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Kontakt adresa: Časopis Acta Medica Medianae, Medicinski fakultet, Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: acta@medfak.ni.ac.rs, Tel+381-18-4533001 lok. 122 fax. +381-18-4534336
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PERCEPTION OF PROTECTIVE MEASURES AMONG PATIENTS TESTED FOR COVID-19: A CROSS SECTIONAL STUDY CONDUCTED IN THE SERBIAN UNIVERSITY CENTER

Aleksandar Višnjić[1,2], Kıvanç Kök[3], Roberta Marković[1,2], Aleksandra Jović Vraneš[4], Zoran Milošević[1,2], Dragan Nikolić[2], Tamara Jovanović[1,2]

The main objective of this study was to explore the practical benefits of precautionary behaviors among general population considering the Coronavirus disease 19 (COVID-19) infection rates. Additionally, sociodemographic aspects, related with the COVID-19 transmission, were also of interest.

For the purposes of this research, we have selected two groups of respondents who voluntarily agreed to participate. The research was conducted in the period from October to December 2020 at the Faculty of Medicine of the University of Niš (Serbia).

A total of 1,035 people underwent a telephone survey. There were 522 (50.4%) women and 513 men; 630 (60.9%) were infected with corona virus. The obtained results indicate that age, level of education, self-assessed health and the existence of chronic diseases have a significant impact on the self-perceived risk of contracting COVID-19 infection. Moreover, the presence of the so-called "fear factor" has a significant impact on infection rates. In contrast, no effect of gender difference and wearing mandatory protective masks was observed on COVID-19 infection rates.

This study yields novel insights into common protective measures against COVID-19, highlighting differences between the studied protective factors. Further efforts in this direction are required in order to develop more elaborate, well-balanced, efficient strategies for containing the ongoing pandemic, especially in the context of the contagion control.

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Key words: *precautionary behavior, face masks, perceived risk, COVID-19, public health*

[1]University of Niš, Faculty of Medicine, Niš, Serbia
[2]Institute of Public Health of Niš, Niš, Serbia
[3]Istanbul Medipol University, International School of Medicine, Istanbul, Turkey
[4]University of Belgrade, Faculty of Medicine, Belgrade, Serbia

Contact: Aleksandar Višnjić
81 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: aleksandar.visnjic@medfak.ni.ac.rs

Introduction

In the Republic of Serbia, the first official case with COVID-19 was reported on March 6th 2020 (1). According to the data of the Public Health Institute of Serbia, a total of 395,263 cases was tested positive among 2,617,473 tested individuals (with PCR test), and 4,020 deaths (1.02% of positives) were reported in the period of almost eleven months, from March 6th 2020 to January 31st 2021 (1). It should be taken into account that the total number of residents in the Republic of Serbia (excluding the Autonomous Province of Kosovo and Metohija) is approximately 7 million people, according to the estimate of the Statistical Office of the Republic of Serbia (2).

The SARS-CoV-2 virus, which causes the COVID-19 disease, is still circulating in the territory of the Republic of Serbia, and beyond. Since Serbia is a country with no entry restriction, it is recommended to follow preventive measures in order to reduce the risk of occurrence and transmission of this infection. The Government of the Republic of Serbia has adopted a number of measures in order to prevent and suppress the pandemic (1). Initially, it was required to wear protective gloves, not to greet people, not to touch the door handles or any other metal surface with bare hands, and to wear face masks indoors (schools, banks, shops, etc.); there was a ban on gathering more than 5 people; catering facilities, gyms, hairdressing salons were closed.

In the meantime, controversial information provided by various sources, through the media, and different networks, made people insecure on how to behave. Many citizens have expressed a serious doubt whether masks protect virus transmission and prevent the spread of the pandemic. It was a period of learning for everyone, and published literature has provoked considerable questions about the individuals' behaviors on the pandemic situation, and links between behavior and the spread of infection (3-11).

Most of the governments have followed the recommendations issued by WHO and mandate the general population to wear a face mask in public locations (12). Therefore, the aim of this study was to explore the perception of protective measures among series of patients tested for COVID-19, and especially tendency of wearing protective masks.

Methods

This is a cross-sectional study on protective measures among the group of patients who were tested for COVID-19 in the period from October to December 2020 at the Faculty of Medicine of the University of Niš (Serbia). A total of 1,078 patients who had previously undergone PCR testing at the Institute of Public Health in Niš, with both positive and negative results, were invited by phone to voluntarily participate in the survey, i.e. telephone interview with a group of doctors from the Institute of Public Health of Niš within 5-7 days after taking the test. Out of the total number of the invited people, 1,035 (96.01%) responded to the calls and agreed to participate in the interview. The study procedures were carried out in accordance with the Declaration of Helsinki (13).

The survey instrument was the structured questionnaire consisting of two sets of questions. With the first set of questions, sociodemographic data, such as gender, age, level of education, marital status and monthly household income, were collected.

Through the second set of questions, patients were asked to assess their own health (as bad, moderate or good), and self-perceived risk of contracting COVID-19 (self-perceived risk to the infection exposure); to declare if they have co-morbidities, and to assess information sufficiency on COVID-19 (with yes or no questions). The follow-up questions were about strict adherence to the pandemic-related instructions of the Ministry of Health of the Republic of Serbia: maintaining hand hygiene, keeping the social distance of two meters, avoiding crowded places, and regular wearing of the protective facial masks - self-report on their behaviors prior to being tested (1).

Among the respondents, 405 were negative and 630 were positive for SARS-CoV-2 virus. In addition, the fear of contracting the COVID-19 (self-perceived risk to the infection exposure) was assessed on the five point Likert scale (where 1 = no fear at all, and 5 = fear the most). For the purposes of this research, the group of respondents were divided into two subgroups (as PCR positive versus PCR negative) based on the corresponding PCR test results, and the COVID-19-related aspects were compared between the resulting subgroups.

Data were analysed using the descriptive statistics (namely, absolute and relative frequency, mean and standard deviation) and analytical statistics, such as binary logistic regression, multiple regression analysis, and correlation tests. The statistical analysis was performed using the SPSS 17.0 program (SPSS Inc., Chicago, IL, USA). The cut-off threshold for statistical significance was set at $p < 0.05$.

Results

General sociodemographic characteristics

A total of 1,035 patients underwent a telephone interview. Mean age of respondents was 48.35 (SD = 17.22). There group of respondents consisted of 522 (50.4%) women and 513 men (Table 1).

More than a half of interviewed patients were with under high school education (55.9%), 340 (32.8%) were with high school diploma, and only 116 (11.3%) with university degree. The majority of patients 784 (75.7%) were married. Approximately 21% of patients had monthly household income (in euro) fewer than 500. More than a half of patients 571 (55.2%) assessed their health as moderate, one third (33.8%) as good, and 114 (11.0%) as bad.

There were 527 respondents who wore masks (surgical or other) regularly, while the remaining 508 respondents wore them only when obliged (e.g. when entering state institutions).

Among other things, it was found that Pearson chi-square statistic for wearing face masks and getting corona infection, with Yates correction, is $\chi^2 = 0.531$ ($p = 0.466$). Therefore, Pearson's analysis did not confirm a statistically supported significance of wearing protective masks. Similarly, the gender-related statistical difference was not observed.

Perceived risks and precautionary behaviors on COVID-19

Table 2. shows that perception of contamination of COVID-19 infection does not correlate to regular wearing a protective facial masks (it is statistically insignificant, $p = 0.526$), while other measures, such as hand hygiene, keeping the distance, and being away from crowded places have statistically significant negative correlation ($p < 0.001$).

Contracted COVID-19 infection has positive statistically significant correlation only to the self-perceived risk to the infection exposure ($r = 0.624$; $p < 0.001$) (Table 2).

The basic idea was to examine what else could have affected the infection rates.

Therefore, we performed a multiple regression analysis, with self-perceived risk of contracting the infection (Likert 1-5) as the main determinant - dependent variable (Table 3).

This analysis showed that only age, level of education, self-assessed health and the existence of some other, chronic diseases (co-morbidities) have a significant impact on the self-perceived risk of contracting this disease (Table 3).

Table 1. Sociodemographic characteristics of the respondents according to the PCR testing results for COVID-19

Sociodemographic characteristics	n = 1,035 **(100%)**	**COVID-19 status (PCR test)**		Pearson's χ^2	*P*
		Negative (n)	**Positive (n)**		
Gender					
Female	522 (50.4)	231	291	1.96	0.16 (ns)
Male	513 (49.6)	204	309		
Education					
Under high school	579 (55.9)	212	367	36.06	< 0.01**
With high school diploma	340 (32.8)	118	222		
With university degree	116 (11.3)	75	41		
Monthly household income (in euro)					
Under 500	216 (20.9)	74	142	11.41	< 0.01**
500–1000	413 (39.9)	157	256		
1000–2000	331 (32.0)	132	199		
≥ 2000	75 (7.2)	42	33		
Self-assessed health					
Bad	114 (11.0)	20	94	144.87	< 0.01**
Moderate	571 (55.2)	160	411		
Good	350 (33.8)	225	125		
Marital status					
Single	251 (24.3)	100	151	0.04	0.85 (ns)
Married	784 (75.7)	305	479		
Information sufficiency on COVID-19					
No	317 (69.3)	84	233	29.85	< 0.01**
Yes	718 (30.7)	321	397		
Presence of comorbidities					
No	837 (80.9)	357	480	22.02	< 0.01**
Yes	198 (19.1)	48	150		
Wearing regularly facial protective masks					
No	508 (49.1)	205	303	0.531	0.47 (ns)
Yes	527 (50.9)	200	327		
Hand hygiene					
No	119 (11.5)	28	91	13.01	< 0.01**
Yes	916 (88.5)	377	539		
Keeping the physical distance of 2 meters					
No	494 (47.7)	90	404	171.84	< 0.01**
Yes	541 (52.3)	315	226		
Avoiding crowded places					
No	155 (15.0)	23	132	108.44	< 0.01**
Yes	880 (85.0)	532	348		

** Chi square (χ^2) is significant at the 0.01 level

Table 2. Correlation between the protective measures, applied by the participants against SARS-CoV-2 and perceived risk of contracting COVID-19

	Self - perceived risk to SARS- CoV-2 infection exposure	Wearing face masks	Hand hygiene	Keeping the distance	Being away from crowded places	COVID-19 - positive PCR test
Self - perceived risk to SARS- CoV-2 infection exposure	1.00	0.624**	0.114**	0.576**	0.132**	0.085*
P		0.000	0.000	0.000	0.000	0.011
Wearing face masks	0.624**	1.00	0.081**	0.461**	0.123**	-0.028
P	0.000		0.009	0.000	0.000	0.526
Hand hygiene	0.114**	0.081**	1.00	0.074*	0.045	-0.077*
P	0.000	0.009		0.017	0.150	0.018
Keeping the distance	0.576**	0.461**	0.074*	1.00	0.030	-0.071*
P	0.000	0.000	0.017		0.337	0.013
Being away from crowded places	0.132**	0.123**	0.045	0.030	1.00	-0.075*
P	0.000	0.000	0.150	0.337		0.016
COVID-19 positive PCR test	0.085*	-0.028	-0.077*	-0.071*	-0.075*	1.00
P	0.011	0.526	0.018	0.013	0.016	

** Spearman correlation is significant at the 0.01 level, and
* at the 0.05 level

Table 3. Multivariate linear regression analysis of perceived risks for COVID-19 of the surveyed participants

Characteristics	**Unstandardized Coefficients**		**Standardized Coefficients**	**T**	***p***	**95% CI**		**Collinearity Statistics**	
Self-perceived risk of contracting the corona virus	**B**	**Std. Error**	**β**			**Lower**	**Upper**	**Tolerance**	**VIF**
Self-Perceived Risk				$R^2 = 0.29$; F = 25.48, df = 8, p < 0.001					
Constant	5.50	0.45		5.493	0.000	1.607	3.395		
Gender	0.002	0.01	0.013	0.261	0.794	-0.01	0.02	0.71	1.41
Age	0.058	0.019	1.417	3.027	0.003	-0.095	-0.020	0.708	1.413
Education	-0.174	0.033	-1.516	-5.33	0.000	-0.238	-0.110	0.543	1.843
Marital status	-0.25	0.366	-0.030	-0.70	0.490	-0.973	0.466	0.752	1.330
Household income	0.084	0.205	0.017	0.412	0.681	-0.318	0.487	0.752	1.330
Self-assessed health	-0.122	0.039	-1.425	-3.165	0.002	0.046	0.198	0.796	1.256
Information sufficiency	0.893	0.455	0.091	1.962	0.050	-0.002	1.788	0.784	1.275
Comorbidities	2.322	0.469	2.208	4.954	0.000	-3.244	-1.401	0.946	1.057

β—Beta coefficient in regression ANOVA analysis of potential predictors;
CI—confidence interval.

Perception of protective measures and infecting with COVID-19 in relation to the examined factors

The last step in our analysis was conducting a binary logistic regression by creating a model with the most consistent variables, among the previously evaluated ones (Table 4). This model consisted of 10 independent variables: gender, age, education, marital status, household income, self-assessed health, information sufficiency on COVID-19, presence of comorbidities, self-perceived risk of getting infected with corona virus, and adherence to wearing face masks.

The whole model was statistically significant - χ^2 (10, N = 1035) = 55.37, p < 0.001. This model explains between 32.5% (r^2 Cox and Snell) and 44.1% (r^2 Nagelkerke) of variance. The assumptions of collinearity and singularity were satisfied.

Infection with corona virus was taken as a dependent variable. Age (OR = 1.45), level of education (OR = 0.84), self-assessment of health (OR = 0.74), existence of comorbidities (OR = 3.02) and perceived risk (OR = 2.54) were shown to have a significant impact on COVID-19 infection. We concluded that, in addition to age, level of education, the existence of chronic diseases and self-assessed health status, the existence of the so-called "fear factor" (self-perceived risk of getting infected with corona virus) has a significant impact on infection (p < 0.001; OR = 2.54) (Table 4).

In this case, our analysis showed that wearing mandatory protective masks had no effect on COVID-19 infection rates (p = 0.103).

Table 4. Binary logistic regression on perception of protective measures and infecting with COVID-19 in relation to the examined factors

Independent Variables	B	df	*P*	OR	95% CI for OR	
					Lower	Upper
Infection with corona virus	Hosmer-Lemeshow test of goodness-of-fit (p = 0.662, for χ^2 = 5.865, df = 8)					
Gender (1)	-0.144	1	0.377	0.866	0.630	1.191
Age	0.253	1	0.002	1.449	1.288	1.434
Education	-0.177	1	0.023	0.838	0.719	0.976
Marital status (1)	0.089	1	0.641	1.093	0.753	1.586
Household income	0.135	1	0.465	1.145	0.796	1.646
Self-assessed health	-0.446	1	0.003	0.740	0.475	0.864
Information sufficiency (1)	0.119	1	0.501	1.126	0.796	1.593
Comorbidities (1)	1.104	1	0.000	3.016	2.742	5.223
Self-perceived risk to the infection exposure	1.265	1	0.000	2.544	2.988	4.203
Wearing face masks (1)	-0.446	1	0.103	0.640	0.374	1.094
Constant	-3.461	1	0.000	0.031	Correctly classified 88.8%	

B - coefficient for the "intercept" in the null model;
OR - odds ratio;
CI - Confidence interval.

Discussion

Maintaining physical distancing, hand hygiene, and avoiding crowds have been shown to be a protective factor in preventing the spread of a pandemic. However, in our group of respondents, the practice of wearing protective masks in public was not confirmed as a protective factor.

According to numerous findings of other authors, mask mandates reduced the COVID-19 infection growth rate. More specifically, they state that over the longer term, mask mandates had a large effect on "flattening the curve" (4-12). In addition, some recent experiments have shown that face masks may provide some protection from the transmission of infective SARS-CoV-2 droplets, but these masks cannot completely block the transmission of virus droplets (5-7). Further, some authors claim that mandating face mask use in public is associated with an immediate decline after being imposed in the daily COVID-19 growth rate (10-11).

Despite the growing body of literature, much remains unknown about the usefulness of mask wearing in the context of the COVID-19 pandemic (14-17). The World Health Organization states that the use of a mask alone, even when it is used correctly, is not sufficient to provide an adequate level of protection against COVID-19 and that masks should be used as part of a comprehensive strategy of measures (17). Also, the researches published so far have mostly suggested that "wearing face masks is likely to be better than wearing no mask at all", or "because COVID-19 is such a serious threat, wearing masks in public should be advised" (6-11).

WHO continues to advise that anyone suspected or confirmed of having COVID-19 or awaiting

viral laboratory test results should wear a medical mask when in the presence of others (18). In health care settings, WHO recommends that health workers providing care to suspected or confirmed COVID-19 patients wear the mask in addition to other personal protective equipment (PPE); in community areas with known or suspected infections, WHO advises universal masking for all persons within the health facility (18). Particularly, surgeons necessarily wear masks to protect themselves and to protect patients from nosocomial infections (19). However, they are designed for a single use and the masks are discarded after each operation.

And finally, WHO advises that the general public should wear a non-medical mask in indoor (e.g. shops, shared workplaces, schools, etc.) or outdoor settings where physical distancing of at least 1 metre cannot be maintained, in areas of known or suspected infections-containing community or cluster SARS-CoV-2 transmission (18).

But what happens when the influences of the previously mentioned measures (other than face masks) are removed? And is the prolonged wearing of the same mask throughout the day a rational approach? Relatedly, in our study wearing protective masks in the general population in order to prevent the spread of COVID-19 did not prove to be an effective measure among the examined population in Serbia.

Evidence-based arguments for wearing face mask in the general population have not yet been proven. Namely, no study has actually shown a real benefit from wearing masks in the general population, i.e. did not single out wearing face masks in direct relation to decreased infection rates.

As we can see, our findings differ, which makes this study noteworthy. Despite the fact that the coronavirus pandemic is not sufficiently researched, our results did not confirm that wearing protective masks were a decisive factor in preventing the transmission of the coronavirus. In addition, most of the "fashion" masks made from cotton are mainly designed for air pollution or pollen allergy, but useless against viruses and bacteria.

According to Nanda et al., there is limited available preclinical and clinical evidence for face mask benefit in SARS-CoV-2. Randomized controlled trials evidence (cited in their review article) for other respiratory viral illnesses shows no significant benefit of masks in limiting transmission but is of poor quality and not SARS-CoV-2 specific (20). On the other hand, Leung et al. strongly indicated in their study that medical face masks could prevent transmission of human coronaviruses and influenza viruses from symptomatic individuals, examining exhaled breath and coughs of patients with acute respiratory illness (21).

Finally, it is not out of place to point out a few disadvantages of regular using face masks by people in the general population. For example, there were discomfort and irritation outcome (22), devastating effect for people with hearing loss (23), as well as collateral consequences for emotional inferences and social judgments (24). Kisielinski et al. (25), in their very detailed and remarkable review, even found that extended mask-wearing by the general population could lead to the general psychological and physical deterioration, which they described as a Mask-Induced Exhaustion Syndrome (MIES) (25). According to the WHO Director-General's remark, which was expressed on the 5th of June 2020, "People can potentially infect themselves if they use contaminated hands to adjust a mask, or to repeatedly take it off and put it on, without cleaning hands in between" (26). Masks reportedly promote the so-called "false sense of security", resulting into neglection of measures against the infection risk. Relatedly, in the letter to the MBJ Journal, published by Lazzarino et al. in May 2020 (27), after listing the known and potential effects of wearing face masks in public, concluded that "It is necessary to quantify the complex interactions that may well be operating between positive and negative effects of wearing surgical masks at population level. It is not time to act without evidence" (27).

Another very interesting thing stood out in our analysis - people who were more afraid of contracting the corona virus (greater self-perceived risk to the infection exposure) were more likely to get it. Is it a psychosomatic effect? This seemingly somewhat strange finding can be related to the so-called infodemia (28). Actually, the Internet and new information and communication technologies have enabled tremendous progress in the organization and delivery of health services, greater access to health information, as well as the involvement of health professionals, patients and the general public in health decision-making. Unfortunately, the same technologies can also be used to spread misinformation, rumors and conspiracy theories. During the COVID-19 pandemic, the amount of information - accurate and inaccurate, coming from various sources - reliable and unreliable, caused an "infodemia" - the rapid spread of large amounts of information that make it difficult for people to make the right decisions about their own health (28).

There were a few limitations to this study which need to be mentioned. We did not consider the types of protective masks in our study. The number of respondents (only 1,035) and research limited to one geographical area (south Serbia) may also be a limiting factor. One of the topics of the study was the use of masks for the prevention of COVID-19 transmission, yet it was not possible to isolate mask use in order to rule out any covariant effect. The period in which the research was conducted was restricted, the end of 2020. Finally, our findings are based on the self-report method. We suggest these aspects are taken into account while viewing our findings and designing future studies.

Conclusion

Despite the growing body of research on measures for protection against the COVID-19 disease, their effectiveness and adoption by the community are not fully understood. Furthemore, sociodemographic aspects underlying the infection risk, remain largely unknown. This study contributed to filling this research gap by investigating the commonly used protection measures in Serbia. The

conducted study suggests that wearing of face masks among the general population has no effect on contracting COVID-19 infection. In contrast, the existence of the so-called "fear factor" (self-perceived risk to the infection exposure) has a significant impact on the infection. The intention of this study is to open the question of the justification of wearing protective masks among the general population, both with public health authorities and with the governments. The obtained findings suggest that the implementation of face mask as a protective measure could be more complex than previously though. Overall, this report yields novel insights into protective measures, which are commonly applied with the purpose of mitigating, containing and ultimately controlling the COVID-19 pandemic. More research in this direction in other countries and a subsequent meta-analysis is expected to provide more accurate conclusions. As our understanding on this complex issue grows, the current practices should be reconsidered and improved in the light of new findings. Further efforts are required in order to develop more elaborate, well-balanced, efficient strategies for combatting the ongoing pandemic.

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PERCEPCIJA MERA PREDOSTROŽNOSTI OD OBOLJEVANJA IZAZVANIH VIRUSOM COVID-19 – STUDIJA POPREČNOG PRESEKA

Aleksandar Višnjić[1,2], Kıvanç Kök[3], Roberta Marković[1,2], Aleksandra Jović Vraneš[4], Zoran Milošević[1,2], Dragan Nikolić[2], Tamara Jovanović[1,2]

[1]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija
[2]Institut za javno zdravlje Niš, Niš, Srbija
[3]Univerzitet Medipol u Istanbulu, Internacionalna škola medicine, Istanbul, Turska
[4]Univerzitet u Beogradu, Medicinski fakultet, Beograd, Srbija

Kontakt: Aleksandar Višnjić
Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: aleksandar.visnjic@medfak.ni.ac.rs

Glavni cilj ove studije bio je da se istraže praktične prednosti predostrožnosti u ponašanju među opštom populacijom u vezi sa zaražavanjem korona virusom COVID-19 i oboljevanjemkoje ovaj virus izaziva. Sociodemografske karakteristike ispitanika u vezi sa prenošenjem COVID-19 virusa takođe su bile praćene.

Za potrebe ovog istraživanja odabrane su dve grupe ispitanika, koji su dobrovoljno pristali da učestvuju. Istraživanje je sprovedeno u periodu od oktobra do decembra 2020. godine na Medicinskom fakultetu Univerziteta u Nišu (Srbija).

Telefonskim pozivima anketirano je ukupno 1.035 ljudi. Ovi pozivi bili su obavezujući za sve testirane individue, a sprovedeni su od strane lekara sa Instituta za javno zdravlje Niš. U ovom istraživanju bile se 522 žene (50,4%) i bilo je 513 muškaraca; korona virusom bilo je zaraženo njih 630 (60,9%). Dobijeni rezultati ukazuju na to da starost, stepen obrazovanja, samoprocenjeno zdravlje i postojanje hroničnih bolesti imaju značajan uticaj na percepciju rizika od zaraze virusom COVID-19. Štaviše, starost, stepen obrazovanja, postojanje hroničnih bolesti i samoprocenjeno zdravstveno stanje, naročito prisustvo takozvanog „faktora straha" imaju značajan uticaj na stopu zaražavanja. Nasuprot tome, nije primećen uticaj razlike među polovima i nošenja obaveznih zaštitnih maski na stope zaražavanja od virusa COVID-19.

Ova studija daje nove uvide u uobičajene mere zaštite od virusa COVID-19, naglašavajući značajne razlike između proučavanih zaštitnih faktora. Neophodni su dalji napori u ovom pravcu, kako bi se razvile razrađenije, dobro izbalansirane, efikasnije strategije za obuzdavanje tekuće pandemije, posebno u kontekstu kontrole zaraze.

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CORRELATION OF CLINICAL AND DEMOGRAPHIC FACTORS WITH THE OCCURRENCE OF MYOCARDIAL INFARCTION AND CARDIAC ARREST IN OLDER PATIENTS AFTER MAJOR ELECTIVE VASCULAR SURGERY

Velimir Perić[1], Mladjan Golubović[1,2], Milan Lazarević[1,2], Tomislav Kostić[2,3], Dragana Stokanović[2], Miodrag Djordjević[2,4], Vesna Marjanović[2,5], Dragan J. Milić[1,2], Biljana Stošić[2,5], Marija Marinković[6], Nemanja Nikolić[7]

The risk stratification as a part of preoperative preparation of patients involves a series of diagnostic and therapeutic procedures with the main objective of reducing peri/post-operative morbidity and mortality. The aim of the study was to identify a wide spectrum of preoperative clinical and demographic characteristics which were significantly associated with the occurrence of myocardial infarction and cardiac arrest (MICA) during six-month period after vascular surgical procedure, during 2017, 2018, 2019, the study included 144 patients (96 men-66.6 % and 48 women-33.3 %) over 65 years of age (average 70 years). MICA in the first six months after the intervention was associated with higher NYHA class ($p < 0.001$), previous coronary artery disease ($p < 0.001$), cardiomyopathy ($p < 0.05$) or previous myocardial infarction ($p < 0.05$), usage of calcium channel antagonists ($p < 0.05$) and antiplatelet drugs ($p < 0.001$), higher ASA score ($p < 0.01$), higher urea concentration ($p < 0.01$), lower ejection fraction ($p < 0.001$) and longer intensive care unit stay ($p < 0.001$). Using binary logistic regression method, multivariate analysis has identified previous coronary artery disease as a predictor of MICA occurrence ($p < 0.01$). In the multivariate Cox-regression model ($\chi^2 = 71.515$, $p < 0.001$), there were six independent predictors of survival without MICA. Previous coronary artery disease is most significant preoperative risk factor for MICA occurrence. Variables related to heart failure and high urea concentration are independent predictors for MICA.

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Key words: *myocardial infarction, cardiac arrest, vascular surgery*

[1]University Clinical Center Niš, Clinic of Cardiovascular Surgery, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Niš, Serbia
[3]University Clinical Center Niš, Clinic of Cardiology, Niš, Serbia
[4]University Clinical Center Niš, Clinic of Endocrine Surgery, Niš, Serbia
[5]University Clinical Center Niš, Clinic of Anesthesiology and Intensive Therapy, Niš, Serbia
[6]University Clinical Center Niš, Clinic of Pulmonary Disease, Niš, Serbia
[7]Policlinic "Dr Nikolić", Niš, Serbia

Contact: Velimir Perić
8 Milovana Jovanovića St., 18000 Niš, Serbia
E-mail: velperic@gmail.com

Introduction

The risk stratification as a part of preoperative preparation of patients involves a series of diagnostic and therapeutic procedures with the main objective of reducing peri/post-operative morbidity and mortality (1). Large elective vascular procedures are distinguished as procedures of the highest cardiac risk, because the frequency of 30-day myocardial infarction (MI) and cardiac arrest (CA) is more than 5% (2). In non-cardiac surgery patients, these two cardiovascular complications are associated with hospital mortality of nearly 60%. The same study Identified, the age over 65 years and vascular surgery procedures as the independent predictors of MICA (3). The importance of risk stratification is due to the aging of the population as well as the increasing frequency of vascular procedures in elderly patients (4). Albeit the most used, easy to implement and the only one given in European (2) and American (5) recommendations, Revised Cardiac Risk Index (RCRI) shows insufficient discriminatory power for cardiovascular complications after vascular surgery (6).

The aim of the study was to identify a wide spectrum of preoperative clinical and demographic charac-teristics which were significantly associated with the occurrence of MICA during six-month period after surgical procedure.

Materials and methods

During 2017, 2018, 2019, the study included 144 patients (96 men – 66.6 % and 48 women – 33.3 %) over 65 years of age (average 70 years). Patients were followed for six months after major elective vascular surgery (abdominal aortic aneurysm repair, carotid endarterectomy and lower limb revascularization). All participants underwent balanced endotracheal anesthesia. Preoperative examination of patients by anesthesiologists included a detailed evaluation of medical history, clinical examination, insight into biochemical, hematological and coagulation tests, electrocardiogram, chest X-ray and transthoracic ultrasonographic examination of the heart. All participants were preoperatively classified according to ASA (American Society of Anesthesiologist) (7) and NYHA (New York Heart Association) classification (8). Patients with decompensated heart failure and unstable coronary disease were not included in the study.

Myocardial infarction was defined as indicative electrocardiogram changes and new-onset cardiac troponin I elevation greater than three times as the upper reference limit. Absence of heart contraction as the consequence of ventricular fibrillation, pulseless ventricular tachycardia and pulseless electric activity were a hallmark for cardiac arrest. The research was accredited by Ethics Committee of the Faculty of Medicine, University of Niš and conducted at the Clinic for Cardiovascular Surgery, University Clinical Center Niš, in accordance with the principles of the Declaration of Helsinki. The obligation of the investigators was to collect the signed informed consents of the study participants before inclusion.

Statistical analysis

We used Statistical Package for Social Sciences (SPSS 21.0; Chicago, IL, USA) for data analysis. Qualitative variables were presented as frequencies in contrast to quantitative variables, which were presented as means with SDs or medians with interquartile ranges. Student's t-test and Mann-Whitney U-test were used for quantitative variables and Fisher's Exact Probability Test was performed for qualitative variables. Prediction of myocardial injury and cardiac arrest were determined by univariate and multivariate binary logistic and Cox-regression modeling. A p-value less than 0.05 was considered to be a measure of statistical significance. Data analyzing was performed using Statistical Package for Social Sciences (SPSS 21.0; Chicago, IL, USA) and a p-value less than 0.05 was recognized as measure of statistical significance.

Results

During the first six months after the intervention, MICA was noted in 16 (11.1%) patients. Ten of these patients (62.5%) had MICA in the first month. Eleven patients (68.8%) had only one event, in two patients two events were observed, and three events in three patients. Three patients (2.1%) died during the follow-up. Both myocardial infarction and cardiac arrest were of equal frequency, each in 8 patients (24.2%).

MICA in the first six months after the intervention was associated with higher NYHA class ($p < 0.001$), previous coronary artery disease ($p < 0.001$), cardiomyopathy ($p < 0.05$) or previous myocardial infarction ($p < 0.05$), usage of calcium channel antagonists ($p < 0.05$) and antiplatelet drugs ($p < 0.001$), higher ASA score ($p < 0.01$), higher urea concentration ($p < 0.01$), lower ejection fraction ($p < 0.001$) and longer intensive care unit stay ($p < 0.001$) (Table 1).

Using binary logistic regression method, multivariate analysis has identified previous coronary artery disease as a predictor of MICA occurrence within six months after the intervention ($p < 0.01$) (Table 2).

Similar predictors were identified for six months survival without MICA. In the multivariate Cox-regression model ($\chi^2 = 71.515$, $p < 0.001$), there were six independent predictors of survival without MICA. Previous coronary artery disease increased the risk 163 times ($p < 0.001$), while previous cardiomyopathy or previous myocardial infarction increased the risk 14 times ($p < 0.05$) and 29 times ($p < 0.01$), respectively. Use of nitrates was associated with 89 times greater risk ($p < 0.01$). Higher ASA score by 1 unit bared 500 times higher risk ($p < 0.05$). Increased uremia, with each unit increased the risk 1.7 times ($p < 0.05$) (Table 3).

Table 1. Correlation of clinical and demographic factors with occurrence of MICA six months after procedure

	With MICA	Without MICA	p-value
Age (years)	71.25 ± 5.46	69.48 ± 3.43	1.269 (0.222)*
Gender (male)	3 (18.8%)	47 (36.7%)	1.311 (0.177) †
Dyspnea (NYHA class)	2.75 ± 0.58	2.09 ± 0.63	4.022 (0.000)*
Atrial fibrillation	0 (0.0%)	7 (5.5%)	0.117 (1.000) †
Previous cerebrovascular insult	2 (12.5%)	40 (31.3%)	1.598 (0.152) †
Previous coronary artery disease	10 (62.5%)	21 (16.4%)	15.262 (0.000) †
Previous cardiomyopathy	5 (31.3%)	13 (10.2%)	4.018 (0.031) †
Prior percutaneous coronary intervention	1 (6.3%)	4 (3.1%)	0.000 (0.450) †
Previous myocardial infarction	6 (37.5%)	19 (14.8%)	3.632 (0.036) †
Prior coronary artery bypass graft	1 (6.3%)	1 (0.8%)	0.396 (0.211) †
Previous hypertension	13 (100.0%)	106 (82.8%)	2.054 (0.132) †
Previous diabetes mellitus	6 (37.5%)	47 (36.7%)	0.000 (1.000) †
Insulin-dependent diabetes mellitus	5 (31.3%)	28 (21.9%)	0.276 (0.527) †
Insulin-independent diabetes mellitus	1 (6.3%)	19 (14.8%)	0.307 (0.700) †
Previous hyperlipidemia	5 (31.3%)	28 (21.9%)	0.276 (0.527) †
Smoking	8 (50.0%)	49 (38.3%)	0.400 (0.421) †
Family history	10 (62.5%)	46 (35.9%)	3.179 (0.056) †
Beta-blocker	13 (81.3%)	91 (71.1%)	0.313 (0.557) †
ACE inhibitor	15 (93.8%)	91 (71.1%)	2.682 (0.070) †
Calcium channel antagonist	9 (56.3%)	30 (23.4%)	6.181 (0.013) †
Antiplatelet therapy	13 (93.8%)	67 (52.3%)	8.328 (0.001) †
Statins	10 (62.5%)	58 (45.3%)	1.067 (0.288) †
Diuretics	2 (12.5%)	22 (17.2%)	0.014 (1.000) †
Nitrates	3 (18.8%)	7 (5.5%)	2.099 (0.083) †
AAAR	6 (37.5%)	25 (19.5%)	4.685 (0.196) †
CE	7 (43.8%)	73 (57.0%)	
AFBP	1 (6.3%)	2 (1.6%)	
FPBP	2 (12.5%)	28 (21.9%)	
ASA score	3.0 (3.0-3.0)	2.0 (2.0-2.0)	2.997 (0.003) ‡
Hemoglobin	12.6 (12.0-14.1)	13.6 (12.4-14.4)	1.139 (0.255) ‡
Creatinine	100.5 (78.9-134.0)	88.2 (79.2-108.0)	1.446 (0.148) ‡
WBC count	7.4 (6.0-9.6)	7.2 (6.0-8.1)	0.579 (0.563) ‡
Platelet count	233.5 (179.2-242.0)	225.5 (183.8-273.8)	0.903 (0.367) ‡
Urea	7.6 (5.5-9.8)	5.6 (5.1-6.8)	2.616 (0.009) ‡
LDL	2.64 ± 0.77	2.82 ± 0.98	0.731 (0.466)*
HDL	1.2 (0.9-1.3)	1.2 (1.0-1.3)	0.118 (0.906) ‡
EF (%)	47.25 ± 4.52	55.35 ± 7.25	6.232 (0.000)*
BMI (kg/m2)	26.10 ± 2.02	25.63 ± 2.63	0.688 (0.492)*
ICU (days)	3.5 (3.0-4.0)	1.0 (1.0-2.0)	4.962 (0.000) ‡

*- t-test,
†- Chi-squared test,
‡-Z-test.

MICA - myocardial infarction and cardiac arrest;
NYHA - New York Heart Association;
ACE - angiotensin converting enzyme;
AAAR, repair of abdominal aortic aneurysm;
CE - carotid endarterectomy;
AFBP - aortobifemoral bypass;
FPBP - femoropopliteal bypass;
ASA - American Society of Anesthesiologist;
WBC - white blood cells;
LDL - low-density lipoprotein;
HDL - high-density lipoprotein;
EF - ejection fraction;
BMI - body mass index;
ICU - intensive care unit.

Table 2. Binary logistic regression model of MICA occurrence in the first 6 months after the intervention

	Univariate		Multivariate	
	OR (95% CI for OR)	p-value	OR (95% CI for OR)	p-value
Previous coronary artery disease	8.492 (2.785-25.897)	0.000	1905.829 (6.175-588189.405)	0.010
Previous cardiomyopathy	4.021 (1.208-13.386)	0.023	0.377 (0.026-5.549)	0.477
Previous myocardial infarction	3.442 (1.119-10.584)	0.031	0.017 (0.000-1.246)	0.063
Positive family history	2.971 (1.014-8.701)	0.047	0.469 (0.072-8.236)	0.828
Calcium channel antagonists	4.200 (1.442-12.233)	0.009	0.571 (0.058-5.581)	0.630
Antiplatelet drugs	13.657 (1.752-106.482)	0.013	11.819 (0.216-647.780)	0.227
Dyspnea (NYHA class)	7.683 (2.439-24.197)	0.000	3.020 (0.193-47.322)	0.431
ASA score	7.689 (1.679-35.212)	0.009	0.002 (0.000-1.024)	0.051
Urea	1.344 (1.096-1.648)	0.005	1.162 (0.820-1.648)	0.399
EF(%)	0.810 (0.727-0.904)	0.000	0.854 (0.723-1.008)	0.0622

MICA - myocardial infarction and cardiac arrest;
NYHA - New York Heart Association;
ASA - American Society of Anesthesiologist;
EF - ejection fraction.

