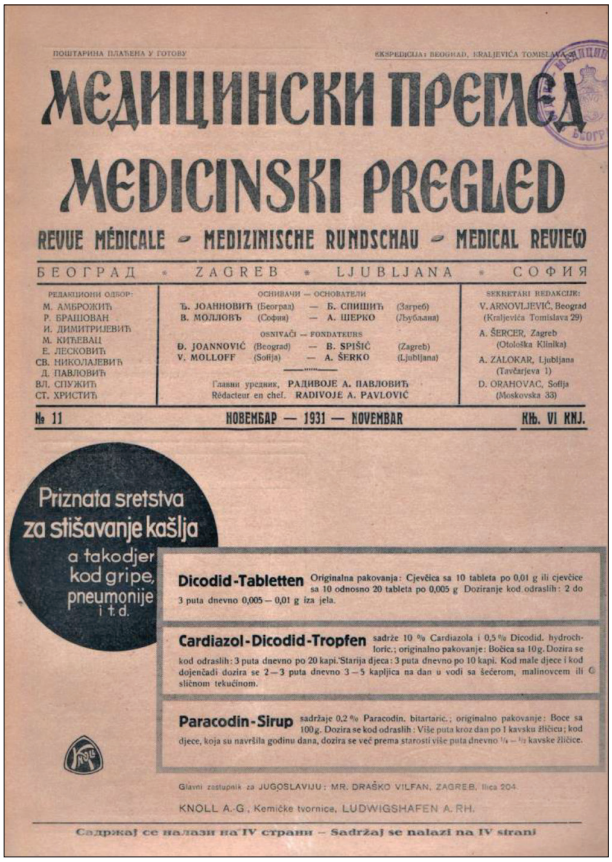
Тегла № III садржи: црева и желудац са садржајем у тежини 700 гр. Узето све у рад - реакција кисела.

Флашица № IV садржи: 10 сс. мутне беле течности реакција је неутралне.

Органи су исецкани на ситну парчад, смеша је закишељена винском киселином и подељена на два једнака дела: у једном делу истраживани су испарљиви и метални отрови, а у другом делу тражени су бибљни отрови.

Испарљиви отрови. Закишељена смеша органа подвргнута је дестилацији у мрачном простору.За време дестилације није примећено никакво светлуцање што искључује присуство фосфора.Дестилата испод 100° није било што значи да лако испарљивих отрова нема.У дестилату истраживана је цијановодонична киселина и фосфор: резултат негативан.

Figure 4. The original report of the State Chemical Laboratory, No. 1803 from December 3, 1929, related to forensic case No. 331 from 1929



МЕДИЦИНСКИ ПРЕГЛЕД
MEDICINSKI PREGLED
REVUE MÉDICALE - MEDIZINISCHE RUNDSCHAU - MEDICAL REVIEW
БЕОГРАД * ZAGREB * LJUBLJANA * СОФИЯ

№ 11 НОВЕМБАР — 1931 — NOVEMBAR КЊ. VI KNJ.

Priznata sretstva za stišavanje kašlja a takodjer kod gripe, pneumonije i t.d.

Dicodid-Tabletten

Cardiazol-Dicodid-Tropfen

Paracodin-Sirup

KNOLL A.-G., Kemische tvornice, LUDWIGSHAFEN A. RH.

Садржај се налази на IV страни – Sadržaj se nalazi na IV strani

Figure 5. Cover of the medical journal Medicinski pregled – Medical Review, in which Professor Milovanović presented his autopsy findings in "the first three fatal cases of chronic cocaine abuse in Belgrade" [1]

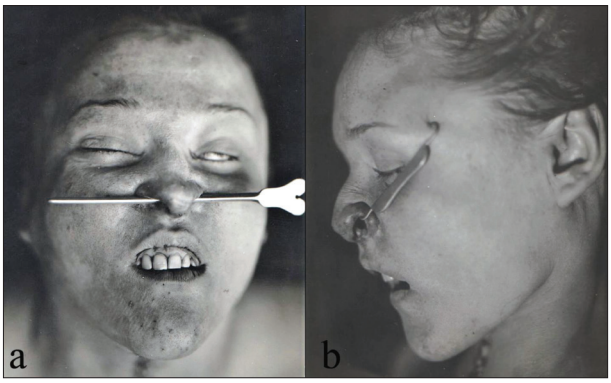


Figure 6. Photographs of fenestration of the nasal septum in a chronic cocaine abuser, taken by Professor Milovan Milovanović; forensic case No. 416 from November 1933: Russian émigré, female, 25 years old, suicidal acute intoxication with barbitone; a: full face; b: lateral aspect

