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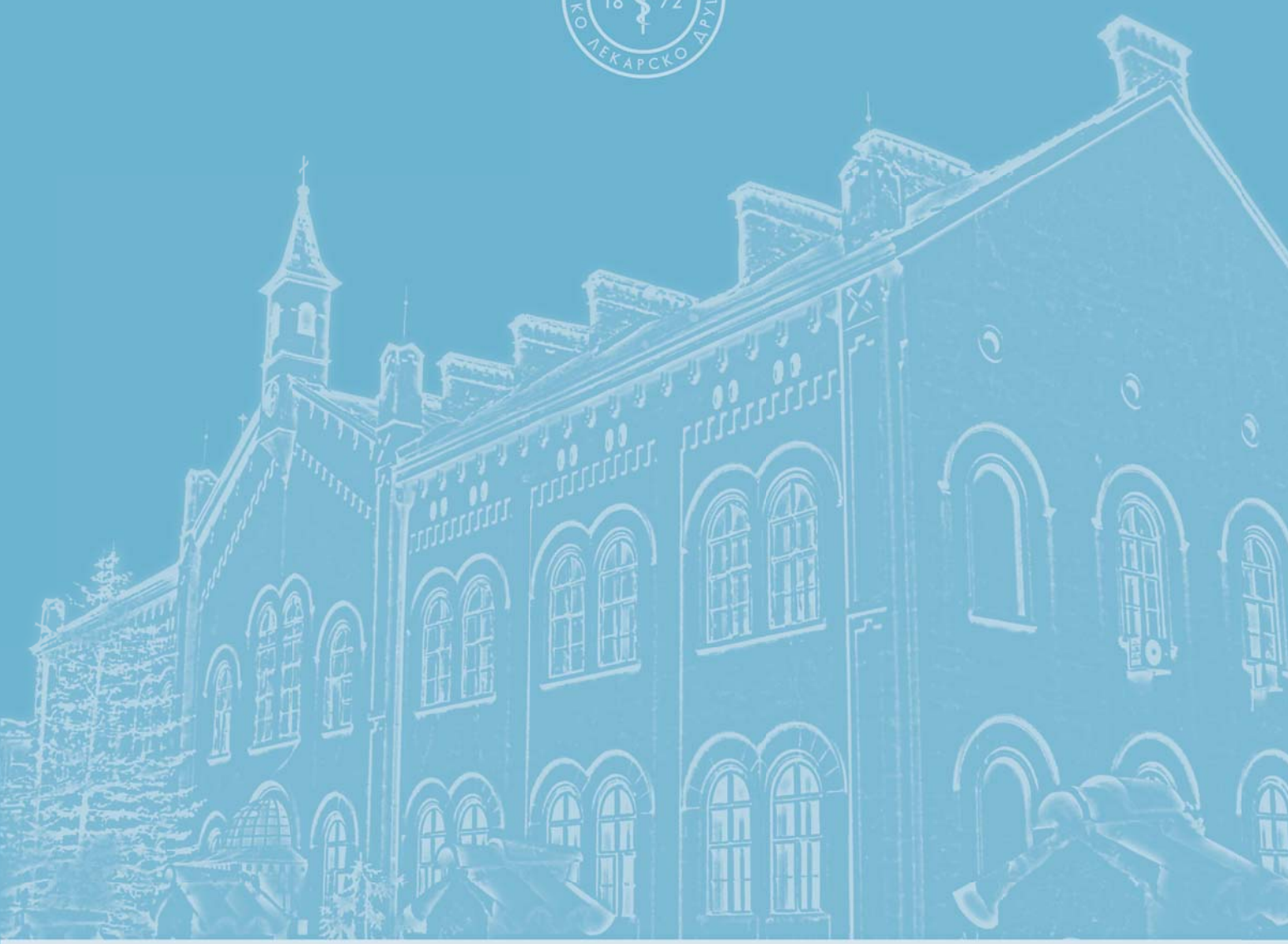
ЧАСОПИС СРПСКОГ ЛЕКАРСКОГ ДРУШТВА ОСНОВАН 1872. ГОДИНЕ

ЗА ЦЕЛОКУПНО ЛЕКАРСТВО



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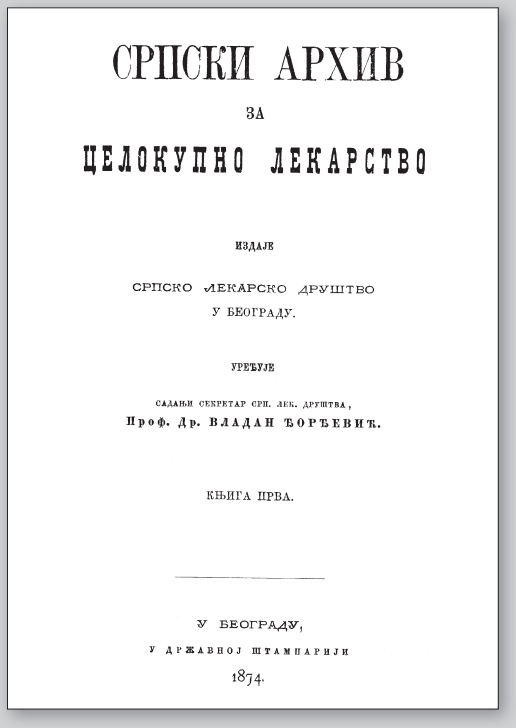

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ЗА

ЦЕЛОКУПНО ЛЕКАРСТВО

ИЗДАЈЕ

СРПСКО ЛЕКАРСКО ДРУШТВО

У БЕОГРАДУ.

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У БЕОГРАДУ,

У ДРЖАВНОЈ ШТАМПАРИЈИ

1874.

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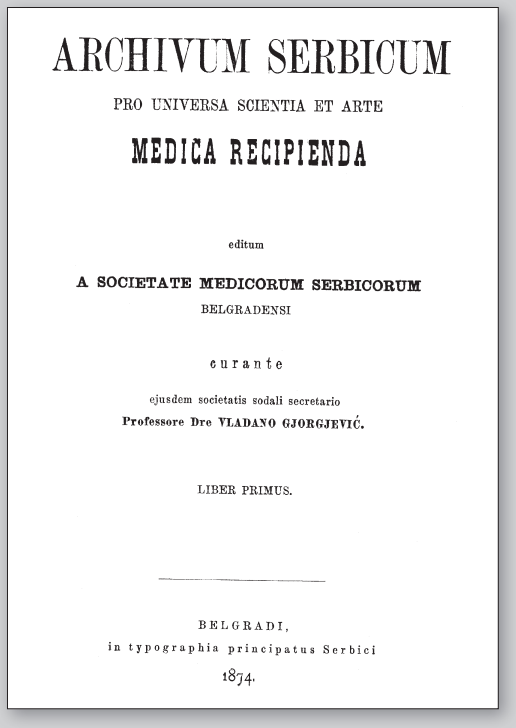

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1874.

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Ocular surface disease incidence in patients with open-angle glaucoma

Marija Radenković[1], Gordana Stanković-Babić[1,2], Predrag Jovanović[1,2], Jasmina Djordjević-Jocić[1,2], Marija Trenkić-Božinović[1]

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SUMMARY

Introduction Ocular surface disease (OSD) is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances, tear film instability with potential damage to the ocular surface, accompanied by increased tear film osmolarity and inflammation of the ocular surface. It is a consequence of disrupted homeostasis of lacrimal functional unit. The main pathogenetic mechanism stems from tear hyperosmolarity and tear film instability. The etiological classification is hyposecretory (Sy-Sjögren and non-Sjögren) and evaporative (extrinsic and intrinsic) form. Delphi panel classification grades disease stages. Antiglaucoma topical therapy causes exacerbation or occurrence of symptoms of dry eye due to main ingredients or preservatives (benzalkonium chloride – BAK), which are dose- and time-dependent. BAK reduces the stability of the lipid layer of tears, the number of goblet cells, induces apoptosis and inflammatory infiltration.

Objective The aim of this study was the analysis of the OSD incidence in open-angle glaucoma patients caused by topical medicamentous therapy.

Methods Retrospective analysis of examined patients with open-angle glaucoma was used.

Results Increased incidence of moderate and advanced OSD Index degrees in the group of primary open-angle glaucoma (POAG) and pseudoexfoliative glaucoma. According to the Delphi Panel Scale the most common grade is IIb (POAG and pseudoexfoliative glaucoma). Evaporative form of OSD prevailed in all treatment groups. High percentage of dry eye in patients with higher concentrations of preservatives applied was noticed.

Conclusion OSD should be timely diagnosed and treated. Dry eye has an impact on surgical outcome and postoperative visual acuity, and in order to improve patient compliance and quality of life, symptoms of dry eye should be addressed and medications with lower concentrations of preservatives should be applied.

Keywords: glaucoma; ocular surface disease; dry eye; preservative

INTRODUCTION

There are many definitions and synonyms explaining entity of dry eye in accordance with the change of knowledge of pathomechanisms referring to these multifactorial disorders. In 1995 Lemp defined dry eye as qualitative and quantitative disorder of the tear film that occurs as a result of the deficiency or increased tear evaporation and results in damaging of interpalpebral surface of the eye, with symptoms of ocular discomfort [1]. Most often used terms in the explanation of dry eye are the following: keratoconjunctivitis sicca, dysfunctional tear syndrome, ocular surface disease (OSD), dry eye syndrome. In accordance with the latest revision of the International Panel of Experts, Dry Eye Workshop (DEWS) 2007, dry eye is a chronic, multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbances, and tear film instability with potential damage of the ocular surface, accompanied by increased osmolarity of the tear film, and inflammation of ocular surface [1].

Dry eye syndrome is a very common disorder in adults, with an average prevalence of about 30% (5.5–57.1%). Etiology is multifactorial and may arise due to the use of medicines (antihistamines), nutritional factors (vitamin A, omega acids), age, diseases of the connective tissue, hormonal deficiencies, surgery of the anterior segment of the eye, trauma, sensory block in contact lens users, Ro therapy [2].

Tear production in healthy eyes depends on neuronal feedback. Disruption of the normal nerve control in tearing causes the dry eye disorder. The proposed mechanism that explains the occurrence of the OSD is feedback model that includes the lacrimal functional unit. It consists of the following three components: 1. ocular surface that is formed by the cornea, conjunctiva and meibomian gland; 2. lacrimal gland; 3. their mutual sensomotor innervation and innervation of the central nervous system.

Environmental stimulus (wind, low humidity) produces afferent impulses from the surface of the eye via the trigeminal nerve (n. V1) to the mesencephalon, cortical synapses, activates the parasympathetic efferent impulses (n. VII) to the lacrimal gland, resulting in tear secretion in a healthy eye. This reflex arc in a healthy eye is an example of positive feedback in response to stimuli from the environment [1, 3].

Damage of any component of the lacrimal functional unit interrupts the reflex arc, which

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results in damaging of the lacrimal gland and/or the surface of the eye. The consequence is a negative feedback with induced damage of the surface of the eye, disrupted secretomotor innervation that cause lacrimal gland dysfunction. Lacrimal gland cytokines continue to damage the conjunctiva and cornea due to activated inflammatory cascade. Released cytokines on the surface of the eye further interrupt signal generation in the secretory component. Secondary disruption of efferent signals to the lacrimal gland causes its further damage due to Ly infiltration, T-cell activation and cytokine release on the ocular surface [4].

Damage to any of the components of the lacrimal functional unit is presented as inflammation and hyperosmolar stress. The inflammatory process is essential in pathophysiology of the OSD. The mediators released in the highest concentration in inflamed lacrimal functional unit are as follows: IL-1, IL-6, IL-8, IL-1β, substance P, TNF-α, and MMP-9. Hyperosmolarity promotes inflammation as the main pathogenetic mechanism in all types of the dry eye. Healthy lacrimal functional unit produces the following protective mediators: androgen-dependent TGF-ß, EGF, IL-1ra, lysozyme and lactoferrin [4, 5].

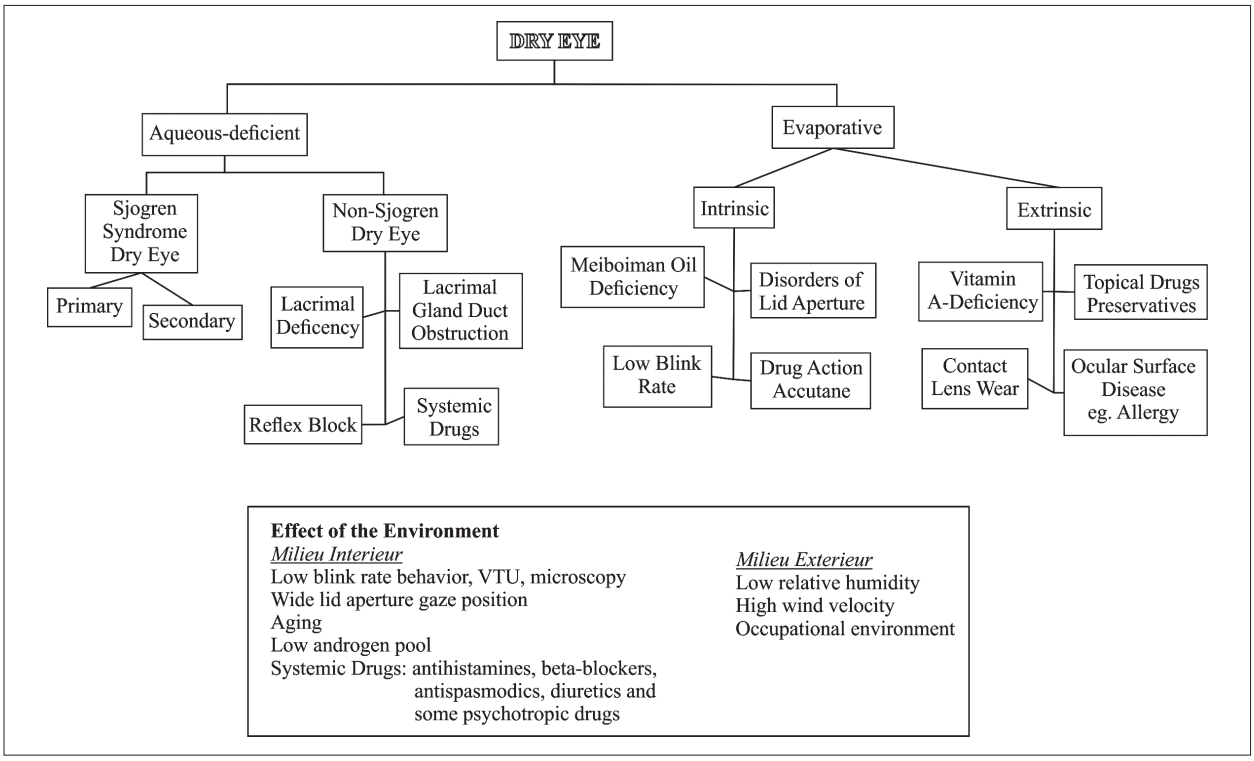
Based on the Triple Classification (SOE 2005, Berlin) and Delphi panels, DEWS in 2007, the three-part classification of dry eye was revised based on etiology, mechanisms and stages of the disease [1]. Etiologic classification system distinguishes two basic categories of dry eye: I. hyposecretory (Sy-Sjögren and non-Sjögren), and II. evaporative (extrinsic and intrinsic mechanism). A more detailed classification is shown in Scheme 1.

Although the scheme differentiates two basic forms of the disease, most people have mixed type, which can have a severe clinical presentation. Meibomian gland dysfunction has a significant role in the development of predominantly evaporative intrinsic form of the disorder. The first definition (Ocular Surface Society Workshop, 2011) states that meibomian gland dysfunction is chronic, diffuse abnormality characterized by obstruction of excretory ducts and/or qualitative and quantitative changes of glandular secretion.

Classification of dry eye toward the pathogenesis includes I. tear hyperosmolarity, and II. tear film instability. Hyperosmolarity (>300 mOsm/l) is essential in pathogenesis in all types of dry eye. Tear film instability in any of the three layers (lipid, aqueous, and mucous) is the key factor, and alteration of the regulatory mechanisms of the ocular surface results in activation of the inflammatory cascade. Inflammation is an underlying pathophysiological process in dry eye but not the primary one – it is a consequence of disrupted, already mentioned homeostatic mechanisms and exacerbation of pre-existing processes [5, 6, 7].

DEWS approved Delphi Panel classification of stages and disease severity according to clinical signs and symptoms is shown in Table 1 [3].

Long-term combined antiglaucoma therapy causes exacerbation of subclinical symptoms of dry eye or their occurrence due to preservatives or main substances. Clinical signs of OSD caused by antiglaucoma medications are not rare and cause suboptimal glaucoma control in non-compliant patients. Hyperemia and irritation due to the combined antiglaucoma therapy worsens OSD, disrupting patient's quality of life. Dry eye "screening" is not a part of glaucoma observation, but provides valuable data in assessing the OSD. POAG patients' complaints about the symptoms of OSD are present in more than 50% of



Scheme 1. Classification of dry eye

Table 1. Delphi Panel Scale grading of dry eye

DEWS Dry Eye Severity Grading Scheme				
Dry eye severity level	1	2	3	4*
Discomfort, severity and frequency	Mild and/or episodic, occurs under environ. stress	Moderate, episodic or chronic stress or no stress	Severe, frequent or constant without stress	Severe and/or disabling and constant
Visual symptoms	None or episodic mild fatigue	Annoying and/or activity limiting episodic	Annoying, chronic and/or limiting activity	Constant and/or possibly disabling
Conjunctival injection	None to mild	None to mild	+/-	+/++
Conjunctival staining	None to mild	Variable	Moderate to marked	Marked
Corneal staining (severity/ location)	None to mild	Variable	Marked/central	Severe punctate erosions
Corneal/tear signs	None to mild	Mild debris, meniscus	Filamentary keratitis, mucus clumping, tear debris	Filamentary keratitis, mucus clumping, tear debris, ulceration
Lid/meibomian glands	Meibomian gland dysfunction (MGD) variably present	MGD variably present	Frequent	Trichiasis, keratinization, symblepharon
Fluorescein tear break-up time	Variable	≤ 10 seconds	≤ 5 seconds	Immediate
Schirmer score	Variable	≤ 10 mm/5 min.	≤ 5 mm/5 min.	≤ 2 mm/5 min.

(Source: The Ocular Surface, April 2007, vol 5, No 2)
* Must have signs and symptoms; ↓ – increased; ↑ – decreased

patients. Also, symptoms increase with each drop containing the preservative benzalkonium chloride (BAK). The most common reason for using preservatives in ophthalmic drugs is the inhibition of microbial growth. BAK is a quaternary ammonium molecule, a cationic surfactant with detergent characteristics that disrupt tear lipid layer. This compound has been shown to cause tear film instability, loss of goblet cells, conjunctival squamous metaplasia and apoptosis, disruption of the corneal epithelium barrier, and damage to deeper ocular tissues [8–12].

A healthy eye surface is an essential finding in successful drug treatment and surgery outcomes (accurate keratometry, IOL calculations, and refractive outcomes). About 50% of patients already have preoperatively manifested dry eye and 50% are asymptomatic [7, 13]. In planning glaucoma surgery as a therapeutic option in the treatment of this chronic progressive optic neuropathy which may result in blindness, diagnosing and treating the problem of dry eye also has a significant role [14, 15].

OBJECTIVE

The aim of this study was retrospective analysis of the OSD incidence in open-angle glaucoma patients treated with topical medicamentous therapy in different disease stages, different combinations of drugs, treatment duration and intraocular pressure compensation as potential candidates for surgical glaucoma treatment in refractory cases and disease progression.

METHODS

The retrospective study included 80 patients or 160 eyes, out of which 40 eyes with diagnosed primary open-angle glaucoma (POAG), 40 eyes suffering from pseudoexfoliative glaucoma (XFG), 40 open-angle glaucoma eyes treated with tafluprost solution, and 40 healthy eyes without topical medicamentous treatment. Inclusion criteria were that all respondents were older than 30 years, previously diagnosed glaucoma in medicamentous treated groups with different duration, and no previous glaucoma surgery. Also, OSD was not previously diagnosed, none of them used artificial drops and there was no concomitant anterior segment pathology, including blepharitis chronica. None of the respondents were contact lens wearers. OSD screening and diagnostic tests were performed during glaucoma patient follow-up.

Performed ophthalmological examination included a questionnaire about experiencing dry eye symptoms that was presented and expressed results through OSD index score. The resulting score of the questions from the three groups related to the presence of symptoms of dry eye (A), symptoms in daily activities (B), and response to environmental factors (C) was added and expressed by the value of D, which is multiplied by 25 and divided with the number of questions with given answers, according to the following formula: OSD index = sum response × 25 / number of questions answered [16] (Figure 1).

The grading was performed according to the displayed color coded map or numerical scale of results as follows: normal finding (0–12), an incipient (13–22), moderate (23–32), and advanced (≥33) dry eye (Figure 2) [16, 17, 18].

The following clinical ophthalmological testing was used in the examination: TBUT test (tear breakup time, time of interrupted precorneal tear film), Schirmer's test I of tear volume production and vital staining (rose bengal) with grading according to Oxford scale after slit-lamp examination [19]. TBUT test is a measure of adequate tear film stability and mucus production. It is expressed as a period of time in seconds from the last blink until the breakup of the fluorescein stained tear film at random positions on the cornea. In evaluation, more than 10 seconds

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Have you experienced any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time
1. Eyes that are sensitive to light?	4	3	2	1	0
2. Eyes that feel gritty?	4	3	2	1	0
3. Painful or sore eyes?	4	3	2	1	0
4. Blurred vision?	4	3	2	1	0
5. Poor vision?	4	3	2	1	0

Subtotal score for answers 1 to 5 (A)

Have problems with your eyes limited you in performing any of the following *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
6. Reading?	4	3	2	1	0	N/A
7. Driving at night?	4	3	2	1	0	N/A
8. Working with a computer or bank machine (ATM)?	4	3	2	1	0	N/A
9. Watching TV?	4	3	2	1	0	N/A

Subtotal score for answers 6 to 9 (B)

Have your eyes felt uncomfortable in any of the following situations *during the last week*?	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
10. Windy conditions?	4	3	2	1	0	N/A
11. Places or areas with low humidity (very dry)?	4	3	2	1	0	N/A
12. Areas that are air conditioned?	4	3	2	1	0	N/A

Subtotal score for answers 10 to 12 (C)

Figure 1. Ocular Surface Disease Index Questionnaire (part I) [16]

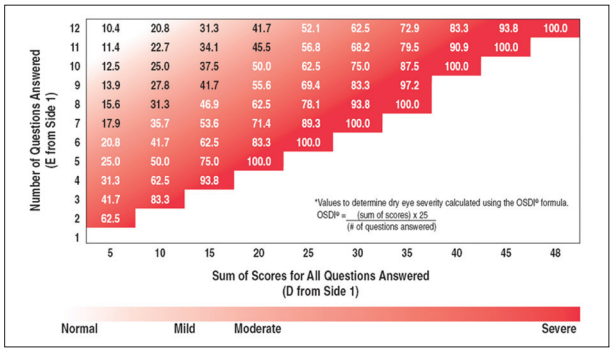

Figure 2. Ocular Surface Disease Index Questionnaire (part II) [16]

is a normal finding, less than 10 seconds is abnormal, and less than five seconds is clearly abnormal. It is standardized that TBUT is taken as mean value of three consecutive measurements.

Schirmer's test I is performed without topical anesthesia via a standardized filter paper strip and measures tear volume (basal and reflex) secretion. According to the test protocol, if more than 15 mm is measured during 5 minutes of testing, the value is considered normal. Length of moisture less than 10 mm is abnormal, and is evaluated as clearly abnormal if less than 5 mm [3, 20, 21, 22].

Ocular surface damage of the exposed eye is assessed by staining with vital dyes and graded against standard charts. There are three standard schemes used to estimate surface damage in dry eye, and in this study Oxford grading scale was used, showed in Figure 3.

Staining is represented by punctate dots on a series of panels (A–E). Staining ranges 0–5 for each panel and 0–15 for the total exposed inter-palpebral conjunctiva and cornea.

Dyes that could be used are lissamine green, fluorescein sodium and rose bengal, which we used. Estimation is made using slit-lamp to observe ocular surface. To grade

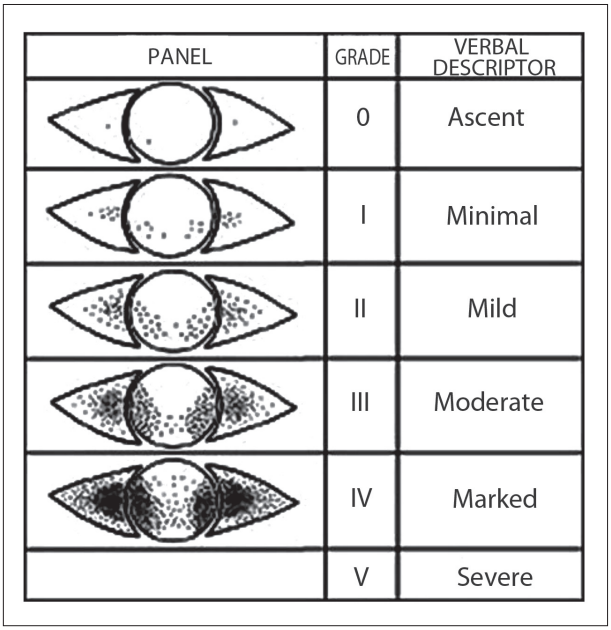

Figure 3. Vital dye (rose bengal) ocular surface disease staining according to Oxford Scale (source: The Ocular Surface, April 2007, vol 5, No 2)

the temporal zone of the conjunctiva, respondent looks towards their nose, and in grading the nasal zone of the conjunctiva, the subject looks to the opposite side, temporal bone [1, 20–23].

Delphi panel grading scale was used in estimation of the disease stages according to Table 1 [1]. More detailed subclassification of stage II is applied and differentiates dry eye as moderate (IIa) and moderately severe (IIb). The main differences are in TBUT values and epithelial integrity. In stage IIa, TBUT values are 10–15 seconds with punctiform epithelial staining; in stage IIb, TBUT values are 5–10 seconds with marked epithelial damage. In both stages, values of Schirmer's test are less than 10 mm [3].

The two groups of treated patients (POAG, XFG) already received antiglaucoma therapy as mono- or therapy combined of two or three drugs with different active substance or contained preservatives in different concentrations available in our country. The drugs contained Purite, which is not harmful, and BAK in different concentrations. These concentrations of preservatives were added if two or three drugs were applied. The third group was treated with monotherapy by tafluprost solution in single-dose containers, comprising EDTA and polysorbate 80. The fourth group did not use any drops, not even artificial tear drops.

Obtained results were analyzed by descriptive statistical analysis, tabular and graphical presentation of cumulative frequency curve in MS Office Excel, and by using SPSS 19 statistical software package (IBM Corp., Armonk, NY, USA) for testing proportions for categorical variables through χ^2 test.

RESULTS

Of all surveyed individuals, 65% were female and 35% were male. Similar sex ratio in POAG and XFG groups

Table 2. Distribution according to sex

Sex	Number of patients (number of eyes)				Total
	POAG group	XFG group	Group on tafluprost	Control group	
Female	12 (24)	9 (18)	16 (32)	15 (30)	52 (65.0%)
Male	8 (16)	11 (22)	4 (8)	5 (10)	28 (35.0%)
Total	20 (40)	20 (40)	20 (40)	20 (40)	80 (100.0%)

POAG – primary open-angle glaucoma; XFG – pseudoexfoliative glaucoma

Table 3. Age structure in tested groups

Age (years of life)	Number of patients (number of eyes)			
	POAG (gl. simplex) group	XFG group	Group on tafluprost	Control group
30–40	1 (2)	/	4 (8)	5 (10)
41–50	/	/	3 (6)	6 (12)
51–60	7 (14)	/	8 (16)	3 (6)
61–70	6 (12)	5 (10)	3 (6)	3 (6)
71–80	5 (10)	8 (16)	2 (4)	3 (6)
81–90	1 (2)	7 (14)	/	/
Total	20 (40)	20 (40)	20 (40)	20 (40)

Table 4. Classification of symptoms according to Ocular Surface Disease Index (OSDI) questionnaire

OSDI grade	Number of patients (%)			
	POAG group	XFG group	Group on tafluprost	Control group
Normal	8 (40.0)	3 (15.0)	18 (90.0)	16 (80.0)
Mild	5 (25.0)	3 (15.0)	2 (10.0)	2 (10.0)
Moderate	3 (15.0)	3 (15.0)	0	2 (10.0)
Severe	4 (20.0)	11 (55.0)	0	0

Table 5. Distribution of dry eye according to the Delphi panel scale

Delphi panel scale	Number of eyes (%)			
	POAG group	XFG group	Group on tafluprost	Control group
Normal	2 (5.0)	/	19 (47.5)	26 (65.0)
Grade I	13 (32.5)	/	15 (37.5)	6 (15.0)
Grade IIa	10 (25.0)	10 (25.0)	4 (10.0)	3 (7.5)
Grade IIb	15 (37.5)	24 (60.0%)	2 (5.0)	5 (12.5)
Grade III	/	6 (15.0)	/	/
Grade IV	/	/	/	/
Total	40 (100)	40 (100)	40 (100)	40 (100)

was found, but more prevalent female sex in the control and the tafluprost treated group is evident (Table 2).

Since glaucoma and OSD frequently occur in the elderly population, age structure in the investigated groups was analyzed and showed population older than 50 years in the POAG group and older than 60 years in the XFG group. In comparison to the tafluprost solution treated group, younger population was noticed, with approximate age distribution of 30–60 years, similar to the results in the control group (Table 3).

According to the symptoms and OSD index, the distribution in the POAG group was as follows (Table 4): 40% normal, 25% incipient, 15% moderate, and 20% had advanced symptoms. In the XFG group, the distribution was the following: 15% normal, 15% incipient, 15% moderate, and 55% of advanced symptoms. In the tafluprost treated group, 90% of the surveyed individuals showed normal findings, and 10% were incipient. OSD index distribution

Table 6. Classification based on types of dry eye

Type of dry eye	Number of eyes (%)			
	POAG group	XFG group	Group on tafluprost	Control group
Normal	16 (40.0)	6 (15.0)	19 (47.5)	26 (65.0)
Hyposecretory	4 (10.0)	6 (15.0)	4 (10.0)	4 (10.0)
Evaporative	10 (25.0)	16 (40.0)	13 (32.5)	6 (15.0)
Mixed type	10 (25.0)	12 (30.0)	4 (10.0)	4 (10.0)
Total	40 (100)	40 (100)	40 (100)	40 (100)

Table 7. Distribution of therapy duration among respondents

Therapy duration	Number of eyes (%)		
	POAG group	XFG group	Group on tafluprost
<1 year	10 (25)	4 (10)	20 (50)
1–5 years	18 (45)	36 (90)	20 (50)
>5 years	12 (30)	/	/
Total	40 (100)	40 (100)	40 (100)

Table 8. Distribution according to the number of drugs (bottles) among respondents

Number of bottles	Number of eyes (%)	
	POAG group	XFG group
1	14 (35)	8 (20)
2	12 (30)	10 (25)
3	14 (35)	22 (55)
Total	40 (100)	40 (100)

in the control group was as follows: 80% normal findings, 10% incipient, and 10% moderate symptoms of dry eye.

According to the Delphi panel scale in POAG (Table 5), the distribution was as follows: 32.5% grade I, 25% grade IIa, and 37.5% grade IIb. In the XFG group the distribution was as follows: 25% grade IIa, 60% grade IIb, and 15% grade III. In the tafluprost group the distribution was the following: 47.5% were healthy eyes, 37.5% grade I, 10% grade IIa, and 5% grade IIb. In the control group the distribution was as follows: 65% were healthy eyes, 15% grade I, 7.5% grade IIa, 12.5% grade IIb.

Classification according to the type of dry eye was done (Table 6). In the POA glaucoma group, 10% of hyposecretory, 25% of the evaporative, and 25% of mixed form was diagnosed. In the XFG group, 15% hyposecretory form, 40% of the evaporative, and 30% of mixed form was diagnosed. In the POAG group treated with tafluprost antiglaucoma solution, 10% hyposecretory, 32.5% evaporative, and 10% of mixed form was determined.

Manifestation of symptoms and signs of OSD depends on the duration of glaucoma therapy, on the number of antiglaucoma agents, and it is also estimated according to the type and concentration of preservatives. Distribution of therapy duration among respondents in therapy groups is shown in Table 7. The most prevalent time interval of applied antiglaucoma drugs in all three treated groups was between one and five years. Relation of applied number of antiglaucoma agents (bottles of drugs) in the treated groups of respondents to the type of glaucoma (POAG; XFG) is presented in Table 8.

Cumulative frequency of BAK preservative-applied combined therapy is illustrated in the diagram (Graph 1)

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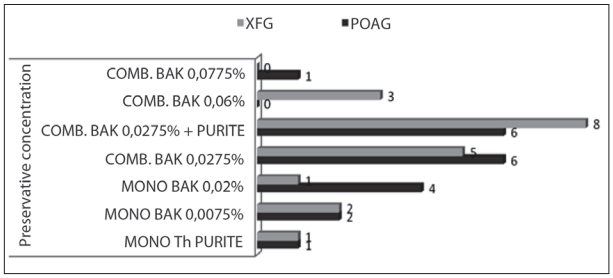

Graph 1. Benzalkonium chloride (BAK) concentrations in primary open angle glaucoma (POAG) and pseudoexfoliative glaucoma (XFG)

Table 9. Benzalkonium chloride (BAK) concentrations in primary open angle glaucoma (POAG) and pseudoexfoliative glaucoma (XFG)

BAK concentrations		Number of patients	
		POAG glaucoma	XFG glaucoma
Monotherapy	Purite	1	1
	BAK 0.0075%	2	2
	BAK 0.02%	4	1
Combined therapy	BAK 0.0275%	6	5
	BAK 0.0275% + Purite	6	8
	BAK 0.06%	0	3
	BAK 0.0775%	1	0

and in Table 9 and correlates to the types of dry eye in both POAG and XFG group and presents most frequently applied 0.0275% of cumulative BAK.

High correlation of dry eye type to different concentrations of applied BAK and number of applied drugs was determined using χ^2 test ($\chi^2 = 0.087$, likelihood ratio $p < 0.021$).

DISCUSSION

Both glaucoma and dry eye are multifactorial and very prevalent diseases. Glaucoma is the second most common cause of blindness in the world; on the other hand, OSD is one of the main diagnoses in ophthalmological practices. OSD prevalence shows large variation in general population of up to 33%, but in glaucoma patients was found to be present in more than 52.6% [2, 24, 25]. In our study group, OSD prevalence and characteristics were analyzed in glaucoma patients, starting with epidemiologic factors, female sex was more prevalent in all respondents (65% vs. 35%), and also more prevalent in the tree groups of glaucoma patients (POAG, XFG and open-angle glaucoma treated with tafluprost) excluding control group, with the ratio of 61.67% of females versus 38.33% of males. A similar sex distribution was found in a comprehensive study by Erb et al. [26] resulting with German Register for Glaucoma Patients with Dry Eye – 60.9% females vs. 39.1% males.

In relation to age, our study based on a small population sample was in accordance with evidence that glaucoma is a disease of elderly population and that dry eye prevalence increases with age. Purpose of the German Register was to determine the links between glaucoma, age, concomitant disease, medication, and dry eye in a large group of glaucoma patients, showing OSD incidence from 31.3% in people younger than 40 years, to 61.6% in patients older than 90 years, according to the German study [26]. Our respondents were mostly older than 60 years in the POAG and the XFG group, but younger than 60 years in open-angle glaucoma group treated with tafluprost, and the control group. Therefore, incidence of dry eye in glaucoma treated groups was expected to be higher than that in the control group.

Questionnaires about presence of dry eye symptoms are included in epidemiologic or clinical research to screen individuals for prevalence of dry eye or in clinical practice to assess the diagnosis, to grade disease severity and estimation of treatment. Increased incidence of moderate and advanced OSD index grades was noticed due to the presence of symptoms of dry eye in the of POAG and XFG groups of our respondents. Open-angle glaucoma group treated with tafluprost (medication without preservative) showed symptoms of dry eye similar to healthy control group, with 90% of normal results and 10% of mild symptoms. In our groups, experienced symptoms of ocular discomfort and dry eye grading to Ocular Surface Disease Index (OSDI) questionnaire were as follows: 60% in the POAG group patients, 85% in the XFG group, 10% in tafluprost treated patients, and 20% in the control group. OSDI score could be a good predictor of preservative toxicity and ocular surface damage, as our treated respondents indicated. Thus, as OSDI score is growing, a patient's quality of life is decreasing. [1, 21, 27].

The most prevalent grades of dry eye according to DEWS grading system are IIa and IIb in both glaucoma groups (POAG and XFG groups). This differs from the most prevalent grade I (37.5%) in tafluprost treated patients, and 65% of normal eyes in the control group. The highest percentages of grade IIb, based on Delphi panel scale in POAG and XFG treated groups, indicate not only high association with dry eye, but these values also indicate the type of glaucoma due to therapy response to topical medications. It is obvious that XFG in all these groups showed the most severe grades (60% IIb), with characteristic clinical signs and changes of the anterior segment of the eye. In addition to exfoliative keratopathy, tear function disorder was analyzed in a study by Kozobolis et al. [28, 29]. Schirmer's and TBUT test show reduced tear production and instability of tear film as disorder of reduced mucin [28]. Electron microscopy indicates that PEX material could be confirmed in conjunctival stroma [29]. This explains why XFG is in low proportion in general population of glaucoma patients (20% of open-angle glaucoma patients, or 5.2% of the population in the German study [26]), but with high incidence of OSD in these treated groups.

Evaporative form of OSD is the most common in all three treatment groups, and indicates disruption of lipid tear film layer, but mixed and evaporative form are more prevalent in therapeutic groups with preservative drops (POAG, XFG).