Table 3. Cox regression model of survival without MICA in the first 6 months after the intervention

	Univariate		Multivariate	
	HR (95% CI for HR)	p-value	HR (95% CI for HR)	p-value
Previous coronary artery disease	6.801 (2.470-18.727)	0.000	163.054 (7.224-3680.348)	0.001
Previous cardiomyopathy	3.363 (1.168-9.685)	0.025	0.071 (0.008-0.618)	0.017
Previous myocardial infarction	2.944 (1.070-8.102)	0.037	0.034 (0.003-0.443)	0.010
Calcium channel antagonists	3.706 (1.379-9.958)	0.009	0.606 (0.121-3.025)	0.541
Antiplatelet drugs	12.152 (1.605-92.016)	0.016	5.230 (0.149-183.324)	0.362
Nitrates	3.576 (1.017-12.573)	0.047	89.035 (3.613-2194.056)	0.006
Dyspnea (NYHA class)	6.649 (2.257-19.586)	0.001	4.785 (0.439-52.131)	0.199
ASA score	6.910 (1.570-30.409)	0.011	0.002 (0.000-0.374)	0.020
Creatinine	1.013 (1.001-1.026)	0.039	0.990 (0.957-1.024)	0.558
Urea	1.294 (1.102-1.520)	0.002	1.674 (1.100-2.547)	0.016
EF(%)	0.843 (0.776-0.916)	0.000	0.907 (0.762-1.081)	0.276

MICA - myocardial infarction and cardiac arrest;
NYHA - New York Heart Association;
ASA - American Society of Anesthesiologist;
EF - ejection fraction.

Discussion

The main predictive factor in our study results is preoperative coronary artery disease. One in five men over the age of 75 suffers from it (9). When it comes to patients who are preparing for major vascular surgery, approximately 60% have advanced or severe coronary artery disease (10). Here we find an explanation for such a high association with the occurrence of MICA in a subgroup of elderly and vascular patients. We believe that CAD is responsible for the connection of many other factors, such as the preoperative use of nitrates and antiplatelet drugs, but also that it is in the etiopathogenesis of various forms of heart failure.

The ASA score has once again been confirmed as a reliable and simple system. Although the ASA scoring system is intended to assess general anesthesia risk, we believe that high ASA scores in vascular surgical patients are associated with the presence and/or sequelae of cardiovascular risk factors. In multicentric study, which included over 200 000 patients from the American College of Surgeons National Surgical Quality Improvement Program database, ASA score was also identified as independent predictor of MICA after non-cardiac surgery (6).

In our study, variables related with heart failure (low ejection fraction, echocardiographic verification of cardiomyopathy and high NYHA class) were associated with the occurrence of MICA. The group of patients with echocardiographically verified cardiomyopathy included asymptomatic patients with preserved EF but echosonography signs indicated diastolic insufficiency. Reasons for the high risk posed by diastolic dysfunction in vascular sur-

gery could be sudden changes in systemic vascular resistance, circulatory overload, impaired tissue perfusion, and ischemic-reperfusion damage (11). Therefore, we emphasize the need for routine and detailed preoperative echocardiographic examination even in asymptomatic patients. Increased urea is an independent predictor of MICA six months after vascular surgery. High urea concentration should not only be interpreted in the context of renal impairment but also increased sympathetic activity and activation of the renin-angiotensin-aldosterone system (12). Increased urea concentration may be a long-term marker of cardiovascular and all-cause mortality in elderly patients with decompensated heart failure (12, 13).

Conclusion

Previous coronary artery disease is most significant preoperative risk factor for MICA occurrence. Variables related to heart failure and high urea concentration are independent predictors for MICA six months after vascular procedure.

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KORELACIJA KLINIČKIH I DEMOGRAFSKIH FAKTORA SA POJAVOM MIOKARDNOG INFARKTA I SRČANOG ZASTOJA KOD STARIJIH BOLESNIKA NAKON VELIKE ELEKTIVNE VASKULARNE HIRURGIJE

Velimir Perić[1], Mlađan Golubović[1,2], Milan Lazarević[1,2], Tomislav Kostić[2,3], Dragana Stokanović[2], Miodrag Đorđević[2,4], Vesna Marjanović[2,5], Dragan J. Milić[1,2], Biljana Stošić[2,5], Marija Marinković[6], Nemanja Nikolić[7]

[1]Univerzitetski klinički centar Niš, Klinika za kardiovaskularnu hirurgiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija
[3]Univerzitetski klinički centar Niš, Klinika za kardiologiju, Niš, Srbija
[4]Univerzitetski klinički centar Niš, Klinika za endokrinu hirurgiju, Niš, Srbija
[5]Univerzitetski klinički centar Niš, Klinika za anesteziju, reanimatologiju i intenzivnu terapiju, Niš, Srbija
[6]Univerzitetski klinički centar Niš, Klinika za pulmologiju, Niš, Srbija
[7]Poliklinika "Dr Nikolić", Niš, Srbija

Kontakt: Velimir Perić
Milovana Jovanovića 8, 18000 Niš, Srbija
E-mail: velperic@gmail.com

Stratifikacija rizika je deo preoperativne pripreme bolesnika, koja uključuje niz dijagnostičkih i terapijskih postupaka sa glavnim ciljem smanjenja perioperativnih, odnosno postoperativnih morbiditeta i mortaliteta. Cilj studije je identifikacija širokog spektra preoperativnih kliničkih i demografskih parametara, koji su značajno povezani sa pojavom infarkta miokarda i srčanog zastoja (MICA), tokom šest meseci nakon vaskularne hirurgije. Tokom 2017., 2018. i 2019. godine studija je obuhvatila 144 bolesnika (96 muškaraca – 66,6% i 48 žena – 33,3%) starosti preko 65 godina (prosečno 70). MICA je u prvih šest meseci povezana sa višom NYHA klasom ($p < 0,001$), prethodnom bolešću koronarnih arterija ($p < 0,001$), kardiomiopatijom ($p < 0,05$), prethodnim infarktom miokarda ($p < 0,05$), upotrebom antagonista kalcijumovih kanala ($p < 0,05$), upotrebom antitrombocitnih lekova ($p < 0,001$), višom ASA klasom ($p < 0,01$), višom koncentracijom uree ($p < 0,01$), nižom frakcijom srčanog izbačaja ($p < 0,001$) i dužim boravkom u jedinici intenzivnog lečenja ($p < 0,001$). Multivarijantnom analizom binarne logističke regresije identifikovana je prethodna bolest koronarnih arterija, kao prediktor pojave MICA ($p < 0,01$). Multivarijantna analiza Koks regresije utvrdila je šest nezavisnih prediktora preživljavanja bez pojave MICA ($\chi^2 = 71,515$, $p < 0,001$). Prethodna bolest koronarnih arterija najznačajniji je faktor preoperativnog rizika za pojavu MICA. Varijable povezane sa srčanom insuficijencijom i visoka koncentracija uree nezavisni su prediktori pojave MICA.

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Ključne reči: *miokardni infarkt, srčani zastoj, vaskularna hirurgija*

Original article

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STUDY ON THE TWO-DIRECTIONAL RELATIONSHIP BETWEEN MYASTHENIA GRAVIS AND PREGNANCY

Gordana Djordjević[1,2], Aleksandar Stojanov[1]

Due to the high prevalence of myasthenia gravis (MG) in women of reproductive age, pregnancy in patients with MG is not uncommon. This requires special clinical and therapeutic caution. There is a two-way relationship between MG and pregnancy: Pregnancy can affect the course of the disease, but MG can affect childbirth and the occurrence of postnatal complications. The purpose of our study was to evaluate the clinical course, delivery, and neonatal outcome of pregnant women with the diagnosis of myasthenia gravis. The clinical course of the disease during pregnancy, labor, and postpartum period was reviewed, as well as the neonatal period in the 23 infants born to 15 MG mothers. Spontaneous abortion was observed in two pregnant women (8%) in the second month of pregnancy. One newborn was diagnosed with transitory neonatal MG. There were no stillbirths or physical anomalies. Clinical worsening was recorded during 10 pregnancies (40%), in 8 pregnant women. The clinical course of MG in pregnancy is variable and unpredictable, but pregnancy does not affect the long-term course of MG. Spontaneous abortion is a potential complication in pregnant women with MG. Cesarean section is a more frequent intervention in pregnant women with MG compared to the general population of women. Thymectomy in mothers with MG before pregnancy could potentially have a positive benefit for the newborn. Neonatal transient myasthenia was uncommon in our patient population. No congenital abnormalities were discovered in the 23 babies delivered at our institution.

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Key words: *myasthenia gravis, pregnancy, clinical course*

[1]University Clinical Center Niš, Neurology Clinic, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Niš, Serbia

Contact: Gordana Djordjević
48 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: gordanadjor@gmail.com

Introduction

Myasthenia gravis (MG) is an antigen-specific autoimmune disease in which antibodies against the nicotinic acetylcholine receptors (nAChR antibodies) or other postsynaptic antigens cause a postsynaptic block of neuromuscular transmission (1). It is characterized by fluctuating weakness and fatigue of the striated musculature while preserving smooth musculature. The disease is rare, with an estimated pooled incidence rate of 5.3 cases per million persons per year, and can occur at any age, in both sexes (2). However, it is twice as common in females, with a peak incidence between the third and fourth decades of life, which coincides with the reproductive period of a woman, which requires special clinical and therapeutic caution.

A review of the existing literature indicates the complexity of the relationship between MG and pregnancy, in terms of the existence of a two-way relationship: pregnancy can affect the course of the disease, but MG can affect childbirth and the occurrence of postnatal complications (3). However, a review of the existing literature has limited extrapolation in current clinical practice (4). Previous studies have been mostly retrospective or individual case reports. The studies differed significantly in the data provided on clinical status before and during pregnancy, disease course, therapy, obstetric interventions, and pregnancy outcome. Many were difficult to evaluate due to sparse details. There was broad agreement on certain points with marked differences in others. In general, what can be concluded is that the course of MG during pregnancy as well as the course and outcome of pregnancy are very variable and difficult to predict.

This study intended to contribute to the further elucidation of the relationship between MG and pregnancy, a thorough evaluation of the clinical course, delivery, and neonatal outcome of pregnant women with the diagnosis of myasthenia gravis,

followed in University Clinical Center Niš which attracts a large number of patients from south-eastern Serbia.

Materials and methods

The study included 15 pregnant women with MG in whom 25 pregnancies were recorded, followed at the University Clinical Center Niš (UCC Niš) in the period 2003-2020. A prospective study was conducted on 7 women (10 pregnancies). In 8 women (15 pregnancies), the required data were obtained by retrospective analysis of medical records. Out of a total of 15 pregnant women, 13 women suffered from acquired MG, and two from juvenile MG. The clinical course of the disease during pregnancy, labor, and postpartum period was reviewed, as well as the neonatal period in the 23 infants born to MG mothers. The condition for this study was that the diagnosis of MG was made before pregnancy. The stage of the disease was determined according to Osserman's classification.

Descriptive variables included: Total number of pregnancies, the average age at the time of pregnancy, the average duration of the disease until pregnancy, outcome (delivery on time, spontaneous abortion, vaginal delivery), obstetric interventions (Caesarean section, Vacuum extractor), the course of MG during pregnancy and puerperium (exacerbation of the disease), neonatal complications (transient neonatal myasthenia gravis (TNMG)).

Clinical variables included clinical characteristics of pregnant women with MG before conception: age at the time of diagnoses (Dg), Osserman classification, anti nAChR antibodies, thymectomy, pharmacological therapy, pharmacological remission, complete remission.

The criteria for improvement, unchanged or worsening of MG during pregnancy were the following:

1) Improvement: patients who had clinical improvement of the symptoms and decrease of the dosage of the medications,

2) No change: patients with no clinical change in their symptoms and same doses of medications compared with before pregnancy.

3) Deterioration: patients who had a deterioration of the disease (worsening of the Osserman's stage) and an increase in the dosages of medications compared with before the pregnancy, or the need for immunosuppressant drugs such as azathioprine and/or prednisone.

Transient neonatal myasthenia gravis was diagnosed based on clinical signs of generalized hypotonia, sucking disturbances, a weak cry, and respiratory difficulties.

Results

The study included 15 patients with MG, mean age 21.13 ± 7.25, in whom 25 pregnancies were observed. The presence of anti nAChR antibodies was confirmed in 13 patients. No antibodies were tested in two patients. Nine patients underwent thymectomy in the same year as Dg, except for one patient where thymectomy was performed after pregnancy. In 9 pregnant women, pharmacological remission was achieved before conception, three pregnant women were in complete remission (without therapy), while three pregnant women had symptoms of the disease before pregnancy. Three patients had three pregnancies each, two of which were twin sisters who got sick from MG at the age of six. Four patients had 2 pregnancies each, while the remaining eight patients had 1 pregnancy each.

The mean age of women at the time of pregnancy was 28 ± 5.5 years (Table 2). The average duration of the disease until pregnancy was 7.7 ± 7.68 years, or 3.58 ± 1.35 years if we exclude two twins with juvenile MG.

Spontaneous abortion was observed in two pregnant women (8%) in the second month of pregnancy (Table 2). The first patient was in complete remission and had three pregnancies. Spontaneous abortion occurred in the second pregnancy with transient exacerbation of the disease. Her previous and next pregnancy passed without any complications, with a vaginal delivery on time. In the second case, it was a patient with juvenile MG who also had three pregnancies, and who was in complete remission before conception. During the first two pregnancies, there was an exacerbation of the disease, but the course of pregnancy and childbirth passed without complications, while the second pregnancy ended in a miscarriage in the second lunar month.

In the remaining cases (92%), childbirth was completed on time (Table 2). Twelve women (52%) gave birth vaginally. In two cases, a vacuum extractor was used (8.7% of the total number of births, and 16.6% of all vaginal births).

One newborn was diagnosed with TNM. There were no stillbirths or physical anomalies. Clinical worsening was recorded during 10 pregnancies (40%), in 8 pregnant women (Table 1, Figure 1, Figure 2).

The largest number of relapses was recorded in the first trimester of pregnancy, but it was also recorded in the last 2 weeks of pregnancy (4%), as well as postpartum (12%). It is important to emphasize that of the total number of pregnancies with MG, one clinical deterioration occurred after a miscarriage in the second month of pregnancy. In most cases (52%), the situation was unchanged. Improvement was recorded in 2 pregnant women in the third trimester (8%).

Table 1. Clinical characteristics of pregnant women with MG before conception

Pregnant women	Age at the time of Dg (years)	Osserman	Anti nAChR antibodies	Thymectomy	Therapy	Remission pharmacol.	Remission complete	Number of pregnancies
1*	18	IIa	/	+		-	+	3
2	17	IIb	/	+		-	+	1
3	24	IIa	+	+		-	+	1
4*	25	IIa	+	+	+	+	-	1
5*	33	IIb	+	+	+	+	-	1
6	25	IIa	+	-	+	-	-	2
7*	6	IIa	+	-	+	+	-	3
8*	6	IIa	+	-	+	+	-	3
9	23	I	+	-	+	+	-	1
10	23	IIa	+	-	+	+	-	1
11*	24	IIa	+	+	+	+	-	2
12	25	IIb	+	+	+	+	-	2
13	24	IIb	+	+	+	-	-	1
14*	26	IIb	+	- (Post partum+)	+	-	-	1
15*	18	IIb	+	+	+	+	-	2

*exacerbation of the disease

Table 2. Characteristics of pregnancy and childbirth

Characteristics	No/years	(%)
Total number of pregnancies	25	
The average age of women at the time of diagnoses	21.13 ± 7.25	
The average age at the time of pregnancy	28 ± 5.5	
The average duration of the disease until pregnancy	3.58 ± 1.35 7.7 ± 7.68*	
Delivery on time	23	92%
Spontaneous abortion	2	8%
Vaginal delivery	12	52%
Caesarean section	11	48%
Vacuum extractor	2	8.7%
Exacerbation of the disease	10	40%
TNMG	1	4.35

* pregnant women with juvenile myasthenia gravis included;
TNMG-transitory neonatal myasthenia gravis

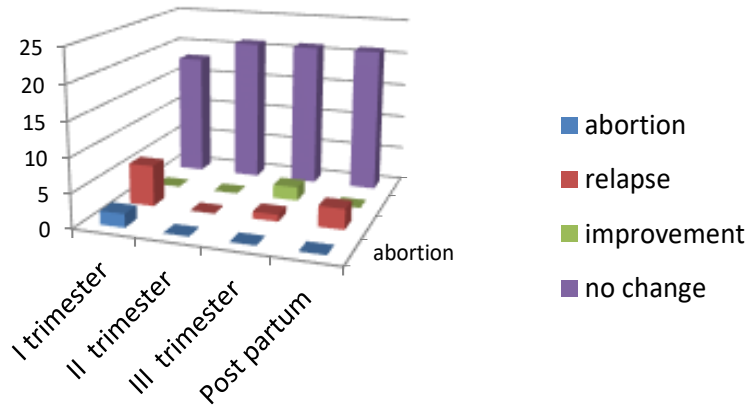

Figure 1. The course of the myasthenia gravis during pregnancy and postpartum

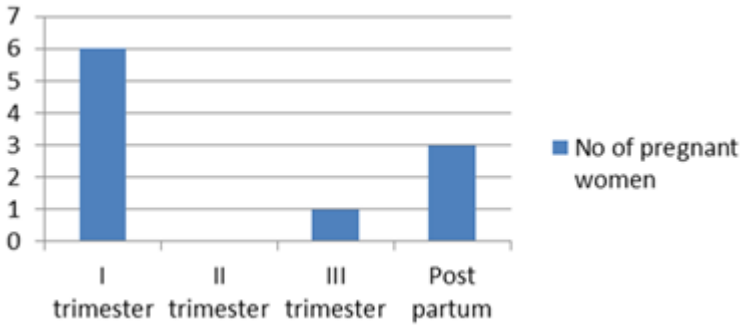

Figure 2. Deterioration of myasthenia gravis symptoms during pregnancy and post partum

Discussion

Due to the high prevalence of MG in women of reproductive age, pregnancy in patients with MG is not uncommon, which requires special clinical and therapeutic caution. In general, the analysis of the outcome of chronic and rare diseases with unpredictable and fluctuating courses is difficult. The clinical course of MG in pregnancy is variable and unpredictable (5-7). Exacerbations, myasthenic crises, but also remissions of the disease are possible. The data in our study show that 10 pregnancies out of a total of 25, were accompanied by a worsening of the clinical course of MG, which means that in our group of patients the percentage of relapses was high (40%). These data coincide with the results of Plush from 1991, where the relapse rate was 41% of a total of 322 pregnancies followed by 225 women with MG (8). However, in other studies, a lower percentage of relapses was noted. For example, in a study conducted by Batocchi et al., clinical worsening of MG was registered during 10 pregnancies (19%), out of a total of 54 (9). Djelmis et al. recorded an even lower relapse rate - 14.5% of a total of 69 pregnancies followed in 65 patients with MG (10). In the remaining pregnant women in our study, the largest number (52%) had an unchanged clinical picture of MG, while in two pregnant women (8%) there was a clinical improvement in the third trimester of pregnancy.

The results of previous researches show that the first trimester and postpartum period appear to be the most critical periods for MG exacerbation, which is associated with reduced progesterone secretion in that period. By that, in our study, the highest percentage of exacerbations was recorded in the first trimester of pregnancy (24%) (11, 12). Worsening in the puerperium was observed in three pregnant women, in three pregnancies (12%), which is a significantly lower percentage compared to other studies. A study by Batocchi et al. recorded postpartum worsening of disease symptoms in 28% of cases. In his study, Hoff recorded a percentage of disease exacerbations in the postpartum period of 29.8% (11). In a study by Djelmis et al., this percentage is slight - 16% (10). Although the critical

period for relapses is the first trimester, worsening of the clinical course is possible even later. In our study, one pregnant woman (4%) showed a slight emphasis on general weakness and fatigue at the end of the third trimester.

The two patients in our study were twin sisters who developed MG at the age of six. The patients achieved complete remission during life. Both patients had three pregnancies each. In all pregnancies, the disease was exacerbated in the first trimester. In one patient, the third pregnancy ended in a miscarriage in the second month of pregnancy. Three patients with MG had two pregnancies each. In one patient, both pregnancies passed without complications. Another patient had a clinical worsening in the second pregnancy. The third patient relapsed in the first pregnancy, while the second pregnancy passed without complications. These results support previous observations that there is no correlation between the clinical course before pregnancy and during pregnancy, as well as that based on the clinical course in one pregnancy, it is not possible to predict the course of subsequent pregnancies. Also, pregnancy did not adversely affect the long-term clinical course of MG in our patients, which is by the data from the literature.

There is not much data in the literature on the influence of thymectomy on the clinical course of MG during pregnancy and after childbirth, as well as on the occurrence of neonatal MG, and these data are contradictory. Although published case reports suggest that the incidence of clinical deterioration is higher in patients who have not been thymectomized, Roth et al. could not confirm this in their study (13). The clinical course of MG was unpredictable during pregnancy and in the postpartum period in both thymectomized and non-thymectomized patients. Thymectomy did not have a significant effect on the course of MG. However, it was observed that women who underwent thymectomy before pregnancy were in better general condition even in the case of disease exacerbation. Our data is consistent with the data of Roth and associates. In our group of patients, there was no significant difference in the occurrence of relapse in thymectomy and non-thymectomy women before conception. Also, the time interval between thymectomy and pregnancy did not affect the occurrence of relapse.

In addition to exacerbations of the course of the disease, other potential complications such as miscarriages or premature births have been described in pregnant women with MG. Spontaneous abortion may occur with a slightly increased frequency in MG (14, 15). In the Batocchi et al. study, 10 pregnancies (15.6%), resulted in abortion (9). In a French study 19.4% of pregnancies were found, 14.8% in a Turkish cohort, 14.3% (16) in Brazil (17). This indicates a rate of around 15%. This is similar to the miscarriage rate in the general population of 10-20% among women who know they are pregnant. In our study, spontaneous abortion was observed in two pregnancies (8%), in two different pregnant women, in both cases in the second month of pregnancy. Both patients had three pregnancies each. In the first patient, the first and third pregnancies ended on time, without complications, while the second pregnancy resulted in a miscarriage and exacerbation of the disease afterward. In the second pregnant woman, the first two pregnancies ended in the term, while the third resulted in a miscarriage. In all other cases, childbirth occurred on time.

In a large retrospective study, Hoff et al. concluded that interventions during childbirth were more frequent in the group of patients with MG than in the reference group and that the percentage of cesarean section was statistically significantly higher than in the reference group (17.3% vs. 8.6 %) (13). The results of Italian authors indicate a higher rate of cesarean section in pregnant women with MG compared to the reference group, but this difference is not statistically significant (30% vs. 14%) (9). In our study, the percentage of cesarean section was 34%, which is a higher percentage compared to the results of Hoff and co-workers, as well as compared to the average rate of cesarean section in Europe (elective cesarean section as elective intervention - 10.7% and 25.2% total number of cesarean section), although large variations have been observed among European countries (18). The disadvantage of our study is the lack of data on the average rate of cesarean section in Serbia.

Neonatal complications in pregnant women with MG include TNMG, as well as congenital anomalies. In general, TNM is a complication that affects around 10% of children born to mothers who have MG but can reach up to 30% (8, 10). This is due to antibodies against AchR or MuSK that are transported from the mother's circulation, across the placenta, and to the fetus (19). No correlation was observed between the severity of the mother's clinical picture in pregnancy and the occurrence of neonatal MG, and similar results were obtained in a study in Italy. Transient neonatal MG was registered in only one of a total of 23 newborns (4.3%) in our study. No thymectomy was performed on the mother of this newborn. These results coincide with the results of a study by Roth et al. where only 2 (16.7%) of the 12 newborns alive showed myasthenic symptoms. Both newborns belonged to the group of non-thymectomized mothers, which denote a possible positive effect of thymectomy. However, previous research has not yet confirmed with certainty the positive benefit in newborns whose mothers were thymectomized before pregnancy. (16, 17). No congenital anomalies were identified in any of the newborns.

Conclusion

The clinical course of MG in pregnancy is variable and unpredictable, but pregnancy does not affect the long-term course of MG. On the other hand, pregnancy in the large majority of MG women is without complications. Spontaneous abortion may occur with a slightly increased frequency in MG. Patients with MG have an increased rate of Cesarean section, mostly as a precaution to avoid exhaustion, which is often unnecessary. Neonatal complications in pregnant women with MG include TNMG. Thymectomy in mothers with MG before pregnancy is not a guarantee for a stable clinical course of MG during pregnancy but could potentially have a po-

sitive benefit for the newborn. To reduce the risk of complications, good cooperation between neurologists, gynecologists, and neonatologists is necessary during pregnancy and childbirth. MG is not a reason to give up motherhood and MG patients should be supported in their desire to conceive.

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STUDIJA DVOSMERNE POVEZANOSTI MIASTENIJE GRAVIS I TRUDNOĆE

Gordana Đorđević[1,2], Aleksandar Stojanov[1]

[1]Univerzitetski klinički centar Niš, Klinika za neurologiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija

Kontakt: Gordana Đorđević
Bulevar dr Zorana Đinđića 48, 18000 Niš, Srbija
E-mail: gordanadjor@gmail.com

Usled velike prevalencije miastenije gravis (MG) kod žena u reproduktivnom periodu, pojava trudnoće kod ovih bolesnica nije neuobičajena. Ovo zahteva poseban klinički i terapijski oprez. Postoji dvosmerni odnos MG i trudnoće: trudnoća može uticati na klinički tok MG, a takođe sama bolest može uticati na porođaj i pojavu postnatalnih komplikacija. Cilj naše studije bio je da se proceni uticaj MG na klinički tok, porođaj i ishod neonatusa kod trudnica. Klinička slika tokom trudnoće, porođaja i u postpartalnom periodu praćena je kod 23 novorođenčeta, rođenih od strane 15 majki sa dijagnozom MG. Spontani abortus primećen je u dva slučaja (8%), u drugom mesecu trudnoće. Kod jednog novorođenčeta dijagnostikovana je tranzitorna neonatalna MG. Nije bilo mrtvorođenih i nije bilo fizičkih anomalija kod novorođenčadi. Kliničko pogoršanje utvrđeno je tokom 10 trudnoća (40%) kod 8 trudnica. Klinički tok MG tokom trudnoće je varijabilan i nepredvidljiv, ali trudnoća ne utiče na dugotrajni ishod MG. Spontani abortus je potencijalna komplikacija kod žena sa MG. Carski rez je intervencija koja se češće sprovodi kod žena sa MG u odnosu na opštu populaciju. Timektomija kod žena sa MG, pre porođaja, može imati pozitivni efekat na novorođenčad. Neonatalna tranzitona MG bila je retka u našoj populaciji. Nije bilo kongenitalnih anomalija kod 23 novorođenčeta u našoj ustanovi.

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Ključne reči: *miastenija gravis, trudnoća, klinički tok*

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ANXIETY, DEPRESSION AND QUALITY OF LIFE IN PATIENTS WITH GLAUCOMA

Suzana Tošić Golubović[1,3], Hristina Jocić[3], Uroš Gugleta[1], Gordana Nikolić[2,3], Nikola Stojanović[4]

Previous studies have shown significant comorbidity between depressive, anxious disorders, and glaucoma, which is the second most common cause of vision loss in the world. This study aimed to determine the presence of depression and anxiety, assess of the quality of life in patients with glaucoma, as well as to compare with the results of the healthy population. Cross-sectional study, was carried out at the Ophthalmology Clinic, of the University Clinical Center Niš. The presence of anxiety, and depression, were evaluated by Zung's self-rating instruments for anxiety, and depression, and the quality of life was evaluated by the WHO Quality of life instrument-Brief version. Sociodemographic and glaucoma-related parameters were gathered. Around 38.64% of patients with glaucoma presented depression, mostly mild and moderate forms, while mild depression was found in 4.17% of respondents in the control group. The number of patients with anxiety was two times higher in the experimental than in the control group. Mentioned results show a statistically relevant difference ($p < 0.01$). We determined a statistically relevant correlation between the frequency of depression and the severity of glaucoma ($p < 0.001$), while no such correlation was found between anxiety and the severity of glaucoma ($p > 0.05$). While comparing the gathered values for each domain as well as the overall QOL we found a statistically relevant difference ($p < 0.001$). A statistically relevant correlation was found between the patients with milder and more severe glaucoma forms. Comorbid depression and anxiety in patients with glaucoma occur in more severe forms of glaucoma, in patients with a longer duration of the disease for medical and psycho-social reasons.

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Key words: *anxiety, depression, quality of life, glaucoma*

[1]University Clinic Center Niš, Psychiatric Clinic, Niš, Serbia
[2]University Clinic Center Niš, Center of Mental Health Protection, Niš, Serbia
[3]University of Niš, Faculty of Medicine, Niš, Serbia
[4]University of Niš, Faculty of Medicine, Department of Physiology, Niš, Serbia

Contact: Nikola Stojanović
81 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: nikola.st90@yahoo.com

Introduction

Glaucoma is a chronic, progressive neuropathy of the optic nerve, which leads to changes in the optic disc and visual field loss. It represents an incurable disease, but with the correct medical treatment, further progression and vision loss could be avoided. It is the second-leading cause of blindness worldwide. About 79.6 million people have glaucoma globally, 11.2 million of which are blind (1). The number of patients estimated in Serbia is about 100 000 (2). Previous studies have shown significant comorbidity between depressive, anxious disorders, and glaucoma. The prevalence of anxiety in glaucoma patients is approximately between 13 and 33%, whereas the prevalence of depression is between 10 and 57% (3-6). Depression and anxiety disorders may arise in glaucoma patients due to the fear of potential blindness, the heavy economic burden caused by multiple medications and surgeries, and also impairments in daily activities such as driving (7) and reading (8). Fear of the consequences of glaucoma (blindness), dysfunctionality in family, social surroundings, and professional, social, and economic difficulties, living with chronic, progressive, and debilitating disease, such as glaucoma, lifelong need for treatment and comorbidity with anxiety and depression lead to the lower quality of life in these patients (9, 10). All of these contribute to the poor compliance of these patients, which leads to the worsening of glaucoma. The functional and psychosocial aspects of glaucoma are often neglected. The social stigma of being diagnosed with a mental disorder and the lack of awareness among patients and ophthalmologists may result in the undertreatment of glaucoma patients for their psychiatric comorbidities. Local studies have found that

nonadherence to glaucoma medications in Singaporean patients is associated with lower quality of life (QOL) (11). Therefore, it may be essential to identify, prevent, and treat glaucoma patients with anxiety and depression to improve their QOL and compliance with medication (12, 13). Inspired by all mentioned findings we conducted this study, which aim was to determine the presence of depression and anxiety, as well as the assessment of the quality of life in patients with glaucoma, and comparison of the obtained results with the same results recorded among the respondents from the healthy population.

Material and methods

This cross-sectional study was performed in the Ophthalmology Clinic, University Clinical Center Niš (UCC Niš), at the Glaucoma department. The experimental group consisted of 44 subjects who had already been diagnosed with glaucoma and who had been treated in the mentioned department for years. The next criterion for inclusion in the study, in addition to the above diagnosis, was the age, range of 20 to 80 years, as well as the completion of at least primary education. Respondents suffering from other ophthalmic diseases, chronic somatic diseases, as well as the ones with current psychiatric disorders or psychiatric disorders in their life history, were excluded from the research. The diagnostic sheet, created by the authors, was used for the purpose of collecting sociodemographic data (gender, age, education, place of residence, partnership status, number of children, employment, socioeconomic conditions), as well as the data on the ophthalmologic disease itself, including the following: the degree of glaucoma, which referred to the presence of only ocular hypertension, a mild degree of glaucoma, moderate, severe without visual impairment, severe with visual impairment; mean value of intraocular pressure-IOP; pattern standard deviation-PSD, which is associated with a localized field of view width; MD - mean deviation, which represents the mean difference between normal sensitivity and measured threshold value at all location test points; the presence of hereditary glaucoma; therapy: mono and polytherapy. The following self-assessment questionnaires were also used: Zung's Anxiety Scale, and Zung's Depression Scale, both completed by the respondents themselves, as well as the WHOQOL-Short Quality Assessment Questionnaire. All questionnaires have been translated into Serbian language and standardized for application. A score less than 50 on Zung's depression assessment questionnaire indicated a state without depression, a score greater than 50, and less than 60 indicated a state with mild depression, a score greater than 60 and less than 70 moderate depression, while a score greater than 70 severe depression. A score less than 36 on Zung's anxiety assessment questionnaire indicated no anxiety, while a score greater than 36 indicated the presence of anxiety symptoms. The control group (belonging to the general population) consisted of subjects who were healthy, without any ophthalmological diseases, psychiatric disorders or disease, as well as any other somatic chronic diseases. We tried to harmonize the control group according to the sociodemographic data of the experimental group. Informed consent has been obtained from all study subjects. The research related to human use has complied with all the relevant national regulations, and institutional policies and is in accordance with the Helsinki Declaration and has been approved by the equivalent Ethics committee.

All statistical analyzes were performed in the online free calculator Social Science Statistics. The values of continuous variables are shown by the mean value with standard deviation, while for categorical variables the frequency is shown. Parametric (Student's t-test) and non-parametric (χ^2-test) correlation tests were used to assess statistical relationships among variables. Statistical significance was determined at the level of $p < 0.05$.

Results

The study involved 68 subjects (44 with glaucoma and 24 in the control group). Sociodemographic data are shown in Table 1.

Fifty-two point twenty-seven percent of respondents had a disease dura-tion of 0-5 years and slightly less disease evolution over 5 years. The largest number of respondents, as many as 90.91%, had a bilateral form of glaucoma. We graded the severity of ophthalmic disease into 5 groups: ocular hypertension, where patients had elevated IOP but without morphological and functional impairments, mild, moderate, severe, and severe with visual impairment. The examined ophthalmic data, which are shown in Table 2, were: mean value of intraocular pressure-IOP; Pattern standard deviation-PSD, which is associated with localized loss of function, MD - Mean deviation, which represents the mean difference between normal sensitivity and measured threshold value at all locational test points.

The average quality of life (QOL) in the group of patients was 80.14 ± 17.93, while in the control group the average QOL was 95.46 ± 11.13. Respondents from the group of patients had lower results in all areas of quality of life (physical and mental health, social relations, environmental factors). The quality of life scores are shown in Table 3.

The number of depressed in the group of patients with glaucoma was 17 (38.64%), while in the control group there was 1 depressed subject (4.17%) (Table 3). A statistically significant association was found between the number of depressed patients and the control group ($p < 0.01$). In the group of glaucoma patients, among depressed patients, there were 12 with mild depression (27.27% and 70.59% of all depressed) and 5 with moderate depression (11.36% and 29.41% of depressed, respectively). In the control group, the subject had a mild form of depression. There were no subjects with severe depression. Also, respondents from the group of patients with glaucoma had higher scores on the scale for self-assessment of depression than those from the control group ($p < 0.01$).

Table 1. Sociodemographic data in the examined groups

	Experimental group (%)	**Control group (%)**
Gender distribution		
Men	59.09	45.83
Women	40.91	54.17
Age distribution (years)		
20-30	13.64	16.67
31-40	2.27	4.17
41-50	11.36	12.5
51-60	18.8	20.83
> 60	54.55	45.83
Employment Status		
Employed	40.91	45.83
Unemployed	20.54	20.83
Retired	38.64	33.33
Number of children		
0	18.18	20.83
1	38.4	50
2	25	16.67
3	18.18	12.5
Partnership status		
Without a partner	11.36	12.5
With a partner	4.55	4.17
Married	70.45	70.83
Divorced	4.55	8.33
Widower/Widow	9.09	4.17
Place of residence		
City	79.55	83.33
Rural area	20.55	16.67
Socioeconomic living conditions		
Good	22.73	45.83
Average	75	45.83
Bad	2.27	8.33

Table 2. Ophthalmic parameters

Glaucoma duration in years (%)	
0-5	52.27
5-10	27.27
More than 10	20.45
Glaucoma type	
Bilateral	90.91 %
Unilateral	9.09 %
Average IOP	20.39 ± 2.5
Avg MD in the worse eye	-11.5 ± 9.44
Avg MD in the better eye	-9.31 ± 10.38
Avg PSD in the worse eye	6.82 ± 5.49
Avg PSD in the better eye	6.26 ± 5.16
Hereditary burden	
Yes	34.09
No	65.91
Severity of disease (%)	
Ocular hypertension	15.91
Mild glaucoma	22.73
Moderate glaucoma	29.54
Severe glaucoma	25
Severe glaucoma with damaged vision	6.82
Therapy	
Monotherapy	38.4
Polytherapy	61.36

Table 3. Quality of life, levels of depression and anxiety, severity of depression in the study groups

	Experimental group	**Control group**
Overall quality of life	80.14 ± 17.93	95.46 ± 11.13
Quality of life domains		
Physical health	23.41 ± 6.18	29.17 ± 3.31
Mental health	18.2 ± 5.35	22.96 ± 3.82
Social relations	10.53 ± 3.03	11.71 ± 1.92
Environmental factors	27.57 ± 5.11	30.45 ± 1.3
Degree of severity of depression (%)		
Mild	27.27	4.17
Moderate	11.36	0
Severe	0	0
Percentage of depressed	38.64	4.17
Number of anxious	68.18	37.5

In the group of glaucoma patients, there were 30 anxious subjects (68.18%), while 14 subjects showed no signs of anxiety (31.82%). In the control group, there were 9 anxious (37.5%) and 15 non-anxious respondents (62.5%) (Table 3). A statistically significant association ($p < 0.05$) was found between the frequency of anxiety in the experimental and control groups. We found a statistically significant relationship between the incidence of depression and the degree of glaucoma ($p < 0.001$). These results are shown in Table 4.