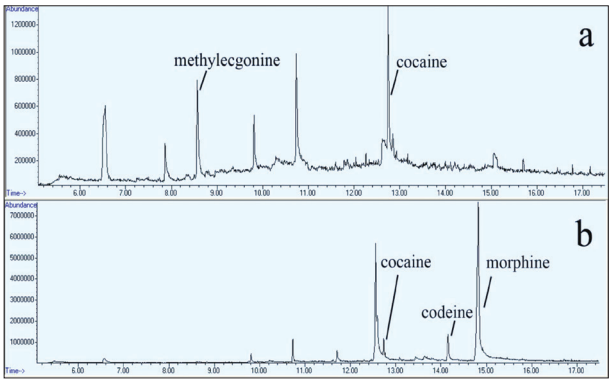


Figure 7. a: chromatogram of the qualitative analysis (GC-MS) of the material obtained from the transparent bottle showing the presence of cocaine; b: the same analysis of the material obtained from one of the three syringes showed the presence of cocaine, morphine, and codeine, meaning the syringe was used for multiple injections of different drugs; these were new, present-day analyses

pregled – Medical Review (Figure 5) [1]. The journal came out monthly from 1926 to 1940 and published papers in four South Slavic languages: Serbian and Bulgarian in the Cyrillic and Croatian and Slovenian in the Latin script, depending on the nationality of the author [4]. In this paper, Professor Milovanović presented the autopsies and other findings in "the first three fatal cases of chronic cocaine abuse in Belgrade," summarizing "the common pathoanatomical changes": cachexia, attenuation, and fenestration of the nasal septum (Figure 6), as well as skin lesions due to repeated cocaine injections. Skin lesions included: multiple needle punctures and bruises, oval infiltration of corium and subcutaneous tissue with necrosis and edema, ruptured and unruptured purulent abscesses, and, finally, oval hollow scars [1], resembling what is nowadays known as skin popping [5]. All these lesions were present on the body of the deceased in typical regions: anterior parts of the thighs and external parts of the left arm, chest, and trunk. The least number of these lesions were present in the lower legs [1] (Figure 3). As opposed to cocaine abusers, such skin lesions

were less numerous and less prominent in chronic morphine abusers [1]: its shorter half-life than morphine and shorter duration of its effects meant that cocaine had to be injected more often [1, 5]. An additional cause of these skin effects also lies in the fact that cocaine is difficult to sterilize – it will decompose in a heated solution [1, 5], which was a common procedure in morphine abuse. According to Professor Milovanović, in its nature, cocaine abuse was "idiopathic and psychogenic," causing a "strong craving," and abusers "got the drugs on the street." In contrast, morphine abuse was "symptomatic and occurred after treatment of chronic pain," and abusers "purchased opium at the pharmacy" legally or illegally [1]. "The previous 17 fatal cases of chronic morphine abuse" pointed to apparent differences in the extent of skin lesions compared to "those three fatal cases of chronic cocaine abuse" [1]. Nowadays, intravenous and subcutaneous cocaine use seems to be quite rare since it is mainly inhaled, while it has largely been supplanted by smoking "crack" [5]. Therefore, skin changes in chronic cocaine users become inconspicuous: nowadays, skin popping is more commonly seen in chronic heroin abusers [5, 6, 7].

The toxicological analyses in the presented case were initially performed in the State Chemical Laboratory (Figure 4), established in 1859. At the end of 1947, this laboratory became an integral part of the Institute of Forensic Medicine, and since late 1979 they have been situated in the same facility. New and modern toxicological equipment (gas chromatography – mass spectrometry or GC-MS) allowed us to take another view of these samples. In forensic toxicology, GC-MS is the most commonly applied method due to the large number of compounds that can be easily quantitated without time-consuming sample preparation. The development, validation, and application of a GC-MS method for separating and determining pharmaceuticals and drugs from different classes have already been tested in many studies [8, 9].

We performed new toxicological analyses, which showed the presence of cocaine in the transparent bottle (Figure 7a) and the mixture of cocaine, morphine, and codeine in one of the three syringes (Figure7b), meaning it was used for multiple injections of different drugs. This way, we confirmed and broadened the results of this 90-year-old case.

ACKNOWLEDGEMENT

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Conflict of interest: None declared.

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Хронична злоупотреба кокаина – да ли се нешто променило током времена? Пример из форензичке колекције

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САЖЕТАК

Увод У раду смо приказали илустративни случај хроничне злоупотребе кокаина из форензичке колекције професора Милована Миловановића (1884–1948). Експонат број 465 из 1929. године састоји се од три стаклена шприца и две мале стаклене бочице са плутаним чеповима: све је ово нађено у џепу капута покојника.

