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MELATONIN AMELIORATES DECREASE IN RAT GASTROCNEMIUS MUSCLE CATALASE ACTIVITY INDUCED BY CARBON TETRACHLORIDE

Ljubiša M. Lilić[1], Rade Z. Stefanović[1], Dragan Toskić[1], Branimir B. Mekić[1], Dušan T. Sokolović[2]

Skeletal muscle tissue is known to be susceptible to oxidative tissue damage that accompanies different disorders. Carbon tetrachloride (CCl_4) is dangerous chemical that is used to mimic disorders, in experimental animals, related to reactive oxygen species induced tissue damage. It is well established that antioxidants, both natural and synthetic ones, are able to alleviate tissue damage caused by reactive oxygen species. The aim of the present study was to determine the effects of melatonin (MLT), a strong naturally occurring antioxidant, on changes in Wistar rat skeletal muscle catalase activity acutely induced by CCl_4. Gastrocnemius muscle tissue, in which catalase activity was determined, was obtained from three groups of animals i.e. control (untreated), CCl_4 treated and MLT and CCl_4 group. The results revealed statistically significant decrease in muscle tissue catalase activity in rats exposed to CCl_4, while in the group that received MLT and CCl_4 this decrease was insignificant. The protective activity of MLT could be contributed to its different mechanisms since it is known to directly scavenge free radicals, increase tissue antioxidant capacity and to up-regulate antioxidant enzyme gene expression.

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Key words: *carbon tetrachloride, gastrocnemius muscle, catalase*

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Introduction

Skeletal muscles (SM) are prone to oxidative tissue damage, apart from different organ underlaying disorders, after a mild/extensive exercise (1-3). One of the major factors that makes SM tissue prone to this type of damage is the presence of myoglobin, a catalyst for formation of reactive oxygen species (ROS) (1). Affected SM tissue is fighting ROS growth by increasing tissue blood flow (perfusion), as well as cell enzymatic antioxidative defences (1). Enzymes involved in defending SM cells from ROS include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). The first two are mainly localized in cytoplasm and mitochondria, while the last one (CAT) can be found predominantly in lysosomes (1). Lysosomes are important cell organelles with main function in polymer molecules degradation, which could be found near cell membrane of almost all animal cells (4). In mitochondria, CAT is thought to act as a "safety device" since it might prevent excessive leakage of generated H_2O_2, thus preventing lipid and protein oxidative damage caused by myoglobin derived ferryl products (1).

Carbon tetrachloride (CCl_4) is a dangerous chemical known to induce different experimental animal tissue damage, which is mediated via trichloromethyl free radicals generated in liver. When applied, it causes liver, kidneys, brain, hearth, muscles, lungs, testis, etc. cell (cell structures) oxidative damage (5, 6). When administered, CCl_4 is metabolised by liver Cytochrome P450 into highly reactive trichloromethyl radicals that further cause damage to different cell macromolecules (lipids, proteins, nucleic acids) (5). Muscle tissue is relativity susceptible to different types of injuries and the process of its regeneration often leads to scar tissue formation. These regeneration processes could be enhanced, thus preventing scaring and loss in muscle strength, by applying different exogenous compound e.g. antioxidants (7).

Melatonin is a neurohormone synthetized by bone marrow cells, gut, lens, pineal gland cells, in the retina, Harderian glands, ovary, testes and skin

(8). Besides its role as a "biological clock" regulator, MLT possesses strong antioxidant activity i.e. acts as a scavenger for hydroxyl and peroxyl radicals (9). Both synthetized and inject MLT exert its activity via three melatonin type receptors (MT1-3) that are distributed throughout the body, where the MT3 is directly related to MLT potential antioxidative defence since it involves enzyme quinone reductase 2 that prevents electron transfer of quinones (10). In rat SM crash injury model MLT was shown to exhibit significant modulation of apoptotic pathways mediated by MT1 receptors, thus preserving SM tissue function (7).

Aim of the study

The aim of the present study was to estimate MLT potential in preventing changes in rat gastrocnemius muscle CAT activity occurring after an acute CCl_4 application.

Methods

Animals and housing

Male Wistar rats, weighting 250-300 g, were obtained from the Vivarium of the Institute of Biomedical Research, Faculty of Medicine, University of Niš, Serbia, housed in groups of 6. The animals were maintained under standard laboratory conditions: temperature 22 ± 2 ºC and humidity 60%, with food and water available ad libitum. All experimental procedures with the animals were conducted in compliance with the Declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010 /63/EU) and were also approved by the local Ethics Committee.

Muscle tissue damage induction

Before the experiment, all animals were divided into three groups of 6 rats each:

- Group I - control group animals were administered with vehicle (olive oil, 10 ml/kg),
- Group II - group treated with CCl_4 (1 ml/kg),
- Group III – group treated with MLT (50 mg/kg) 1 h prior to CCl_4 application.

All animals were treated by an intraperitoneal injection and all were sacrificed by an overdose of ketamine 24 h after the last injection. Skeletal muscle tissue samples collected for tissue biochemical analyses included the gastrocnemius (GCM) muscle.

Tissue biochemical analysis

Collected GCM tissue samples were homogenized in distilled water (10%, w/v) and centrifuged afterward (4000 rpm, 10 min, 4 °C) in order to obtain supernatant. Total protein content in GCM homogenates was determined using standard Lowry method (11). The calculations were performed based on albumin standard curve (0-12 mg/ml).

Catalase activity determined following standard method previously described (12). Briefly, equal volumes of tissue homogenates and substrate, H_2O_2, dissolved in phosphate buffer saline were incubated for 5 minutes at room temperature. Afterward, the same volume of ammonium molybdate was added to reaction mixture in order to stop the reaction. The intensity of formed yellow dye was measured at 405 nm and the results were expressed as U/g of proteins.

Statistical analysis

The obtained results were given as mean values ± standard deviation (SD) and were further compared using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons (GraphPad Prism, ver. 5.03; San Diego, CA). Probability values (p) less than 0.05 were considered to be statistically significant.

Results

The results obtained for CAT activity in GCM of animals from Group I-III are given in Table 1. Acute administration of CCl_4 caused statistically significant decrease in GCM CAT activity. Application of MLT 1h before CCl_4 prevented a significant decrease in CAT activity (Table 1).

Table 1. Gastrocnemius muscle catalase activity in rats acutely administered with CCl_4 and MLT

Biochemical parameter/ Group (treatment)	Group I (Vehicle)	Group II (CCl_4)	Group II (MLT + CCl_4)
Gastrocnemius muscle catalase activity (U/g of tissue proteins)	134.4 ± 11.9	115.7 ± 2.2*	146.9 ± 17.2#

The results are presented as mean value ± SD (n=6); *p<0.001 *vs*. Group I; #p<0.001 *vs*. Group II.

Discussion

Different disorders affecting SM, as well as some intense exercise protocols are strongly related to free reactive oxygen species (ROS) damaging effects (13). Generation of ROS can be traced back to either SM cells and/or interstitial inflammatory cells, where over time myofibrils are losing their ability to cope with excessive ROS (13). At physiological levels ROS are needed for proper SM functioning, however their excessive production leads to contractile dysfunction (13). Enzymatic antioxidant capacities of SM exposed to significant increase in ROS is reported to be both increased and decreased by different authors (14-16). All cells, including SM cells, have their own antioxidative enzyme systems that precisely regulate the amounts of ROS in physiological conditions (13). However, these enzymes could be significantly altered and their deficiencies lead to vide range of different disorders such as metabolic and autoimmune disorders (17).

The presence of CAT in skeletal muscle tissue cells has been pointed out during 1950s (18) and enzyme localisation and function have not been completely understood. Namely, the loss in rat GCM wet weight, seen after animals were exposed to starvation, is reported to linearly follow an increase in tissue catalase activity, suggesting that CAT might be used as a biomarker for muscle waist (18). On the other hand, more recent study revealed that progressive sarcopenia (loss of SM mass), a frequent disorder seen in older people, is followed by an increase in H_2O_2 and decrees in tissue CAT activity (14). Also, they postulated that H_2O_2 represents the most important ROS involved in the onset of sarcopenia (14).

Since the CAT is reported to be an important enzyme involved in different neuromuscular disorders (13, 14) the present study evaluated its activity in CCl_4 induced SM damage model. The activity of the CAT, an important enzyme involved in ROS metabolism, was found to be significantly altered in animals that received CCl_4, whereas the activity was found to be increased in liver and hearth, while in brain and kidneys its activity was decreased (19). The results of our study suggest that CCl_4 acutely administered to rats leads to CAT activity decrease in the investi-gated SM (Table 1).

Melatonin is proven to be powerful antioxidant both in *in vivo* and *in vitro* conditions, where it is able to scavenge ROS and to affect tissue enzymatic and non-enzymatic antioxidant defences (20). There are studies reporting that MLT restorative effect on oxidative status is related to its capability to diminish H_2O_2 and lipid peroxidation (20). One of the suggested mechanisms of MLT antioxidant action is its potential to directly scavenge ROS, where some authors suggest that its activity could be complementary to the function of catalase and glutathione peroxidase (21). Namely, MLT was found to directly scavenge H_2O_2 forming N(1)-acetyl-N(2)-formyl-5-methoxykynura-mine that can further be transformed by CAT (21).

Also, previous studies revealed that MLT is able to increase SOD and CAT activity in skin cells and lymphocytes by up-regulating their gene expression (17, 22). Such activity is proposed to be related to the melatonin ability to prevent nuclear factor-E2-related factor (Nrf2) degradation, thus enabling Nrf2 to stimulate the transcription of enzymes involved in cell antioxidant defenses (17). Melatonin is known to modulate tissue antioxidant activity via MT1 receptors (17) that are expressed in rat skeletal muscle cells as well (23). Additionally, in an *in vivo* model of doxorubicin induced rat cardiac muscle damage melatonin lead to statistically significant increase in tissue catalase activity (24).

Conclusion

The results of the present study revealed that acutely administered CCl_4 caused significant decrease in rat gastrocnemius muscle catalase activity. Additionally, when melatonin was administered prior to CCl_4 such drastic decrease in cahttalase activity was prevented. The activity of melatonin could possibly be attributed both to its ability to directly scavenge H_2O_2 and to increase catalase production by enhancing mRNA synthesis.