When comparing the obtained values between the study and control group, for each domain as well as for the total QOL, we obtained a statistically significant difference (QOL: $p < 0.001$; QOL domain 1: $p < 0.001$; QOL domain 2: $p < 0.001$; QOL domain 3: $p < 0.05$; QOL domain 4: $p < 0.05$). We further calculated the quality of life, total, in each domain in patients with varying degrees of glaucoma severity, where we also obtained a statistically significant difference between the quality of life of patients with mild (ocular hypertension, mild and moderate glaucoma) and severe glaucoma and severe with visual damage. The results are shown in Table 4.

We found that there is a statistically significant relationship between the examined ophthalmic parameters and the presence of depression, and the results are shown in Table 5.

Table 4. Scores for quality of life, total and in each individual domain, percentage of depressed in patients with varying degrees of glaucoma

	Ocular hypertension	Mild glaucoma	Moderate glaucoma	Severe glaucoma without visual damage	Severe glaucoma with visual damage
Overall quality of life	84.86 ± 17.54	90.2 ± 13.39	88.85 ± 5.97	64.18 ± 16.44	56.33 ± 6.13
Quality of life domains					
Physical health	25 ± 6.59	26.9 ± 4.08	25.46 ± 2.79	19.09 ± 6.67	15 ± 1.41
Mental health	19.85 ± 5.44	22.3 ± 4.03	21.15 ± 1.56	14.36 ± 4.68	11 ± 2.45
Social relations	11 ± 2.98	11.2 ± 2.4	12.77 ± 2.23	8 ± 1.65	6.67 ± 0.47
Environmental factors	29 ± 3.7	29.8 ± 4.19	30 ± 2.52	22.73 ± 5.54	23.67 ± 2.05
Percentage of depressed	42.86	10	7.69	81.8	100

Table 5. Average ophthalmic parameters among depressed and non-depressed subjects with glaucoma

	Depressed subjects	Non-depressed subjects	Level of statistical significance (p)
Avg MD in the worse eye	-18.35 ± 9.9	-6.62 ± 9.9	< 0.001
Avg MD in the better eye	-14.36 ± 13.04	-5.6 ± 5.04	< 0.01
Avg PSD in the worse eye	10.51 ± 5.84	4.49 ± 3.68	< 0.001
Avg PSD in the better eye	9.73 ± 5.63	3.69 ± 2.9	< 0.001
Avg IOP	21.94 ± 2.94	19.41 ± 1.5	< 0.001

Ophthalmic parameters, such as the mean value of MD in the eye with the most significant changes, and the mean value of PSD in the better and worse eye, were related to the degree of anxiety, as shown in Table 6. The values of MD in the better eye did not show a statistically significant association with anxiety. IOP values did not show a statistically significant association with the incidence of anxiety.

The duration of the disease was associated with a higher incidence of depression and anxiety, as shown in Table 7. Gender, level of education, and socioeconomic status in this study did not show a statistically significant association with the occurrence of depression and anxiety. A statistically significant relationship was found between the age of the subjects, employment, and the frequency of depression and anxiety, which is shown in Table 7. The degree of glaucoma did not show a statistically significant association with anxiety ($p > 0.05$). The number of drugs used by patients in therapy did not show a statistically significant association with the incidence of depression and anxiety ($p > 0.05$).

Table 6. Average ophthalmic parameters among anxious and non-anxious subjects with glaucoma

	Anxious subjects	Non-anxious subjects	Level of statistical significance (p)
Avg MD in the worse eye	-13.07 ± 10.04,	-7.03 ± 6	< 0.005
Avg PSD in the worse eye	7.94 ± 5.80	4.41 ± 3.73	< 0.05
Avg PSD in the better eye	7.35 ± 5.52	3.72 ± 2.91	< 0.05
Avg IOP	21.94 ± 2.94	19.41 ± 1.5	< 0.001

Table 7. Prevalence of depressed and anxious subjects depending on the duration of glaucoma, age structure, marital and occupational status

	Depressed subjects	**Anxious subjects**	Level of statistical significance (p)	
			depressed	anxious
Duration of illness (years)				
0-5	17.39	52.17	< 0.01	< 0.05
6-10	50	75	< 0.01	< 0.05
>10	77.78	100	< 0.01	< 0.05
Age (years)				
20-30	0	33.33	< 0.05	< 0.05
31-40	0	0	< 0.05	< 0.05
41-50	20	80	< 0.05	< 0.05
51-60	25	50	< 0.05	< 0.05
> 61	58.33	83.3	< 0.05	< 0.05
Professional status				
Employed	16.67	40	< 0.01	< 0.05
Unemployed	33.33	75	< 0.01	< 0.05
Retired	64.77	88.24	< 0.01	< 0.05

Discussion

Anxiety and depression are the most common psychiatric disorders that accompany chronic and progressive somatic diseases. The rate of these disorders in glaucoma patients, according to data from the literature, is often significantly higher compared to other chronic somatic diseases (4-6). The results of our study also showed a statistically significant rate of depression among glaucoma patients in relation to the general population, 38.64% vs. 4.17%. In the group of glaucoma patients, more than two-thirds showed mild depression, and one-third showed moderate, while there were no subjects with severe depression. These results can be explained by the fact that, in the group of the affected, half of them had a disease that lasted from 0 to 5 years, which represents a shorter evolution of the disease, which is claimed in the literature to be associated with a lower degree of depression (14-17). The intensity of glaucoma is associated with the intensity of manifested depression and anxiety (4), which was reported in our study. All subjects with severe glaucoma with visual impairment showed depression, and this prevalence decreased with decreasing degree of glaucoma severity, i.e. in the group with mild glaucoma the incidence of depression was the lowest. The duration of the disease is associated

with a higher incidence of depression and anxiety (12). In our study, the prevalence of depression was highest (77.78%) among subjects who had suffered from glaucoma for more than 10 years. High anxiety was presented by all subjects with a long evolution of the disease. In the group of glaucoma patients, high anxiety was reported in almost twice as many as in the control group, 68.18% vs. 37.5%, which was a statistically significant difference and is in line with data from the literature (3-6).

The average quality of life (QOL) in the group of patients was statistically significantly lower compared to the control group, which is also in line with data from the literature (3, 13, 15). Respondents from the group of patients had lower results in all areas of quality of life (physical and mental health, social relations, environmental factors). We found a statistically significant relationship between the frequency of depression and the degree of glaucoma, as well as the examined ophthalmic parameters that are indicators of disease severity. The intensity of glaucoma causes consequent problems in everyday family and social functioning, as well as the appearance of anxiety related to the future, which is the basis of the appearance of depression. The obtained results are in line with the statement in the literature that the loss of ability to live everyday life (drive a car, read, move independently in and out of the house) is the greatest risk factor for depression in glaucoma patients (7).

Gender of respondents, level of education, and socioeconomic status in our study did not show a statistically significant association with the occurrence of depression and anxiety. A statistically significant relationship was found between the age of the study subjects, employment, and the frequency of depression and anxiety, which is in line with data from the literature (4, 12, 13). Age, sex, poor results when examining the visual field, and the intensity of glaucoma are objective risk factors for depression, which the patient cannot influence (4, 12, 16). Factors of subjective nature, such as compliance, healthy living habits, education about one's illness, and illness acceptance, could be changed and would have positive prognostic and outcome effects on glaucoma (4, 10, 17).

Functional and psychosocial aspects of glaucoma are often underestimated by ophthalmologists, as well as significant comorbidity between glaucoma, anxiety, and depressive disorders, all resulting in a worse prognosis and therapeutic outcome. Therefore, prevention is of extraordinary importance, as is the recognition and treatment of glaucoma patients with comorbid depression, and anxiety, as well as improving the quality of life and thus compliance (17-19).

Conclusion

Comorbid depression and anxiety in glaucoma patients occur in more severe forms of glaucoma, those with a longer duration of the disease due to medical and psychosocial nature. Recognition and treatment of these disorders have a positive effect on improving the quality of life and compliance, and, thus, on the course and outcome of glaucoma.

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ANKSIOZNOST, DEPRESIJA I KVALITET ŽIVOTA KOD BOLESNIKA SA GLAUKOMOM

Suzana Tošić Golubović[1,3], Hristina Jocić[3], Uroš Gugleta[1], Gordana Nikolić[2,3], Nikola Stojanović[4]

[1]Univerzitetski klinički centar Niš, Klinika za psihijatriju, Niš, Srbija
[2]Univerzitetski klinički centar Niš, Centar za zaštitu mentalnog zdravlja, Niš, Srbija
[3]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija
[4]Univerzitet u Nišu, Medicinski fakultet, Katedra za fiziologiju, Niš, Srbija

Kontakt: Nikola Stojanović
Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: nikola.st90@yahoo.com

Prethodne studije pokazale su značajan komorbiditet između depresivnih i anksioznih poremećaja i glaukoma, koji je drugi najčešći uzrok gubitka vida u svetu. Ova studija imala je za cilj utvrđivanje prisustva depresije i anksioznosti, kao i procenu kvaliteta života bolesnika sa glaukomom, uz upoređivanje ovih rezultata sa rezultatima zdrave populacije. Studija preseka sprovedena je na Klinici za oftalmologiju, Univerzitetskog kliničkog centra Niš. Prisustvo anksioznosti i depresije procenjeno je pomoću Zungovih instrumenata za samoocenjivanje anksioznosti i depresije, a kvalitet života procenjen je pomoću SZO instrumenta za kvalitet života – kratka verzija. Prikupljeni su sociodemografski parametri i parametri vezani za glaukom. Oko 38,64% bolesnika sa glaukomom imalo je depresiju, uglavnom blage i umerene forme, dok je blaga depresija konstatovana kod 4,17% ispitanika u kontrolnoj grupi. Broj bolesnika sa anksioznošću bio je dva puta veći u eksperimentalnoj nego u kontrolnoj grupi. Navedeni rezultati pokazuju statistički relevantnu razliku ($p < 0,01$). Takođe utvrđena je statistički relevantna korelacija između učestalosti depresije i težine glaukoma ($p < 0,001$), dok između anksioznosti i težine glaukoma ($p > 0,05$) takva korelacija nije pronađena. Upoređujući prikupljene vrednosti za svaki domen, kao i ukupni kvalitet života, pronašli smo statistički relevantnu razliku ($p < 0,001$). Utvrđena je statistički značajna korelacija između bolesnika sa blažim i težim oblicima glaukoma. Komorbidna depresija i anksioznost kod bolesnika sa glaukomom javljaju se kod težih oblika glaukoma, kod bolesnika sa dužim trajanjem bolesti iz medicinskih i psiho-socijalnih razloga.

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Ključne reči: *anksioznost, depresija, kvalitet života, glaukom*

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BETAINE CONTENT IN RAW COW AND SHEEP MILK

Jelena V. Živković[1], Nataša Trutić[1], Slavica Sunarić[1], Slavoljub Živanović[2], Tatjana Jovanović[3], Gordana Kocić[4], Radmila Pavlović[5]

Betaine (trimethylglycine) exists at a physiological pH value in a zwitterionic form. It acts as a methyl group donor, an osmolyte, and a lipotropic agent. Although this micronutrient is a valuable ingredient of a healthy diet, there is limited data on its content in various foods.

The aim of this study was to determine the betaine content in raw, unprocessed cow and sheep milk from household farms in southeastern Serbia. The content of fat and protein in raw cow milk was (4.20 ± 0.38%) and (3.25 ± 0.12%), respectively. Furthermore, the content of fat and protein in raw sheep milk was (6.67 ± 0.33%) and (5.58 ± 0.16%), respectively. The content of betaine in raw cow and sheep milk was (7.51 ± 0.66 mg/l) and (15.68 ± 3.52 mg/l), respectively.

Given the importance of betaine as a significant micronutrient, its twice as high content as in cow milk contributes to the high nutritional value of sheep milk.

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Key words: *betaine, cow milk, sheep milk, HPLC method*

[1]University of Niš, Faculty of Medicine, Department of Chemistry, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Research Centre for Biomedicine Niš, Serbia
[3]University of Niš, Faculty of Medicine, Department of Physics, Niš, Serbia
[4]University of Niš, Faculty of Medicine, Department of Biochemistry, Niš, Serbia
[5]University of Milan, Department of Veterinary Science and Public Health, Milan, Italy

Contact: Jelena V. Živković
81 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: jelena.zivkovic.hemija @medfak.ni.ac.rs

Introduction

Betaine (trimethylglycine) consists of three methyl groups attached to the nitrogen atom of the amino acid glycine. At a physiological pH value, this small, highly water-soluble molecule exists in a zwitterionic form with positively charged quaternary ammonium and negatively charged carboxyl functional groups (Figure 1) (1).

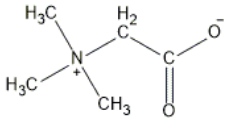

Figure 1. Chemical structure of betaine (2)

Betaine was first isolated in the nineteenth century from sugar beets (*Beta vulgaris*) and hence the name betaine (3). It could be synthesized/transported and accumulated in bacteria, fungi, animals, and plants (4). In mammals, betaine can be synthesized endogenously by two-step oxidation of choline in the liver or readily absorbed through dietary intake obtained from the diet (5, 6). Choline and its metabolite betaine, both quaternary ammonium compounds, actively participate in one-carbon (7) and lipid metabolism (8).

The amount of betaine is considerably more abundant in the kidneys and liver than in other mammalian organs. Its main role in the kidneys is osmoprotectant in the cells of the medulla. As a vital intracellular osmolyte, betaine regulates cell volume by countering changes in extracellular tonicity and stabilizing macromolecules against a variety of physiological perturbations. Its concentration in blood plasma is about 0.1 mmol/l, derived from the diet and choline metabolism (9). According to Obeid (10), plasma concentrations of betaine are highly individual. Women have lower plasma concentrations of betaine than men, whereas younger adults show lower concentrations than older ones.

Another biological function of betaine is that it is one of the essential methyl-group donors included in many biochemical pathways (11). Betaine and micronutrients such as folate, choline, vitamin B12, and other B vitamins contribute to DNA methylation as methyl donors and cofactors. The status of these nutrients correlates with DNA methylation and represents potential preventive and therapeutic targets in pathological conditions such as cancers, in which atypical DNA methylation is frequently presented (12).

As a direct methyl-group donor, betaine provides a methyl group for the conversion of homocysteine into methionine, catalyzed by betaine-homocysteine methyltransferase (BHMT), increasing the plasma and tissue concentrations of methionine while reducing homocysteine levels (3). Betaine lowers plasma levels of total homocysteine (tHcy), a possible risk factor in several age-related pathologies such as cardiovascular disease, osteoporosis, dementia, stroke, cancer, and mortality. Betaine has been used clinically to lower plasma homocysteine levels in pediatric and adult patients and has proven to be safe and efficient (13).

The effects of betaine on blood and tissue lipids have been documented in animal models, suggesting that betaine supplementation has a useful impact on obesity-related health risk factors. Although it is a lipophobic compound, betaine has physiological interactions with lipids. The intensive animal industry uses additional betaine in animal food to produce leaner meat with lower fat content and decrease obesity. In a study with patients with acute coronary syndrome, betaine and lipid concentrations in plasma suggested that low plasma betaine concentrations correlated with an unfavourable lipid profile (10, 14). Higher betaine intake was also related to atherosclerosis and fatty liver (15). The hepatoprotective effects of betaine were examined in many experimental animal models of liver diseases, including alcoholic liver disease and bile acid-induced liver injury, with different mechanisms involved. Promising therapeutic effects of betaine supplementation on non-alcoholic fatty liver disease (NAFLD) have also been investigated and reported in both clinical and experimental studies (16).

Betaine is a significant methyl-group donor, osmolyte, and lipotropic agent. Although this micronutrient is a valuable ingredient of a healthy diet, there is still only limited data on its content in various foods. As far as the authors are aware, there are no published results on the betaine content in sheep milk, with this study being the first with this finding.

The aim

The aim of this study was to determine the betaine content in raw, unprocessed cow and sheep milk from household farms in southeastern Serbia. The previously developed HPLC method for betaine determination in commercial milk was used for its quantification. The content of macronutrients - fat and protein in samples was also measured. It was examined whether there was a statistically significant difference in the level of analyzed compounds in milk samples.

Materials and methods

Sample collection

Raw cow and sheep milk samples were collected from household farms in southeastern Serbia in July 2019. The cow farms were located in Pasi Poljana (a suburb of the city of Niš) (F1), Aleksinac municipality (F2), and Lipovica (a village in Leskovac municipality) (F3), whereas sheep farms were located in Bela Palanka (F4 and F5) and Gadžin Han (F6) municipalities.

The ruminants were healthy, bread on the same farm, and manually milked. Samples were taken from the farm bulk tank and milk cans and put into clean plastic sampling flasks of 100 ml, cooled at a temperature of 4 ^{O}C, and transported to the laboratory. Commercial ultra-high temperature (UHT) processed cow and goat milk, produced by dairy companies in Serbia, was purchased from local shops in Niš in July 2019. The flasks with raw milk and UHT milk carton packaging were thoroughly shaken for 2 min before the analysis to ensure the homogeneity of the samples. Each milk sample was analyzed at least in triplicate.

Determination of milk fat content

Milk fat content was determined using the UV spectrophotometric method according to Forcato et al. (17). An aliquot of milk (30 µl) was added to 3 ml of absolute ethanol (Zorka-Pharma, Šabac, Serbia, 99.5%), and all vials were hermetically capped and stored at −20 ^{O}C for 1 hour. This procedure enables the precipitation of proteins and hydrophobic peptides that interfere with UV measurement. The samples were centrifuged (15 min at 13,000 rpm) and allowed to reach room temperature. The aliquots of the supernatants were directly transferred to a quartz cuvette 1 cm path length and the absorbance was measured by the Evolution 60 UV/Vis scanning spectrophotometer (Thermo Scientific, USA) at 208 nm. A blind probe was absolute ethanol. The calibration curve for milk fat content quantification was performed using a series of specimens with increasing fat content by adding proper amounts of cow milk cream (20% fat content) to 0.5% fat cow milk.

Determination of milk protein content

The protein content in the milk samples was determined according to the procedure prescribed by Lowry et al. (18) and later modified by Polberger and Lönnerdal (19). Proteins first react with alkaline cupric sulfate and sodium-potassium tartrate to form a complex. The produced copper(1+) ions then reduce phosphomolybdic-phosphotungstic acid, Folin-Ciocalteu (FC) phenol reagent, and result in a complex with intense blue color. After 30 min

incubation at room temperature, the absorbances of the samples were measured with a spectrophotometer at 750 nm. Bovine serum albumin (Serva Feinbiochemica, Heidelberg, Germany) was used as the protein standard for calibrating absorbance values. A commercially available FC reagent (2.0 mol/l) (Merck, Germany) was applied.

Determination of content of betaine

The betaine content in milk samples was estimated using the isocratic HPLC method that we previously developed and used for betaine quantification in commercial cow and goat milk (20). The sample pretreatment included deproteinization with 0.3% trifluoroacetic acid in acetonitrile. As a derivatization agent, 4-bromophenol bromide was used. Chromatography was performed on the Agilent Technologies 1200 Series high-performance liquid chromatography (HPLC) system (Agilent, Santa Clara, CA, USA) with a diode-array detector (DAD) SL (Agilent Technologies Inc.) and stationary phase Spherisorb SCX column (5 μm, 4.6 × 150 mm) (Waters, USA).

Statistical analysis

All examinations for each sample were done in triplicate, and the data were expressed as the mean value ± standard deviation (SD). The one-way analysis of variance (ANOVA) was used for testing significant differences between the mean, followed by Tukey's honest significant difference (HSD) post hoc comparison. The correlation was analyzed using the Pearson correlation coefficient, and the level of significance was set at $p < 0.05$. IBM Corp. SPSS Statistics 21.0 statistical software was applied for data analyses (21).

Results

Betaine content in raw cow milk

Table 1 shows the content of fat, protein, and betaine in raw cow milk.

The fat content in raw cow milk ranged from 3.86 (F3) to 4.69% (F1), and the average value was (4.20 ± 0.38%). There was a significant difference in fat content among the samples ($p < 0.05$). A statistically significant difference (95%) was found between the protein content in sample F3 with the lowest protein content (3.11%) and other tested milk. The average protein content in raw cow milk was (3.25 ± 0.12%). Sample F3 had the highest betaine content (8.29 mg/l), whereas the lowest content was determined in sample F1 (7.07 mg/l). There was a statistically significant difference in betaine content between those samples ($p < 0.05$). The average betaine content was (7.51 ± 0.66 mg/l).

Betaine in raw sheep milk

The content of fat, protein, and betaine in raw sheep milk is presented in Table 2.

Sample F4 had the highest fat content (7.03%), and a statistically significant difference (95%) was found between the fat content in samples F4 and F6, with 7.03 and 6.34% fat, respectively. The average fat content in raw sheep milk was (6.67 ± 0.33%). There was no statistically considerable difference between the protein content in the samples of raw sheep milk ($p > 0.05$), and the average value for this compound was (5.58 ± 0.16%). The betaine content ranged from 12.25 mg/l (F4) to 20.13 mg/l (F6), and there was a significant difference in the betaine content between the samples ($p < 0.05$). The average betaine content in raw sheep milk was (15.68 ± 3.52 mg/l).

Table 1. Content of fat, protein, and betaine in raw cow milk

Farm	Fat (%)	RSD (%)	Protein (%)	RSD (%)	Betaine (mg/l)	RSD (%)
F1	4.69 ± 0.04a*	0.85	3.36 ± 0.08a	2.38	7.07 ± 0.33b	4.67
F2	4.04 ± 0.08b	1.98	3.28 ± 0.04a	1.22	7.16 ± 0.40ab	5.59
F3	3.86 ± 0.07c	1.81	3.11 ± 0.05b	1.61	8.29 ± 0.27a	3.26
Average	4.20 ± 0.38		3.25 ± 0.12		7.51 ± 0.66	

* Data are shown as mean ± standard deviation of three replicates. Values in the column followed by different lowercase letters are significantly different according to Tukey's test ($p < 0.05$).

Table 2. Content of fat, protein, and betaine in raw sheep milk

Farm	Fat (%)	RSD (%)	Protein (%)	RSD (%)	Betaine (mg/l)	RSD (%)
F4	7.03 ± 0.20a*	2.84	5.67 ± 0.14a	2.47	12.25 ± 0.47c	3.84
F5	6.65 ± 0.09ab	1.35	5.64 ± 0.12a	2.13	14.67 ± 0.63b	4.29
F6	6.34 ± 0.16b	2.52	5.43 ± 0.14a	2.57	20.13 ± 0.35a	1.74
Average	6.67 ± 0.33		5.58 ± 0.16		15.68 ± 3.52	

* Values in the column followed by different lowercase letters are significantly different according to Tukey's test ($p < 0.05$)

The HPLC chromatograms of betaine in standard, raw cow, and raw sheep milk are presented in Figure 2 (A, B, and C, respectively). The peak of betaine content was determined in the milk samples at the retention time of (9.38 ± 0.01 min). Also, the peak area and height significantly increased in the chromatogram of raw sheep milk (Figure 2C) compared to the corresponding peak in raw cow milk (Figure 2B).

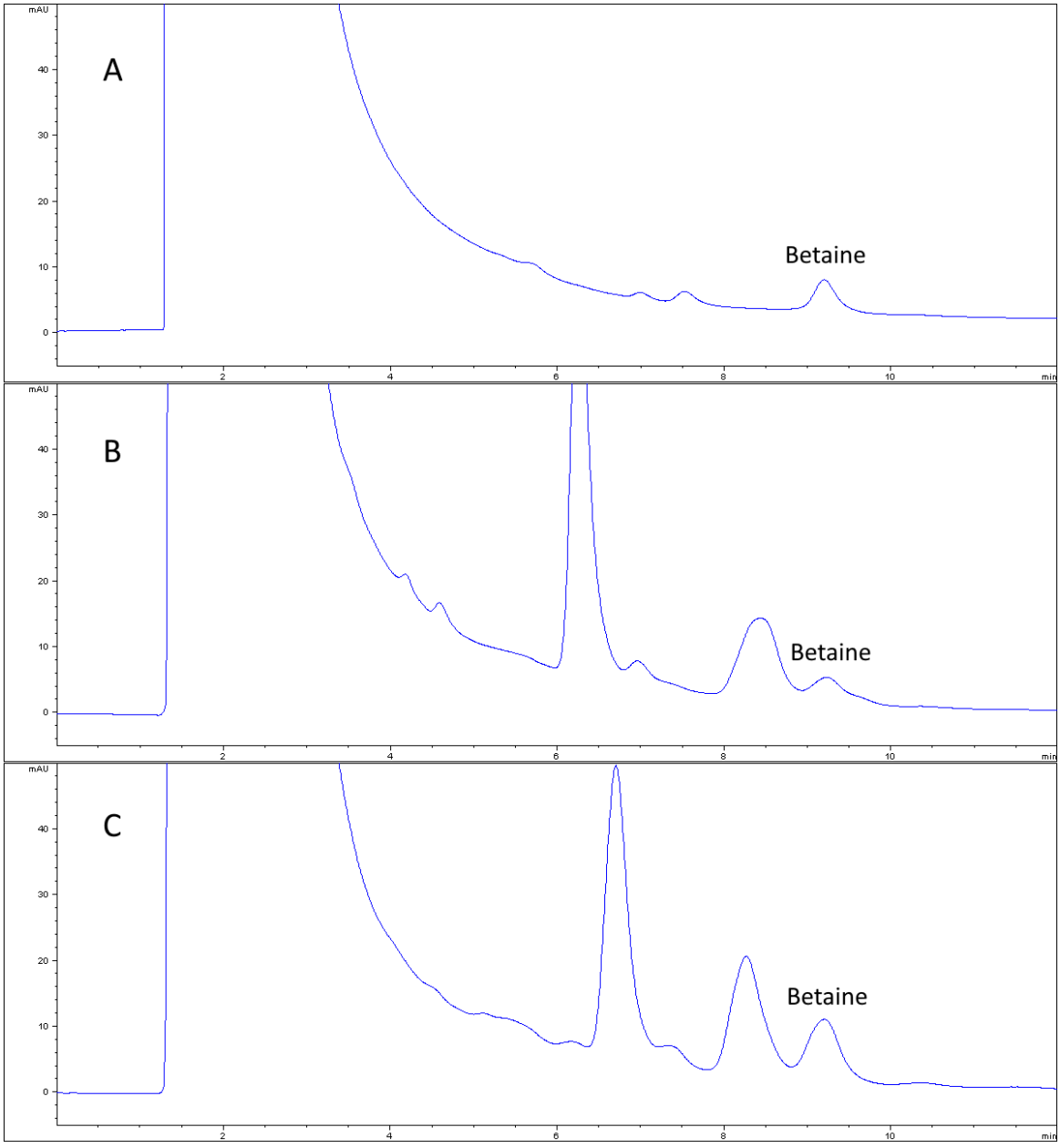

Figure 2. HPLC chromatograms of (A) standard solution of betaine hydrochloride (5.86 mg/l),
(B) betaine (7.16 mg/l) in F2 raw cow milk, and
(C) betaine (14.67 mg/l) in F5 raw sheep milk.

Betaine in commercial milk

Table 3 shows the content of fat, protein, and betaine in commercial milk.

There was a statistically significant difference between the samples in the analyzed parameters (fat, protein, and betaine) ($p < 0.05$).

The precision of quantification of the compound expressed as the relative standard deviation (RSD) for fat and protein varied from (0.85 to 2.84%) and (0.60 to 2.57%), respectively. RSD for betaine ranged between 0.57 and 5.59% (Tables 1, 2, and 3).

Correlation matrix

The correlation between the fat, protein, and betaine content for all analyzed milk samples is shown in Table 4.

The betaine and fat and betaine and protein content in the samples ($r = 0.790$ and 0.857, respectively) correlated highly and significantly. Also, the correlation between the fat and protein content was highly significant ($r = 0.977$).

Table 3. Content of fat, protein, and betaine in commercial milk

Milk	Fat (%)	RSD (%)	Protein (%)	RSD (%)	Betaine (mg/l)	RSD (%)
Cow	3.20 ± 0.03b*	0.94	3.00 ± 0.05b	1.67	7.04 ± 0.04b	0.57
Sheep	6.00 ± 0.07a	1.17	5.00 ± 0.03a	0.60	14.35 ± 0.34a	2.37

* Values in the column followed by different lowercase letters are significantly different according to Tukey's test ($p < 0.05$)

Table 4. Pearson correlation coefficients (r) between fat, protein, and betaine content

	Fat	Protein
Protein	0.977**	
Betaine	0.790*	0.857*

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level

Discussion

Given that betaine can readily and quickly be metabolized from choline, it is considered a non-essential nutrient. There are currently no recommended betaine dietary intake values, and the average daily intake varies considerably (3). Choline and betaine are found in many different types of food. Animal-based food, including red meat, poultry, milk, and eggs, is the leading source of choline, whereas betaine is found in grain products, cruciferous vegetables, beets, and seafood. In a study of dietary intake of these nutritions conducted by Cho et al. (15), the mean choline and betaine intake was (313 ± 61 mg/day) and (208 ± 90 mg/day), respectively. The betaine intake was slightly higher in women (216 mg/day) than in men (200 mg/day). Yonemori et al. (22) studied dietary choline and betaine intakes in the adult multiethnic population. The resulting mean intake estimated for betaine varied by gender and amounted to 154 mg/day in men and 128 mg/day in women. The main betaine intake was from grains (58.4%), followed by dark-green vegetables (12.6%) and alcohol (7.3%; beer mostly), and was similar among ethnic groups. The obtained intake of betaine from milk was 1.3% due to foods of animal origin, such as cow milk being a scarce source of betaine. In the average New Zealand diet, the mean betaine intake was (298 ± 4 mg/day). Men exhibited higher betaine intake values than women, which decreased with age. Betaine intake is dominated by grain products (67% of the total intake), with bread contributing approximately one-third of the total betaine intake. Significant non-grain contributors to betaine intake include tea and beer, while dairy products contribute only 1% (23). On the other hand, daily intake of milk varies much among regions, with an estimated average value of approximately (200-240 g/day) in Western Europe and North America, (130-300 g/day) in Latin America, (100-200 g/day in Africa), and (20-150 g/day) in Asia (24).

Milk contains a wide range of relevant nutrients and therefore plays a significant role in assisting both children and adults in meeting their nutrient requirements. From the earliest times, ruminant milk and milk products have formed an integral component of human diet. More than 95% of dairy products consumed in developed countries originate from bovine milk (25). After cow milk, goat and

sheep milk are the two most produced and consumed types of ruminant milk. Cow milk dominates in the total milk production in Serbia (96.84%), whereas goat (2.20%) and sheep milk (0.96%) constitute a smaller share (26). Even though researchers are primarily focused on bovine milk, milk obtained from small ruminants, including goats and sheep, remains an appealing area of study (27). Milk composition varies depending on the species (cow, goat, sheep), with fat, protein, lactose, and mineral compounds being the major milk components (28). The average and SD values for fat and protein in raw cow milk obtained from household farms in southeastern Serbia were (4.20 ± 0.38%) and (3.25 ± 0.12%), respectively (Table 1), which is in line with the results presented in the literature. According to Roy et al. (28), the fat and protein range in cow milk was (3.3-5.4%) and (3.0-3.9%), respectively. The average fat and protein content in raw sheep milk was (6.67 ± 0.33%) and (5.58 ± 0.16%), respectively (Table 2). Similar results were found in a study by Ferro et al. (29), in which the average milk fat value amounted to (6.9 ± 1%), i.e., (5.4 ± 0.4%) for milk protein. In addition, a close range to our study, (5.0-9.0%) of the fat and (4.5-7.0%) of the protein content in sheep milk, was obtained by Roy et al. (28).

Sheep milk contains higher total solids (protein and fat) and more necessary nutrients than goat and cow milk. The beneficial effects of this kind of milk originate from a high content of fatty acids, immunoglobulins, proteins, hormones, vitamins, and minerals. Compared to cow milk, it has more than twice as much Vitamin C, double or triple other essential vitamins, especially B vitamins which are significant in the functioning of the nervous system. In addition, among animal milk, only buffalo milk has more fat content than sheep milk (30). Sheep milk has high concentrations of fat globules, which are smaller than in cow milk. Their size and dispersion make it easier to digest and confer greater milk consistency. Sheep milk also has the highest linoleic acid content of all ruminant milk due to sheep milk being effective in preventing obesity and type 2 diabetes. Also, it has more than twice as much high-quality protein (casein and whey proteins) as cow milk. Many bioactive peptides in sheep milk have proven antiviral, antibacterial, and anti-inflammatory properties (31, 32).

The average content of betaine in raw cow and sheep milk was (7.51 ± 0.66 mg/l) and (15.68 ± 3.52 mg/l), respectively (Tables 1 and 2). The betaine content in commercial (UHT) cow and sheep milk was (7.04 ± 0.04 mg/l) and (14.35 ± 0.34 mg/l), respectively (Table 3). The result obtained for (UHT) cow milk (7.04 mg/l) was in line with the average betaine concentration in commercial cow milk (7.21 mg/l) that we determined in our previous study (20). Zeisel et al. (33) reported a slightly higher betaine content (8.40 mg/kg) in 2% fat cow milk. In both raw and processed forms, sheep milk contains more than twice as much betaine compared to cow milk. A high and significant correlation was determined between the betaine and fat ($r = 0.790$) and the betaine and protein ($r = 0.857$) content in milk, while fat and protein correlate highly significantly ($r = 0.977$) (Table 4). Comparing betaine levels in raw and pasteurized (UHT) cow milk (7.51 and 7.04 mg/l) and sheep milk (15.68 mg/l and 14.35 mg/l), a lower betaine value was measured in processed milk (Tables 1, 2, and 3). We could not attribute a decrease in the betaine content in commercial milk types compared to raw to the pasteurization because the same samples did not process from a raw to a commercial form. Yet, according to Zeisel et al. (33), given that betaine is a small, highly water-soluble molecule, losses during some food processing and cooking were not unexpected.

Although cow milk is the most abundant type of milk (with 83% of global production), the use of milk from other animals has increased in recent years. Sheep milk is processed manly into cheese, yoghurt, and other dairy products. A notable advantage is that most dairy sheep production systems are environmentally friendly and play a significant role in developing rural communities (34).

Conclusion

Currently, sheep milk is considered a delicacy in many countries, including Serbia. It is known to have high concentrations of fat and proteins, and this was confirmed by the results obtained from the analysis of raw sheep milk samples from southeastern Serbia. The high fat content in sheep milk highlights that this type of milk is a valuable energy source. In addition, bearing in mind the importance of betaine as an influential micronutrient, the high nutritional value of this foodstuff provides twice more betaine than cow milk. The current study is the first with the betaine content in sheep milk and is valuable for the estimation of this compound with a wide range of actions.

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SADRŽAJ BETAINA U SIROVOM KRAVLJEM I OVČIJEM MLEKU

Jelena V. Živković[1], Nataša Trutić[1], Slavica Sunarić[1], Slavoljub Živanović[2], Tatjana Jovanović[3], Gordana Kocić[4], Radmila Pavlović[5]

[1]Univerzitet u Nišu, Medicinski fakultet, Katedra za hemiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Naučnoistraživački centar za biomedicinu, Niš, Srbija
[3]Univerzitet u Nišu, Medicinski fakultet, Katedra za fiziku, Niš, Srbija
[4]Univerzitet u Nišu, Medicinski fakultet, Katedra za biohemiju, Niš,
[5]Univerzitet u Milanu, Departman za veterinarske nauke i javno zdravlje, Milano, Italija

Kontakt: Jelena V. Živković
Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: jelena.zivkovic.hemija@medfak.ni.ac.rs

Betain (trimetilglicin) postoji na fiziološkoj pH vrednosti u cviterjonskom obliku. Deluje kao donor metil grupa, osmolit i lipotropni agens. Iako je ovaj mikronutrijent vredan sastojak zdrave ishrane, još uvek postoje samo ograničeni podaci o njegovom sadržaju u različitim namirnicama. Cilj ovog istraživanja bio je da se utvrdi sadržaj betaina u sirovom, neprerađenom kravljem i ovčijem mleku sa gazdinstava u Jugoistočnoj Srbiji. Sadržaj masti i proteina u sirovom kravljem mleku iznosio je 4,20% ± 0,38% i 3,25% ± 0,12%, respektivno. Zatim, sadržaj masti i proteina u sirovom ovčijem mleku bio je 6,67% ± 0,33% i 5,58% ± 0,16%, respektivno. Sadržaj betaina u sirovom kravljem i ovčijem mleku iznosio je 7,51 mg/l ± 0,66 mg/l i 15,68 mg/l ± 3,52 mg/l, respektivno. S obzirom na značaj betaina, kao važnog mikronutrijenta, njegov dvostruko veći sadržaj, u ovčijem nego u kravljem mleku, doprinosi visokoj nutritivnoj vrednosti ovčijeg mleka.