The duration of glaucoma also plays a role in OSD occurrence. OSD prevalence in POAG (60%), XFG (85%)

and tafluprost group (52.5%) in all treated patients corresponds to more than one year of therapy, respectively (75% vs. 90% vs. 50%), in treated groups. Similar distribution is in the applied number of drugs, more precisely bottles, because each bottle contains its own preservative in different concentration, which means that applied monotherapy and combined therapy affects ocular surface differently in POAG and XFG.

OSD prevalence increases with the number of antiglaucoma drugs and duration of their application.

Prevalence of OSD increases in relation to number of antiglaucoma agents due to active substance and mostly preservative containing drops. Preservatives could be analyzed by the type and concentration of preservative contained in a bottle of an antiglaucoma drug. Apart from preservative-free agents, most of glaucoma drops contain variable degrees of BAK, whose harmful effects – disturbed tear film, inflammation of conjunctiva, cytotoxicity to cornea – are well known. In a study by Leung et al. [2] after correcting for age and gender, every additionally administered BAK-containing anti-glaucoma agent resulted in a two-fold increase in the incidence of ocular surface lesions found on lissamine green staining.

A high percentage of dry eye in the treatment groups with high concentration of applied preservatives (mostly BAK) detects the causative factor. All our treated groups showed more than 50% prevalence of dry eye, similar to other authors and the mentioned German study [2, 26, 30, 31]. In our treated groups (POAG, 60%; XFG, 85%; tafluprost group, 52.5%), incidence of OSD correlates with higher BAK level, number of applied drugs (drops), and therapy duration.

Clinical and experimental studies have demonstrated that OSD is common in glaucoma patients receiving glaucoma drops, and that the preservatives in these drops play a major role in the occurrence of OSD [32].

CONCLUSION

Ocular surface changes may induce both symptoms reported by patients and anterior segment clinical signs, detected by ophthalmologist, and should be systematically assessed during examination in all glaucoma patients. Preservative-free drops are associated with lower OSD incidence. In patients with OSD, reducing the amount of preservatives administered using fixed drug combinations should be advised. Adding artificial tear drops could be useful. According to the DEWS recommendations, preserved medications should be replaced by those without preservatives whenever possible. Generally, in order to improve compliance of patients, quality of life, and treatment, combinations of drugs with lower BAK concentrations, less toxic formulations (Purite, polyquaternium) or drops without preservatives are recommended. Also, timely diagnoses and treatment of dry eye is very important as well-timed indication for surgery treatment of glaucoma in certain situations for successful surgical outcome.

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NOTES

The paper is part of Marija Radenković's master thesis and presents some of the results that will be processed and published in the thesis.

A portion of the results presented in the paper was also presented at the Second Congress of Glaucomatologists of Serbia, held on May 15–16, 2015, Kopaonik, Serbia.

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Инциденца болести површине ока код пацијената са глаукомом отвореног угла

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КРАТАК САДРЖАЈ

Увод Болест површине ока (БПО) мултифакторијелно је обољење суза и површине ока које резултује симптомима дискомфора, сметњама вида и нестабилношћу сузног филма са потенцијалним оштећењем површине ока. Ово обољење праћено је повећаном осмоларношћу сузног филма и инфламацијом површине ока. Последица је нарушене хомеостазе лакрималне функционалне јединице. Основни патомеханизам настанка је услед хиперосмоларности суза и нестабилности сузног филма. Етиолошки су класификоване две форме: хипосекреторна (Су Сјогрен и нон-Сјогрен) и евапоративна (*extrinsic* и *intrinsic*). Стадијуме градира Делфи панел класификација. Антиглаукомна терапија проузрокује егзацербацију или настанак симптома сувог ока услед дејства основне супстанце или конзерванса (бензалконијум – БАК), која је дозно и временски зависна. БАК редукује стабилност липидног слоја суза, број пехарастих ћелија, индукује апоптозу и инфламаторну инфилтрацију.

Циљ рада Циљ истраживања је била анализа инциденце БПО код пацијената са глаукомом отвореног угла на топикалној медикаментозној терапији.

Методе рада Ретроспективна анализа прегледаних пацијената са глаукомом отвореног угла.

Резултати Повећана је инциденца индекса БПО и то умерених и узнапредовалих градуса у групи примарног глаукома отвореног угла и псеудоексфолијативног глаукома. Делфи панел скала: најзаступљенији је градус *IIb* (у *POAG* и псеудоексфолијативном глаукому). Евапоративна форма БПО је најзаступљенија у све три терапијске групе. Високи проценат сувог ока потврђен је код пацијената са апликованом највишом концентрацијом конзерванса.

Закључак БПО би требало правовремено дијагностиковати и лечити. Суво око има утицаја на постоперативни хирургшки исход и видну оштрину, те ради побољшања комплијансе и квалитета живота пацијената треба кориговати знаке сувог ока и применити лекове без конзерванса.

Кључне речи: глауком; болест површине ока; суво око; конзерванс

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Useful histological findings in incisional biopsies of oral squamous cell carcinoma

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SUMMARY

Introduction Oral squamous cell carcinoma (OSCC) is one of the most common head and neck cancers.

Objective The aim of this study was to investigate the histopathological features of OSCC specimens obtained from incisional biopsies and to alert clinicians to the importance of more representative biopsies.

Methods Forty-eight OSCC samples were obtained from incisional biopsies and classified by Bryne's score. The following morphological features were analyzed: invasive front, invasiveness, apoptotic cells, atypical mitosis, giant cells, acantholysis, ulceration, necrosis, calcification, surface epithelium, granulation tissue, desmoplasia, tissue invasions, inflammatory infiltrate and tumor thickness.

Results Ten (21%) cases were classified as high grade malignancies and 38 (79%) as low grade. Apoptotic cells (n = 26), atypical mitosis (1–2/20×; n = 38), giant cells (n = 8), acantholysis (n = 5), necrosis (n = 5), calcification (n = 1), granulation tissue (n = 32), desmoplasia (n = 4), perineural invasion (n = 2), muscular invasion (n = 8), invasion of salivary gland tissue (n = 3), vascular invasion (n = 10), and chronic inflammation (n = 33) were observed. Vascular invasion (p = 0.04, Pearson's χ^2 test) and necrosis (p = 0.04, Pearson's χ^2 test) were significantly associated with cases of high-grade malignant tumors. Atypical mitosis was associated with a greatest tumor thickness (p = 0.04, Fischer's exact test).

Conclusion This study suggests that incisional biopsies may be useful and significant as they can show histopathological variables that are important to classify oral squamous cell carcinomas into low grade and high grade according to Bryne's score, which was used in this study. Thus, more representative biopsies might be useful to achieve this and allow a more accurate planning.

Keywords: oral neoplasms; oral cancer, diagnosis; histopathological staging; carcinoma, squamous cell/pathology; carcinoma, squamos cell/surgery.

INTRODUCTION

Squamous cell carcinoma (SCC) is one of the most common head and neck cancers. Traditional risk factors such as smoking and alcohol consumption are frequently associated with this cancer [1]. Local invasion, regional lymph node involvement and distant metastasis are clinical parameters used to define the TNM stage (T: size or extent of the primary tumor; N: number of lymph nodes involved; M: presence or absence of metastases). This staging system continues to be the best predictor of prognosis and a fundamental tool for the definition of therapy [1, 2]. However, the TNM system does not consider individual histological features of tumors [3]. In this respect, tumors classified as being in an early stage and treated adequately may present an unfavorable evolution and may be fatal, whereas patients with more advanced tumors that have a poor prognosis can exhibit a favorable evolution, demonstrating a lack of accuracy of the TNM system in establishing a prognosis [3].

Careful evaluation of the histopathological features of SCC is of fundamental importance for its diagnosis. In addition, it provides an important parameter for the adoption of therapeutic management and to predict the possible clinical course of the disease [4]. Several histological grading systems designed to predict the biological behavior of oral squamous cell carcinoma (OSCC) have been proposed [3, 5–8]. Furthermore, various histological parameters of prognostic value are being studied: inflammatory infiltrate, tumor thickness, degree of keratinization, depth of tumor invasion, pattern of invasion, perineural invasion, vascular invasion, eosinophilia and foreign body reaction, among other morphological features [4, 6, 9, 10].

According to Foschini et al. [11], incisional biopsies from early oral SCCs could provide prognostic parameters to assess metastatic potential and avoid unnecessary dissection.

OBJECTIVE

The objective of the present study was to investigate retrospectively the histopathological features of 48 cases of OSCC obtained from incisional biopsies, diagnosed at the Laboratory of Surgical Pathology in the School of Dentistry

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of the Federal University of Bahia (Universidade Federal de Bahia – UFBA), in order to permit oral pathologists to issue a more detailed histopathological report and to alert clinicians to the importance of more representative biopsies.

METHODS

Specimens

In a retrospective study, OSCC specimens stored from 2002 to 2011 in the archives of the Surgical Pathology Service, School of Dentistry, UFBA, were analyzed. The sample consisted of 48 formalin-fixed paraffin-embedded (FFPE) oral SCC specimens obtained from incisional biopsies. The inclusion criterion was sufficient biological material in good condition in the paraffin blocks. Sufficient biological material was defined as stromal tissue infiltrated by an evident squamous carcinoma and useful for histopathological diagnosis. Clinical data such as gender, age, tumor location and gross pathology of the tumor were obtained from the anatomopathological records. This study was approved by the Ethics Committee of the School of Dentistry (Protocol # 235.138), Federal University of Bahia (FOUFBA).

Histopathological analysis

For histological analysis, 5 µm thick sections were cut from all FFPE specimens. The slides of each case were stained with hematoxylin-eosin and submitted to a light microscope histological assessment by two different examiners. The cases were evaluated according to the system proposed by Bryne et al. [8], which takes into consideration four morphological features: degree of keratinization, nuclear pleomorphism, pattern of invasion, and inflammatory infiltrate. The mean score was obtained by the sum of scores attributed to each morphological feature divided by the number of parameters used, in order to prevent possible bias. A score of 1.0 to 2.5 was classified as low grade of malignancy and a score of 2.6 to 4.0 as high grade of malignancy. Furthermore, the following morphological features were analyzed as described by Woolgar and Triantafyllou [12]: invasive front (obvious/confusing), and invasiveness (microinvasive/invasive), giant cells, desmoplasia, perineural invasion, muscular invasion, glandular invasion, vascular invasion, presence of apoptotic cells, atypical mitosis (1–2/20×), acantholysis, ulceration, necrosis, calcification, surface epithelium and granulation tissue. The presence of stromal and parenchymal eosinophils [9], lymphocytes and neutrophils [13] was also evaluated. All of histopathological features were compared with histological grade of malignancy. In addition, the tumor thickness [14] of the incisional biopsies was compared with all histological findings. The tumor thickness was measured from de surface of the tumor to the deepest point of invasion.

Statistics

Differences between groups were evaluated using Pearson's χ^2 test or Fisher's exact test. All data were analyzed with the Minitab® 15 (Minitab Inc., State College, PA, USA) statistical software. A $p < 0.05$ was set to be statistically significant.

RESULTS

Thirty-two (66.7%) of the 48 OSCC cases occurred in men, and 16 (33.3%) occurred in women. Patient age ranged from 27 to 92 years (58.12 ± 13.47 years). Twenty-nine patients were 60 years old or younger (49.67 ± 6.20), and 19 were older than 60 years (72.45 ± 9.85). The most common site of OSCC was the floor of the mouth (n = 14), followed by the tongue (n = 13), palate (n = 9), gingiva (n = 4), lower lip (n = 1), buccal mucosa (n = 3) and vestibule mucosa (n = 1). Some SCCs did not have any site data (n = 3). The gross pathology ranged from 0.8 mm to 22 mm (mean: 9.1 mm).

A summary of histopathological findings is presented in Table 1, and Figures 1A–D highlight several histopathological findings. Using the classification of Bryne et al. [8], 10 (21%) cases were classified as high grade of malignancy and 38 (79%) as low grade (Tables 1 and 2).

Table 1. Morphological features according to the system proposed by Bryne et al. [8]

Histopathological features	n = 48	(%)
Keratinization (grade)		
1 – intense	13	27.1
2 – moderate	5	10.4
3 – minimal	20	41.7
4 – absent	10	20.8
Nuclear pleomorphism (grade)		
1 (>75% mature cells): minimal	20	41.7
2 (50–70% mature cells): moderate	15	31.2
3 (25–50% mature cells): abundant	7	14.6
4 (0–25% mature cells): extreme	6	12.5
Pattern of invasion (grade)		
I	14	29.2
II	24	50
III	9	18.7
IV	1	2.1
Lymphoplasmacytic infiltrate (grade)		
1 – intense	16	33.4
2 – moderate	10	20.8
3 – mild	21	43.7
4 – absent	1	2.1

n – number of cases

With respect to greatest tumor thickness and histological findings, only atypical mitosis was significantly associated with greater tumor thickness, in which those OSCCs with a tumor thickness greater than 9 mm showed a high incidence of mitosis ($p = 0.04$, Fisher's exact test) (Table 3).

This study found a chronic inflammatory infiltrate in 33 cases in addition to mixed (n = 12) and acute (n = 2)

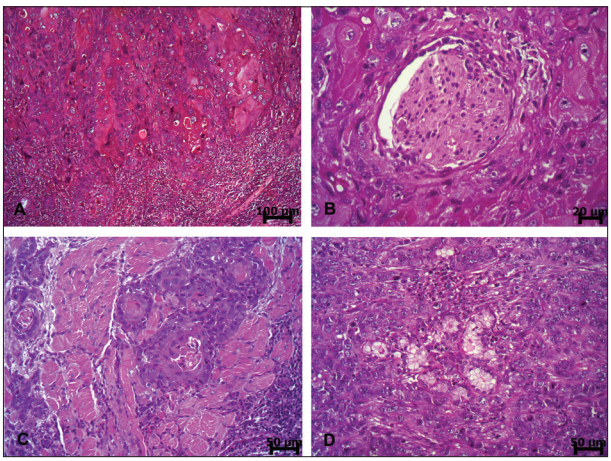

Figure 1. A – low grade OSCC with obvious invasive front (hematoxylin and eosin staining); B – low grade OSCC exhibiting perineural invasion (hematoxylin and eosin staining); C – high grade OSCC exhibiting muscular invasion (hematoxylin and eosin staining); D – high grade OSCC invading salivary gland (hematoxylin and eosin staining)

Table 2. Histopathological features of the OSCCs and malignancy grade

Histopathological features	High grade (n = 10)	(%)	Low grade (n = 38)	(%)	p*
Necrosis	2	4.2	3	6.3	0.04
Calcification			1	2.11	
Acantholysis			5	10.4	0.19
Ulceration	3	6.2	14	29.2	0.68
Surface epithelium	8	16.7	35	72.9	0.13
Granulation tissue	8	16.7	28	58.3	0.68
Giant cells	1	2.1	9	18.7	0.34
Vascular invasion	4	8.4	6	12.5	0.04
Perineural invasion	1	2.1	1	2.1	0.56
Glandular invasion			3	6.2	0.16
Muscle invasion	1	2.1	7	14.6	0.52
Desmoplasia	1	2.1	3	6.2	0.62
Atypical mitoses	7	14.6	26	54.2	0.1
Apoptotic cells	2	4.2	24	50	0.72
Lymphocytes	4	8.4	13	27.1	0.73
Neutrophils	1	2.1	3	6.2	0.79
Eosinophils	2	2.1	5	10.4	0.58

* Pearson's χ^2 test
n – number of cases

inflammatory infiltrates and a confusing front in 34 cases. We did not observe microinvasive cancer. In addition, there was no association between clinical data and histological features ($p > 0.05$, Fisher's exact test).

Association between the morphological parameters and malignancy grade

Inflammatory infiltrate and malignancy grade

Lymphocytes invading the tumor parenchyma were observed in 17 (35.4%) cases, most of them low-grade malignant tumors (n = 13, 76.5%), but the result was not statistically significant ($p = 0.73$, Pearson's χ^2 test). Similarly, neutrophils invading the tumor parenchyma were observed in four (8.3%) cases, three of them low-grade malignant tumors ($p = 0.79$, Pearson's χ^2 test). In addition, eosinophils were detected in seven (14.6%) cases, most of them (n = 5) low-grade malignant tumors ($p = 0.58$, Pearson's χ^2 test).

Table 3. Histopathological features of the OSCCs and tumor thickness.

Histopathological Features	Tumor Thickness		p*
	>3, ≤9 m (n)	>9 mm (n)	
Inflammatory infiltrate			
Present	18	26	0.28
Absent		4	
Keratinization			
Present	14	24	0.14
Absent	1	9	
Necrosis			
Present	1		
Absent	13	34	1.00
Calcification			
Present	1		0.29
Absent	13	34	
Acantholysis			
Present	3	2	0.14
Absent	11	32	
Ulceration			
Present	6	11	0.52
Absent	8	23	
Surface epithelium			
Present	13	30	1.00
Absent	1	4	
Granulation tissue			
Present	11	25	1.00
Absent	3	9	
Giant cells			
Present	4	6	0.45
Absent	10	28	
Vascular invasion			
Present	5	6	0.26
Absent	9	28	
Perineural invasion			
Present		2	1.00
Absent	14	32	
Glandular invasion			
Present		3	0.54
Absent	14	31	
Muscular invasion			
Present	3	4	0.40
Absent	11	30	
Desmoplasia			
Present	1	3	1.00
Absent	13	31	
Atypical mitosis			
Present	8	30	0.04
Absent	6	4	
Apoptotic cells			
Present	2	24	0.00
Absent	12	10	

*Fisher's exact test
n – number of cases

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Vascular, glandular, muscle, and perineural invasion and malignancy grade

No significant association was observed between grade of malignancy and glandular (p = 0.16), muscle (p = 0.52) or perineural invasion (p = 0.56, Pearson's χ^2 test for all associations). However, vascular invasion was significantly associated with cases of high-grade malignant tumors (p = 0.04, Pearson χ^2 test).

Apoptotic cells and malignancy grade

Apoptotic cells were detected in 26 (54.1%) cases; only two of them were high-grade malignant SCCs, and 24 were low-grade tumors. There was no statistically significant association (p = 0.72, Pearson's χ^2 test).

Giant cells and malignancy grade

Giant cells were detected in 10 (20.8%) cases, including only one high-grade malignant tumor. No association was observed between the presence of giant cells and grade of malignancy (p = 0.34, Pearson's χ^2 test).

Necrosis and malignancy grade

Necrosis was observed in five (10.4%) cases and was associated with high grade of malignancy (p = 0.04, Pearson's χ^2 test).

DISCUSSION

Although oral pathologists encounter difficulties in the histopathological analysis of incisional biopsies of OSCCs due to their size and depth, it is important that the histopathological reports include detailed morphological information with more precise definitions and descriptions rather than only the proposed diagnostic. Careful description of the observed histopathological features can be of high prognostic value and may contribute to the choice of treatment in some cases. Sometimes the incisional biopsy may not be representative of the entire tumor or may not comprise the more advanced part of the tumor, and a new biopsy may therefore be required. In view of these considerations, the present study evaluated a series of cases of OSCC, focusing on these individual histopathological features in incisional biopsies, in an attempt to associate these features with the histological grade of malignant tumors and other aspects such as the greatest diameter of the incisional biopsies. These histopathological features are discussed below.

The invasive front of the tumor is not always obvious [12], as observed in the present study, in which most cases exhibited an invasive front with a confusing pattern. According to Woolgar and Triantafyllou [12], it is important that an incisional biopsy be of sufficient size and depth to include part of the advancing invasive front of the tumor. Ideally, the deep invasive front should be included, but if not, as in the case of large tumors, the peripheral (lateral) front is often sufficiently representative to permit provisional evaluation. In the present study, the advancing front was not always included, with many biopsies not having the ideal size or depth to permit visualization of the invasive front.

Different regions of the same tumor frequently exhibit different patterns of invasion, but the most aggressive pattern should be recorded. For this purpose, Bryne et al. [8] proposed a traditional classification evaluating the predictive value of the pattern of invasion at the tumor interface on a scale from I to IV: pattern I, well-demarcated, broad tumor invasion; pattern II, finger-like tumor invasion or through separate tumor islands, with a stellate appearance, forming strands, cords or solid bands; pattern III, invasive islands of the tumor with more than 15 cells per island; pattern IV, invasive tumor islands with fewer than 15 cells per island, including a single invasive cell. This scale was expanded by Brandwein-Gensler et al. [6], who included pattern V, defined by the presence of dispersed satellites at a distance of 1 mm or more from the tumor. In the present study, the invasion patterns I and II were predominated.

Some authors suggested the classification of a tumor as microinvasive if it is confined to the papillary lamina propria, or as superficially invasive if it remains confined to the reticular (deep) lamina propria without involvement of submucosal tissues. However, the term "superficially invasive" cannot be applied to tumors located in the alveolar process of the mandible or in the gingival mucosa due to the absence of submucosa [12]. In the present study, there were only four cases of OSCC in the gingival mucosa and all of them were low-grade tumors.

Accumulation of giant cells in SCCs is a rare finding but has been reported in cutaneous SCCs [4, 15]. In the present study, giant cells were detected in eight cases of OSCC. Despite the non-specificity of giant cells observed in this study, it was not possible to associate the presence of these cells with the reactive nature of the tumor, although macrophages were frequently observed in the center of some tumor islands, and a foreign body-type giant cell reaction may occur near areas of keratinization.

Desmoplasia is a histological pattern characterized by the presence of hyaline stroma and a minimal cell infiltrate consisting of spindle-shaped tumor cells or, occasionally, by a fascicular pattern [12, 16]. In non-cutaneous tumors, the presence of desmoplasia has been associated with a malignant phenotype [16]. In the present study, desmoplasia was observed in four cases of OSCC. Despite the importance of this finding, little attention has been given to desmoplasia in histological grading systems of OSCCs. However, its presence seems to be important for tumorigenesis and metastasis [12].

Perineural invasion is an important predictor of survival in patients with SCC of the oral cavity and oropharynx [17]. According to Chatzistamou et al. [14], the shape of the tumor (ill- or well-defined) is also associated with the presence of perineural invasion, as its incidence is almost twice as high for ill-defined tumors as for well-defined ones. Other authors report that tumors presenting peri-

neural invasion are more likely to develop metastases [18]. The depth of the biopsy may have influenced this finding, as only two cases exhibited perineural invasion. However, in 202 tongue SCCs, Rodrigues et al. [2] observed perineural invasion in almost 15% of samples.

There is extensive discussion about whether sialadenotropism should be regarded as invasion, considering its association with increased local recurrence [19]. It is therefore important to include the depth and extent of SCCs in the histopathological report [12]. In the present study, glandular invasion was observed in only three cases of SCC. These were low-grade malignant tumors that occurred in the floor of the mouth, tongue border, and retromolar region, sites where well-differentiated glandular tissue is usually found.

Vascular invasion can be identified when tumor cell aggregates are found within clear spaces that are completely lined by endothelial cells. Invasion of thin-walled vessels is more common, whereas invasion of muscular vessels is rare [12]. According to Rodrigues et al. [2], vascular invasion is more frequent than perineural invasion in tongue SCC, and this was also seen in this study in incisional biopsies. In the present study, four (36.3%) of the 11 cases with vascular invasion were high-grade malignant tumors and this difference was significant. According to Chandler et al. [20], muscular invasion influences the type and extent of treatment of OSSC. In the present study, muscular invasion was observed in only eight cases of OSCC, most commonly in the tongue.

In this study, apoptotic cells were detected in 26 (54.1%) cases of OSCC. Apoptosis plays an important role in the removal of aberrant cells that can lead to the development of tumors. The relationship between cell growth and cell death (apoptosis) in cancer will predict the rate of tumor growth. Apoptosis is increased in pre-malignant and malignant oral lesions and is the main pathway of cell regulation [21]. On the other hand, according to Aoyama et al. [22], the presence or absence of mitosis is a significant indicator of neoplastic atypical squamous lesion of the uterine cervix. In the present study, atypical mitosis was found in 38 cases of OSCC and their presence was found significant in those cases with tumor thickness larger than 9 mm.

In the present study, 10.4% (n = 5) of OSCCs exhibited tumor islands with acantholysis. Honings et al. [23] found no association between the presence of acantholysis and tumor prognosis in SCC of the trachea.

The degree of keratinization of oral SCCs is a parameter of the histological grading system proposed by Bryne et al. [8]. According to Matos et al. [10], keratinization tends to be low in metastatic SCCs. This seems to be true since in the present study no keratinization was observed in 70% (n = 7) of high-grade malignant SCCs.

A surface epithelium was seen in most of the OSCC cases studied (n = 43). This finding was significant when associated with well-differentiated OSCC. Occasionally, the origin of the overlying surface epithelium is less obvious in some tumors present in the lamina propria or submucosa. This is often the case when the area biopsied is an intact area adjacent to the tumor [12]. On the other hand, in a 2013 study of 36 OSCCs, 50% showed ulceration [24], but this feature was present in 37 cases of the present study. Other minor findings included calcification and necrosis. Necrosis was observed in five (10.4%) of the cases studied and was associated with high grade of malignancy. With respect to necrosis, Honings et al. [23] found no association between the presence of this histological feature and tumor prognosis in SCC of the trachea.

The chronic inflammatory response influences the prognosis of SCC, with a high-density [6, 9, 14] or low-density [10] infiltrate being associated with a better prognosis or regional metastases, respectively. According to Chatzistamou et al. [14], the presence of a stromal chronic inflammatory infiltrate is associated with the shape of the tumor (ill- or well-defined). In this respect, well-defined tumors exhibit an intense chronic inflammatory infiltrate and are associated with a favorable prognosis. On the other hand, tumors not associated with stromal inflammation exhibit a more aggressive growth pattern accompanied by a more expansive shape (ill-defined). However, there is clear evidence that the immune and inflammatory cells can invoke both tumor-promoting and tumor-antagonizing effects, depending on the context and cell types [2].

The role of eosinophils in carcinogenesis is still a matter of discussion. In the present study, eosinophils were more frequently detected in low-grade malignant tumors (74.1%), but the difference was not significant. Despite this, Goldsmith et al. [25] showed eosinophilia to be the variable that most influenced a less aggressive clinical course of SCCs. Lundqvist et al. [9] found no prognostic value in the presence of eosinophils in most of the SCC cases studied, and there was no correlation with treatment response or recurrence.

Tumor-infiltrating neutrophils have been associated with a poor clinical course of different types of cancer [18]. In the present study, neutrophils invading the tumor parenchyma were observed in four cases of SCC, three of them low-grade malignant tumors. These findings differ from those found by Trellakis et al. [13], who found that a high neutrophil infiltration was independently associated with a poor prognosis of head and neck SCCs. Further studies are necessary to clarify this matter.

In conclusion, this study suggests that incisional biopsies may have considerable significance as they can show histopathological variables that are important to classify OSCCs into low grade and high grade in accordance with Bryne's score used in this study. Thus, more representative biopsies might be useful to achieve this and allow a more accurate planning.

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Значај инцизионе биопсије оралног сквамоцелуларног карцинома за валидност хистопатолошког налаза

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КРАТАК САДРЖАЈ

Увод Орални сквамоцелуларни карцином (ОСЦК) један је од најчешћих канцера главе и врата.

Циљ рада Циљ ове студије био је да се истраже хистопатолошке особине узорака ОСЦК добијених инцизијалним биопсијама, те да се скрене пажња клиничарима на важност репрезентативнијих биопсија.

Методе рада Обухваћена су 48 узорка ОСЦК добијена инцизијалним биопсијама и класификованих према Бринеовој скали. Анализиране су следеће карактеристике: инвазивни фронт, инвазивност, апоптотичне ћелије, атипична митоза, џиновске ћелије, акантолиза, улцерација, некроза, калцификација, површински епителијум, гранулационо ткиво, десмоплазија, инвазије ткива, инфламаторни инфилтрат и дебљина тумора.

Резултати Десет (21%) случајева класификовано је као малигнитет високог степена, а 38 (79%) као малигнитет ниског степена. Уочени су апоптотичне ћелије ($n = 26$), атипична митоза (1–2/20×; $n = 38$), џиновске ћелије ($n = 8$), акантолиза ($n = 5$), некроза ($n = 5$), калцификација ($n = 1$), гранулационо ткиво ($n = 32$), десмоплазија ($n = 4$), перинеурална инвазија ($n = 2$), мускуларна инвазија ($n = 8$), инвазија ткива пљувачне жлезде ($n = 3$), васкуларна инвазија ($n = 10$) и хронично запаљење ($n = 33$). Васкуларна инвазија ($p = 0,04$, Пирсонов χ^2-тест) и некроза ($p = 0,04$, Пирсонов χ^2-тест) значајно су повезани са случајевима тумора високог степена малигнитета. Атипична митоза је повезана са великом дебљином тумора ($p = 0,04$, Фишеров егзактни тест).

Закључак Ова студија сугерише да инцизијалне биопсије могу имати велику важност, јер могу указати на хистопатолошке чиниоце који су значајни за поделу ОСЦК на оне високог и ниског степена према Бринеовој скали, коришћеној у овом раду. Стога би репрезентативније биопсије могле бити од користи како би се постигао наведени циљ и како би било омогућено боље планирање.

Кључне речи: оралне неоплазме; орални карцином, дијагноза; хистопатолошко градирање; сквамоцелуларни карцином, патологија; сквамоцелуларни карцином, хирургија

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Modified Risdon approach using periangular incision in surgical treatment of subcondylar mandibular fractures

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SUMMARY
Introduction No consensus has been reached yet on the surgical approach for treatment of condylar fractures.
Objective The aim of this study was to present modified Risdon approach (without facial nerve identification) in the treatment of subcondylar mandibular fractures.
Method This is a retrospective study of a period 2005–2012. During this seven-year period, 25 condylar mandibular fractures in 22 men and three women (19–68 years old) were treated by modified Risdon approach without identifying the facial nerve. The main inclusion criterion was subcondylar fracture according to Lindahl classification.
Results No additional morbidity related to postoperative complications, such as infection or salivary fistula, was observed in this series. Only two (8%) patients developed temporary weakness of the marginal branch of the facial nerve, which resolved six weeks postoperatively. Each patient achieved good mouth opening postoperatively. Scar was camouflaged in the first cervical wrinkle. Two patients developed temporomandibular joint dysfunction. No patient had postoperative occlusal disturbance. In all of the patients good aesthetic result was achieved in a two-year follow-up.
Conclusion In comparison with techniques described in the literature, the main advantages of the modified Risdon approach are the following: no need for facial vessels identification; direct, fast, and safe approach to mandibular angle and subcondylar region; relatively simple surgical technique and good cosmetic result – due to aesthetically placed incision. This approach could be recommended for subcondylar fracture as a simplified and safe procedure.
Keywords: subcondylar fracture; extraoral approach; open reduction

INTRODUCTION

Open approaches for treating patients with mandibular fractures have been used in the modern era of maxillofacial traumatology since the implementation of internal fixation [1]. No consensus has been reached yet over the surgical approach for treatment of condylar fractures. However, many authors support an opinion that open reduction and internal fixation provide good functional outcome [2, 3].

Beside intraoral endoscopic-assisted surgical procedures, the conventional extraoral treatment approaches, such as the submandibular, retromandibular (transparotid and retroparotid), preauricular and modified Risdon approach are still in use. Some of these approaches, like transparotid, are very complex, and require identification and preservation of certain anatomical structures like buccal and marginal branches of the facial nerve, which is time consuming surgery [4]. On the other side, postoperative neuropraxia of the mandibular branch of the facial nerve (MBFN) has been reported as the main complication of the classic Risdon's procedure and care should be taken to minimize this risk [5]. In order to avoid this complication conventional approaches have been modified [6, 7, 8].

In order to reduce this risk, some authors recommend direct access to the mandible through submandibular gland without identification of the MBFN. This procedure involves elevating the superior flap and can be used for mandibular fractures as well [1, 5]. The routine exploratory identification of the MBFN nerve is still a matter of debate.

OBJECTIVE

The aim of this study is to describe modified Risdon approach (without facial nerve identification) in surgical treatment of subcondylar mandibular fractures and to examine the effectiveness of this method in open reduction and internal fixation.

METHODS

Surgical technique

Modified Risdon approach to the subcondylar region was performed under general anesthesia. To control bleeding, the surgical site was infiltrated with a solution of lidocaine and epinephrine (1:80,000).

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There are several important anatomical landmarks for this procedure: 1. platysma layer; 2. superficial layer of deep cervical fascia (SLDCF) – investing fascia; 3. anterior superior border of sternocleidomastoid muscle (SCMM), and 4. posterior border of submandibular gland (SG).

The periangular incision was placed into the first cervical crease 1.5–2 cm from the gonion, being about 5 cm long (Figure 1). After skin and subcutaneous tissue incision, platysma was dissected carefully in order to preserve SLDCF because the MBFN is just above or adherent to the mentioned fascia (Figure 2). SLDCF was incised meticulously at the precise determined localization, 2 cm below the mandibular angle and above the anterior border of the SCMM (Figure 3). The subfascial space (under SLDCF) was entered using blunt scissors between the anterior border of the SCMM (landmark #3) and posterior border of the SG (landmark #4). This space is avascular and safe – it represents a keyhole for this approach. Dissection, which could be carried out either by scissors or fingertip, was continued further towards the angle of the mandible. It is very easy to identify lower border of the mandibular angle by palpation and elevation of SLDCF with overlying tissue. This manoeuvre provided exposure of pterygo-masseteric sling. It has to be highlighted that this approach does not require visualization and identification of the MBFN. Mild retraction of submandibular gland anteriorly and sternocleidomastoid muscle posteriorly provides enough space for safe incision of periosteum along the lower border of the mandibular angle (Figure 4). Masseter muscle was then stripped from the angle and along the posterior border of the ramus to identify fracture line and bony fragments. Adequate subperiosteal dissection over the mandibular

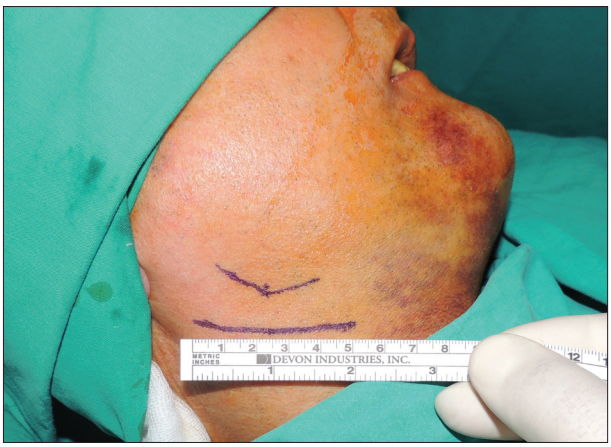

Figure 1. Placement of periangular incision

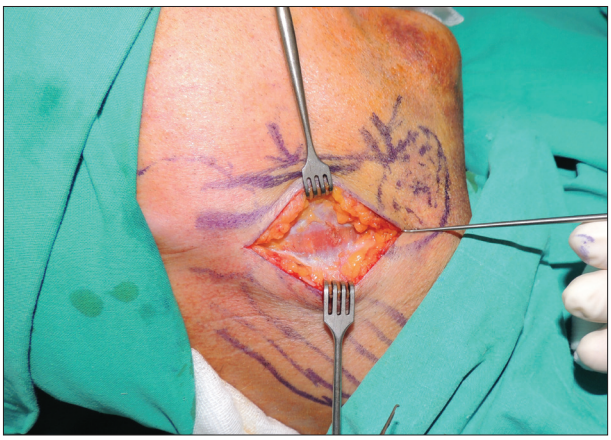

Figure 2. Platysma layer

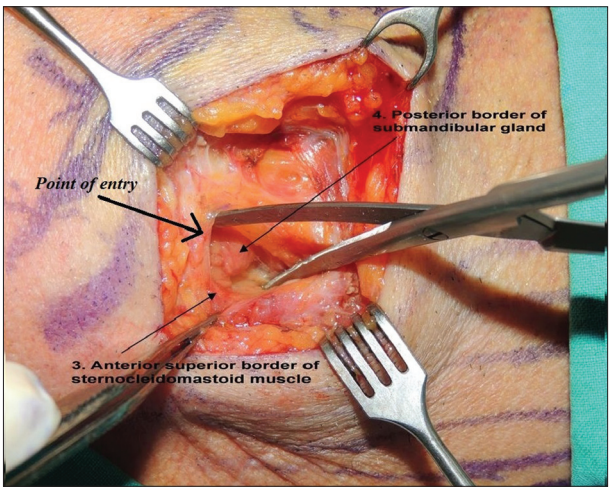

Figure 3. Cutting of the superficial layer of deep cervical fascia – point of entry into subfascial space

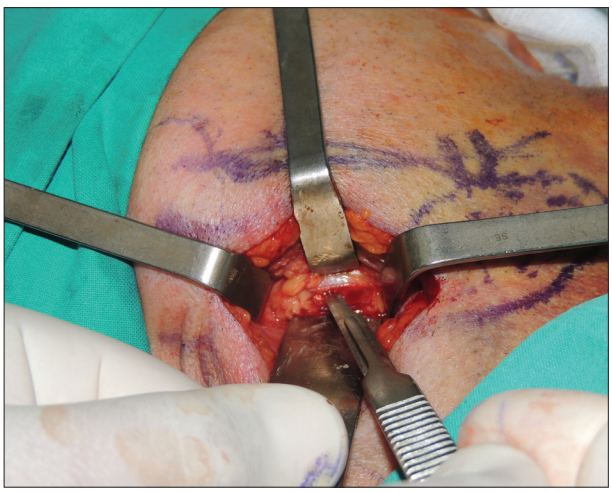

Figure 4. Safe incision of periosteum along the lower border of the mandibular angle

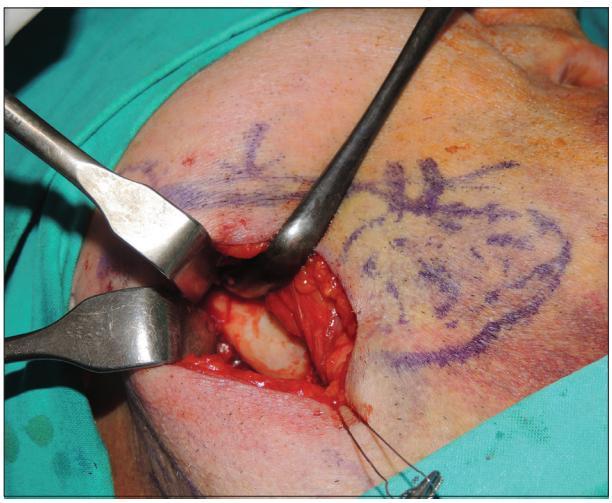

Figure 5. Bony fragments after reduction

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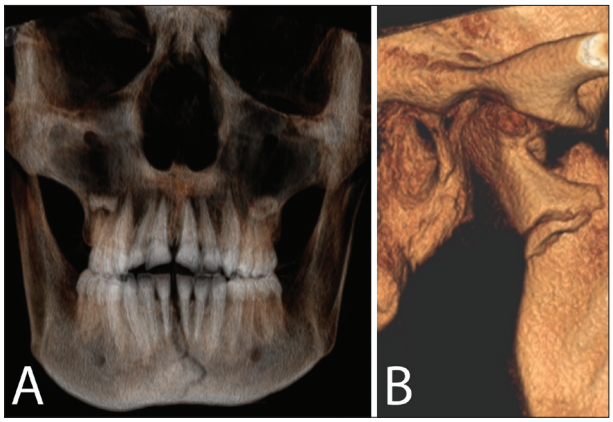

Figure 6. Preoperative cone beam computed tomography (SCANORA® 3Dx, SOREDEX, Tuusula, Finland): a) preoperative postero-anterior radiogram 3D; b) preoperative 3D picture of left the right subcondylar fracture

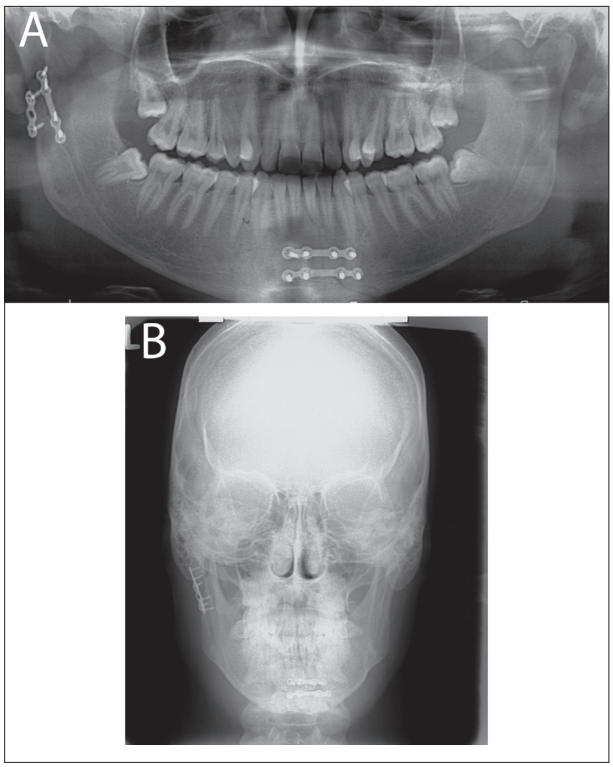

Figure 7. a) Postoperative ortopantomogram; b) Postoperative postero-anterior radiogram

ramus provided good exposure of the fracture site in order to reduce the fracture fragments and to place internal fixation (Figure 5). Retromandibular vein could be visualized posteriorly and should be preserved.