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MELATONIN SPREČAVA SMANJENJE AKTIVNOSTI KATALAZE INDUKOVANO APLIKACIJOM UGLJEN-TETRAHLORIDA U *M. GASTROCNEMIUS*-U PACOVA

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Poznato je da je skeletno mišićno tkivo podložno oštećenjima koja su indukovana reaktivnim vrstama kiseonika, koje prate različite poremećaje skeletnih mišića. Ugljen-tetrahlorid (CCl_4) je opasna supstanca koja se koristi u eksperimentalne svrhe za indkuciju oštećenja tkiva i posredovana je reaktivnim kiseoničnim vrstama. Takođe, utvrđeno je da antioksidansi, prirodni ili sintetički, mogu da spreče/ublaže oštećenja izazvana kiseoničnim vrstama. Cilj ove studije bio je da utvrdi uticaje melatonina (MLT), snažnog antioksidansa, na promene u aktivnosti mišićne katalaze kod Wistar pacova koji su akutno tretirani CCl_4. Tkivo *m. gastrocnemius*-a, u kome je određivana aktivnost katalaze, uzeto je iz životinja koje su svrstane u tri grupe, i to iz životinja kontrolne grupe (netretirane), grupe tretirane CCl_4 i iz grupe pacova koja je tretirana MLT i CCl_4. Dobijeni rezultati ukazuju na statistički značajno smanjenje u aktivnosti katalaze kod životinja koje su tretirane CCl_4, dok ovako značajan pad aktivnosti katalaze nije primećen kod životinja koje su primile MLT i CCl_4. Protektivna aktivnost MLT može biti posledica različitih mehanizama dejstva s obzirom da je poznato da MLT direktno vezuje reaktivne kiseonične vrste, povećava antioksidativni kapacitet tkiva, ali i povećava gensku ekspresiju antioksidantnih enzima u tkivima.

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Ključne reči: *ugljen-tetrahlorid, m. gastrocnemius, katalaza*

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PROTEIN ENERGY WASTING IN PERITONEAL DIALYSIS PATIENTS

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Among many risk factors that affect outcomes of patients with chronic kidney disease and the ones on maintenance dialysis, the state of metabolic and nutritional derangements called "protein-energy wasting" (PEW) plays a major role. Most guidelines recommend that nutritional status should be evaluated by using a combination of valid, complementary measures.

The aims of this study were to analyze body composition, to detect the presence of undernutrition, and to establish the relationship between parameters of body composition, body mass index (BMI) and laboratory parameters routinely used as indicators of nutritional status in PD patients. Body composition was examined by a body composition monitor (BCM) that expresses body weight in terms of lean tissue mass (LTM) and fat tissue mass (FTM). BMI is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2). All the patients had blood samples taken for biochemical analysis (creatinine, albumin, cholesterol).

Our study included 37 peritoneal dialysis patients (23 female and 14 male). Our study was able to show high prevalence of undernutrition (56.76% of patients) expressed by low LTI, in our group of patients. Most of the patients had normal FTI (83.78%). Body mass index was not a good marker of nutritional status of our patients and according to this parameter there were no malnourished patients in our study group. The levels of creatinine and cholesterol showed positive correlation with LTM, proving to be good markers of nutritional status while the levels of albumin failed to show any correlation with other parameters of nutritional status. The 3 year follow up showed that the LTI has a positive correlation with survival.

Our conclusions are that PEW is common in dialysis patients. It should be assessed by complementary methods, and patients at risk should be treated adequately to reduce the risk of morbidity and mortality.

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Key words: *protein energy wasting, body composition, body mass index, peritoneal dialysis*

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Introduction

Among many risk factors that affect outcomes of chronic kidney disease (CKD) patients, especially the ones with end-stage renal disease (ESRD) and on maintenance dialysis, the state of metabolic and nutritional derangements called "protein-energy wasting" (PEW) of chronic kidney disease plays a major role(1). PEW is defined as a state of gradual and non-functional loss of muscle and fat tissue, eventually resulting in cachexia. The described state is not caused only by an inadequate dietary intake but is rather the result of metabolic disturbances in chronic renal failure such as derangements in fat metabolism, acidosis, inflammation-driven catabolism, nutrient losses in the dialysate, along with endocrine disturbances, such as hyperparathyroidism, hyperglucagonemia, and peripheral insulin resistance. A number of studies have shown that PEW is related to an increased number of hospitalizations, increased morbidity, and mortality (2).

While consensus about the importance of identifying and treating malnourished dialysis patients exists (3), the assessment of their nutritional status is not simple. Over the years, many methods have been proposed to identify malnourished and wasted CKD patients. However, there is currently no single tool that can be used to identify protein-energy wasting in these patients. On the contrary, most guidelines recommend that nutritional status should be evaluated by using a combination of valid, complementary measures (4, 5).

The aims of this study were to analyze body composition, to detect the presence of undernutri-

tion, and to establish the relationship between parameters of body composition, body mass index (BMI) and laboratory parameters routinely used as indicators of nutritional status in PD patients.

Patients and methods

We conducted a study in 37 patients that had been on peritoneal dialysis (PD) program in Clinical Center Niš, Clinic for Nephrology, during 2013. Survival of the patients was followed until the end of 2016. All the patients had biochemical parameters important for their nutritional assessment done (creatinine, cholesterol, and albumin). Body mass index was calculated as body weight (kg) to square of the body height (m) ratio. Body composition was assessed by a portable device (a body composition monitor - BCM Fresenius Medical Care D GmbH) that expresses body weight in terms of lean tissue mass (LTM) and fat tissue mass (FTM) independent of hydration status. The BCM Fresenius device measures the impedance of body tissues over wide frequency ranges and differentiates normally hydrated (NH) LTM from NH FTM regardless of the degree of extracellular fluid and it offers a reliable measure of both hydration status and nutritional status in our patients [+]. Undernutrition was defined as having a lean tissue index (LTI = LTM/height^2) below the 10th percentile of a reference population derived from BCM measurements of 1000 healthy adult subjects aged 18-75. This reference population is age and gender-specific, as body composition varies throughout life and between genders (6). Measurements were taken with the patient calm, supine, and relaxed for 2 minutes after the electrodes had been attached to the hand and foot on the same side of the body. All measurements were performed by a trained nurse. Exclusion criteria were dictated by the device and included the history of a pacemaker, defibrillator, stent implantation or amputation of a major limb.

The Excel program from Microsoft Office was used for quantitative statistical analysis, and a software package was used for the tabular presentation of data. Calculations were performed using the SPSS program version 10.0. The limits of statistical significance were estimated at 0.01 or 1% and 0.05 or 5% as the default error in all analysis. Chi-squared test was used to determine whether there was a significant difference between the expected frequencies and the observed frequencies in one or more categories. Spearman's rank correlation coefficient was used as a nonparametric measure of rank correlation (statistical dependence between the rankings of two variables), and, Mann–Whitney *U* test was used as a nonparametric test of the null hypothesis that it is equally likely that a randomly selected value from one sample will be less than or greater than a randomly selected value from a second sample.

Results

We conducted a study in 37 patients (pts), 14 (37.84%) male and 23 (62.16%) female, and their average age was 63.32 ± 12.61 years. The average age of male pts was 66.84 ± 9.93 years and the average age of female pts was 61.42 ± 13.70 years.

The frequency of patients on PD with qualitatively defined LTI and FTI values is given in table 1. A slight majority of patients, 21 (56%) had low LTI, while the rest of the study group had a normal LTI. None of them had high LTI. The majority of patients - 31 (83.78%) had normal values of FTI, while 3 of them (8.11%) had low FTI, and 3 of them (8.11%) had high FTI. There were significantly more PD patients with normal than abnormal values of FTI ($p < 0.001$, χ^2 test) in this study.

Table 1. Qualitatively defined LTI and FTI indexes in PD patients

Parameter	LTI	FTI
Normal	16 (43.24%)	31 (83.78%)
Low	21 (56.76%)	3 (8.11%)
High		3 (8.11%)
Σ	37 (100.00%)	37 (100.00%)

The nutritional status of PD patients based on BMI values is shown in Table 2. Based on BMI, there were no malnourished patients in our group, while 18 of them (48.65%) were normally nourished, and 19 of them (51.35%) were obese.

Table 2. Nutritional status of PD patients according to BMI values

Nutrition		
Normal	18	(48.65%)
Obese	19	(51.35%)
Σ	37	(100.00%)

Table 3 presents comparison of LTI and FTI values in adequately nourished and obese pts in PD (estimated by BMI), and the following results were established: FTI values are statistically higher in obese than in normally nourished PD pts (student t-test, $p < 0.001$).

Table 3. Values of LTI and FTI compared to the nutrition of the PD patients

	Normal weighted n = 18	Overweighted n = 19
LTI	12.03 ± 2.20 (11.60)	11.19 ± 1.94 (11.40)
FTI	8.93 ± 2.42 (9.00)	15.52 ± 4.85 (14.60)

The correlation of continual values of LTI and FTI with BMI is shown in Table 4. BMI shows a negative and week correlation with LTI, but a very strong and positive correlation with FTI (*Spearman correlation index*, $p < 0.001$).

Table 4. Correlation of BMI with LTI and FTI in PD patients

	LTI	FTI
BMI	-0.23	0.82***

There is a positive correlation between LTI and all the biochemical parameters of nutrition, but the statistically significant ones are with creatinine ($r = 0.34$, $p < 0.05$) and cholesterol ($r = 0.34$, $p < 0.05$).

There is positive but statistically non significant correlation between FTI and albumin (Table 5).

Table 5. Correlation of LTI and FTI with the biochemical parameters of nutritional status

	Creatinine		Albumin	Chol
LTI	0.34*(r)	r	0.27 (r)	0.39*(r,ρ)
FTI	-0.12 (ρ)	ρ	0.16 (ρ)	-0.30(ρ)

* - $p < 0.05$,
r – Pearson correlation coefficient,
ρ - Spearmancorrelation coefficient

There was statistically significant positive correlation between LTI and survival in our group of pts ($p < 0.01$). On the other hand no statisticaly significant correlation was found after comparing FTI and survival (Table 6).

Table 6. The correlation of LTI and FTI index and mortality in PD patients

	Survived n=19	Decided n=18
LTI	12.64 ± 1.91 (13.30)**	10.49 ± 1.68 (11.15)
FTI	10.84 ± 4.14 (9.70)	13.87 ± 5.59 (12.70)

** $p < 0.01$ (Mann-Whitney test)

Discussion

Our study was able to show high prevalence of undernutrition, as expressed by low LTI, in our group of patients. These findings are consistent with several other studies in which the prevalence of undernutrition differed according to the method used to define nutritional status. The French study (7) which included 7123 patients, showed undernutrition in 62% of the studied population, according to the lean body mass index. Woodrow et al. (8) have also found a significantly lower LTM measured with dual X-ray absorptiometry (DXA) and bioelectrical impedance analysis (BIA) in patients with advanced chronic renal failure, peritoneal dialysis, and HD compared with the healthy group.

Body mass index has not adequately presented the nutritional status of our patients, and according to this parameter, there were no malnourished patients in our study group, while 18 of them (48.65%) were normally nourished, and 19 of them (51.35%) were obese. This fact supports the view that BMI does not represent an adequate parameter of nutrition for patients with chronic renal failure, as gross imbalance in fluid status in these patients may cloud the results. Also, the loss of muscle mass is characteristic in PEW; however, a relatively well-preserved fat mass still usually remains, resulting in small changes in BMI that can be disguised by imbalances in fluid homeostasis (9). Finally, BMI can't estimate the distribution of fat tissue which has significant metabolic implications.