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Ključne reči: *betain, kravlje mleko, ovčije mleko, HPLC metoda*

Review article

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CT GUIDED TRANSTHORACIC BIOPSY OBTAINED WITH *CORE* BIOPSY TECHNIQUE: SAFETY AND SUCCESS OF THE PROCEDURE

Aleksandar Tasić[1], Dragan Stojanov[1,2], Miloš Stamenković[1], Bojan Ilić[3], Marija Topalović[4]

Percutaneous Transthoracic Biopsy (TTB) is a minimally invasive method of obtaining tissue specimens from a previously detected thoracic lesion for further analysis in order to reach a definite diagnose.

The study aimed at determining the role of percutaneous transthoracic biopsy in the light of current international recommendations for performing the intervention, and presenting our experiences - success rate, and complications.

The study included 57 patients (17 women and 40 men) of average 64.4 years, who underwent biopsy procedures from January 2016 to November 2019. The procedure was performed using the cutting biopsy technique, using an automated BARD MAGNUM Reusable Core Biopsy System with 14-18 G diameter needles, under the guidance of GE 16 and GE 64 MDCT, with a postprocedural scan for complication evaluation. The material was sent to the Pathology Clinic, Clinical Center Niš.

The procedure was successful in 53 patients (92.98%). Of the complications, pneumothorax was reported in 14 patients (24.56%), hemoptysis in 4 patients (7%), and intrapulmonary hemorrhage in 10 patients (17.54%). Only 4 cases of pneumothorax (7%) required drainage. The smallest lesion was 20 mm in diameter and the longest pathway through the lung parenchyma was 50 mm.

Based on our results, we can conclude that CT-guided transthoracic biopsy with core biopsy technique is a minimally invasive inexpensive procedure, with high rates of diagnostic accuracy, and acceptably low complication rates, and therefore one of the mandatory procedures to be considered in diagnosis of thoracic masses.

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Key words: *biopsy, percutaneous, transthoracic, computed tomography (CT), pneumothorax*

[1]University Clinical Center Niš, Centre of Radiology, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Niš, Serbia
[3]University Clinical Center Niš, Clinic of Thoracic Surgery, Niš, Serbia
[4]University Clinical Center Niš, Clinic of Lung Diseases, Niš, Serbia

Contact: Aleksandar Tasić
75/37 Vojvode Mišića St., 18000 Niš, Serbia
E-mail: dr.aleksandartasic@yahoo.com

Introduction

With increased availability of spiral CT, a growing number of thoracic tumors is detected and they are still a diagnostic and therapeutic challenge- in management of these lesions histological diagnosis is a prerequisite for treatment planning (1). While mediastinal tumors (most common being lymphomas, thymomas and metastases) and thoracic wall tumors are relatively rare (2, 3), lung cancer represents a growing problem, being second most frequently diagnosed cancer, accounting for 17% of all cancers in men and 9% in women, and a leading cause of cancer-related death worldwide (4).

Since percutaneous needle biopsy has yielded excellent results in multiple organ systems followed by few complications (5), it is expected to be very successful for thoracic lesions. Transthoracic biopsy is a minimally invasive procedure used to obtain samples from previously detected thoracic mass for further analyses, leading to definite diagnosis. While bronchoscopic lung biopsy is highly accurate for cytologic and histologic diagnosis of centrally located lesions, it is of limited value for peripheral lesions (6).

Therefore, often following inconclusive findings of bronchoscopy or having a peripherally located lesion, imaging guided transthoracic biopsy is used as a standard procedure. Image guidance is necessary for precise sampling, to make sure the needle is placed in correct position when obtaining the sample. While various imaging modalities can be used for image-guided interventions (fluoroscopy,

ultrasound, computed tomography and magnetic resonance), CT guidance is most frequently used, due to high spatial and contrast resolution of spiral multidetector CT, and availability of multiplanar and 3D reformations (7).

Although two basic methods are in use for obtaining samples, based on needle type, cutting needle biopsy provides higher diagnostic accuracy of benign lung lesions and equal diagnostic accuracy of malignant lung lesions compared to fine needle aspiration, risk of complications being within the acceptable range (8).

While considered minimally invasive and very safe, there are some complications reported following this procedure. Most common complications are pneumothorax (incidence ranging up to 54%, mean value 20%) (9) and major bleeding (incidence being 2.8%) (10), followed by rare complications - systemic air embolism (ranging from 0.01% to 0.21%) (11) and tumor seeding via biopsy tract. Most common complications often spontaneously resolve, but sometimes pneumothorax and major bleeding require prolonged hospitalization and additional intervention.

Having in mind reported diagnostic accuracy rates for cutting needle lung biopsy ranging from 76% to 93% (12), in this retrospective study we sought to evaluate CT-guided transthoracic biopsy results and safety, as performed in our institution.

Materials and methods

From January 2016 to November 2019, transthoracic CT-guided cutting needle biopsy procedures were performed in 60 patients.

Prior to transthoracic biopsy procedures, all patients sent to biopsy were surveyed for contraindications. Since there are no absolute contraindications (8, 13), medical documentation was searched and patients with suspicion of vascular structure and hydatid cyst, and those unable to cooperate (positioning, cough control) were not included, while based on recorded platelet counts, prothrombin time, and activated thromboplastin time some of the patients were subjected to correction of coagulopathy.

Detailed information considering performing the procedure, possible complications, and treatment of these complications were explained to each subject.

Written informed consent was obtained from all patients.

Of the initial 60 subjects, 3 were excluded from the analysis because their records were incomplete or unavailable (since they had been referred from other centers for the biopsy procedure). The records of included patients were retrospectively evaluated.

All patients who underwent percutaneous transthoracic lung biopsy had one from the list of indications defined by Manhire et al. (13) in BTS guidelines:

1) new or enlarging solitary nodule or mass on the chest radiograph/CT which is unlikely to be accessible by bronchoscopy/following insufficient bronchoscopy,

2) multiple nodules in a patient without known primary malignancy/with known primary malignancy and a prolonged remission,

3) persistent infiltrates, without diagnosis after sputum or blood culture analyses, serology or bronchoscopy,

4) hilar mass following negative bronchoscopy.

Thoracic CT scans of 57 patients were evaluated, and the size of the targeted lesions (the longest diameter) was recorded. Lesions were divided into two groups based on size (largest measured diameter ≤ 30 mm, and larger than 30 mm), and into two groups based on localization (peripheral and non-peripheral, lesion considered as peripheral if partly within 20 mm distance from visceral pleura).

All biopsies were performed by 2 interventional radiologists, on GE multidetector systems for computed tomography - GE 16 and GE 64.

Patients were positioned on the tomography table regarding the targeted lesion, to enable the shortest and safest route of the needle, in prone, supine position or lying on the side. CT scan was obtained with images of 1.25 to 2.5 mm thickness, and as narrow a field of view as possible while encompassing the targeted lesion. Appropriate measurements were taken, and after determining appropriate image slice, laser guidance system was used to define the exact site of puncture on the skin. Following cleaning and sterilization of the skin, local anesthetic was applied, and minimal incision was made to provide passage of the needle. A 14 G to 18 G needle was introduced at an appropriate angle to the lesion, with a tip of the needle at the border of the lesion. Samples were obtained by an automated BARD MAGNUM Reusable Core Biopsy System (C. R. Bard, Inc. Bard Medical, Covington, Georgia) with compatible Core Biopsy Needles (C. R. Bard, Inc. Bard Medical, Covington, Georgia) - three samples per patient, if possible, that were placed in a container with formalin solution and sent to the pathology laboratory for analysis.

Immediately after the biopsy procedure, control CT scans were performed, and a control chest X-ray 4 hours after the procedure. Complications were noted in reports.

If patients had no symptoms and no increase in the pneumothorax on follow-up, no intervention was performed. If a significant or rapidly enlarging pneumothorax was found, a chest tube was placed into the pleural space by a thoracic surgeon.

Complications (pneumothorax, bleeding, hemoptysis, etc.) and interventions necessary to treat them were noted. Frequency of complications was compared considering size and localization of lesions.

Transthoracic biopsy results were classified into 2 groups: diagnostic, and nondiagnostic results. The procedure was deemed successful if samples taken from the lesion provided enough material to reach the histologic diagnosis.

Results

Transthoracic biopsy using a core biopsy system was performed on 60 patients. Complete records were available in 57 patients. The mean age of the patients was 64.4; 17 (28.33%) were female and 40 (66.67%) were male.

The longest axial diameter of targeted lesions varied from 20 mm to 65 mm.

Pathology results of transthoracic biopsy were achieved in all 57 patients (100%), of whom the results were diagnostic in 53 (92.98%). The pathology results were nondiagnostic in 4 patients (7%).

From lesions identified on thoracic CT, a mean of 2.5 biopsy specimens were obtained from each patient. In 1 patient with severe emphysema, pneumothorax developed on our second attempt, but the biopsy specimen was already obtained and it turned out that it was sufficient for diagnosis.

Complications were observed in 21 (36.84%) of 57 patients in whom a transthoracic biopsy was performed (several patients had combined complications). Pneumothorax developed in 12 patients (21.05%); a drainage tube was needed in 4 of them (7%), and procedure was performed by thoracic surgeon - surgery was not needed.

In patients with bleeding and hemoptysis, no additional therapeutic intervention was needed on follow-up. Intrapulmonary hemorrhage occurred in 10 (17.54%) and hemoptysis in 5 patients (8.77%). The complications detected after transthoracic biopsy are summarized in Table 1 compared to thresholds of good clinical practice and meta-analysis that included the largest number of transthoracic biopsies.

Statistical analysis used chi square test to correlate complications with lesion localization (Table 2) and size (Table 3), with contingency coefficient to assess intensity of correlation. Lesion size and lesion localization turned out to be in positive correlation with occurrence of pneumothorax major and minor, and intrapulmonary hemorrhage, while diagnosis was not affected by the presence or absence of complications.

Table 1. The complications detected after transthoracic biopsy

Complications of TTB		Good Clinical Practice (%)	Meta-Analysis (Heerink et al.) (%)	Our results	
				%	n
Pneumothorax		45	25.3	21.05	12
	Major PNX Thoracic Drainage	20	5.6	7.02	4
Hemorrhage	Hemoptysis	2	4.1	8.77	5
	IP Hemorrhage		18	17.54	10

Table 2. Lesion localization

		Localization		Total	χ^2	Sig	C
		Non peripheral	Peripheral				
Pneumothorax Minor	No	11 (64.71%)	38 (95%)	49 (85.96%)	9.074	.003	.371
	Yes	6 (35.29%)	2 (5%)	8 (14.04%)			
Pneumothorax Major	No	14 (82.35%)	39 (97.5%)	53 (92.98%)	4.195	.041	.262
	Yes	3 (17.65%)	1 (2.5%)	4 (7.02%)			
Hemoptysis	No	15(88.24%)	37 (92.5%)	52 (91.23%)	.271	.603	.06
	Yes	2 (11.76%)	3 (7.5%)	5 (8.77%)			
Intrapulmonary Hemorrhage	No	9 (52.94%)	38 (95%)	47 (82.46%)	14.588	.000	.451
	Yes	8 (47.06%)	2 (5%)	10 (17.54%)			

Table 3. Lesion size

		Size		Total	χ^2	Sig	C
		≤ 30 mm	> 30				
Pnx Minor	No	14 (70%)	35 (94.59%)	49 (85.96%)	6.509	.011	.320
	Yes	6 (30%)	2 (5.41%)	8 (14.04%)			
Pnx Major	No	16 (80%)	37 (100%)	53 (92.98%)	7.958	.005	.350
	Yes	4 (20%)	0 (0%)	4 (7.02%)			
Hemoptysis	No	19 (95%)	33 (89.19%)	52 (91.23%)	.548	.459	.098
	Yes	1 (5%)	4 (10.81%)	5 (8.77%)			
Intrapulmonary Hemorrhage	No	13 (65%)	34 (91.89%)	47 (82.46%)	6.490	.011	.320
	Yes	7 (35%)	3 (8.11%)	10 (17.54%)			

Discussion

Following inconclusive findings of bronchoscopy or used as an initial procedure in patient workup, CT-guided transthoracic biopsy is a well established procedure to characterize thoracic, especially pulmonary lesions. Even though the results of both TTB and bronchoscopy depend on lesion size and location, and on individual skills of the physician performing the procedure (14-16), TTB has a higher sensitivity and specificity than bronchoscopy, being preferable test in diagnosing solitary nodules.

While reported success of fine-needle aspiration biopsies was up to 99% (17, 18) with a few complications, limitations were low sensitivity for benign lesions (being under 50%) (19), and inadequate or insufficient samples in up to 20% (20-22). Core biopsy is preferred by many authors as it provides high quality tissue samples more adequate for electron microscopy, immunohistochemistry and analysis of tumor-markers, enhancing diagnostic specificity (19, 23, 24). Therefore, we used cutting technique with automated core biopsy systems.

In our study, all lesions were equal to or larger than 20 mm (in measured diameter) and sufficient histologic material was successfully obtained from core specimens, even from lesions with pleural distance of more than 40 mm. Overall diagnostic accuracy was reported to be very high with 3 specimens obtained during every biopsy procedure (25), as in our study. In our study overall accuracy observed was 92.98 %, in accordance with numerous authors and appropriateness criteria. In 7% of our cases tissue samples were not diagnostic, reasons thought to be sampling mistakes - missed biopsies, sampling from necrotic center or peritumoral inflammatory zone.

CT-guided transthoracic cutting needle biopsy is safe; however, an overall complication rate was recorded to be 39.1% (26), encompassing pneumothorax, bleeding (pulmonary hemorrhage, hemoptysis), air embolism, tumor seeding via biopsy tract, infection and mortality.

Pneumothorax is the most common complication of CT-guided lung biopsy, varying from 8% to 64% (27), due to different factors, most important being depth of the lesion, emphysema, higher sensitivity of CT for diagnosis of pneumothorax. In our study pneumothorax was observed in 24.56% of patients, finding that was consistent with various authors (28, 29). Chest tube was placed in 4 (7%) of our patients, and it was also in agreement with previous results (8).

Hemorrhage remains the second most common complication of transthoracic biopsy, which can be life-threatening. Hemorrhage occurs in approximately 5% to 16.9% of patients undergoing percutaneous biopsy, rates going as high as 30% (29). Hemoptysis may follow, in 1.25% to 5% (30, 31). In our study there were 5 patients with hemoptysis (8.77%) and 10 patients with intrapulmonary hemorrhage (17.54%). No major bleeding complication or hematothoraces occurred, and no transfusion, bronchoscopic tamponade, arterial embolization, or surgery were necessary.

While infection may follow in 2% of patients, air emboli (0.012-0.61%), tumor seeding via biopsy tract (0.02-0.4%) and mortality (0.16%) are extremely rare complications (32). None of these events was recorded in our study.

Previous studies have reported higher rates of complications, especially hemorrhage, for core biopsy systems than for fine-needle aspirates (33). Meta-analysis performed by Heerink et al. (32) confirmed higher rates of pneumothorax and hemorrhage (both intrapulmonary and hemoptysis) if using core biopsy system, and defined risk factors leading to complications, naming size of the needle, number of pleural passes and coaxial technique as very important. The most important yield of the meta-analysis was reporting that even though complications in general were more numerous in cutting biopsy group, the rate of major complications was similar to FNA biopsies. Heenrik also concluded that, apart from needle size, common risk factors for major complications were size and depth of the targeted lesion. Our results revealed statistically significant correlation of complications (pneumothorax major and minor, intrapulmonary hemorrhage) with lesion localization (Table 2) and size (Table 3), suggesting non-peripheral lesions with diameter of 30 mm and smaller are associated with higher risk for developing complications after biopsy.

Apart from being accurate and safe procedure, it is also 6.3 and 10.9 times less expansive, compared to the costs of thoracoscopic surgery and thoracotomy, respectively (34). As a means to definite diagnosis, result of transthoracic biopsy proves to influence patient management, allowing for avoiding surgery in approximately 75% of patients (29).

Conclusion

In our study, we showed that even though we used somewhat larger needles (14-18 G) compared to other authors (18-20 G), and no coaxial needle, complication rates were in agreement with previous studies. Diagnostic accuracy in our study was very high (92.98%). Based on our results and previous studies, we can conclude that CT-guided transthoracic biopsy with core biopsy technique is a minimally invasive inexpensive procedure, with high rates of diagnostic accuracy, and acceptably low complication rates, and therefore one of the mandatory procedures to be considered in diagnosis of thoracic masses.

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CT-OM VOĐENA TRANSTORAKALNA BIOPSIJA IZVEDENA TEHNIKOM *CORE* BIOPSIJE: BEZBEDNOST I USPEŠNOST PROCEDURE

Aleksandar Tasić[1], Dragan Stojanov[1,2], Miloš Stamenković[1], Bojan Ilić[3], Marija Topalović[4]

[1]Univerzitetski klinički centar Niš, Centar za radiologiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija
[3]Univerzitetski klinički centar Niš, Klinika za grudnu hirurgiju, Niš, Srbija
[4]Univerzitetski klinički centar Niš, Klinika za pulmologiju, Niš, Srbija

Kontakt: Aleksandar Tasić
Vojvode Mišića 75/37, 18000 Niš, Srbija
E-mail: dr.aleksandartasic@yahoo.com

Perkutana transtorakalna biopsija (PTTB) predstavlja minimalno invazivnu metodu, kojom se pribavlja uzorak tkiva iz uočene promene grudnog koša za dalju analizu, u cilju postavljanja dijagnoze.

Cilj ovog rada je utvrđivanje uloge perkutane transtorakalne biopsije, u svetlu trenutnih međunarodnih preporuka za obavljanje intervencije, kao i predstavljanje naših iskustava – stepena uspeha i komplikacija.

Studijom je obuhvaćeno 57 bolesnika (17 žena i 40 muškaraca) proseče starosti 64,4 godine, koji su bili podvrgnuti procedurama biopsije od januara 2016. do novembra 2019. godine. Postupak je izvršen tehnikom CORE biopsije, korišćenjem automatizovanog BARD MAGNUM systema za CORE biopsiju za višestruku upotrebu, iglama prečnika 14 G – 18 G, pod vođstvom GE 16 i GE 64 aparata za višerednu komjuterizovanu tomografiju, uz postproceduralno skeniranje za procenu komplikacija. Materijal je potom upućivan na Kliniku za patologiju Kliničkog centra Niš.

Procedura je bila uspešna kod 53 bolesnika (92,98%). Od komplikacija zabeležen je pneumotoraks kod 14 bolesnika (24,56%), hemoptizije kod 4 bolesnika (7%) i intrapulmonalno krvarenje kod 10 bolesnika (17,54%). Samo 4 slučaja pneumotoraksa (7%) zahtevali su plasiranje drenažnog katetera. Najmanja lezija bila je prečnika 20 mm, a najduži put kroz plućni parenhim bio je 50 mm.

Na osnovu naših rezultata možemo zaključiti da je transtorakalna biopsija vođena CT-om i tehnikom CORE biopsije minimalno invazivna, jeftina procedura, sa visokom stopom dijagnostičke tačnosti i prihvatljivo niskom stopom komplikacija, te je stoga jedan od obaveznih koraka koji treba razmotriti u dijagnostici torakalnih masa.

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Ključne reči: *biopsija, perkutana, transtorakalna, kompjuterizovana tomografija (CT), pneumotoraks*

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DIAGNOSIS AND SURGICAL TREATMENT OF MELANOMA: A MINI REVIEW

Goran Stevanović[1,2], Stefan Momčilović[2]

Melanoma is a rare but the deadliest form of skin cancer. In the early stages, melanoma can be treated successfully by surgery and survival rates are high. However, in patients with metastases, survival rates drop significantly. Therefore, early and accurate diagnosis, prompt referral and proper management are crucial for ensuring the best prognosis. Also, multidisciplinary approach is necessary.

The diagnosis of melanoma is usually made by dermoscopic examination of suspected pigmented lesions. Misdiagnosis of melanoma is one of the most common reasons for initiating lawsuits for wrong treatment, when it comes to pathologists and dermatologists, immediately after breast cancer.

Regarding the treatment of metastatic melanoma, in the last 10 years several new drugs have been developed that have significantly improved the prognosis of patients suffering from this disease. However, a majority of patients do not show a lasting response to these treatments. Thus, new biomarkers and drug targets are needed to improve the accuracy of melanoma diagnosis and treatment.

This article discusses the current state of melanoma diagnosis and treatment based on the generally accepted consensus in this area and current national guidelines for treatment recommendations. The parts that remained open in the treatment algorithm (i.e. insufficiently clearly defined) are also mentioned.

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Key words: *melanoma, diagnosis, treatment, surgery, dermoscopy*

[1]University of Niš, Faculty of Medicine, Niš, Serbia
[2]University Clinical Center Niš, Plastic and Reconstructive Surgery Clinic, Niš, Serbia

Contact: Goran Stevanović
48 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: gste66@yahoo.com

Introduction

Cutaneous melanoma is a malignant tumor of the melanocytes, cells that are localized in the basal layer of the epidermis and produce the pigment melanin which is responsible for skin color (1).

Despite the fact that the incidence of many types of tumors is decreasing at the beginning of the 21st century, melanoma still remains a potentially fatal malignancy which incidence continues to increase. Currently, this malignant tumor is regarded as the fifth most common cancer in men and the sixth most common cancer in women in the United States. Furthermore, it has been estimated that 1 in 63 Americans will develop melanoma during their lifetime. On the other hand, the incidences of cutaneous melanoma vary greatly between European countries and are highest in Switzerland and the Scandinavian countries (2). However, the highest incidence and mortality rates from this cancer in the world have been noted in Australia and New Zealand (3).

Different incidence patterns of melanoma can be explained by variations in racial skin phenotype, as well as differences in sun exposure. In addition, as opposed to other solid tumors, melanoma mostly affects young and middle-aged individuals (4). Furthermore, patients with previous history of melanoma have an approximately 7% chance of developing a second primary melanoma. To date, it has been shown that exposure to UV radiation represents the predominant environmental risk factor for melanoma. Moreover, chronic sun exposure (especially in an intermittent pattern) and baseline history of severe sunburns are both suspected as causative (5, 6). In most patients, melanoma arises as a new lesion in the skin, while in less than 30% of cases this cancer occurs as a result of malignant transformation of pre-existing melanocytic nevi. Nevertheless, previous studies have shown that the number of common nevi and the presence of atypical nevi (irregular edges, uneven color, diameter

greater than 5 mm) represent important independent risk factors for melanoma. Finally, a positive family history has been documented in about 5-15% of patients, suggesting that genetic predisposition may contribute significantly to disease development and progression (7).

Melanoma treatment has always been a major challenge in plastic surgery. Although the basic concepts are well known, advances in basic research, as well as numerous clinical studies, are changing traditional treatment paradigms.

Diagnosis

The prognosis in patients with melanoma is closely related to the depth of the tumor, which in turn increases with time. Consequently, timely recognition and diagnosis, as well as prompt treatment, are crucial given the fact that five-year survival in the early stages of the disease exceeds 80% (IA-IIA) (2, 5). In comparison with other malignant tumors, cutaneous melanoma is, due to its localization, easily accessible to physicians for examination and therefore can be early detected by non-invasive approaches such as inspection of the patient's skin, dermoscopy and reflectance confocal microscopy (2). Routine clinical examination of suspected pigmented lesions usually includes the "ABCDE rule" which should indicate the presence of A: asymmetry, B: irregular borders, C: color variations, D: diameter > 6 mm, and E: elevated surface (Figure 1).

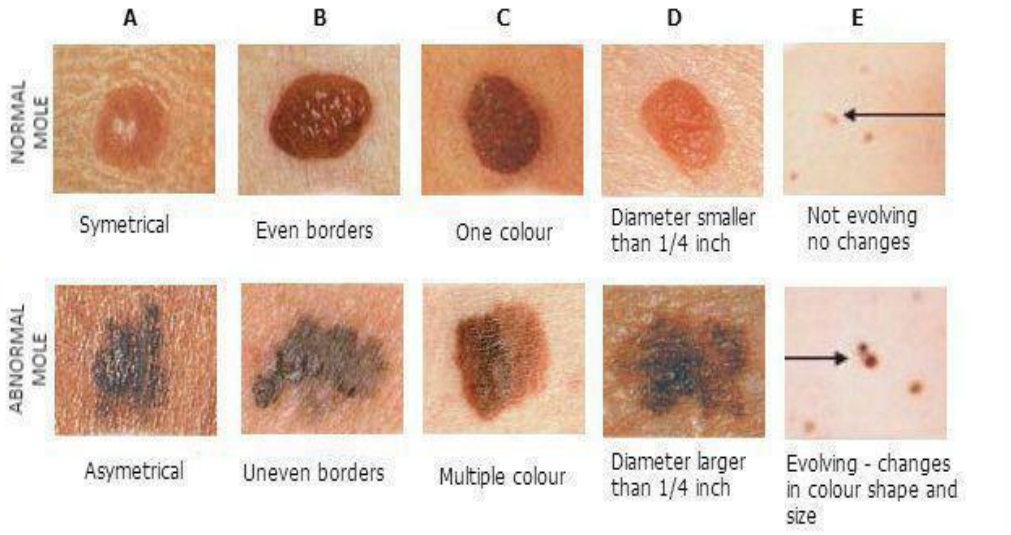

Figure 1. "ABCDE rule" for clinical examination of suspected mole lesions

However, a clinical diagnosis based on naked eye inspection is accurate in approximately 80% of cases if determined by dermatologists, while this rate is significantly lower (about 30%) in non-dermatological specialists (1). Therefore, in order to improve diagnostic efficacy and limit the number of false negative cases, skin surface microscope or a dermoscope can be used for better visualization of the lesion including subsurface features such as deeper pigment and microstructures of the epidermis, dermo-epidermal junction, papillary dermis and vascular structures (1, 8, 9). Although dermoscopy has been shown to be more sensitive and specific in classifying skin lesions than clinical examination with the naked eye alone, it remains limited by significant inter-physician variability and diagnostic accuracy is highly dependent on user experience. Even more, in the hands of untrained practitioners, this diagnostic method may result in poorer performance compared to routine clinical examination and there is a danger of increased excisions, over-referral or false reassurance (8, 10). On the other hand, an artificial intelligence algorithm categorizing photographs of pigmented lesions has been recently developed and it has been shown that it is capable of classifying melanoma with a level of competence comparable to that of dermatologists. However, further evaluation in a real-world, clinical setting is necessary in order to validate this potential diagnostic modality across the full distribution and spectrum of lesions encountered in typical practice (11, 12). Finally, although it is far from perfect, pathohistological analysis of suspected pigmented lesions after excisional biopsy still remains the gold standard for melanoma diagnosis (13, 14). For certain types of lesions there is large inter- and intra-observer variability among community-based pathologist whether a given lesion is benign or malignant.

Surgical treatment

Surgery remains the most appropriate treatment option and gives the best chances for curing patients with local melanoma, as well as disease

control in those with regional spread. Numerous randomized studies have provided better insight into the course of the disease and established certain recommendations for surgical treatment of primary melanoma.

Current, generally accepted algorithm for the surgical treatment of melanoma and "*conditio sine qua non*", begins with a biopsy of skin lesions that are clinically and dermoscopically suspicious of melanoma in order to establish a definitive pathohistological diagnosis (15). Compared to other sampling techniques such as "shave", "punch" and incisional biopsy that can lead to misjudgement of the depth of tumor invasion and, subsequently, inadequate initial surgical treatment, excisional biopsy (with a 1-3 mm surgical margins of clinically unaltered skin) remains the gold standard in diagnosis. The use of magnification, strong light, and, if available, Wood's light or confocal microscope during preoperative marking of the lesion significantly improves the precision level of excisional biopsy in defining detectable tumor margins (16).

In pathohistologically verified primary melanoma of the skin, it is necessary to perform a broad local excision with appropriate margins as recommended by national guidelines. The optimal time interval for performing the final surgical treatment is up to 4 weeks after excisional biopsy, and the recommended width of the re-excision from the middle of the postbiopsy scar depends on the thickness of the tumor according to Breslow (15):

- *in situ* melanoma - resection margins 0.5 cm,
- ≤ 2 mm - resection margins 1 cm,
- 2 mm - resection margins 2 cm.

Reducing safety margins is acceptable for melanoma "*in situ*" which is localized in the facial area, as well as for maintaining function in acral melanoma (the "slow Mohs" technique can be performed), although prospective randomized trials are lacking. In the case of acral lentiginous melanoma, "functional" amputation is indicated, while in subungual melanoma amputation can be avoided only if melanoma is diagnosed "*in situ*" or stage IA (15).

Following local excision, sentinel lymph node biopsy (SLNB) is the next step performed in patients with clinically and ultrasound-undetectable lymph nodes. This diagnostic standard in many patients with invasive melanoma and subsequent (elective) lymph node dissection remains controversial due to the conflicting results of several large clinical studies. According to current recommendations, SLNB is recommended for all patients with moderate melanoma (1.0 to 4.0 mm) (17) and for 0.75 to 1 mm thick high-risk lesions (ulceration and/or high mitotic index on pathohistological findings) (18).

SLNB with subsequent lymph node dissection may increase cure rates for patients with advanced melanoma. However, many patients who have died from melanoma have been shown to have negative SLNB. Also, lymph node dissection itself was not superior in terms of survival from clinical and radiological monitoring of lymph nodes in patients with nodal micrometastases identified by SLNB. It is expected that modern molecular diagnostics will have a more significant impact on the identification of specific risk groups of patients with SLNB in the future. If lymph node metastases are clinically evident or confirmed by ultrasound or computed tomography (CT) scan, radical dissection of the regional lymphatic basin is considered as standard therapy (15).

Melanomas diagnosed during pregnancy can be treated by preoperative lymphoscintigraphy and extensive local excision under local anesthesia, with SLNB under general anesthesia which is delayed until parturition (19).

In patients who have local recurrences (up to 2 cm from the primary tumor), in transit metastases (lesions localized more than 2 cm from the primary tumor to the regional lymphatic basin) or satellitosis that are surgically accessible, radical excision including clinically healthy, protective skin edges should be performed (15).

A deeper understanding of the molecular pathogenesis of melanoma in the last ten years has led to a significant shift in the treatment of patients with metastatic disease. As a result, diagnostic tests, risk assessment, and melanoma treatment are changing faster than ever. In addition to surgery, based on the results of large international studies, "target" treatments and immunotherapy have been introduced, which restored hope to this group of patients with a poor prognosis to fight and cope with a serious illness.

Conclusion

In the era of systemic treatments that have improved the prognosis for patients with advanced disease, surgery combined with early and accurate diagnosis remains the cornerstone of effective treatment of malignant melanoma. Also, given the fact that in many countries patients with suspicious skin lesions first consult a primary care physician, it is of great significance to develop training programs that will facilitate the uptake and use of dermoscopy in primary care. Finally, early prevention of melanoma through educating young people, using sunscreen, wearing protective clothing, limiting sun exposure, as well as identifying high-risk populations, such as those with a potential familial predisposition or gene mutation, is essential to reduce melanoma incidence rates.

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DIJAGNOZA I HIRURŠKO LEČENJE MELANOMA – KRATKI PREGLED

Goran Stevanović[1,2], Stefan Momčilović[2]

[1]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija
[2]Univerzitetski klinički centar Niš, Klinika za plastičnu i rekonstruktivnu hirurgiju, Niš, Srbija

Kontakt: Goran Stevanović
Bulevar dr Zorana Đinđića 48, 18000 Niš, Srbija
E-mail: gste66@yahoo.com

Melanom je redak, ali najsmrtonosniji oblik karcinoma kože. U ranim fazama, melanom se može uspešno lečiti hirurški i stope preživljavanje su visoke. Međutim, kod bolesnika sa prisustvom metastaza, stopa preživljavanja značajno opada. Stoga su rana i ispravna dijagnoza, pravovremeno upućivanje i pravilno lečenje ključni za obezbeđivanje najbolje moguće prognoze bolesnika. Takođe, multidisciplanirni pristup veoma je važan.

Dijagnoza melanoma uglavnom se postavlja dermoskopskim pregledom suspektnih, pigmentnih lezija. Pogrešna dijagnoza melanoma jedan je od najčešćih razloga pokretanja tužbi za pogresno lečenje, kada su patolozi i dermatolozi u pitanju, odmah posle raka dojke.

Što se tiče lečenja metastatskog melanoma, u poslednjih 10 godina razvijeno je nekoliko novih lekova, koji su značajno poboljšali prognozu bolesnika koji boluju od ove bolesti. Međutim, većina bolesnika ne pokazuje trajni odgovor na ove tretmane. Stoga su potrebni novi biomarkeri i nova ciljna mesta za delovanje lekova, kako bi se poboljšali tačnost dijagnoze i lečenje melanoma.

Ovaj članak govori o trenutnom stanju dijagnoze i lečenja melanoma kod nas i u svetu, na osnovu opšte prihvaćenog konsenzusa u ovoj oblasti i aktuelnih vodiča sa preporukama o lečenju. Takođe, pomenuti su i delovi koji su u algoritmu lečenja ostali otvoreni,tj. nedovoljno jasno definisani.

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Ključne reči: *melanom, dijagnoza, terapija, hirurgija, dermoskopija*

Review article

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TODAY'S CHALLENGES - TREATMENT OF ANEMIA IN PATIENTS WITH RENAL FAILURE IN COVID-19 CIRCUMSTANCES

Branka Mitić[1,2], Zorica Dimitrijević[1,2], Radmila Veličković Radovanović[1,2]

A high rate of severe anemia wasobserved in patients with acute kidney injury (AKI) and also in patients on dialysis or chronic kidney disease (CKD) who contracted anew infectious disease caused by SARS-CoV-2. The most severe anemia in COVID-19 occurs in people with severe systemic inflammation, which may occur during illness. Recent studies showed that elevated concentrations of D-dimer are associated with lower hemoglobin and higher serum ferritin. A controversial aspect of therapy in patients with kidney diseases and COVID-19 infection is observed in both populations (with AKI or CKD) about the use of erythropoiesis-stimulating agents (ESA) for the treatment of anemia.

Erythropoiesis stimulating agents represent a revolution in the treatment of anemia in patients with kidney disease. But, he combined interaction of the inflammatory and immune systems with the coagulation system is extremely pronounced in patients with COVID-19 infection. The question is how to treat anemia in patients with COVID-19, whether ESAs are potentially harmful or beneficial, what encourages us to continue the treatment of anemia in patients with COVID-19 usingESA and what are the possibilities to reduce or exceed the risks, as well as whether this therapeutic approach is a new challenge in the treatment of Covid-19 infection.

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Key words: *kidney disease, anemia, Covid-19*

[1]University of Niš, Faculty of Medicine, Niš, Serbia
[2]University Clinical Center Niš, Clinic of Nephrology, Niš, Serbia

Contact: Branka Mitić
48 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: miticdrbranka@gmail.com

Introduction

The SARS-CoV-2 virus has caused an insufficiently known infectious disease in the human population, clinically presented as an acute respiratory disease with pneumonia (but also with involving kidney, heart, digestive tract, blood and nervous system). In most cases, the infection either does not produce any symptoms or causes a mild disease similar to flu, which resolves spontaneously. About 16-20% of cases are classified as "serious" or "critical" (1-3). Recent studies (4-10) indicate transmission of the infectious agent in the period before the onset of any symptoms or asymptomatic cases. The infectious period before the onset of symptoms is not well defined, and according to available data (11-13) lasts on average 2 days before the onset of symptoms. It is suggested that the presence of asymptomatic or infectious patients may be a key epidemiological problem to prevent the spread of COVID-19 in dialysis units.

There is no evidence that COVID-19 infection "damages" the kidneys in those with mild to moderate infection. However, when a serious infection develops and requires hospitalization, kidney abnormalities are noticed in 25-50% of the subjects (proteinuria and erythrocyturia).

A small percentage (less than 15%) develops a decrease in renal filtration function (acute kidney damage).The incidence of acute renal impairment (AKI) with SARS COV-2 is mainly due to acute tubular necrosis (ATN) and rhabdomyolysis.

Studies show that the prevalence of chronic kidney disease among patients infected with Covid-19 is about 0.09%-47.05%. There are no reliable data on the long-term consequences of kidney disease in patients who have survived COVID-19 infection (14-16). However, in patients with acute kidney injury (AKI), as well as in those with pre-existing chronic kidney disease and on dialysis, the presence of a high degree of severe anemia is observed. The most severe degree of anemia is observed in people who develop severe systemic inflammation during the course of the COVID-19 disease, with lower hemoglobin values and high

serum ferritin levels being associated with high concentrations of D-dimer, as confirmed by numerous studies.There is a considerable controversy about the use of erythropoiesis-stimulating agents (ESAs) in the treatment of anemia in patients with either acute kidney injury or chronic kidney disease and COVID-19 infection (17).

The relationship between anemia and inflammation is referred to as chronic disease anemia syndrome or inflammatory anemia. Hepcidin, on the one hand, limits the absorption of iron from the intestine, and on the other hand, the release of iron from the depot in the spleen and the liver, and thus modulates the ferrokinetic response in the presence of inflammation. As a result of limiting the entry of iron into the circulation and tissues, the organism is protected from infection, bearing in mind the fact that most microorganisms significantly use iron as a necessary nutrient. Decreased hemoglobin concentrations, with concomitant increases in serum ferritin levels, are clinical signs of hepcidin induction due to iron trapped in depots, even when there is low circulating iron concentration and low transferrin saturation (18).