Приказ случаја У питању је био младић стар 30 година, некадашњи студент медицине, чије је тело нађено у шупи у дворишту једне кафане. Као официр руске војске, пратећи генерала Врангела, дошао је као емигрант у Београд 1921. године. Био је алкохоличар, наркоман и члан тзв. четворочлане кокаинске руске банде. У обдукционом записнику професор Миловановић приказао је књишки пример изгледа хроничног наркомана. Обдукција је показала и масно измењену јетру, запаљење плућа и малокрвност. Токсиколошку анализу ткива унутрашњих органа урадила је Државна хемијска лабораторија и анализа је показала квалитативно присуство кокаина у органима покојника, као и присуство морфијума у једној од бочица нађених у џепу капута покојника.

Закључак Накнадна токсиколошка анализа материјала из једног од шприцева и друге бочице која својевремено није анализирана, урађена на савременом апарату (*GC-MS*), показала је присуство наркотика у траговима, чак деведесет година после прве анализе у Државном хемијској лабораторији, далеке 1929. године.

Кључне речи: обдукција; музејска збирка; 1920-е; кокаин; морфијум; токсикологија

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LETTER TO THE EDITOR / ПИСМО УРЕДНИКУ

Direct adsorption of LDL cholesterol – one center experience

Dear Editor,

Low-density lipoprotein (LDL)-apheresis is a method of extracorporeal elimination of the particles containing apolipoprotein B (ApoB)100. Table 1 lists the currently available LDL-apheresis techniques [1]. LDL-apheresis is applied in patients, where there has been no satisfactory reduction in lipoprotein levels, despite medical diet, physical activity, and the application of pharmacotherapy have been used [1].

Table 1. LDL-apheresis techniques

HELP (Heparin-induced Extracorporeal LDL Precipitation)	The precipitation of ApoB by forming complexes with other proteins
DALI (Direct Adsorption of Lipoproteins)	Positively charged ApoB binds to negatively charged polyacrylate anions
Liposorber Dextran Sulfate	Positively charged ApoB binds to negatively charged dextran sulfate
MONET	Lipoprotein size-based elimination
TheraSorb	Filter columns containing ApoB antibodies

LDL – low-density lipoprotein; ApoB – apolipoprotein B

The American Society for Apheresis guideline states the following indications for LDL-apheresis: homozygous form of familial hypercholesterolemia (HoFH) with serum cholesterol level > 9 mmol/l or heterozygous form (HeFH) with LDL cholesterol level > 5.0 mmol/l [2, 3, 4].

The Dutch Lipid Clinic Network criteria is most frequently used for diagnosis familial hypercholesterolemia (FH) and consider: family history (severe HoFH, premature coronary artery disease), physical examination (e.g., tendon xanthoma), LDL-C levels and DNA mutation. Our center uses the Dutch Lipid Clinic Network criteria that are recognized by health care insurance system [5, 6].

Our center treats patients:

– with or without major adverse cardiac events (MACE),
– with the score of Dutch Lipid Clinic Network criteria ≥ 8, who do not achieve lowering LDL-C by more than 40% with the maximum tolerated dose of statin [4, 7].

The direct adsorption of lipoproteins (DALI) method reduces the total cholesterol by 54.1%, LDL-C by 62.3%, triglycerides by 52.3% and high-density lipoprotein (HDL)-C by 2.8% [8]. The appearance of the adsorber during the apheresis of a person with hyperchylomicronemia is shown in Figure 1, while the appearance of a filter of a person without hyperchylomicronemia is shown in Figure 2. In Figure 1 is clearly seen an example of non-homogeneous adsorption in the filter, or inadequate filter efficiency, caused by the blocking of pore with chylomicrons.

Within the Clinic for Endocrinology, there is an LDL-apheresis cabinet, which applies the

Figure 1. The appearance of the filter during the apheresis of a person with hyperchylomicronemia

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Figure 2. The appearance of an adsorber during the apheresis of a person without hyperchylomicronemia

Table 2. Results of the five-year implementation of LDL-apheresis

N = 365	Before apheresis	After apheresis	Reduction level after each treatment (%)
Total cholesterol (mmol/l)	13.6	6.41	56%
Triglycerides (mmol/l)	0.85	1.31	↑54%
LDL cholesterol (mmol/l)	6.49	2.11	67%
Non-HDL cholesterol (mmol/l)	7.16	2.66	63%
ApoA (g/l)	1.35	1.20	11%
ApoB (g/l)	1.64	0.62	62%

N – total number of procedures; LDL – low-density lipoprotein; HDL – high-density lipoprotein; ApoA – apolipoprotein A; ApoB – apolipoprotein B

DALI method. In the past five-year period, 365 well-documented LDL-apheresis were performed. Based on this, a retrospective analysis of the results was made. The study has been approved by the local ethics committee and was conducted in accordance with the Helsinki Declaration. The results are presented in Table 2. [5, 9].