The ability of the Body Composition Monitor to separate fat from lean tissue allowed us to examine the correlation between BMI and LTI as well as BMI and FTI. In healthy subjects BMI depends on both fat and lean (muscular) tissue (10). In our patients, BMI clearly expressed the changes in the fat deposits but failed to show lean mass status. Similar results were obtained by Honda et al. (9) who have examined the relationship between BMI, body composition parameters, and PEW in 328 patients who started dialysis treatment. Their study showed high incidence of PEW in obese patients, which correlated with high FTI and low LTI, a state which is defined as "obese sarcopenia".

Biochemical parameters have an important role in routine assessment of the nutritional status of dialysis patients. The most commonly used biochemical parameters are: creatinine, albumin and cholesterol. However, many studies have shown that they are not always reliable.

In our study the level of albumin has shown no correlation with LTI and a week positive correlation with no statistical significance with FTI. These results are in line with other studies that have demonstrated the uselessness of serum albumin levels as an accurate marker of nutritional status in dialysis patients (11, 12). There are several reasons for that: first, the level of albumin is influenced by factors such as overhydration, proteinuria and losses into the dialysate in PD patients. Additionally, albumin has a relatively long half-life, limiting the impact of a decreased protein intake on serum concentrations (13). Also, albumin is an acute phase reactant; thus, s-albumin levels in dialysis patients are strongly associated with inflammation rather than insufficient food intake (14).

Creatinine has shown a positive and statistically significant correlation with LTI, proving to be a good marker of muscle mass. These results are in accordance with several other studies which have shown that even in stable chronic dialysis patients in whom kidney function is minimal to nonexistent serum creatinine level is a reliable surrogate marker of muscle mass (15, 16).

Serum cholesterol has also been proposed as an assessment criterion for malnutrition and PEW (5). Our study has shown statistically significant positive correlation between serum cholesterol levels and LTI, but no correlation between s-cholesterol and FTI. Contrary to the general population (17), a high s-cholesterol level in the CKD and dialysis population is associated with improved survival (18).

The three year follow up of our patients showed that there was a statistically significant positive correlation between LTI and survival in our study group, while, on the other hand, no statistical significance was found after comparing FTI and survival. These findings are similar to those published in the study of Slovakian population which showed that low LTI was a strong independent predictor of mortality (19).

Conclusion

Protein energy wasting is common and very significant problem in dialysis patients. It is not always easy to assess, and it usually requires complementary measures to be done. However, since it has a significant impact on our patients lives by increasing the number of hospitalizations, morbidity, and mortality it should be carefully monitored and treated when needed.

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GUBITAK PROTEINSKE ENERGIJE KOD BOLESNIKA PERITONEALNE DIJALIZE

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Među brojnim faktorima rizika koji utiču na ishod lečenja bolesnika sa hroničnom bubrežnom slabošću i onima na dijalizi, stanje metaboličkih i nutritivnih poremećaja označeno kao "proteinsko-energetska pothranjenost" zauzima značajno mesto. Brojni vodiči predlažu da se stanje uhranjenosti naših bolesnika procenjuje kombinovanjem komplementarnih merenja.

Ciljevi ispitivanja bili su analiza telesnog sastava, utvrđivanje učestalosti pothranjenosti i korelacije između parametara telesnog sastava, indeksa telesne mase (ITM) i laboratorijskih parametara koji se najčešće koriste za procenu stanja uhranjenosti bolesnika na dijalizi. Analiza telesnog sastava vršena je na osnovu podataka dobijenih Body composition monitorom – aparatom koji meri masu masnog (fat tissue mass FTM) i nemasnog (lean tissue mass LTM) tkiva. ITM je izračunavan kao odnos telesne težine u kilogramima i kvadrata visine u metrima (kg/m^2). Svim bolesnicima uzimana je krv za opšte laboratorijske analize (kreatinin, holesterol, albumini).

U našem ispitivanju učestvovalo je 37 bolesnika sa programa peritonealne dijalize (23 žene i 14 muškaraca). Naše ispitivanje pokazalo je veliku učestalost pothranjenosti bolesnika (56,76%) izraženu kroz nizak indeks nemasnog tkiva. Najveći broj bolesnika imao je normalan indeks masnog tkiva (83,78%). Indeks telesne mase (ITM) nije se pokazao kao dobar marker nutritivnog statusa i na osnovu ovog parametra nije bilo pothranjenih bolesnika u našoj grupi. Nivo kreatinina i holesterola pokazao je pozitivnu korelaciju sa masom nemasnog tkiva, čime su se ovi parametri izdvojili kao dobri markeri nutritivnog statusa, za razliku od albumina koji nije pokazao nikakvu korelaciju sa drugim parametrima uhranjenosti. Trogodišnje praćenje ovih bolesnika pokazalo je da postoji pozitivna korelacija između indeksa nemasnog tkiva i preživljavanja bolesnika.

Naši zaključci su da je proteinsko-energetska pothranjenost česta kod bolesnika na dijalizi. Treba je procenjivati kombinovanjem komplementarnih metoda i bolesnike sa rizikom treba adekvatno lečiti kako bi se smanjio morbiditet i mortalitet.

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Ključne reči: *proteinsko-energetska pothranjenost, telesni sastav, indeks telesne mase, peritonealna dijaliza*

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PREPARATION AND INTEGRITY EXAMINATION OF FREEZE DRIED KIT OF TRASTUZUMAB-IMMUNOCONJUGATES AND COLD LABELED IMMUNOCONJUGATES BY APPLYING SDS-PAGE ELECTROPHORESIS

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Radioimmunoconjugates are promising agents in diagnostics and treatment of different types of cancers. The aim of this study was the formulation of stable freeze dried kits of trastuzumab with three types of bifunctional chelators for further radiolabeling. The integrity of the antibody in formulated conjugates was examinated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Trastuzumab is a humanized monoclonal antibody used in the therapy of aggressive HER2 positive breast cancer. Conjugation of trastuzumab was made with various chelators: p-SCN-Bn-DTPA (1:10; 1:20; 1:50), p-SCN-Bn-DOTA (1:20), and 1B4M-DTPA (1:10; 1:20; 1:50). The purified immunoconjugates were lyophilized by applying two day protocol in order to produce the stable freeze dried kits. Cold labeling with nonradioactive isotopes $LuCl_3$ and YCl_3 was performed to examine the possible modifications of secondary structure after radioactive labeling. SDS-PAGE electrophoresis was used to estimate the purity and integrity of the antibody before and after conjugations, lyophilization and labeling under reducing conditions.

The obtained results show that there is no degradation of the examined antibody. The trastuzumab-conjugates and cold labeled formulations migrated in two bands (~50 kDa and ~25 kDa), in the same way as IgG1 antibodies and unmodified trastuzumab.

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Key words: *Bifunctional chelators, conjugations, electrophoresis, trastuzumab*

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Introduction

The human epidermal growth factor receptor (HER2) is overexpressed on a surface of the cells in aggressive breast cancer. HER2 positive breast cancers without anti-HER2 therapy lead to metastasis and very low survival rates of the patients (1). Trastuzumab is the first approved humanized monoclonal antibody. It's a potent anti-HER2 antibody which originates from murine 4D5 antibody (2). Carter et al., 1992 (3) cloned hypervariable regions from 4D5 in plasmids which are encoding formation of constant regions from human IgG1 antibody. A vector that is encoding formation of chimeric antibody, which is additionally humanized, was successfully generated. Trastuzumab is binding to the IV subdomain of the receptor and manifests the effect through antibody-dependent cellular cytotoxicity (4).

Conjugated antibodies provide high expectation for development of cancer-specific cytotoxic reagents. Each immunoconjugate has an enhanced therapeutic specificity due to the conjugation with antibody which shows specificity to a particular antigen on the surface of tumor cells (5). Radioimmunoconjugates are step forward in diagnostic and treatment of different types of cancers (6). Until now, there is no FDA registration of trastuzumab-radioimmunoconjugates for commercial use. A series of clinical and preclinical trials of radioimmunoconjugates for treatment and imaging of breast cancer with different radionuclides ^{111}In (7-9), ^{99}mTc (10), ^{67}Ga (11), ^{90}Y, ^{86}Y (12), ^{177}Lu (13, 14) have been made in the past few years. Creation of stable immunocon-jugate with bifunctional chelators (BFCAs) is required in order to obtain succsessful labeling. This chelator allows binding to the antibody on the one side and coordinative binding of radioisotopes on the opposite. The commonly investigated chelators are: DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), DTPA (diethylene triamine pentaacetic acid),

DTPA derivate 1B4M-DTPA (2-(4-isothi-ocyanatobenzyl)-6-methyl-diethylene-triaminepenta-acetic acid) and EDTA (ethylenediamine tetraacetic acid) (15). Due to the easy chemical and physical degradations, monoclonal antibodies are not stable in water solutions. The commonly processes of preparations are denaturation, deamidation, oxidation, deglycolisation, aggregation, precipitation and adsorption. In order to prolong the life of protein pharmaceuticals, it is necessary to remove the water from the product. The commonly used method is lyophilization which obtains stable freeze dried kits (16).

Examinations with non-radioactive isotopes (Lu and Y) are done with intention to confirm that there are no changes in the secondary structure of the antibody after coordinative binding of the metal, because these studies are impossible with radioactive isotopes (17). Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is important for the determination of molecular mass (Mr) as well as the possible degradations of trastuzumab, trastuzumab-immunoconjugates and cold labeled kits too (18).

The aim of this study was formulation of stable freeze dried kit of trastuzumab with p-SCN-Bn-DOTA (2-(4-izothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), p-SCN-Bn-1B4M-DTPA (2-(4-isothiocyanatobenzyl)-6-methyldiethylene-triaminepentaacetic acid) and p-SCN-Bn-DTPA (2-(4-izothiocyanatobenzyl)-diethylenetriaminepentaacetic acid) and subsequently labeling with cold isotopes (Lu and Y). Protein integrity determination and possible aggregations were investigated by using reducing one-dimensional SDS-PAGE.

Material and methods

Purification of trastuzumab. Trastuzumab was purified from Herceptin® (Hoffmann-La Roche, Basel, Switzerland) with Amicon® Ultra-4 (Sigma Aldrich, Missouri, USA), 30 KDa for ultrafiltration for 1 hour at 5000 rpm, by washing the antibody in six cycles with 0.1 M PBS, pH = 8, using HuMax 4k (Human, Wiesbaden, Germany) centrifuge.

Conjugation of trastuzumab. The conjugations were made by mixing the antibody with 10 mg/mL solution of BFCAs in different molar ratio (p-SCN-Bn-DTPA – 1:10; 1:20; 1:50, 1B4M-DTPA – 1:10; 1:20; 1:50 and p-SCN-Bn-DOTA – 1:20) and 18 hours incubation on 4 °C with gentle shaking. The immunoconjugates were purified with six cycles of ultrafiltration (Amicon® Ultra-4, 30 KDa), by rinsing with 0.05 M ammonium acetate, pH = 7. The concentration was adjusted to 1 mg/mL using the 6715 UV/Vis Spectrophotometer Jenway® (Stafford-shire, UK).