The treatment of anemia in patients with COVID-19 and renal insufficiency, as well as what potential effects use of ESA may have, beneficial or harmful, are key issues. The interaction of the inflammatory/immune system with the coagulation process in people with COVID-19 infection was emphasized. The presence of blood clots in the venous and arterial systems of patients is often proven in COVID-19 infection. There is also a danger of more frequent clotting of hemodialysis filters, especially when applying the therapy of continuous replacement of renal function. Pulmonary thromboembolism has also been reported more frequently (19, 20). Experience has shown that for many patients with COVID-19, who are in hospital treatment, it is necessary to use more aggressive protocols for thromboprophylaxis.

There is an intense inflammatory phase in patients with severe COVID-19 infection. Characteristically, the effectiveness of agents for stimulating erythropoiesis is very limited in the inflammatory state (21). It is expected that in the state of inflammation during COVID-19 infection, there will be no adequate response to the use of ESA in patients with anemia, regardless of whether renal disease is present or not. Recent studies announce a new class of stabilizers of factors that cause hypoxia. These are oral preparations that, by their effects, stimulate the production of erythropoietin and increase the availability of iron, which can be considered as potentially more effective than ESA in the treatment of anemia in conditions of intense inflammation (22).

Erythropoiesis stimulating agents represent a revolution in the treatment of anemia in patients with kidney disease. They improve the quality of life and at the same time reduce the need for blood transfusions in this patient population (23).

However, it should be noted that the use of ESA enhances platelet aggregation in hemodialysis patients, which can lead to a prothrombotic condition (24). The side effects of ESA to increase the tendency to thrombosis, with the concomitant prothromboticstatin COVID-19 infections, further increase the unfavorable treatment outcome of these patients (25).

COVID-19 infection is often accompanied by a very low hemoglobin concentration, and, in order to maintain systemic oxygenation, often requires the use of blood transfusions, both in patients without and in those with acute renal injury. The use of erythropoiesis stimulating agents in these individuals is not recommended due to the potentially high risk of side effects, which outweigh the potential benefits.

The production of erythropoietin (EPO) in patients with chronic renal failure and those on dialysis, is significantly reduced, which makes these patients incapable to tolerate the anemic effects of COVID-19 infection. Recommendations for the use of erythropoiesis stimulating agents in these conditions differ. For the patients who are hospitalized due to the severe clinical course of COVID-19 infection, and have already received the appropriate dose of ESA during outpatient dialysis treatment, it is recommended to continue with the same dose to achieve 8 to 9 g/di instead of the recommended 110 to 115 g/dl without COVID-19 infection. It is also recommended that the dose of ESA should not be increased if the target hemoglobin cannot be achieved.

Recent data show that in people with COVID-19 infection, high levels of interleukins (such as IL-1β and IL-6) are accompanied by a more severe clinical course and a higher mortality rate. The results of recent studies suggest that the application of therapies focused on the effects of IL-1β and IL-6 can give promising results (26). In connection with these observations, the immunoregulatory effects of EPO have been shown, which include inhibition of monocytes to produce IL-1β and IL-6 and enable the survival of regulatory T-cells (27). There is also growing evidence of the establishment of global anti-apoptotic effects protection of tissues by the action of erythropoietin, especially in the target organs of COVID-19 (28).

The question is whether this encourages us to continue the treatment of anemia in patients with COVID-19, the use of ESA and what are the possibilities to reduce or exceed the risks, as well as whether this therapeutic approach is a new challenge in the treatment of Covid-19 infection.

Hannelore Ehrenreich, a scientist from the Institute of Experimental Medicine Max Planck investigates the effect of endogenous growth factors over 30 years. "For example, we found that dialysis patients extremely well tolerated Covide-19 - and these patients regularly receive erythropoietin" says Ehrenreich.

The production of endogenous erythropoietin, mostly in the kidney tissue, is stimulated by a reduced concentration of oxygen in the tissues. This cytokine is a signaling molecule for erythrocyte precursors in the bone marrow, and its increased

production enables an adequate supply of oxygen to the brain and muscles. Athletes who take synthetic EPO as a doping agent also use a higher degree of oxygenation that erythropoietin causes. EPO, as a pleotropic hormone, acts not only on erythrocytes but also on many other tissues (29, 30).

The possibility of using EPO as a supportive therapy for severe COVID-19 infection is based on beneficial pleotropic effects on respiratory function, acting on several levels:

1) brainstem and respiratory center,

2) lungs, including protection of overall tissue homeostasis, and

3) n.phrenicus, facilitating respiratory motor control. Erythropoietin, as a proinflammatory cytokine, is responsible for inhibiting the expression of NF-κB in lung tissue, inhibits IL-6 and TNF-alpha and improves the level of the anti-inflammatory cytokine IL-10, thus showing a protective effect on the lung parenchyma. By inhibiting erythrocyte precursor apoptosis, EPO increases red blood cell mass and thus improves tissue oxygenation. In addition, beneficial cytoprotective effects in another various tissues, such as heart muscle, endothelial cells, nervous system, retina, kidney, pancreas, include antiischemic, regenerative and anti-apoptotic effects (31, 32).

Experimental models of EPO effect on liver damage shows reduction of cellular edema (caused by LPS, lipopolysaccharide) in liver lobules, infiltration by lymphocytes and necrosis of hepatocytes (33).

The beneficial effects of erythropoietin in patients with acute renal impairment in sepsis have been accompanied by decreased microvascular damage (34), a reduction in renal inflammation and an improvement in renal tissue oxygenation by a reduction in HIF-1 alpha, iNOS and NF-κB, and an improvement in EPO-R, PeCAM-1, VEGF and VEGFR-2 expression (35).

EPO has cardioprotective effects that are manifested by a decrease in the inflammatory response of the myocardium, it reduces the decrease in the potential of mitochondrial membrane and has antiapoptotic effects on heart muscle cells via the mitochondrial pathway and also alters the expression of NF-κB p65 (a major factor in many inflammatory pathways) (36).

EPO can block the activation of NF-κB and thus affect the modulation of cytokines and its regenerative and anti-apoptotic effects, which can prevent the worsening of the clinical course of the disease COVID-19 (37).

The use of EPO against Covid-19 may reduce severe disease progression.

In a condition where brain damage has occurred during Sars-CoV-2 infection, EPO, acting as a growth factor, can prevent progressive disease and long-term adverse neurological effects, as confirmed by recent studies, EPO improves breathing in case of lack of oxygen

It could protect against neurological symptoms and long-term effects of diseases such as headaches, dizziness, loss of smell and taste and seizures.

The effects of EPO, which lead to a decrease in the levels of IL-6 and modulators of the ferrokinetic response in inflammation, such as hepcidin, result, on the one hand, in an increase in the release of iron from macrophages, and on the other, in an increase in iron absorption in the bone marrow. This reduces the availability of iron as an important nutrient for intracellular organisms, such as Coronavirus, and their enzymatic activities (38, 39).

This possibility of the potential antiviral effect of EPO suggests useful use in human viral infections such as HCV, HIV-1, HBV and CMV, and currently COVID-19 infections.

Conclusion

More clinical studies are needed to answer the question of whether the use of ESA is a reasonable choice in critically ill patients with COVID-19 and what is the optimal dose for the treatment of anemia, with maximum cytoprotective and anti-apoptotic effects and minimal toxicity potential.

The therapeutic approach in patients with kidney disease, especially dialysis patients, requires flexibility in treatment strategy, balancing the potential benefits and harms of ESA therapy, in line with the growing knowledge of the pathophysiology of COVID-19 and its treatment.

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LEČENJE ANEMIJE KOD BOLESNIKA SA BUBREŽNOM INSUFICIJENCIJOM U USLOVIMA PANDEMIJE VIRUSA COVID -19 – IZAZOVI DANAS

Branka Mitić[1,2], Zorica Dimitrijević[1,2], Radmila Veličković Radovanović[1,2]

[1]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija
[2]Univerzitetski klinički centar Niš, Klinika za nefrologiju, Niš, Srbija

Kontakt: Branka Mitić
Bulevar dr Zorana Đinđića 48, 18000 Niš, Srbija
E-mail: miticdrbranka@gmail.com

Kod bolesnika sa akutnim oštećenjem bubrega (AOB), kao i kod bolesnika na dijalizi ili sa hroničnim bolestima bubrega (HBB), koji su oboleli od nove zarazne bolesti SARS-CoV-2, primećena je visoka stopa teške anemije. Najteža anemija, kada je u pitanju virus COVID-19, javlja se kod osoba sa teškom sistemskom upalom, koja se može razviti tokom bolesti, koju ovaj virus izaziva. Nedavna istraživanja pokazala su to da su povišene koncentracije D-dimera povezane sa nižim hemoglobinom i većim serumskim feritinom. Kontroverzni aspekt terapije kod bolesnika sa bubrežnim oboljenjima i infekcijom izazvanom virusom COVID-19 primećen je u obe populacije (sa AOB ili HBB), a odnose se na primenu agenasa za stimulaciju eritropoeze (ESA) u lečenju anemije. Agensi za stimulaciju eritropoeze predstavljaju revoluciju u lečenju anemije kod bolesnika sa bubrežnim bolestima. Ali, interakcija upalnog/imunološkog sistema sa koagulacijom izuzetno je naglašena kod bolesnika sa infekcijom izazvanom virusom COVID-19. Postavljaju se pitanja kako lečiti anemiju kod bolesnika sa virusom COVID-19, da li su ESA potencijalno štetne ili korisne, šta nas ohrabruje da nastavimo lečenje anemije kod bolesnika sa virusom COVID-19 upotrebom ESA i koje su mogućnosti:smanjiti ili premašiti rizike, kao i to da li je ovaj terapijski pristup novi izazov u lečenju infekcije izazvane virusom COVID-19.

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Ključne reči: *bolesti bubrega, anemija, COVID-19*

Review article

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SMOOTH MUSCLE TISSUE OF THE NIPPLE-AREOLA COMPLEX

Aleksandar Petrović[1], Maja Milentijević[2,3], Ivan Ilić[2,3], Tijana Denčić[2,3], Nataša Vidović[2,3], Milica Lazarević[1], Ivan Rančić[1]

The center of the breast integument is characterized by the roundish, glabrous (hairless) musculocutaneous specialization, nipple-areola complex, enriched with the integumentary class of smooth muscle tissue, which bundles are intimately intermingled with fibro-elastic connective tissue of the reticular dermis, with the openings of the distal, ending parts of excretory mammary gland ductal system, situated at the tip of the nipple. Inside this specific breast skin complex composed of two anatomically recognizable regions, muscle tissue is continuous, extending through the areola and nipple, nevertheless, functioning as one anatomic unit. Although present in both genders, in female physiology during the reproductive part of life, it is significantly more developed, and beside sexual arousal reaction, the major function of this structure is transitory contractile activity as essential part of physiological mechanisms necessary for regulated milk releasing during the period of breastfeeding. Nevertheless, analyzed in details and well defined from 19th century, latter, the topic of the nipple-areola complex muscular system in usual textbooks of anatomy and histology conceived unjustifiably a prejudice of marginality. Understanding the necessity for didactical recapitulation and systematization of data about the musculature of the complex and its associated structures, we reviewed the available literature, especially sources which were important for the theme and less often noticed in contemporary reviews.

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Key words: *areola, nipple, smooth muscle tissue, Sappey, Meyerholtz*

[1]University of Niš, Faculty of Medicine, Department of Histology and Embryology, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Department of Pathology, Niš, Serbia
[3]University Clinical Center of Niš, Pathology and pathological anatomy Center, Niš, Serbia

Contact: Aleksandar Petrović
81 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: aleksandar.petrovic@medfak.ni.ac.rs

Introduction

The center of the breast integument is characterized by roundish glabrous (hairless) skin specialization, nipple-areola complex, musculocutaneous specialization whose fibro-elastic connective tissue of reticular dermis is enriched with abundant smooth muscle bundles, and in its center, at the tip of the nipple, are situated openings of the distal, ending parts of excretory mammary gland ductal system (1-6). Although present in both genders, in female physiology, during the reproductive part of life, the major function of this structure is transitory contractile activity as essential part of physiological mechanisms necessary for regulated milk releasing during period of breastfeeding (5, 7-9). Nevertheless, composed of two anatomically recognizable regions, having specific characteristics (nipple and areola), muscle of the complex is continuous, extending through areola and nipple, functioning as one anatomic unit (10). The topic of the nipple-areola complex muscular system is somehow neglected, or suffered from simplifications or fragmentations, and it seems that is better elaborated in older, however often less available sources, so we understand that a review of the available literature should be needed for the purpose of recapitulation and systematization of data about the musculature of the complex and its associated structures.

In the nipple-areola complex, anatomically two major constituents could be recognized:

a) outer, peripheral, roundish, discoid, flattened area – areola (*areola mammae*) (Figure 1 – A) and

b) centrally positioned, elevated, conically shaped, nipple (*papilla mammae*, breast papilla, *mammilla*, mammary papilla, teat) (Figure 1 – N).

Well pigmented, hairless integument of the nipple-areola complex, from the limbus (Figure 1 – L) of areola, where abruptly transitions into the thin hirsute skin of the breast periphery (Figure 1 – obs)

extends toward the openings (Figure 1 – pl) of the ending parts of mammary glands ductal system (Figure 1 – dp), situated at the tip of the nipple. Major, and peripheral part of the complex, areola (Figure 1 – A), is delimited by shallow papillary furrow (Figure 1 – pf) from centrally positioned, nipple (Figure 1 – N). The average areola measures 15 to 60 mm in diameter (5, 11), while the nipple has a range of dimensions, on average 10 to 12 mm in width, and 9 to 10 mm in height, on whose tip there are observable 5-12 openings of the excretory mammary gland ducts (1-6, 11).

Histologically, it is possible to recognize two major structural components of the nipple-areola complex:

1) glabrous skin, with reticular dermis enriched with smooth muscle tissue bundles, and

2) terminal parts of excretory mammary gland ducts, which are opening at the tip of nipple. The skin of the whole complex is composed of epidermis, papillary dermis, and reticular dermis. Dermis of the nipple and areola skin is rich in fibroelastic connective tissue, and its reticular part is enriched with intermingled bundles of smooth muscle tissue. Skin of the areola is also characteristic by specific skin adnexa, Montgomery glands; and the nipple by sebaceous glands of its tip, associated with openings of mammary gland ductal system (1-6, 11). Remaining part of breast integument is represented by thin hirsute skin extending from outer areola limit – limbus of areola (Figure 1 – L), covering the rest of the breast, being continued with skin of thorax at the breast periphery (Figure 1 – obs). The structure of this unspecific skin resembles mostly to the skin of other parts of thorax integument; it is less pigmented than the skin of the areola-mammilla complex, it has hair follicles of vellus type, and hypodermis below, extending toward outer leaf of superficial fascia (1-3, 8, 12-15).

The epidermis (Figure 1 – e) of the nipple and areola have well developed ridges (more pronounced than in the rest of the breast skin), which are interdigitated with large and numerous dermal papillae of the papillary dermis (Figure 1 – dp). This jagged epidemo-dermal junction is considered as morphological adaption to the mechanical demands, present during possible breastfeeding (1, 3, 6, 7, 11, 16). Melanin pigmentation of epidermis is developing after menarche, in nulliparous Caucasian woman is of pinkish nuance, however in pregnant woman, during second month of pregnancy, this complex enlarges (specially areola) and develops brownish, almost black pigmentation, and remains partially maintained lifelong (5, 7, 8, 17-19).

Specific characteristics of the areolar part of the integument

The skin of the areola is characterized by small, papular, nodular structures, Morgagni`s tubercles (Figure 1 – Mt) (sometimes designated as Montgomery`s tubercles) (1, 18). These Morgagni`s tubercles, closer to, and around nipple base have random distribution, and on areolar periphery, are distributed in form of regular circle, parallel to inner side of limbus. Morgagni`s tubercles contain Montgomery`s glands (Figure 1 – Mg), considered as accessory areolar glands (1, 6). Montgomery`s glands are large modified sebaceous glands, developed in range from one alveolus to multilobular racemose glands (Figure 1 – asg), with their own (in 97% of cases), associated, miniature, branched, lactiferous duct (Figure 1 – ald), ending blindly in sub-areolar region (11, 20, 21). Associated sebaceous glands are positioned highly, and their sebaceous ducts are separately opening at the tip of Morgagni`s tubercles or are tributaries to Montgomery`s gland lactiferous duct, just before fusion of its epithelium with epidermis (11, 21). Hair roots (Figure 1 – h) may be the integral part of peripherally positioned Morgagni`s tubercles, near to limbus of the areola. If present, hair follicles, are individualy developped and active, and from them grow hair shafts, vellus or even terminal (8, 22). Lactiferous duct of Montgomery`s gland (Figure 1 – ald) appears to be a miniature of the major mammary system, and may develop fully functional lobules of the mammary gland. These mammary ducts are lined with two layers of cuboidal to columnar cells (11, 21). During lactation, secretions of these glands of areola are involved in lubrication of the areola and nipple skin, reducing irritation during breastfeeding. These glands may produce small quantities of milk, however mostly producing natural substance which has emollient and antiseptic properties. It is believed that the areolar glands may have a role in favorable beginning of breastfeeding and establishing the psychological bond between child and mother (16, 23, 24).

Specific characteristics of the nipple part of the integument

The surface of the nipple tip is coblestone shaped, and criss-crossed by shallow sulci, in whose bottoms there are observable five to dozen openings (*pori lactiferi*, lactiferous porus, Figure 1 – pl) (8, 11, 25) of the most peripheral (ending) portions of the mammary gland excretory system (papillary ducts, *ductus papillares*, Figure 1 – pd) (1, 14, 23). Just before its opening, the papillary duct (Figure 1 – pl) becomes funnel shaped, the part known as infundibulum (Figure 1 – inf), covered with stratified squamous keratinized epithelium, which is continued peripherally with the epidermis of the nipple tip, at the level of the duct oppening (3, 5, 18). Inside the dermis, in the close neighborhood of these infundibula, characteristic, large sebaceous glands of the nipple tip are present (Figure 1 – nsg). The excretory ducts of these sebaceous glands, covered by epidermis, open mostly separately onto the tip of the nipple, or less often into the ending part of the papillary ducts (1, 14). These sebaceous glands are the proof of the mammary gland development phylogenetic association with mechanisms observable during the development of "pilar complex", apo-pilosebaceous (or more specific mammolobular-pilosebacous) units (24). The eccrine sudoriparous glands are rarely, or not present in the skin of the areola and nipples (8, 22).

Often, in the literature sources, the most distal mammary ducts, present inside nipples, for

sake of simplifying explanation, are equilibrated with the lactiferous ducts. However, it is necessary to elucidate that these ducts, by their location, morphology and function, differ significantly from the lactiferous ducts. Namely, these peripheral, ending parts of the mammary gland ductal system are elsewhere known as papillary ducts (*ductus papillares*) (8), and are present only in the nipples. Two or few of them may be merged just before their openings (*pori lactiferi*) at the nipple tip, and toward the base of the nipple, may be continued as single, or arborized in few ductal structures (11, 26). Below the plane of the areola, each of these ducts is afterward continued with a single lactiferous duct (Figure 1 – mld), main draining ductal structure of one lobus of milk gland. At this plane, each lactiferous duct is widened (2-4.5 mm) into cone shaped lactiferous sinus (ampulla) (Figure 1 – ls) (3, 6). The lactiferous sinuses (ampullae) are predestined to function as temporary milk containers during a breastfeeding, however by some authors disputed as the structural element (3, 27). The major difference between papillary and lactiferous ducts is that former are lined by stratified squamous non-keratinized epithelium, and the ending with one funnel shaped infundibulum (Figure 1 – inf) (3), is covered by epidermis (1, 6, 23), and on the contrary, the lactiferous ducts are lined with two-stratified columnar epithelium. The smooth transition from lactiferous ducts toward papillary ducts epithelium is provided by the presence of stratified cuboidal in proximal parts, and stratified squamous non-keratinized, in lactiferous sinuses distal parts (28).

In available literature, one could encounter a versatility of the reported number of mammary glands excretory ducts, integral part of the nipples interior. The recorded numbers of ductal structures present inside the nipple goes from lesser 7 to 12 (21, 29), intermediate 15 to 25 (1, 3, 11, 12, 30) to higher values 27 to 48 (12, 26, 28). Additionally, mentioned versatility is combined with the significantly lesser number of their openings at the tip of the nipple (*pori lactiferi*), differently recorded, 5-10 (8, 11) or 7-12 (25). It is assumed that this ambiguity is based on two major factors:

1) limitations of different research methods applied (canaliculation, casting, histology, radiologic methods) (12, 25), and

2) on the individual variability and complexity of the anastomosing patterns between these ductal structures present inside the nipple (3, 6, 8, 12, 14).

Nevertheless, it is much more important to highlight that all of these ducts show tendency of quasi longitudinal position through the length of the medial, axial zone of the nipple, with three different patterns of their extension (straight, convergent, divergent) (31). Finally, this loose "bundle" of ducts, enters, extends longitudinally inside muscular "cylindric box" of the nipple (will be explained later in detail), and exits from it, finalizing with ductal openings at the tip of the nipple.

Mentioned parts of the excretory ductal system, lactiferous ducts, lactiferous sinuses, and papillary ducts, outside their epithelial lining, possess their own lamina propria (5), composed of fibro-elastic connective tissue (5, 32), and do not have own muscular tunic (33, 34). Ducts of nipple (altogether with their lamina propria), are crenulated structures, approximately 0.5 mm in diameter (12, 28, 30). The lamina propria of these ductal structures is composed of fibro-elastic tissue, in females in reproductive part of life, rich in numerous, coarse, mostly longitudinally oriented elastic fibers (6, 11, 14, 19, 32).

General organization of the nipple-areola complex smooth muscle tissue

Areolar-nipple muscle belongs to class of integumentary smooth muscle tissue (35), which bundles are intimately intermingled with bundles of fibro-elastic tissue of reticular dermis skin of the complex (5, 7-9). The smooth muscle cells may lie in bundles or singly and establish close connection with connective tissue bundles. Connective tissue of the areola and nipple is abundant in elastic fibers, with individual variations (14). Contractions of the smooth muscle cells are transferred to the closest reticular fibers, and then by elastic and collagen fibers of reticular dermis toward papillary dermis of regional skin. The presence of elastic tissue fibers associated with the smooth muscle cells is evidenced in some bundles up to 25% of the cross-sections (6, 14, 36). Besides individual variability, generally elastic fibers are at the height of their development and functionality during the reproductive phase of female life, before are less present, and after menopause degenerate (6). They are the most abundant inside the papillary dermis at the tip of the nipples, where many bundles spray out into extremely delicate fibers which advance and terminate in the vicinity of the basal epidermal stratum, sometimes almost completely fill the corial papillae. Elastic fibers are less present on the lateral sides of the nipples, and in the areola fewer still (6, 14, 37). In two places, namely at the papillary furrow (groove, Figure 1 – pf) and at the tip of the nipple, the musculature enters into a more intimate relationship with the skin (33, 34).

The muscle of the nipple-areola complex extends approximately from the outer border of the complex, the limbus of the areola, and under the base of the nipple continually extending into it, as the conically (dome) shaped muscle part of the complex. Symbolically, it could be said, that the nipple and areola together form an elastic, muscular hollow body in relation to the skin, as Dabelow (19) cites Nagel (38), which could be compared to the shape of a wide-brimmed hat. Marcacci (10) explained, that it is possible to distinguish two faces of the nipple-areola muscle: an upper or cutaneous (toward papillary dermis of integument), and an inferior or glandular (toward mammary gland). The cutaneous surface, follows as the flat structure the superficial parts of the areolar skin, and the other conical, elevated, which follows the structure of the nipple skin including the tip part of it. Here only should be added, that previously mentioned glandular face of the muscle, in areolar portion, is turned toward the mammary gland, and the glandular face of the nipple portion of the muscle, generally facing something which could be considered as the

extension of the mammary gland, the connective tissue of the axial (middle) zone, in which are aggregated and embedded the nipple papillary ducts.

In the nipple-areola complex, bundles of smooth muscle tissue have largely "circular", and to lesser extent radial, orientation inside the reticular dermis, forming planes parallel to the surfaces of the nipple and areolar skin (5, 7-9). It is necessary to highlight that areolar-nipple muscle, in both its parts is dominantly composed of circularly oriented smooth bundles, which could be observed in histologic sections, organized in several layers (33). However, rather than strictly circular, the orientation of smooth muscle bundles is tangential, helicoidal, antiparallel, criss-crossed, and furthermore organized in several physically continuous layers, building tri-dimensional muscular meshwork (19, 38). In explanation of multilayered tri-dimensional meshwork organization of "circular" parts of the nipple areola muscle perfectly fits the description of regional smooth muscle cells morphology given by Gairns et al. (36). Besides the common spindle shaped, they also described the smooth muscle cells of versatile morphology; as a larger, and a very branched, which varied from spindle forms with small spurs, through Y shapes and star shapes, to very irregular forms, showing overlapping, criss-crossing cells. Exactly the branched smooth muscle cell types would be necessary for the formation of complex, tri-dimensional meshwork muscular mass of the nipple and areola.

Specific morphology of areola part of the muscle

Rieffel (39) describes the areola part of the muscle as flattened, white-greyish, continuous layer, with average muscle thickness of 2 mm, and sectioned antero-posteriorly has a triangular profile, or as rhomboid (33), which narrows and extends with its tip to the areola limbus, and its wider part positioned medially, is continued by the muscle tissue of the nipple from its base (Figure 1).

Lactiferous ducts (Figure 1 – mld), from all parts of the mammary gland converge toward the center of the areola, above which is the base of the nipple, and just before, become significantly widened into lactiferous sinuses (Figure 1 – ls). The lactiferous sinuses proximally give rise to narrower ductal structures (35), papillary ducts (Figure 1 – dp), of which one or two may merge with each other, and at the level of their penetration through the openings of the nipple base smooth muscle meshwork, become highly grouped, even "bundled" (26), which is known as a "waist" (12, 28).

Dabelow (19) defines zonal organization of the areolar part of the muscle:

1) the inner muscular ring (at the base of the nipple) (Figure 1 – ic),

2) the wide, tangential, intermediate zone (Figure 1 – it), and

3) the outer muscular ring (Figure 1 – oc), whose muscular bundles, in their distribution, detours around Montgomery`s glands (19).

Vorherr, (3) as a successor of Dabelow, explained: "The muscle fibers around the nipple (base) show a circular course, and from the nipple basis inner tangential, stronger fibers (intermediate muscle layer) leave to form a circumferential fiber meshwork at the periphery of the tissue fibers, thus, creating counter-directed double spirals before inserting as tendinous fibers on papillae of the adjacent corium. This fiber arrangement allows a concentric diminution of the size of the areola mammae as necessary".

The innermost part of the areolar muscle, which latter Dabelow (19) classified as inner muscular ring, previously described by Eggeling (33) as individually varying, circularly oriented smooth muscle tissue, also implying the association with the so-called papillary furrow (Figure 1 – pf), a shallow, ring-shaped furrow of skin, which often separates the root of the wart from the areola.

Additionally, Marcacci (10), Henle (40), Henning (41), Rieffel (39) and Eggeling (33), also described the continuation of areolar smooth muscle bundles, and their extension under the nipple base, taking tangential and slightly curved direction, criss-crossed, positioned just peripherally (above) to lactiferous sinuses, and therefore forming the basket-woven, diaphragm-like plate, through which, without direct contact, papillary ducts are passed. To this diaphragm-like muscular meshwork in the center of the areola, we would like to propose the term, "central areolar feltwork" (Figure 1 – caf), composed of smooth muscle bundles which are extending tangentially between different parts of the areola muscular inner circle, located just between the superficial parts of the lactiferous sinuses, and the proximal parts of the papillary ducts. The papillary ducts continuing distally from the lactiferous sinuses aggregate below this region giving the aspect of narrow "waist" (12, 28) before are passed through this "central areolar feltwork" toward the structure of the nipple.

Often, in numerous textbooks, as well as in introductory texts of research articles, eponyms are used: Sappey`s muscle (Figure 1 – Sm) for circularly organized, and superficially positioned, as well as Meyerholtz`s muscle (Figure 1 – Mm) for deeper located, and radially oriented bundles of the areolar smooth muscle tissue. However, it is interesting that in newer and contemporary literature, the referencing is almost always circumstantial, without quotation of the primary sources. The Meyerholtz`s surname was associated with the radial component of the areolar muscle by Henle in his textbook of Anatomy (40), who worked in the same Institute where Meyerholtz did his investigations. Again, the name was established as the eponym also by Rieffel (39, 42). Neither Henle nor Rieffel quote a published source (42). Furthermore, Marcacci in his article (10) very meticulously analyzed and discussed the structure of the nipple-areolar muscle, and also cited Henle`s credit to Meyerholtz (40). However, it is interesting to mention here, that Marcacci in that article cited Meyerholtz`s surname with transliteration, as Mayerholz. Meyerholtz`s surname was also occasionally cited, through the literature of 20th up to the articles of 21st century, as "Meyerholz". In the

case of Sappey`s eponym, the original description of the areolar muscle was recorded in Sappey`s textbook of anatomy *"Traité d'Anatomie descriptive"* (35). However, in specialized literature dedicated to systematization of eponyms, direct explanation who and when introduced Sappey`s surname as the eponym associated with the structure is not given by specialized publications (42, 43). It seems to us that most probably the use of Sappey`s surname as the eponym for circularly oriented bundles of the areolar muscle was firstly introduced by Rieffel in Poirier`s textbook of Anatomy (39). Finally, we also should be grateful to Rieffel (39), for his precise systematization of the literature results associated to the topic of the nipple-areolar complex muscle tissue, during which he introduced the third eponym "Marcacci`s muscle" for designation of smooth muscle tissue of the nipple-areola complex, which Marcacci (10) analyzed in details, and advocated that the muscle of the complex (areola and nipple) was one inseparable anatomic unit. The term was also later cited by Dobson (42).

Specific morphology of the nipple part of the muscle

The nipple part of the complex muscle is a conical structure, as Marcacci (10) stated, nothing other than the areolar portion molded into the interior of the nipple, so that the muscle part of the areola, at the base of the nipple is introduced (like a finger in a finger cot) into the skin of the nipple, taking, like the latter, a conical shape. However, the muscular component of the nipple has its own specific complexity which could be classified in three different entities:

1) the bulk of the musculature which is composed of circularly oriented smooth muscle bundles, organized in multilayered meshwork, positioned in the reticular dermis of the lateral sides of the nipple integument (muscular wall, muscular columns) (Figure 1 – mc),

2) the nipple tip sphincter feltwork (Figure 1 – smf), and

3) the axial (middle, inner) nipple zone (Figure 1 – anz), with longitudinally (Figure 1 – lb) and transversely (Figure 1 – tb) oriented, scarce smooth muscle bundles.

The fibers which form the external part of the muscle, or the external muscular wall (Figure 1 – mc), intersect and place themselves on the top of each other in a very regular way, forming quite compact nipple muscle cone well accentuated especially in women, where it goes from the base to the upper third of the nipple, being thicker at the bottom than the top (10).

In the upper portion, that is to say at the tip of the nipple, muscular nipple is organized as the muscular mesh (feltwork), leaving spaces which are traversed by the papillary ducts, where these conduits may be tightened by the muscular mesh as by a sphincter (Figure 1 – smf). At this point, the relations of the skin with the muscle and the milk ducts are very close. This plate-like feltwork is supported by the proximal two-thirds of the exterior muscular wall (10). Muscular sphincter feltwork is composed of ramified and ten times narrower bundles than those in lateral parts of the nipple muscle (19, 44).

The muscular wall (Figure 1 – mc) of the nipple appears as a thick muscular cylinder, which is continuation of the innermost part of the areolar muscle at the base of the nipple, and in the region of the nipple tip, "covered" with plate-like part of the muscle (Figure 1 – smf), arranged in a sphincter-like fashion (1), intermingled between ending parts of the papillary ducts. When taken into consideration that the center of the nipple base is also bordered with diaphragm-like muscular meshwork (Figure 1 – caf), the muscular part of the nipple could be described as a barrel like structure through middle part of which, papillary ducts are traversing longitudinally. For this central part of the nipple, encircled with the nipple muscles on lateral sides (Figure 1 – mc), distally with the sphincter feltwork of nipple tips (Figure 1 – smf), and toward the base with "central areolar feltwork" (Figure 1 – caf), we would like to propose the term "axial nipple zone". This "axial nipple zone" (Figure 1 – anz), is occupied by papillary ducts, surrounded by stretchable and mobile fibro-elastic connective tissue (3, 11), in which are disposed longitudinal (Figure 1 – lb) and transverse (Figure 1 – tb) bundles of smooth muscle tissue. Longitudinal bundles of the axial nipple zone (Figure 1 – lb) extending from the base of the nipple (and its areolar "diaphragm" – "central areolar feltwork") toward the nipple sphincter feltwork. The transverse bundles (Figure 1 – tb) are short, extending from the inner side of the muscular wall, and traversing perpendicularly between papillary ducts of the nipple (10, 19, 32, 34, 37). None of the smooth muscle bundles positioned in feltworks (of the areola or nipple) take close physical relation to or are integrated in the walls of the papillary ducts, rather leaving the openings through which these ducts are passed. It is the same for the longitudinal or the transverse bundles of the axial nipple zone (10, 19, 34). A mechanism consisting of smooth muscle and elastic fibers acting as a sphincter at the end of the ducts in the nipple appears to prevent most unwanted loss of milk (1, 11). Musculature, fibro-elastic tissue, and the rest of the complex integument of the complex are involved in processes of breastfeeding as well as in the protection of ductal structures from its shearing stress. The axial zone of the nipple is less "muscular" and its fibro-elastic tissue provides a degree of elastic deformation, which combined with contactless passage of papillary ducts trough the openings of the muscular feltworks, permits partial axial mobility of papillary ducts. As early dissection results evidenced (19, 38), the "loose" interior of the nipple, provides that after the nipple tip is severed, ductal structures of the nipple could be stretched for few millimeters. The fact that papillary ducts are "stretchable" inside muscular "box" of the nipple, limited anteriorly with muscular sphincter feltwork of the tip, and posteriorly, above lactiferous sinuses, with central areolar feltwork, may indicate to the biomechanical system which, during breastfeeding functions as double, unidirectional valve.

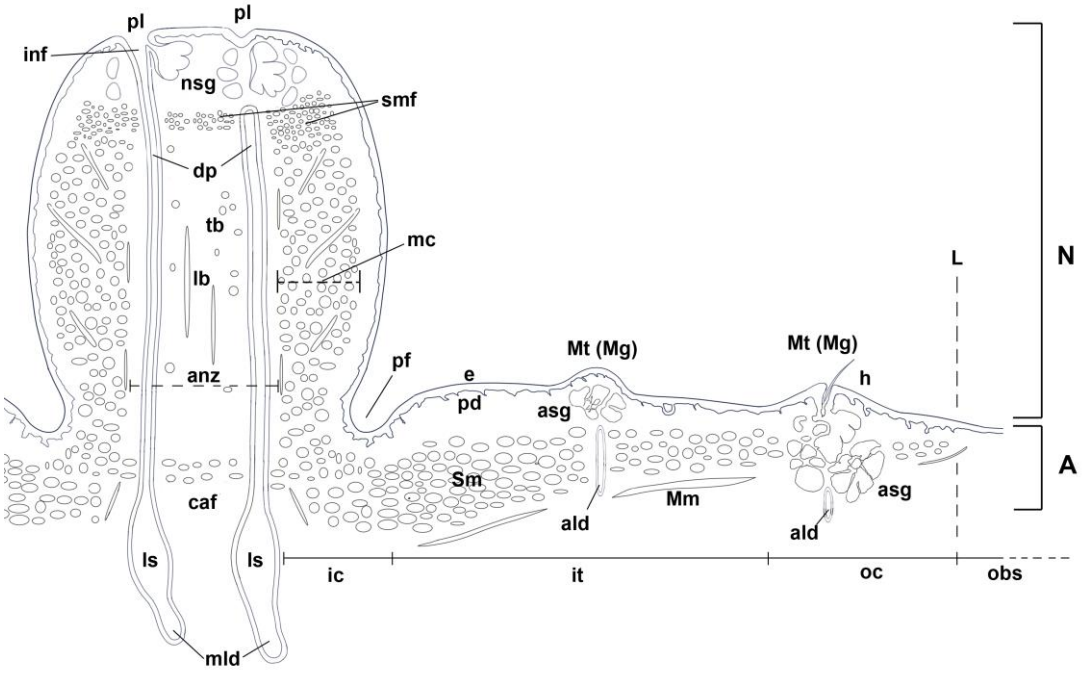

Figure 1. Areolar-nipple complex

A - areola;
N - nipple;
pf - papillary furrow;
L – areolar limbus;
obs – unspecific thin hirsute skin of a breast;
e – epidermis;
pd – papillary dermis;
Mt -Morgagni`s tubercles and Mg - Montgomery`s glands (with own lactiferous ducts – ald);
asg - sebaceous glands of Montgomery`s glands;
h - hair;
mld - lactiferous ducts of mammary gland(s);
ls - lactiferous sinus (ampulla);
dp - papillary duct;
inf - infundibulum of papillary duct;
pl – opening of nipple ducts;
nsg - sebaceous glands of nipple tip;
ic – inner,
it – intermediate;
oc - outer areolar zone;
Sm - circularly oriented bundles of smooth muscle tissue in areola;
Mm - radially oriented bundles of smooth muscle tissue in areola;
caf – central areolar feltwork, smooth muscle bundles of the nipple base;
anz - axial nipple zone;
lb - longitudinal muscle bundles of axial nipple zone;
tb - transversal (interstitial) muscle bundles of axial nipple zone;
mc – nipple muscle wall (circular bundles of muscle columns, tangential bundles of muscle columns);
smf - smaller bundles of papillary sphincter smooth muscle tissue feltwork.