The results of treatment in our center, with a reduction of 67% in LDL-C and 62% in ApoB, coincide with data from retrospective studies, such as in Germany, which included 15,527 subjects [10].

The study by Schettler et al. [10] also showed a reduction of 78% in MACE. During the 5-year period of treatment in our center, there were no new MACE. This can be explained by the fact that the patients treated in our center were younger (average 54.81 years), and some of them without previous MACE, while Schettler et al. [10] presented the results of apheresis in patients with previous MACE, which are patients with higher cardiovascular risk, majority were women, in age range of 60–90 years. We considered the cumulative LDL-C level, and thus patients with HeFH undergo early interventions, which certainly contributes to better results in terms of MACE incidence [11].

We believe that there is insufficient awareness of the existence of familial HoFH [12–15]. Patients with FH have rapid progression of atherosclerosis with a high incidence of MACE. For this reason, strict control of lipid parameters is necessary in these patients [16–19].

Previous findings suggest that LDL-apheresis is an effective method of reducing LDL-C in those patients who do not achieve the target values with pharmacotherapy and lifestyle changes [20]. The method is well tolerated and, according to the previously published data, it stops the progression of atherosclerotic.

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Пре подношења рукописа Уредништву часописа „Српски архив за целокупно лекарство“ (СА) сви аутори треба да прочитају Упутство за ауторе (*Instructions for Authors*), где ће пронаћи све потребне информације о писању и припреми рада у складу са стандардима часописа. Веома је важно да аутори припреме рад према датим пропозицијама, јер уколико рукопис не буде усклађен с овим захтевима, Уредништво ће одложити или одбити његово публиковање. Радови објављени у СА се не хонорарашу. За чланке који ће се објавити у СА, самом понудом рада Српском архиву сви аутори рада преносе своја ауторска права на издавача часописа – Српско лекарско друштво.

ОПШТА УПУТСТВА. СА објављује радове који до сада нису нигде објављени, у целости или делом, нити прихваћени за објављивање. СА објављује радове на енглеском и српском језику. Због боље доступности и веће цитираности препоручује се ауторима да радове свих облика предају на енглеском језику. У СА се објављују следеће категорије радова: уводници, оригинални радови, претходна и кратка саопштења, прикази болесника и случајева, видео-чланци, слике из клиничке медицине, прегледни радови, актуелне теме, радови за праксу, радови из историје медицине и језика медицине, медицинске етике, регулаторних стандарда у медицини, извештаји са конгреса и научних скупова, лични ставови**,** наручени коментари, писма уреднику, прикази књига, стручне вести, *In memoriam* и други прилози. Оригинални радови, претходна и кратка саопштења, прикази болесника и случајева, видео-чланци, слике из клиничке медицине, прегледни радови и актуелне теме, публикују се искључиво на енглеском језику, а остале врсте радова се могу публиковати и на српском језику само по одлуци Уредништва. Радови се увек достављају са сажетком на енглеском и српском језику (у склопу самог рукописа). Текст рада куцати у програму за обраду текста *Word*, фонтом *Times New Roman* и величином слова 12 тачака (12 *pt*). Све четири маргине подесити на 25 *mm*, величину странице на формат А4, а текст куцати с двоструким проредом, левим поравнањем и увлачењем сваког пасуса за 10 *mm*, без дељења речи (хифенације). Не користити табулаторе и узастопне празне карактере (спејсове) ради поравнања текста, већ алатке за контролу поравнања на лењиру и *Toolbars*. За прелазак на нову страну документа не користити низ „ентера“, већ искључиво опцију *Page Break*. После сваког знака интерпункције ставити само један празан карактер. Ако се у тексту користе специјални знаци (симболи), користити фонт *Symbol*. Подаци о коришћеној литератури у тексту означавају се арапским бројевима у угластим заградама – нпр. [1, 2], и то редоследом којим се појављују у тексту. Странице нумерисати редом у доњем десном углу, почев од насловне стране.

При писању текста на енглеском језику треба се придржавати језичког стандарда *American English* и користити кратке и јасне реченице. За називе лекова користити искључиво генеричка имена. Уређаји (апарати) се означавају фабричким називима, а име и место произвођача треба навести у облим заградама. Уколико се у тексту користе ознаке које су спој слова и бројева, прецизно написати број који се јавља у суперскрипту или супскрипту (нпр. ^{99}Tc, *IL*-6, O_2, $Б_{12}$, *CD*8). Уколико се нешто уобичајено пише курзивом (*italic*), тако се и наводи, нпр. гени (*BRCA1*).