The access to the fracture site with this approach usually takes between five and 10 minutes and there is no significant bleeding. In all cases a plating system was used for rigid internal fixation. The wound was irrigated, checked for hemostasis, and closed in the following four layers: the periosteum, superficial cervical fascia with platysma, subcutaneous layer, and skin. Skin was closed with intradermal suture. A suction drain was usually removed after two days. X-rays showed preoperative findings and appropriate anatomic reduction (Figures 6 and 7).

Patients

Twenty-five patients with mandibular fractures at 34 sites including subcondylar enrolled in this study. There were 22 male and three female patients, ranging from 19 to 68 years of age. They were surgically treated at the Department of Maxillofacial Surgery between 2005 and 2012 using a modified Risdon approach without identifying the facial nerve (Table 1). The other fracture sites were treated using open reduction with an intraoral approach, or combined approaches in some complex cases. The routine preoperative investigation comprised clinical examination and digital ortopanoramic and posterior–anterior radiograms of the mandible. All the patients selected for open reduction and internal fixation had posttraumatic malocclusion and showed radiological signs of the displaced fracture. The main inclusion criteria in this study group were displaced subcondylar fractures according to Lindahl classification with occlusal disturbance and deflection [9]. In all patients postoperative review of above mentioned radiograms were compared with the preoperative ones and the following clinical signs were noted: mouth opening, occlusion, temporomandibular joint (TMJ) dysfunction and facial nerve damage.

RESULTS

In all patients good anatomical position of bone fragments were achieved. No postoperative complications, such as infection or salivary fistula, were observed in this series. Only two (8%) patients developed temporary weakness of the MBFN, which resolved six weeks postoperatively. Each patient achieved good mouth opening with no deflection. Two patients developed TMJ dysfunction, clicking that was treated conservatively (Table 1). No patient had postoperative occlusal disturbance. In all the patients good aesthetic results were achieved.

DISCUSSION

Risdon has described submandibular approach for TMJ ankylosis in 1934, which has been traditionally used for many years [10]. In 1963, Ginestet et al. [11] illustrated a curve-shaped incision at the mandibular angle for extraoral ectopic wisdom teeth extraction, without detailed description of the procedure. Using Risdon approach, Nam et al. [12] described treatment of subcondylar fracture trough submandibular incision placed 3 cm or more below the inferior border of the mandible because of the close vicinity of the MBFN. This is a classic submandibular approach that is technically demanding because of longer route to fracture site and more difficulties in fragment reducing and plating. The use of periangular incision provides direct access to the angle of the mandible and is closer to the subcondylar area. Furthermore, surgical anatomy of the MBFN presents great variability in the literature [13, 14, 15]. Ziarah and Atkinson [16] pointed out that the

Table 1. Open reduction group

ID	Sex	Age	Lindahl	Location	Etiology	Occlusion	Opening	TMJ dysfunction	VII damage	Follow-up (months)
1	M	33	L/D	Left	IPV	Normal	Good	No	no	16
2	M	44	L/D	Bilateral	IPV	Normal	Good	No	no	17
3	M	20	L/D	Left	IPV	Normal	Good	No	no	20
4	M	52	L/D	Left	IPV	Normal	Good	No	no	18
5	M	27	L/D	Left	IPV	Normal	Good	NO	yes	18
6	M	41	L/D	Left	IPV	Normal	Good	Right TMJ click	yes	72
7	M	21	L/D	Left	IPV	Normal	Good	No	no	60
8	M	19	L/D	Left	IPV	Normal	Good	No	no	65
9	F	41	L/D	Left	FFH	Normal	Good	No	no	60
10	M	19	L/D	Left	IPV	Normal	Good	No	no	60
11	M	68	L/D	Bilateral	IPV	Normal	Good	Deviation	no	57
12	M	54	L/D	Left	IPV	Normal	Good	No	no	56
13	M	41	L/D	Left	FFH	Normal	Good	No	no	62
14	M	32	L/D	Right	IPV	Normal	Good	No	no	15
15	M	28	L/D	Right	FFH	Normal	Good	No	no	18
16	M	29	L/D	Right	IPV	Normal	Good	No	no	32
17	F	48	L/D	Right	TA	Normal	Good	No	no	24
18	M	31	L/D	Right	IPV	Normal	Good	No	yes	30
19	M	21	L/D	Right	IPV	Normal	Good	No	no	66
20	M	24	L/D	Right	IPV	Normal	Good	No	no	48
21	M	39	L/D	Right	IPV	Normal	Good	No	no	36
22	M	27	L/D	Right	IPV	Normal	Good	No	no	48
23	M	28	L/D	Left	FFH	Normal	Good	No	no	60
24	F	29	L/D	Left	TA	Normal	Good	No	no	48
25	M	56	L/D	Left	WRI	Normal	Good	No	no	48

ID – patient ID; Lindahl – classification (M – medial overlap, L – lateral overlap, NO – no overlap, ND – no displacement, SD – slight displacement, MD – moderate displacement, D – dislocation); TMJ – temporomandibular joint; IPV – interpersonal violence; FFH – fall from height; TA – traffic accident; WRI – work related injury

MBFN could be traced 0.2–1.4 cm below the mandibular angle. Hayes Martin manoeuvre, described within submandibular approach, may not always be considered safe [17]. Because of that, other authors suggest identifying and dissecting the MBFN for more safety [12, 15]. It has been even reported that MBFN can occasionally cross under the facial artery and vein [15]. Some surgeons have pointed out the utility of the surgical approach underneath the submandibular gland fascia without the routine identification of the mandibular nerve, which actually minimizes the risk of nerve damage [10, 18, 19]. Nevertheless, using submandibular approach, surgeons usually face long tunnel to the subcondylar area and stronger tissue traction is always necessary to reach fracture site, which can cause neuropraxia of mandibular branch of facial nerve. However, using a simplified technique described in this study, the tunnel is significantly shorter. Furthermore, the surgical layer of the subfascial flap, which is elevated in order to access the mandibular border, can be identified precisely using various anatomic landmarks mentioned where surgical technique is described. There is no need for facial blood vessels identification and ligation. Comparing to other approaches, this method does not require identification and visualization of the MBFN [12]. It is crucial to avoid dissection or tissue traction directly over the masseter muscle due to variability of the route of the MBFN [16]. It is absolutely necessary to respect each layer and to dissect "one by one," as described (Figure 3). The crucial step is to perforate SLDCF near anterior border of the SCMM and 2 cm below the mandibular angle and dissect it from the posterior border of the SG. This distance of 2 cm should be reached due to variability of the rout of the MBFN [16]. It provides the safe zone, which is best to enter by blunt finger dissection. It is a very important manoeuvre, needed in order to avoid blood vessels and nerves, and is a very quick way in approaching mandibular angle and visualizing pterygo-masseteric sling. This also provides less tissue traction and manipulation during reduction and fixation of bone fragments.

In this series, only two (8%) patients developed temporary facial nerve weakness. The frequency of temporary facial palsy after extraoral approaches to the subcondylar region varies in the range 0–30% [20–24]. Hou et al. [25] found that after modified transparotid approach, 22% of patients suffered from temporary facial palsy. Using retromandibular approach, two authors described two cases of permanent facial palsy after five years of follow-up, although damage to the facial nerve frequently produces weakness of the depressors of the mandible [25]. In all cases, the neuropraxia is transient, and the cause might be compression of the nerve by retractors or nerve manipulation.

According to literature, submandibular approach showed the worst outcome regarding permanent palsy of the facial nerve and hypertrophic/visible scarring. Additionally, the

extraoral scars of the submandibular and preauricular approaches were significantly longer than those of the retromandibular approach [26]. Using the modified Risdon approach, the scars were barely noticeable and always hidden. There were no signs of hypertrophic scaring in this study.

CONCLUSION

The main advantages of the modified Risdon approach described in this study were the following: low risk for the mandibular branch of facial nerve damage; no identification and visualization of the mandibular branch facial nerve; no need for blood vessel identification (facial artery and vein); direct, fast, and safe approach to the mandibular angle and subcondylar region; relatively simple surgical technique and good cosmetic result – due to aesthetically placed incision.

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Модификовани Ризданов приступ кроз периангуларну инцизију у хируршком лечењу субкондиларних прелома доње вилице

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Увод У литератури не постоји консензус у погледу хируршког приступа за лечење прелома кондиларног наставка доње вилице.

Циљ рада Циљ рада је био да прикаже модификовани Ризданов приступ (без идендификације фацијалног живца) у лечењу субкондиларног прелома доње вилице.

Методе рада Ова ретроспективна студија односи се на период од 2005. до 2012. године. Током седам година, 25 субкондиларних прелома доње вилице код 22 пацијента мушког пола и три женског пола (19–68 година) третирано је модификованим Риздановим приступом без идентификације фацијалног живца. Основни критеријум за укључење пацијената у студију био је присуство субкондиларног прелома доње вилице према Линдахловој класификацији.

Резултати У постоперативном току нису биле присутне саливарне фистуле и инфекција. Код два пацијента (8%) била је присутна привремена пареза маргиналне гране фацијалног живца, која се повукла у року од шест недеља. Код свих пацијената је успостављено нормално отварање уста постоперативно. Ожиљак је добро скривен у првој вратној бори. Два пацијента су испољила симптоме дисфункција темпоромандибуларног зглоба. Код свих пацијената постигнут је добар естетски резултат у двогодишњем периоду праћења.

Закључак У поређењу са техникама описаним у литератури, основне предности модификованог Ризданового приступа су следеће: одсуство потребе за идентификацијом фацијалних крвних судова; директност, што омогућава брз и безбедан приступ углу доње вилице и субкондиларној регији; релативна једноставност хируршке технике, као и естетски позиционирана инцизија. Овај приступ може бити препоручен за лечење субкондиларног прелома доње вилице, као једноставан и безбедан.

Кључне речи: субкондиларни прелом; екстраорални приступ; отворена редукција

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Thyroglobulin value in patients surgically treated for differentiated thyroid carcinoma

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SUMMARY

Introduction Thyroglobulin is composed glycoprotein, and it is synthesized by follicular cells of the thyroid gland. Treatment of differentiated thyroid carcinomas involves total thyroidectomy followed by radioiodine ablation of a potential remaining tissue. The measurement of thyroglobulin in the postoperative follow-up can serve as an indicator of tumor growth or recurrence of the disease.

Objective The aim of this paper is to examine the value of thyroglobulin in patients surgically treated for differentiated thyroid cancer who had metastases in the lymph nodes of the neck, as well as in operated on patients without any evident metastasis.

Methods Thyroglobulin values in the serum of 58 patients were analyzed. Two groups were formed. The thyroglobulin value was established with the use of IRMA-hTg (^{125}I) system. Normal levels of thyroglobulin were from 2 ng/ml to 65 ng/ml. For all of 58 patients, thyroglobulin was determined three times. The first, so-called pre-ablation thyroglobulin was determined immediately before the application of ^{131}I ablation dose. The second and the third measurements were conducted six to eight months and one year, respectively, after the application of the ablation dose respectively.

Results The first group consisted of 14 patients with histologically proven metastases in the lymph nodes of the neck, while the second group consisted of 44 patients without any evident metastases. The average thyroglobulin value of pre-ablation in the patients from the first group was 43.45 ng/ml, while in the second was 7.57 ng/ml. Levene's test (with $p = 0.00$, i.e $p < 0.05$), demonstrated a statistically significant difference. Furthermore, in both groups, there was statistically significant difference between pre-ablation and post-ablation thyroglobulin values (Student's t-test with $p < 0.05$).

Conclusion It can be concluded that the average value of thyroglobulin was significantly higher in patients with lymph node metastases in the neck. We are of the opinion that the determination of thyroglobulin, despite individual variations, may serve as a good indicator to assist in monitoring of patients surgically treated for differentiated thyroid cancer.

Keywords: thyroglobulin; total thyroidectomy; ablation; thyroid gland cancer

INTRODUCTION

Thyroglobulin (Tg), which weighs 660 kilodaltons, is a glycosylated protein. It is synthetized by follicular cells of the thyroid gland and "inserted" into the lumen of thyroid follicles. The secretion of thyroid hormones triiodothyronine (T3) and tiroxin (T4) starts with micropinocytosis of the iodinated thyroglobulin from the follicular lumen, and regresses into thyrocytes. Thyroglobulin has a tendency to hydrolyze under the influence of lysosomal enzymes and subsequently, by the diffusion through the basement membrane, formed molecules of T3 and T4 hormones move to the capillary network [1]. Thyroglobulin molecules can avoid hydrolysis and together with hormones, through the basement membrane, enter the blood circulation [2]. High thyroglobulin values were found both in patients with follicular adenoma and in differentiated thyroid carcinoma (DTC). The abovementioned has led to the conclusion that tumor cells of papillary or follicular cancer, presumably like a normal thyrocyte, produce and secrete thyroglobulin. The amount of thyroglobulin in the circulation can increase along with the growth of tumors, greater invasion of healthy thyroid tissue, as well as with the emergence of metastases [3]. Nevertheless, thyroglobulin does not have a special significance in the diagnosis of thyroid cancer. High thyroglobulin value is often found in patients with diffuse goiter and patients with Graves–Basedow disease [4]. Furthermore, some patients with histologically confirmed DTC preoperatively had normal thyroglobulin values.

The first step in the DTC therapy is thyroidectomy, i.e. surgical removal of the entire thyroid tissue [5]. Complete elimination of the thyroid tissue would consequently cause the absence of thyroglobulin in the serum. Such cases are rare in practice. In most cases, we come across certain thyroglobulin values, which raises the question of quality of the declaratively performed thyroidectomy. In cases of partial thyroidectomy, such as extended lobectomy and subtotal resections, different thyroglobulin values are expected in the patients' serum. The measured thyroglobulin value can help with assessing the types of residual tissue, i.e. whether the latter is normal or tumoral [6, 7]. In order to eliminate the functionally residual thyroid tissue, a pharmacological ablation is conducted. Radioactive iodine (^{131}I), in a capsule or liquid, is administered *per os*.

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Most commonly, it is administered in a dose of 3.7 MBq according to the prescribed procedure [8]. In the first six months after the ablation, thyroglobulin values in the serum decline to low or undetectable values, and with an adequate hormone substitution (levothyroxine), they remain as such during regular, routine check-ups. For most authors, subsequent increase of thyroglobulin values is the most reliable sign of aggravation of the disease, that is, the appearance of local recurrence, lymph nodes metastasis or distant metastasis. Most authors believe that the post-ablation thyroglobulin is an important tumor marker for DTC [9].

In contrast to the classical prognostic factors, mathematically derived value thyroglobulin doubling time (TDT) is a more valid indicator of carcinoma recurrence. The base for the calculation of TDT consists of at least four consecutive measurements of thyroglobulin in defined time intervals [10]. Giovanella et al. [11] assert that the TDT is a reliable indicator of carcinoma recurrence even in cases when 131I-scan is negative.

In cases when thyroglobulin values remain relatively high after the ablation, it is necessary to assess possible causes. Poorly performed ablation (small dose of ^{131}I or omissions during the procedure) may be the only cause. The resistance of postoperative residual tissue should not be neglected as the second or additional cause. Interestingly, some authors have noted weaker effects of ablation in cases where the pre-ablation thyroglobulin value was relatively higher (discriminant 10 ng/ml) [12].

OBJECTIVE

The aim of this study is to determine whether the thyroglobulin values in the serum of the patients surgically treated for differentiated thyroid carcinoma could be sufficient and accurate indicators of disease progression.

METHODS

Thyroglobulin values in the serum of 58 patients were analyzed. The thyroglobulin value was established with the usage of IRMA-hTg (^{125}I) system and in the range of 0 to 250 ng/ml. Normal levels of thyroglobulin were from 2 ng/ml to 65ng/ml. All patients underwent total thyroidectomy. The existence of the papillary or follicular DTC was histologically proven. Two groups were formed. The first group consisted of 14 female patients, of average age of 49 years, with papillary thyroid cancer and metastases that were detected in the lymph nodes of the neck and were classified as T(2-3)N1M0. All the patients received the ablation dose ^{131}I of 3.7 GBq.

The second group comprised 44 patients without detected metastasis, classified as T(2-3)N0M0. There were 38 female patients with an average age of 44.5 years, and six male patients, with an average age of 41 years. In the majority of patients, that is in 43 patients, papillary carcinoma was diagnosed, and only one patient (m) had follicular carcinoma. In accordance with the decision made by the authorized consultation team, a dose of ^{131}I in the range of 1.5 to 3.7 GBq was administrated.

Thyroglobulin was determined three times. The first determination was conducted immediately before the administration of the ablation dose of ^{131}I, pre-ablation thyroglobulin in conditions of high TSH level. The second determination was conducted six to eight months after the application of ^{131}I, and the third one during the second year after the ablation. At the time of the second and third thyroglobulin determination, the patients were under the appropriate substitution therapy (levothyroxine). In the monitoring period, there were no changes in physical findings. Apart from clinical examinations, all the patients underwent neck ultrasonography, whole body scintigraphy, and in several cases thorax and neck computed tomography scan. Patients in whom the presence of high thyroglobulin-antibody titer was detected were not included in the analysis.

RESULTS

The first group consisted of 14 patients. The average pre-ablation thyroglobulin value was 43.45 ng/ml, and the individual values were in the range of 2–155 ng/ml (Table 1).

In the second measurement, the average value was 1.50 ng/ml, and the individual values ranged from 0.1 ng/ml to 7.0 ng/ml. The statistical analysis, Student's t-test ($t = 2.481$, $p = 0.035$) showed a significant difference ($p < 0.05$) between the pre-ablation and the post-ablation thyroglobulin values. The average value in the third measurement was slightly higher than the previous one, i.e. 2.13 ng/ml. The individual values ranged from 0.1 ng/ml to 4.4 ng/ml.

The second group comprised 44 patients. The average pre-ablation thyroglobulin value was 7.58 ng/mL, and the individual values ranged from 0.5 ng/ml to 37.0 ng/ml. In the second measurement, the average value was 0.49 ng/ml in the range of 0.1 ng/ml to 2.5 ng/ml (Table 2). There was a significant difference between the pre-ablation and post-ablation thyroglobulin values ($t = 5.473$, $p = 0.000$, i.e. $p < 0.05$). The third measurement had the average value of 0.29 ng/ml, and the individual values were from 0.1 ng/ml to 2.3 ng/ml.

Table 1. Overview of the individual and average thyroglobulin (Tg) values in the first group: T(2-3) N1 M0

Group I Tg measurement	Minimum value	Maximum value	Average value	Standard deviation
First	2.0	155.0	43.45	55.31
Second	0.1	7.0	1.50	2.17
Third	0.1	4.4	2.13	1.85

Table 2. Overview of the individual and average thyroglobulin (Tg) values in the second group: T(2-3) N0 M0

Group II Tg measurement	Minimum value	Maximum value	Average value	Standard deviation
First	0.5	37.0	7.58	8.11
Second	0.1	2.5	0.49	0.43
Third	0.1	2.3	0.29	0.39

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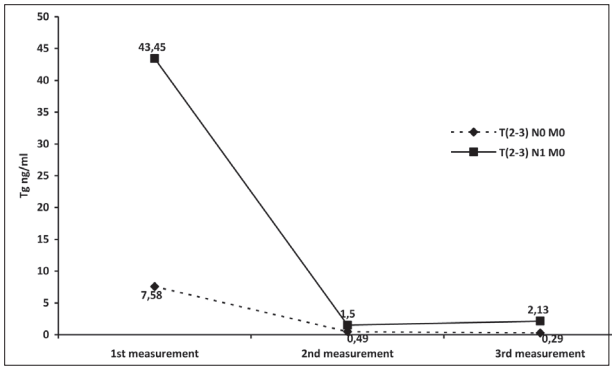

Graph 1. Comparative overview of the average thyroglobulin (Tg) values

Basic comparison between the average pre-ablation thyroglobulin values of the first group (43.45 ng/ml) and the analog value of the second group (7.57 ng/ml) presented a clear difference (Graph 1). Statistically, group variances were significantly different (Levene's test: F = 32.681, p = 0.000, i.e. p < 0.05).

DISCUSSION

The importance of the thyroglobulin as a prognostic factor in patients treated for DTC is interpreted differently. Some authors believe that the pre-ablation thyroglobulin in combination with the post-ablation scintigraphy has a significant prognostic value [12]. Most authors believe that the elevated pre-ablation thyroglobulin values themselves have a significant prognostic value [13]. In this study, the average value of the pre-ablation thyroglobulin was significantly higher in the first group of patients in comparison with the second group. Unfortunately, we have no plausible explanation for the observed difference. In contrast, the range of individual values did not show such a relationship. Namely, in the first group there were four female patients who had relatively low thyroglobulin values without regard to the tumor with extrathyroidal expansion (about 2 ng/ml). On the other hand, in the second group there were three female patients with relatively high thyroglobulin values (22–30 ng /ml). A compatible attitude on normal concentration of serum thyroglobulin after the conducted thyroidectomy cannot be found in literature. Lin and associates conducted a retrospective analysis of patients who received ablation doses of ^{131}I after the thyroidectomy [13]. They decided to divide patients solely according to the ablation thyroglobulin values, and thereby the patients were divided into three groups as follows: group A (Tg < 1 ng/mL), group B (1 ng/ml < Tg < 10 ng/ml), and group C (Tg > 10 ng/ml). A significant difference in the incidence of recurrence was noted, i.e. in group A 4.2%, group B 23.6% and in group C even 62.9%. Hence, they generally gave a greater prognostic significance to the pre-ablation thyroglobulin. Although it is not the aim of this study, we find it useful to note that in several cases post-ablation scintigraphy did not display focal accumulation, although it was expected to be found due to the high pre-ablation thyroglobulin values.

In the first assessment after the administration of radioactive iodine, a decrease of average thyroglobulin values in both groups was noticed. The higher thyroglobulin value in comparison with the pre-ablation thyroglobulin value was not detected at all. In all patients, the application of radioiodine therapy after the surgery was still inconsistent [14]. The above-cited results in this group support the proposition that radioiodine ablation reduces the remaining tissue. What is more, we are closer to believe that a successful ablation therapy leads to better prognosis [15,16].

In the first group, the second measurement after the application showed an increase in average values in relation to the previous measurement. Bearing in mind individual values, there was a slight increase of the thyroglobulin values in six female patients, although loco-regional changes were not observed with the use of conventional examination. Kaneko et al. [17] claim that an increase of thyroglobulin value is a sufficient indication for the use of more complicated procedures such as positron emission tomography (PET) or computed tomography (CT) in order to locate metastasis. Unexpectedly, in some patients who underwent ^{18}F-FDG-PET/CT, the results were negative [17, 18].

The second measurement in the second group showed that the average thyroglobulin value was lower in relation to the previous measurement. Individual values did not show the value higher than the previously measured one. In our case, both post-ablation thyroglobulin values were measured in terms of suppressed TSH. We believe that the post-ablation thyroglobulin values below 1 ng/ml indicate a favorable course of the disease, while in all other cases it is necessary to be alert and predict metastasis. Some authors support the notion that patients with thyroglobulin value higher than 2 ng/ml should be subjected to re-ablation, despite the fact that there are no clear, physical elements of the disease progression [19]. The thyroglobulin value would be more sensitive if it was to be determined in conditions of high TSH. Unfortunately, for patients this would mean uncomfortable exclusion of substitution for a period of two to three weeks or application of rTSH [20].

CONCLUSION

The average pre-ablation thyroglobulin value was significantly higher in the group of patients with the lymph nodes metastases of the neck when compared to the patients without metastases. The individual pre-ablation thyroglobulin values showed significant variations.

In all cases, the post-ablation thyroglobulin value was lower than the pre-ablation thyroglobulin value. In terms of adequate suppression, the post-ablation thyroglobulin monitoring has greater practical significance.

We believe that systematic thyroglobulin monitoring is needed in all patients surgically treated for differentiated thyroid carcinoma.

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Вредност тиреоглобулина код болесника оперисаних због диферентованих карцинома штитасте жлезде

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КРАТАК САДРЖАЈ

Увод Тиреоглобулин је по саставу гликопротеин, а синтетишу га фоликуларне ћелије штитасте жлезде. Лечење диферентованих тиреоидних карцинома подразумева тоталну тиреоидектомију, након које следи радиојодна аблација евентуално преосталог ткива. Мерење тиреоглобулина у постоперативном праћењу може послужити као показатељ туморског раста или рецидива болести.

Циљ рада Циљ рада јесте испитати вредности тиреоглобулина у серуму болесника оперисаних због диферентованих карцинома штитасте жлезде који су имали метастазе у лимфним нодусима врата и оперисаних болесника без евидентних метастаза.

Методе рада Анализирана је вредност тиреоглобулина у серуму 58 болесника. Формиране су две групе. Тиреоглобулин је одређиван радиоимунолошким сетовима, *IRMA-hTg* (^{125}I). Нормалне вредности су од 2 *ng/ml* до 65 *ng/ml*. Код свих 58 болесника тиреоглобулин је одређен три пута. Први, преаблациони тиреоглобулин је одређен непосредно пред апликацију аблационе дозе ^{131}J. Друго одређивање је вршено 6–8 месеци након аблације, а треће након једне године.

Резултати Прву групу чини 14 болесника са патохистолошки доказаним метастазама у лимфним нодусима врата, а другу 44 болесника без евидентних метастаза. Просечна вредност преаблационог тиреоглобулина код пацијената прве групе је била 43,45 *ng/ml*, док је у другој групи била 7,57 *ng/ml*. Левинов тест (при чему је $p = 0,00$, тј. $p < 0,05$) доказује значајну статистичку разлику. Такође, у обе групе је утврђена значајна статистичка разлика између преаблационих и постаблационих вредности тиреоглобулина (*t*-тест, при чему је $p < 0,05$).

Закључак Може се закључити да је просечна вредност тиреоглобулина значајно виша у групи болесника са метастазама у лимфним нодусима врата. Сматрамо да одређивање тиреоглобулина, упркос појединачним одступањима, може послужити као добар индикатор у праћењу болесника оперисаних због диферентованог карцинома штитасте жлезде.

Кључне речи: тиреоглобулин; тотална тиреоидектомија; аблација; карцином штитасте жлезде

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Correlation between the season, temperature and atmospheric pressure with incidence and pathogenesis of acute appendicitis

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SUMMARY

Introduction There is very little literature data on the correlation between the seasons, temperature and atmospheric pressure, and pathogenesis of acute appendicitis (AA).

Objective The aim of this research is to investigate the association between the seasons, changes in atmospheric temperature and pressure, and patients' age and severity of the clinical form of AA in the city of Niš.

Methods This study included 395 patients diagnosed with AA, who, during the two-year period, from July 1st 2011 to June 30th 2013, were hospitalized and operated on at the Department of General Surgery, Clinical Center in Niš, Serbia.

Results The increased average daily values of barometric pressure by 1 millibar on the day when the event took place was associated ($p < 0.05$) with the decrease of total risk of the occurrence of appendicitis by 2.2% (0.2–4.1%). In all observed patients, each increase of the mean daily temperature by 1°C three days before the event took place (Lag 3) was associated ($p < 0.05$) with the increase of total risk of the occurrence of appendicitis by 1.3% (0.1–2.5%).

Conclusion According to the results of this research, we can conclude that patients' sex, age and severity of the clinical form of AA are not in connection with the seasons, while there are certain connections between appendicitis occurrence and atmospheric temperature and pressure.

Keywords: seasons; temperature; atmospheric pressure; acute appendicitis

INTRODUCTION

Acute appendicitis (AA) is the most frequent cause of acute surgical abdomen. Its course is marked as a nonspecific bacterial process. The complications of AA can be the forming of abscess and perforation, which lead to localized or generalized peritonitis with possible fatal outcome [1].

The incidence of appendicitis varies according to country, geographical region, race, sex, age, food culture, and seasons [2–5]. Most of literature data confirms the seasonal pattern of AA occurrence [3, 4, 6–12], though there are data that denies the correlation [13].

There are no literature data on the correlation between patients' age, gender, and severity of the clinical form of AA and the seasons in the city of Niš. Also, there are no literature data on the influence of changes in atmospheric temperature and pressure on the hospitalization of AA.

OBJECTIVE

The aim of this research is to investigate the association between the seasons, changes in atmospheric temperature and pressure and patients' age and severity of the clinical form of AA in the city of Niš.

METHODS

This retrospective study included 395 patients diagnosed with AA, who, during a two-year period, from July 1st 2011 to June 30th 2013, were hospitalized and operated on at the Department of General Surgery, Clinical Center in Niš, Serbia.

The research included only patients who were residents of Niš, whose symptoms of AA started on the day of their operation.

The patients were divided into groups according to sex, age, and severity of the clinical form of AA. According to age, they were divided into two groups: the group of patients under the age of 30 years, and the group of patients aged 30 years or older. The research included only patients over the age of 18.

The severity of the clinical form of AA was defined intra-operatively. Less severe form included catarrhal and phlegmonous AA, while more severe forms included gangrenous and perforated forms of AA.

The mean daily temperature and atmospheric pressure values for the city of Niš, Serbia, were obtained from the National Meteorological Department. The range of average daily values of temperature was 13.25 ± 9.73°C. The range for the daily average values of barometric pressure was 992.49 ± 6.43 millibars (mBar).

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Statistical analysis

The data presentation for this article consists of descriptive statistics including means and standard deviations for continuous variables and frequency counts, and percentages for categorical variables. To compare frequencies, χ^2 test was performed. To compare patients' years of age across four seasons, one-way analysis of variance (one-way ANOVA) was used.

To investigate the association between the appendicitis related hospitalizations and the barometric pressure, we used the negative binomial regression model. A negative binomial model was used to account the overdispersion (mean daily number of appendicitis related hospitalizations for the investigated period was 0.48 ± 0.50), as well as the number of days with zero cases (from 823 days of investigated period there were 429 days without appendicitis related hospitalizations). This model included daily appendicitis counts as the dependent variable, and average daily values of barometric pressure as the independent variable:

$$daily\ appendicitis\ counts = e^{intercept\ +\ b\ (average\ daily\ values\ of\ barometric\ pressure)}$$

where e is a natural number (approximately equal to 2.72) while b is a regression coefficient.

Appendicitis occurrence rate ratios for a 1 mBar change in daily average values of barometric pressure are obtained by exponentiation of the regression coefficients, that is, $\exp[b]$. For ease of interpretation, expression $100 \times (\exp[b] - 1)$ tells us the percentage change in risk of appendicitis for each unit increase in barometric pressure.

The relationship between barometric pressure and the appendicitis risk after lags for 0, 3, and 7 days, as well as the cumulative lags for 0–3 and 0–7 days, was examined.

To investigate the association between the appendicitis related hospitalizations and the temperature, we used the negative binomial regression model that included daily appendicitis counts as the dependent variable and daily values of maximum, minimum or mean temperature as the independent variable:

$$daily\ appendicitis\ counts = e^{intercept\ +\ b\ (average\ daily\ values\ of\ temperature)}$$

The relationship between temperature and the appendicitis risk after lags for 0, 3, and 7 days, as well as the cumulative lags for 0–3 and 0–7 days, was examined.

Analyses were performed using R, a language and environment for statistical computing, version 2.12.0 (R Foundation for Statistical Computing, Vienna, Austria). A p-value less than 0.05 was considered significant in all comparisons.

RESULTS

Relationship between average age, sex, and severity of appendicitis and seasons, regarding patients with appendicitis, is shown in Table 1.

Table 1. Relationship between average age, sex, and severity of appendicitis and seasons, in patients with appendicitis

Characteristic	Total	Winter	Spring	Summer	Autumn
Age (years)	35.38 ± 17.86	35.71 ± 17.84	36.85 ± 18.74	33.68 ± 16.89	34.98 ± 17.84
Male	181 (45.8%)	39 (44.8%)	56 (47.9%)	40 (42.1%)	46 (47.9%)
Female	214 (54.2%)	48 (55.2%)	61 (52.1%)	55 (57.9%)	50 (52.1%)
Less severe	231 (58.5%)	50 (57.5%)	62 (53.0%)	61 (64.2%)	58 (60.4%)
More severe	164 (41.5%)	37 (42.5%)	55 (47.0%)	34 (35.8%)	38 (39.6%)

Table 2. Appendicitis occurrence rate ratios (95% confidence interval) for 1 mBar increase in barometric pressure by age group

Age and Lag period	Total	Less severe	More severe
All ages			
Lag 0	0.978 (0.959–0.998)*	0.994 (0.973–1.017)	0.975 (0.951–0.999)*
Lag 3	0.988 (0.968–1.008)	0.999 (0.977–1.021)	0.983 (0.959–1.008)
Lag 7	0.995 (0.976–1.015)	0.994 (0.973–1.016)	1.001 (0.977–1.026)
Lag 0–3	0.974 (0.952–0.997)*	0.992 (0.967–1.017)	0.972 (0.944–0.999)*
Lag 0–7	0.979 (0.954–1.005)	1.000 (0.972–1.028)	0.970 (0.939–1.002)
<30 years			
Lag 0	0.979 (0.960–0.999)*	0.983 (0.958–1.009)	0.978 (0.941–1.016)
Lag 3	0.997 (0.974–1.021)	1.003 (0.977–1.030)	0.986 (0.949–1.026)
Lag 7	0.995 (0.973–1.018)	0.991 (0.966–1.017)	1.005 (0.967–1.045)
Lag 0–3	0.981 (0.955–1.007)	0.988 (0.958–1.017)	0.974 (0.931–1.018)
Lag 0–7	0.985 (0.956–1.015)	0.993 (0.960–1.027)	0.973 (0.926–1.023)
≥30 years			
Lag 0	0.992 (0.969–1.015)	1.011 (0.979–1.044)	0.977 (0.949–1.007)
Lag 3	0.985 (0.962–1.009)	0.991 (0.959–1.023)	0.984 (0.955–1.014)
Lag 7	0.998 (0.975–1.021)	0.997 (0.965–1.029)	0.999 (0.970–1.029)
Lag 0–3	0.984 (0.957–1.010)	0.999 (0.962–1.036)	0.975 (0.942–1.009)
Lag 0–7	0.986 (0.957–1.017)	1.006 (0.965–1.049)	0.973 (0.936–1.011)

*$p < 0.05$

Table 3. Appendicitis occurrence rate ratios (95% confidence interval) for 1 mBar increase in barometric pressure by gender

Gender and Lag period	Total	Less severe	More severe
Females			
Lag 0	0.982 (0.960–1.005)	0.989 (0.964–1.014)	0.976 (0.941–1.013)
Lag 3	0.994 (0.971–1.017)	0.996 (0.970–1.022)	0.991 (0.955–1.029)
Lag 7	0.998 (0.976–1.020)	0.995 (0.970–1.020)	1.005 (0.969–1.043)
Lag 0–3	0.981 (0.956–1.007)	0.984 (0.956–1.014)	0.982 (0.941–1.024)
Lag 0–7	0.986 (0.958–1.016)	0.993 (0.961–1.026)	0.978 (0.933–1.026)
Males			
Lag 0	0.990 (0.967–1.014)	1.007 (0.974–1.041)	0.979 (0.949–1.009)
Lag 3	0.990 (0.966–1.014)	1.004 (0.971–1.038)	0.981 (0.951–1.011)
Lag 7	0.997 (0.973–1.020)	0.995 (0.963–1.028)	0.999 (0.969–1.029)
Lag 0–3	0.985 (0.959–1.013)	1.008 (0.970–1.047)	0.969 (0.941–0.998)*
Lag 0–7	0.987 (0.957–1.018)	1.011 (0.969–1.055)	0.970 (0.932–1.009)

*p < 0.05

Table 4. Appendicitis occurrence rate ratios (95% confidence interval) for 1°C increase in mean daily temperature by age group

Age and Lag period	Total	Less severe	More severe
All ages			
Lag 0	1.010 (0.997–1.022)	1.008 (0.994–1.021)	1.004 (0.989–1.019)
Lag 3	1.013 (1.001–1.025)*	1.007 (0.993–1.020)	1.010 (0.995–1.025)
Lag 7	1.010 (0.998–1.022)	1.001 (0.988–1.015)	1.013 (0.997–1.028)
Lag 0–3	1.013 (1.001–1.026)*	1.010 (0.996–1.024)	1.007 (0.992–1.023)
Lag 0–7	1.013 (1.001–1.026)*	1.006 (0.992–1.021)	1.010 (0.994–1.027)
<30 years			
Lag 0	1.007 (0.993–1.021)	1.007 (0.991–1.023)	1.004 (0.981–1.028)
Lag 3	1.006 (0.992–1.020)	1.004 (0.989–1.020)	1.008 (0.984–1.032)
Lag 7	1.009 (0.995–1.023)	1.002 (0.987–1.018)	1.021 (0.997–1.046)
Lag 0–3	1.010 (0.995–1.024)	1.009 (0.992–1.025)	1.008 (0.984–1.033)
Lag 0–7	1.009 (0.994–1.024)	1.006 (0.990–1.023)	1.012 (0.987–1.037)
≥30 years			
Lag 0	1.005 (0.991–1.019)	1.006 (0.986–1.026)	1.003 (0.985–1.021)
Lag 3	1.010 (0.996–1.024)	1.008 (0.988–1.028)	1.009 (0.991–1.028)
Lag 7	1.003 (0.988–1.017)	0.998 (0.979–1.017)	1.006 (0.988–1.024)
Lag 0–3	1.007 (0.993–1.022)	1.008 (0.988–1.028)	1.005 (0.987–1.024)
Lag 0–7	1.007 (0.992–1.022)	1.004 (0.983–1.024)	1.008 (0.989–1.027)

*p < 0.05

There was no statistically significant association between the patients' age, sex, and severity of the clinical form of AA and the seasons.