Conclusion

The nipple-areola complex is a unique anatomical structure, characterized by the glabrous skin with a specific adnexa (Montgomery`s glands, and the nipple tip sebaceous glands), equipped with the integumentary class of the smooth muscle tissue, intermingled with the fibro-elastic connective tissue of the reticular dermis, with a major role in regulation of milk through the ending components of the mammary gland ductal system (papillary ducts), opening at the tip of the nipples. The nipple areola muscle is one impartible anatomic unit, organized as a multilayered muscular meshwork, however having specific topographical organization. In the areola, there are recognizable peripherally positioned, with circularly oriented smooth muscle bundles (Sappey`s muscle), and deeper are radially oriented bundles of Meyerholtz`s muscle. Peripherally to the papillary furrow, the areolar muscle could be divided into three zones:

1) inner muscular ring (at the base of the nipple),
2) wide, tangential, intermediate zone, and
3) outer muscular ring.

Additionally, just below the nipple's center, bundles of areola smooth muscle tissue form a basket-woven, diafragm-like plate, through which, without direct contact, papillary ducts are passed, and to which we propose the term "central areolar feltwork".

The nipple part of the complex is organized in three components:

1) the nipple muscular wall (in the reticular dermis of the lateral sides of the nipple integument),
2) the nipple tip sphincter feltwork, and
3) the axial nipple zone, with longitudinally and transversely oriented scarce smooth muscle bundles.

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GLATKOMIŠIĆNO TKIVO AREOLARNO-MAMILARNOG KOMPLEKSA

Aleksandar Petrović[1], Maja Milentijević[2,3], Ivan Ilić[2,3], Tijana Denčić[2,3], Nataša Vidović[2,3], Milica Lazarević[1], Ivan Rančić[1]

[1]Univerzitet u Nišu, Medicinski fakultet, Katedra za histologiju i embriologiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Katedra za patologiju, Niš, Srbija
[3]Univerzitetski klinički centar Niš, Centar za patologiju i patološku anatomiju, Niš, Srbija

Kontakt: Aleksandar Petrović
Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: aleksandar.petrovic@medfak.ni.ac.rs

Centralna pozicija integumenta dojke se karakteriše prisustvom, kružne, glabrozne (bezdlake), muskulo-kutane specijalizacije, areolarno-mamilarnim kompleksom, specifičnim po prisustvu integumentarne klase glatkomišićnog tkiva, čiji su snopovi utkani u fibro-elastično tkivo retikularnog derma, a otvori distalnih, završnih delova ekskretornog duktalnog sistema mlečne žlezde, smešteni u vršnom delu bradavice dojke. Unutar ovog specifičnog kompleksa kože dojke, sačinjenog od dva anatomski prepoznatljiva činioca, mišićno tkivo je kontinualnog prostiranja, i pruža se kroz areolu i bradavicu, funkcionišući kao jedinstvena anatomska jedinica. Iako prisutna kod osoba oba pola, u fiziologiji ženskog organizma, tokom reproduktivnog perioda života, značano biva razvijenija i pored uloge u reakcijama polnog uzbuđenja, glavna funkcija ove strukture je tranzitorna kontraktilna aktivnost, kao deo fiziološkog mehanizma kojim se obezbeđuje kontrolisano oslobađanje mleka tokom procesa dojenja. Još tokom istraživanja sprovedenih tokom devetnaestog veka, ova struktura je analizirana i definisana do detalja, ali je kasnije, u uobičajenim udžbenicima anatomije i histologije zapostavljena zbog predrasude o marginalnosti značaja. Shvatajući potrebu za didaktičkom rekapitulacijom i sistematizacijom podataka vezanih za muskulaturu i pridružene strukture ovog kompleksa, ovde je predstavljen pregled dostupne literature, naročito iz onih izvora, koji su bili od značaja za razvoj teme, ali su ređe bili predstavljani u savremenoj naučno-stručnoj publicistici.

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Ključne reči: *areola, bradavica, glatkomišićno tkivo, Sappey, Meyerholtz*

Case report

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MULTICENTRIC SPINAL CORD AND BRAIN GLIOBLASTOMA: A CASE REPORT

Bojan Stanojević[1], Jovan Ilić[1], Aleksandar Igić[1], Vesna Nikolov[1,2], Aleksandra Aracki Trenkić[3], Marija Djordjević[2], Slavko Živković[1], Stefan Todorović[4]

Multicentric glioblastomas, which simultaneously involve supra- and infratentorial areas, are rare. In our patient, the magnetic resonance imaging (MRI) of cervical and thoracic spine was performed, which verified the spinal intramedullary tumor at the level of the C6 and from Th1 to Th4 segment. During surgery, the tumor, which had macroscopic characteristics of glioblastoma was encountered and it was partially resected. Pathohistological findings verified that the tumor was IDH-wild type glioblastoma. The MRI of the brain was performed after surgery, which showed the right temporoparietal glioblastoma. The patient underwent the postoperative chemoradiation therapy and came for regular check-up examinations for 6 months, however, the patient's neurological signs and symptoms have gradually worsened to this day. Although diagnostic advancements in neuro-oncology have led to more sensitive and specific diagnosis of multicentric gliomas, this topic is still insufficiently researched and requires our attention.

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Key words: *glioblastoma, surgical oncology, neurosurgery*

[1]University Clinical Center of Niš, Department of Neurosurgery, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Niš, Serbia
[3]University Clinical Center of Niš, Department of Radiology, Niš, Serbia
[4]University Clinical Center of Niš, Department of Neurology, Niš, Serbia

Contact: Jovan Ilić
112/12 Byzantine Blvd., 18000 Niš, Serbia
E-mail: jovanilic94@gmail.com

Introduction

Glioblastoma multiforme (GBM) represents circa 45.6% of primary malignant brain tumors (PMBT), as well as about 54% of all glial tumors (GT), and infratentorial presentation is unusual (1, 2). Moreover, when it comes to the frequency and aggressiveness, it takes the first place of all PMBT. The average survival rate of treated is only 14.6 months (2). As a grade IV astrocytoma, it consists of tumor cells with rapid and infiltrative growth, while histological methods show characteristic malignant morphology, necrosis of tumor cells and neovascularization (3). Multicentric glioblastomas (MCGB) represent spatially and temporally separated GBM, which incidence ranges between 0.15 and 10% among GBM. MCGB, which simultaneously involve supra- and infratentorial localization, are rare. Moreover, they represent isolated tumor masses, located in different parts of the central nervous system (CNS), which cannot be explained by local spread, dissemination via cerebrospinal fluid (CSF), blood or through commissural pathways (1, 2). Continuity cannot be demonstrated between tumor masses microscopically or macroscopically, and these do not represent satellite lesions of the primary tumor (3, 4). If the conditions above are not met, then the tumor most likely represents a multifocal glioblastoma (MFGB) (3, 5).

In this case report, we present a patient with MCGB, with consideration of appropriate therapeutic options.

Case report

A 38-year-old male patient was admitted to the Clinic of Neurosurgery because of the interscapular pain with propagation towards the right shoulder. He also stated that, during the last month, the pain was accompanied by a change in the walking pattern as well as bilateral leg numbness, predominantly in the left. The magnetic resonance imaging (MRI) of the cervical and thoracic spine was performed, which confirmed an intramedullary tumor at the level of the C6 and from Th1 to Th4 medullar

segment, which pushed the rest of the spinal medulla to the right (Figure 1, 2).

At the admission, the neurological examination showed the Glasgow Coma Scale score of 15. Postural tremor of the right hand was noted, while the walking pattern was bizarre. Lazarević's sign was positive bilaterally at about 40 degrees. The patellar and ankle reflexes were amplified on both sides. Ankle and knee clonus were positive. Babinski's sign was bilaterally positive, and hypoesthesia was also recorded for dermatomes from L1 to S3 on the left.

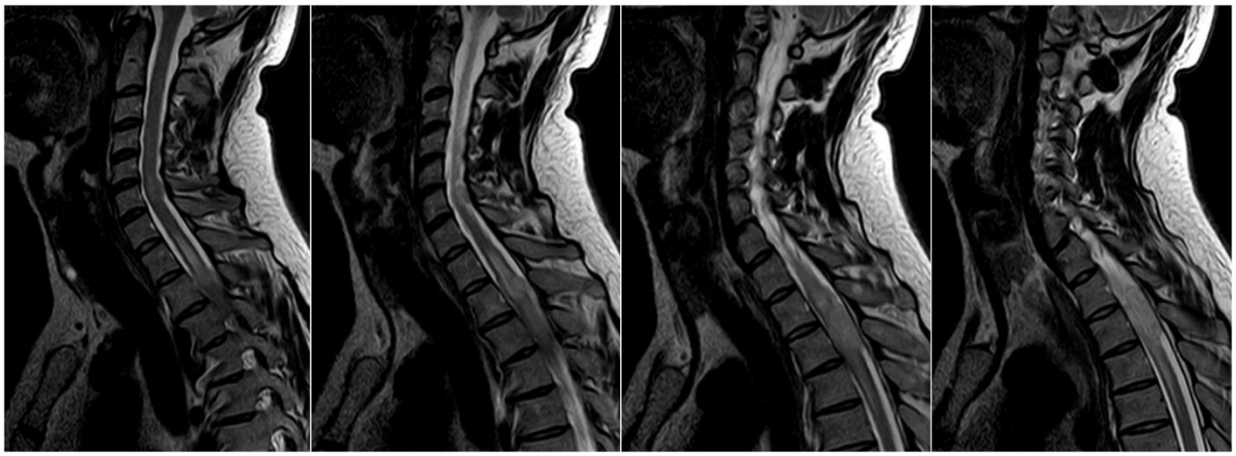

Figure 1. Preoperative sagittal T2W tomograms detect an intradural and predominantly extramedullary lesion at the C6 level, on the left posterolateral side, which cannot be clearly delineated from the spinal cord;
from levels Th1 to Th4, predominantly on the left posterolateral side, an intradural tumor lesion is detected with consequent compression of the spinal cord, dislocating it contralaterally in the cranial aspect, while the caudal part of the tumor infiltrates and expands the spinal cord.
Diffuse myelopathic type changes of medullary signal in the cranial and caudal part of the tumor are noted.

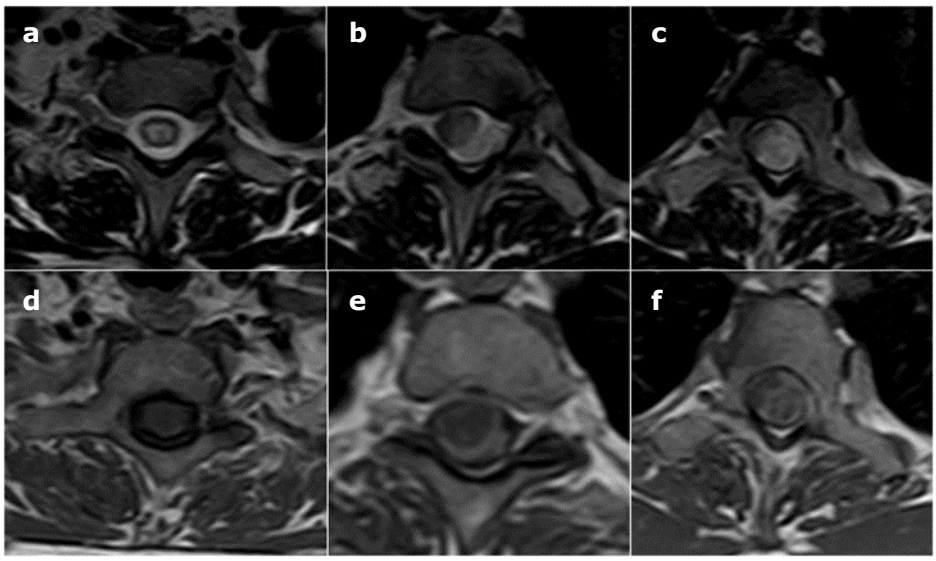

Figure 2. Preoperative axial T2W (a, b, c) and T1W postcontrast (d, e, f) tomograms indicate intradural and intramedullary lesions with extramedullary propagation

After adequate preoperative examination, surgical treatment of the patient was planned. Laminectomy was performed from the Th1 to Th4 vertebrae and the dura matter was opened in the middle. The tumor was encountered, which was adherent to the spinal cord and without noticeable borders towards the spinal cord, and was partially resected. The roots of the Th2 and Th3 spinal nerves were resected as well. The postoperative course went with severe spastic paraparesis and loss of sensitivity for all types of somatosensory senses, from the dermatome Th2 below. After two weeks of conservative treatment and physical therapy, the patient's neurological status improved, achieving the MRC (Medical Research Council scale) grade of 3 for both legs. Deep sensibility recovered gradually, while hypoesthesia persisted at the previously mentioned level. Control computed tomography (CT) of the thoracic spine showed a subcutaneous hematoma in the operative region, which resolved spontaneously. Thereafter, a control MRI of the cervical and thoracic spine was performed, which showed vaguely tumor masses at the level of the C2 and C3 vertebrae, measuring about 7 x 3 mm, as well as tumor mass at the level of the C6 vertebra, measuring 17 x 8 x 5 mm, which could not be clearly differentiated from the spinal medulla (Figure 3, 4).

Afterwards, the patient underwent a rehabilitation treatment for one month with moderate paraparesis. The MRI of the brain was performed, which recorded the right temporoparietal tumor with transcallosal invasion, as well as the signs of Wallerian degeneration (Figure 5). Pathohistological findings verified that the tumor was IDH-wild type Glioblastoma (WHO grade IV). Consequently, daily concomitant temozolomide at 75 mg/m^2 as an adjunct to craniospinal radiotherapy (302 Gy¼60 Gy of the involved field) followed by up to six cycles of temozolomide at 150-200 mg/m^2 on 5 out of 28 days was indicated. The patient came for regular checkup examinations for 6 months, however, the patient's neurological signs and symptoms have gradually worsened to this day.

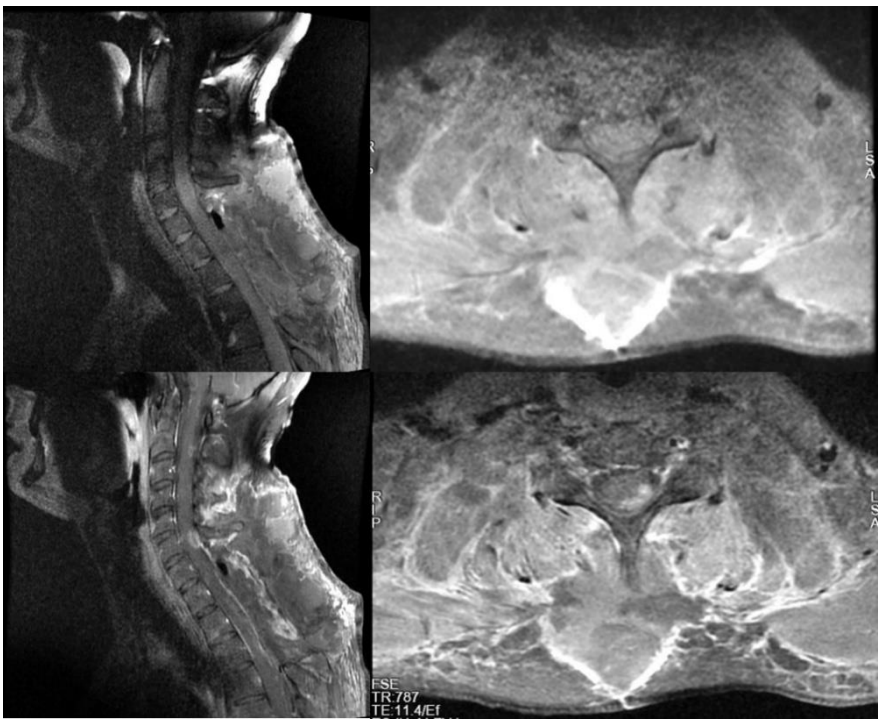

Figure 3. Postoperative sagittal and axial pre- and postcontrast T1W tomograms with fat suppression of the cervicothoracic segment of the spine indicate the presence of intradural, extramedullary lesions in the posterolateral left segment of the canal with marginal contrast capture, without delineation in relation to C2 and C6 medullary level; on the segment of Th1-Th4 spinal cord, in the operative field, is an expanded lesion, with T1W hyposignal, as well as discrete linear and "patchy" post-contrast signal amplification, in favor of probable remnant and post-therapeutic sequelae.

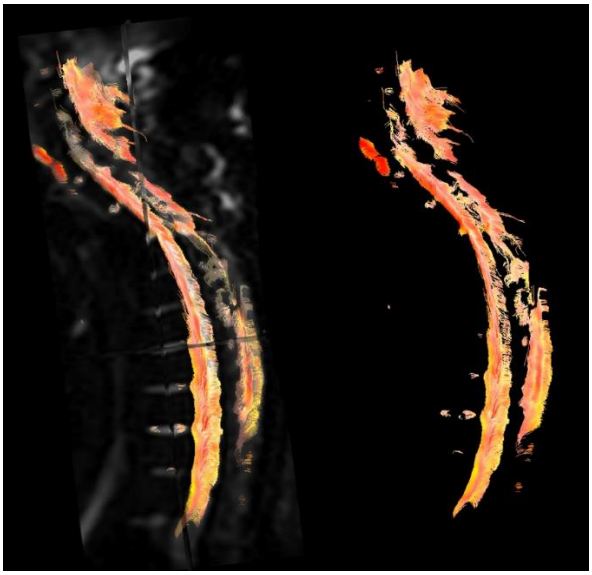

Figure 4. Postoperative cervical and thoracic MRI tractography shows the preservation and continuity of most of the fibers with partial destruction in the posterior part, predominantly at the levels of Th2 and Th3 of spinal cord.

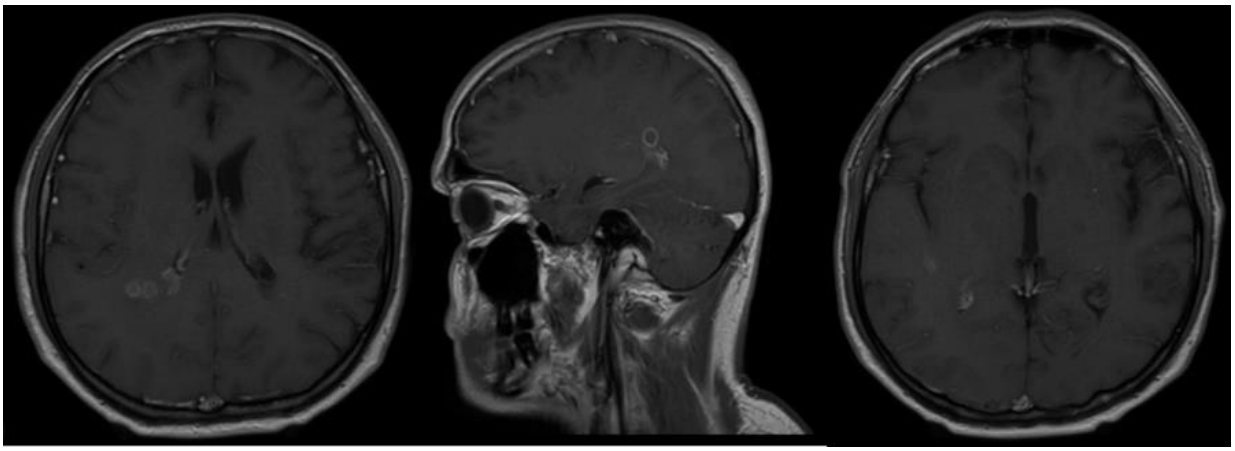

Figure 5. Postcontrast postoperative T1W tomograms show marginal pathological signal enhancement of oval confluent lesions, typical for high-grade gliomas.

Discussion

Most MCGB are localized supratentorially, and simultaneous supra- and infratentorial localizations are less common, while infrateritorial MCGB mostly occur in the cerebellum or brainstem (4). According to some authors, MCGB are defined by supra- and infratentorial localization (5, 6).

To the best of our knowledge and according to the literature we reviewed, this is the first described case of MCGB that simultaneously affects the brain, cervical and thoracic part of the spinal cord.

Various hypotheses and theories on the origin of multicentric gliomas have been proposed, but the etiopathogenesis has not yet been fully elucidated (7, 8). Conhein et al. hypothesized that multicentric gliomas occur when there are multiple embryonic residues in different parts of the CNS (9). Willis et al. considered the possibility of developing multicentric gliomas in two phases. During the first phase, there is a neoplastic transformation of CNS tissue, which covers a large area, whereas in the second phase, there is a neoplastic proliferation at different sites of the CNS (10, 11). Other authors believe that multicentric gliomas are actually metastatic in nature, but

the spreading path from primary glioma to secondary tumor mass has not yet been proven (12). Some authors have highlighted the importance of the calcium-binding protein Mts1/S100A4 in migration, invasiveness, dissemination and interactions of high-grade glioma cells with the surrounding brain tissue (13). They obtained the results that supported the hypothesis that higher levels of Mts1/S100A4 protein in glioma cells and astrocytes positively correlated with the invasiveness and dissemination of glioma cells (13).

The spread of tumor cells via the cerebrospinal fluid (CSF) has been considered as a potential mechanism for the development of MCGB (14), but cytological examination of the CSF in our patient did not reveal the presence of tumor cells. Existing techniques for revealing tumor cells in CSF are known to have limitations in terms of sensitivity and specificity, so other techniques are being developed, such as immunohistochemistry, flow cytometry, PCR as well as non-cellular biomarkers and other *in vivo* methods, which could provide more credible results in the future (15). Jomin et al. considered that multicentric gliomas were of low-grade malignancy, while high-grade gliomas metastasized early and represented multifocal gliomas (16). Contrary to this, our patient with multicentric glioma had pathohistologically confirmed IDH-wild type glioblastoma.

Some authors have described MCGB on the MRI as tumor masses outside the cortical-subcortical boundaries, or localized in the deep white matter of the brain. The same authors believe that these MCGB are presented as solid tumor masses of irregular nodular shape without central necrosis, which differs significantly from metastasis (12). Contrary to this, the brain MRI in our patient showed the presence of oval and confluent lesions with necrotic central part within the deep white mass, as well as extensive surrounding vasogenic edema extending along the corpus callosum and contralaterally, with signs of Valerian degeneration and mild mass effect. Some other authors support the fact that MCGB appear deeper within the white matter of the brain on MRI images, bind contrast more densely, and that compared to metastasis, the surrounding vasogenic edema is more extensive, which is in accordance with our results.

Earlier, neuroimaging methods for the detection of MCGB had serious limitations and over time have further developed and become more sensitive. There is a reasonable suspicion that many cases of MCGB would have been diagnosed as multifocal by using modern neuroimaging, including the FLAIR MRI sequence. Probably because of this, some authors considered that there was no practical value of differentiation between multicentric and multifocal gliomas (17, 18).

Multiple tumor masses, that are also spatially separated, reflect on the patient's performance status, and treatment with aggressive tumor resection is less common. Since these tumors are more often located in the deep white matter of the brain or in the posterior fossa, and they can affect the opposite hemisphere, the survival prognosis is poor. Furthermore, patients with multiple tumor masses limited to one brain compartment have a similar survival prognosis as patients with a solitary lesion. Moreover, maximal tumor resection is rarely achieved in patients with multifocal and multicentric gliomas, so these patients have a worse prognosis (18, 19).

Since on the preoperative MRI of the cervical and thoracic spine, the tumor masses were presented intradurally and extramedullary, we opted for a total resection of these tumors. After laminectomy from the level of Th1 to Th4 vertebrae, we found a tumor mass that was presented differently intraoperatively compared to the MRI presentation, as a vaguely limited intramedullary tumor that was predominantly necrotic and macroscopically corresponded to a high-grade glioma. Therefore, during surgery, we changed the decision on treatment and decided on the maximum reduction of tumor mass only at that level, without operating on at the level of C6 segment of the spinal cord, until the results of pathohistological analyzes were delivered. Considering that this is a very rare localization of MCGB, more precisely, that we have not been able to find such a case described in the literature, MRI of the brain was performed only after obtaining the pathohistological results. We did not opt for preoperative CT or MRI of the brain because the initial clinical presentation could have been explained by the localization of MCGB in the cervical and thoracic part of the spinal cord. Had we performed a preoperative MRI of the patient’s brain, our team of neurosurgeons would have probably opted for other approach, such as a biopsy. However, it turned out that we made the right treatment decision because the patient had short-term clinical and neurological improvement after the operation. Finally, we believe that comprehensive diagnostic analyzes and procedures are necessary when diag-nosing MCGB.

Conclusion

Although advances in diagnostic technology of brain tumors have led to more sensitive and specific diagnosis of multicentric and multifocal gliomas, this topic is still insufficiently researched and requires our attention. The clinical presentation and prognosis of patients with MCGB, in comparison with patients with solitary GBM, is worse.

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Prikaz bolesnika

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MULTICENTRIČNI GLIOBLASTOM SPINALNE I KRANIJALNE LOKALIZACIJE: PRIKAZ SLUČAJA

Bojan Stanojević[1], Jovan Ilić[1], Aleksandar Igić[1], Vesna Nikolov[1,2], Aleksandra Aracki Trenkić[3], Marija Đorđević[2], Slavko Živković[1], Stefan Todorović[4]

[1]Univerzitetski klinički centar Niš, Klinika za neurohirurgiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Srbija
[3]Univerzitetski klinički centar Niš, Klinika za radiologiju, Niš, Srbija
[4]Univerzitetski klinički centar Niš, Klinika za neurologiju, Niš, Srbija

Kontakt: Jovan Ilić
Vizantijski bulevar 112/12, 18000 Niš, Srbija
E-mail: jovanilic94@gmail.com

Multicentrični glioblastomi, koji se istovremeno nalaze supratentorijalno i infratentorijalno, retko nastaju. Kod našeg bolesnika, urađena je magnetna rezonanca (MR) vratne i torakalne kičme, kojom je otkriven spinalni intramedularni tumor na nivou C6 i od Th1 do Th4 segmenta. Tokom operacije, prikazan je tumor, koji je imao makroskopske karakteristike glioblastoma i obavljena je parcijalna resekcija istog. Patohistološkim nalazom potvrđeno je da se radi o glioblastomu IDH divljeg tipa. Nakon operacije,odrađena je MR mozga, kojiom je evidentiran desni temporoparietalni glioblastom. Bolesnik je potom podvrgnut postoperativnoj hemioradijaciji i redovno su obavljani kontrolni pregledi tokom 6 meseci, ali su se, do danas, neurološki znaci i simptomi kod bolesnika postepeno pogoršavali. Uprkos napretku dijagnostike u neuroonkologiji, a posledično i u postavljanju dijagnoze multicentričnih glioblastoma, ova tema je još uvek nedovoljno istražena i smatramo da su potrebna dodatna istraživanja u ovoj oblasti.

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Ključne reči: *glioblastom, onkološka hirurgija, neurohirurgija*

Case report

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TREATMENT OF A LARGE LENTIGO MALIGNA AND LENTIGO MALIGNA MELANOMA WITHIN THE LESION WITH INCISIONAL BIOPSY AND 5% IMIQUIMOD

Milica Gajić[1,2], Dejan Ogorelica[1,3], Milana Ivkov Simić[1,3], Sonja Prćić[1,4], Milan Matić[1,3], Branislava Gajić[1,3]

Lentigo maligna melanoma (LMM) is an invasive melanoma most commonly occurring on the head and neck. The diagnosis is aided by specific dermoscopic criteria and confirmed by biopsy. The treatment of LMM is surgical excision. There are alternative therapies for its precursor lesion lentigo maligna (LM, also known as Hutchinson's melanotic freckle) – melanoma in situ, and they include the application of topical 5% imiquimod cream. Our patient had a 7 x 4 cm lesion with dermoscopic features of both LM and LMM. The diagnosis was confirmed by pathohistological examination of the incisional biopsy. The patient, concerned about the aesthetic outcome, refused surgical treatment and was treated by 5% imiquimod cream. Dermoscopy aided the clinical diagnosis, it allowed for a non-invasive follow-up and tailoring of the treatment in order to attain satisfactory results – evanescence of dermoscopic features suggestive of LM and LMM and an aesthetically acceptable outcome after treatment.

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Key words: *Hutchinson's melanotic freckle, melanoma, biopsy, dermoscopy, imiquimod*

[1]University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia
[2]Policlinic "Novakov i sar.", Novi Sad, Serbia
[3]University Clinical Center Novi Sad, Clinic of Dermatovenereology Diseases, Novi Sad, Serbia
[4]Institute for Child and Youth Health Care of Vojvodina, Pediatric Clinic, Novi Sad, Serbia

Contact: Milica Gajić
2 Trg neznanog junaka, 21000 Novi Sad, Serbia
E-mail: milica.gajicns@gmail.com

Introduction

The most common type of melanoma *in situ* is lentigo maligna (LM, also known as Hutchinson's melanotic freckle), a precursor lesion of the invasive lentigo maligna melanoma (LMM) (1, 2). The diagnosis of both entities is based on the clinical characteristics and dermoscopic features. The diagnosis is confirmed by a histopathologic examination of the excision or biopsy of the lesion. Dermoscopy aids in selection of the site of biopsy for an adequate diagnosis and in the complex management of LM and LMM (3). Surgical excision remains the standard treatment for all stages of primary cutaneous melanoma; however, when surgery is not a reasonable option because of the size of the lesion, patient comorbidities or preferences, the alternative therapies are considered for the treatment of LM (4). Alternative therapies supported by the results of a systemic review of non-surgical treatment of LM include radiotherapy and topical imiquimod cream (5). Still there are no review studies supporting alternative therapies in the treatment of LMM.

Case report

An 80-year-old female patient presented with an irregularly shaped, ill-defined, flat pigmented, two colored skin lesion on chronically sun-damaged skin. The lesion covered more than 60% of her right cheek at the time she was referred to our clinic. The lesion was 7 x 4 cm, the size had been reached by slow growing during the last ten years. The patient was otherwise healthy with no history of chronic or malignant disease. Dermoscopic examination revealed a pseudonetwork, presence of a number of colors – light brown, dark brown, black, grey, as well as structureless areas. Also some obliterated hair follicles, increased density of the vascular network and gray circles were among the key dermoscopic features for the dermoscopic diagnosis of lentigo maligna melanoma (Figure 1). The dermoscopic findings were suggestive of LMM within the lesion of LM. The part of the lesion that was suspicious for LMM was marked before the incisional biopsy (Figure

2). The pathologist report staged the melanoma with Breslow depth of 0.2 mm as stage I A. Also, apart from the LMM, there was a lentigo solaris on the lower part of the cheek.

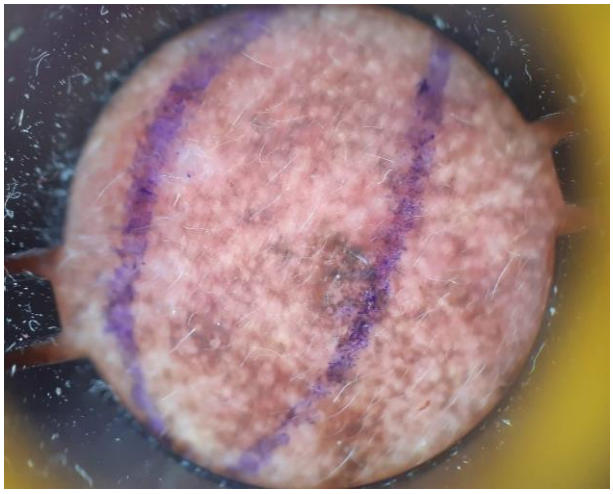

Figure 1. Dermoscopy of the part of the lesion marked up for the incisional biopsy.
The picture was taken by a smartphone camera lens leaned into a Derm Lite DL100 dermatoscope (10 x magnification).

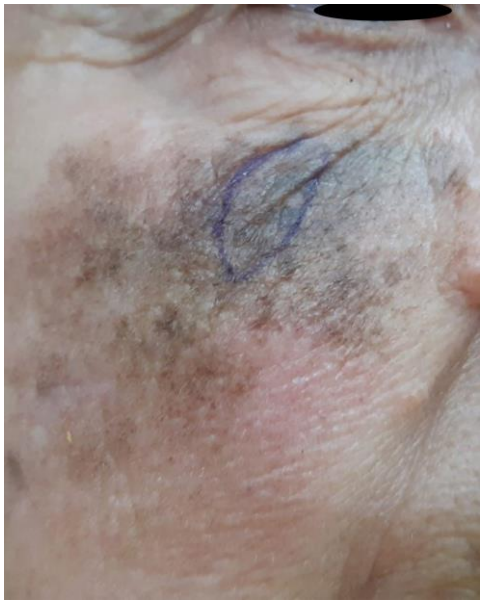

Figure 2. LMM of the cheek, the marked region is the planned site of biopsy.

The patient refused to undergo surgical treatment of the melanoma. After a detailed discussion of alternative treatments of LMM with the patient and the patient's family, the decision was made to start with the daily application of 5% imiquimod cream.

After the ninth month of the topical therapy a pinkish papule of 2 mm in diameter, located in the central part of the lesion in close proximity to the scar of the incision biopsy, was noticed. The papule had been present there for four weeks. It was excised at the follow-up and the pathologists report

showed hyperkeratosis with no atypical or malignant cells.

The last follow-up took place three years after the introduction of topical treatment. Upon clinical and dermoscopic evaluation, a mild erythema was seen in the upper part of the treated field, beneath the lower eyelid, indicating that there is still some ongoing inflammatory reaction to 5% imiquimod cream (Figure 3). Treatment is being continued, in order to be assessed at the next follow-up in a month.

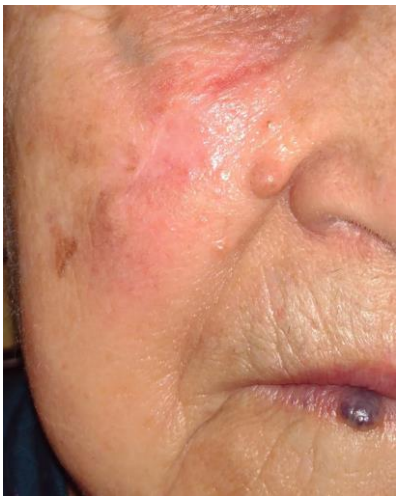

Figure 3. Ongoing reaction to 5% imiquimod cream at 36 months of therapy.

Discussion

LM and LMM most commonly occur on the head and neck, on chronically sun-damaged skin (6). LM arises on the cheeks with a significant female predominance (7); this was the case with the LMM in our patient. LMM arising in the facial skin presents with a different dermoscopic pattern from those observed in melanoma on non-acral skin (8). Dermoscopy is an indispensable tool for the diagnosis and treatment, since it not only aids the diagnosis, but allows for a non-invasive follow-up during treatment, particularly for topical treatments (3). For an accurate and timely diagnosis, being up to date with dermoscopic features of LMM is a necessity (8). The Tiodorovic-Zivkovic et al. study from 2013, highlights that the presence of the gray color in facial lesions is the single most sensitive feature for the dermoscopic recognition of early facial melanoma. Its presence should always prompt the clinician to perform a biopsy (9). We were guided by this clinical thinking during the diagnosis, treatment and follow-up of our patient, too. The classical follicular invasion criteria defined by Stolz et al. were confirmed by Pralong et al. in the study "Dermoscopy of lentigo maligna melanoma: report of 125 cases". The study draws attention to the utility of original new features for the diagnosis of LMM: increased vascular density, red rhomboidal structures, target-like pattern and darkening at dermoscopic examination (8). One of the new original features was present in our patients' lesions – increased vascular density.

The final diagnosis of cancer is based on the pathologist's report, so a biopsy is a mandatory step after strong clinical suspicion. When the clinical diagnosis is cutaneous melanoma, one should ideally perform a narrow excisional biopsy that encompasses the entire breadth of the lesion with clinically negative margins to a depth sufficient to ensure that the lesion is not transected (4). Our patient refused excisional biopsy of the LMM, and consented to an incisional biopsy.

In our clinical case, the LMM was classified as stage IA melanoma. The alternative treatment modalities to surgical excision and their risks were discussed in detail with the patient. Therefore, the decision to start with the topical application of 5% imiquimod cream was made.

Topical imiquimod is a synthetic imidazoquinoline amine that has the ability to increase the production of inflammatory cytokines and chemokines; it induces tumor cell apoptosis and has an anti-angiogenic effect (10). There is no high-quality evidence supporting the use of imiquimod as a single therapy for LMM. However, the results of a systemic review study from 2017, might be of relevance to those patients with LM who refuse to undergo or are not eligible for surgery or radiotherapy. Evidence suggests complete clinical clearance rates of 78.3% and histological clearance rates of 77% after the application of imiquimod cream (11). The proposed

treatment schedule to achieve clinical and/ or histological clearance consisted of a cumulative dose of > 60 applications and a treatment intensity of > 5 applications per week (11). In our case the treatment lasted twelve times longer. No new clinical or dermoscopic features of LMM were observed and the final result was complete resolution of clinical and dermoscopic features of LM.