Уколико је рад део магистарске тезе, односно докторске дисертације, или је урађен у оквиру научног пројекта, то треба посебно назначити у Напомени на крају текста. Такође, уколико је рад претходно саопштен на неком стручном састанку, навести званичан назив скупа, место и време одржавања, да ли је рад и како публикован (нпр. исти или другачији наслов или сажетак).

КЛИНИЧКА ИСТРАЖИВАЊА. Клиничка истраживања се дефинишу као истраживања утицаја једног или више средстава или мера на исход здравља. Регистарски број истраживања се наводи у последњем реду сажетка.

ЕТИЧКА САГЛАСНОСТ. Рукописи о истраживањима на људима треба да садрже изјаву у виду писаног пристанка испитиваних особа у складу с Хелсиншком декларацијом и одобрење надлежног етичког одбора да се истраживање може извести и да је оно у складу с правним стандардима. Експериментална истраживања на хуманом материјалу и испитивања вршена на животињама треба да садрже изјаву етичког одбора установе и треба да су у сагласности с правним стандардима.

ИЗЈАВА О СУКОБУ ИНТЕРЕСА. Уз рукопис се прилаже потписана изјава у оквиру обрасца *Submission Letter* којом се аутори изјашњавају о сваком могућем сукобу интереса или његовом одсуству. За додатне информације о различитим врстама сукоба интереса посетити интернет-страницу Светског удружења уредника медицинских часописа (*World Association of Medical Editors – WAME; http://www.wame.org*) под називом „Политика изјаве о сукобу интереса“.

АУТОРСТВО. Све особе које су наведене као аутори рада треба да се квалификују за ауторство. Сваки аутор треба да је учествовао довољно у раду на рукопису како би могао да преузме одговорност за целокупан текст и резултате изнесене у раду. Ауторство се заснива само на: битном доприносу концепцији рада, добијању резултата или анализи и тумачењу резултата; планирању рукописа или његовој критичкој ревизији од знатног интелектуалног значаја; завршном дотеривању верзије рукописа који се припрема за штампање.

Аутори треба да приложе опис доприноса појединачно за сваког коаутора у оквиру обрасца *Submission Letter*. Финансирање, сакупљање података или генерално надгледање истраживачке групе сами по себи не могу

оправдати ауторство. Сви други који су допринели изради рада, а који нису аутори рукописа, требало би да буду наведени у Захвалници с описом њиховог доприноса раду, наравно, уз писани пристанак.

ПЛАГИЈАРИЗАМ. Од 1. јануара 2019. године сви рукописи подвргавају се провери на плагијаризам/аутоплагијаризам преко *SCIndeks Assistant* – Cross Check (iThenticate). Радови код којих се докаже плагијаризам/аутоплагијаризам биће одбијени, а аутори санкционисани.

НАСЛОВНА СТРАНА. На првој страници рукописа треба навести следеће: наслов рада без скраћеница; предлог кратког наслова рада, пуна имена и презимена аутора (без титула) индексирана бројевима; званичан назив установа у којима аутори раде, место и државу (редоследом који одговара индексираним бројевима аутора); на дну странице навести име и презиме, адресу за контакт, број телефона, факса и имејл адресу аутора задуженог за кореспонденцију.

САЖЕТАК. Уз оригинални рад, претходно и кратко саопштење, преглед литературе, приказ случаја (болесника), рад из историје медицине, актуелну тему, рад за рубрику језик медицине и рад за праксу, на другој по реду страници документа треба приложити сажетак рада обима 100–250 речи. За оригиналне радове, претходно и кратко саопштење сажетак треба да има следећу структуру: Увод/Циљ рада, Методе рада, Резултати, Закључак; сваки од наведених сегмената писати као посебан пасус који почиње болдованом речи. Навести најважније резултате (нумеричке вредности) статистичке анализе и ниво значајности. Закључак не сме бити уопштен, већ мора бити директно повезан са резултатима рада. За приказе болесника сажетак треба да има следеће делове: Увод (у последњој реченици навести циљ), Приказ болесника, Закључак; сегменте такође писати као посебан пасус који почиње болдованом речи. За остале типове радова сажетак нема посебну структуру.

КЉУЧНЕ РЕЧИ. Испод Сажетка навести од три до шест кључних речи или израза. Не треба да се понављају речи из наслова, а кључне речи треба да буду релевантне или описне. У избору кључних речи користити *Medical Subject Headings – MeSH (http://www.nlm.nih.gov/mesh)*.