Appendicitis occurrence rate ratios for 1 mBar increase in barometric pressure by age group are shown in Table 2.

In all observed patients each increase of the average daily value of barometric pressure by 1 mBar on the day when the event took place was associated ($p < 0.05$) with the decrease of total risk of the occurrence of appendicitis by 2.2% (0.2–4.1%), as well as with the occurrence of more severe appendicitis ($p < 0.05$) by 2.5% (0.1–4.9%). The increase of the average barometric pressure by 1 mBar on the day when the event took place and three days before it (Lag 0–3) was associated with the decrease of the risk of the occurrence of appendicitis ($p < 0.05$) by 2.6% (0.3–4.8%), as well as of the occurrence of more severe appendicitis ($p < 0.05$) by 2.8% (0.1–5.6%).

In the <30 years age group each increase of the average daily values of barometric pressure by 1 mBar on the day when the event took place was associated ($p < 0.05$) with the decrease of total risk of the occurrence of appendicitis by 2.1% (0.1–4.0%). In the ≥30 years age group no significant association between barometric pressure and appendicitis occurrence risk was confirmed.

Appendicitis occurrence rate ratios for 1 mBar increase in barometric pressure by sex are shown in Table 3.

In males, the increased average daily values of barometric pressure by 1 mBar on the day when the event took place and three days before it (Lag 0–3) was associated with the decrease of the risk of the occurrence of more severe appendicitis ($p < 0.05$) by 3.1% (0.2–5.9%). In females, no significant association between barometric pressure and appendicitis occurrence was confirmed.

Appendicitis occurrence rate ratios (95% confidence interval) for 1°C increase in mean daily temperature by age group are shown in Table 4.

In all observed patients, each increase of the mean daily temperature by 1°C three days before the event took place (Lag 3) was associated ($p < 0.05$) with the increase of total risk of the occurrence of appendicitis by 1.3% (0.1–2.5%). The increase of the mean daily temperature by 1°C on the day when the event took place and three days before it (Lag 0–3), as well as mean daily temperature on the day when the event took place and seven days before it (Lag

Table 5. Appendicitis occurrence rate ratios (95% confidence interval) for 1°C increase in mean daily temperature by gender

Gender and Lag period	Total	Less severe	More severe
Females			
Lag 0	1.012 (0.998–1.026)	1.012 (0.996–1.028)	1.007 (0.985–1.030)
Lag 3	1.010 (0.996–1.024)	1.008 (0.992–1.024)	1.010 (0.987–1.033)
Lag 7	1.008 (0.994–1.022)	1.004 (0.988–1.019)	1.014 (0.991–1.037)
Lag 0–3	1.015 (1.001–1.030)*	1.014 (0.998–1.031)	1.011 (0.988–1.035)
Lag 0–7	1.013 (0.998–1.028)	1.010 (0.994–1.027)	1.013 (0.989–1.037)
Males			
Lag 0	0.999 (0.985–1.014)	0.997 (0.977–1.017)	1.001 (0.983–1.019)
Lag 3	1.006 (0.992–1.021)	1.002 (0.982–1.022)	1.008 (0.990–1.027)
Lag 7	1.004 (0.990–1.019)	0.997 (0.977–1.017)	1.010 (0.991–1.028)
Lag 0–3	1.001 (0.987–1.016)	0.999 (0.978–1.020)	1.003 (0.984–1.022)
Lag 0–7	1.003 (0.988–1.018)	0.997 (0.976–1.018)	1.007 (0.988–1.027)
Lag 0	0.999 (0.985–1.014)	0.997 (0.977–1.017)	1.001 (0.983–1.019)

*$p < 0.05$

0–7) were associated with the increase of the risk of the occurrence of appendicitis ($p < 0.05$) by 1.3% (0.1–2.6%).

Appendicitis occurrence rate ratios (95% confidence interval) for 1°C increase in mean daily temperature by sex are shown in Table 5.

In females, each increase of the mean daily temperature by 1°C on the day when the event took place and three days before it (Lag 0–3) was associated with the increase of the risk of the occurrence of appendicitis ($p < 0.05$) by 1.5% (0.1–3.0%). In males, no significant association between appendicitis occurrence and mean daily temperature was confirmed.

DISCUSSION

The conducted research did not show statistically significant correlation between the patients' age, sex, and severity of the clinical form of AA and the seasons. The lack of the seasonal pattern of AA in this research matches the results of Wolkomir et al. [13], while most of literature data show the summer pattern of the occurrence of AA [3, 4, 8–11]. Also, there are studies that suggest the tendency of the occurrence of appendicitis during winter [14, 15, 16]. The reason for this trend is unclear, but it has been reported that several factors may play a part: 1) the varying effects of bacterial or viral pathogens that cause infections at different temperatures, 2) the effect of allergens during warmer months, 3) nutritional variations, and 4) the effect of migration of tourists during summer [6, 17, 18].

The results of the conducted research showed that changes in daily values of atmospheric pressure on the day of the occurrence of AA, as well as three days before, were accompanied by decreased frequency of AA in all examined patients, including the patients with severe forms of AA. In all examined patients, changes in daily values of atmospheric temperature three days before the occurrence of AA, as well as changes in temperature three and seven days prior to the day of the occurrence of AA, were accompanied by increased occurrence of AA, with no connection to the severity of clinical form. The research conducted by Gallerani et al. [8] in Italy showed a seasonal pattern of the occurrence of AA, which depended on the clinical form of AA. In this research, acute appendicitis accompanied by peritonitis occurred more frequently during the summer [8]. Deng et al. [10] showed in their research that perforative AA had a winter pattern of incidence.

Appendix is classified as a lymphatic organ due to its structure rich in lymph follicles. There are about 200 lymph follicles in human body between the ages of 12 and 20 years. After the age of 30, the number of lymph follicles decreases by half, and after the age of 60, lymph follicles are present only in traces [19, 20]. The lymph follicles play an important role in pathogenesis of AA. Therefore, different pathogenetic mechanisms are expected among different age groups. The results presented in the research show that in patients with AA who were under the age of 30, a change in the average value of atmospheric pressure on the day of the event was accompanied by decreased occurrence of AA, with no connection with the clinical form of AA. In patients with AA aged 30 and over, there was no correlation between a change in the average value of atmospheric pressure and the occurrence and clinical form of AA. The age of patients with AA was not related to changes in the average values of atmospheric temperature. The research of Gallerani et al. [8] showed that in patients under the age of 19, the occurrence of AA was most frequent during the winter, while in patients aged 20 and over, the occurrence of AA was more frequent in the summer.

The research conducted by Sulu et al. [21] shows that AA is most common in males 10–19 years of age, while the research conducted by Noudeh et al. [9] shows that AA is most common in the summer, in men aged 20–29. Körner et al. [22] showed that the occurrence of perforated AA is not gender related. In the conducted research, it is presented that in men a change in the average daily value of atmospheric pressure on the day of the occurrence of AA, as well as three days before it, was accompanied by decreased frequency of only severe forms of AA, while the same correlation was not found in women. In women, a change in the average daily value of atmospheric temperature three days prior to the day of the occurrence of AA, was accompanied by increased occurrence of AA, with

no connection to the clinical form of AA, while in men, there was no correlation between a change in the average value of atmospheric temperature and the occurrence and clinical form of AA.

Khaavel' et al. [6] reported the increase of appendicitis occurrence rate during the periods of vast fluctuations of air temperature, increase in air humidity and decrease of actual sun radiance duration. The increase of acute appendicitis occurrence was also noted during the months of great and extremely great magnetic storms [6].

There are no literature data on the mechanism involved in the influence of atmospheric temperature and pressure on the pathogenesis of AA. The results of the study suggest that an increase of atmospheric pressure decreases the occurrence of AA and vice versa. Hypothetically, an increase in temperature, which is always accompanied by peripheral vasodilatation and decreased volume of blood circulating through internal organs, results in decreased blood irrigation of the appendix [23]. Thus, the supply of defense cells like leukocytes in appendix is decreased [23]. This results in decreased perfusion and defense ability of appendix, which further increases the occurrence of AA.

In humans, an increase in hydrostatic pressure of only 5 atmosphere absolute (ATA) causes a significant bradycardia [24]. Mulkey et al. [25] found that neurons in the dorsal motor nucleus of vagus and nucleus tractus solitarius are stimulated by ≤4 ATA of helium. These cardiovascular changes are likely mediated, in part, by a hyperbaric-induced increase in catecholamine secretion [26, 27]. Human studies at relatively high pressures have shown an augmentation of sympathetic nervous system activity, as measured by plasma epinephrine and norepinephrine levels [26]. Hypothetically, we can presume that an increase of pressure affects the sympathetic nervous system, which leads to peripheral vasodilatation under the influence of catecholamine, all of which increases perfusion of internal organs, including the appendix. The increased perfusion, as well as the increased supply of defense cells, could affect the decrease in the occurrence of AA.

It is necessary to point out that this is the very first research dealing with correlation between atmospheric temperature and pressure and pathogenesis of AA. Further research should be aimed at additional clarification concerning the mechanisms of the influence that atmospheric temperature and pressure have on pathogenesis of AA, as well as at examination of association between climatic factors and already known etiological risk factors concerning the occurrence of AA. The data would attribute to additional clarification of pathogenesis of AA.

CONCLUSION

According to the results of this research, we can conclude that patients' sex, age, and severity of the clinical form of AA are not in connection with the seasons, while there are certain connections with changes in atmospheric temperature and pressure.

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Корелација годишњих доба, температуре и атмосферског притиска с патогенезом акутног апендицитиса

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КРАТАК САДРЖАЈ

Увод Постоји врло мало литературних података о повезаности годишњих доба, температуре и атмосферског притиска са патогенезом акутног апендицитиса (АА).

Циљ рада Циљ овог истраживања је да се утврди повезаност годишњих доба, температуре и атмосферког притиска са патогенезом акутног апендицитиса на територији града Ниша.

Методе рада Истраживањем је обухваћено 395 пацијената са дијагнозом АА, који су у периоду од две године, од 1. јула 2011. до 30. јуна 2013, хоспитализовани и оперисани на Клиници за општу хирургију КЦ Ниш.

Резултати Повећање просечног дневног атмосферског притиска за један милибар на дан када су се јавили први симптоми апендицитиса било је повезано са смањењем ризика од појаве апендицитиса за 2,2%. Код свих пацијената свако повећање средње дневне температуре за 1°C три дана пре појаве симптома било је повезано са повећањем ризика од појаве апендицитиса за 1,3%.

Закључак Према нашим резултатима можемо да закључимо да пол, старост пацијента и тежина клиничке форме АА нису повезани са годишњим добима, али постоји повезаност између броја хоспитализација због апендицитиса и атмосферског притиска и температуре.

Кључне речи: годишња доба; температура; атмосферски притисак; акутни апендицитис

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Rising incidence of childhood type 1 diabetes in Montenegro

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Introduction The incidence rate of childhood type1 diabetes continues to rise across Europe by an average of approximately 3–4% per annum.
Objective The aim of this study was to examine incidence and trends of type 1 diabetes in children aged 0–14 years in Montenegro from 1997 to 2011.
Methods This was a prospective study. Primary case ascertainment was from a diabetes register, and a secondary independent data source was from hospital notifications. Case ascertainment was 100% complete using the capture-recapture method. Standardized incidence rates were calculated and trends estimated using the Poisson regression.
Results A total of 298 children (157 boys and 141 girls) were diagnosed with type 1 diabetes before 15 years of age during 1997–2011. The mean age-standardized incidence was 15.0/100,000 persons (95% CI: 12.3–17.6) during this period, increasing from 11.7/100,000 in 1997 to 18.8/100,000 in 2011. The age-specific rates per 100,000 persons per year were 10.7, 17.2, and 18.2 at ages 0–4 years, 5–9 years, and 10–14 years, respectively. A significant linear trend in incidence ($p = 0.002$) has been observed over time, with an average annual increase of 4.2%. The increase in incidence was present in both genders, with the largest relative increase in the 0–4 years age group for boys (11.0%; $p = 0.006$).
Conclusion The incidence of type 1 diabetes in Montenegro children continues to increase. We need further monitoring and additional research in order to explain the cause.
Keywords: type 1 diabetes, incidence; type 1 diabetes, children; Montenegro

INTRODUCTION

The prevalence of type 1 diabetes (T1D) varies greatly between countries. The lowest incidence (<1/100,000 persons per year) was reported in the populations from China and South America, and the highest incidence (>20/100,000 per year) was reported in Sardinia, Finland, Sweden, Norway, Portugal, the United Kingdom, Canada, and New Zealand [1]. The incidence rate of childhood T1D continues to rise across Europe by an average of approximately 3–4% per annum. Recent incidence rate trends in childhood T1D have been described in publications by the EURODIAB [2], DIAMOND Project [3], and the SEARCH for Diabetes in Youth (SEARCH) study [4].

While some studies in Europe have found that the greatest increase trend in the incidence of T1D occurred in children under five years of age [5], others showed that the trend of T1D in this age group increased to a certain point of time after which it remained stable [6]. The increased trend in T1D incidence and wide variations in the incidence could be explained by the change of environmental factors as well as of lifestyle, and their influence on persons with suitable genetic predisposition [2].

In the Balkan and Mediterranean countries, the incidence rates of T1D also show wide differences, whereas for some of them, there are still no relevant data [7].

The incidence of childhood-onset T1D mellitus among Montenegro children under 15 years of age during 1996–2006 was 13.4/100,000 per year. The annual increase rate for this period was 4.6% [8].

OBJECTIVE

This study aimed to update the previous 10-year research on the incidence and trends of type1 diabetes in 0–14-year-old children in Montenegro.

METHODS

Geographical and population data

Montenegro is located in South-Eastern Europe, with an area of 13,812 km^2 and a population of 620,145 (according to the census of 2011), including 118,751children (19%) in the 0–14 years group. The climate is continental, Mediterranean and mountainous. Montenegro is a country with slow but sustainable economic growth. Gross domestic product per capita in 2000 was €1,750, whereas in 2011 it was €5,211. The infant mortality rate in 2000 was 11.7; in 2011 it was 5.1 per 1,000 live births [9].

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Data collection

Diabetes onset in children and adolescents aged <15 years was documented according to the EURODIAB criteria [2]. All Montenegro children with newly diagnosed T1D were admitted to the University Children's Hospital in Podgorica, where they have been further monitored in the children's endocrinology outpatient clinic.

Children with newly diagnosed T1D in Montenegro who were listed on the National Public Health Institute diabetes register and Hospital Discharge Register in 1997–2011 were included in a cohort study. Data collected for each case for the diabetes register include name, date of birth, sex, place of residence, and date of diagnosis taken as the date of first insulin administration. Completeness of ascertainment was estimated with the capture–recapture method [10].

Population data

Children population was obtained from 2003 and 2011 census data, as well as the mid-year population estimates for other years in the study, which have been published by the Statistical Office of Montenegro [9].

Statistical analysis

Age and sex specific incidence rates were calculated from the number of new cases divided by the estimated number of person-years at risk in the 0–14 age group (0–4, 5–9, and 10–14 years) for each sex. Age and sex standardized incidence rates were obtained for the 0–14 years age group using the direct method with a world standard population. Poisson regression models were used to analyze the changes in incidence of T1D mellitus for 1997–2011 period and to examine the incidence differences among the age groups, and between girls and boys, as well as to estimate the temporal trend in incidence. P-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 17 (SPSS Inc., Chicago, IL, USA).

RESULTS

Incidence

In the period from 1997 to 2011, 298 children (157 boys and 141 girls) were diagnosed with T1D mellitus. Seven of them were found by primary source only, and 291 (97.7%) were identified by both sources providing 100% ascertainment. The largest number of patients was recorded in the 10–14 age group – 129 (43.3%) – while the smallest number was recorded in the 0–4 age group – 65 (21.8%).

The mean age and sex standardised incidence rate over the 15–year period was 15.0/100,000/year (95% CI: 12.3–17.6). The incidence rate ranged from 11.7/100,000/year (95% CI: 6.2–17.1) at the beginning of the study in 1997 to 18.8/100,000/year (95% CI: 11.1–26.6) at the end of the study in 2011. The peak annual incidence rate occurred in 2007 (21.8/100,000/year).

Age group

The mean incidence was 10.7 (95% CI: 7.3–14.0) for the 0–4 age group, 17.5 (95% CI: 13.1–21.9) for 5–9 age group, and 17.9 (95% CI: 14.7–21.0) per 100,000 children/year for 10-14 age group (Table 1). Differences between the groups were statistically significant (p = 0.001). The peak annual incidence rate occurred in 2007 in 5–9 age group – 21.8/100,000/year (95% CI: 13.7–30.0), more precisely in 5–9-year-old boys – 25.3/100,000/year (95% CI: 13.2–37.5).

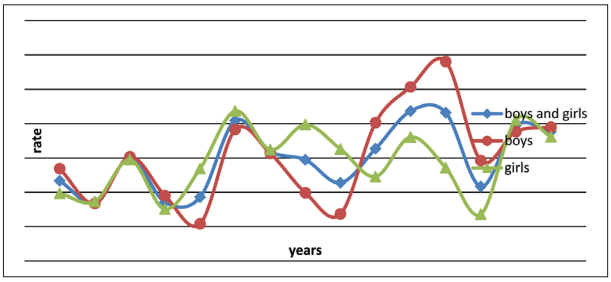
Sex

The mean age standardised incidence rate for the 15-year study period was 15.4 per 100,000/year (95% CI: 11.6–19.1)

Table 1. Mean age- and sex-specific incidence rates of type 1 diabetes mellitus in Montenegro during 1997–2011 and percentage increase incidence per year

Sex	Age groups	Population	Number of cases	Annual increases of incidence % (95% CI)	p-value	Mean incidence rate (95% CI)
Boys	0–4	21,437	35	11.0 (3.2–18.9)	**0.006**	11.9 (6.2–17.6)
	5–9	22,184	49	7.7 (3.4–14.3)	**0.021**	15.1 (9.0–21.2)
	10–14	23,843	74	1.9 (-3.4–7.1)	0.487	20.9 (15.8–25.9)
	0–14*	67,464	158	5.2 (1.5–8.8)	**0.005**	15.4 (11.6–19.1)
Girls	0–4	19,772	30	0.0 (-8.2–8.3)	0.994	10.1 (7.5–12.8)
	5–9	20,647	61	6.3 (0.5–12.2)	**0.035**	20.1 (14.1–26.0)
	10–14	22,380	49	0.8 (-5.6–7.3)	0.801	14.7 (11.4–17.9)
	0–14*	62,800	140	3.0 (-0.8–6.8)	0.123	14.5 (11.9–17.1)
All	0–4	41,209	65	4.8 (-0.8–10.4)	0.096	10.7 (7.3–14.0)
	5–9	42,832	110	7.0 (2.6–11.3)	**0.002**	17.5 (13.1–21.9)
	10–14	46,223	123	1.5 (-2.6–5.6)	0.479	17.9 (14.7–21.0)
	0–14**	130,264	298	4.2 (1.5–6.8)	**0.002**	15.0 (12.3–17.6)

* age- and sex-standardized incidence rate
** age-standardized rate
CI – confidence interval



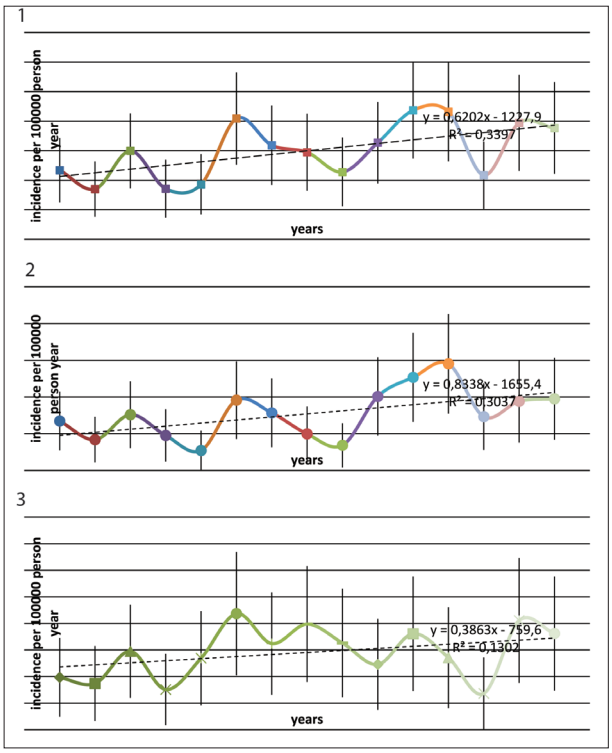
Graph 1. Mean age-specific incidence of type 1 diabetes in Montenegro boys and girls, 0–14 years old (1997–2011)

in boys and 14.5 (95% CI: 11.9–17.2) in girls. There was no significant difference between incidence in boys and girls (p = 0.671) (Graph 1).

Incidence trends

The incidence has increased by an average of 4.2% per year (95% CI: 1.5–6.8; p = 0.002), in boys 5.2% per year (95% CI: 1.5–8.8; p = 0.005), in girls 3% per year (95% CI: -0.8–6.8; p = 0.123) (Graph 2).

The highest annual increase of 11% (95% CI: 3.2–18.9; p = 0.006) was recorded in 0–4-year-old boys, but the lowest was in 0–4-year-old girls – 0% (95% CI: -0.8–6.8; p = 0.123).



Graph 2. Trends in sex-specific annual incidence (95% confidence interval) of type 1 diabetes mellitus in 0–14-year-olds in Montenegro from 1997 to 2011; observed rates in boys and girls (1), boys (2), and girls (3).

DISCUSSION

The mean age- and sex-standardized incidence rate of T1D in Montenegro for the study period of 1997–2011 was 15.0/100,000/year. The previous study performed in 2009 had shown T1D incidence of 13.4/100,000/year [8]. Montenegro belongs to the group of countries with high risk for development of T1D [3]. This rate is close to the rates in some European countries such as Austria [11], Hungary [12], Luxemburg [2], some parts of Poland [13], and Belgium [14].

The frequency of T1D is rising in almost every population. The prevalence estimates show that there are about 500,000 children under the age of 15 with T1D worldwide [15].

Most studies report an increase in incidence with increasing age, with the highest incidence occurring in the 10–14-year-old age group [3, 16]. We also found the highest incidence in this age group during the whole study period: 18.2 (95% CI: 14.9–21.4).

In this study there was no difference in the incidence between the sexes. Some studies have also shown no difference in the incidence of T1D mellitus between boys and girls [17, 18], but some countries show a female preponderance [19]. A significant male excess was found in Italy and Spain [20, 21], as well as in countries/regions with the highest incidence of T1D, such as Sardinia [22].

The increase in incidence of T1D in Montenegro is statistically significant, with a yearly average increase of 4.2% (p = 0.002). In boys, the yearly average increase was 5.2% (p = 0.005), but in girls the yearly average increase of incidence was not statistically significant – 3% (p = 0.123). The increase in incidence trend in Montenegro is approximately equal to the yearly increase in incidence of T1D in Slovenia [2].

Publications by the EURODIAB registries in Europe showed acceleration in the incidence trend in: Austria, Germany (Baden-Württemberg), Hungary, and Lithuania, and deceleration in the rate of increase in the Czech Republic, Poland (Katowice), UK (Oxford and Yorkshire), and Germany (Dusseldorf/North Rhine-Westphalia) in 1999–2008 [2].

Our findings suggest that the largest annual increase incidence is seen in boys less than five years of age, which is consistent with previously published data [23, 24], some of which assume that male sex is a risk factor for early manifestation of the disease [17].

The incidence rate of T1DM in children (age range 0–14 years) varies widely in Mediterranean and neighboring countries/regions [7], ranging from 5.8/100,000/year in the Former Yugoslav Republic of Macedonia [2], to 44.8/100,000/year in Sardinia [22].

There are great variations in incidence rates across Europe and even within various regions in an individual country. This demonstrates the interplay between genes and environment [3, 16].

There are also differences in the incidence of T1D in the seven states of the former Yugoslavia. In comparison to other former Yugoslav countries, Montenegro has the highest T1D mellitus incidence. The standardized incidence (depending on the follow-up period) of T1D (age

group 0–14 years) in Serbia was 10.4–15.5/100,000 [24, 25], in Croatia 8.2–17.23/100,000 [2, 26], in Slovenia 11.1–14.6/100,000 [2], Bosnia and Herzegovina (Republic of Srpska) 7.5/100,000 [27], Bosnia and Herzegovina (Tuzla Canton) 7.1/100,000 [28], Former Yugoslav Republic of Macedonia 5.6–5.8/100,000 children per year [2].

The incidence of T1D mellitus in Montenegro increased continuously during the 15-year survey. The raise of incidence per year was noticed in all age groups except in 0–4-year-old girls. We have no reasonable explanation for this. Recent studies demonstrate that many of the environmental triggers are associated with T1D [29]. According to some authors, breastfeeding, nicotinamide, zinc, and vitamins C, D, and E have been reported to possibly have a protective effect against T1D, whereas N-nitroso compounds, cow milk, increased linear growth, and obesity may increase the risk [30].

During the last 15 years, the country has undergone significant changes as a result of wars in the region, isolation, economic crisis, and the abrupt transition to the western lifestyle. Recent studies in Montenegro show that significant changes occurred in people's diet and that there is more frequent occurrence of obesity among schoolchildren [31]. We also have the migration of population from villages to towns. Therefore, further genetic, immunologic, and ecologic researches are required to explain the rapid growth of the T1DM incidence in Montenegro.

CONCLUSION

The incidence of T1D in Montenegro children continues to increase. We need further monitoring and additional research in order to explain the cause.

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Раст инциденце дијабетеса типа 1 код деце у Црној Гори

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КРАТАК САДРЖАЈ

Увод Годишњи пораст инциденце дијабетес мелитуса типа 1 у Европи креће се од 3% до 4%.

Циљ рада Циљ студије је утврдити инциденцу и тренд инциденце дијабетес мелитуса типа 1 код деце узраста 0–14 година у Црној Гори за период од 1997 до 2011.

Методе рада Спроведена је проспективна студија. Као први извор послужили су подаци о новооболелим пацијентима из Регистра за дијабетес, а као други извор коришћена су отпусна писма деце са новооткривеним дијабетесом типа 1. Поузданост података испитана је *capture-recapture* методом. За рачунање стандардизиване инциденце коришћена је метода директне стандардизације на стандардну светску популацију за узраст 0–14 година. За анализу промена у инциденци за период од 1997. до 2011. примењиван је *Poisson log* линеарни регресиони модел.

Резултати У испитиваном периоду регистровано је 298 деце оболеле од дијабетес мелитуса типа 1 (157 дечака и 141 девојчица). Стандардизована инциденца за целу старосну групу била је 15,0/100.000 по години (95% *CI*: 12,3–17,6) и повећавала се од 11,7/100.000 (1997. год.) до 18,8/100.000 (2011. год.). Инциденца према старосним групама кретала се од 10,7/100.000 у узрасту 0–4 године, 17,2/100.000 у узрасту 5–9 година до 18,2/100.000 и узрасту 10–14 година. Забележен је статистички значајан узлазни тренд инциденце ($p = 0,002$) са годишњим порастом од 4,2%. Повећање инциденце је присутно код оба пола са највећим годишњим порастом код дечака узраста 0–4 године (11,0%; $p = 0,006$).

Закључак Инциденца дијабетеса типа 1 у Црној Гори и даље расте. Потребни су даље праћење и допунска испитивања да би се разјаснио узрок.

Кључне речи: Црна Гора; дијабетес типа 1, инциденца; дијабетес типа 1, деца

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Lymphoblastic lymphomas in children – A single-center experience from Serbia

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SUMMARY

Introduction Intensive treatment protocols used for non-Hodgkin lymphoma in children lead to event-free survival rates ranging from 80% to 90%. However, the results are less successful in developing countries. Lymphoblastic lymphoma (LBL) is the second most frequent type of lymphoma in children, contributing with about one third to all non-Hodgkin lymphoma in childhood.

Objective The aim of the study was to evaluate the results of LBL treatment in University Children's Hospital (UCH), Belgrade.

Methods A retrospective analysis of patient records at UCH from 1997 to 2015 was carried out in patients aged 0–18 years, in whom the diagnosis of LBL had been established. Twenty-two children were included in the analysis.

Results Mean age at diagnosis was 10 years, with preponderance of male patients. All patients were treated according to Berlin-Frankfurt-Münster-based chemotherapy protocols. With median follow-up of 91.5 months, five-year probability of event-free survival was 79.5% for all patients, while overall survival was 81.8%.

Conclusion Our results, although slightly inferior to those of leading international groups, reflect a good treatment outcome in our patients.

Keywords: pediatric; lymphoblastic lymphoma; Serbia; children

INTRODUCTION

About 7% of all cancers in children are non-Hodgkin lymphomas (NHL). Lymphoblastic lymphoma (LBL) is the second most frequent type after mature B-cell lymphoma, contributing with about one third to all childhood NHL. LBL comprises a group of tumors of immature lymphoid cells that share the same morphology and immunophenotype with the lymphoblasts of precursor-cell acute lymphoblastic leukemia (ALL). Distinction between LBL and ALL is based on the extent of bone marrow infiltration by lymphoblasts, i.e. infiltration with 25% or more defines ALL, while less than 25% may be seen in LBL [1]. Great majority of pediatric LBL are T-cell LBL [2]. The initial presentation of T-LBL is an anterior mediastinal mass often leading to superior mediastinal syndrome, as well as tumor lysis syndrome [2]. Using modern chemotherapy protocols similar to those for ALL, T-LBL became a highly curable disease, with cure rates as high as 80% [3–6]. Incomplete regression of the mediastinal tumor can be seen in patients with LBL. However, absence or presence of residual tumor after induction chemotherapy could not predict the subsequent course of the disease. For that reason, most authors prefer not to change the treatment in case of partial tumor volume reduction [7]. On the other hand, patients with no response to first-line treatment have a very poor prognosis [8]. Literature reports on LBL in children in Serbia are scarce [9].

OBJECTIVE

Our study presents initial and follow-up clinical data, as well as treatment results of pediatric patients with LBL, treated in a single pediatric center in Serbia.

METHODS

We performed a review of medical records of newly diagnosed pediatric patients with LBL at the Hematology/Oncology Department of University Children's Hospital, Belgrade, from January 1st, 1997 until June 1st, 2015. A diagnosis of LBL was made in 22 children among the total of 93 patients with non-Hodgkin lymphoma. Histopathological analysis was performed by one of the authors (DB). Diagnosis of LBL in most (9/10) patients presenting with pleural effusion was established by flow cytometric analysis of cells in effusion fluid. Disease staging was performed according to the St. Jude classification. We also analyzed the initial complications of the disease. Superior mediastinal syndrome – signs and symptoms caused by compression or obstruction of superior vena cava and/or tracheobronchial compression was noted. Tumor lysis syndrome (TLS) was defined as laboratory and/or clinical TLS as per Cairo–Bishop criteria [10]. The patients were treated according to NHL-BFM-90 and EURO-LB-02 protocols [11, 12, 13]. Clinical and radiological reassessment

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of the patients was performed on day 15 and day 33 in order to evaluate treatment response, as well as after induction treatment in patients who failed to achieve complete remission (CR) on day 33. Complete remission was defined as complete resolution of tumor mass confirmed by imaging studies (X-ray and/or computed tomography). Written informed consent for the treatment was obtained from patients' parents or guardians. Probability of event-free survival (pEFS) was calculated using the Kaplan–Meier method. The differences in pEFS were compared by Mantel–Cox log-rank test. The pEFS was calculated from the date of diagnosis until the first event (death of any cause, disease progression, relapse or secondary malignancy) or until the date of last follow-up. Time was censored at last follow-up date if no failure occurred or if patient was lost to follow-up after completing therapy. Follow-up was updated to June 1st, 2015. Overall survival was calculated as the percentage of patients who were still alive at the aforementioned date of the last follow-up. Statistical calculations were performed using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corporation, Redmond, WA, USA) operating system.

RESULTS

Among 22 children, male to female ratio was 2.6:1, and mean age at diagnosis was 10 years (range 3.5–17.9, median 8.7 years).

Presenting symptoms, stage, primary localization of the disease, significant initial complications and treatment results are shown in Table 1. Half of the patients presented with breathing difficulties, while palpable tumor masses and cough were seen in more than one third. Duration of symptoms before presentation was two to 60 days (median 30 days) and about 80% of patients had two or more presenting symptoms on admission. Hepatomegaly was detected in five and splenomegaly in three patients. Most patients were diagnosed with advanced stage disease, stage III in 14 (63.6%) and stage IV in seven patients (31.8%). Only one patient had localized, stage II disease, in the neck region. Sixteen patients had mediastinal tumor only, while three patients had mediastinal tumor mass associated with other affected sites: one patient had left tonsillar affection, another patient had a tumor in the right tonsil, while one patient had infiltration of testicles, kidneys and intestines. Three patients presented with extra-mediastinal involvement, namely right temporal fossa, cervical lymphadenopathy, as well as left breast and left axilla. Four (18.2%) had initial bone marrow infiltration. Initial central nervous system infiltration was recorded in two patients (9.1%). Patients affected by tumor lysis syndrome were successfully treated by usual conservative measures such as forced alkaline diuresis with furosemide and allopurinol, preceding or parallel to initial chemotherapy. Superior mediastinal syndrome was also successfully treated after starting cytotoxic treatment in all of the patients. Malignant pleural effusion enabled the diagnosis of LBL by flow cytometry in nine patients (40.9%). In other patients, the diagnosis was established by tumor biopsy. Other initial complications were noted in seven patients, namely: pericardial effusion in four patients; atelectasis, pneumothorax, and hematothorax in one patient each. Eight patients had LDH level above 500 U/L, and four patients above 1,000 U/L. Almost all patients (21/22) were diagnosed with T-LBL, while one patient had precursor B-LBL. Ten patients (45.4%) received previous glucocorticoid treatment in local/regional hospitals at some time during the four weeks before admission.