Lyophilization of trastuzumab-immunoconjugates. The volume of 1 mL of the solution was transferred to type I glass vials and lyophilized to solid state. The immunoconjugates were lyophilized with cryoprotectant and bulking agent, 1% mannitol (50 µL in each vial). The lyophilization was performed with Labconco Free Zone Stoppering Tray Dryer (Kansas City, Missouri, USA), using the protocol by Gjorgieva Ackova et al., 2014 (19), with several modifications. The samples were equally placed on the shelves after reaching the temperature of 4 °C. The first step was freezing the samples. The temperature was decreased at -40 °C, 1 °C/min (rate of cooling, from 4 °C to -40 °C) and the time of keeping was 5 hours. The second phase of preparation was primary drying at the temperature of -10 °C, 0.15 °C/min (rate of heating, from -40 °C to -25 °C) for 28 hours and pressure of 0.133 mBar. The third part was secondary drying on 25 °C, 0.2 °C/min (rate of heating, from -25 °C to 25 °C) for 14 hours. The whole protocol of lyophilization is shown in Figure 1. After completing freeze drying, the vials were closed and kept at 4 °C in order to perform the following examinations.

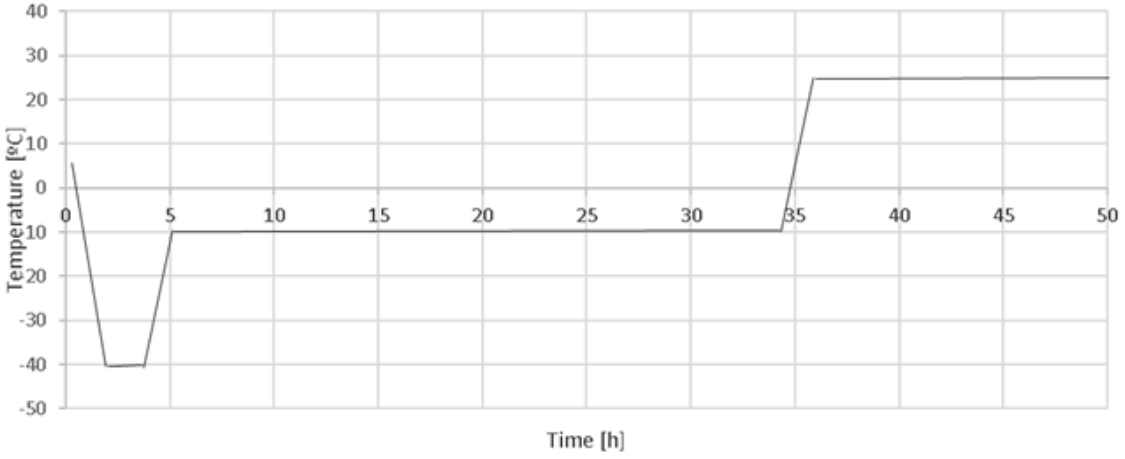

Figure 1. Lyophilization protocol

Labeling trastuzumab-immunoconjugates with non-radioactive Lu and Y

The solutions of non-radioactive Lu and Y were prepared by dissolving of $LuCl_3$ to concentration of 1.0709 μg/μL (equivalent to maximum tolerated dose (MTD) of ^{177}Lu (4377,1 MBq)) and YCl_3 to concentration of 1.1555 mg/μL (equivalent to MTD of ^{90}Y (1049,69 MBq)). The immunoconjugates were dissolved with sterile 0.9% solution of NaCl and then labeled with 1 μL of prepared solution of cold Lu and Y to total volume of 1 mL. The labeled DTPA and 1B4M-DTPA conjugates were incu-bated for 30 min at 25 ºC as well as DOTA conjugates were incubated for 1 hour at 40 ºC.

Integrity examinations with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis was performed under reducing conditions with Enduro® Modular Vertical Gel Electrophoresis System (Labnet, Edison, NJ, USA), using mercaptoethanol. In this process, 12% separating acrylamide/bisacrylamide gel and 4% stacking gel was prepared according to the protocol of the manufacturer manual instructions. Electrophoresis was performed three times, under the same conditions. Concentration of each sample was 1 mg/mL. At the first gel, unmodified trastuzumab and freeze dried immunoconjugates were applied after reconstitution with 0.9% NaCl. At the second and the third gel, immunoconjugates were applied labeled with non-radioactive Lu and Y. A mass of 30 μg of sample was added in 10 μL of loading buffer. Then the mixtures were heated for 5 min at dry bath at 98.5 ºC and centrifuged for 5 min at 5000 rpm. 25 μL of each sample and 10 μL of SigmaMarkerTM, Wide Range, Molecular Weight (Saint Louis, MO, USA) were applied in wells of stacking gel. According to our previous studies, constant voltage at 150 V and approximately current at 23 mA has been used as optimum condition. After finishing the entire process (which lasted approximately an hour and a half), the gel was placed for Coomassie staining for 20 hours. Discoloration was observed in the solution composed of water, methanol and acetic acid for 7-8 hours. All used reagents were from Sigma-Aldrich (Missouri, USA). The prepared gels were scanned with Glite 900 BW Gel Scanner (PacificImage, Torrance, CA).

Results

Stable freeze dried kits of trastuzumab-immunoconjugates were formulated. The structures of used BFCAs are shown in Figure 2. There are various groups (anhydride, bromoacetamid, isothiocyanate) that can be attached to the BFCAs molecules, to achieve an easier conjugation (17). The chelators have 4-izothiocyanatobenzyl groups in theirs structures which participate in the process of binding to the antibody (Figure 3). Thiourea linkage is created due to reaction between amino groups of lysine residues of trastuzumab and isothiocyanate groups of chelators (20). To remove the water and increase the stability, the immunoconjugates were lyophilized using the previously described protocol. The whole process included freezing of the sample and then removing the water by sublimation in a vacuum environment. After performing the freeze drying process solid formulations with a homogeneous structure were obtained. For further examinations, the stable freeze dried cakes were used. The integrity of the protein, purity and possible formation of aggregates after lyophilization and after cold labeling were established with SDS-PAGE. For comparison, unmodified trastuzumab in a concentration of 1 mg/mL was used. The electrophoresis was performed under reducing conditions. Disulfide bonds were reduced with 2-mercaptoethanol and antibody was separated in two fragments: two heavy chains ~50 kDa and two light chains ~25 kDa (Figure 4) (21). The gel with unmodified trastuzumab and trastuzumab-immunoconjugates with different molar ratio of BFCAs is shown in Figure 5. The gels with cold labeled immunoconjugates are shown in Figure 6 and Figure 7, as well.

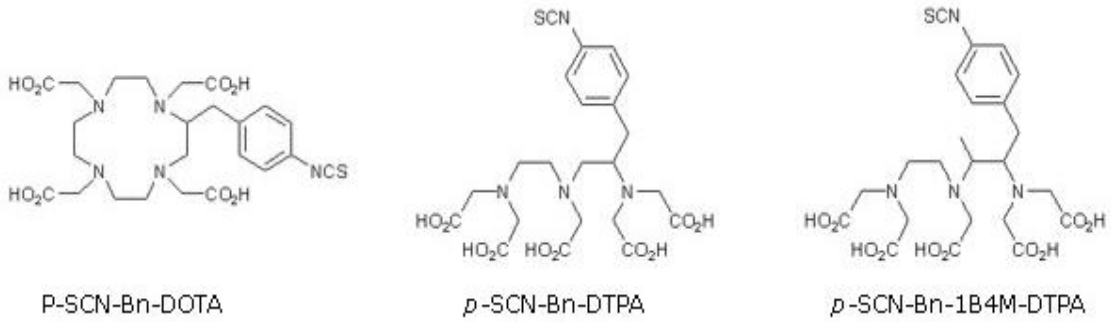

Figure 2. Structure of BFCAs

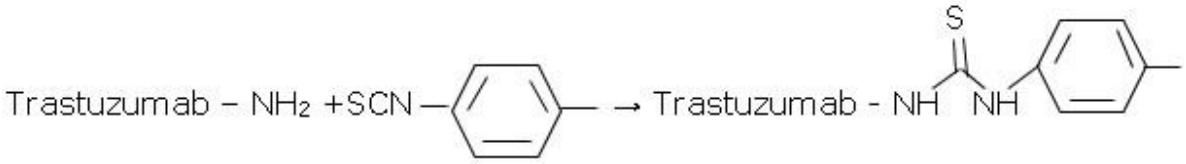

Figure 3. Reaction of conjugation

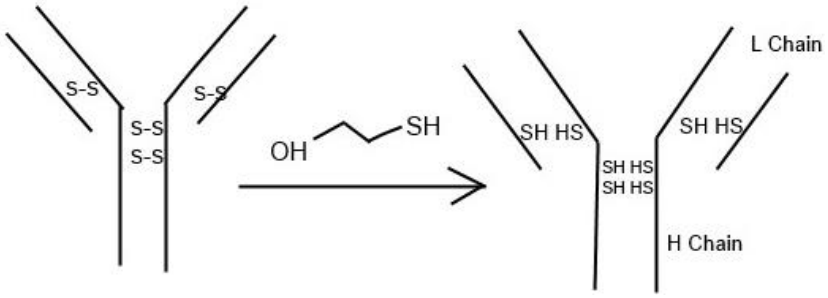

Figure 4. Reducing SDS-PAGE

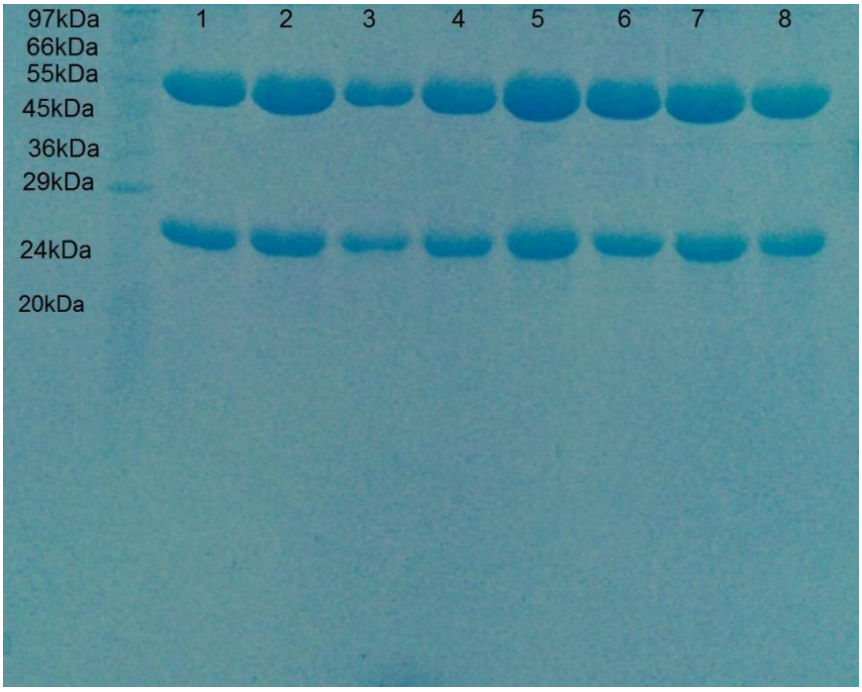

Figure 5. Reducing SDS-PAGE of Trastuzumab 1 mg/mL (1);
DTPA-Trastuzumab (1:10) (2); DTPA-Trastuzumab (1:20) (3);
DTPA-Trastuzumab (1:50) (4);
DOTA-Trastuzumab (1:20) (5);
1B4M-DTPA-Trastuzumab (1:10) (6);
1B4M-DTPA-Trastuzumab (1:20) (7);
1B4M-DTPA-Trastuzumab (1:50) (8).