ПРЕВОД НА СРПСКИ ЈЕЗИК. На трећој по реду страници документа приложити наслов рада на српском језику, пуна имена и презимена аутора (без титула) индексирана бројевима, званичан назив установа у којима аутори раде, место и државу. На следећој – четвртој по реду – страници документа приложити сажетак (100–250 речи) с кључним речима (3–6), и то за радове у којима је обавезан сажетак на енглеском језику. Превод појмова из стране литературе треба да буде у духу српског језика. Све стране речи или синтагме за које постоји одговарајуће име у нашем језику заменити тим називом. Уколико је рад у целости на српском језику, потребно је превести називе прилога (табела, графикона, слика, схема) уколико их има, целокупни текст у њима и легенду на енглески језик.

СТРУКТУРА РАДА. Сви поднаслови се пишу великим масним словима (болд). Оригинални рад и претходно и кратко саопштење обавезно треба да имају следеће поднаслове: Увод (Циљ рада навести као последњи пасус Увода), Методе рада, Резултати, Дискусија, Закључак, Литература. Преглед литературе и актуелну тему чине: Увод, одговарајући поднаслови, Закључак, Литература. Првоименовани аутор прегледног рада мора да наведе бар пет аутоцитата (као аутор или коаутор) радова публикованих у часописима с рецензијом. Коаутори, уколико их има, морају да наведу бар један аутоцитат радова такође публикованих у часописима с рецензијом. Приказ случаја или болесника чине: Увод (Циљ рада навести као последњи пасус Увода), Приказ болесника, Дискусија, Литература. Не треба користити имена болесника, иницијале, нити бројеве историја болести, нарочито у илустрацијама. Прикази болесника не смеју имати више од пет аутора.

Прилоге (табеле, графиконе, слике итд.) поставити на крај рукописа, а у самом телу текста јасно назначити место које се односи на дати прилог. Крајња позиција прилога биће одређена у току припреме рада за публиковање.

СКРАЋЕНИЦЕ. Користити само када је неопходно, и то за веома дугачке називе хемијских једињења, односно називе који су као скраћенице већ препознатљиви (стандардне скраћенице, као нпр. ДНК, сида, ХИВ, АТП). За сваку скраћеницу пун термин треба навести при првом навођењу у тексту, сем ако није стандардна јединица мере. Не користити скраћенице у наслову. Избегавати коришћење скраћеница у сажетку, али ако су неопходне, сваку скраћеницу објаснити при првом навођењу у тексту.

ДЕЦИМАЛНИ БРОЈЕВИ. У тексту рада на енглеском језику, у табелама, на графиконима и другим прилозима децималне бројеве писати са тачком (нпр. 12.5 ± 3.8), а у тексту на српском језику са зарезом (нпр. 12,5 ± 3,8). Кад год је то могуће, број заокружити на једну децималу.

ЈЕДИНИЦЕ МЕРА. Дужину, висину, тежину и запремину изражавати у метричким јединицама (метар – *m*, килограм (грам) – *kg* (*g*), литар – *l*) или њиховим деловима. Температуру изражавати у степенима Целзијуса (°*C*), количину супстанце у молима (*mol*), а притисак крви у милиметрима живиног стуба (*mm Hg*). Све резултате хематолошких, клиничких и биохемијских мерења наводити у метричком систему према Међународном систему јединица (*SI*).

ОБИМ РАДОВА. Целокупни рукопис рада који чине – насловна страна, сажетак, текст рада, списак литературе, сви прилози, односно потписи за њих и легенда (табеле, слике, графикони, схеме, цртежи), насловна страна и сажетак на српском језику – мора износити за оригинални рад, рад из историје медицине и преглед литературе до 5000 речи, а за претходно и кратко саопштење, приказ болесника, актуелну тему, рад за праксу, едукативни чланак и рад за рубрику „Језик медицине“ до 3000 речи; радови за остале рубрике могу имати највише 1500 речи.
Видео-радови могу трајати 5–7 минута и бити у формату *avi, mp4*(*flv*). У првом кадру филма мора се навести: у наднаслову Српски архив за целокупно лекарство, наслов рада, презимена и иницијали имена и средњег слова свих аутора рада (не филма), година израде. У другом кадру мора бити уснимљен текст рада у виду апстракта до 350 речи. У последњем кадру филма могу се навести имена техничког особља (режија, сниматељ, светло, тон, фотографија и сл.). Уз видео-радове доставити: посебно текст у виду апстракта (до 350 речи), једну фотографију као илустрацију приказа, изјаву потписану од свег техничког особља да се одричу ауторских права у корист аутора рада.