Nineteen patients (86.4%) achieved CR after induction treatment; one child (4.5%) achieved partial remission, while one patient died before achieving CR due to tumor lysis syndrome and consequent multi-organ failure. Another patient succumbed to a toxic complication (sepsis) during intensification treatment. The patient who achieved partial remission developed disease progression and died after second-line high-dose chemotherapy with radiotherapy and autologous stem cell rescue. One patient developed testicular relapse and was treated with second-line chemo-

Table 1. Patients' characteristics and outcome

Characteristics		N
Symptoms on admission	Difficulty breathing	12
	Tumor	9
	Cough	8
	Fever	7
	Pain	5
	Vomiting	2
	Weight loss	3
	Painful swallowing	2
	Sweating	2
Stage (total = 22)	I	0
	II	1
	III	14
	IV	7
Localization	Mediastinum	16
	Mediastinum + other localization	3
	Other than mediastinum	3
CNS		2
Bone marrow		4
Initial complications	Tumor lysis syndrome	3
	Superior mediastinal syndrome	13
	Pleural effusion	10
	Other	7
Treatment results	CR	19
	PR	1
	Death before CR	1
	Toxic death	1
Relapse	Local	0
	BM	0
	BM + CNS	0
	CNS	0
	Testicular	1
Secondary malignancy		0
Survival	OS	81.8%
	five-year pEFS	79.5%

LBL – lymphoblastic lymphoma; CNS – central nervous system; CR – complete remission; PR – partial remission; BM – bone marrow; OS – overall survival; pEFS – probability of event-free survival

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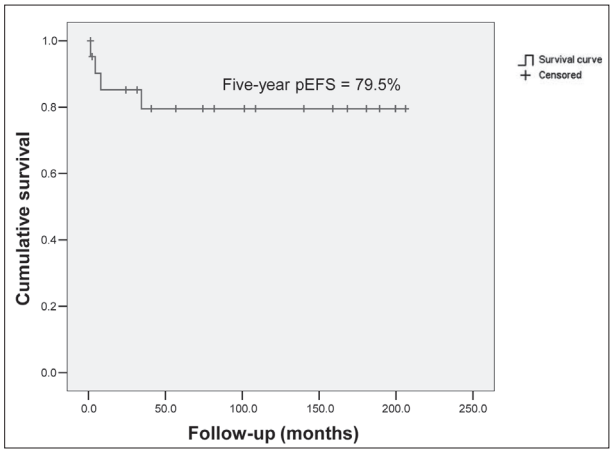

Graph 1. Kaplan–Meier estimate of event-free survival for all evaluable patients (n = 22; median follow-up 91.5 months)

pEFS – probability of event-free survival

therapy and radiotherapy. This patient then progressed to develop the second relapse presenting with cutaneous and medullary infiltration and died due to cardiac failure after salvage therapy with nelarabine, without achieving another CR. Mean follow-up was 91.5 months (range 1.3–206.1, median 78.1 months). Five-year pEFS was 79.5% for all patients (Graph 1), while overall survival was 81.8%.

DISCUSSION

Analysis of clinical presentation, treatment results and survival of childhood LBL has not been previously published in Serbia. However, there are published single-center experiences of pediatric NHL, including patients with LBL [14]. All our patients, both children and adolescents, were treated in a similar way using BFM-based protocols for LBL.

General features of our patient group were similar to those described in larger patient series in the literature [13, 15]. The incidence of LBL is generally higher in boys than in girls, which was also reflected in our results (male to female ratio 2.6:1). Median age at diagnosis of LBL was comparable to published data. However, most of our patients were diagnosed with advanced stage disease and more patients were stratified to stage IV disease; also, there were more CNS-affected patients than expected from literature data. This may be due to the fact that symptoms of lymphoblastic lymphoma are not well recognized by primary care physicians, leading to late referral of patients. In our series, median time from presentation to diagnosis was about 30 days. In addition, misrecognition of symptoms leads to an unacceptable frequency of pretreatment by systemic glucocorticoids, further complicating the diagnostic process. In our series, as many as 45% of the patients were pretreated in this way. The heavy burden of disease could also be ascertained by the high percentage of patients (59%) with elevated LDH level of more than 500 IU/L. A high proportion of patients with superior mediastinal syndrome also supports this observation.

Three patients had tumor lysis syndrome as one of the initial complications. One of these patients died before reaching CR due to tumor lysis syndrome as well as septic complication and disseminated intravascular coagulation. Two other patients were successfully managed by conservative treatment; however, urate oxidase was not used since it is not available in Serbia.

Pleural effusion was present in 10 patients (45.5%). In nine of these patients (90%) we established the immunophenotypic diagnosis by flow cytometric analysis of effusion fluid. This diagnostic method offers a considerable advantage of being far less invasive than tumor biopsy [15], as well as the added benefit of reducing time to diagnosis, since results are available in a matter of hours.

The patients were treated according to the BFM-based protocols and stratified accordingly. Our results are comparable to those of other groups that use same or similar protocols. Five-year EFS of 79.5% is slightly inferior to the largest multicenter BFM study (85 ± 1%) [13]. We lost two patients during the initiation treatment, one of whom died of sepsis, and the other of TLS complications. This early mortality (9.1%) is certainly poorer than <6% that has been reported in developed countries [7, 16, 17]; however, it does not exceed reported early mortality in other countries with limited healthcare resources [18–21]. Social and economic conditions and living standards proved to be a significant factor that influences overall treatment outcome [22]. In spite of these difficulties, our results essentially do not fall behind those in developed countries.

CONCLUSION

Although inferior to leading international groups, our treatment results reflect a mostly favorable outcome in our patients affected by LBL. However, management of early treatment complications proves to be highly challenging in a country with limited financial and medical resources such as ours. Many patients were admitted and treated in a tertiary care institution with considerable delay, and some even received glucocorticoids in primary care centers prior to being referred, significantly compromising treatment success. In countries with poor socio-economic conditions, a well-considered balance between the intensity of systemic chemotherapy, avoiding unnecessary surgical procedures, and ability of timely and appropriate detection and treatment of early complications appears to be crucial for further improvement of treatment results. Additionally, improved education of primary health care physicians, as well as multi-institutional inter-disciplinary team efforts need to be strengthened in order to improve country-level data collection, treatment results and scientific research of pediatric LBL.

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Лимфобластни лимфоми код деце – искуство једног центра у Србији

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КРАТАК САДРЖАЈ

Увод Терапија нехочкинског лимфома (НХЛ) код деце заснована на интензивним протоколима лечења доводи до преживљавања без нежељених догађаја које се креће између 80 и 90%. Међутим, успех је нешто слабији у земљама у развоју. Лимфобластни лимфом (ЛБЛ) други је по учесталости тип лимфома код деце, чинећи око једне трећине свих НХЛ у детињству.

Циљ рада Циљ рада био је евалуација резултата лечења ЛБЛ у Универзитетској дечјој клиници (УДК) у Београду.

Методе рада Спровели смо ретроспективну анализу историја болести пацијената УДК код којих је постављена дијагноза ЛБЛ у периоду 1997–2015. године, уврстивши пацијенте узраста 0–18 година. У анализу је укључено укупно двадесет двоје деце.

Резултати Средњи узраст при дијагнози био је 10 година, уз преовладавање мушког пола. Сви пацијенти су лечени хемиотерапијом заснованом на протоколу Берлин–Франкфурт–Минстер (БФМ) за ЛБЛ. Уз средњу дужину праћења од 91,5 месеци, вероватноћа петогодишњег преживљавања без нежељених догађаја износила је 79,8% за све пацијенте, док је вероватноћа укупног преживљавања била 81,8%.

Закључак Иако нешто слабији од резултата водећих међународних група, наши резултати суштински одражавају задовољавајући исход лечења наших пацијената.

Кључне речи: педијатријски; лимфобластни лимфом; Србија; деца

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Search for the presence of occult hepatitis C in patients with treatment-induced viral clearance using an ultrasensitive assay

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SUMMARY
Introduction Occult hepatitis C is defined by the presence of virus in the peripheral blood mononuclear cells (PBMCs) and/or liver cells, in the absence of serum viremia.
Objective To detect the persistence of occult hepatitis C in hemodialysis (HD) patients and patients without renal disease (non-renal) with treatment-induced clearance of hepatitis C virus (HCV) infection, using assays with a very low detection limit of viremia.
Methods A group of 13 HD patients and a group of 43 non-renal patients, with treatment-induced HCV infection clearance were investigated in the study. The HD patients were treated with pegylated interferon alpha (PEG-IFN-α) only, while the non-renal patients were treated with a combination therapy of PEG-IFN-α and ribavirin. Detection of a possible persistence of HCV RNA in the PBMCs and plasma samples was assessed by an ultrasensitive reverse transcription polymerase chain reaction (RT-PCR) assay (2 IU/ml).
Results HCV RNA was not detected in the PBMCs and plasma samples of HD patients and of non-renal patients, when assessed by the ultrasensitive RT-PCR assay.
Conclusion When a sensitive RT-PCR assay was applied, to determine if treatment induced clearance of HCV infection had been successful, occult hepatitis C could not be detected by an ultrasensitive assay, neither in HD nor in non-renal patients.
Keywords: hepatitis C virus; treatment, pegylated interferon α; ribavirin; hemodialysis; mononuclear leukocytes

INTRODUCTION

Occult hepatitis C has been described as a different pathological entity than chronic hepatitis C. The presence of detectable hepatitis C virus (HCV) RNA in the liver cells and peripheral blood mononuclear cells, in the absence of serum HCV RNA, with or without presence of antibodies against HCV, defines occult hepatitis C [1–3]. Occult HCV infection can be presented with the following two clinical settings: (i) seronegative occult hepatitis C in patients without serological evidence of a prior exposure to HCV (HCV antibodies and serum HCV RNA negative) with persistently elevated liver enzymes, and (ii) seropositive occult hepatitis C in patients who had spontaneous or treatment induced clearance of HCV infection (HCV antibodies positive and serum HCV RNA negative). In both clinical presentations of occult hepatitis C, there is a persistence of HCV RNA in the liver cells and peripheral blood mononuclear cells [4–7].

The diagnosis and monitoring of HCV infection is based on the serologic detection of antibodies to HCV, followed by the detection of HCV RNA in serum with commercial diagnostic assays [8]. In the occult HCV infection, HCV RNA can be detected in the liver cells and peripheral blood mononuclear cells (PBMCs) but not in the serum. The reason for the absence of viral RNA in the serum of patients, whose virus replicated in their liver cells and PBMCs, could be explained by the persistence of very low levels of viremia not detectable with commercial diagnostic assays [9–11]. The PBMCs represent an extra hepatic site for HCV replication [12–14]. Testing for the presence of HCV RNA in PBMCs proved to be a reliable method of identification of occult HCV infection, without the need of a liver biopsy because of its invasive nature [15–18].

The presence of occult HCV infection represents a potential risk for the spread of HCV through transfusions, nosocomial transmission of the virus in dialysis units [19, 20] and also a risk for recurrence of the HCV infection after liver or renal transplantation, in patients treated successfully with antiviral therapy prior to transplantation [21, 22].

The standard treatment of HCV infection consists of a combination therapy of pegylated interferon alpha (PEG-IFN-α) and ribavirin (RBV) [23]. Monotherapy with a reduced dose of PEG-IFN-α is a standard treatment of chronic hepatitis C in hemodialysis patients, because

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ribavirin induced hemolytic anemia in this patient population [24–26]. Several small studies have demonstrated that the use of low-dose RBV in combination with interferon based therapy was feasible for treating chronic hepatitis C in hemodialysis patients. However, the use of RBV in hemodialysis patients has not been well studied and it should be used with extreme caution and close follow-up. [18, 26, 27]

OBJECTIVE

The aim of the study was to detect the persistence of occult hepatitis C in patients with treatment-induced clearance of the virus, using diagnostic assays with very low detection limit of viremia.

METHODS

Patients

A group of 13 hemodialysis patients and a group of 43 patients without a renal disease with treatment-induced clearance of hepatitis C virus infection were investigated in the study. The study was approved by the local Ethics Committee and a written informed consent was obtained from each study participant. The 13 patients on maintenance hemodialysis were treated only with pegylated interferon α-2a. It was administered subcutaneously, once a week at a standard dose of 135 μg. The group of 43 patients without renal disease (non-renal) was treated with a combination therapy of pegylated interferon α-2a or α-2b and ribavirin. The PEG-IFN-α was administered subcutaneously once a week at a standard dose of 180 μg for PEG-IFN-α-2a and 1.5 μg/kg for PEG-IFN-α-2b. The ribavirin was administered daily at a dose of 1,000 mg for patients with body weight of less than 75 kg, and 1,200 mg for patients with body weight over 75 kg when combined with PEG-IFN-α-2a. When combined with PEG-IFN-α-2b, the ribavirin dose was 800 mg for patients with body weight of less than 65 kg, 1,000 mg for patients with body weight 65–85 kg, and 1,200 mg for patients with body weight greater than 85 kg. The treatment duration was 48 weeks for both groups of patients infected with HCV genotype 1, and 24 weeks for the patients infected with HCV genotype 2. All the patients had completed the antiviral treatment at least six months prior to enrollment in the study and they all achieved sustained viral response. The sustained viral response (SVR), defined as an absence of serum HCV RNA, six months after completion of the antiviral treatment [28], was confirmed by a reverse transcription polymerase chain reaction (RT-PCR) assay with a detection limit of 20 IU/ml. Peripheral blood samples in EDTA as an anticoagulant were obtained from each patient enrolled in the study.

HCV quantification

Hepatitis C virus RNA was extracted from plasma using QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR assay for HCV quantification was done with conventional HCV Real-TM Quant (Sacace Biotechnologies, Como, Italy) on Stratagene MX3005P real-time PCR system (Agilent Technologies, Edinburgh, UK) according to the manufacturer's instructions. Detection limit of the assay was 20 IU/ml.

Ultrasensitive detection of HCV RNA in plasma

All plasma samples which tested HCV RNA negative using the conventional real-time HCV Real-TM Quant test were re-extracted with QIAmp UltraSens Virus kit (Qiagen), following the manufacturer's instructions. The RNA extracted from 1 ml of plasma was afterwards precipitated with ethanol, suspended in RNase-free water, and placed into a single reaction to obtain maximum sensitivity. HCV RNA was detected by nested amplification of the 5' untranslated region (UTR), the region of the HCV genome [29]. The following primer pairs were used: 5'-GCAGAAAGCGTCTAGCCATGGCGT-3' (sense, KY-80) and 5'-CTCGCAAGCACCCTATCAGGCAGT-3' (antisense, KY-78) for the RT-PCR round; and 6FAM-5'-CGGGAGAGCCATAGTGG-3' (sense, R-130fam) and 5'-CGGGAGAGCCATAGTGG-3' (antisense, R-290) for the nested round. The RT-PCR step was performed using Superscript III one-step PCR kit (Invitrogen) under the following conditions: reverse transcription at 58°C for 30 minutes, initial denaturation at 94°C for two minutes, and 55 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 68°C for one minute. For the nested round, 1 μl of the RT-PCR product was used in a total volume of 25 μl including 2 mM Mg^{2+}, 200 μM of each dNTP, 2 μM of both primers, and 1.25 U Taq Gold polymerase (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The following conditions were used: initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 10 minutes at 72°C. Both positive and negative controls were included in each round. A total of 1 μl of the nested PCR product was diluted in formamide (total volume 10μl), denaturized at 95°C for three minutes and separated with a capillary electrophoresis on ABI 3500 Genetic Analyzer (PE Applied Biosystems) using Gene Scan 500 LIZ Size Standard (Applied Biosystems). The sensitivity of the nested one-step RT-PCR amplification was determined by testing stepwise dilutions of the quantified HCV RNA plasma (4th World Health Organization international standard for HCV RNA, National Institute for Biological Standards and Control, code 06/102). The low HCV RNA dilutions (10, 5, 2, and 1 IU/ml) were tested in triplicates. The detection limit of the assay was 2 IU/ml.

Isolation of PBMCs

PBMCs were isolated with a density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and homogenized in Tri Reagent Solution (Ambion, Thermo Fisher Scientific).

Ultrasensitive detection of HCV RNA in PBMCs

HCV RNA was extracted from about 5×10^6 cells using the RNeasy Mini kit (Qiagen), following the manufacturer's instructions. The total RNA extracted from the PBMCs was precipitated with ethanol, suspended in RNase-free water, and used in a single reaction to maximize the sensitivity. HCV RNA was detected with a nested amplification of the 5' UTR region of the HCV genome by RT-PCR using the same materials and protocols as for the ultrasensitive detection in plasma. The detection limit of the assay was 2 IU/ml.

RESULTS

HCV genotype 1 was the cause of infection in 12 hemodialysis patients, while one patient was infected with HCV genotype 2 (Table 1). The demographic data of the hemodialysis patients and the distribution of HCV genotypes are presented in Table 1.

Sustained viral response after the antiviral treatment of HCV infection in hemodialysis patients was confirmed by a conventional RT-PCR assay with low detection limit of viremia (sensitivity: 20 IU/ml). HCV RNA was not detected in the PBMCs and plasma samples of hemodialysis patients either, by the use of the ultrasensitive RT-PCR assay (sensitivity: 2 IU/mL).

The group of 43 non-renal patients with SVR was also studied. HCV genotype 1 was the cause of infection in 95.3% of the patients, while dual infection with HCV genotype 1 and 3 was found in two patients (Table 2). The demographic data of the non renal patients and the distribution of HCV genotypes are presented in Table 2.

Achievement of sustained viral response in the non-renal patients was confirmed by the conventional RT-PCR assay with low detection limit of viremia (sensitivity: 20 IU/ml). When tested with the ultrasensitive RT-PCR assay (sensitivity: 2 IU/ml), HCV-RNA in the PBMCs and the plasma samples, was also not detected in any of the non-renal patients.

The hemodialysis patients and the patients without renal disease experienced treatment-induced clearance of the HCV infection confirmed by the ultrasensitive RT-PCR assay, which showed no detection of HCV RNA in the PBMCs and in the plasma samples. Thus, there was no detection of occult hepatitis C in the hemodialysis patients or in the patients without renal disease with achieved sustained viral response.

Table 1. Demographic data and HCV genotypes of the hemodialysis patients

Variable	Hemodialysis patients
Number, No. (%)	13
Sex, No. (%)	
Men	13 (100%)
Women	/
Age, year, mean ± SD	47.7 ± 10.2
HCV genotype, No. (%)	
G1	12 (92.3%)
G2	1 (7.7%)

Table 2. Demographic data and HCV genotypes of the non-renal patients

Variable	Patients without a renal disease
Number, No. (%)	43
Sex, No. (%)	
Men	30 (69.8%)
Women	13 (30.2%)
Age, year, mean ± SD	40.0 ± 10.6
HCV genotype, No. (%)	
G1	41 (95.3%)
G1 + G3	2 (4.7%)

DISCUSSION

In the current clinical practice, patients with achieved sustained viral response following antiviral treatment, confirmed by commercial assays, are considered to have cleared the infection with HCV. However, data accumulated in the last decade demonstrate that the only effective approach to the diagnosis of HCV infection and its long-term persistence is the implementation of more sensitive HCV RNA diagnostic assays, as well as examination of the presence of the virus in the cells of the immune system. Searching for the presence of HCV viremia with more sensitive and ultrasensitive diagnostic assays, several investigator groups found that HCV RNA persisted at very low levels in the serum and PBMCs of patients with apparently spontaneous or treatment-induced resolution of the HCV infection [7, 30]. The study of Pham et al. [7] included 16 patients (five with spontaneous and 11 with treatment-induced resolution of HCV infection) who had undetectable serum HCV RNA tested with a commercial RT-PCR assay (sensitivity: 500 IU/ml). Low levels of viremia were detected in the serum and PBMCs of all patients using a highly sensitive RT-PCR-nucleic acid hybridization assay (sensitivity: 2 IU/ml). Radkowski et al. [30] investigated 11 HCV antibody positive patients who tested negative for serum HCV RNA with the commercial assay (sensitivity: 50 IU/mL). In the same study, low levels of HCV RNA were detected in the serum samples of six (55%) patients and in PBMCs of 11 (100%) patients, using a high-sensitivity RT-PCR assay (sensitivity: 2 IU/mL).

Our results did not reveal the presence of occult hepatitis C or persistence of HCV RNA at very low levels in the plasma or PBMCs of HD patients who achieved sustained viral response following antiviral treatment. Similar to our results, two studies which included hemodialysis patients who underwent renal transplantation after a successful treatment of HCV infection showed no presence of residual HCV RNA in their plasma samples and PBMCs. The first study included 16 patients who had achieved SVR after treatment with interferon-α during the HD [31]. At kidney transplantation, 38 months (range: 2–57 months) after interferon-α therapy, HCV viremia was negative in all patients. At the last follow-up after kidney transplantation, i.e. 22.5 months (range: 2–88 months), HCV viremia remained negative in all patients despite the immunosuppressive treatment. There

was no detection of HCV RNA in the examined PBMCs of eight transplanted patients. The second study assessed the persistence of HCV infection in 26 renal transplant patients on immunosuppressive treatment, who had spontaneous (No. = 10) or treatment-induced clearance (No. = 16) of the HCV infection while on HD [29]. No biochemical or viral relapse was detected in the studied patients during the median post-transplant follow-up of 10.5 years (range: 2–16 years). HCV RNA was not detected in any of the patient's plasma samples, repeatedly taken during the follow–up period (average: 5 times; range: 1–15 times), using a conventional RT-PCR assay (sensitivity: 15 IU/mL). Residual HCV RNA was also not detected in their plasma samples and PBMCs (unstimulated and stimulated) with an ultrasensitive RT-PCR assay (sensitivity: 2 IU/mL).

Our study results also confirmed effective clearance of HCV from the plasma and PBMCs of patients without renal disease who achieved SVR following standard antiviral treatment. Occult hepatitis C was also not detected in other studies involving patients without renal disease with achieved SVR after antiviral treatment. George et al. [32] investigated 150 patients with SVR after HCV infection treatment and followed them up for five years, for evidence of viral relapse. The presence or absence of HCV RNA in their sera was determined by RT-PCR methodology with a sensitivity of 29 IU/mL. There was no detection of HCV RNA in the patients' serum samples, confirming that there was no evidence of HCV RNA persistence. Morisco et al. [33] evaluated the long-term eradication of HCV infection in 150 treated patients with achieved SVR. The median follow-up was 8.6 years (range 2–19.9 years). Presence of HCV RNA in the serum was not detected in all study participants, using the RT-PCR assay with sensitivity of less than 50 IU/mL, when testing at least four blood samples taken at different time points.

The concept of occult hepatitis C is still a matter of debate. Some studies detected occult hepatitis C and other studies did not detect it in patients with treatment-induced clearance of HCV infection. The disagreement between these studies could be associated with the differences of the criteria used to define sustained viral response after antiviral treatment. There were differences in the detection limit of the RT-PCR assays used to determine the sustained viral response, ranging from 15 IU/ml to 500 IU/ml. Therefore, HCV RNA would have probably been detected in the serum of patients with occult hepatitis C, if RT-PCR assays with a low detection limit of viremia had been used to check achievement of sustained viral response.

CONCLUSION

Occult hepatitis C was described as a phenomenon characterized by undetectable HCV RNA in the serum tested with commercial assays, but with detection of viral RNA in PBMCs and/or liver cells. The "absence" of HCV RNA in the serum could be explained with the persistence of very low levels of viremia, frequently escaping detection by commercial assays. When HCV RNA was undetectable in the serum using assay with high sensitivity, there was also absence of HCV RNA in the peripheral blood mononuclear cells in patients with treatment-induced viral clearance.

The implementation of assays with high sensitivity, capable of detecting low levels of viral RNA, for clinical and population-based examination, should be the only effective approach to determine a long-term sustained viral response in patients treated with antiviral therapy.

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Претрага за присуство окултног хепатитиса Ц код пацијената са терапијском елиминацијом вируса применом ултрасензитивног теста

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КРАТАК САДРЖАЈ

Увод Окултни хепатитис Ц је дефинисан присуством вируса хепатитиса Ц у мононуклеарним ћелијама периферне крви и/или хепатоцитима, при негативним налазом вирусне рибонуклеинске киселине (РНК) у серуму.

Циљ рада Циљ овог рада је утврдити присуство окултног хепатитиса Ц код пацијента на хроничној хемодијализи и код пацијента без бубрежне болести, код којих је постигнута терапијска елиминација вируса, помоћу примене ултрасензитивног теста за детектовање вирусне РНК.

Методе рада У студији је било укључено 13 пацијента на хемодијализи и 43 пацијента без бубрежне болести, код којих је постигнута елиминација вируса хепатитиса Ц помоћу антивирусног третмана. Пацијенти на хемодијализи били су лечени само пегилираним интерфероном алфа, док су пацијенти без бубрежне болести били лечени комбинацијом пегилираног интерферона алфа и рибавирина. Потенцијално присуство вирусне РНК у мононуклеарним ћелијама периферне крви и у плазми пацијента је испитивано помоћу ултрасензитивног теста за детектовање вирусне РНК (сензитивност од 2 *IU/ml*).

Резултати Применом ултрасензитивног теста, вирусна РНК није била откривена ни у мононуклеарним ћелијама периферне крви нити у плазми хемодијализних пацијента и пацијента без бубрежне болести.

Закључак Применом ултрасензитивног теста за детектовање вирусне РНК, који се користио за утврђивања успешности терапије код хепатитиса Ц, окултни хепатитис Ц није био откривен ни код хемодијализних, нити код пацијената без бубрежне болести.

Кључне речи: вирус хепатитиса Ц; лечење; пегилиран интерферон алфа, рибавирин; хемодијализа; мононуклеарни леукоцити

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Factors associated with inconsistent condom use with clients among female sex workers in Podgorica, Montenegro

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SUMMARY
Introduction Female sex workers (FSWs) are a group at increased risk for human immunodeficiency virus (HIV) infection, and inconsistent condom use with clients is a known risk factor for infection in this group.
Objective The aim of the study was to determine factors associated with inconsistent condom use with clients among female sex workers in Podgorica, Montenegro.
Methods We conducted an HIV bio-behavioral cross-sectional study in a sample of female sex workers recruited by snowball sampling.
Results A total of 142 FSWs were recruited. Eighty-one (57.0%) of them used condoms consistently with clients. HIV prevalence was 0.0%. In the multivariate analysis inconsistent condom use with clients in the previous month was associated with clients' negative personal attitude [age-adjusted odds ratio (AOR) = 22.7, 95% confidence interval (CI) = 2.3–228.0] or client's indifference (AOR = 13.0, 95% CI = 1.4–118.9) towards using condom during sex with sexual workers, decision making by clients or by mutual agreement with client about using a condom (AOR = 10.2, 95% CI = 3.7–28.0), and early age of first sex (AOR = 5.4, 95% CI = 1.6–18.5).
Conclusion Our results suggest not only the need for further promotion of condom use, information and education for FSW but also the need to strengthen negotiation skills of FSWs with clients on regular use of condoms, as well as the need to extend prevention programs to clients of FSWs.
Keywords: female sex workers; paid sex; HIV; consistent condom use

INTRODUCTION

In order to track the human immunodeficiency virus (HIV) epidemic and to target limited resources for prevention and care programs, ongoing collection of accurate data on populations at increased risk for HIV is needed. Female sex workers (FSWs) are at increased risk due to exposure to multiple sexual partners, thus increasing the probability of exposure to an HIV-infected partner, as well as due to high prevalence of bacterial and viral sexually transmitted infections (STI) that increase the probability of HIV infection once exposure occurs [1, 2]. Globally, in Europe and North America, as well as in Asia and Latin America, injection drug use either by FSWs or their steady male partners also increases risk of infection [1, 3].

Prevention of sexually transmitted HIV infection in FSWs hinges on consistent condom use (CCU) both with clients and other partners. CCU is one of the most effective and efficient ways of preventing sexual transmission of HIV and other STIs and slowing the spread of HIV where the epidemic has not yet extended into the general population [4, 5, 6]. Among female sex workers, the prevalence of CCU with clients varies in different regions and countries, from 5.8% in Indonesia, to 75.3%, 81%, and 93.4% in Croatia, the Netherlands and Chile, respectively [7–13]. To date, there have been two studies of HIV risk and condom use among FSWs in Montenegro, which found that 44% and 58% of FSWs, respectively, reported consistent condom use with clients in the previous month [13, 14].

The sampling of a FSW population poses a unique challenge. In Podgorica, as well as in the whole of Montenegro, there is no registration of FSWs and most of sex work is not based in brothels. As a result, there is no feasible framework for drawing a representative population-based sample. Montenegro's small population size has implications on the practice of sex work as, unlike in other more populated European cities, both sex workers and clients express concern about being recognized. Low density of FSWs, lack of contact between FSWs and local health care services, and a high level of mistrust towards "official" agencies have made respondent-driven sampling difficult in populations of FSWs in Montenegro and other countries of Eastern Europe [15]. For these reasons, most of the studies of FSW in this area have been conducted using snowball sampling.

OBJECTIVE

The objective of this study was to determine factors associated with inconsistent condom use with clients among FSWs in Podgorica, Montenegro.

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METHODS

Setting

Montenegro, a former Yugoslav republic, is a small, newly independent country in southeastern Europe with a population of around 620,000. Podgorica, with around 200,000 inhabitants, is the capital of Montenegro. Existence of FSWs is only moderately visible in Podgorica. HIV prevalence in general population of Montenegro is 0.018% [16–18]. The annual incidence of reported HIV/AIDS cases from 2005 to 2013 varied from 1.1 to 2.2/100,000 persons. Cumulative HIV incidence between 1989, when the first case was registered, and 2013, as reported in the Annual Report on HIV/AIDS in Montenegro, is 153 persons [17]. The predominant route of HIV transmission is sexual – homosexual, bisexual, and heterosexual [17]. Targeted interventions for HIV prevention and harm reduction have been in existence in Podgorica since 2004. Currently, there is one drop-in center for FSWs in Podgorica (established in 2010) and one Center for Voluntary and Confidential HIV Counseling and Testing. The drop-in has been established and managed by non-governmental organizations (NGOs), while the Center for Voluntary and Confidential HIV Counseling and Testing is operated by the Institute for Public Health.

Sampling procedure and data collection

We conducted a bio-behavioral cross-sectional survey among FSWs in Podgorica from April through July 2012. Eligible participants were women, at least 18 years of age, who had at least one commercial sex partner in the previous 12 months, and who resided in Podgorica for at least three months during the previous 12 months.

We identified and recruited participants using snowball sampling. With the assistance of a local NGO that provides prevention services to FSWs (distribution of free condoms and HIV/STI educational materials and referral to HIV voluntary counseling and testing centers), we recruited six initial participants who operated in different parts of the city. These initial participants referred other FSWs from various parts of the city. Each participant received primary incentive for completing the questionnaire and for providing a blood sample to be tested for HIV, and secondary incentives for each additional person recruited into the study. We gave each participant a detailed explanation of study procedures and objectives prior to enrolment. In order to maintain confidentiality, we obtained verbal informed consent and collected no individually identifying information. We gave each respondent an identification code that linked their questionnaires and laboratory tests. Participants used the same code to obtain their HIV test results at voluntary testing and counseling centers. Participants could withdraw from the study at any stage of the study without any consequences except not getting incentive.

Measurements

We gathered data from all respondents using a standardized questionnaire, which contained questions on demographic and behavioral characteristics related to potential HIV and STI exposure [19]. Respondents completed the questionnaire by themselves, but could ask an interviewer for assistance if they had not understood a question or were unable to fill in the questionnaire by themselves.

Our main outcome measure was inconsistent condom use with a commercial sex partner (client) in the previous month. Our predictor variables were demographic characteristics (age, marital status, employment status), behavioral factors (age at first sexual intercourse, age at first paid sex, how clients are contacted, number of clients in the past week, condom use during last sex with a client, observed attitude of clients towards using condom, observed process of decision making of condom use during commercial sex, number of persons other than clients with whom participant had sex during the previous 12 months, consistency of condom use with a non-commercial sexual partner(s) during the previous month, experience with drug use, prior history of an STI, visits to a gynecologist in the previous 12 months, previous HIV testing, knowledge about HIV transmission (four questions), knowledge about HIV prevention (two questions), self-assessment of risk for HIV infection, receipt of free condoms and information from a local NGO about HIV transmission and prevention. We recoded knowledge variables to three categories: knowledgeable about HIV transmission and prevention (correct answer to all six questions), knowledgeable about HIV transmission (correct answer to all four transmission question), and knowledgeable about HIV prevention (correct answer to two questions).

Laboratory methods

We collected blood samples from all participants and tested them to detect HIV 1/2 antibodies using a rapid test with immune-chromatography technology (HIV1/2, Rapid Device, HIV Rapid, Ab1&2 Cassette, BIOTEC, UK). Confirmatory HIV 1/2 testing was done by western immunoblot (NEW LAW BLOT I/II, BIO-RAD, Marnes-la-Coquette, France).

Blood samples were tested in the laboratory of the Center for Medical Microbiology of the Institute for Public Health, which serves as a national referral center for HIV testing.

Statistical analysis

We analyzed data using SPSS/PC version 12 (SPSS Inc., Chicago, IL, USA) and used χ^2 tests and unadjusted odds ratio to assess associations between each of the defined predictors and inconsistent condom use with clients in the previous month. We included all significant predictors ($p < 0.05$) and known confounders in a multivariate logistic regression and tested the overall fit of the final model using the Hosmer and Lemeshow test.

Ethical consideration

All survey procedures were conducted by trained personnel. Ethical approval was granted by the Ethical Committee of the Institute for Public Health in Podgorica.

RESULTS

We recruited 142 FSW to participate in the study. The median age of the participants was 27 years (range: 18–50 years); 63.4% had elementary school education or less; 63.4 were single, and 88% were unemployed in the formal sector (Table 1). About one-fourth had had first paid sex before the age of 18 years. The most frequent ways of contacting clients were through friends (52.8%), bars/clubs/restaurants (16.9%), through phone calls (16.9%) or in the streets/parks (5.6%). Almost one fifth reported that they had injected drugs in the previous year; 7.7% reported having STI during the previous year. Three out of five had visited a gynecologist in the previous 12 months.

Almost three quarters (73.2%) reported condom use during their last sexual contact with a client, but fewer (57.0%) reported that they had always used condoms with clients in the past month; 64.8% of participants reported that they were decision makers about condom use with client. Majority of participants (88.7%) reported sexual intercourses with one or more partners other than clients in the previous 12 months. Only 23.0% of these reported consistent condom use with such partners during the month preceding the study.

Prior to participation in this survey, 33.1% of the participants reported that they had been tested for HIV. None of the FSWs in our sample were HIV positive. Only 16.2% were able to correctly answer all six HIV knowledge questions, but 69.7% understood that proper and regular condom use was protective against HIV infection, and 31.7% had received free condoms and prevention information from local NGOs; 61.3% considered themselves to be at moderate or higher risk of HIV infection.

In univariate logistic regression analysis, client's indifference or his negative personal attitudes towards using condom during sex with sexual workers, decision about condom use made by client or with mutual agreement, lack of knowledge about HIV prevention measures, not receiving HIV prevention information and free condoms from a local NGO, never being tested for HIV, early age at first sexual intercourse, and being single were all associated with inconsistent condom use with clients (Table 2).

In multivariate logistic regression analysis inconsistent condom use with clients in the previous month were associated with client's negative personal attitude [age-adjusted odds ratio (AOR) = 22.7, 95% confidence interval (CI) = 2.3–228.0] or client's indifference (AOR = 13.0, 95% CI = 1.4–118.9) towards using condom during sex with sexual workers, decision making by clients or by agreement with client about using of condom (AOR = 10.2, 95% CI = 3.7–28.0) and early age of first sex (AOR = 5.4, 95% CI = 1.6–18.5) (Table 3).

DISCUSSION

We found that 57% of FSWs in Podgorica reported that they had used condoms during each episode of commercial sex in the prior month. We also found that client's indifference or his negative personal attitude towards using condom during sex with sexual workers, decision about condom use made by client or with mutual agreement and early age at first sexual intercourse, all predicted inconsistent condom use with clients.

In earlier studies among FSWs conducted in Montenegro, the prevalence of consistent condom use in the month preceding the survey was 44% and 58%, respectively, suggesting a stagnancy of trend in risk reduction [13, 14].

The association we found between inconsistent condom use with client's indifference or negative personal attitude towards condom use during commercial sex, as well as with deciding on condom use done by clients or by mutual agreement between FSWs and client, were consistent with the results of the studies among FSWs in China, Cambodia, Korea, Sri Lanka, Ghana, and Vietnam, which found similar associations with inconsistent condom use [20–28]. Factors involved in the frequent acceptance of commercial sex without condoms probably include lack of authority, fear of losing clients and weak negotiation skills. This could be likely compounded by lack of knowledge about HIV prevention measures since 30.3% of FSW did not know that proper and regular condom can reduce risk of HIV infection, comparing with FSW population in Belgrade in neighboring Serbia, where only 15.9% of street sex workers and 11.5% of "indoor" sex workers had no knowledge of this fact [29].

Early age of first sex was found as a risk factor for inconsistent condom use in Uganda [30]. This finding could be explained by possible prevention fatigue or lower self esteem. Unfortunately, our data did not allow us to explore further this range of possible explanations.

There are a few limitations to this study. Our study is limited by imperfection of snowball sampling design and its relatively small sample size, which may lead to reduced representativeness and lack of statistical power. Nonetheless, based on independent estimates of the FSW population size in Podgorica, we assumed that our sample included the majority of the active commercial sex workers in the city. Our study also relies on self-reports of sexual practices and behaviors, which are subject to recall bias and are difficult to be verified. We hope that this study will help in gaining trust from FSWs for future bio-behavioral studies that may be able to employ different sampling methodologies, such as respondent-driven sampling or time-location sampling.