Conclusion

Being well-acquainted with the dermoscopic features for LM and LMM is the key to an accurate and timely diagnosis. Dermoscopy is a powerful tool for the diagnosis of LM and LMM, it aids biopsy such as selection of the site of biopsy, precise staging of cutaneous cancer and allows for non-invasive follow-up of patients during treatment.

The patient studied in our case refused the complete excision of the lesion fearing the potential facial damage to an aesthetically sensitive region. Based on our experience in this case, 5% imiquimod cream was an acceptable alternative to surgical excision. The treatment was tailored to the patient, and the response to treatment was satisfactory both in respect to the regression LMM, and in the aesthetic outcome after the treatment. Further studies should be performed in cases similar to this one in order to evaluate the effectiveness of the topical application of imiquimod on LMM lesions.

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Prikaz bolesnika

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TERAPIJA VEĆE LEZIJE LENTIGO MALIGNA I LENTIGO MALIGNI MELANOM U OKVIRU LEZIJE INCIZIONOM BIOPSIJOM I 5% IMIKVIMODOM

Milica Gajić[1,2], Dejan Ogorelica[1,3], Milana Ivkov Simić[1,3], Sonja Prćić[1,4], Milan Matić[1,3], Branislava Gajić[1,3]

[1]Univerzitet u Novom Sadu, Medicinski fakultet, Novi Sad, Srbija
[2]Poliklinika „Novakov i sar.", Novi Sad, Srbija
[3]Univerzitetski klinički centar Novi Sad, Klinika za dermatovenerološke bolesti, Novi Sad, Srbija
[4]Institut za zdravstvenu zaštitu dece i omladine Vojvodine, Pedijatrijska klinika, Novi Sad, Srbija

Kontakt: Milica Gajić
Trg neznanog junaka 2, 21000 Novi Sad, Srbija
E-mail: milica.gajicns@gmail.com

Lentigo maligni melanom (LMM) je invazivni melanom koji se najčešće javlja na glavi i vratu, čija se klinička dijagnoza, potpomognuta specifičnim dermoskopskim karakteristikama, potvrđuje biopsijom. Lečenje LMM podrazumeva eksciziju lezije, dok za njegovu prekursorsku leziju lentigo maligna (LM, poznata i kao Hačinsonova melanocitna pega) – melanoma in situ – postoje alternativni terapijski modaliteti, kao što je lokalna primena 5% imikvimod kreme. Naša bolesnica imala je leziju veličine 7 cm x 4 cm sa dermoskopskim karakteristikama LM i LMM. Bolesnica je odbila hirurško lečenje, zbog potencijalne deformacije estetski osetljive regije. Dijagnoza je postavljena patohistološkim pregledom materijala incizone biopsije. Lečena je 5% imikvimod kremom. Dermoskopija je pomogla u postavljanju kliničke dijagnoze, omogućila neinvazivno praćenje i prilagođavanje treapije radi postizanja zadovoljavajućeg rezultata – nestanak dermoskopskih karakteristika, koje sugerišu na LM i LMM i estetski zadovoljavajući rezultat.

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Ključne reči: *Hačinsonova melanocitna pega, melanom, biopsija, dermoskopija, imikvimod*

Review article

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MYCOBACTERIOSIS: THE PAST AND PRESENT

Zoran Stamenković[1], Lidija Ristić[1,2], Ivana Stanković[1,2], Milan Radović[1,2], Slavica Golubović[1], Vesna Ivanović Djordjević[1], Ivan Matejić[3]

Non-tuberculous mycobacteria (NTM) are ubiquitous organisms, they are found everywhere in the vicinity. Humans are in everyday contact with these microorganisms. Although tuberculosis (TB) cases have been declining worldwide, there is a growing incidence of NTM infections. NTM may cause both asymptomatic infection and symptomatic disease in humans. The most common are pulmonary infections of varying severity. Accurate diagnosis is of crucial importance because the treatment medications may have serious adverse effects, among other things. The treatment of mycobacteriosis is not directly analogous to the treatment of tuberculosis. Empiric therapy is not recommended. *In vitro* susceptibility of many NTM does not correlate with clinical response to antimycobacterial drugs.

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Key words: *mycobacteriosis, epidemiology, diagnostics, treatment*

[1]University Clinical Center Niš, Clinic of Lung Diseases, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Department of Internal Medicine, Niš, Serbia
[3]University Clinical Center Niš, Clinic of Thoracic Surgery, Niš, Serbia

Contact: Zoran Stamenković
48 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: zokinis@live.com

Introduction

Although tuberculosis (TB) cases have been declining worldwide, there is a growing incidence of non-tuberculous mycobacterial (NTM) infections. Recent studies have shown an increase of NTM lung infections at an annual rate of 8.2%. The reasons of the increase have not been fully understood yet, but they are probably multifactorial, including environmental factors, host, and microbes. It is generally accepted that increasing prevalence of mycobacteriosis is a consequence of demographic changes, such as aging of the population that weakens immunity and causes a series of predisposing diseases, and increased exposure to, for example, chlorine water while bathing. Increased detection rate is the result of increased awareness of this pathogen and improved detection techniques. The availability of gene sequencing techniques improved the taxonomy of mycobacteria, with significant increase in the number of validly recognized NTM species (1-8). When guidelines of The American Thoracic Society-ATS and Infectious Disease Society of America-IDSA about NTM were published in 1997, there were about 50 NTM species identified. Until the ATS/IDSA guidelines in 2007 more than 125 NTM species have been identified. Currently, there are 233 NTM species and 23 subspecies described. This dramatic increase is not only in the number of new species, but also in the number of clinically important species. There are at least 60 mycobacterial species that cause disease in humans (9-11).

History

First reports on mycobacteria isolation, other than Koch's human tubercle bacilli, date back to 1885, when Alvarez and Tavel isolated smegma bacillus. Probably the earliest case of the disease caused by NTM was reported by Pappenheim in 1898, who described a young woman having 'gangrene of the lung'. In 1908, Duvall reported first, fully docu-mented case of disseminated infection (12). Runyon and Timpe were the first to classify and describe NTM in the 1950s. Runyon observed mycobacteria as a biologist. He divided them into four groups according to their rate of growth, morphology of the colonies, and pigment production in the presence of light into slow-growers (non-photochromogens, photochromogens, scotochromogens) and fast-growers (13). This classification is less useful today since pigment production may vary, serotyping reveals closely related distant species and differences among close categories (14). In 1989, Davidson divided NTM according to their

clinical relevance into conditional pathogens, opportunistic mycobacteria, and pure saprophytes. It is not always easy to define boundaries between them, so it is believed that saprophytes may become pathogenic in certain conditions (9).

Epidemiology

Humans are in everyday contact with these microorganisms. NTM are ubiquitous organisms, isolated from water samples (natural waters – lakes and streams; pipeline systems), soil, dust, raw milk and other animal products. They can also be found in throat swab, sputum culture, gastric content, and urine in healthy persons. From human samples they can be isolated as:

1) an accidental isolate with low number of bacilli isolated once;

2) prolonged saprophyte colonization, especially in lower respiratory tract in patients with chronic pulmonary disease, but it is not known when local invasion into a tissue or the disease progression may occur;

3) real pathogens (12, 15).

Until recently, it has been believed that NTM infection originate from the environment without evidence on human-to-human or animal-to-human transmission, being the reason of less public importance because of not reporting it to epidemiological services, so the prevalence is unknown. However, owing to sequencing of NTM isolates, recent literature data suggest that indirect cross infection with M. abscessus is possible in patients with cystic fibrosis (CF), leading to changes in infection control standards in this group of patients (15).

The rate of disease due to NTM in developed, industrialized countries is in the range of 1.0 to 1.8/100000. The prevalence of NTM pulmonary infections in the United States of America (USA) and Australia range from 3.2 to 9.8/100000, whereas a study registered annual prevalence estimates in Hawaii up to 44/100000. In Europe, the prevalence estimate is generally lower, up to 3.3/100000. The prevalence is significantly higher in certain regions and groups of patients, so the prevalence in persons over 65 years of age in the USA in an 11-year period has been over 100/100000. Such approximate prevalence is calculated according to NTM isolates reports, conducted studies and medical insurance records (6, 10, 16).

The distribution of species varies according to regions. M. avium complex (MAC - M. avium, M. intracellularae) is predominant in North America and East Asia, but not in Europe, where M. kansasii, M. xenopi and M. malmoense are predominant (17). Pathogenicity of NTM species may differ by different geographic regions, and it significantly varies among species, from M. gordonae that rarely causes the disease in humans, to M. kansasii that is usually considered pathogenic (18, 19).

Inhalation of environmental aerosol particles is a primary transmission route of the infection. Infections are also possible by drinking contaminated water, or by using contaminated medical and surgical equipment, hospital-acquired infections. High tolerance to different noxious substances is one of the main reasons of their pathogenicity in humans. Owing to the presence of lipid-rich outer membrane they develop resistance to desinfectants, primarily chlorine and ozone. It is believed that water conditioning and treatment with chlorine negatively selected resistant species. Also, they have the ability of biofilm formation (10, 15).

The mortality rate of NTM disease in HIV uninfected persons in the USA has increased in the period 1999-2014, especially in white, older women. Considering the fact that there has been a simultaneous decline in TB-related deaths, these findings show a change in fatal mycobacterial infections in the USA (20). The mortality rate of NTM pulmonary disease is significant, according to Denmark's population data, five-year mortality rate was 40.1%. In this study, patients with M. xenopi had the worst prognosis (21). The largest population-based cohort based on epidemiologic characteristics of NTM infection aimed at evaluating incidence, prevalence and mortality of NTM infection in Korea showed a rapid increase in incidence and prevalence in the last two decades, higher in women and elderly people. The mortality rate in persons with NTM infection was almost as twice as high than in general population. This trend should be closely monitored in order to provide optimal healthcare policies and treatment strategies for NTM infections (22).

Clinical forms of the disease

NTM may cause both asymptomatic infection and symptomatic disease in humans (10). It is believed that NTM-human relationship is mostly a transient colonization that goes away on its own, since the immune system in the majority of a population kills bacilli (9). The most common and distinct clinical problems include pulmonary disease, lymphadenitis, and disseminated infection, but the infection and disease can occur in other tissues, such as soft tissues, bones, joints, and genitourinary tract. Skin and soft tissue infections are usually iatrogenic, while visceral and disseminated infections are associated with severe immunosuppression (23-25). There are isolated cases of the diseases, such as meningitis, keratitis, mastoiditis, endocarditis, hepatitis, caused by different NTM species (12).

The most common are pulmonary infections of varying severity, from extremely progressive, destructive, to hardly visible changes with minimal physical signs of the disease. There are no specific features to differentiate NTM pulmonary disease from pulmonary TB. Coughing and expectorating are common symptoms and can be accompanied by hemoptysis, high temperature, night sweats, general fatigue, and weight loss, but all of these are much less seen than in TB (14). The most common pulmonary pathogens are: slowly growing - MAC, M. kansasii, M. malmoense, rapidly growing - M. abscessus, M. xenopi, M. chelonae, M. fortuitum (3).

Predisposing diseases for NTM infections are mostly chronic pulmonary diseases, such as chronic

obstructive pulmonary disease (COPD), or bronchiectasis, especially in the elderly. In a prospective cohort in COPD patients with frequent exacerbations, 22% of subjects had positive NTM culture (26). The association of previous TB, pneumoconiosis, CF, pulmonary alveolar proteinosis and silicosis, with NTM infection has become clear in time (3, 9). Studies have shown that prevalence in NTM patients with CF is 4-20%, and increases over time and with older age. Namely, when NTM pulmonary infection was studied in patients with CF over 40 years of age, the rates were close to 50% (3, 27). The risk is also higher in patients with rheumatoid arthritis, diabetes, in alcohol consumers, extrapulmonary malignancies, and gastrectomy patients as well. Chest deformities, such as scoliosis, or pectus excavatum present favourable environment for the development of mycobacteriosis. A slightly higher tendency in the NTM infections increased incidence is in cases of connective tissue diseases, as well as in patients with mitral valve prolapse (6, 9, 15). Rather common association can be seen between mycobacteriosis and immune deficiency syndrome. The best known is association with Acquired Immune Deficiency Syndrome (AIDS), with up to 40% of HIV positive patients' deaths due to NTM infections. NTM infections occur more commonly in immunocompromised patients as well, especially in leukemia, as well as in patients on long-term corticosteroid treatment, or some other immunosuppressive therapy (9). It has been identified that the incidence of NTM infection is increasing in lung transplant recipients (28).

Pulmonary syndrome due to NTM with a presentation similar to hypersensitivity pneumonitis, has also been recognized. The syndrome was previously referred to as 'hot tub lung'. It may also be related to occupational industrial metalworking fluids exposure. Exposure to such aerosols may result in a hypersensitivity-like pneumonitis syndrome similar to 'hot tub lung' that occurs from exposure to hot water from hot tubs, but is almost only associated with M. immunogenum, a fast-growing mycobacterium. These patients are usually younger than patients with MAC or other mycobacteriosis, non-smokers, similar to patients with other forms of hypersensitivity pneumonitis. The disease course is subacute. Although there are still no reports on chronic form of NTM hypersensitivity-like pneumonitis, it may be possible.

Health Care-and Hygiene-associated Disease and Disease Prevention: avoid chlorine-based disinfectants, as it allows M. abscessus growth. Endoscopes cleaned in tap water and clinical samples contaminated with tap water or ice are unacceptable (10).

Diagnosis

Accurate diagnosis is of crucial importance because the treatment medications may have serious adverse effects, among other things (10). Good communication between clinicians and microbiologist is of key importance. A clinician should ensure that adequate specimens are taken and sent to the microbiologist regarding the type of specimen and clinical details (23).

According to ATS/IDSA guidelines published in 1997 and updated in 2007, establishing the diagnosis of mycobacteriosis depends on the fulfillment of clinical, microbiological and radiological signs of the disease (Figure 1).

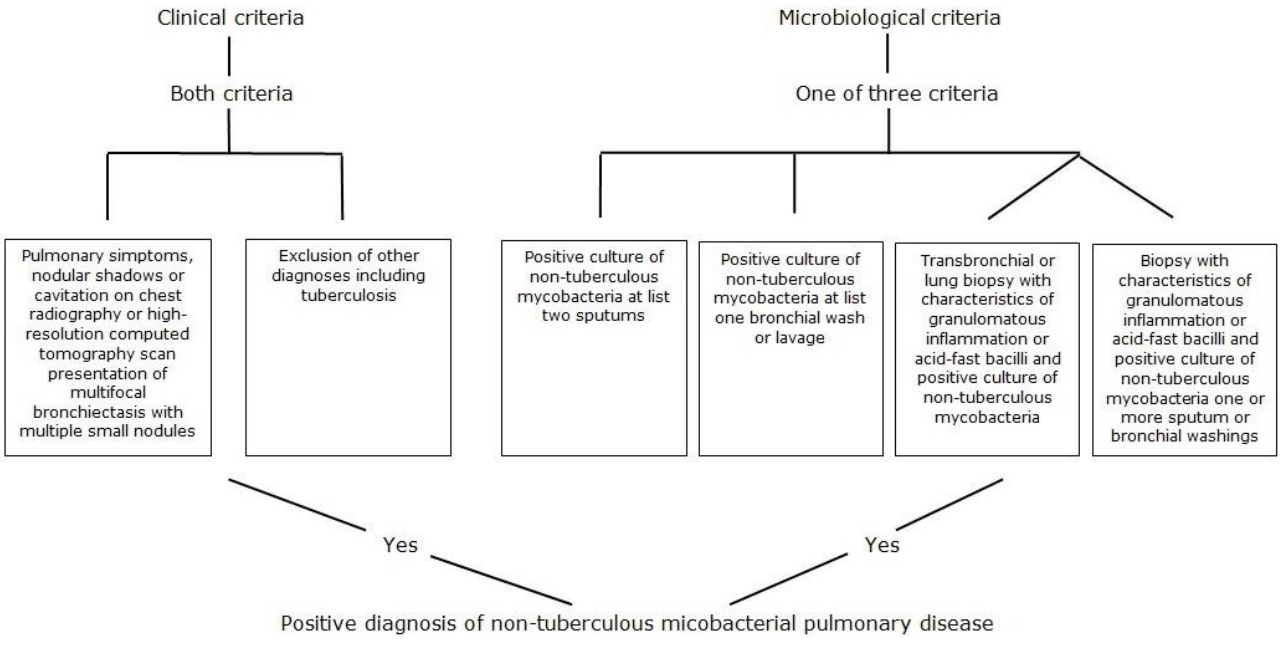

Figure 1. NTM pulmonary diseases diagnostic criteria

The onset of radiological signs has to be at the same time as NTM isolation, and the absence of some other etiologically recognized pathogenic microorganism or a disease is also important (3, 10).

Clinical suspicion is the first step in establishing the diagnosis. The minimum evaluation includes:

1) chest radiography or, in the absence of cavitation, chest high resolution computed tomography (HRCT);

2) three or more sputum specimens for acid-fast bacilli (AFB) analysis;

3) exclusion of other potential causes of the disease, such as TB or malignancies.

Both clinical criteria, along with one microbiological criterion must be met for NTM pulmonary disease. Such established criteria completely apply to only certain NTM isolated species and represent guidelines for pulmonary forms of the disease. These criteria are applicable best for MAC, M. kansasii and M. abscessus, and little is known about other species (10). In most cases a lung biopsy or bronchoscopy are not necessary for a diagnosis of pulmonary mycobacteriosis (3).

The diagnosis of NTM lymphadenitis is based on histopathological finding of necrotizing granulomas, with or without AFB within, and with negative tuberculin test in most cases. The definitive diagnosis is established by NTM finding in lymph nodes culture (10).

Disseminated NTM infection is seen among severely immunosuppressed patients, most often HIV-related. Patients at risk of the disease development are those with gastrointestinal and respiratory tract colonization. In more than 90% of cases infections are caused by MAC, with more than 90% due to M. avium. Other most frequent agents include M. kansasii, then M. scrofulaceum, M. gordonae, M. haemophilum, and others. Diagnosis of disseminated disease is most commonly established noninvasively, with over 90% of patients with disseminated MAC disease having positive blood cultures (10).

Microbiologic data are of key importance in establishing the diagnosis of MAC hypersensitivity-like disease, but not isolated, nor without clinical, radiologic, or pathologic findings characteristic for MAC hypersensitivity-like disease. Cultures obtained from sputum, bronchial lavage, tissue biopsy, and hot-tub water show MAC isolate. Matching of MAC isolates from patient specimens and isolates from hot-tub water have been proven by analyzing DNA genes and enzyme electrophoresis (10).

In comparison to previous ones, ATS/IDSA guidelines from 2007 modified microbiologic criteria for diagnosing (one NTM culture from bronchial lavage in properly selected patients category, or two positive sputum cultures, are now sufficient for establishing the diagnosis), making clinical criteria more specific at the same time (3).

Diagnostic ATS/IDSA criteria for pulmonary mycobacteriosis in HIV positive and HIV negative cases from 1997:

● if three sputum/bronchial wash results were isolated in previous 12 months:

1) three positive cultures with negative direct microscopy results, or

2) two positive cultures and one positive direct microscopy finding;

● if only bronchial wash is available:

1) positive culture with 2+, 3+ or 4+ positive smears (direct microscopy), or

2) 2+ to 4+ growth on solid surface;

● if sputum/bronchial washes are not diagnosed, or another disease cannot be excluded:

1) transbronchial or lung biopsy obtained NTM isolate, or;

2) pathohistologic finding of granulomatous inflammation and/or AFB, and one or more sputums or bronchial washing positive for NTM, even with low number increase 1+.

Aforementioned criteria are applied in symptomatic patients with infiltrates, nodular or cavitary disease, or in patients with lung computed tomography (CT) scan showing multifocal bronchiestasis bronchiectasis and/or multiple small nodules (29).

Diagnostic criteria for pulmonary mycobacteriosis according to BTS guidelines from 1999 are similar. The diagnosis of pulmonary disease caused by M. kansasii, MAC, M. malmoense and M. xenopi established after positive multiple cultures develop in non-sterile specimens obtained during 7 days according to radiographically raised suspicion of mycobacteriosis in patients with or without symptoms, and one sample in primarily sterile sample with positive pathohistological finding. Isolated strain should be identified to the species level in order to distinguish pathogenicity between the species. Different skin tests are not reliable for accurate diagnosis, so they are not recommended. The diagnosis of NTM lymphadenitis is made by complete extirpation of the involved lymph node and culture of the material obtained. A decision on definite diagnosis and treatment is made by close cooperation between pulmonologist, pediatrician, and other specialists, including otorhinolaryngologist, surgeon and microbiologist. M. fortuitum or M. chelonae may cause skin or soft tissue infections after trauma or surgery, forming abscesses or fistulas. M. marinum infection known as 'swimming pool granuloma' or 'fish tank granuloma' is acquired in the swimming pool or fish tanks after a trauma. M. ulcerans may be a causative agent of chronic necrotic skin ulceration, known as Buruli ulceration, rarely occurring outside Africa. Infections of bones, joints, genitourinary tract are not common (23).

In the absence of strong evidence to support an alternative definition, as well as due to significant clinical and research advantages in using a uniform definition, BTS guidelines from 2017 recommend definition of NTM pulmonary disease issued by ATS/IDSA in 2007. In case of high clinical suspicion of NTM infection, but negative sputum culture, CT-guided bronchial washing is recommended in order to get targeted sample. If patients have been taking antibiotic therapy that can damage NTM growth (aminoglycosides, macrolides, tetracyclines, cotrimoxazole, linezolid), discontinuation of two weeks should be considered before collecting the samples. In high clinical suspicion, but negative specimen cultures, an option is discussion with reference laboratory microbiologist about the possibility of using alternative media, at different cultivation tempera-

ture, extended time of cultivation, or the use of molecular techniques (30).

Recent joint guidelines released by European Respiratory Society-ERS, European Society of Clinical Microbiology and Infectious Diseases-ESCMID and ATS/IDSA also recommend the utility of diagnostic criteria for NTM pulmonary disease, shown in Figure 1. But, it is pointed out that fulfillment of NTM pulmonary disease diagnostic criteria does not necessarily mean that antibiotic treatment should be started. A careful assessment of the pathogenicity of the organisms, symptoms, risk and benefits of the treatment, as well as the possibility of receiving the treatment and its goals, and the patient's wish about the therapy should be considered before initiating the treatment. In some cases 'watchful waiting' may be a rational procedure in treatment course (31).

In the end, the importance of NTM isolate in patients during pulmonary TB treatment is uncertain. The importance of two NTM isolates is also unknown (10).

Therapy

The treatment of mycobacteriosis is not directly analogous to the treatment of TB. Empiric therapy is not recommended (10). A fundamental rule is never to use only a single antibiotic, since it can result in negative selection of mutants from mycobacterial population. It is very important to note that majority of NTM are resistant to pyrazinamide (Z), so it should not be used in mycobactereiosis treatment. In general, isoniazid (H) and (Z) are not effective in slow-growing mycobacteria, and sensitivity is different to rifampicin (R), quinolones and macrolides. The spectrum of effective drugs against rapidly growing mycobacteria is broader and includes ciprofloxacin, clarithromycin, tobramycin, amikacin, cefoxitin, imipenem, and sul-famethoxazole. Some studies have pointed out the synergy between R and ethambutol (E) in NTM that showed resistance to individual aforementioned drugs (9).

In vitro susceptibility of many NTM does not correlate with clinical response to antimycobacterial drugs (32). A clinician may utilize *in vitro* susceptibility, having in mind its limitations. According to the ATS/IDSA guidelines from 2007, clarithromycin susceptibility testing is recommended for new, previously untreated MAC isolates. It is a standard for testing of the newer macrolides because clarithromycin and azithromycin have cross-susceptibility and cross-resistance. The importance of first-line anti-tuberculous agents testing using methods for MAC is still unknown. Previously untreated M. kansasii isolates should be treated *in vitro* only to R, because those that show susceptibility to R will also show susceptibility to rifabutin. M. kansasii isolates resistant to R should be tested against a panel of secondary drugs, including rifabutin, E, H, clarithromycin, fluoroquinolones and sulfonamides. M. marinum isolates do not require susceptibility testing, except in case of treatment failure after several months. There are no current recommendations for a specific method of *in vitro* susceptibility testing of fast-growing NTM isolates and some not so common NTM isolates (10). Susceptibility testing to NTM drugs is useful, but only for antibiotics well documented in having correlation between *in vitro* activity and microbiological response to treatment, as recommended by the latest ATS/ERS/ESCMID/IDSA guidelines. They include macrolides, (clarithromycin and azithromycin) and amikacin for MAC and M. abscessus, and R for M. kansasii. In patients with M. xenopi pulmonary disease, the board members believe that there is not enough evidence to make a recommendation 'for' or 'against' susceptibility-based treatment (31).

Treatment recommendations by ATS/IDSA guidelines from 2007 for MAC pulmonary diseases are given in Table 1, and for treatment and prevention of disseminated MAC disease in HIV positive patients in Table 2. Treatment recommendations for not so common NTM are made on the basis of only a few reported cases. Having in mind this limitation, the duration of treatment for the most of pulmonary disease NTM pathogens is based on treatment recommendations for most commonly isolated NTM, such as MAC and M. kansasii, negative culture for at least 12 months while on therapy.

Table 1. Therapy for MAC pulmonary disease – recommendations depending on the disease status and/or its severity

	Initial Th for nodular/ bronchiectatic form	Evidence quality	Initial Th for cavitary disease	Evidence quality	Advance (severe) forms, or previously treated disease	Evidence quality
Macrolide	Claritromycin 1000 mg T or Azithromycin 500/600 mg T	B, II	Claritromycin 500/1000 mg daily or Azithromycin 250-300 mg daily	A, II	Claritromycin 500*/1000 mg daily or or Azithromycin 250-300 mg daily	B, II
Ethambutol	25 mg/kg T		15 mg/kg daily		15 mg/kg daily	
Rifampicin	600 mg T		450*-600 mg daily		450*-600 mg or Rifabutin 150-300mg daily	
IV Aminoglycoside	none		Streptomycin or Amikacin 25 mg/kg T, first 2-3 months		Streptomycin or Amikacin 25 mg/kg T, first 2-3 months	

Th - therapy; IV - intravenous; T - three times a week; * lower dose for weight under 50 kg

Table 2. Recommendations for the treatment and prevention of disseminated MAC disease in HIV positive patients

Recommended therapy (A, I)	Alternative therapy (B, I)
Clarithromycin 500 mg 2x a day	Azithromycin 500 mg daily
+	
Ethambutol 15 mg/kg daily	Ethambutol 15 mg/kg daily
+/-	
Rifabutin 300 mg daily	Rifabutin 300-450 mg* daily
Prevention+	
Azithromycin 1200 mg orally weekly	Clarithromycin 500 mg 2x a day or Rifabutin 300 mg* daily

* possibility of Rifabutin dose modification due to interactions with other drugs
\+ preventive therapy indicated in less than 50 CD4+ cells/nl, may be discontinued if > 100 cells/nl

In disseminated infection, the duration of treatment of most NTM pathogens is the same as in disseminated MAC infection, the treatment can be discontinued with the withdrawal of symptoms and reconstruction of cellular immunity. Recommendations for the treatment of hypersensitive pneumonitis associated with NTM include corticosteroids and short-term antibiotic therapy. There are no widely accepted criteria for selecting patients with mycobacteriosis for resection surgery. In general, more severe cases are treated medically, milder cases should be considered for surgical treatment, having in mind its risks and benefits. Expert opinion is of great importance. Resection of a solitary MAC pulmonary nodule is believed to be curative. The best response to treatment options is the first time it is applied, so it is very important to have a complete recommended therapeutic regime the first time the treatment is introduced. In case the treatment response is not satis-factory, expert consultation is needed (10).

BTS guidelines from 1999 are shown in Tables 3 and 4. Treatment with R and E was recommended as sufficient for most patients with M. kansasii pulmonary disease for 9 months, but in immunocompromised patients it should continue until 15-24 months, or until sputum cultures have been negative for 12 months.

Table 3. Suggested treatment for HIV negative patients with NTM disease

	Treatment	Duration
Pulmonary disease:		
M. kansasii	Rifampicin 450 mg < 50 kg; 600 mg > 50 kg daily Ethambutol 15 mg/kg daily	9 months
M. avium complex	as listed +/- Isoniasid 300 mg daily	2 years
M. malmonoese	as listed	
M. xenopi	as listed	
Lymphadenitis:		
M. kansasii M. malmoense M. xenopi	Excision. In relapse, excision + Rifampicin and Ethambutol (in aforementioned doses)	2 years
M. avium complex	Excision. In relapse, excision + Rifampicin and Ethambutol (in aforementioned doses) and Clarithromycin 500 mg daily	2 years
Intolerance to Rifampicin or Ethambutol	substitution with Clarithromycin and/or Ciprofloxacin	

Table 4. Suggested treatment for HIV positive patients with NTM disease

	Treatment	Duration
Pulmonary or disseminated disease:		
M. avium complex M. kansasii M. malmoense M. xenopi	Rifampicin 450 or 600 mg (or Rifabutin 300 mg) daily, Ethambutol 15 mg/kg daily, and Clarithromycin 500 mg daily	Lifelong
Prophylaxis MAC:		
Possibility	Azithromycin 1200 mg, orally, weekly Clarithromycin 500 mg daily Azithromycin 1200 mg orally weekly + Rifabutin 300 mg daily	Undefined

Table 5. Suggested antibiotic treatment for MAC pulmonary disease in adults

	Treatment	Duration
MAC pulmonary disease, mild to Moderate stage:	Rifampicin 600 mg 3x per week Ethambutol 25 mg/kg 3x per week Azithromycin 500 mg 3x per week or Clarithromycin 1g (2x500 mg) 3x per week	12 months after culture conversion
Severe form:	Rifampicin 600 mg daily Ethambutol 15 mg/kg daily Azithromycin 250 mg daily or Clarithromycin 500 mg x2 daily and consider intravenous Amikacin for up to 3 months or nebulised Amikacin	12 months after culture conversion
Clarithromycin-resistant MAC pulmonary disease	Rifampicin 600mg and Ethambutol 15mg/kg and Isoniazid 300mg (+pyridoxine 10mg) daily or Moxifloxacin 400mg daily and consider intravenous Amikacin for up to 3 months or nebulised Amikacin	12 months after culture conversion

Table 6. Suggested antibiotic treatment for M. kansasii pulmonary disease

	Treatment	Duration
Rifampicin-sensitive M. kansasii pulmonary disease:	Rifampicin 600 mg daily Ethambutol 15 mg/kg daily Isoniazid 300 mg (with pyridoxine 10 mg) daily or Azithromycin 250 mg daily or Clarithromycin 500 mg x2 daily	12 months after culture conversion

Table 7. Suggested antibiotic treatment for M. malmoensae pulmonary disease

	Treatment	Duration
Mild to moderate M. malmoensae pulmonary disease:	Rifampicin 600 mg daily Ethambutol 15 mg/kg daily Azithromycin 250 mg daily or Clarithromycin 500 mg x2 daily	12 months after culture conversion
Severe form:	Rifampicin 600 mg daily Ethambutol 15 mg/kg daily Azithromycin 250 mg daily or Clarithromycin 500 mg x2 daily and consider intravenous Amikacin for up to 3 months or nebulised Amikacin	12 months after culture conversion

Table 8. Suggested antibiotic treatment for M. xenopi pulmonary disease

	Treatment	Duration
Mild to moderate form:	Rifampicin 600 mg daily Ethambutol 15 mg/kg daily Azithromycin 250 mg daily or Clarithromycin 500 mg x2 daily and Moxifloxacin 400 mg daily or Isoniazid 300 mg (+ pyridoxine 10 mg) daily	12 months after culture conversion
Severe form:	Rifampicin 600 mg daily Ethambutol 15 mg/kg daily Azithromycin 250 mg daily or Clarithromycin 500 mg x2 daily and Moxifloxacin 400 mg daily or Isoniazid 300 mg(+pyridoxine 10 mg) daily and consider intravenous Amikacin up to 3 months or nebulised Amikacin	12 months after culture conversion

Table 9. Suggested antibiotic treatment for M. abscessus pulmonary disease

	Treatment	Duration
Clarithromycin sensitive isolates or inducible macrolide resistant isolates:	Initial phase: 1month or more[+] IV Amikacin 15 mg/kg daily or 3x per week[$] IV Tigecycline 50 mg x2 daily and if well tolerated IV Imipenem 1 g x2 daily and if well tolerated Clarithromycin 500 mg daily or Azithromycin 250-500 mg daily Continuation phase: Nebulised Amikacin[$] and oral Clarithromycin 500 mg x2 daily or Azithromycin 250-500 mg daily and 1-3 antibiotics based on their susceptibility and tolerance: Clofazimine 50-100 mg daily[&] Linezolid 600 mg daily or x2 daily Minocycline 100 mg x2 daily Moxifloxacin 400 mg daily Cotrimoxasole 960 mg x2 daily	12 months after culture conversion
Constitutive macrolide-resistant isolates:	Initial phase: 1 month or more[+] IV Amikacin 15 mg/kg daily or 3x per week[$] and IV tigecycline 50 mg x2 daily and if tolerated well IV Imipenem 1 g x2 daily Continuation phase: Nebulised Amikacin[$] and 2-4 antibiotics based on susceptibility and tolerance: Clofazimine 50-100 mg daily[&] Linezolid 600 mg or x2 daily Minocycline 100 mgx2 daily Moxifloxacin 400 mg daily Cotrimoxazole 960 mg x2 daily	12 months after culture conversion

[+] Due to poor response in patients with inducible or constitutive macrolide-resistant isolates and high efficacy of antibiotics administered intravenously, prolonging the duration of intravenously administered antibiotic to 3-6 months in patients with good antibiotic tolerance may be the most appropriate treatment strategy in this subgroup of patients

[$] Substitute IV/nebulised amikacin with an alternative antibiotic if M. abscessusis resistant to amikacin

[&] Start clofazimine during the initial phase if tolerated, since serum concentrations cannot be achieved until 30 or more days of treatment

IV – intravenous

Patients with no response to R and E treatment have been treated with adjuvant prothionamide 1 gr/daily orally and streptomycin (S) 0.75-1 gr/daily. The aforementioned treatment was also recommended for extrapulmonary disease caused by M. kansasii (23).

BTS recommendations from 2017 for treatment of more common forms of NTM pulmonary diseases are given in Tables 5, 6, 7, 8 and 9. Interferon gamma is not recommended as adjuvant therapy in patients with NTM pulmonary disease without a clearly defined immunodeficiency resulting in its decrease. Follow-up of patients means that microbiological sputum samples should be tested every 4-12 weeks during the treatment and 12 months upon the treatment completion. In patients who do not expectorate sputum, a CT-directed bronchial wash can be performed after 6 and 12 months of treatment in order to assess microbiological response. As for radiological response, it is necessary to perform a CT scan at the end of the treatment (30, 32). In patients with MAC-susceptible pulmonary disease, ATS/ERS/ESCMID/IDSA guidelines recommend a three-drug regimen (Table 10). Due to less interactions, azithromycin is preferred over clarithromycin. For patients with cavitary or advanced/ severe bronchiectatic or macrolide-resistant MAC pulmonary disease, parenteral amikacin or S are recommended for the initial treatment regimen. A parenteral drug is usually administered for 2-3 months at least. In patients with nodular/bronchiectatic form of the disease, drug administration 3 times a week is recommended, and a daily macrolide-based regimen is recommended in patients with cavitary disease. Recommended treatment duration is at least 12 months after culture conversion. If culture conversion fails after 6 months of recommended therapy administration, amikacin inhalation form of the drug is recommended for further treatment. In patients with macrolide-resistant MAC, expert consultation is needed. Pulmonary disease caused by R sensitive M. kansasii, should be treated according to the suggested regimen: R, E and H or macrolide (Table 10).