ПРИЛОЗИ РАДУ су табеле, слике (фотографије, цртежи, схеме, графикони) и видео-прилози.

Свака табела треба да буде сама по себи лако разумљива. Наслов треба откуцати изнад табеле, а објашњења испод ње. Табеле се означавају арапским бројевима према редоследу навођења у тексту. Табеле цртати искључиво у програму *Word*, кроз мени *Table–Insert–Table*, уз дефинисање тачног броја колона и редова који ће чинити мрежу табеле. Десним кликом на мишу – помоћу опција *Merge Cells* и *Split Cells* – спајати, односно делити ћелије. Куцати фонтом *Times New Roman*, величином слова 12 *pt*, с једноструким проредом и без увлачења текста. Коришћене скраћенице у табели треба објаснити у легенди испод табеле. Уколико је рукопис на српском језику, приложити називе табела и легенду на оба језика. Такође, у једну табелу, у оквиру исте ћелије, унети и текст на српском и текст на енглеском језику (никако не правити две табеле са два језика!).

Слике су сви облици графичких прилога и као „слике“ у CA се објављују фотографије, цртежи, схеме и графикони. Слике означавају се арапским бројевима према редоследу навођења у тексту. Примају се искључиво дигиталне фотографије (црно-беле или у боји) резолуције најмање 300 *dpi* и формата записа *tiff* или *jpg* (мале, мутне и слике лошег квалитета неће се прихватати за штампање!). Уколико аутори не поседују или нису у могућности да доставе дигиталне фотографије, онда оригиналне слике треба скенирати у резолуцији 300 *dpi* и у оригиналној величини. Уколико је рад неопходно илустровати са више слика, у раду ће их бити објављено неколико, а остале ће бити у е-верзији чланка као *PowerPoint* презентација (свака слика мора бити нумерисана и имати легенду).
Видео-прилози (илустрације рада) могу трајати 1–3 минута и бити у формату *avi, mp4*(*flv*). Уз видео доставити посебно слику која би била илустрација видеоприказа у *e*-издању и објављена у штампаном издању. Уколико је рукопис на српском језику, приложити називе слика и легенду на оба језика.

Слике се у свесци могу штампати у боји, али додатне трошкове штампе сносе аутори.

Графикони треба да буду урађени и достављени у програму *Excel*, да би се виделе пратеће вредности распоређене по ћелијама. Исте графиконе прекопирати и у *Word*-ов документ, где се графикони означавају арапским бројевима према редоследу навођења у тексту. Сви подаци на графикону куцају се у фонту *Times New Roman*. Коришћене скраћенице на графикону треба објаснити у легенди испод графикона. У штампаној верзији чланка вероватније је да графикон неће бити штампан у боји, те је боље избегавати коришћење боја у графиконима, или их користити различитог интензитета. Уколико је рукопис на српском језику, приложити називе графикона и легенду на оба језика.

Цртежи и схеме се достављају у *jpg* или *tiff* формату. Схеме се могу цртати и у програму *CorelDraw* или *Adobe Illustrator* (програми за рад са векторима, кривама). Сви подаци на схеми куцају се у фонту *Times New Roman*, величина слова 10 *pt*. Коришћене скраћенице на схеми треба објаснити у легенди испод схеме. Уколико је рукопис на српском језику, приложити називе схема и легенду на оба језика.

ЗАХВАЛНИЦА. Навести све сараднике који су допринели стварању рада а не испуњавају мерила за ауторство, као што су особе које обезбеђују техничку помоћ, помоћ у писању рада или руководе одељењем које обезбеђује општу подршку. Финансијска и материјална помоћ, у облику спонзорства, стипендија, поклона, опреме, лекова и друго, треба такође да буде наведена.

ЛИТЕРАТУРА. Списак референци је одговорност аутора, а цитирани чланци треба да буду лако приступачни читаоцима часописа. Стога уз сваку референцу обавезно треба навести *DOI* број чланка (јединствену ниску карактера која му је додељена) и *PMID* број уколико је чланак индексиран у бази *PubMed/MEDLINE*.

Референце нумерисати редним арапским бројевима према редоследу навођења у тексту. Број референци не би требало да буде већи од 30, осим у прегледу литературе, у којем је дозвољено да их буде до 50, и у метаанализи, где их је дозвољено до 100. Број цитираних оригиналних радова мора бити најмање 80% од укупног броја референци, односно број цитираних књига, поглавља у књигама и прегледних чланака мањи од 20%. Уколико се домаће монографске публи-

кације и чланци могу уврстити у референце, аутори су дужни да их цитирају. Већина цитираних научних чланака не би требало да буде старија од пет година. Није дозвољено цитирање апстраката. Уколико је битно коментарисати резултате који су публиковани само у виду апстракта, неопходно је то навести у самом тексту рада. Референце чланака који су прихваћени за штампу, али још нису објављени, треба означити са *in press* и приложити доказ о прихватању рада за објављивање.