CONCLUSION

As the most important factors affecting the inconsistent condom use during sex with clients, our study has identified the following: clients' insistence on not using condom or clients' indifference towards condom use; allowing clients to have bigger influence over the decision on

Table 1. Demographic and behavioral characteristics of female sex workers, Podgorica, Montenegro, 2012

Variables		n	%
Age group (years)	18–24	50	35.2
	25–30	70	49.3
	31–40	17	12.0
	41+	5	3.5
Level of completed education	Elementary school or less	90	63.4
	Secondary	47	33.1
	University	5	3.5
Marital status	Married	12	8.4
	Regular partner	40	28.2
	Single	90	63.4
Employment status (formal sector)	Full time	3	2.1
	Part time	14	9.9
	Unemployed	122	88.0
Age at first sex (years)	≤14	32	22.6
	15–17	79	55.6
	≥18	31	21.8
Age at first paid sex (years)	≤14	7	4.9
	15–17	29	20.4
	18–24	64	45.1
	≥25	42	29.6
Number of clients during last week	≤2	55	38.7
	3–4	40	28.2
	5–7	29	20.4
	≥8	18	12.7
Use of condom during last sex with client	Yes	104	73.2
	No	38	26.8
Consistent use of condom with clients during previous month	Yes	81	57.0
	No	61	43.0
Attitude of most clients towards using condoms	Insist on condom use	23	16.2
	Prefer condom but willing to have sex without condom if not available	33	23.2
	Do not care	58	40.8
	Insist on sex without condom	28	19.7
Decision maker on using a condom during sex with client	Sexual worker by herself	92	64.8
	Client	7	4.9
	Agreement (together with client)	43	30.3
Number of persons other than clients with whom she had sex during last year	0	16	11.3
	1	56	39.4
	2–3	42	29.6
	4–7	23	16.2
	≥8	5	3.5
Consistent use of condom with other than clients whom she had sex during previous month	Yes	29	23.0
	No	97	77.0
Used illegal drugs by injection route during previous 12 months	Yes	26	18.3
	No	116	81.7
Had symptoms of a sexually transmitted infection during last year	Yes	11	7.7
	No	131	92.3
Visited a gynecologist during previous 12 months	Yes	84	59.2
	No	58	40.8
Ever tested for HIV	Yes	47	33.1
	No	95	66.9
Correctly answered all six questions about HIV transmission and prevention	Yes	23	16.2
	No	119	83.8
Correctly answered all four questions about HIV transmission	Yes	49	34.5
	No	93	65.5
Correctly answered both questions about HIV prevention	Yes	60	42.3
	No	82	57.7
Knows that proper and regular condom use can reduce risk of HIV	Yes	99	69.7
	No	43	30.3
Self-assessment of risk for HIV	No or low risk	55	38.7
	Moderate risk	40	28.2
	High or very high risk	47	33.1
Received free condoms and information about HIV transmission and prevention from an NGO	Yes	45	31.7
	No	97	68.3
Result of HIV rapid screening test	Reactive (positive)	0	0
	Nonreactive (negative)	142	100.0

n – number of participants (female sex workers) in sample

Table 2. Correlates of inconsistent condoms use during last month by female sex workers with clients, Podgorica, 2012 (univariate logistic regression)

Variables		n/N	Sample prevalence (%)	Unadjusted OR (95% CI)	p-value
Attitude of most clients towards using condoms	Insist on condom use#	1/23	4.3	1.00	
	Prefer condom but willing to have sex without condom if not available	19/33	57.6	29.9 (3.6–248.6)	0.002
	Do not care	25/58	43.1	16.7 (2.1–132.1)	0.008
	Insist on sex without condom	16/28	57.1	29.3 (3.4–249.1)	0.002
In general, the decision on using a condom during sex with client is made by	Sexual worker by herself#	23/92	25.0	1.00	
	Client or mutual agreement	38/50	76.0	9.5 (4.2–21.2)	0.000
Correctly answered both questions about HIV prevention§	Yes#	20/60	33.3	1.00	
	No	41/82	50.0	2.0 (1.0–3.9)	0.049
Received free condoms and HIV information from an NGO	Yes#	13/45	28.9	1.00	
	No	48/97	49.5	2.4 (1.1–5.1)	0.023
Ever tested for HIV	Yes#	14/47	29.8	1.00	
	No	47/95	49.5	2.3 (1.1–4.8)	0.027
Age at first sex (years)	≥18#	8/31	25.8	1.00	
	≤17	53/111	47.7	2.6 (1.1–6.4)	0.033
Marital status	Married or regular partner#	16/52	30.8	1.00	
	Single	45/90	50.0	2.2 (1.1–4.6)	0.027
Age group (years)	18–24#	20/50	40.0	1.00	
	≥25	41/92	44.6	1.2 (0.6–2.4)	0.600

OR – odds ratio; CI – confidence interval
Referent value
§Composite indicator consisted of the following two questions: 1. Can sexual intercourse with only one, faithful and uninfected partner, reduce risk of HIV infection?; and 2. Can proper and regular use of condoms reduce risk of HIV infection?

Table 3. Independent correlates of inconsistent condom use in the previous month by female sex workers with clients, Podgorica, Montenegro, 2012 (multiple logistic regression)

Variables		AOR	95% CI	p-value
Attitude of most clients towards using condoms	Insist on condom use #	1.00		
	Prefer condom but willing to have sex without condom if not available	27.3	2.8–271.2	0.005
	Do not care	13.0	1.4–118.9	0.023
	Insist on sex without condom	22.7	2.3–228.0	0.008
In general, the decision on using a condom during sex with client is made by	Sexual worker by herself #	1.00		
	Client or mutual agreement	10.2	3.7–28.0	0.000
Age at first sex (years)	≥18#	1.00		
	≤17	5.4	1.6–18.5	0.007
Correctly answered both questions about HIV prevention§	Yes#	1.00		
	No	2.4	0.9–6.2	0.060
Received free condoms and HIV information from an NGO	Yes#	1.00		
	No	2.1	0.5–8.2	0.284
Ever tested for HIV	Yes#	1.00		
	No	0.8	0.2–3.3	0.760
Marital status	Married or regular partner#	1.00		
	Single	1.7	0.6–4.5	0.292
Age group (years)	18–24#	1.00		
	≥25	2.4	0.9–6.9	0.093

AOR – adjusted odds ratio
Referent value
§Composite indicator consisted of the following two questions: 1. Can sexual intercourse with only one, faithful and uninfected partner reduce risk of HIV infection?; and 2. Can proper and regular use of condoms reduce risk of HIV infection?

condom use; making decision on condom use based on mutual agreement between FSWs and clients, as well as the early age of FSWs at the time of first sexual intercourse. Although the last mentioned factor can hardly be influenced by any kind of intervention, according to similar studies and experiences from Thailand, all other above mentioned factors can be influenced primarily through better health education of both FSWs and clients about the importance of regular and proper use of condoms in preventing HIV and other STIs, as well as through expanding specific health education programs among FSWs, targeting improving negotiation skills with clients in order to stress the importance of condom use or even to make it a precondition to sexual intercourse with clients.

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Фактори повезани са недоследном употребом кондома приликом сексуалних односа са клијентима међу сексуалним радницама у Подгорици у Црној Гори

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КРАТАК САДРЖАЈ

Увод Сексуалне раднице у југоисточној Европи представљају групу лица која су изложена повећаном ризику за ХИВ инфекцију. Недоследна употреба кондома током сексуалних односа са клијентима представља фактор ризика за ХИВ инфекцију за припаднице ове групе.

Циљ рада Циљ овог рада је био утврђивање фактора повезаних са недоследношћу коришћења кондома током сексуалних односа с клијентима у популацији сексуалних радница у Подгорици.

Методе рада Спроведена је биобихејвиорална студија пресека у којој су испитанице из узорка регрутоване методом грудве снега.

Резултати Укупно су регрутоване 142 испитанице. Њих 81 (57,0%) током последњих месец дана пре испитивања конзистентно је користило кондоме током сексуалних односа са клијентима. Међу испитаницама нису идентификована лица са ХИВ инфекцијом. Мултиваријатна анализа је показала да је недоследна употреба кондома током сексуалних односа са клијентима повезана са негативним ставом (*AOR* = 22,7, 95% *CI* = 2,3–228,0) или равнодушношћу клијента (*AOR* = 13,0, 95% CI = 1,4–118.9) према коришћењу кондома током сексуалних односа са сексуалним радницама, одлучивањем од стране клијента или договарањем са клијентом о коришћењу кондома (*AOR* = 10,2, 95% *CI* = 3,7–28,0), као и раним узрастом у време првог сексуалног односа (*AOR* = 5,4, 95% *CI* = 1,6–18,5).

Закључак Резултати наше студије сугеришу да је, поред даљег здравственог просвећивања и промовисања доследне употребе кондома, неопходно ојачати преговарачке вештине сексуалних радница са клијентима око обавезне употребе кондома. Такође, требало би проширити здравствено-едукативне програме на клијенте сексуалних радница.

Кључне речи: сексуалне раднице; плаћени секс; ХИВ; доследна употреба кондома

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A case of severe type of cerebro-costo-mandibular syndrome

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SUMMARY

Introduction Cerebro-costo-mandibular syndrome (CCMS) is a rare disorder, with only 75 cases described in the literature to date. CCMS is characterized by association of micrognathia and specific multiple rib defects. It is accompanied by mental deficiency in considerable number of cases. Sometimes, there are associated anomalies and problems, such as spine deformities, brain, heart, kidney or ear anomalies, feeding difficulties, delayed psychomotor development, and growth impairment. Depending on severity of deformities and consecutive respiratory insufficiency, in about 35–50% of CCMS cases, death occurs during the first year of life. These cases are referred to as severe types of CCMS.

Case Outline In this paper we present a female infant with severe type of CCMS. Diagnosis was established in the first day of life, based on micrognathia and findings of posterior rib-gap defects on the chest X-ray, accompanied by dyspnea. Progressive severe respiratory insufficiency caused by chest and air-way deformities and exacerbated by episodes of pneumonia, led to respiratory failure and death at the age of 7.5 months.

Conclusion CCMS should be considered in every infant with micrognathia and rib-gap defects on chest X-ray.

Keywords: cerebrocostomandibular syndrome; rib-gap defects with micrognathia; micrognathism; respiratory insufficiency; ribs; congenital abnormalities

INTRODUCTION

Cerebro-costo-mandibular syndrome (CCMS) is a rare disorder, originally described by Smith et al. [1] in 1966. Seventy-five cases have been published in the literature since then [2]. Most published cases are from Western European countries, Asian countries, and the USA. To the best of our knowledge, this is the only one published from Western Balkans countries.

The main characteristics of this syndrome are specific rib defects and micrognathia [2, 3]. It is accompanied by mental deficiency in a considerable number of cases. Sometimes, there are associated anomalies and problems, such as spine deformities, brain, heart, kidney or ear anomalies, feeding difficulties, delayed psychomotor development, and growth impairment.

The etiopathogenesis of CCMS is still not fully explained. A significant number of cases occur sporadically [2, 3]. Cases associated with consanguinity have also been described, as well as those with CCMS in siblings, suggesting autosomal recessive inheritance [4, 5, 6]. Finally, cases of CCMS in parents and their offspring have also been published, indicating autosomal dominant type of inheritance with variable gene expression [7, 8]. Recently, significant progress in genetic basis of CCMS has been achieved. In 2014, Lynch et al. [9] published a paper on genetic testing performed in a cohort of ten families with CCMS members – seven families with sporadic CCMS cases, two with parent-child transmission, and one with CCMS in siblings of unaffected parents. Heterozygous mutations in the small nuclear polypeptids B and B1 (SNRPB) gene were found in probands [9]. SNRPB belongs to non-coding conserved elements of the human genome. These elements have an important regulatory role through their impact on mechanism of alternative splicing, process of great importance for creation of additional protein diversity. Mutations in SNRPB gene, with consecutive defect in the splicing machinery, cause developmental disorders characteristic of CCMS. Mutation analysis suggested that CCMS is an autosomal dominant disorder with possibility of non-penetrance, as well as a high rate of de novo mutations [9]. Deregulation of SNRPB expression as the main mechanism for CCMS was confirmed by Bacrot et al. [10], through results of genetic testing of five CCMS patients.

We present a case of an infant with CCMS diagnosed on the first day of life. The authors showed the clinical course of the severe type of CCMS, and pointed to evolution of findings observed at birth and their clinical implications.

CASE REPORT

A female infant was born as the second child of a 22-year old healthy mother, from a non-consanguineous marriage. The course of this pregnancy was uneventful, with appropriate prenatal care. The family history was unremarkable. The older sibling is a healthy girl.

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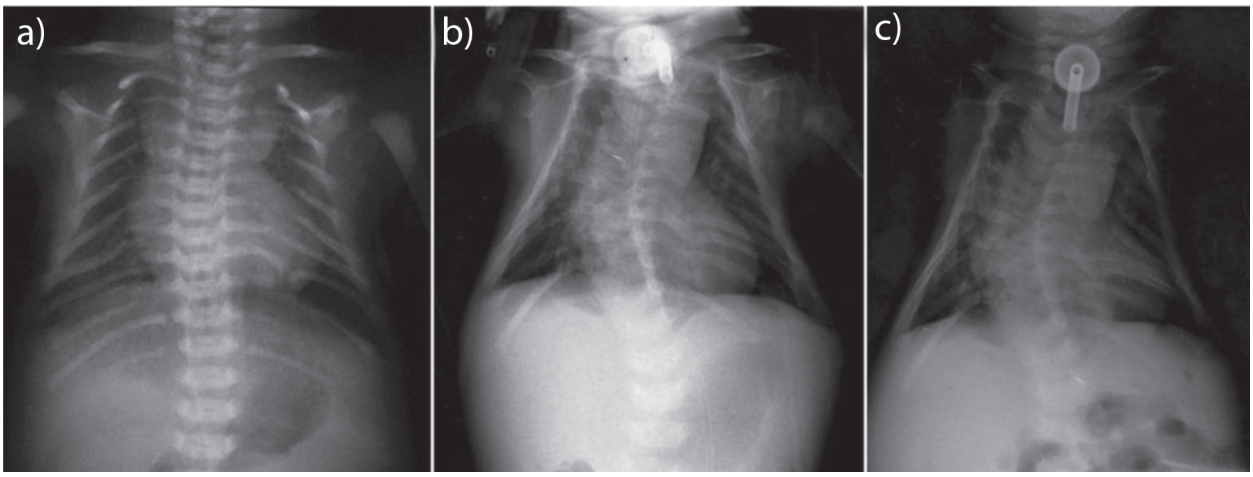

Figure 1. Chest X-rays of the infant showing 11 pairs of ribs, posterior rib-gap defects on the 2nd–9th rib bilaterally, narrow ribcage and spine deformity. Photographs were taken at the age of (a) one day, (b) four months, and (c) 6.5 months.

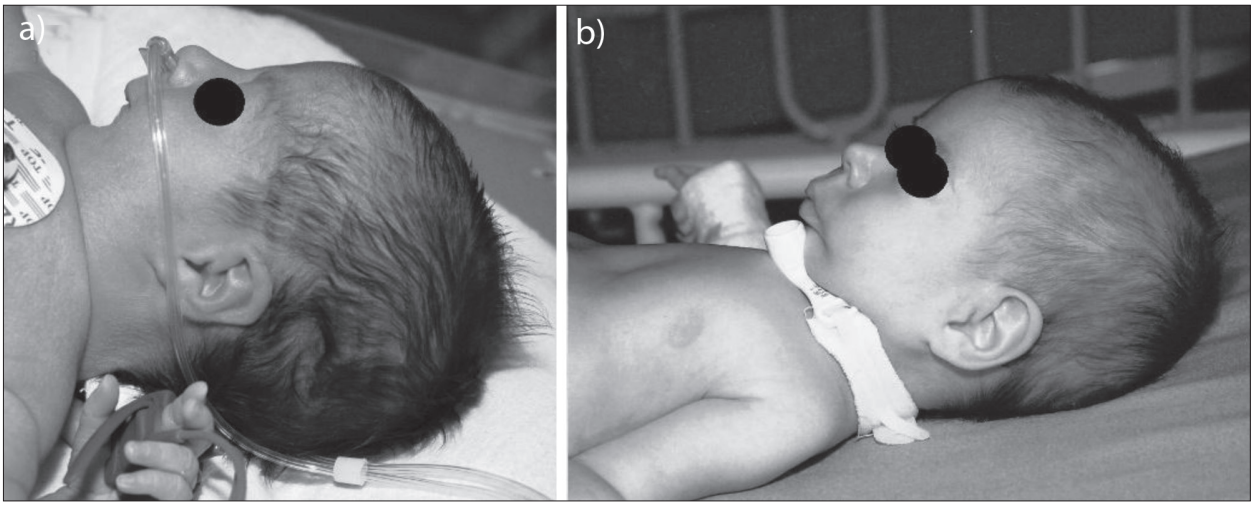

Figure 2. Infant's face profile with micrognathia and retrognathia. Photographs were taken when the infant was at the age of (a) two days and (b) 5.5 months; on photograph (b) tracheal stoma is present.

The pregnancy was terminated at 38 weeks of gestation, with spontaneous vaginal delivery at a local maternity hospital. Apgar score was 4, 6, and 7 at 1, 5, and 10 minutes after birth, respectively. The birth weight was 2,800 g (25 p), birth length was 49 cm (50 p), head circumference was 36 cm (>90 p), and chest circumference was 32 cm (25 p). The baby was resuscitated at birth, since she did not show spontaneous respiratory effort. Respiratory support via nasal continuous positive airway pressure was applied afterwards. During the first day of life she was transferred to our neonatal intensive care unit for further evaluation and treatment.

On admission, several malformations were observed: retromicrognathia, high-arched palate, hypertelorism, low-set malformed ears, narrow chest, wide-set nipples, redundant skin and muscle hypotonia.

Chest X-ray on the first day of life showed a narrow, bell-shaped thorax with eleven pairs of ribs, with posterior rib-gap defects of the second to ninth rib on both sides (Figure 1a). This finding, accompanied with retromicrognathism (Figure 2a), pointed to cerebro-costo-mandibular syndrome.

Cranial ultrasound showed signs of brain hypoxia. Karyotype and laboratory tests including initial laboratory screening for immunodeficiency (complete blood count with differential, measurement of serum immunoglobulin and complement levels), echocardiography, ultrasound of abdominal organs and hips, electroencephalography and ophthalmologic tests were unremarkable.

From the second day of life, the infant breathed spontaneously, with oxygen therapy (30% oxygen), but was occasionally tachypneic and dyspneic. At the age of five weeks, respiratory insufficiency appeared. The underlying cause was pneumonia. The infant was endotrachealy intubated, and mechanically ventilated, with appropriate antibiotic therapy and supportive measures. In further course, clinical, ultrasonographic and radiologic sings of pneumonia resolved, but attempt to wean the infant from respiratory support repeatedly failed. Since there was a prolonged need for mechanical ventilation, tracheotomy was performed at the age of two months. During the intervention, it was noticed that tracheal rings were rather thick, with narrower tracheal air-path than expected according to the body size. Two more occasions of pneumonia accompa-

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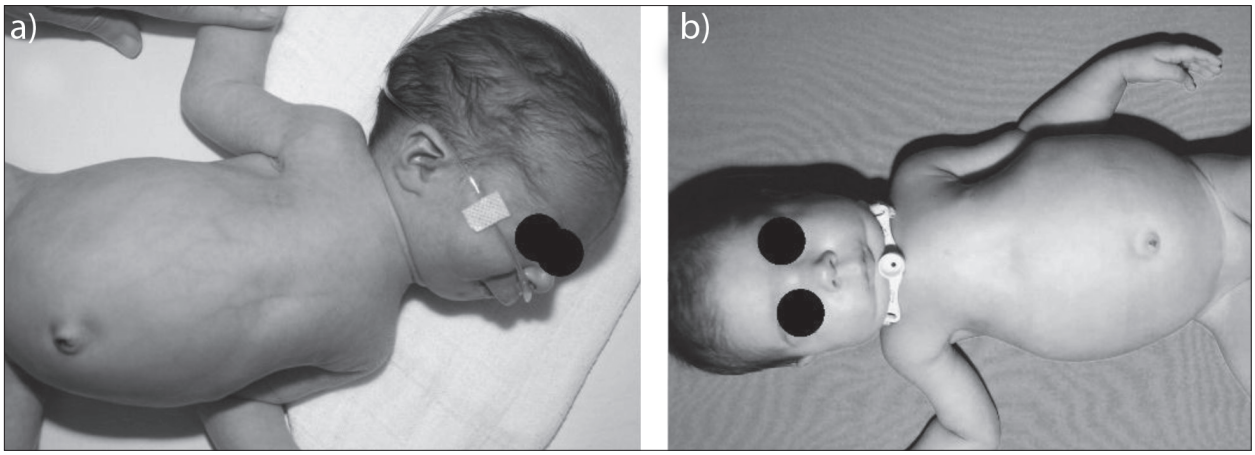

Figure 3. Infant's body with narrow chest. Photographs were taken when the infant was at the age of (a) two days, and (b) 5.5 months; on photograph (b) tracheal stoma is present.

nied with pleural effusions were diagnosed until the age of five months. After resolving of infections, the respiratory function improved, despite even more gracile appearance of the narrow thorax. Respiratory physiotherapy was initiated on a regular basis.

At the age of 5.5 months, mechanical ventilation was discontinued, with oxygen applied via tracheal stoma. At this age, the infant's body weight was 4,180g (< 3 p), body length was 56 cm (< 3 p), head circumference was 38 cm (< 3 p), and chest circumference 33 cm (< 3 p). Monitoring of anthropometric parameters confirmed further discrepancy between chest and head circumference, which rose from 4 cm at birth to 5 cm at the age of almost six months (Figure 3). Micrognathia also seemed more pronounced over time (Figure 2). Subsequent chest X-rays showed a more pronounced deformity of the chest, which took on a triangular shape, with reduced lung surface (Figure 1b, c). Also, scoliosis of the spine was observed. Its exacerbation over time was noticed as well (Figure 1b, c). Examination of psychomotor development showed a significant delay with pronounced muscle hypotonia at the age of six months. Nourishment was implemented through a gastric tube. Hypercaloric diet was given because of malnutrition, but weight gain was still poor. Neuromotor stimulation treatment was implemented, in order to improve infant's development as well as respiratory muscles' strength.

At the age of seven months, another episode of pneumonia appeared, caused by multiresistant strain of *Pseudomonas aeruginosa*. Over the next few days, global respiratory insufficiency developed gradually. Respiratory support was reinitiated, accompanied with already started antimicrobial and supportive therapy. Despite all the measures taken, clinical condition was further deteriorating and the infant passed away at the age of 7.5 months.

Autopsy was not performed, on parents' request.

DISCUSSION

Clinical manifestation of CCMS may vary, but rib-gaps and micrognathia are mandatory findings. Typical posterior rib-gap defects are pathognomonic finding in CCMS [11, 12]. These basic clinical and X-ray findings are sufficient for the diagnosis. Micrognathia can be accompanied by cleft palate, but is always of significant degree. Rib abnormalities originate from replacement of the bone tissue between costovertebral junction and the lateral arch with undifferentiated fibrous and muscle tissue, or cartilage [7, 13].

In differential diagnosis one should take into consideration other entities accompanied by micrognathia and thoracic and rib abnormalities [14, 15, 16]. Thoracic X-ray may mimic multiple costal fractures at birth, especially when resuscitation is applied, as in our patient. Spontaneous rib fractures were also described in some cases of vaginal delivery [17]. Concomitant presence of micrognathia and posterior rib-gap defects which are present on subsequent X-rays without callus formation are clues for correct diagnosis. In trisomy 18, 11 pairs of thin and hypoplastic ribs and micrognathia are often present [15]. Other typical features and organ abnormalities, as well as karyotype reveal the true diagnosis. Robin sequence is characterized by micrognathia, but without accompanied rib abnormalities. More than 50% of infants born with Robin sequence have associated syndrome or anomalies, including skeletal dysplasias [14], but not specific posterior rib-gap defects, except in the case of CCMS. Infants born with atelosteogenesis type I and campomelic dysplasia have also small, narrow thorax at birth [16, 18], but very distinctive other clinical and radiological features.

Literature data indicate that in about 35–50% of CCMS cases, death occurs during the first year of life [12]. Nagasawa et al. [2] classified CCMS according to severity into the following three types: lethal CCMS, where death occurs during the first month of life; severe type, where death occurs between the first and 12th month of life; and mild type, where patients live more than a year. Analyzing the descriptions of previously published cases, the same authors concluded that patients with severe type of CCMS had respiratory infections significantly more often than patients with a mild type [2]. Poor respiratory functions and frequent respiratory infections present the main causes of death in infants with severe CCMS. Their origin lies in underventilation of lungs, lying position in bed, and often microaspirations because of feeding difficulties. Special

issues are infections with multiresistant hospital strains in hospitalized patients with CCMS [19]. In our patient, there were deformities of tracheal cartilages and of thoracic spine. Combination of micrognathia, rib and spine deformities, and deformities of tracheal cartilages caused air-way disturbances and flail chest, with consequent respiratory distress. Exacerbations coincided with respiratory infection, causing a vicious circle, which eventually led to fatal outcome.

In several other case reports of CCMS, there were some other anomalies: ear anomalies [21], conductive hearing loss [7], cardiac anomalies [12], renal cysts [12], central nervous system anomalies [5], mycrocephaly [5], occult spina bifida [2], other bone deformities – except ribs and mandible [5], arthrogryposis [21], and urogenital anomalies [5]. All these deformities are sporadic and have low incidence [6, 11].

Mental deficiency was found in a considerable number of cases with CCMS [4, 7, 8, 9, 11], but there were also reports of normal intelligence [8, 12]. At first, it was considered that mental retardation is inherited, and, as such, a part of this syndrome; hence the "cerebro" part of the acronym. Recently, most authors see mental deficiency as a consequence of neonatal hypoxic brain injury [8], with the exception of cases accompanied with mycrocephaly, cerebral abnormalities or extensive perivascular calcification as a basis for mental retardation [5, 20].

At birth, head circumference is normally greater than chest circumference, and equalization of these two measures takes place at the age of about six months [22]. In our patient, chest circumference was considerably smaller than head circumference at birth. This difference was even greater at the age of six months. Chest deformity played an important role in reduction of respiratory capacity. This is obvious from subsequent chest X-rays (Figure 1), as well as from simple observation of the infant over time (Figure 3).

Figueroa et al. [23] postulated that a certain number of infants with micrognathia show accelerated growth of the mandible during the first year of life, thus resolving airway obstruction. In our patient, micrognathia seemed to be even more pronounced at the age of six months than at birth (Figure 2). In some other cases of CCMS, authors also noticed lack of mandibular catch-up growth over time [2].

Maintenance of airway patency is a key point in early treatment of infants with CCMS. Depending on the nature and severity of respiratory distress, one should try conservative methods first – prone positioning, nasopharyngeal airway, laryngeal mask [24], or continuous positive airway pressure via nasal mask, as applied in our patient. If and when these measures become insufficient, surgical interventions must be considered. Tracheotomy was performed in our patient, considering artificial ventilation dependence and pneumonia present at the time. Finding of the abnormalities of tracheal cartilages during the intervention confirmed that it was a right decision. In several other reported cases, tracheotomy was performed in patients with CCMS [12, 13, 21, 25]. In some other cases, a tongue-lip adhesion operation [24] and bilateral mandibular osteostomy and osteogenic distraction [8] were succesfully performed, but in infants without airway narrowing below the tongue base.

In conclusion, CCMS should be considered in every infant with micrognathia and rib-gap defects on chest X-ray. These infants need careful monitoring of respiratory functions, and surveillance of respiratory infections. Furthermore, their physical and psychomotor development requires frequent assessment. Rapid and accurate diagnosis is basic for proper information for parents' expectations regarding health problems and life span of the child, as well as for genetic counseling in subsequent pregnancies.

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Случај тешког облика цереброкосто-мандибуларног синдрома

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КРАТАК САДРЖАЈ

Увод Цереброкосто-мандибуларни синдром (*CCMS*) редак је поремећај. До сада је описано 75 случајева. Главна карактеристика овог синдрома је удружено присуство микрогнатије и мултиплих специфичних дефеката ребара. У значајном броју случајева *CCMS* је удружен са менталним дефицитом, а понекада са неким другим аномалијама и проблемима: деформитети кичме, аномалије мозга, срца, бубрега и ува, тешкоће у храњењу, успорен психомоторни развој, заостатак у расту. У зависности од тежине деформитета и последичне респираторне инсуфицијенције, *CCMS* у око 35–50% случајева доводи до смртног исхода у првој години живота. Такви случајеви спадају у тежак облик *CCMS*.
Приказ болесника У овом раду приказујемо женско одојче са тешким обликом *CCMS*. Дијагноза је постављена у првом дану живота, на основу постојања микрогнатије и специфичних дефеката ребара на рендгенском снимку грудног коша, праћених диспнејом. Прогресивни поремећај респираторне функције, узрокован деформитетима грудног коша и дисајних путева и погоршан епизодама пнеумоније, довео је до респираторне инсуфицијенције и смрти у узрасту од 7,5 месеци.
Закључак Дијагнозу *CCMS* треба имати на уму код сваког одојчета са микрогнатијом и специфичним дефектима ребара на рендгенском снимку грудног коша.

Кључне речи: цереброкосто-мандибуларни синдром; дефекти ребара са микрогнатијом; микрогнатизам; респираторна инсуфицијенција; ребра; урођене абнормалности

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Primary reconstruction of neck defect after excision of metastatic melanoma of unknown primary site with regional pectoral myocutaneous flap

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SUMMARY

Introduction Metastatic melanoma of unknown primary (MMUP) is already a well described oncologic phenomenon in the literature, whereas tissue defects' reconstructions on the neck region always present a challenge for the reconstructive surgeon. Two cases of giant metastatic, skin infiltrative neck tumor masses are presented. In both cases MMUP was diagnosed. Both intraoperative tissue defects were reconstructed using pectoralis major (PM) regional flap.

Outline of cases The first patient was admitted with giant tumor mass on the right side of the neck. The fast growing mass appeared two months prior to the admission. Thorough examination showed no signs of primary tumor. Removal surgery was performed and the defect was reconstructed using the PM musculocutaneous flap. The second patient was admitted with large tumor mass on the left side of the neck. Thorough examination displayed no signs of any primary tumor. After the excision, the tumor mass and subsequent neck dissection, reconstruction followed, using the pedicled PM muscle flap and partial thickness skin transplants. There were no major complications in either case. The histopathological examinations presented metastatic melanoma diagnoses.

Conclusion Clinical outcome of MMUP described in literature is rather variable. Different studies have shown that prognosis in patients with MMUP is better than that in patients with diagnosed primary melanoma with metastatic disease. Therefore, the best initial course of action in those cases would be surgery, according to oncological principles, if possible. Neck defects' reconstructions should fulfill both functional and esthetic demands. Due to the reliability and low cost of the procedure, PM regional flap presents a very good and trustworthy reconstruction modality.

Keywords: metastatic melanoma of unknown primary; pectoralis major flap; surgery; reconstruction; neck tumor

INTRODUCTION

Primary reconstruction of massive neck defects presents a continuously challenging question in the field of reconstructive surgery. The reconstruction's goals primarily include acceptable coverage for the underlying tissue, with protection of important anatomical structures, and, secondly, acceptable and furthermore desirable aesthetic appearance if possible.

Metastatic melanoma of unknown primary (MMUP) presents a specific entity with many characteristics that underlies the need for adequate therapeutic approach. It is broadly described in literature. The capricious presentations of the disease itself can be found in different reports, for example: 1.7 kg lymph node axillary metastasis [1], lung metastasis [2], right atrial metastasis with pericardial effusion [3], midbrain und inguinal metastases [4], skin – colored skin-fixed noduli with inguinal mass, rectal wall metastasis, lung metastasis and liver metastases [5], adrenal metastasis with subcutaneous metastatic focus [6], or simply in form of inguinal swelling that presents itself via enlarged lymph node. All of these are advocates of either the melanoma regression theory, or transformation theory, which includes the appearance of aberrant melanocyte within the lymph node [7]. As all of the reports described, despite the meticulous diagnostic procedures, primary melanoma was not diagnosed.

We present two cases of large neck tumor mass that were surgically treated in the Clinic for Plastic and Reconstructive Surgery, Clinical Center Niš, Serbia, which were diagnosed with MMUP.

CASE REPORTS

Case 1

A 47-year-old male was admitted to the clinic with a large tumor mass on the right side of the neck. The patient reported that tumor mass appeared two months before and grew until it reached the preoperative size (Figure 1). At the time of admission, general health condition was inconspicuous, without concomitant diseases. A thorough examination was performed, including physical examination of the skin, anus, genitalia, and adnexae; ophthalmoscopy, otorhinolaryngology examination, rectoscopy,

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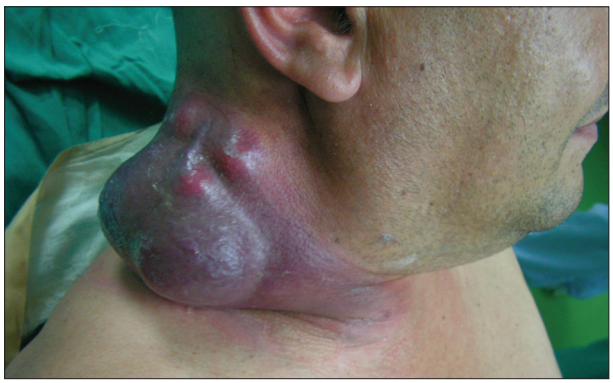

Figure 1. Large tumor mass on the right side of the neck; notable infiltration of the skin dermis is present, with peritumoral congestion

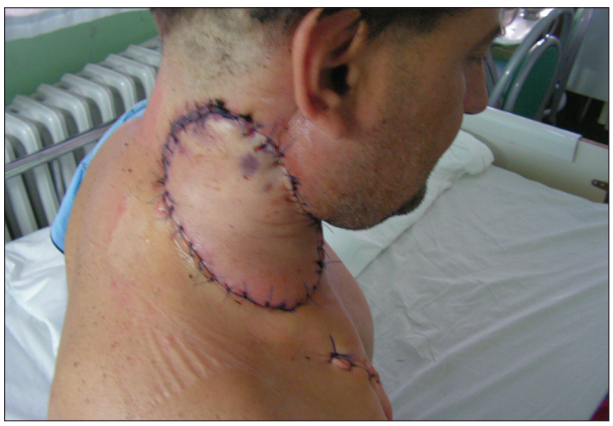

Figure 2. Postoperative appearance during the dressing change on the sixth postoperative day; the pectoralis major flap remains vital

thoracic X-rays, abdominal ultrasound, computed tomography (CT) scans of the thorax and abdomen. Anamnestically, no surgical procedures had been performed prior to the admission. The CT-scans showed that no major blood vessels were affected by the tumor mass. Primary melanoma or other cutaneous lesions, as well as other pathological findings were not diagnosed.

The surgery was performed under general anesthesia. Surgery planning was thorough, because of the relations with the vital structures in the neck. The excision was methodically performed, continued by a neck dissection. The dimensions of the excised tumor mass were 12 × 9 × 8 cm. Afterwards, ipsilateral pectoralis major (PM) myocutaneous pedicled flap was harvested, raised und placed into the defect. The suturing was performed in two layers, over a suction drainage. The secondary defect was covered with split-thickness skin grafts harvested from the right thigh. Subsequent histopathological examination showed an enlarged lymph node with metastatic melanoma including the dermis infiltration of the skin. The postoperative care of the patient was performed in elevated head position, with the head rotated ipsilaterally. Marginal epidermolysis of the flap was the only complication noted during the initial postoperative period (Figure 2). The drainage was removed on the third postoperative day; the sutures were removed on the 13th postoperative day. On one-month follow-up examination the patient described the aesthetic outcome as very-good. Additional radiotherapy was recommended, which the patient rejected. Three months after the surgery he developed local neck relapse, including the carotic artery wall infiltration. Seven months after the surgery the patient died of multiple visceral metastases.

Case 2

A 54-year-old male was admitted with a large tumor mass on the left supraclavicular region. The patient reported the appearance of a small tumor mass two and a half months prior to the admission, which successively grew until it reached the preoperative size. Venous skin congestion surrounding the tumor mass was also noted by the admission check-up (Figure 3). At the time of admission patient was not suffering from any concomitant diseases. The step-by-step diagnostic procedures as in the first case were performed. Primary melanoma or other cutaneous lesions, as well as other pathological findings, were not diagnosed. The surgery was performed under general anesthesia. The tumor mass resection was performed, followed by ipsilateral lower neck dissection. The dimension of the excised tumor mass was 15 × 15 × 10 cm. Using the mid-clavicular incision line, lateral margin of the left PM muscle was approached; the flap was dissected and raised (Figure 4), and subsequently turned into the defect. The flap was sutured and covered with split-thickness skin grafts harvested from the right thigh. The chest wound was sutured over the suction drain. Initial postoperative period was uneventful. Following histopathological examination of the excised tumor mass showed enlarged lymph node with melanoma metastasis. The drainage was removed on the fourth postoperative day; approximately 95% of the skin grafts healed and small areas healed by secondary epithelization (Figure 5). The sutures were removed on the 13th postoperative day.

On examination three months post-surgery, the patient described the aesthetic outcome as good. Minor problems with arm movements were nonetheless reported by the patient.