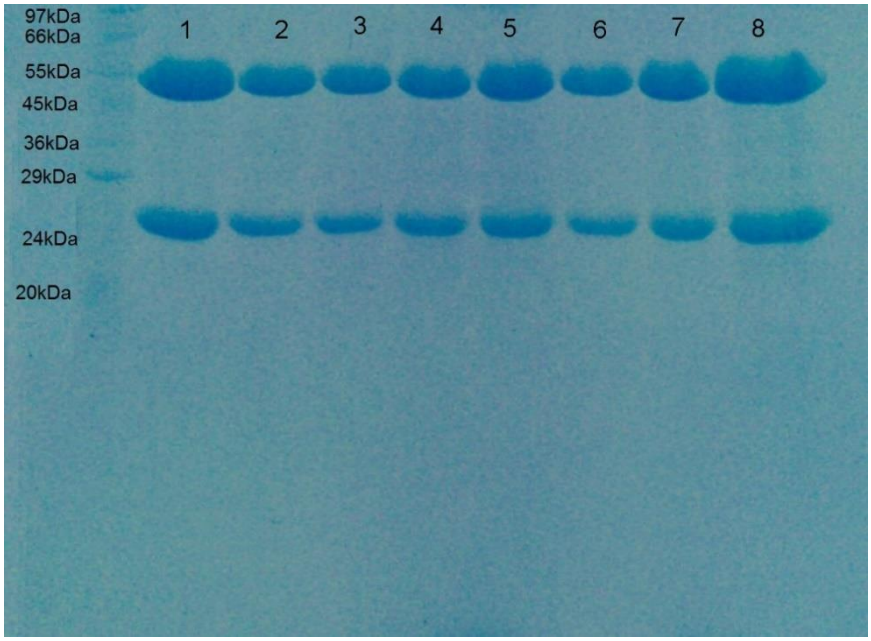

Figure 6. Reducing SDS-PAGE of Y-DTPA-Trastuzumab (1:10) (1);
Lu-DTPA-Trastuzumab (1:10) (2);
Y-DTPA-Trastuzumab (1:20) (3);
Lu-DTPA-Trastuzumab (1:20) (4);
Y-DTPA-Trastuzumab (1:50) (5);
Y-DTPA-Trastuzumab (1:50) (6);
Y-DOTA-Trastuzumab (1:20) (7);
Lu-DOTA-Trastuzumab (1:20) (8).

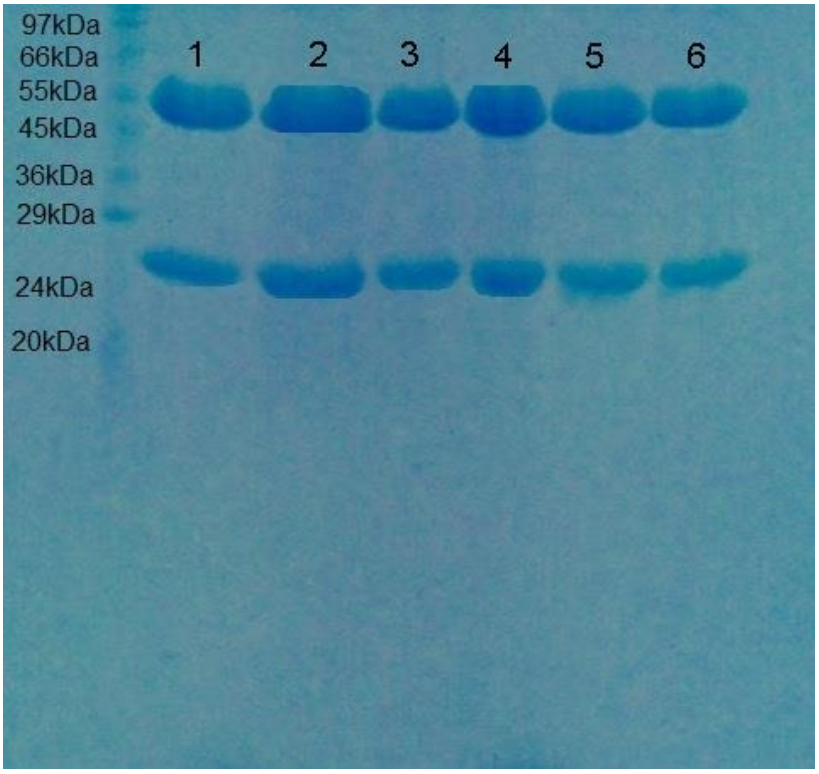

Figure 7. Reducing SDS-PAGE of Y-1B4M-DTPA-Trastuzumab (1:10) (1);
Lu-1B4M-DTPA-Trastuzumab (1:10) (2);
Y-1B4M-DTPA-Trastuzumab (1:20) (3);
Lu-1B4M-DTPA-Trastuzumab (1:20) (4);
Y-1B4M-DTPA-Trastuzumab (1:50) (5);
Lu-1B4M-DTPA-Trastuzumab (1:50) (6).

Discussion

Lyophilization has a major significance in the process of formulation of stable pharmaceutical products (vaccines, monoclonal antibodies and protein formulation). Properly selected conditions are essential for complete removal of water and retention of the structural integrity of the proteins. They are necessary procedures for obtaining stable, homogeneous cakes with flat surfaces which retain the properties after reconstitution. The first step, freezing, is important to the process of separation of the water from the solution and leads the protein to crystalline state, which is more stable. Control of crystallization and size of crystals during the freezing are critical parameters and the appropriate selection of cooling rate is very important. It is already known that slow cooling provides larger crystals, while faster freezing provides smaller crystals. On the other side, the faster cooling can lead to aggregations and denaturation of the antibody. During preparation of aqueous formulations, it is necessary to place them on the cooled shelves to 4-5 ºC (22). With an intention to improve the crystallization, the annealing step is often included (23). Our research team has already some experience with freeze drying of monoclonal antibody rituximab. Namely, Gjorgieva Ackova et al., 2014 (19) used a three day protocol with included annealing process. The obtained results were promising, since stable cakes with no aggregates and denaturation of the antibody were produced. But, our goal was to shorten the length of procedures in two days with exclusion of the annealing step. Also, we tried to show that with the application of cooling rate of 1 º C/min we could obtain cakes with the same quality. The choice of the mentioned cooling rate was done based on results of the study of Tang and Pikal, 2004 (24). Our improvement of procedure of a super cooling without phase of separation and a uniform of ice structure was reached with the same rate of freezing.

The primary drying is the second step of lyophilization when the majority of frozen bulk water is removed by sublimation in a vacuum environment. Instead of 25 hours, the time was prolonged to 28 hours. Also, the secondary drying time was extended to 14 hours. At this phase some bound unfrozen water is removed at temperatures higher than 25 ºC (23). Many sugars (glucose, mannitol or trehalose) can be used like cryoprotectants, to protect the proteins from denaturation and inactivation during the process of freezing (25). Nounou et al., 2005 (26) have shown in their study that obtained freeze dried cakes without any cryoprotectant possessed a compact structure but also demonstrated difficulties in process of reconstitution. This is probably the result of using sugars as bulking agents in production of fluffy cakes for easy reconstruction. In our studies to protect the antibody from stress temperature and to obtain elegant cakes for easy reconstitution 1% mannitol was used. After the dissolving of the cakes with 0.9% NaCl for less than a minute, clear to opalescent solutions were obtained without presence of visible solid particles and colloids.

The processes of conjugation and freeze drying of the antibodies can lead to changes in protein integrity (aggregations and fragmentations). It is important to show that after cold labeling with Y and Lu, the metal does not change the structure of antibody. One of the most important methods for examination of protein integrity and purity is SDS-PAGE in reducing conditions (size-based method, where separation is based on the size of the molecules) (17, 27). Many stability studies of trastuzumab were made using a SDS-PAGE in reducing or non-reducing conditions. Under reducing conditions, trastuzumab was migrated as two bands a ~50 kDa and ~25 kDa (Mr of heavy and light chain), while under non-reducing conditions only one band at ~150 kDa was observed (Mr of whole antibody) (18, 28, 29). Our group has already used the applied method of reducing electrophoresis for integrity examinations of rituximab after conjugation and lyophilization (17, 19).

Almost the same conditions were used for exammination of trastuzumab, with several modifications. In order to achieve reducing conditions, instead of dithiothreitol, 2-mercaptoethanol was used. The SDS -PAGE examinations were performed after six months storage of the freeze dried kits at the temperature of 4 ºC. The cold labeling was done before starting the process of electrophoresis. The obtained results of migration of formed samples correspond to already published results for other IgG1 monoclonal antibodies (18, 28). Under reducing conditions, migration of the trastuzumab provided separation of two bands of fragments with molecular weight of 25 kDa for light chain and 50 kDa for heavy chain, proven with the Wide Range, molecular weight marker. The same intensity of the fragments of lyophilized and labeled conjugates with the fragments of pure commercial (unmodified) trastuzumab indicated that there was no degradation of the antibody. The results have shown that the conjugation, lyophilization and label-ing were successful, without any damage to the anti-body, with absence of fragmentation, denaturation and aggregation of the trastuzumab.

Conclusion

The results of electrophoretic examination have shown that stable freeze dried conjugates of trastuzumab were formulated with BFCAs in different molar ratios. The integrity and purity of the antibody were retained after cold labeling with Y and Lu. Therefore, these kits will be used for further characterization of the secondary structure of the antibody and determination of the number of the chelators with Infra Infrared Spectroscopy (IR), Raman Spectroscopy and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (MALDI-TOF-MS). Moreover, immunoconjugates are good basis for radiolabeling with application of ^{177}Lu and ^{90}Y and therefore further researches will be done in order to evaluate the potential role of these conjugates in diagnostics and therapy of metastatic breast cancer.

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PRIPREMA I ISPITIVANJE INTEGRITETA LIOFILIZIRANIH KITOVA TRASTUZUMAB-IMUNOKONJUGATA I HLADNO OBELEŽENIH IMUNOKONJUGATA PRIMENOM SDS-PAGE ELEKTROFOREZE

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Radioimunokonjugati su agensi za koje se smatra da imaju veliki potencijal u dijagnostici i lečenju različitih tipova kancera. Cilj ove studije je formulacija stabilnih liofiliziranih kitova trastuzumaba korišćenjem tri vrste bifunkcionalnih helatora, radi daljeg radioaktivnog obeležavanja. Integritet antitela u formulisanim konjugatima ispitivan je primenom natrijum dodecil sulfat poliakrilamid gel elektroforeze (SDS-PAGE).