Table 10. Recommended treatment for MAC, M. kansasii, M. xenopi pulmonary disease – depending on the disease status and/or its severity

Organism	Initial Th for nodular/bronchiectatic form	Initial Th for cavitary disease	Refractory form of the disease
MAC	Azithromycin 500 mg T (Clarithromycin)	Azithromycin 250/500 mg (Clarithromycin) daily Azithromycin 500 mg T (with aminoglycosides)	Azithromycin 250/500 mg (Clarithromycin) daily Azithromycin 500 mg T (with aminoglycosides)
	Rifampicin 450/600 mg T (Rimfabutin)	Rifampicin 10 mg/kg max 600 mg (Rifabutin) daily Rifampicin 600 mg T (with aminoglycosides)	Rifampicin 10 mg/kg max 600 mg (Rifabutin) daily Rifampicin 600 mg T (with aminoglycosides)
	Ethambutol 25 mg/kg T	Ethambutol 15 mg/kg daily Ethambutol 25 mg/kg T (with aminoglycosides) Amikacin IV 15-25 mg/kg T (Streptomycin)	Ethambutol 15 mg/kg daily Ethambutol 25 mg/kg T (with aminoglycosides) Amikacin IV 15-25 mg T (Streptomycin) or ALIS[&] 590 mg daily
M. kansasii	Azithromycin 250-500 mg daily (Clarithromycin) Rifampicin 10 mg/kg max 600 mg (Rifabutin) daily Ethambutol 15 mg/kg daily	Azithromycin 500 mg T (Clarithromycin) Rifampicin 600 mg T (Rimfabutin) Ethambutol 25 mg/kg T	Isoniazid 5 mg/kg max 300 mg daily Rifampicin 10 mg/kg max 600 mg (Rifabutin) daily Ethambutol 15 mg/kg daily
M. xenopi	Azithromycin 250/500 mg (Clarithromycin) daily and/or Moxifloxsacin 400 mg daily Rifampicin 10 mg/kg max 600 mg (Rifabutin) daily Ethambutol 15 mg/kg daily	Azithromycin (Claritromycin) 250/500 daily mg and/or Moxifloxacin 400 mg daily Azithromycin 500 mg T (with aminoglycosides) Rifampicin 10 mg/kg max 600 mg (Rifabutin) daily Rifampicin 600 mg T (with aminoglycosides) Ethambutol 15 mg/kg daily Ethambutol 25 mg/kg T (with aminoglycosides) Amikacin IV 15-25 mg/kg T	

IV - intravenous; T - three times a week; & amikacin liposome inhalation suspension

Amikacin and S are not recommended for routine use. In patients with nodular/bronchiectatic form of the disease macrolide-based treatment is suggested, R and E 3 times per week, but in patients with cavitary disease a daily regimen of this treatment is suggested. In patients with R resistant M. kansasii or intolerance to one of the first-line antibiotics, fluoroquinolone (e.g. moxifloxacin) is suggested as a part of second-line regimen. Unlike ATS/IDSA guidelines from 2007, suggested duration of treatment regimen is 12 months, not 12 months after sputum conversion. As usual time for sputum conversion based on R in these patients is 4 months, if there is no conversion in this period of time, expert consultation is recommended. In patients with M. xenopi pulmonary disease, a daily regimen of at least three drugs is suggested: R, E and macrolide and/or fluoroquinolone (e.g. moxifloxacin) (Table 10). In severe form of M. xenopi pulmonary disease, addition of parenteral amikacin to the treatment regimen is recommended, as well as expert consultation if needed, given the poor prognosis of treatment outcome. The treatment should be continued for 12 months after culture conversion. The optimal drugs, treatment regimen and treatment duration of M abscessus pulmonary disease are not known. If the disease is caused by strains without inducible (typically M. massiliense) or mutational macrolide resistance, a multidrug treatment containing macrolide is suggested, including at least three active drugs (guided by *in vitro* susceptibility) in the initial phase of treatment (the phase including intravenous agents) (Table 11), and opposite, if the disease is caused by strains with inducible or mutational macrolide resistance, a regimen of at least 4 active drugs is recommended if possible. A macrolide-containing regimen is recommended due to its immunomodulatory features, although macrolide is not considered to be an active drug in the multidrug regimen. For the continuation phase of therapy (after discontinuation of parenteral component), at least 2 to 3 active drugs are administered. Some experts suggest a multidrug intermittent therapy regimen instead a transition to prolonged treatment phase, although almost all published studies reported treatment duration of > 12 months. In the absence of data that support shorter or longer treatment regimen of M. abscessus pulmonary disease, the panel members suggest expert consultation before the initiation of the therapy in order to help in designing the regimen and in determining whether a shorter or longer treatment regimen should be applied (31). In selected patients with NTM pulmonary disease, surgical resection adjuvant to medical therapy is possible after expert consultation.

Table 11. Suggested antibiotic treatment for M. abscessus pulmonary disease

Macrolide susceptibility	Initial phase	Continuation phase
Mutational and inducible macrolide sensitive isolates:	perenteral (choose 1-2) Amikacin 10-15 mg daily (Amikacin 15-25 mg T) Imipenem 500-1000 mg 2-3x daily (or Cefoxitin) Tigecycline 25-50 mg 1-2x daily oral (choose 2) Azitromycin 250-500 mg (Clarithromycin)# Clofazimine 100-200 mg daily Linezolid 600 mg 1-2x daily	oral/inhaled (choose 2-3) Azithromycin 250-500 mg (Clarithromycin)# daily Clofazimine 100-200 mg daily Linezolid 600 mg 1-2x daily Inhaled amikacin 590 mg daily
Mutational sensitive, inducible macrolide resistant isolates:	perenteral (choose 2-3) Amikacin Imipenem (or Cefoxitin) Tigecycline oral (choose 2-3) Azithromycin (Clarithromycin)* Clofazimine Linezolid	oral/inhaled (choose 2-3) Azitromycin (Clarithromycin)* Clofazimine Linezolid Inhaled amikacin
Mutational resistant, inducible sensitive or resistant isolates:	perenteral (choose 2-3) Amikacin Imipenem (or Cefoxitin) Tigecycline oral (choose 2-3) Azithromycin (Clarithromycin)* Clofazimine Linezolid	oral/inhaled (choose 2-3) Azithromycin (Clarithromycin)* Clofazimine Linezolid Inhaled amikacin

Dosage: daily (aminoglycosides may be administered 3 times a week).
Mutational resistance: none, when isolate phenotypic sensitivity is detected at 3-5 days of incubation in culture.
Present: when isolate phenotypic resistance is detected at 3-5 days of incubation or rrl mutation, known to be responsible for the resistance, identified on sequencing.
Inducible resistance: functional erm (41) gene: isolate phenotypic resistance identified after 14 days of incubation or functional gene sequence identified on sequencing.
Non-functional erm (41) gene: isolate identified to be resistant after 14 days of incubation or truncated sequence or C28 mutation (abscessus subgroup) on sequencing. Initial phase refers to the timing that the parenteral agents are given. Continuation phase refers to the next phase of the treatment, usually including oral antimicrobial agents sometimes combined with inhaled agents.
* azithromycin (clarithromycin) is active in this environment and should be used whenever possible.
# azithromycin (clarithromycin) activity is questionable, but it can be added for its immunomodulatory effects, although it should not be taken into consideration as active against M. abscessus with functional erm (41) gene. In this situation, frequent sputum cultures should be taken to detect a potentially new organism like MAC.

Since the treatment duration is based on culture conversion, it is necessary to collect culture specimens every 1-2 months to confirm the recommended treatment duration. Apart from microbiological response, clinical and radiological responses to treatment should also be monitored.

Possible adverse effects of applied treatment regime are numerous: hepatitis, fever, rash, peripheral neuropathies, nausea, vomiting, diarrhea, anemia, thrombocytopenia, leukopenia, pancytopenia, renal failure, polymyalgia, polyarthralgia, vertigo, ataxia, tinnitus, headache, insomnia, anxiety and others, so it is necessary to individualize monitoring approach, based on concurrent drugs, age, comorbidities, and possible drug interactions. Determination of therapeutic blood levels of drugs may be beneficial in patients with sputum conversion or treatment effect failure not due to drugs resistance or non-adherence, and for reducing the risk of ototoxicity and nephrotoxicity in those receiving aminoglycosides, or in patients with comorbidities, such as renal failure (10, 31).

Conclusion

Owing to demographic changes, modern lifestyle and living conditions, development of modern technologies, along with current trends in spreading the infection caused by M. tuberculosis, and increased understanding of this type of pathogen, mycobacteriosis importance in human pathology has been growing.

Accurately and timely diagnosis, as well as adequate therapy, are of great importance, having in mind numerous treatment-related adverse effects as well.

Future studies and randomized control trials are needed to further optimize the treatment.

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MIKOBAKTERIOZE – NEKAD I SAD

Zoran Stamenković[1], Lidija Ristić[1,2], Ivana Stanković[1,2], Milan Radović[1,2], Slavica Golubović[1], Vesna Ivanović Đorđević[1], Ivan Matejić[3]

[1]Univerzitetski klinički centar Niš, Klinika za plućne bolesti, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Katedra za internu medicinu, Niš, Srbija
[3]Univerzitetski klinički centar Niš, Klinika za grudnu hirurgiju, Niš, Srbija

Kontakt: Zoran Stamenković
Bulevar dr Zorana Đinđića 48, 18000 Niš, Srbija
E-mail: zokinis@live.com

Netuberkulozne mikobakterije (NTM) su ubikvitarne i nalaze se svuda u okolini. Ljudi su svakodnevno izloženi kontaktu sa ovim mikroorganizmima. Dok broj slučajeva obolelih od tuberkuloze (TB) u celom svetu opada, učestalost infekcija izazvanih NTM je u porastu. NTM mogu uzrokovati kako asimptomatsku infekciju, tako i simptomatsku bolest kod ljudi. Najčešće su plućne infekcije različitog stepena težine. Tačna dijagnoza veoma je važna, između ostalog i zbog toga što lekovi koji se koriste u lečenju ove bolesti mogu imati značajne sporedne efekte. Terapija mikobakterioza, uopšteno, nije direktno analogna terapiji tuberkuloze. Empirijska terapija se ne preporučuje. *In vitro* osetljivost mnogih NTM nije u korelaciji sa kliničkim odgovorom na antimikobakterijske lekove.

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COENZYME Q10 ATTENUATES METHOTREXATE-INDUCED LIVER INJURY IN RATS

Sonja Ilić[1], Natalija Mitić[2], Slavica Stojnev[3], Mladen Stojanović[4], Natalija Stojiljković[5]

Main goal of this research was investigation the protective effects of coenzyme Q10 on methotrexate-induced liver damage. Study was performed on 32 Wistar rats divided in 4 groups, whereas first group received normal saline, second received coenzyme Q10, third received methotrexate alone and fourth group received concomitantly coenzyme Q10 and methotrexate. Morphological and functional changes in liver tissue were performed by biochemical analysis of serum, histopathological analysis of liver tissue sections and determination of parameters of oxidative stress in liver tissue. Administration of methotrexate in rats caused a significant increase of the concentrations of AST, ALT and γ-GT and significant decrease of amount of total proteins in the serum compared with C group of animals. Also, methotrexate significantly increased MDA and AOPP levels in and decreased catalase activity in hepatic tissue. Histopathological analysis showed pronounced liver damage with cellular derangement of hepatic cordons and significant cell swelling, vacuolar degeneration and signs of inflammatory response after methotrexate administration.In group of rats that received coenzyme Q10 8 days after methotrexate administration, injury of liver tissue was significantly decreased with mild disorder of normal radial arrangement of the hepatocytes and only discretely uneven distribution of hepatic glycogen content. In same group, biochemical analysis showed significantly decreased concentrations of serum parameters of liver injury and changes of parameters of oxidative stress were statistically significantly ameliorated compared with results in group that received methotrexate alone. Our results confirmed coenzyme Q10 as a protective agent in methotrexate-induced hepatotoxicity probably due to its antioxidant effects.

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Key words: *methotrexate, coenzyme Q10, hepatotoxicity, oxidative stress*

[1]University of Niš, Faculty of Medicine, Department of Physiology, Niš, Serbia
[2]University of Niš, Faculty of Medicine, Niš, Serbia
[3]University of Niš, Faculty of Medicine, Department of Pathology, Niš, Serbia
[4]University Clinical Centre Niš, Clinic for Orthopaedic Surgery and Traumatology, Niš, Serbia
[5]University of Niš, Faculty of Medicine, Department of General Education, Niš, Serbia

Contact: Sonja Ilić
81 Dr Zoran Djindjić Blvd., 18000 Niš, Serbia
E-mail: sonjaili@yahoo.com

Introduction

Methotrexate (MTX) is a folic acid antagonist and it belongs to the group of cytostatics known as antimetabolites. Its use is widespread, considering that it is used as a therapeutic drug in the treatment of various types of cancer, rheumatoid arthritis and other autoimmune diseases (1). Side effects of MTX are numerous, some of which are myelosuppression, various infections, gastrointestinal disorders, and kidney and liver disorders.Considering liver, frequent side effects include an increase in liver enzymes, fatty liver, liver cirrhosis, a decrease in serum albumin, while acute hepatitis and reactivation of chronic hepatitis occur very rarely (2). The mechanisms by which MTX causes liver damage have not yet been fully investigated and clarified, but there are several assumptions about the pathophysiology of these injuries. Among many, the occurrence of oxidative stress in the tissue is the most common (3). Therefore, several antioxidant agents have been used to reduce its side effects (4).

Coenzyme Q10 (CQ10) is an endogenous lipid-soluble substance that is a strong antioxidant. Antioxidant properties of CQ10 are demonstrated trough its ability to scavenge reactive oxygen species (ROS) and prevent the lipid peroxidation in cellular membranes. Also, CQ10 has antiapoptotic and anti-inflammatory effects due to its ability to reduce secretion of proinflammatory cytokines (5). In earlier studies, the administration of CQ10 has shown a significant preventive effect in several models of oxidative and inflammatory damages of renal

or liver tissue induced by cisplatin and acetaminophen (6, 7). Considering that some studies have shown a protective effect of CQ10 in cases of nephrotoxicity and hepatotoxicity, the main goal of this research is to investigate the beneficial effects of CQ10 on methotrexate-induced liver damage.

Materials and methods

In our investigation we used adult male Wistar rats, of an average weight of 270 grams. The rats were kept in a standard conditions with controlled temperature (20 ± 2 ^{O}C) and humidity (60%) and 12 hours light/12 hours dark cycle. The animals had free access to food and water. All experiments were conducted at the Institute of Biomedical Research, Medical Faculty, Niš, Serbia, in accordance with all ethical regulations of European Union (EU Directive of 2010; 2010/63/EU) and principles for the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011), and approved by the Ministry of Environmental Protection of the Republic of Serbia (No. 323-07-00073/2017-05/1).

Experimental protocol

A total of 32 animals were divided into four groups of 8 animals. The methotrexate group of animals or M-group, received MTXEBEWE Pharma (Ges.m.b.H.NFG.KG, Austria) intraperitoneally (i.p.) at a dose of 20 mg/kg on the first day of the experiment. Control group or C-group received 1 ml/kg i.p. of normal saline daily for eight days. Coenzyme Q10 (Q) group of animals received CQ10 Sigma-Aldrich (St. Louis, MO, USA) dissolved in corn oil (10 mg/kg) for 8 days(i.p.). Methotrexate - coenzyme Q10 (MQ) group of animals received MTX (20 mg/kg) on day 1, and CQ10 dissolved in corn oil (10 mg/kg) for 8 days. On the ninth day of the experiment, all animals were anesthetized with ketamine in a dose of 80 mg/kg and sacrificed. We took blood from aorta (separated serum immediately) to perform biochemical analysis, and we removed liver to determine tissue biochemical and histopathological studies.

Serum biochemical analysis

Biochemical analysis of serum included determining the concentrations of alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyl transferase (γ-GT) and total protein levels. All parameters were determined by Olympus AU680 Chemistry-Immuno Analyzer (A25 Biosystems, Barcelona, Spain).

Histopathological analysis

One part of liver tissue from each animal first was fixed in 10% paraformaldehyde at room temperature for 48 hours, than dehydrated in alcohol and embedded in paraffin. We cut tissue samples at a thickness of 5 mm (model: LKB 2218, LKB-Produkter AB, Bromma, Sweden) and stained using haematoxylin and eosin (HE) and periodic acid-Schiff (PAS) methods and analysed using Olympus BH2 light microscope.

Tissue biochemical analysis

We cut liver tissue samples into small pieces and homogenized in ice cold water by a homogenizer (VELP Scientifca, Italy). We prepared homogenates and separated supernatant using the same method as in our previous research (8). Protein content in the supernatants was determined according to the Lowry's method (9).

Levels of malondialdehyde (MDA) as a marker of lipid peroxidation in liver tissue were determined by method described by Ohkawa (10). After measuring of homogenate absorption, concentration of MDA was calculated and expressed as g/protein.

Determination of the concentration of advanced oxidation protein products (AOPP) in tissue homogenates was described in our previous study (8). The concentrations of AOPP were expressed as mmol/g of proteins.

Determination of the activity of catalase (CAT) was performed by the method described by Goth (11). We measured homogenate absorption at 405 nm and expressed activity of this enzyme as international units (IU) per gram of protein.

Statistical analysis

For statistical analysis we expressed results of examined parameters as the mean value ± standard deviation (SD). To determine statistically significant differences we performed one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (GraphPad Prism version 5.03, San Diego, CA, USA). Probability values (p) ≤ 0.05 were considered to be statistically significant.

Results

Biochemical analysis

Administration of MTX in rats caused a significant increase ($p < 0.001$) in the concentrations of AST, ALT and γ-GT in M group compared to the values of the same parameters in the C group of rats. The amount of total protein in the serum was significantly decreased in the M group compared to the C group of animals ($p < 0.001$) (Figure 1). Rats that received CQ10 combined with MTX showed a significant decrease in AST, ALT andγ-GT compared to that when receiving MTX alone ($p < 0.001$) (Figure 1),while the serum total protein levels were significantly increased in the MQ group compared to the M group ($p < 0.05$) (Figure 1).

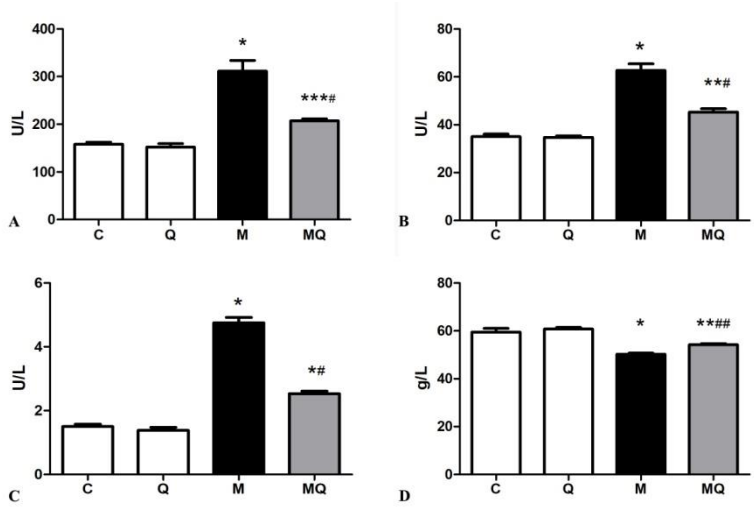

Figure 1. The concentrations of AST (A), ALT (B), γ-GT (C) and total protein (D) in serum of experimental animals. Data are given as mean ± SD, ANOVA followed by Tukey post hoc test.
*p < 0.001 vs. C group; **p < 0.01 vs. C group; ***p < 0.05 vs. C group;
#p < 0.001 vs. M group; ## p < 0.05 vs. M group

Histopathological analysis

Liver sections from the control group showed the normal morphology of hepatic lobules. Features of a regular tissue structure, including adequate localization of central veins, normal composition of portal tracts and streaming of blood sinusoids are presented in Figure 2A. Orderly distribution of hepatocytes in hepatic plates is also retained in the Q group, where no significant changes in histologic architecture were observed (Figure 2B). Sections from the methotrexate-treated group demonstrated liver injury associated with cellular derangement of hepatic cordons and significant cell swelling. Hepatotoxic damage reflected in the reversible and irreversible cellular alterations ranged from vacuolar degeneration, nuclear reactive changes, to apoptosis of hepatocytes. Individual hepatocytes or cell clusters showed cytoplasmic hypereosinophillia and condensation, and nuclear hyperchromasia and pyknosis.

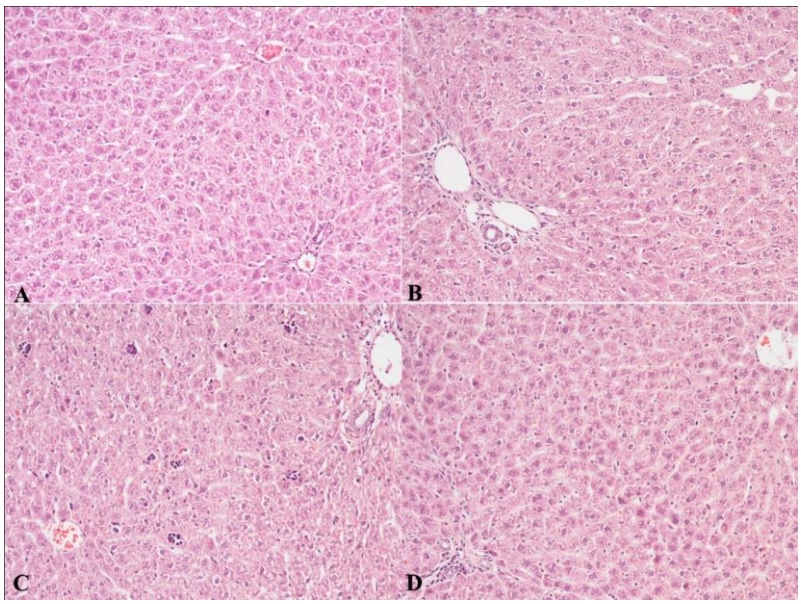

Figure 2. Photomicrographs of hematoxylin and eosin (H&E)-stained liver sections of:
A) C group, B) Q group, C) M group and D) CQ group of animals (Original magnification 200x)

Histologic signs of inflammatory response were prominent in this group. An infiltrate composed of mononuclear cells, predominantly lymphocytes, and, in lesser extent, polymorphonuclear leukocytes, was observed within the portal tract, surrounding the billiary ducts and portal vein branches. Moreover, the aggregates of the immune cells had a quite conspicuous intralobular distribution. Portal veins and sinusoid capillaries showed marked congestion (Figure 2C). In the group MQ, where animals received CQ10 8 days after the administration of methotrexate, the extent of tissue damage was visibly less striking than in the M group. Liver sections showed a disturbance of the hepatic lobule and a mild congestion of the portal area associated with scarce inflammatory infiltration. There has been some cellular edema and degeneration resulting in mild architectural distortion, but irreversible injury with cell loss was not noted (Figure 2D). Inflammatory infiltrate was restricted to portal tracts, with no significant intralobular activity. Control groups sections showed a normal hepatic architecture and cell cytoplasm rich with PAS-positive glycogen granules (Figure 3 A and B). The abundance of glycogen content significantly deteriorated in methotrexate treated animals. In addition to massive hepatic degeneration, depletion of PAS-positive intracellular content was striking. Hepatocytes appeared empty and displayed clear, transparent cytoplasm, while residual glycogen granules where shifted against the cell membrane. Generally, the distribution of PAS-positive granules was uneven and varied between areas of hepatic tissue (Figure 3C). In the CQ group changes were associated with moderate amelioration of hepatocyte degeneration with apparently near normal distribution of PAS-positive granules (Figure 3D).

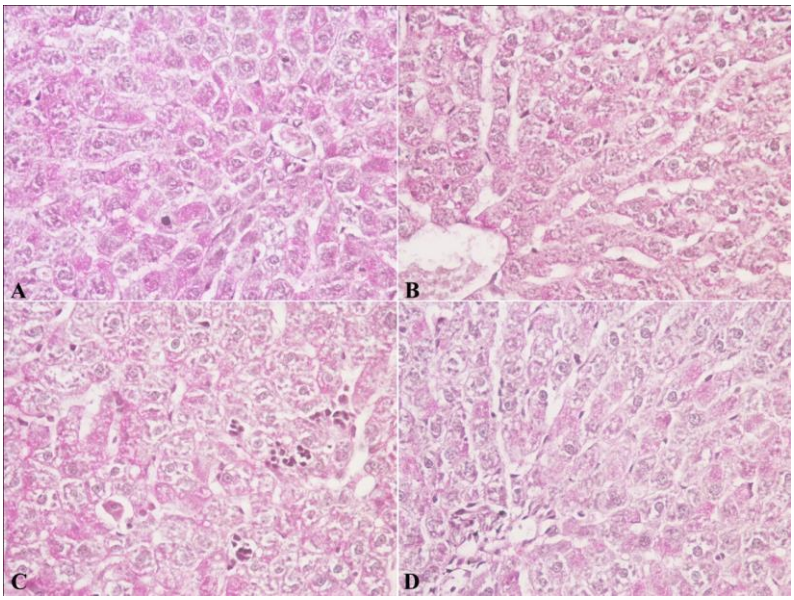

Figure 3. Photomicrographs of PAS - Periodic acid–Schiff-stained rat liver sections of:
A) C group, B) Q group, C) M group and D) CQ group of animals (Original magnification 400x)

Tissue biochemical analysis

The statistical analysis showed a significant increase in MDA and AOPP levels in tissue homogenates in the M group when compared with the C-group ($p < 0.001$), while the CAT activity in liver tissue was significantly decreased ($p < 0.001$) in the M-group of animals in comparison with the C-group (Table 1). Concomitant administration of CQ10 with MTX in the MQ group attenuated oxidative stress induced by MTX, so in this group levels of MDA and AOPP in liver tissue were significantly decreased compared to the M group of animals ($p < 0.05$), while CAT activity was significantly increased compared to the M group ($p < 0.01$).

Table 1. Parameters of oxidative stress in liver tissue

Group/Parameter	**AOPP** (mmol/g proteins)	**MDA** (mmol/g proteins)	**CAT** (IU/g proteins)
C	17.44 ±3.540	5.002 ± 1.766	26.71 ± 3.376
Q	17.45 ±4.645	4.672 ± 1.524	26.72 ± 4.083
M	30.15 ±1.422*	10.45 ± 1.110*	10.40 ± 4.236*
MQ	23.92 ±3.680***#	7.998 ± 0.4312**#	19.12 ± 1.821**##

Data are given as mean ± SD, ANOVA followed by Tukey post hoc test
*p< 0.001 vs. C group; **p< 0.01 vs. C group; ***p< 0.05 vs. C group
#p< 0.05 vs. M group; ##p< 0.01 vs. M group

Discussion

The antineoplastic drug MTX is a very effective drug in the treatment of various types of cancers and other diseases such as rheumatoid arthritis (12, 13). However, its use leads to side effects that include myelosuppression, gastrointestinal disorders, kidney damage, acute injury of liver, hepatic fibrosis and cirrhosis (14). In order to reduce the side effects, the administration of some non-toxic natural substances could significantly ameliorate the damages caused by MTX with the preservation of its chemotherapeutic efficacy. In our research, we performed histopathological and biochemical analyses of liver tissue to determine structural and functional alterations induced by MTX in the liver and to determine potential protective effects of CQ10 on MTX-induced liver injury. In our research, we found that a single dose of MTX caused a significant increase in AST, ALT and γ-GT levels and a decrease in the total protein ($p < 0.001$) compared to the control group. Similar effects of MTX were shown in study published by Kelleni et al. (14). We showed that administration of CQ10 (10 mg/kg), 8 days after a single dose of MTX had protective effect on liver treated with MTX. This protective effect was evidenced by significantly reduced levels of AST, ALT and γ-GT and decreased levels of total protein in the MQ group in comparison with the group of animals that received only MTX (Figure 1).

A histopathological analysis was performed in order to determine morphological injuries. We showed that MTX-treated group showed pronounced liver damage with cellular derangement of hepatic cordons and significant cell swelling, vacuolar degeneration and signs of an inflammatory response. Portal veins and sinusoid capillaries showed congestion. Small foci of lobular inflammation could also be found (Figure 2C). Also, on PAS stained liver tissue sections there was significant abundance of glycogen content and hepatocytes appeared empty and displayed clear, transparent cytoplasm (Figure 3C).

Our biochemical and histopathological analysis showed that MTX induced damage of liver tissue. Rats treated with CQ10 and MTX revealed an amelioration of histopathological alterations. In the MQ group comprised a mild congestion of the portal area with scarce inflammatory infiltration was observed. Irreversible injury with cell loss was not noted. In PAS stained tissue sections the distribution of PAS positive granules was near normal (Figures 2D and 3D). These results were in accordance with the previous findings that showed that CQ10 has protective effects against cisplatin, fructose and acetaminophen-induced kidney and liver injury (7, 15, 16).

Despite numerous studies with MTX, the main mechanism of MTX-induced liver injury has not yet been clarified fully yet. Coleshowers et al. (4) and Goudarzi et al. (17) suggested that one of the most important mechanisms in MTX-induced liver injury is the generation of the ROS and reduction of the antioxidant defence system. In order to determine if MTX causes oxidative stress, and if CQ10 is able to attenuate possible disturbances of oxidative stress parameters, we examined levels of MDA and AOPP as well as catalase activity in liver tissue homogenates. Our results showed that levels of MDA and AOPP were significantly increased while catalase activity was significantly decreased in the liver tissue after only one single dose of MTX (Table 1). Dalaklioglu et al (18) suggested that MTX induces generation of ROS such as superoxide anion and hydroxyl radicals and also strongly stimulates occurrence of lipid peroxidation in liver tissue. Lipid peroxidation is an autocatalytic process that most often ends with irreversible damage of the function and structure of the cell membrane. Lipid peroxidation products, especially MDA, can damage the membranes of lysosomes, which leads to the release of hydrolytic enzymes, as well as the damage of the mitochondrial membranes, which causes the release of Ca ions and the activation of enzymes dependent on this ion (19). Increased levels of AOPP in the liver tissue in the M group indicate that oxidative protein modification has occurred. Oxidative modification of proteins leads to structural alterations in the primary, secondary and tertiary structure of proteins due to changes in amino acid residue molecules, as well as a functional inactivation of many enzymes (20). Our results were consistent with the assumptions that part of the mechanism of MTX hepatotoxicity is related to the depletion of the antioxidant system (21, 22). Catalase activity in the M group was statistically significantly reduced compared with theCgroup (Table 1). Catalase is one of the endogenous antioxidant enzymes that play a key role in

reducing the oxidative modification of lipids and the propagation of lipid peroxidation (4).

CQ10 is an endogenous liposoluble benzoquinone that contains 10 isoprene side chains and it functions as a transporter of electrons in the mitochondrial respiratory chain where it plays a key role in aerobic cellular respiration to produce ATP. In addition to participating in the elimination of free radicals, it also prevents the initiation of lipid peroxidation, i.e. lipid damage in cell membranes under the influence of free radicals (23). In our study, the levels of MDA and AOPP in liver homogenates of the MQ group of rats were significantly decreased compared to the same in M group (Table 1). Administration of CQ10 significantly increased catalase activity in liver homogenates in the MQ group compared to catalase activity in group of animals that received MTX only once. Our results showed that CQ10 ameliorated liver injuries caused by MTX probably through its antioxidative effects. We can indicate the key role of CQ10 in the scavenging of free radicals produced by the MTX, as well as in the protection of lipids and proteins from oxidative modification, and a significant role in preserving the activity of antioxidant enzymes. These effects of CQ10 certainly greatly contribute to the protection from structural liver damage caused by the MTX, which we confirmed by histopathological and biochemical analysis in our study.In recent studies, it has been confirmed that CQ10 has protective effects on proteins, DNA and lipids in membranes from oxidative damage, primarily by strong inhibition of oxidative stress (24). These effects of CQ10 might be the result of its redox activity in mitochondrial respiratory chain. Earlier studies showed that CQ10 primarily acts in the mitochondria, where it is able to transfer electrons in mitochondrial respiration. When CQ10 is acting as an antioxidant in the mitochondria it is able to neutralize both, free radicals in the cytoplasm and ROS produced in the mitochondria (23).

Conclusion

Our results confirm that MTX damages lipids of the cell membrane and proteins due to increased production of ROS. Probably, lipid peroxidation and damaging of structure of proteins contribute further to DNA damage and apoptosis of cells. According to our results, administration of CQ10 significantly ameliorates oxidative and histological injury of liver tissue caused by MTX. These results indicate that CQ10 can be very useful in the prevention of structural and functional liver injury caused by MTX. Considering the widespread use of MTX in the treatment of a several pathological conditions, the use of CQ10 should be further study in order to standardize its application.

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KOENZIM Q10 UBLAŽAVA OŠTEĆENJE JETRE IZAZVANO METOTREKSATOM KOD PACOVA

Sonja Ilić[1], Natalija Mitić[2], Slavica Stojnev[3], Mladen Stojanović[4], Natalija Stojiljković[5]

[1]Univerzitet u Nišu, Medicinski fakultet, Katedra za fiziologiju, Niš, Srbija
[2]Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija
[3]Univerzitet u Nišu, Medicinski fakultet, Katedra za patologiju, Niš, Srbija
[4]Univerzitetski klinički centar Niš, Klinika za ortopedsku hirurgiju i traumatologiju, Niš, Srbija
[5]Univerzitet u Nišu, Medicinski fakultet, Departman za opšte obrazovanje, Niš, Srbija

Kontakt: Sonja Ilić
Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: sonjaili@yahoo.com

Glavni cilj ovog istraživanja bio je ispitivanje zaštitnih efekata koenzima Q10 na oštećenje jetre izazvano metotreksatom. Studija je obavljena na 32 pacova Vistar podeljenih u 4 grupe, pri čemu je prva grupa primala normalni fiziološki rastvor, druga koenzim Q10, treća samo metotreksat i četvrta grupa istovremeno koenzim Q10 i metotreksat. Morfološke i funkcionalne promene u tkivu jetre urađene su biohemijskom analizom seruma, histopatološkom analizom preseka tkiva jetre i određivanjem parametara oksidativnog stresa u tkivu jetre. Primena metotreksata kod pacova izazvala je značajno povećanje koncentracija AST, ALT i g-GT i značajno smanjenje količine ukupnih proteina u serumu u poređenju sa C grupom životinja. Takođe, metotreksat je značajno povećao nivoe MDA i AOPP i smanjio aktivnost katalaze u tkivu jetre. Histopatološka analiza je pokazala izraženo oštećenje jetre sa ćelijskim poremećajem jetrenih kordona i značajnim oticanjem ćelija, vakuolnom degeneracijom i znacima inflamatornog odgovora nakon primene metotreksata. U grupi pacova koji su primali koenzim Q10 8 dana nakon primene metotreksata, značajno je smanjena povreda tkiva jetre. Blagi poremećaj normalnog radijalnog rasporeda hepatocita i samo diskretno neravnomerna raspodela sadržaja hepatičnog glikogena. U istoj grupi, biohemijska analiza je pokazala značajno smanjene koncentracije serumskih parametara oštećenja jetre, a promene parametara oksidativnog stresa su statistički značajno poboljšane u poređenju sa rezultatima u grupi koja je primala samo metotreksat. Naši rezultati su potvrdili da je koenzim Q10 zaštitni agens kod hepatotoksičnosti izazvane metotreksatom, verovatno zbog njegovih antioksidativnih efekata.

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Ključne reči: *metotreksat, koenzim Q10, hepatotoksičnost, oksidativni stres*

JEDINSTVENI KRITERIJUMI ZA OBJAVLJIVANJE NAUČNIH RADOVA U BIOMEDICINSKIM ČASOPISIMA

Ideja o postavljanju jedinstvenih kriterijuma za objavljivanje radova u časopisima za biomedicinske nauke iskristalisana je 1978. godine u Vankuveru. Ovi kriterijumi za rukopise, uključujući pravila za pisanje bibliografije, prvi put su objavljeni 1979. godine. Vankuverska grupa je vremenom prerasla u Međunarodni komitet urednika medicinskih časopisa — International Committee of Medical Journal Editors (ICMJE). Trenutno je na snazi peta revizija kriterijuma za objavljivanje radova u biomedicinskim časopisima, doneta 1997. godine.

Kriterijumi za citiranje i navođenje referenci

Reference se obeležavaju arapskim brojevima u zagradama, pri čemu se reference obele-žavaju brojevima onim redosledom kojim se pojavljuju u tekstu. Reference citirane jedino u tabelama ili legen-di moraju se obeležiti brojem u skladu sa redosledom pojavljivanja u tekstu.

Naslove medicinskih časopisa treba pisati u skraćenom obliku onako kako su navedeni u poglavlju **List of Journals Indexed in Index Medicus**. Lista skraćenih naziva medicinskih časopisa objavljuje se svake godine u januarskom broju ***Index Medicusa***. Ova lista se takođe može naći na adresi **www.nlm.nih.gov**

Izbegavati upotrebu apstrakata kao referenci, već koristiti samo izvorne tekstove (*in extenso* članci). Reference koje se odnose na radove koji su prihvaćeni, ali još nisu odštampani, treba označiti sa "u štampi", pri čemu autor mora imati pismeno odobrenje da citira takve radove i da priloži pismeni dokaz da je citirani rad prihvaćen za štampu. Informacije iz rukopisa koji nisu prihvaćeni za štampanje mogu se citirati u tekstu kao "neobjavljeni rezultati", ali sa pismenom dozvolom autora.

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Kriterijumi za pisanje referenci korišćenih u radu

U ovom pregledu biće obrađena pravila za pi-sanje literaturnih referenci samo za najčešće korišćene tipove publikacija.

Članci u časopisima

1. Standardni članak u časopisu

Navesti prvih šest autora, ukoliko ih je više iza šestog dodati **et al.** ukoliko je referenca na engleskom jeziku ili **i sar.** ukoliko je referenca na srpskom jeziku.

Vega KJ, Pina I, Krevsky B. Heart transplantation is associated with an increased risk for pancreatobiliary disease. Ann Intern Med 1996; 124(11):980-3.

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The Cardiac Society of Australia and New Zealand. Clinical exercise stress testing. Safety and performance guidelines. Med J Aust 1996;164:282-4.

3. Članak bez poznatih autora

Cancer in South Africa (editorial). S Afr Med J 1994;84:15.

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Smith P, Golladay K. Payment for durable medical equipment billed during skilled nursing facility stays. Final report. Dallas (TX): Dept. of Health and Human Services (US), Office of Evaluation and Inspections; 1994 Oct. Report No.: HHSIGOEI69200860.

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