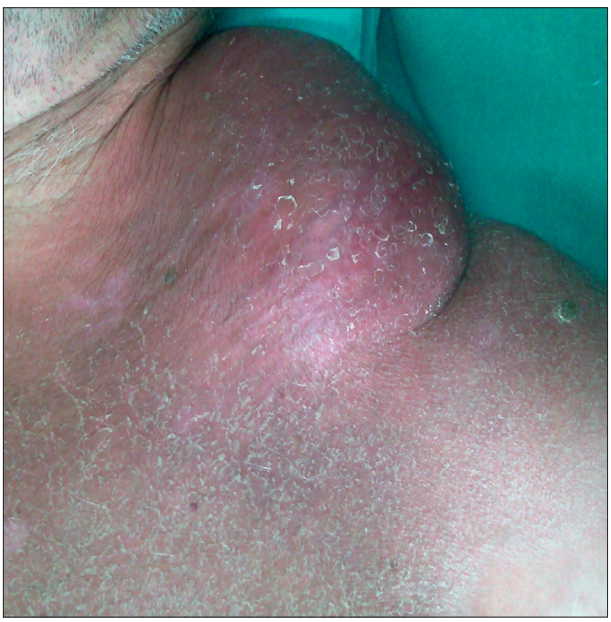

Figure 3. Large tumor mass on the left supraclavicular region; peritumoral blood stasis with skin exfoliation is noted

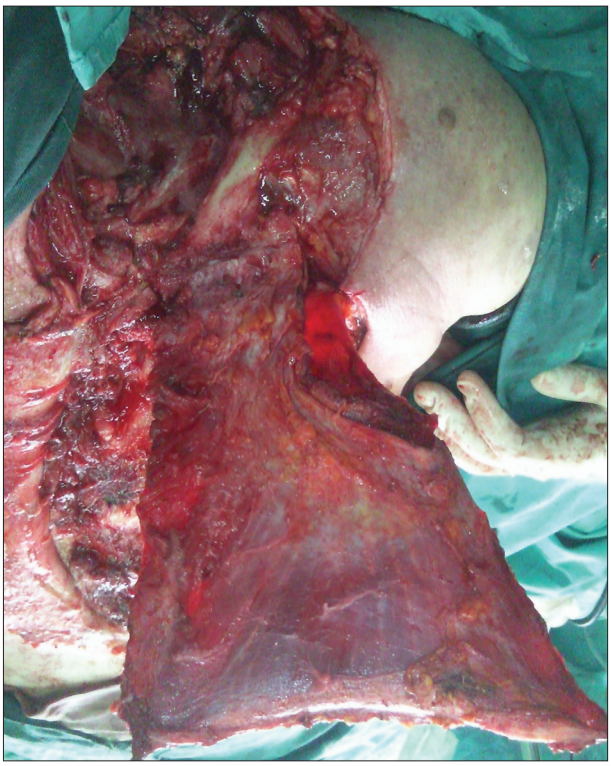

Figure 4. Tissue defect medial of the left shoulder; after the excision and neck dissection, the pedicled pectoralis major muscle flap is dissected from distal and raised. The clavicle is notable in the middle of the tissue defect; proximal to the clavicle, altered anatomical tissue organization is demonstrable. Major blood vessels are not recognizable in this photograph

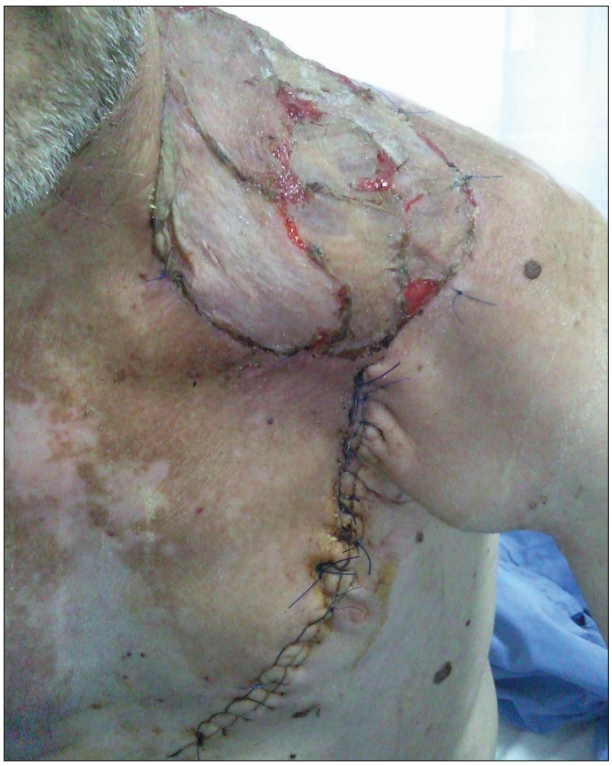

Figure 5. Postoperative appearance during the dressing change, skin grafts are healed, the venous congestion has declined; the wound shows no signs of irritation or infection

Despite the applied radiotherapy, twelve months after the surgery the patient developed generalized disease with multiple cerebral metastases. The patient died fourteen months after the primary surgical treatment.

DISCUSSION

MMUP presents a clinically completely different entity compared to melanoma of known primary (MKP); nevertheless, genetic researches show rise in BRAF and NRAS mutations, which resemble the genotype of cutaneous melanoma [8–10] and not of the mucosa [9]. Both mutations have no significant prognostic impact on the clinical outcome [9, 10]. The number of metastatic lymph nodes remains the most significant prognostic factor for overall survival [10]. One study has suggested that AJCC stage and time to disease progression, and not the initial metastatic load, nor the mutational status, displays the important prognostic factors [11].

MMUP presents a clinical entity that has a different prognosis to that of the MKP. As mentioned before, the number of involved lymph nodes involved presents a negative prognostic factor, but the prognosis itself is also influenced by the clinical form of the disease. In comparison to patients with MKP, the patients with MMUP showed better prognosis [12]; however, in-transit or satellite metastases present an additional unfavorable effect [13]. To date, surgical treatment remains the initial therapeutic modality, unless absolute contraindications for surgical treatment are present.

The role of appropriate reconstruction presents an open question in the field of reconstructive surgery. The role of regional flaps remains, to date, unquestioned. A number of papers on this topic only underline the significance of the regional PM flap. Many advantages using this flap are mentioned in the literature, e.g. vitality of the flap, reasonably short time of recovery, favorable aesthetic outcome at the donor site [14], versatility and excellent reach in the neck region [15], cost of surgery of the regional vs. free flaps. Also, minor but notable or even no postoperative complications using PM flap were mentioned in the latest literature [16, 17].

Providing that regional PM muscle flap dates from the second third of the 20th century, as described in the literature, and that it has until now been used as a reliable modality for treatment of diverse head and neck defects, it presents a good modality for the treatment of the defects following the surgery of MUP. The significance of free flaps stays undisputed, but the economical aspect of the surgery costs and the recovery time could be also be considered, especially when it comes to use of the PM muscle flap in the developing societies. A thorough patient examination remains of foremost significance, because small or unrecognized skin or adnexae lesions could present the primary site of the later diagnosed metastatic disease [18].

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Примарна реконструкција дефекта на врату после ексцизије метастатског меланома коже непознате примарне локализације пекторалним мишићнокожним режњем

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КРАТАК САДРЖАЈ

Увод Метастаски меланом непознате примарне локализације (ММНПЛ) у литератури је већ добро описан онколошки феномен; реконструкције ткивних дефеката на врату представљају увек изазов за реконструктивног хирурга. Представљена су два случаја великих метастаских туморских маса са инфилтрацијом коже. У оба случаја је дијагностикован ММНПЛ. Оба интраоперативна ткивна дефекта су реконструисана помоћу *pectoralis major* (*PM*) регионалног режња.

Приказ болесника Први пацијент је регистровао брзо растућу масу на десној страни врата два месеца пре болничког пријема. Обављени детаљни физикални преглед и допунске дијагностичке методе нису показали постојање примарног тумора. Уследило је хируршко лечење ексцизијом и реконструкција дефекта *PM* мишићнокожним режњем. Други пацијент је примљен на стационарно лечење са великом туморском масом на левој страни врата. Детаљни преглед и дијагностика нису потврдили постојање примарног тумора. Након ексцизије туморске масе и дисекције врата уследила је реконструкција ткивног дефекта петаљкастим *PM* мишићним режњем. Оба случаја су протекла без значајних хируршких компликација. Хистопатолошка дијагностика је у оба случаја показала метастаски меланом.

Закључак Клинички исход код пацијената са ММНПЛ описан у литератури је различит. Различите студије су показале да је прогноза пацијената са ММНПЛ боља него пацијената са дијагностикованим примарним меланомом са метастатском болешћу. Последично, најбољи правац деловања би било хируршко лечење, уколико је могуће по онколошким принципима. Реконструкција дефеката на врату би требало да испуни функционалне и естетске захтеве. Због поузданости и релативно ниских трошкова хируршког лечења, *PM* регионални режањ представља веома добру опцију за хируршку реконструкцију.

Кључне речи: метастаски меланом непознате примарне локализације; *pectoralis major* режањ; хирургија; реконструкција; тумор на врату

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ПРИКАЗ БОЛЕСНИКА / CASE REPORT
UDC: 616.24-005.1

Subendocardial hemorrhages in a case of extrapercardial cardiac tamponade – A possible mechanism of appearance

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SUMMARY

Introduction Subendocardial hemorrhages are grossly visible bleedings in the inner surface of the left ventricle, the interventricular septum, and the opposing papillary muscles and adjacent columnae carneae of the free wall of the ventricle. These are commonly seen in sudden profound hypotension either from severe blood loss from "shock" in the widest sense and, even more often, in combination with brain injuries.

Case Outline We present a case of a 38-year-old man, injured as a car driver in a frontal collision, who died c. 45 minutes after the accident. The autopsy revealed severe chest trauma, including multiple right-sided direct rib fractures with the torn parietal pleura and right-sided pneumothorax, several right lung ruptures, and a rupture of one of the lobar bronchi with pneumomediastinum, and prominent subcutaneous emphysema of the trunk, shoulders, neck and face. The patchy subendocardial hemorrhage of the left ventricle was observed. The cause of death is attributed to severe blunt force chest trauma.

Conclusion We postulate pneumomediastinum leading to extrapericardial tamponade as the underlying mechanism of this subendocardial hemorrhage.

Keywords: forensic pathology; subendocardial hemorrhage; extrapericardial cardiac tamponade; pneumomediastinum; subcutaneous emphysema; lung injury

INTRODUCTION

Autopsy reveals superimposed images of both injuries and organ changes generated by the injuries. Our task is to put these images in chronological order, and to make the reconstruction of the injury event. In order do it properly, a good understanding of the pathophysiological mechanisms and injury patterns is required.

Subendocardial hemorrhages are grossly visible bleedings in the inner surface of the left ventricle, the interventricular septum, and the opposing papillary muscles and adjacent columnae carneae of the free wall of the ventricle. The hemorrhages are flame-shaped and confluent, not petechial. They can appear extremely rapidly, within a few heartbeats [1], and can even be found in trauma deaths with nearly immediate circulatory arrest [2]. These are commonly seen in sudden profound hypotension, either from severe blood loss or from "shock" in the widest sense [1, 3], and, even more often, in combination with brain injuries [4]. These hemorrhages were sometimes termed "shock lesions", or named after Sheehan [5].

In the case under review here, we present an intriguing mechanism of subendocardial hemorrhages appearance.

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CASE OUTLINE

A 38-year-old man was injured as a car driver in a frontal collision. He died c. 45 minutes after the accident, on the way to the hospital. An autopsy was performed the following day.

The deceased was 176 cm tall and weighed approximately 75 kg. Along with multiple small skin excoriations and bruises of the extremities, the external examination revealed prominent subcutaneous emphysema of the trunk, shoulders, neck and face (Figure 1). The internal examination showed multiple right-sided direct rib fractures (*fracture en dedans*) with the torn parietal pleura and right-sided tension pneumothorax. The right lung was partially loose and collapsed. On the lateral side of the right lung, there were three ruptures up to c. 2 cm in depth, which corresponded to the fractured ribs. A relatively small amount of free blood (c. 300 ml) was found in the right pleural cavity. There was a rupture in the right lung hilus with one of the lobar bronchus lacerated (Figures 2a and 2b), while the mediastinal connective tissue was crepitant to the touch. Left lung, pericardium, heart and great thoracic vessels were intact and without any visible injuries. Patchy subendocardial hemorrhage of the left ventricle was observed (Figure 3). The autopsy also revealed a longitudinal liver laceration, on the lower side of the right lobe with c. 500 ml of free blood in the abdominal cavity, and a right-sided parietal skin laceration with the isolated contrecoup fracture of the left bony orbit and several left frontal lobe brain contusions. The victim's blood and urine alcohol concentrations were found to be 1.01 g/l and 1.17 g/l, respectively. The toxicological analysis

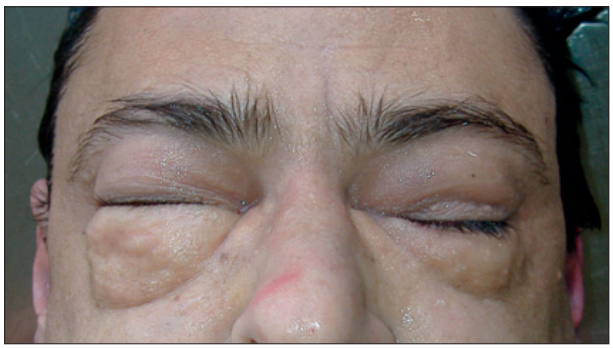

Figure 1. Subcutaneous emphysema extended to the face. Eyelids were inflated.

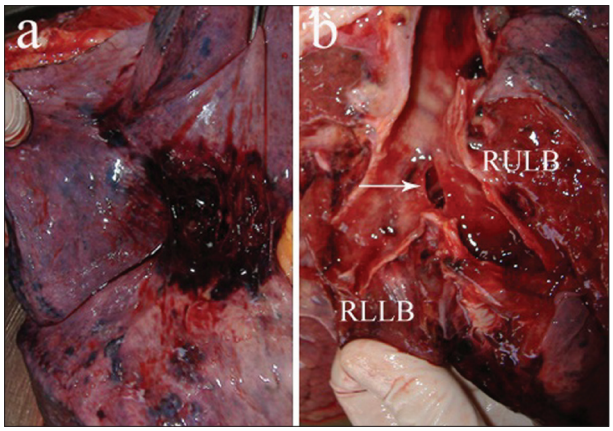

Figure 2. a) The rupture in the right lung hilus; b) The laceration of the lobar bronchus (arrow); RULB – right upper lobe bronchus, RLLB – right lower lobe bronchus.

of the blood, urine, and vitreous humor showed no traces of drugs (head-space gas chromatography).

The cause of death was attributed to severe blunt force chest trauma.

DISCUSSION

Initially, it was thought that subendocardial hemorrhages might be due to rupture of congested subendocardial blood vessels in cases of sudden hypotension: the intraventricular pressure drops precipitously, and the existing blood pressure in the coronary system is then unsupported across the endocardium by the equal pressure within the ventricular lumen, which in turn leads to the rupture of the superficial vessels [1]. Also, mechanical damage to the left ventricular endocardium caused by the vigorous contractions of the relatively empty left ventricle seems to be a possible causative factor in the origin of subendocardial hemorrhages [6]. Recent studies suggest that subendocardial hemorrhages are mediated by hypersecretion of catecholamines [3, 7]. Finally, some animal experiments suggest a combination of these two mechanisms [2]. The subendocardial hemorrhages are a manifestation of a generalized cardiovascular lesion occurring in various conditions which in varying ways lead to systemic hypoxia [8].

In cases of cardiac tamponade, the pressure exerted by the pericardial fluid will eventually equal diastolic pressure within the heart chambers. The first structures to be affected

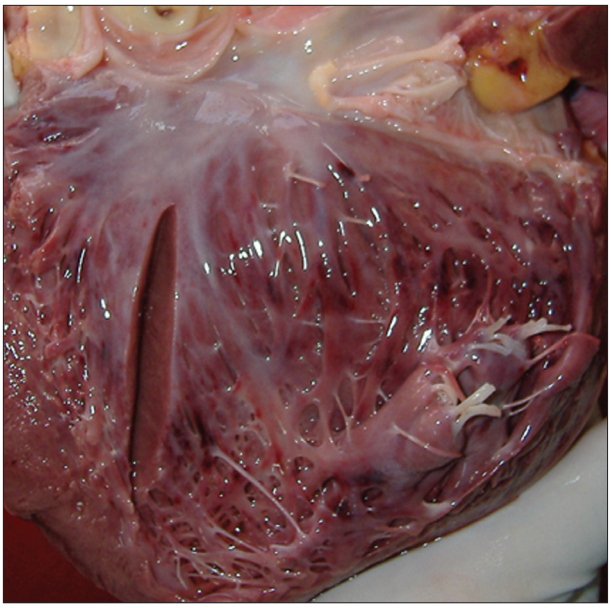

Figure 3. The patchy subendocardial hemorrhage of the left ventricle

are the right atrium and the right ventricle, where diastolic pressures are normally the lowest. Compression by pericardial fluid interferes with the right atrial filling during diastole, resulting in systemic venous congestion and symptoms of right-sided heart failure. Decreased atrial filling leads to decreased ventricular filling, decreased stroke volume, and reduced cardiac output, which may result in life-threatening circulatory collapse [9]. However, cardiac tamponade can also occur due to the extrapericardial compression of the heart. The most common cause of this are large pleural effusions. The increased intrapleural pressure is transmitted to the pericardial space and impairs ventricular filling, thus mimicking the hemodynamic abnormalities of cardiac tamponade. There are case reports of extrapericardial cardiac compression resulting from anterior mediastinal hematomas, masses, ascites, and pneumomediastinum [10, 11, 12].

Pneumomediastinum or mediastinal emphysema is the presence of extra-alveolar air in the mediastinum. Most commonly, free air leaks from ruptured trachea or bronchi, dissecting along the bronchovascular sheaths towards the mediastinum, and eventually into the subcutaneous tissue. Posttraumatic pneumomediastinum can originate from the mediastinal propagation of subcutaneous emphysema, or due to a direct air leak from a tension pneumothorax through a small tear in the mediastinal pleura [13]. Mediastinal emphysema is accompanied by massive subcutaneous emphysema, and *vice versa* in a quarter of cases, and it can compress the venae cavae, causing extrapericardial tamponade and shock [13]. The tension pneumothorax produced by bronchial rupture also compresses the large veins with the same effects of extrapericardial tamponade as mediastinal emphysema [13].

In the presented case, the laceration of lobar bronchus (Figure 2b) is followed by right-sided tension pneumothorax, mediastinal and subcutaneous emphysema, all exacerbated by cardiopulmonary resuscitation attempts. The tension pneumothorax and mediastinal emphysema

compressed the large heart veins, causing extrapericardial tamponade. This condition led to decrease of atrial and ventricular filling, stroke volume, and reduction in cardiac output and shock, which most probably was the cause of subendocardial hemorrhage that occurred. Also, the blood in the right pleural space, as well as in the abdomen, would have contributed to the shock, primarily caused by pneumomediastinum.

It is possible that the rise of coronary pressure due to the excessive endogenous secretion of the catecholamines stemming from the accident, administration of adrenaline during resuscitation attempt, as well as coincided abrupt drop of intraventricular pressure due to acute extrapericardial cardiac compression, and relatively mild traumatic brain injury, could have been the contributing pathophysiological mechanisms of subendocardial hemorrhages in the presented case.

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Могући механизам настанка субендокардних крварења код екстраперикардне тампонаде срца – приказ случаја

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КРАТАК САДРЖАЈ

Увод Субендокардна крварења у пределу леве коморе најчешће виђамо у случајевима где је узрок смрти акутни хиповолемијски шок – пре свега искрварење, или повреда мозга. Наравно, и неке друге повреде могу бити узрок настанка ових крварења.

Приказ случаја Ради се мушкарцу старом 38 година, који је повређен као возач у чеоном судару. Обдукцијом је установљена тешка повреда грудног коша: серијски прелом десних ребара и руптура десног плућног крила и бронха, са развојем тензионог пнеумоторакса, пнеумомедијастинума и поткожног емфизема. Обдукцијом су установљена и субендокардна крварења у левој комори.

Закључак Могући патофизиолошки механизам настанка ових крварења у срцу, у конкретном случају, јесте и настанак и развој ектраперикардне тампонаде срца.

Кључне речи: судска медицина; субендокардна краварења; екстраперикардна тампонада срца; пнеумомедијастинум; поткожни емфизем; повреда плућа

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Glomus tumor – A case report

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SUMMARY
Introduction Glomus tumor is a neuromyoarterial tumor. It is a rare tumor which accounts for about 2% of all hand tumors. The diagnosis is based on the triad of symptoms, clinical examination which includes three tests, magnetic resonance imaging, and ultrasound imaging. The most common treatment is surgical excision, using transungual or lateral subperiosteal approach. Sclerotherapy and radiotherapy may be the treatments of choice, but they are less effective. The recurrence rate is high – from 5% to 50%.
Case Outline We diagnosed a glomus tumor of 1 cm in diameter in the distal phalanx of the fourth finger of the right hand in a 30-year-old woman. She had been visiting different physicians for more than two years and had been variously diagnosed. We performed a biopsy of the tumor, which was bleeding profusely during the procedure. Upon biopsy results, the tumor was excised with transungual approach. Two and a half months after the procedure the patient was feeling well.
Conclusion There should be higher awareness of this tumor in order to diagnose it more easily and treat it accordingly, and thus alleviate the severe pain which the tumor causes. When it is considered as the possible cause of the lesion, the diagnosing is easier and treatment is immediate.
Keywords: glomus tumor; excision, transungual; soft-tissue

INTRODUCTION

A glomus tumor is a rare benign neuromyoarterial tumor. Seventy percent of glomus tumors are located in digital, i.e. subungual region [1]. The tumor arises from the glomus body, which controls blood pressure and temperature (thermoregulatory shunt) by regulating blood flow through cutaneous vasculature [2]. Glomus tumors account for 1–4.5% of all hand tumors [3]. The tumor was first described by Wood in 1812, while histopathological description and terminology was given by Masson in 1824 [4]. The triad of glomus tumor symptoms includes severe pain, pinpoint pain, and cold sensitivity [5]. The patient is diagnosed with glomus tumor after physical examination and magnetic resonance (MR) imaging and ultrasound (US) scanning. Clinical examination includes Love's pin test, Hildreth's test and cold sensitivity test. Love's pin test means that the patient is experiencing severe pain when the skin over the suspected area is pressed with a pinhead. In Hildreth's test, transient ischemia along the arm is induced by tying a tourniquet, which leads to pain in the affected area, which can be outlined. Cold water or ice cubes can be applied to the affected area while performing the cold sensitivity test. The test is positive when increased pain is elicited during the procedure [6]. US and MR imaging scans are also used to support clinical diagnosis.

CASE REPORT

A 30-year-old female was referred from dermatology department, with a three-year history of pain in the fourth finger of the right hand. She had numerous visits to different healthcare providers in the past. Exposure to the cold or temperature changes provoked occasional pain. Even a minor trauma used to provoke a severe bout of pain. A soft mass with no skin impairment appeared at the tip of the finger in the last several months. No discoloration either of the nail plate or of the proximal nail fold was observed. The mass felt mucous when palpated. Meanwhile, the pain grew constant. Routine laboratory results were within normal limits. The radiographic testing did not reveal any abnormalities. A biopsy specimen of skin tissue was taken under local anesthesia. The wound was bleeding profusely, which immediately indicated the vascular origin of the lesion (Figures 1a and 1b). The bleeding occurred at each

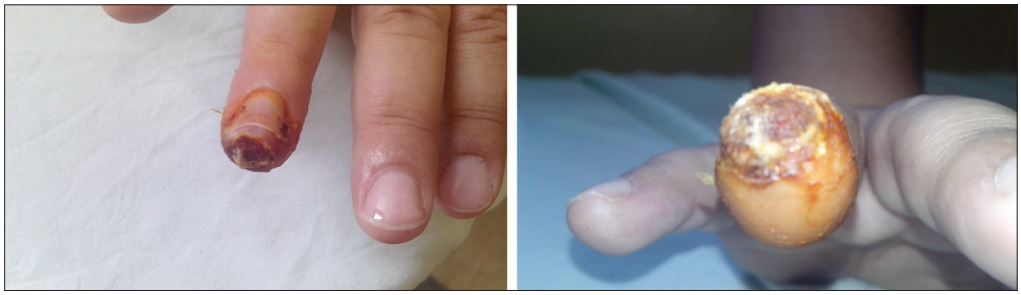

Figure 1. Glomus tumor: a) before biopsy; b) after biopsy

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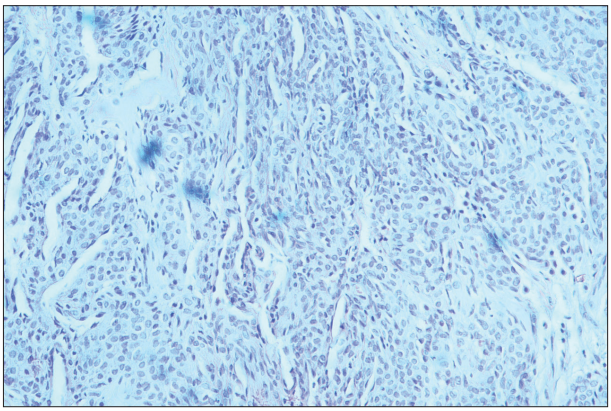


Figure 2. Glomus tumor composed of branching vascular spaces, overlaid with normal endothelial cells, separated by stroma which contains glomus cells nest-like aggregates

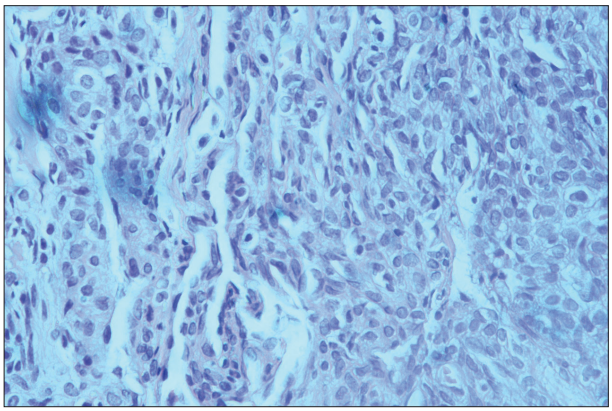
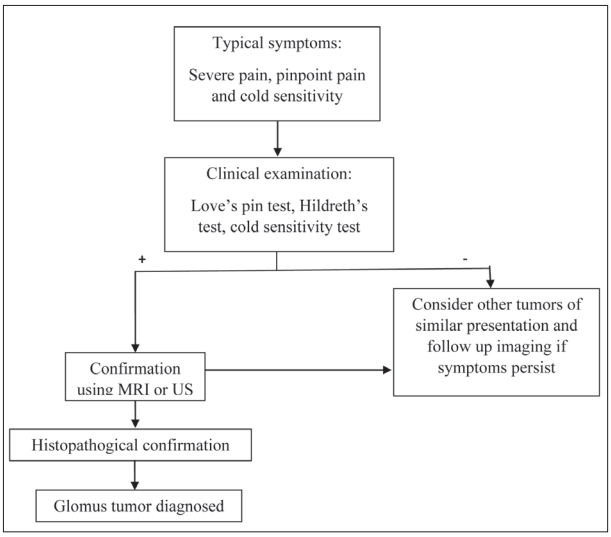


Figure 3. Glomus cells arrayed around vascular space; the cells are epithelioid and they have small, regular, round nuclei and very low mitotic activity



Scheme 1. Original algorithm of the glomus tumor diagnosis (on Tang's idea [7])

dressing change. Histopathological examination revealed a "glomus tumor." The tumor was excised, and biopsy results were confirmed (Figures 2 and 3). Two and a half months after the procedure the patient was feeling no pain, while occasional numbness at the incision site occurred.

DISCUSSION

Algorithm in Scheme 1 shows simple diagnostic steps when glomus tumor is suspected as a possible condition. Sometimes it takes several years and various differential diagnoses before accurate diagnosis is made and the tumor excised [3, 7]. Furthermore, the nomenclature is confusing – the term "glomus tumor" refers to tumors in the head and neck (glomus caroticum, glomus jugularae), while it also refers to tumors mainly located in digital and subungual regions. The peripheral glomus tumor is called ganglioma. The tumor can be composed of prominent glomus cells (solid glomus tumors, 25%), or prominent vascular components (ganglioma, 60%), or prominent smooth muscle components (glomangiomyoma, 15%) [8]. Paraganglioma of the peripheral nerves (not located in the head or neck), which originates from sympathetic or parasympathetic ganglia, are also classified as glomus tumors. The last group of tumors also includes pheochromocytoma [9]. A few cases of glomus tumor of digital nerve have been reported [10].

Our patient presented with a lesion the size of a half a distal phalanx, about 10 mm in diameter. Some of the common symptoms were present (pain and hypersensitivity in the tumor area). The first and the working diagnosis was the benign tumor of the distal phalanx extensor tendon. Routine examination and X-ray scanning were done, while MR imaging and US scanning were not performed. It is thought that the latter types of scanning are not necessarily needed in practice. There are two reasons for this: the surgical excision is urgent, and the mentioned scans are costly. [11]. In case of erosion of the lateral part of the bone, evident on an X-ray scan, glomus tumor can be suspected [12]. MR imaging and US are used to delineate the size and precise location of the tumor.

A tissue sample from the fingertip was taken and sent for evaluation. The histopathological findings confirmed "glomus tumor." It was an indication for excision in local or general anesthesia. After consulting a plastic surgeon in a tertiary institution, the excision was done with transungual approach, which is most frequently performed [13]. According to Vasisht, surgical excision using lateral subperiosteal approach can be done as well [14]. The use of low power lens proved to be useful. Sclerotherapy and argon cryotherapy were found to be ineffective.

Follow-up examinations are quick and they are done every three to six months. Two and a half months after the procedure the patient was feeling well. There was mild paresthesia, but the patient felt no pain. The recurrence of the tumor is frequent (5–40%) and is caused by the tumor growth *de-novo* or the incomplete excision [13, 15].

Glomus tumor is a rare disease, as such it is frequently misdiagnosed, and it is necessary to suspect it when presented with the triad of symptoms, MR imaging and US scans, or biopsy results. Early diagnosis is also important from the aspect of pain relief. The treatment is surgical. Relevant scientific studies report a high incidence of recurrence.

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Гломус тумор – приказ болесника

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КРАТАК САДРЖАЈ

Увод Гломус тумор је неуромиоартеријални тумор. Спада у групу ретких тумора. Од свих тумора шаке чини око 2%. Дијагностика се базира на тријасу симптома, клиничком прегледу (три теста), магнетној резонанци и ултразвуку. Терапијски стандард је хируршка ексцизија, која може бити трансунгуална и латерално субпериостална. Спроводи се и склеротерапија, као и радиотерапија, али са мање успеха. Проценат рецидива је висок – од 5 до 50%.

Приказ болесника Дијагностиковали смо гломус тумор величине око 1 *cm* у пречнику на дисталној фаланги десног 4. прста, код жене у тридесетим годинама. Више од две године је посећивала различите лекаре и имала више различитих дијагноза. Ми смо урадили биопсију тумора, који је доста крварио при томе. По добијању резултата урађена је трансунгуална ексцизија. После два и по месеца осећа се добро.

Закључак Потребно је мислити на овај тумор да би лакше био дијагностикован и лечен, а самим тим и уклоњени јаки болови које изазива. Када се мисли на њега као узроку тегоба, дијагноза се поставља лакше и терапија је промптна.

Кључне речи: гломус тумор; ексцизија, трансунгуална; мекоткивни

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Allogeneic fetal stem cell transplantation to child with psychomotor retardation – A case report

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SUMMARY

Introduction The consequences of autologous and allogeneic stem cell transplantation (stem cells of hematopoiesis), applied in adults and children suffering from leukemia or some other malignant disease, are well-known and sufficiently recognizable in pediatric clinical practice regardless of the indication for the treatment. However, the efficacy of fetal stem cell transplantation is unrecognizable when the indications are psychomotor retardation and epilepsy.

Case Outline With the exception of neurological psychiatric problems, a boy aged 9.5 years was in good general health before transplantation with allogeneic fetal stem cells. The main aim of allogeneic fetal stem cell transplantation was treatment of psychomotor retardation and epilepsy. After 13 months of treatment, he was admitted to hospital in a very serious, life-threatening condition due to sepsis and severe pleuropneumonia. The humoral immunity in the boy was adequate, unlike cellular immunity. The immune imbalance in terms of predominance of T-suppressor lymphocytes contributes to delayed and late development of sepsis and severe pleuropneumonia. The boy still shows the same severity of psychomotor retardation, dyslalia, epilepsy, strabismus and amblyopia.

Conclusion Implementation of fetal stem cell therapy for unconfirmed indications abuses the therapeutic approach, harms patients, misleads parents, and brings financial harm to the healthcare system of any country, including Serbia.

Keywords: stem cells; transplantation; homologous; epilepsy; psychomotor disorders; costs

INTRODUCTION

The purpose of this appeal is to contribute to knowledge about the delayed consequences of the treatment with allogeneic fetal stem cell transplantation in children, and about the relevance of the indications for this therapy. The consequences of different types of stem cell transplantations applied in adults and children suffering from leukemia [1], some other malignant disease [1], ataxia telangiectasia [2], or spinal injury [3] are known, but consequences of the treatment with human embryonic stem cells do not include severe infections [2, 4].

To date, some basic groups of stem cells (totipotent, pluripotent, multipotent, oligopotent, unipotent, adult) are known [5]. Fetal stem cells are pluripotent, they are seven to 12 weeks old, there are two types of them (fetal proper, extraembryonic), and they have very high differentiation potential, thus they can differentiate into wide range of cell types within a certain germ layer (ectodermal, endodermal, mesodermal), which is considered to be an advantage. Modern therapy recognizes division of stem cells [6], and their relevant applicability, so that 1) embryonic stem cells are used for treatment of patients with age-related macular degeneration, 2) tissue stem cells are used for treatment of blood, skin, genetic blood diseases, heart attack, critical limb ischemia, damage of the cornea, and 3) induced pluripotent stem cells are used for treatment of several neurological disorders (Down's syndrome, Parkinson's disease). However, the efficacy of fetal stem cell transplantation is unrecognizable when the indications are psychomotor retardation and epilepsy, and consequences of this therapy in the context of severe infection are not known [3]. In addition, treatment by transplantation of plant stem cells is still considered homeopathic in some countries [7, 8], and for this reason it deserves to be considered from a different standpoint.

We found that the damage of allogenic fetal stem cell transplantation in a child suffering from psychomotor retardation and epilepsy is greater than the benefits. It is necessary to set clear and precise indications for fetal stem cells treatment for both healthcare professionals and parents, in order to prevent misuse of the cell therapy, until there are completed controlled clinical studies available.

CASE REPORT

A boy aged 9.5 years with body weight of 32 kg was hospitalized in a serious general condition due to staphylococcal sepsis and inva-

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Table 1. Difference of immunology parameters and blood count before and 13 months after fetal stem cell transplantation

Immunology parameters and blood count	Reference ranges	Before transplantation of fetal stem cells	13 months after transplantation of fetal stem cells and one month after sepsis
B-lymphocytes – CD19+	9 ± 6% 0.21 ± 0.08 × 10^9/l	14.4% 0.7853 × 10^9/l	13.26% 0.3912 × 10^9/l
T-lymphocytes – CD3+	60 ± 9% 1.34 ± 0.02 × 10^9/l	59.3% 3.2337 × 10^9/l	63.92% 1.8856 × 10^9/l
T-helpers – CD4+	39 ± 5% 0.86 ± 0.01 × 10^9/l	35.6% 1.9413 × 10^9/l	37.2% 0.7015 × 10^9/l
T-suppressors – CD8+	23 ± 4% 0.52 ± 0.01 × 10^9/l	23.7% 1.2924 × 10^9/l	31.7% 0.5979 × 10^9/l
NK – CD16+	12 ± 6% 0.39 ± 0.01 × 10^9/l	10.3% 0.5617 × 10^9/l	10.26% 0.3027 × 10^9/l
Helper-suppressor ratio CD4+/CD8+	2.4 ± 0.9	1.5	1.17
White blood cells	4.0–8.8 × 10^9/l	13.3 × 10^9/l	5.85 × 10^9/l
Lymphocytes	19-37% 1.2–3.0 × 10^9/l	41% 5.453 × 10^9/l	50% 2.95 × 10^9/l
Monocytes	0.0–9% 0.00–0.80 × 10^9/l	9% 0.49 × 10^9/l	10% 0.59 × 10^9/l
Red blood cells	3.8–5.3 × 10^{12}/l	4.03 × 10^{12}/l	3.688 × 10^{12}
Hemoglobin	110–170 g/l	133 g/l	125 g/l
Platelets	120–380 × 10^9/l	273 × 10^9/l	202 × 10^9/l

NK – natural killers
Reference ranges are for a boy 9.5 years old [13].

sive pneumococcal pleuropneumonia. The admission took place 13 months after fetal stem cell transplantation, performed routinely in the Center for Transplantation of Organs, Tissues and Cells in Ukraine, with two successive doses intravenously and subcutaneously, one day after the other [3]. The quantity of stem cells was defined as the total amount of 2.1 ml per dose, while there is no data in the discharge list regarding the exact number of given cells. On the official website of this institution it is stated that the quantity of stem cells is one for every 10,000–15,000 cells in bone marrow and one for every 100,000 cells in the bloodstream [9, 10]. Additional data are not known to the patient's parents. Both samples of cell suspension are certified with a series of tests in terms of absence of bacteria and viruses (HIV1, HBV, HCV, HGV, CMV, EBV, HHV6, HSV1,2, rubella, parvovirus B19) and intracellular infections (*Treponema pallidum*, *Toxoplasma gondii*, *Chlamydia trachomatis*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum parvum*) [8, 9]. Immediately prior to fetal stem cell transplantation, normal cytology-representation in the blood was determined immunologically by flow cytometry, as shown in Table 1. There is no information that the patient received immunosuppressants or corticosteroids, either before or after the treatment. With the exception of neurological psychiatric problems, the child was in good general health before treatment with allogeneic fetal stem cells.

Thirteen months after the fetal stem cell transplantation and a month after healed staphylococcal sepsis and invasive pneumococcal pleuropneumonia, there were significant differences in the immune cytology representation in the blood, while the relationship between T-helper and T-suppressor lymphocytes (1.5 vs. 1.2 helper-suppressor ratio CD4+/CD8+) were significantly disrupted in favor of T-suppressor lymphocytes (23.7% vs. 31.7%). The representation of B-lymphocytes was unchanged. Due to our suspicions regarding development and genesis of hematological or immunological diseases in the patient, bone marrow aspiration and biopsy were performed during the sepsis, and showed cellularity of the fourth degree, with increased presence of megakaryocytes and overproduction of myeloid lineage. Immunoglobulin levels were normal, as well as C3 and C4 complement components, which means that humoral immunity in the boy was adequate, unlike cellular immunity.

DISCUSSION

To date it has been known that the predominance of T-suppressor lymphocytes contributes to delayed and late development of stem tumors, teratomas or teratocarcinomas [2], abnormal growth of the brain and the spinal canal [11], but not to weakening of immunity, which clinically presents as life-threatening sepsis and severe pleuropneumonia. The patient continues to show the same severity of psychomotor retardation, dyslalia, epilepsy, strabismus, amblyopia, and regularly uses valproic acid at a dose of 35 mg/kg/day, and optionally diazepam – which proves to be an unsuccessful and costly treatment with fetal stem cells. The transplantation was performed using fetal stem cells in the case of an indication such as psychomotor retardation, which has been unknown so far, and by using a number of cells undisclosed to the public, all of which should be criticized. Such innovative treatments also carry substantial risks with them and the potential not only for well-known malignant transformation of transplanted cells [2], but for severe infections, such as sepsis or severe pneumonia and pleuritis, as shown in this case report. It is likely that this delayed immune imbalance in terms of the up-regulation

of cellular immunity presents a risk of severe infections as adverse effects of treatment with fetal stem cells.