Trastuzumab je humanizovano monoklonsko antitelo koje se koristi u terapiji agresivnog HER2 pozitivnog kancera dojke. Konjugacija trastuzumaba izvršena je korišćenjem različitih helatora: p-SCN-Bn-DTPA (1:10; 1:20; 1:50), p-SCN-Bn-DOTA (1:20), i 1B4M-DTPA (1:10; 1:20; 1:50). Prečišćeni imunokonjugati liofilizirani su primenom dvodnevnog protokola kako bi se dobili stabilni liofilizirani kitovi. Hladno obeležavanje neradioaktivnim izotopima $LuCl_3$ i YCl_3 izvršeno je da bi se uočile potencijalne modifikacije sekundarne strukture nakon radioaktivnog obeležavanja. SDS-PAGE elektroforeza u uslovima redukcije korišćena je za utvrđivanje čistoće i integriteta antitela pre i posle konjugacije, liofilizacije i obeležavanja.

Na osnovu dobijenih rezultata nije uočena degradacija ispitivanog antitela. Trastuzumab-konjugati i hladno obeležene formulacije migriraju u dve trake (~50 kDa i ~25 kDa), na isti način kao IgG1 antitela i nemodifikovani trastuzumab.

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Ključne reči: *bifunkcionalni helatori, konjugati, elektroforeza, trastuzumab*

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BIOLOGICAL ACTIVITY OF EXTRACTS AND ESSENTIAL OILS OF TWO *ERYNGIUM* (*APIACEAE*) SPECIES FROM THE BALKAN PENINSULA

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The present study describes antioxidant and antimicrobial activity of water, methanol, acetone and ethyl acetate extracts from *Eryngium maritimum* L. from Greece and *Eryngium serbicum* Pančić, growing wild in Serbia. Also, antimicrobial activity of essential oils from aerial parts was analysed. Spectrophotometric methods were used for measuring of total phenols, total flavonoids, as well as for antioxidant potential, using DPPH and ABTS methods. The total phenolic content in the extracts was determined using Folin-Ciocalteu reagent and their amounts ranged between 7.47 and 121.35 mg GAE/g. The concentrations of flavonoids in the extracts varied from 8.98 to 48.68 mg QU/g. Antioxidant activity ranged from 1.247 to 31.19 IC_{50} (mg/ml) and from 0.109 to 3.36 mg AA/g when tested with the DPPH and ABTS reagents, respectively. The antimicrobial activity of the extracts and essential oils was investigated using a micro well-dilution assay against the most common human gastrointestinal pathogenic bacterial strains. The most resistant bacterium was *Srteptococcus pyogenes*, while *Staphylococcus aureus* showed high sensitivity in presence of all tested extracts exept on water extract of E. maritimum. Essential oil of *E. serbicum* showed better antimicrobial activity than *E. maritimum* oil. This finding suggests that investigated *Eryngium* species may be considered as a natural source of antioxidant and antimicrobial agents.

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Key words: *Eryngium maritimum, E. serbicum, extracts, essential oils, antioxidant, antimicrobial activity*

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Introduction

The genus *Eryngium*, belonging to the subfamily Saniculoideae of the Apiaceae family, is represented by 317 accepted taxa worldwide, known by thier high content of acetylenes, flavonoids, coumarins and triterpene saponins (1). Among them, several *Eryngium* species have been used as ornamental plants, condiments (2) or in traditional medicine (3, 4).

Eryngium maritimum L. (sea holly) is a perennial plant (30-60 cm high) with mauve flowers growing wild on the sandy beaches of West Europe, Mediterranean basin and Black Sea (5). Young roots and shoots of *E. maritimum* are eaten as a vegetable and young leaves are consumed as a salad in northern Europe and Greece. Candied roots of *E. maritimum*, have been valued as an aphrodisiac tonic especially in England (6). *E. maritimum* have also been reported to exhibit different therapeutic uses in folk medicine as diuretic or hypoglycemic (7). *E. maritimum* was in the past a widely used medicinal herb and in modern phytotherapy it is considered a remedy in renal disorders (3, 8). The main secondary metabolites isolated from *E. maritimum* extracts were glycosides of kaempferol, isoquercetin and astragalin (9).

Eryngium serbicum Pančić is a perennial plant growing to a height of 40-75 cm. This species is distributed in Serbia and considered a regional endemic (10, 11).

In this work the antioxidant activity of four different extracts for *E. maritimum* and *E. serbicum* is reported. Also, we compared antimicrobial effect of essential oils and extracts from aerial parts of this species. To our knowledge, the comparative biological effect of this two species has not been previously reported.

Material and methods

Chemicals

Organic solvents were purchased from "Zorka pharma" Šabac, Serbia. Gallic acid, 3-tert-butyl-4-hydroxyanisole (BHA) and 2,2-dyphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent was purchased from Merck, Darmstadl, Germany. Sodium carbonate anhydrous (Na_2CO_3), potassium acetate ($C_2H_3KO_2$), potassium peroxidisulphate ($K_2O_8S_2$) and L(+) - Ascorbic acid (Vitamin C) were purchased from AnalaR Normapur, VWR, Geldenaaksebaan, Leuven Belgium. Aluminium nitrate nonahydrate ($Al(NO_3)_3x9H_2O$) was purchased from Fluka Chemie AG, Buchs, Switzerland. ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) and quercetin hydrate were obtained from TCI Europe NV, Boerenveldsweg, Belgium. All other solvents and chemicals were of analytical grade.

Plant material

Aerial parts from wild growing species of *E. maritimum* (Glyfada, Corfu - Greece) and *E. Serbicum* (coast of the Ibar river – Serbia) were collected in July 2015. A voucher specimen (10876, 10877) was deposited in the "Herbarium Moesiacum Niš", University of Niš.

Preparation of plant extracts

Plant material was air dried in the dark and ground to powder. The aerial plants parts (10 g) were extracted with 100 ml water (H_2O), methanol (MeOH), acetone (Acet) or ethyl acetate (EtOAc).

Table 1. The extracts yield for investigated Eryngium species

Yield of extract (%, mg per g)	H_2O	MeOH	Acet	EtoAc
E. maritimum	0.945	0.958	0.336	0.382
E. serbicum	0.993	1.043	0.275	0.186

The mixture was exposed to ultrasound bath for 30 min and after 24 h standing in the dark was filtered. MeOH, EtOAc and Acet solvents were removed by evaporation under the reduced pressure, at maximum temperature of 40ºC. H_2O extract was frozen and later dried by freeze-drying. After evaporation of the solvent the crude extract was subjected to subsequent analysis. Extracts concentration was 2 mg/ml. The extracts yields for *E. maritimum* and *E. serbicum* are presented in Table 1.

Essential oils isolation

Essential oils were obtained separately by hydro-distillation for 3 h, using a Clevengertype apparatus, from 470 g of dried aerial parts of *E. Maritimum* and *E. serbicum.* Anhydrous sodium sulfate was used for desiccation of oils. Oils were stored at temperature of 4ºC. The yield of essential oils calculated from dried plant material was 0.094% and 0.091%, respectively.

Determination of total phenolic content

The total phenolic content of extracts was determined spectrophotometrically by Folin-Ciocalteu method according to slightly modified procedure of Singleton et al. (1999) (12). Briefly, 300 µl of extracts solution and 1500 µl of 1:10 Folin-Ciocalteau reagent were mixed and after 6 minutes in the dark 1200 µl of sodium carbonate (7.5%) was added. After 2 h of incubation in the dark at room temperature, the absorbance at 740 nm was measured. The total phenolic concentration was calculated from a gallic acid (GAE) calibration curve (10-100 mg/g).

Determination of flavonoid content

The total flavonoid content was evaluated using aluminium nitrate nonahydrate according to the procedure reported by Woisky and Salatino (1998) with some modifications (13). The sample for determination was prepared by mixing a 600 µl of extracts solution and 2580 µl of mixture (80% C_2H_5OH, 10% $Al(NO_3)_3x9H_2O$ and 1M $C_2H_3KO_2$). After 40 min of incubation at room temperature, the absorbance at 415 nm was measured. The total flavonoid concentration in extracts was calculated from a quercetin hydrate (Qu) calibration curve (10-100 mg/g).

Evaluation of DPPH scavenging activity

The antioxidant activity of extracts was evaluated by means of the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method. This spectrophotometer assay uses stable radical DPPH as reagent (14).

Absorbance of remaining DPPH radical was measured on 517 nm after that time (A_1) on Shimadzu, UV-Visible PC 1650 spectrophotometer. Every concentration was done in triplicate and the same was done with Vitamin C and BHA, known antioxi-

dants. Blank probes were done in the same way using MeOH instead of investigated solution (A_0). The decrease of absorption of DPPH solution is calculated by equation:

Percentage of absorption decrease (on 517 nm) = (A_0-A_1) x100/ A_0

Concentrations which decrease absorption of DPPH solution for 50% (IC_{50}) were obtained from the curve dependence of absorption of DPPH solution on 517 nm from concentration for each compound and standard antioxidant.

Evaluation of ABTS radical scavenging activity

For ABTS radical-scavenging activity, the procedure followed the method of Miller and Rice-Evans (1997) with some modifications (15). The $ABTS^+$ solution was prepared by mixing 19.2 mg of ABTS with 5 ml of potassium persulfate (2.46 mM). The solution was held at room temperature in the dark for 12-16 h before use. The $ABTS^+$ solution (1 ml) was diluted with 100-110 ml H_2O, in order to obtain an absorbance 0.7 ± 0.02 at 734 nm. Fresh $ABTS^+$ solution was prepared for each analysis. Antioxidant or standard solutions, 75 µl, were mixed with 3 ml of diluted $ABTS^+$ solution and incubated at 30ºC for 30'. The absorbance at 734 nm was measured. ABTS radical scavenging activity in different extracts was calculated from the Ascorbic acid calibration curve (0-2 mg/g).

Antimicrobial activity
Microbial cultures

The antimicrobial activity of the investigated samples was evaluated using laboratory control strains obtained from the American Type Culture Collection: Gram (-) bacteria - *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 10031, *Proteus mira-bilis* ATCC 12453; Gram (+) bacteria: *Strepto-coccus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 19433, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228 and yeast *Candida albicans* ATCC 24433. Bacterial strains were maintained on Nutrient Agar (NA) at 37 °C and yeast on Sabouraud Dextrose Agar (SDA) at 30 °C at the Microbiology Laboratory (Department of Biology, Faculty of Science and Mathematics, University of Niš).