Референце се цитирају према Ванкуверском стилу (униформисаним захтевима за рукописе који се предају биомедицинским часописима), који је успоставио Међународни комитет уредника медицинских часописа (*http://www.icmje.org*), чији формат користе *U.S. National Library of Medicine* и базе научних публикација. Примере навођења публикација (чланака, књига и других монографија, електронског, необјављеног и другог објављеног материјала) могу се пронаћи на интернет-страници *http://www.nlm.nih.gov/bsd/uniform_requirements.html*. Приликом навођења литературе веома је важно придржавати се поменутог стандарда, јер је то један од најбитнијих фактора за индексирање приликом класификације научних часописа.

ПРОПРАТНО ПИСМО (*SUBMISSION LETTER*). Уз рукопис обавезно приложити образац који су потписали сви аутори, а који садржи: 1) изјаву да рад претходно није публикован и да није истовремено поднет за објављивање у неком другом часопису, 2) изјаву да су рукопис прочитали и одобрили сви аутори који испуњавају мерила ауторства, и 3) контакт податке свих аутора у раду (адресе, имејл адресе, телефоне итд.). Бланко образац треба преузети са интернет-странице часописа (*http://www.srpskiarhiv.rs*).

Такође је потребно доставити копије свих дозвола за: репродуковање претходно објављеног материјала, употребу илустрација и објављивање информација о познатим људима или именовање људи који су допринели изради рада.

ЧЛАНАРИНА, ПРЕТПЛАТА И НАКНАДА ЗА ОБРАДУ ЧЛАНКА. Да би рад био објављен у часопису *Српски архив за целокупно лекарство*, сви аутори који су лекари или стоматолози из Србије морају бити чланови Српског лекарског друштва (у складу са чланом 6. Статута Друштва) и измирити накнаду за обраду чланака (*Article Processing Charge*) у износу од 3000 динара. Аутори и коаутори из иностранства су у обавези да плате накнаду за обраду чланака (*Article Processing Charge*) у износу од 35 евра. Уплата у једној календарској години обухвата и све наредне, евентуалне чланке, послате на разматрање у тој години. Сви аутори који плате ову накнаду могу, уколико то желе, да примају штампано издање часописа. Треба напоменути да ова уплата није гаранција да ће рад бити прихваћен и објављен у *Српском архиву за целокупно лекарство*. Обавеза плаћања накнаде за обраду чланка не односи се на студенте основних студија и на претплатнике на часопис.

Установе (правна лица) не могу преко своје претплате да испуне овај услов аутора (физичког лица). Уз рукопис рада треба доставити копије уплатница за чланарину и претплату / накнаду за обраду чланка, као доказ о уплатама, уколико издавач нема евиденцију о томе. Часопис прихвата донације од спонзора који сносе део трошкова или трошкове у целини оних аутора који нису у могућности да измире накнаду за обраду чланка (у таквим случајевима потребно је часопису ставити на увид оправданост таквог спонзорства).

СЛАЊЕ РУКОПИСА. Рукопис рада и сви прилози уз рад достављају се искључиво електронски преко система за пријављивање на интернет-страници часописа: *http://www.srpskiarhiv.rs*

НАПОМЕНА. Рад који не испуњава услове овог упутства не може бити упућен на рецензију и биће враћен ауторима да га допуне и исправе. Придржавањем упутства за припрему рада знатно ће се скратити време целокупног процеса до објављивања рада у часопису, што ће позитивно утицати на квалитет чланака и редовност излажења часописа.

За све додатне информације, молимо да се обратите на доле наведене адресе и број телефона.

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The papers are always submitted with Summary in both English and Serbian, included in the manuscript file. The text of the manuscript should be typed in *MS Word* using the *Times New Roman* typeface, and font size 12 pt. The text should be prepared with margins set to 25 mm and onto A4 paper size, with double line spacing, aligned left and the initial lines of all paragraphs indented 10 mm, without hyphenation. Tabs and successive blank spaces are not to be used for text alignment; instead, ruler alignment control tool and *Toolbars* are suggested. In order to start a new page within the document, *Page Break* option should be used instead of consecutive enters. Only one space follows after any punctuation mark. If special signs (symbols) are used in the text, use the *Symbol* font. References cited in the text are numbered with Arabic numerals within parenthesis (for example: [1, 2]), in order of appearance in the text. Pages are numbered consecutively in the right bottom corner, beginning from the title page.

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