It should be noted that the representation of B-lymphocytes was unchanged, which coincides with data presented in literature [10]. In the patient, humoral immunity was preserved and balanced – that is, transplantation of fetal stem cells did not affect the number and activity of B-lymphocytes.

Modern recommendations concerning the techniques and methods of stem cell transplantation, regardless of their origin, are that the localization of the affected tissues defines the required number of transplanted cells [12]. In current literature there are no precise recommendations on the calculated number of fetal cells for transplantation for patients with epilepsy and psychomotor retardation, nor are there any recommendations according to the patients' age [5, 9, 12].

This paper will hopefully deliver a strong message, a criticism, and raise a question among professional public about the benefit–risk balance of allogeneic fetal stem cell transplantation in children with psychomotor retardation, epilepsy, a condition after intraventricular/periventricular hemorrhage, and hypoxic ischemic encephalopathy. At the same time, there is a requirement set before hematologists and neurologists to determine clear and precise indications for fetal stem cell transplantation in children, and to describe the limits for the therapy's possibilities. The therapy should not be an uncontrolled experiment with sick children, and controlled clinical studies approved by ethics committees need to be performed. Implementation of any type of stem cell therapy for unconfirmed indications is an abuse of the therapeutic approach, does harm to patients, misleads parents, and brings financial harm to the healthcare system of any country, including Serbia.

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Алогена трансплантација феталним матичним ћелијама код детета са психомоторном ретардацијом – приказ болесника

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КРАТАК САДРЖАЈ

Увод Последице аутологне и алогене трансплантације матичних ћелија (матичне ћелије хематопоезе), које се примењују код одраслих и деце оболеле од леукемије и неких других малигних болести, добро су познате и довољно препознатљиве у педијатријској клиничкој пракси без обзира на то шта је индикација за лечење. Али ефикасност трансплантације феталним матичним ћелијама је непрепознатљива када су индикације психомоторна ретардација и епилепсија.

Приказ болесника Са изузетком неуропсихијатријских проблема, дечак узраста девет и по година био је доброг здравственог стања пре трансплантације алогених феталних матичних ћелија. Главни циљ алогене трансплантације феталних матичних ћелија био је лечење психомоторне ретардације и епилепсије. После 13 месеци, он је примљен у болницу у веома тешком, животно угроженом стању због сепсе и тешке плеуропнеумоније. Хуморални имунитет код дечака био је адекватан за разлику од ћелијског имунитета. Имунолошки дисбаланс у смислу превласти Т-супресорских лимфоцита доприноси одложеном и касном развоју сепсе и тешке плеуропнеумоније. Дечак још увек показује исту тежину психомоторне ретардације, дислалије, епилепсије, страбизма и слабовидости.

Закључак Примена терапије матичним ћелијама фетуса за непотврђену индикацију значи злоупотребу терапијског приступа, штету за пацијента, заблуду за родитеље и финансијску штету у здравственом систему у било којој земљи, укључујући и Србију.

Кључне речи: матичне ћелије; трансплантација; хомологна; епилепсија; психомоторни поремећаји; трошкови

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Capture the fracture – Use of bone turnover markers in clinical practice

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SUMMARY
Bone is a living tissue, metabolically very active, with the level of turnover of about 10% per year. Bone remodeling is a well-balanced process of bone resorption, induced by osteoclasts and bone formation-maintained osteoblasts. Loss of bone remodeling balance, with increased bone resorption, leads to osteoporosis. Bone turnover markers are classified as markers of bone formation and of bone resorption. During the growth and development of skeleton, bone turnover markers show higher levels of activity than in the adult period. The increase in biochemical markers peaks again in the postmenopausal period, indicating accelerated bone remodeling. Bone mineral density is an important predictor of an osteoporotic fracture. Timely assessment of risk factors of osteoporosis and bone markers can detect subjects with accelerated bone remodeling and osteoporosis. This may introduce adequate therapy and prevent fracture.
Keywords: bone; bone markers; osteoporosis; fracture

INTRODUCTION

Bone is metabolically active tissue that undergoes continuous remodeling by two counteracting processes, namely bone formation and bone resorption. These processes rely on the activity of osteoclasts (resorption), osteoblasts (formation), and osteocytes (maintenance). Bone cells secrete enzymes and proteins, detected in serum and urine, named bone turnover markers (BTMs) [1]. Under normal conditions, the resorption phase takes approximately 10 days, which is then followed by a formation phase that can last for up to three months. Bone turnover is the principal factor that controls both the quality and the quantity of bone in the adult skeleton. High bone turnover, characterized by increased activity of osteoclasts, leads to bone loss, abnormal bone micro architecture and potential deterioration in bone quality. Low bone turnover, characterized by a reduction of both bone formative and bone resorptive activities may result in increased bone mass, accumulation of micro-damage and bone fragility [2]. Unbalanced bone turnover leads to osteoporosis, a systemic skeletal disease characterized by low bone mass and micro architectural bone deterioration, with consequent increase in bone fragility.

Bone comprises about two thirds mineral and one third osteoid, most of which is type I collagen. Pyridinium's cross-linking molecules stabilize the collagen triple helix in mature bone. During resorption, fragments of type I collagen enter the circulation, and are then cleared in the urine. The collagen-derived bone resorption markers in clinical use do not reflect dietary intake and are relatively specific for bone. Changes in BTMs, which include bone resorption and bone formation markers, are dynamic. The aim of this paper is to review the application of BTMs in the management of osteoporosis.

What are the bone turnover markers?

BTMs are biochemical products, measured usually in blood or urine, which reflect the metabolic activity of bone. Pioneering work in the mid-1990s indicated that measurement of BTMs provided insight into bone turnover rates, which correlate well with bone resorption and formation [3]. BTMs can be divided into bone resorption and bone formation markers, as shown in Table 1. They are used in identifying subjects with an increased risk of fractures and for monitoring treatment of osteoporosis or bone metastases.

Table 1. Markers of bone remodeling in serum

Bone resorption markers	Tartrate-resistant acid phosphatase (TRAP)
	C-terminal telopeptide fragment of type I collagen (CTP)
	β-crosslaps (βCTX)
	N-terminal telopeptide fragment of type I collagen (NTX)
Bone formation markers	Total alkaline phosphatase (ALP)
	Bone alkaline phosphatase (bsALP)
	Osteocalcin (OC)
	C-terminal propeptide of protocollagen type I (P1CP)
	N-terminal propeptide of protocollagen type I (P1NP)

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Markers of bone formation

Bone formation markers in serum are released from osteoblasts, during bone matrix synthesis. They consist of matrix proteins (osteocalcin), products of posttranslational processing of type I collagen molecules (procollagen type I N- or C-terminal propeptides) and enzymes (alkaline phosphatase).

Alkaline phosphatase has been clinically available for several years as a marker for bone metabolism. Total serum alkaline phosphatase consists of several dimeric isoforms that originate from various tissues, such as liver, bone, intestine, spleen, kidney, and placenta. In adults with normal liver function, approximately 50% of the total alkaline phosphatase activity arises from the liver and 50% from the bone [4]. Higher serum values can be found in osteoporosis, hyperparathyroidism, osteosarcoma, bone metastases, Paget's disease; mild elevation can be found in liver disease, Hodgkin's disease, ulcerative colitis. The development of immunoassay-based markers with monoclonal antibodies directed to the bone-specific isoform of alkaline phosphatase has improved specificity and sensitivity [5]. Bone-specific alkaline phosphatase is more specific to represent function of osteoblasts and is elevated in bone mineralization [6]. Changes in bone-specific alkaline phosphatase can lag by several weeks. The half-life of bone-specific alkaline phosphatase is two days, and is less sensitive to circadian variation. The proposal is to measure and monitor alkaline phosphatase at the beginning of the treatment of osteoporosis and after three and six months.

Osteocalcin (OC) or bone Gla-protein (glutamic acid) is the main non-collagen protein of the bone matrix, which is primarily synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes. It contains 49 amino acids (5.8 kDa), of which there are three gamma-carboxyl glutamic acids (post-translational, K vitamin-dependent enzyme carboxylation) at positions 17, 21, and 24, and they are responsible for calcium-binding characteristics of this protein. Osteocalcin binds to hydroxyapatite and constitutes 15% of non-collagenous osteoid. It is a late marker of bone formation. Elevated OC values are described in patients suffering from increased bone formation – hyperparathyroidism, Paget's disease, significant remodeling due to osteoporosis, hyperthyroidism, renal osteodystrophy, fractures, and acromegaly. It is also elevated in postmenopausal women with high bone turnover [7]. This peptide is rapidly degraded in serum; its fragments can be detected by antibody-based assays. Because it is very quickly secreted through kidneys, the half-life of circulating OC is around four to five minutes. OC and its fragments accumulate and their concentration in serum increases when kidney function is changed. Osteocalcin has circadian rhythm activity, being higher in the morning. Hence, it is recommended to take serum samples of osteocalcin at the same time, in the morning, best before 9 a.m. [8]. Osteocalcin values may be affected by anticoagulant drugs. Osteocalcin should be monitored at the start of osteoporotic therapy and after three and six months.

Procollagen type-1 N-terminal propeptide (P1NP) represents a marker of type I collagen synthesis, circulates as a trimeric structure and soon exceeds in the monomeric form. It primarily appears from osteoblasts, but small amounts derive from skin, tendon, dentin and cartilage [9]. It follows a low diurnal rhythm, higher in early morning; concentration is not dependent of renal function. P1NP is cleared by the mannose receptor in endothelial cells of the liver, regulated by growth hormone and thyroid hormones. Interpretation of results is complicated in patients with pituitary or thyroid diseases [10]. P1NP is inversely associated with bone mineral density (BMD) even after controlling for age, body mass index, and years since menopause. The utility of P1NP in the management of metabolic bone diseases remains a subject of debate since the reference ranges are not rigorously established [11].

Markers of bone resorption

Markers of bone resorption are generated from type I collagen, by the protease, cathepsin K, in osteoclasts. Type I collagen comprises more than 90% of the protein of the bone. The most commonly used markers of bone resorption are C-terminal cross-linked fragment of type I collagen (CTX-1) and N-terminal cross-linked fragment of type I collagen (NTX). Two types of CTX-1 may be analyzed as the α and β type. αCTX-1 is generated by cathepsin K cleavage during osteoclast-mediated bone resorption of young collagen. It is elevated in cases with very high bone turnover, such as Paget's disease, osteolytic metastases, osteoarthritis, and rheumatoid arthritis. βCTX-1 is generated during the resorption of mature collagen. The index of αCTX/βCTX represents the ratio of bone remodeling. Both may be used as a potential indicator of risk of osteonecrosis of the jaw in patients receiving oral bisphosphonates [12].

Since the levels of serum NTX and CTX are significantly reduced during anti-resorptive therapy, both are helpful in monitoring the treatment of osteoporosis. The recommendation is to analyze them before treatment and after three and six months. These markers follow the circadian rhythm, being higher early in the morning. Hence, plasma samples should be taken before 9 a.m. These values are not dependent on food intake [13]. The importance of bone resorption markers is underlined by the fact that changes can be observed markedly earlier than the BMD changes, i.e. CTX level measured at three months is predictive of a three-year change in BMD [14]. However, low levels of βCTX may be maintained long after the cessation of therapy.

Pyridinium cross-links and deoxypyridinoline are found in mature collagens and are involved in the cross-linking between collagen polypeptides. They can be detected in urine and serum [15]. These markers also follow circadian rhythm and are higher early in the morning and scarcely influenced by diet. Their use in clinical practice is limited due to low sensitivity.

Tartrate-resistant acid phosphatase 5b (TRACP-5b) and cathepsin K are enzymes produced by osteoclast during

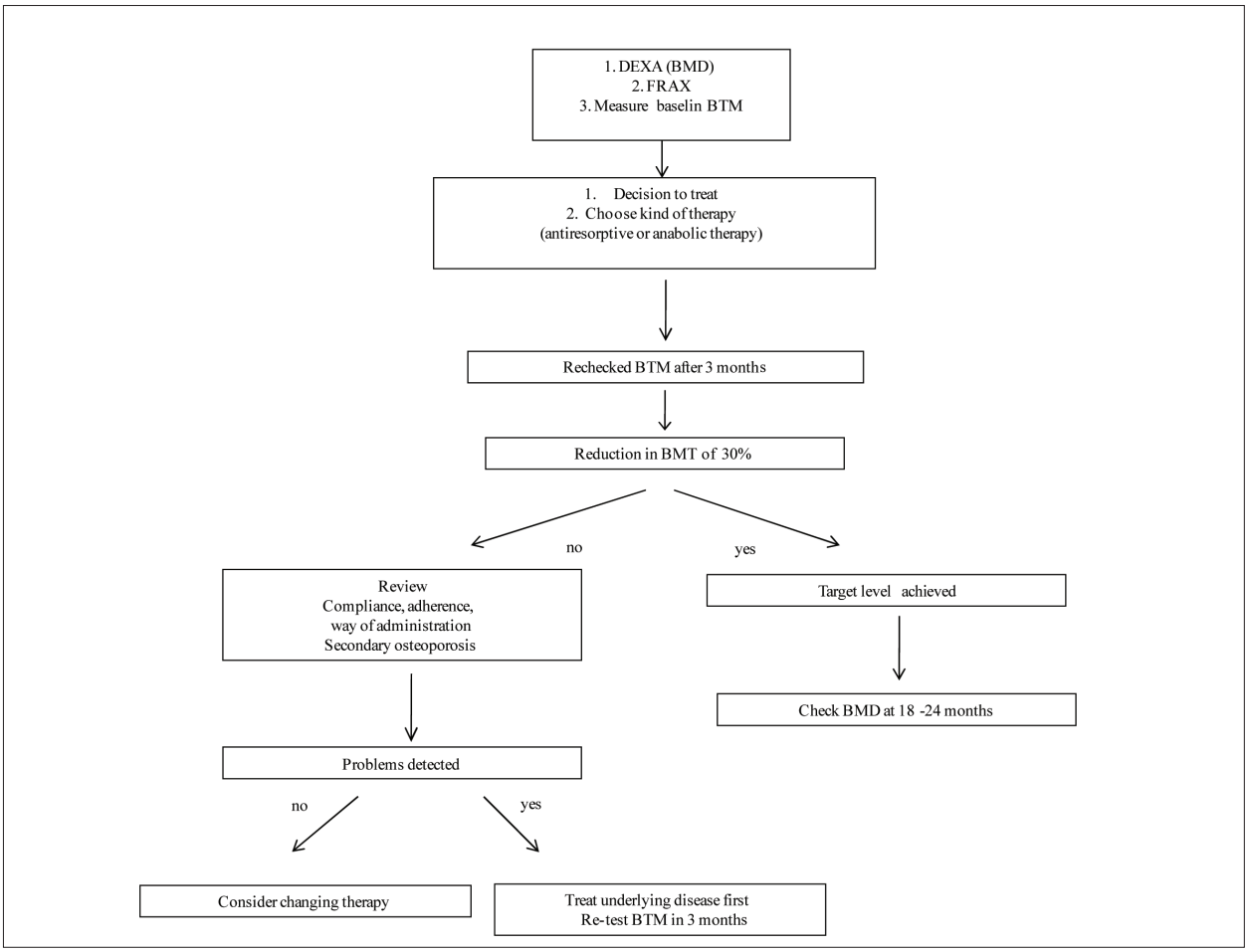
bone resorption. They reflect osteoclast number. TRACP-5b is typically increased in high bone turnover conditions, such as Paget's bone disease, bone metastases, multiple myeloma and after ovariectomy [16]. Its sensitivity to report changes in bone turnover after anti-resorptive therapy has not been as consistent as with other markers, so it has not been viewed as reliable in monitoring osteoporosis.

Bone sialoprotein is the most important non-collagenous extracellular matrix protein, in addition to osteocalcin and osteonectin. It is involved in the mineralization of newly deposited bone matrix and in tissue extra-skeletal calcification. It is a highly phosphorylated acidic glycoprotein with high affinity for hydroxyapatite crystals. Its level reflects the level of bone resorption [17].

USE OF BONE TURNOVER MARKERS IN DIAGNOSIS AND FRACTURE RISK ASSESSMENT

Bone turnover markers are increased in women after menopause, correlating well with accelerated bone turnover. However, the diagnosis of osteoporosis is made by BMD, as biomarkers of tissue turnover do not provide a stand-alone diagnostic value. An inverse correlation, dependent on skeletal site and age, has been observed between BMD and BTMs, but this is stronger for resorption than for formation markers. BTMs are a helpful tool in assessment of bone remodeling in patients with osteopenia (low normal BMD). In osteopenic patients, assessment of risk factors for osteoporosis and measurement of BTMs may detect those with an increased fracture risk, who would benefit from early therapy [18]. The OFELY study has shown a10-year expectancy of fracture in osteopenic women with one risk factor of 26%, compared to 6% in women with no risk [19].

High levels of bone turnover markers are associated with an increased risk of fracture, independent of BMD. Several studies have shown that independent risk factors for spine and hip fractures are elevated CTX. Women with osteoporosis of hip and high serum CTX levels have a fracture risk of 55% in five years. When there is low BMD with normal CTX, the risk is 39%, and when only isolated high CTX levels are present, the relative risk is 25% [19]. High urinary deoxypyridinoline positively correlates with hip fracture risk [20]. A meta-analysis of several randomized clinical trials, evaluating the treatment of osteoporosis, has shown that a reduction in bone resorption markers of 70% reduces non-vertebral fracture risk for 40%. A 50% decrease in bone formation markers was associated with a 44% reduction for non-vertebral fracture risk [21].



Scheme 1. An algorithm for the use of bone turnover markers (BTMs) in treatment and monitoring of osteoporosis (adapted from and based on Bergmann et al. [31])

DEXA – dual energy X-ray absorptiometry; BMD – bone mineral density; FRAX – fracture risk assesment tool; BTM – bone turnover markers

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Table 2. Uncontrollable and controllable sources of variability on bone turnover markers (BTMs) and their importance (adapted from and based on Bergmann et al. [31])

	Very important		Important	
Uncontrollable source of variability on BTMs	Age	BTMs increase with age in men and women	Fractures	BTMs increase after a fracture (maximal at 2–12 weeks, but effect lasts for up to 52 weeks)
	Menopause	BTMs increase within a few months after the last menstrual period	Pregnancy, lactation	BTMs are increased during pregnancy; highest levels during third trimester, even higher postpartum
	Sex	BTMs are higher in older women than in older men	Drugs (corticosteroids, anticonvulsants, heparin, GnRH agonists)	BTMs may be decreased (glucocorticoids) or increased (anticonvulsants)
			Disease (thyroid disease, diabetes, renal impairment, liver disease)	BTMs often increased (thyrotoxicosis, chronic kidney disease)
			Bed rest/immobility	Bone formation markers decrease and resorption markers increase
Controllable source of variability on BTMs	Circadian	Most striking for bone resorption markers; highest values in second half of night and on waking; lowest values in afternoon and evening	Fasting status	Feeding results in a decrease in BTMs; for example, s-CTX decreases by 20% after breakfast
			Exercise	Changes occur but depend on type of exercise and age of subjects

In clinical practice, the use of BTMs should be carefully considered, and all their causes should be taken into account in their interpretation (Table 2). Assessment of parameters of bone metabolic activity is important to differentiate physiological from pathological bone metabolism. For example, an increase in BTMs of more than 150% of the upper reference range is not typical of postmenopausal osteoporosis and should prompt a search for another cause. Often, these levels are indicative of other diseases, even metastases [22].

BONE TURNOVER MARKERS IN MONITORING THERAPEUTIC EFFICACY AND ADHERENCE TO THERAPY

Bone resorption markers rapidly decrease by over 40% after three months of bisphosphonate therapy. This is followed by a reduction in bone formation markers in the next six months [23]. Changes in BTMs predict changes in BMD. A decrease in CTX after three months on ibandronate is predictive of a significant increase in BMD of lumbar spine after one and two years [24]. Recent data for zoledronic acid have shown good result in reduction in alkaline phosphatase, P1NP, CTX levels, which precedes the reduction in vertebral fractures [25]. Bone resorption markers remained low even after six years of treatment [26]. Similar result has been shown with denosumab, which produces a very rapid fall in resorption markers and a smaller change in formation markers. Low levels are maintained on therapy [27, 28]. In contrast, teriparatide treatment stimulates new bone formation. Bone formation markers are doubled during the first month of treatment and continue to increase in the following six months [29].

Lack of efficacy may be the reason for no change in BTMs on anti-resorptive therapy. Sometimes, the reason for non-reduction of BTMs is poor compliance with therapy, secondary osteoporosis, malabsorption, or eating too soon after taking oral bisphosphonates. This problem can be solved by switching to parenteral form of therapy or changing to a different class of medication [30]. An algorithm has been proposed for the use of bone turnover markers in treatment and monitoring of osteoporosis (Scheme 1).

Adherence to bisphosphonate therapy is not easy. Taking a weekly or monthly medication can easily be forgotten. Compliance and persistence in osteoporosis is not significantly different from other asymptomatic chronic conditions. Most of the poor medication behavior with osteoporosis medication is probably intentional rather than unintentional. Low suppression of BTMs can be indicative of non-compliance. Some studies have shown improved adherence to treatment when turnover marker results were provided to patients [32].

There is still no consensus on the duration of bisphosphonate therapy. A "drug holiday" in low-risk patients after five years of continuous treatment with bisphosphonates has been proposed [33]. In clinical practice, recheck of BTMs at six-month intervals of drug holiday may indicate the need to start the therapy again. This is done when BTMs increase to the upper limit of the premenopausal range.

PRECAUTION AND SELECTION OF SPECIFIC THERAPY

BTM variation includes diurnal and circadian variability. Diurnal variability of bone markers may be as high as 40–70%, whereas the circadian is 7–17%. Levels of BTMs are the highest in the early morning and the lowest in the afternoon and evening. It is worth mentioning that an increase in dietary calcium intake can lower the levels of BTMs, particularly in people with low calcium intake [34]. The range for BTMs must be adjusted for sex and age. Physical activity can increase BTMs [35]. The

lack of assay standardization is still a matter of concern, making difficult the comparison of results obtained by different methods or laboratories. This is the reason why monitoring should always be done in the same laboratory. Theoretically, better response to anti-resorptive therapy is expected in patients with high bone turnover (higher levels of BTMs), while an anabolic agent would be more appropriate in case of low bone turnover. However, the use of BTMs in selection of the optimal treatment for osteoporosis is not recommended [36].

CONCLUSION

Bone turnover markers have clinical applicability in decision making on early treatment of women with osteopenia, fracture risk assessment, and monitoring of therapy. They could identify "fast bone losers", which could benefit the most from the therapy, and "non-responders", which could benefit from the change of therapy. To capture the fracture is possible with timely measurements of bone mineral density and bone turnover markers.

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Спречити фрактуру – употреба коштаних маркера у клиничкој пракси

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КРАТАК САДРЖАЈ

Кост је живо ткиво, метаболички врло активно, са коштаним обртом од око 10% годишње. Коштано ремоделовање представља равнотежу између процеса ресорпције и формирања кости, регулисану активношћу остеокласта и остеобласта. Губитком равнотеже између ова два процеса, са преовладавањем процеса ресорпције у односу на процес формирања кости, настаје остеопороза. Маркери коштаног промета су класификовани у маркере формирања и маркере ресорпције кости. Током раста и развоја скелета, биохемијски маркери показују виши ниво активности него код одраслих. Пораст нивоа коштаних маркера јавља се поново у постменопаузалном периоду, услед недостатка естрогена, што индикује убрзање коштаног ремоделовања. Минерална густина кости је важан предиктор ризика за прелом. Важно је на време испитати факторе ризика за остеопорозу и тестирати коштане маркере. Правовремено откривање особа са убрзаним коштаним ремоделовањем и увођење терапије може спречити настанак остеопоротичне фрактуре.

Кључне речи: кост; коштани маркери; остеопороза; фрактура

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Can we always take analysis of complete blood count by automated blood cell analyzer as absolutely correct?

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Dear Editor,

Automated blood cell analyzer is a rapid and cost-effective method for complete blood cell count (CBC) and leukocyte differential count (LDC), and the results are very precise and reliable. However, in certain situations where results do not correspond to the clinical findings, it may be advisable to perform a manual LDC [1]. We recently experienced such a case.

Before travelling abroad for several months, a 76-year-old female patient, in whom only monocytosis and granulocytopenia were found in November 2013 (CBC – hemoglobin 130 g/l, white blood cells 4.77×10^9/l, platelets 280×10^9/l; LDC – neutrophils 11.2%, lymphocytes 37.8%, monocytes 36.2%, basophils 0.9%, eosinophils 4.3%, and large unstained cells 9.6%) underwent a regular control of all laboratory data, including a complete blood count. On subsequent controls, performed in respectable laboratories in November 2014, April 2015, and October 2015, LDC showed presence of severe granulocytopenia and monocytosis, which was constantly above 40%. The patient remained without any complaints throughout the stated period of time, with normal physical findings and in completely good health. Biochemistry data were within normal limits all the time. On abdominal ultrasonography, both liver and spleen were homogenous and of normal size. The accessory spleen in the splenic hilum of 2.5 cm in diameter was seen. Small nodes of up to 3 mm in diameter were seen on ultrasonography in both lobes of the thyroid gland. There was no lymphadenopathy.

The patient was referred to the hematologist in a highly respectable hematology institution in Belgrade where CBC, LDC, and biochemistry were reexamined. After checking the results, the hematologist expressed suspicion that the patient might have had a myelodysplastic syndrome, CMML type (chronic myelomonocytic leukemia) and suggested an examination of bone marrow cytology and histology. Being completely symptom-free, the patient did not accept the examination as she had to travel again and stay abroad for several months. Hence, the patient was advised to undergo regular CBC monitoring abroad and to take 1,000 mg of vitamin C, two tbl (5 mg) of folic acid, 1,000 mg of vitamin B_{12} daily. As she stayed asymptomatic throughout her stay abroad, the patient did not follow the advice on monitoring CBC. After the patient returned, another CBC checkup was performed, again showing the same results as before: granulocytopenia and monocytosis above 40%.

On October 5, 2015, the patient came to the Laboratory Department of the Clinic of Hematology, Clinical Center of Serbia, Belgrade, where CBC and LDC were checked, both using automated blood cell analyzer and manual differential leukocyte formula, both showing almost exactly the same data: neutrophils 50%, lymphocytes 39%, monocytes 8%, eosinophils 2%, and basophils 1%. The dilemma about the cause of "severe granulocytopenia and monocytosis" was solved as error of automated blood cell analyzer. Apparently, the number of the previous machine count had been incorrect for some reason, taking most of the granulocytes as monocytes.

What can be a possible explanation for repeated mistakes of the automatic blood cell analyzer?

The blood monocytes are the largest cells in normal blood. They are in transit between the bone marrow and tissues, where they transform into macrophages. They participate in virtually all inflammatory and immune disorders, and thus their concentration may be increased in many such conditions, including autoimmune diseases, gastrointestinal disorders, sarcoidosis, and several viral and bacterial infections [2]. All such conditions could have been excluded as the patient has stayed well for more than two years now, without any symptom and with normal all other laboratory data. As profound monocytosis may represent a hematologic disorder or malignancy, such as myelodysplastic and myeloproliferative syndromes or monocytic leukemia, a further, more sophisticated and aggressive analyses should be performed in such a case. All these possibilities were taken into consideration, but were very unlikely in our patient. That's why we considered the monocytosis as probable automated blood cell analyzer error. Hence, before undertaking

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these invasive procedures in the patient, we performed a classical manual blood film examination, which proved to be the right decision.

We believe that, in absence of clinical symptoms and signs of any disease, an "abnormal" CBC and LDC alone should be interpreted within the context of an individual's health condition and sometimes checked using classical methods before performing invasive diagnostic procedures. At the end, one has to be aware of the well-known fact that as much as 5% of the general population without disease may display laboratory values outside the statistically assigned "normal" reference range [3].

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Пре подношења рукописа Уредништву часописа „Српски архив за целокупно лекарство“ 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 може одложити објављивање рада или чак одбити његово публиковање. Стога се ауторима и потенцијалним сарадницима саветује да пажљиво прочитају ово упутство пре него што приступе припреми рукописа за штампу у „Српском архиву за целокупно лекарство“.

ОПШТА УПУТСТВА. Оригинални и стручни радови се у целини достављају на енглеском језику са сажетком на енглеском и српском. 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, $Б_{12}$, CD8).

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.

КЛИНИЧКА ИСТРАЖИВАЊА. Клиничка истраживања се дефинишу као истраживања која се односе на једну здравствену интервенцију или на више њих, а ради испитивања утицаја на здравствени исход. Регистарски број истраживања треба да се наведе у последњем реду Кратког садржаја.

ЕТИЧКА САГЛАСНОСТ. Рукописи о хуманим медицинским истраживањима или историјама болести пацијената 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 локалним 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 рада, наравно, уз писани пристанак.

НАСЛОВНА СТРАНА. На првој страници рукописа треба навести следеће: наслов рада без скраћеница; 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.

КРАТАК САДРЖАЈ. Уз оригинални рад, саопштење, преглед литературе, приказ болесника, рад из историје медицине, рад за рубрику „Језик медицине“ и рад за праксу, на другој по реду страници документа треба приложити кратак садржај рада обима 100–250 речи. За оригиналне радове кратак садржај 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. За приказе болесника кратак садржај треба да има следеће делове: Увод, Приказ болесника, Закључак; сегменте такође писати као посебан пасус који почиње болдованом речи. За остале типове радова сажетак нема посебну структуру.

КЉУЧНЕ РЕЧИ. Испод Кратког садржаја 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 кратак садржај (100–250 речи) с кључним речима (3–6), и то за радове у којима је обавезан кратак садржај 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 (нпр. рад из историје медицине, језика медицине и др.), 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 обавезно треба да има следеће поднаслове: Увод, Циљ рада, Методе рада, Резултати, Дискусија, Закључак, Литература. Преглед литературе чине: Увод, одговарајући поднаслови, Закључак, Литература. Првоименовани аутор прегледног рада мора да наведе бар пет аутоцитата (референце у којима је био први аутор или коаутор рада) радова публикованих у часописима с рецензијом. Коаутори, уколико их има, морају да наведу бар један аутоцитат радова такође публикованих у часописима с рецензијом. Приказ болесника чине: Увод, Приказ болесника, Дискусија, Литература. Не треба користити имена болесника, иницијале, нити бројеве историја болести, нарочито у илустрацијама. Прикази болесника не смеју имати више од седам аутора.

Прилоге (табеле, графиконе, слике итд.) сместити на крај рукописа, а у самом телу текста назначити место које се односи на дати прилог. Крајња позиција прилога пре штампања рада зависиће од прелома текста.

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 кратком садржају, али ако су неопходне, сваку скраћеницу поново 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 – *kg*, 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 РУКОПИСА. Целокупни рукопис рада – који чине насловна страна, кратак садржај, 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, саопштење, рад из историје медицине и преглед литературе до 5.000 речи, а за приказ болесника, рад за праксу, едукативни чланак и рад за рубрику „Језик медицине“ до 3.000 речи; радови за остале рубрике могу имати највише 1.500 речи.

Провера броја речи у документу може се извршити у програму *Word* кроз подмени *Tools–Word Count* или *File–Properties–Statistics*.

ТАБЕЛЕ. 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. Слике се означавају арапским бројевима према редоследу навођења у тексту. Примају се искључиво дигиталне фотографије (црно-беле или у боји) резолуције 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 као *Grayscale* (или у боји) у резолуцији 300 *dpi* и снимити их у оригиналној величини.

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. 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.

Уколико је рукопис на српском језику, приложити називе графикона и легенду на оба језика.

СХЕМЕ (ЦРТЕЖИ). Схеме цртати 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. 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 публикације и чланци могу уврстити у референце, аутори су дужни да их цитирају. Већина цитираних научних чланака не треба да буде старија од пет година. Избегавати коришћење апстракта као референце, а апстракте старије од две године не цитирати. 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. Примери 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 И ПРЕТПЛАТА. Да би рад био објављен у часопису *Српски архив за целокупно лекарство*, сви аутори морају бити чланови Српског лекарског друштва (у складу са чланом 6. Статута Друштва), док први аутор мора бити и претплатник на часопис, за годину у којој се рад предаје Уредништву. 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, као доказ о уплатама. Аутори из иностранства нису дужни да буду чланови Српског лекарског друштва, нити претплатници на часопис за текућу годину. Додатне информације о чланарини и претплати могу се добити на телефоне 011/3245-149 и 011/3346-963, односно имејлом (*srparhiv@bvcom.net*) и на интернет-страници часописа (*http://www.srpskiarhiv.rs*).

СЛАЊЕ РУКОПИСА. Рукопис рада и сви прилози уз рад могу се доставити имејлом (*srparhiv@bvcom.net*), електронски преко система за пријављивање на интернет-страници часописа (*http://www.srpskiarhiv.rs*), препорученом пошиљком или лично, доласком у Уредништво. Уколико се рад шаље поштом или доноси у Уредништво, рукопис се доставља одштампан у три примерка и нарезан на *CD* (снимљени материјал треба да је истоветан оном на папиру).

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 свезака.

За све додатне информације, молимо да се обратите на доленаведене адресе и број телефона.

АДРЕСА:
Српско лекарско друштво
Уредништво часописа „Српски архив за целокупно лекарство“
Ул. Краљице Наталије 1
11000 Београд
Србија

ТЕЛЕФОН: 011/3245-149
E-MAIL: *srparhiv@bvcom.net*
ИНТЕРНЕТ АДРЕСА: *http://www.srpskiarhiv.rs*

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GENERAL INSTRUCTIONS. Original and professional papers should be written entirely and exclusively in the English language with a summary and title page in both English and Serbian. 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.

When writing text in English, linguistic standard American English should be observed. Write short and clear sentences. Generic names should be exclusively used for the names of drugs. Devices (apparatuses, instruments) are termed by trade names, while their name and place of production should be indicated in the brackets. If a letter-number combination is used, the number should be precisely designated in superscript or subscript (i.e., ^{99}Tc, IL-6, O_2, B_{12}, CD8).

If a paper is a part of a master's or doctoral thesis, or a research project, that should be designated in a separate note at the end of the text. Also, if the article was previously presented at any scientific meeting, the name, venue and time of the meeting should be written.

CLINICAL TRIALS. Clinical trial is defined as any research related to one or more health related interventions in order to evaluate the effects on health outcomes. The trial registration number should be included as the last line of the summary.

ETHICAL APPROVAL. Manuscripts with human medical research, or patients case histories should contain a statement that the subjects' written consent was obtained, according to the Declaration of Helsinki, the study has been approved by competent ethics committee, and conforms to the legal standards. Experimental studies with human material and animal studies should contain statement of the institutional ethics committee and meet local legal standards.

CONFLICT OF INTEREST STATEMENT. The manuscript must be accompanied by a disclosure statement from all authors (contained within the Submission Letter) declaring any potential interest or stating that the authors have no conflict of interest. For additional information on different types of conflict of interest, please see World Association of Medical Editors (WAME, *www.wame.org*) policy statement on conflict of interest.

AUTHORSHIP. All individuals listed as authors should be qualified for authorship. Every author should have participated sufficiently in writing the article in order to take responsibility for the whole article and results presented in the text. Authorship is based only on: crucial contribution to the article conception, obtaining of results or analysis and interpretation of results; design of manuscript or its critical review of significant intellectual value; final revision of the manuscript being prepared for publication.

The authors should enclose the description of contribution to the article of every co-author individually (within the Submission Letter). Funding, collection of data or general supervision of the research group alone cannot justify authorship. All other individuals having contributed to the preparation of the article should be mentioned in the *Acknowledgment* section, with description of their activities, and their written consent.

TITLE PAGE. The first page of the manuscript (cover sheet) should include the following: title of the paper without any abbreviations; each author's full names and family names (no titles), indexed by numbers; official name, place and country of the institution in which authors work (in order corresponding to the indexed numbers of the authors); at the bottom of the page: name and family name, address, phone and fax number, and e-mail address of a corresponding author.

SUMMARY. Along with the original article, communication, review article, case report, article on history of medicine, article for "Language of medicine" and article for practitioners, the summary not exceeding 100–250 words should be typed on the second page of the manuscript. In the original article, the summary should have the following structure: Introduction, Objective, Methods, Results, Conclusion. Each segment should be typed in a separate paragraph using boldface. The most significant results (numerical values), statistical analysis and level of significance are to be included. In case reports, the summary should consist of the following: Introduction, Case Outline (Outline of Cases), Conclusion. Each segment should be typed in a separate paragraph using boldface. In other types of papers, the summary has no special outline.

KEYWORDS. Below the summary, 3 to 6 keywords or frases should be typed. The keywords need not repeat words in the title and should be relevant or descriptive. *Medical Subject Headings – MeSH* (*http://www.nlm.nih.gov/mesh*) are to be used for selection of the keywords.

TRANSLATION INTO SERBIAN. The third page of the manuscript should include: title of the paper in the Serbian language; each author's full name and family name (no titles), indexed by numbers; official name, place and country of the institution in which authors work. On the fourth page of the manuscript the summary (100–250 words) and keywords (3–6) should be typed, but this refers only to papers in which a summary and keywords are compulsory. The terms taken from foreign literature should be translated into comprehensible Serbian. All foreign words or syntagms that have a corresponding term in Serbian should be replaced by that term.

If an article is entirely in Serbian (e.g. article on history of medicine, article for "Language of medicine", etc.), captions and leg-

ends of all enclosures (tables, graphs, photographs, schemes) – if any – should be translated into English as well.

STRUCTURE OF THE MANUSCRIPT. All section headings should be in capital letters using boldface. An original article should have the following section headings: Introduction, Objective, Methods, Results, Discussion, Conclusion, References. A review article includes: Introduction, corresponding section headings, Conclusion, References. The first named author of a review article should cite at least five auto-citations (references of which he was the author or co-author of the paper) of papers published in peer-reviewed journals. Co-authors, if any, should cite at least one auto-citation of papers also published in peer-reviewed journals. A case report should consist of: Introduction, Case Report, Discussion, References. No names of patients, initials or numbers of medical records, particularly in illustrations, should be mentioned. Case reports cannot have more than seven authors.

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