Micro-well Dilution Assay

Antimicrobial activity was evaluated using a broth microdilution method (16). Overnight cultures (18 h) were used for making cell suspensions standardized to 0.5 McFarland turbidity, as measured on McFarland Densitometer (DEN-1, Biosan). Dimethyl sulfoxide (100%) was used for making stock solutions of the prepared plant extracts and essential oils. The solutions of the extracts were further diluted with sterile distilled H_2O (dilution factor 10) in order to achieve 10% solution of the solvent, confirmed by preliminary experiments as non-harmful to the test microorganisms. These solutions were further serially diluted (the diluting factor 2) with sterile PBS in the concentration range 0.001-30 mg/ml. Temperature of incubation was 37 °C and period of inoculation was 24 h. Controls included chloramphenicol and nystatin as the positive controls, while wells without inoculum and test substance represented the negative control, including test sterility of the medium. Visual reading of the bacterial growth was performed after the addition of triphenyltetrazolium chloride (TTC, 0.5%) aqueous solution. The lowest concentration of the test compound that inhibited growth was represented by red colored medium in the wells and considered the minimal inhibitory concentration (MIC). All experiments were done in triplicate.

Statistical analysis

All values were done in triplicate and presented as average of those values ± standard deviation. These results were calculated by using Microsoft Excel 2007®. The IC_{50} values obtained in the antioxidant assays were determined by reg-ression equation, including the concentration of samples and the scavenging effect. Software used for analyzing the results was OriginPro 8.0. The results were also analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test ($p \leq 0.05$). This analysis was carried out using the Minitab®17 software.

Results

Total phenolic content

The results of the total phenolic content determination of the examined plant extracts are presented in Table 2. The content of total phenols in different extracts, expressed as gallic acid equivalents (GAE), ranged between 7.47 to 121.35 mg GAE/g. The highest phenolic content was found in Acet extract and the lowest in EtOAc for both species. The values for *E. maritimum* and *E. Serbicum* extracts were in the next order:

Acet>H_2O>MeOH>EtOAc.

Flavonoid concentrations

The summary of quantities of flavonoids identified in the tested extracts is shown in Table 2. The concentration of flavonoids in H_2O, MeOH, Acet and EtOAc extracts of aerial parts *E. maritimum* and *E. serbicum* were determined using spectrophotometric method with aluminium nitrate nonahydrate. The content of flavonoids was expressed in terms of quercetin hydrate equivalents. The concentrations of flavonoids in plant extracts ranged from 8.98 to 48.68 mg Qu/g. The highest flavonoid content was identified in Acet extracts for both species. The lowest content for *E. maritimum* was identified in MeOH extracts, while the lowest content for *E. serbicum* was in H_2O extract.

DPPH scavenging activity

DPPH is a very stable free radical. The effect of an antioxidant on DPPH radical scavenging is due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form diphenylpicrylhydrazine with the loss of its violet color (17).

Free radical scavenging capacities of the tested extracts were measured by DPPH assay and results are shown in Table 2. According to the results obtained, different extracts were found active with IC_{50} value for extracts between 1.247 to 31.19 mg/ml of solution. IC_{50} values of the synthetic antioxidant BHA 0.093 mg/ml and ascorbic acid 0.054 mg/ml were determined in parallel experiments. A lower IC_{50} value indicates higher antioxidant activity. H_2O extract of aerial parts from *E. maritimum* and *E. serbicum* possessed the strongest antioxidant activity compared to others. For both species, EtOAc extracts showed the lowest activity.

ABTS scavenging activity

The results from the ABTS assay are shown in Table 2. The amount ranged from 0.109 to 3.36 mg AA/g. The higher ABTS values present the stronger antioxidant activity. The highest content was identified in H_2O extract and the lowest in EtOAc extract for both species.

Table 2. Comparative analysis of TPC, TFC, ABTS and DPPH tests between extracts from two Eryngium species

Antioxidant activity	TPC (mg GAE/g)	TFC (mg Qu/g)	ABTS (mg AA/g)	DPPH IC_{50} (mg/ml)
E. maritimum (H_2O)	50.82±0.008^{d}	9.11±0.004^{e}	1.84±0.023^{c}	2.82±0.033^{e}
E. maritimum (MeOH)	49.01±0.016^{d}	8.98±0.007^{e}	1.058±0.026^{b}	4.82±0.012^{d}
E. maritimum (Acet)	77.08±0.138^{bc}	48.68±0.026^{a}	0.769±0.045^{b}	12.52±0.033^{b}
E. maritimum (EtOAc)	7.47±0.005^{f}	17.55±0.014^{d}	0.109±0.003^{a}	31.19±0.051^{a}
E. serbicum (H_2O)	90.1±0.004^{b}	17.49±0.004^{d}	3.36±0.007^{e}	1.247±0.005^{h}
E. serbicum (MeOH)	71.41±0.005^{c}	18.08±0.002^{d}	2.34±0.023^{d}	2.062±0.023^{f}
E. serbicum (Acet)	121.35±0.01^{a}	37.18±0.001^{b}	2.44±0.011^{d}	1.838±0.013^{g}
E. serbicum (EtOAc)	23.056±0.003^{e}	28.964±0.003^{c}	0.544±0.006^{e}	10.376±0.03^{c}
BHA	63.31±0.001	/	2.66±0.005	0.093±0.018
Ascorbic Acid	40.91±0.002	/	/	0.054±0.002

Each value in the table was obtained by calculating the average of three analyses ± standard deviation. Different letters above bars indicate statistically significant differences only among the treatments performed for each assay according to the Tukey test ($p \leq 0.05$)

Antimicrobial activity

Results obtained for the extract's antimicrobial activity are presented in Table 3. Two antimicrobial commercial agents, namely Chloramphenicol (antibacterial) and Nystatin (antifungal) were used as references for comparison of the investigated extract's activities. Essential oils of the two *Eryngium species* showed activity at concentrations ranging from <0.001-15 mg/ml, while the solvent extract's activities ranged from 0.15-30 mg/ml. In the case of the two essential oils, the one isolated from *E. serbicum* plant material showed significantly higher antimicrobial potential (inhibition from < 0.001-> 2.5 mg/ml) in comparison to the oil isolated from *E. maritimum* (3.75-15 mg/ml). The menti-oned essential oil showed very intensive inhibitory activity, exhibited against all tested microbial strains with the exception of *S. pyogenes*. This activity was especially significant against the two Gram negative human pathogenic bacteria (*K. pneumoniae* and *P. mirabilis*), where it was determined that inhibitory action can be achieved even at concentrations lower than 1 µg/ml, which is close to the activity of chloramphenicol (0.39 µg/ml). On the other hand, *E. Maritimum* essential oil showed moderate antimicrobial potential, where the two species (*K. pneumoniae* and *P. mirabilis*) especially sensitive to the action of the previous oil, as well as *S. pyogenes* demonstrated resistance to the highest tested concentration of this oil.

From the results obtained for the tested solvent extracts, once again, it is clearly visible that *E. serbicum* extracts possessed much higher antimicrobial potential. In the case of *E. maritimum* extracts, the highest activity has been demonstrated by Acet extract, followed by EtOAc and MeOH extracts, while H_2O extract showed no activity against any of the

Table 3. Comparative antimicrobial activity of extracts and essential oils from two Eryngium species

Antimicrobial activity (mg/ml)	*S. aureus*	*S. pyogenes*	*E. faecalis*	*S. epidermidis*	*E. coli*	*K. pneumoniae*	*P. mirabilis*	*P. aeruginosa*	*C. albicans*
E. maritimum (H_2O)	/	/	/	/	/	/	/	/	/
E. maritimum (MeOH)	7.5	/	30	/	/	/	/	30	30
E. maritimum (Acet)	7.5	/	15	3.75	15	/	/	15	7.5
E. maritimum (EtOAc)	10	/	10	1.25	/	/	/	/	5
E. serbicum (H_2O)	10	>10	2.5	2.5	>10	5	>10	10	>10
E. serbicum (MeOH)	1.25	1.25	0.31	1.25	0.15	1.25	0.62	2.5	0.31
E. serbicum (Acet)	2.5	>2.5	>2.5	2.5	0.31	2.5	0.31	>2.5	0.25
E. serbicum (EtOAc)	2.5	>2.5	>2.5	2.5	1.25	>2	1.25	>2.5	>2.5
E. maritimum (Essential oil)	7.5	/	15	3.75	15	/	/	15	15
E. serbicum (Essential oil)	0.01	>2.5	0.015	1.25	0.62	<0.001	<0.001	0.62	0.31
Chloramphenicol (μg/ml)	0.39	0.19	0.39	0.19	0.78	0.39	0.39	0.39	/
Nystatin (μg/ml)	/	/	/	/	/	/		/	0.09

tested strains. On the other hand, H_2O extract of *E. serbicum* showed limited activity, against five out of nine panel strains. EtOAc extract exhibited activity against only four strains, but at lower concentrations (1.25-2.5 mg/ml), which was similar to the activity of Acet extract (6 sensitive strains, MIC = 0.31-2.5 mg/ml). Among the tested extracts of this species, the MeOH one showed the highest potential, where all strains showed sensitivity to its action at concentrations 0.15-2.5 mg/ml.

Discussion

Comparative biological study for *E. maritimum* and endemic *E. serbicum* is largely unknown. Different solvents such as H_2O, MeOH, Acet, EtOAc, (ranged from higher polarity to lower polarity) extracts were used for the study of antioxidant activity. Various solvents were used to achieve extraction of active substances with diversity in their polarity. For extraction, the solvent is chosen as a function of the type of required phenol or flavonoid. According to given results, H_2O extract showed high antioxidant activity while EtOAc extract possessed the lowest antioxidant activity (DPPH, ABTS assays). In comparison of the species, *E. serbicum* possessed higher percentage of total phenol and flavonoid content and equivalently to those results this species have better antioxidative activity.

In a recent study, Tunisian *E. maritimum* leaf extracts displayed the strongest H_2O_2 scavenging activity (IC_{50} = 76.83 μg/mL) and the highest DPPH scavenging activity value (IC_{50} = 47.87 μg/mL) compared to other extracts. Good relationships were observed between antioxidant activities and the total phenolic and flavonoid contents. Nine bioactive compounds were detected in *E. maritimum* extracts: six phenolic acids (gallic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid and cinnamic acid) and three flavonoids (rutin, quercetin and luteolin) (18). In our study, IC_{50} value for all *E. maritimum* extracts was lower, indicating better antioxidative activity. Radical scavenging activity of *E. maritimum* MeOH extract revealing IC_{50} = 0.28 mg/ml in the ABTS assay was earlier investigated as well (19).

In previous studies (20), Germacrene-D and three uncommon oxygenated sesquiterpenes: 4*β*H-cadin-9-en-15-*al*, 4*β*H-cadin-9-en-15-*ol* and 4*β*H-muurol-9-en-15-*al* were reported as major component of *E. maritimum* essential oil from Corsica. The main constituents of the *E. serbicum* essential oil from Serbia were germacrene D, *β*-elemene and spathulenol (21). Dominant compounds in essential oils have significant part in biological activity.

Previous studies demonstrated that extracts from leaves and roots of *Eryngium species* (*E. Planum*, *E. campestre* and *E. maritimum*) showed antibacterial and antifungal activity, expecially against dermatophytes (22, 23).

Ethanol extracts of *E. planum*, *E. campestre* and *E. maritimum* leaves acted inhibitory in range MIC=0.4-1.9 mg/ml on *S. aureus* strain (23). Ethanol extract of the *E. maritimum* against the same

bacterium exhibited inhibition of growth at 0.7 mg/ml, while the same study reported activity of same extract against *C. albicans* at 1.3 mg/ml. In the present investigation, it has been determined that the extracts showing antimicrobial potential had much lower potential than the ethanol one in the mentioned study. Investigation of Meot-Duros et al. (2008) investigated antimicrobial potential of chloroform (non-polar) and MeOH (polar) extracts of *E. Maritimum* (19). The results showed much higher activity than those obtained here, where polar fraction (MeOH extract) showed activity only against *P. aeruginosa* at only 1 µg/ml. In the same study, chloroform extract inhibited *S. aureus* at 10 µg/ml, while it was active at 2 µg/ml against *P. aeruginosa* and considering remaining strains mostly at 100 µg/ml. Such low active concentrations can be explained by much lower inoculum size added into the test wells of the microtitre plate in the mentioned study, which was 10 000 times lower than our (10^2 and 10^6, respectively). In another study, MeOH extract, Acet extract, EtOAc fraction and butanol fraction extracted from *E. maritimum* were investigated for antimicrobial activity. The results showed that all of them were active against *Listeria monocytogenes, Escherichia coli*, *Staphylococcus aureus and Bacillus cereus,* while MeOH and butanol extract were the only active against *Pseudomonas aeruginosa* (24). Due to the different methods of antimicrobial activity determination (disc diffusion method), concentrations cannot be compared between this and the present investigation.

There is no clear selectivity considering cell wall structure of the treated microorganisms (bacteria/fungi; Gram-positive/Gram negative). Among the tested microorganisms, the most resistant was *S. pyogenes*, followed by *K. pneumoniae* and *P. mirabilis*, while *S. aureus* showed the highest sensiti-vity by being resistant only to the action of the H_2O *E. maritimum* extract.

Conclusion

All extracts evaluated from *E. maritimum* and *E. serbicum* could be used as protective against oxidative stress based on conducted DPPH and ABTS assays. Essential oil isolated from *E. serbicum* possesses strong antimicrobial activity. Also, all type of extracts inhibited the growth of tested microorganisms. Polyphenolic compound is responsible for the antioxidant and antimicrobial activity. Regular consumption of secondary metabolites isolated from this two species may provide positive consequences for human health.

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BIOLOŠKA AKTIVNOST EKSTRAKATA I ETARSKIH ULJA DVE VRSTE RODA *ERYNGIUM* (*APIACEAE*) SA BALKANSKOG POLUOSTRVA

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U ovoj studiji opisana je antioksidativna i antimikrobna aktivnost vodenih, metanolnih, acetonskih i etil-acetatnih ekstrakata dobijenih od vrsta *Eryngium maritimum* L. iz Grčke i *Eryngium serbicum* Pančić, samonikle u Srbiji. Takođe, analizirana je antimikrobna aktivnost etarskih ulja izolovanih iz nadzemnih delova ovih vrsta. Spektrofotometrijske metode korišćene su za merenje koncentracije ukupnih fenola, flavonoida, kao i za određivanje antioksidativnog potencijala uzoraka upotrebom DPPH i ABTS metoda. Ukupna količina fenola u ekstraktima određena je korišćenjem Folin-Ciocalteu reagensa, a vrednosti su se kretale u opsegu od 7,47 mg GAE/g do 121,35 mg GAE/g. Koncentracija flavonoida u ekstraktima je bila od 8,98 mg GAE/g do 48,68 mg QU/g. Antiokstidativna aktivnost kretala se u opsegu od 1,247 IC_{50} do 31,19 IC_{50} (mg/ml) i od 0,109 mg VitC/g do 3,36 mg VitC/g za DPPH test i ABTS test. Antimikrobna aktivnost ekstrakta i etarskih ulja ispitivana je pomoću mikrodilucione metode na patogene gastrointestinalnog trakata. Najotpornija bakterija bila je *Streptococcus pyogenes*, dok je vrsta *Staphylococcus aureus* pokazala visoku osetljivost na prisustvo svih testiranih ekstrakta osim u slučaju vodenog ekstrakta *E. maritimum*. Etarsko ulje vrste *E. Serbicum* pokazalo je bolju antimikrobnu aktivnost u odnosu na ulje izolovano iz vrste *E. Maritimum*.

Na osnovu dobijenih rezultata može se zaključiti da vrste roda *Eryngium* mogu biti potencijalni prirodni izvori antioksidativnih i antimikrobnih agenasa.

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Ključne reči: *Eryngium maritimum, E. serbicum, ekstrakti, etarska ulja, antioksidativna i antimikrobna aktivnost*

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QUALITY OF SLEEP IN PATIENTS WITH MYASTHENIA GRAVIS

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Myasthenia gravis (MG) is a chronic neuromuscular disease that leads to progressive weakness, fatigue of the skeletal muscles, and is often associated with psychological changes, especially with poorer quality of sleep.

To evaluate the quality of sleep in patients suffering from MG in relation to socio-demographic and clinical characteristics of the dissease.

A total of 70 adult patients have been classified according to Myasthenia Gravis Foundation of America classification and divided into groups with regard to the age of onset, gender, employment status and type of work, presence or absence of pathological changes on thymus and presence or absence of anti-nAchr antibodies. Severity of clinical manifestations was evaluated by using quantitative MG scores and MG composite scores. Pittsburgh questionnaire was used to assess the subjective quality of sleep. In addition, Hamilton's anxiety and depression scales and questionnaires for quality of life assessment were also implemented.

The results of our research show a correlation between poor quality of sleep and prolonged duration of the disease, pathological changes on thymus, positive anti-nAchr antibodies. The correlation between poor quality of sleep with more severe clinical presentation, poor quality of life, anxiety and depression was confirmed.

Quality of sleep is impaired in patients with MG, especially in the case of severe clinical manifestations and prolonged duration of the disease. Considering the lack of literature on the subject, a better understanding of the prevalence and severity of sleep disorders in MG requires further research.

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Key words: *myasthenia gravis, quality of sleep*

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Introduction

Myasthenia gravis (MG) is a chronic neuromuscular disease that causes a disturbance in transmission of nerve impulses to the muscles, which leads to progressive weakness and fatigue of the skeletal musculature (1). MG is a rare neurological disorder, with the prevalence ranging from 15 to 200 out of 1,000,000 persons (2). In Serbia, prevalence is 188.8 out of 1,000,000 persons, which is one of the highest prevalence reported worldwide (3). It can occur at any age and is more frequent in women (1.3:1) (3). MG can lead to fatal outcome if it affects respiratory muscles, which as initial manifestation occurs in 1-4% of patients, and the occurrence of this symptom increases to 30-40% at a later stage of the disease (4). Weakness of the intercostal and diaphragmatic muscles leads to the development of dyspnea at rest and sleep disordered breathing (SDB) (5). Obstructive sleep apnea (OSA) is a sleep disorder more common in MG than in other neuromuscular diseases (6). The prevalence of SDB among patients with MG ranges up to 60%, and for OSA is 21.1% (7). Symptoms of breathing disorders develop gradually; they are often unrecognized by patients and are a major cause of mortality in patients with neuromuscular diseases (5, 8). Previous research found a correlation between severity of clinical presentation of MG, accompanied with difficulties in performing daily activities, with poorer quality of life (9, 10).

As a disease with an unpredictable outcome, MG is accompanied by a justified psychological reaction and an increased risk of psychiatric disorders (11, 12). Quality of sleep includes duration and latency of sleep, as well as the degree of rest after

sleep. Sleep quality disturbances are very often present in nearly all psychiatric disorders, particularly in depression and anxiety disorders (13). Little research was done on the relationship between the MG and the quality of sleep, and the interconnectedness is still not clear. It is a fact that evening exacerbations are more frequent in patients with MG, and that affects sleep. In addition, the impaired quality of sleep affects many domains of life in patients with MG. Therefore, it is very important to evaluate the quality of sleep and recognize early manifestations of sleep disorders in these patients.

Aim

The aim of this research was to evaluate the quality of sleep in patients suffering from MG, with regard to socio-demographic and clinical characteristics.

Methods

Study included 70 adult patients treated at the Neurology Clinic, Clinical Center Niš, from December 2016 to December 2017. The diagnosis of MG was made based on data from the patient history, examination, prostigmin and repetitive nerve stimulation test results, as well as by exclusion of other possible causes of symptoms presented. Data on sex, age of onset and current age, employment status, type of work, pathologically altered thymus and presence of anti-nAchr antibodies were collected. Severity of clinical manifestations was evaluated by using quantitative MG scores (QMG) (14) and MG composite scores (MGC) (15). The QMG is a 13-item scale that measures ocular, bulbar, respiratory and limb function. It evaluates each finding individually and results range from 0 (no myasthenic findings) to 39 (maximal myasthenic deficits). MGC consists of 10 items and ranks the severity of MG at five levels in relation to the symptomatology and disorder of the affected muscle group function. The maximum score is 50, and the higher score reflects the more difficult form of clinical manifestation.

Patients were divided into groups according to the Myasthenia Gravis Foundation of America (MGFA) classification. Patients from MGFA group V on artificial ventilation were excluded from the study, as well as patients with other chronic illness that could affect the quality of sleep (diabetes, asthma, hypertension, heart failure, renal and hepatic insufficiency).

PSQI Quality of Sleep Assessment questionnaire consists of 19 self-rated questions. Components of the questionnaire measure quality of sleep, duration and latency of sleep, common efficacy of sleep, and functionality during the day. It assesses a 1-month interval and provides data useful both in clinical and scientific work. In addition to assessing the quality of sleep it provides clinically useful evaluation of variety of factors that might affect quality of sleep. These 19 questions are grouped into seven groups, each scored from 0-3. Obtained global PSQI score is from 0-21, where higher score indicate lower quality of sleep. It could be used as a tool for measuring interaction of sleep disturbances and depression, as well as relationship between sleep quality and other variables such as age, gender, health status, psychiatric and other medical conditions (13). It is so far the most used quality of sleep questionnaire (16), which is translated and standardized in Serbia (17). PSQI is easy to apply and understandable to patients, and it can potentially be used in everyday clinical practice.

In addition, the Hamilton scales for anxiety (HAM-A) and depression (HAM-D) and questionnaires "Questionnaire of Life Quality Specific for Myasthenia Gravis - 15 items" - revised version (MGQOL15r) and Short Form Survey (SF-36), were used.

HAM-D measures the intensity of depression, and the values are interpreted as follows: 0-9 (without depression), 10-13 (mild depression), 14-17 (mild to moderate depression) and 18 or more (moderate to severe depression). HAM-A measures intensity of anxiety, where the ultimate values below 17 indicate absence or mild anxiety, values between 18-24 mild to moderate anxiety, and values between 25-30 moderate to severe anxiety.

The MGQOL15r questionnaire measures the quality of life specifically associated with myasthenia gravis. There is no pre-specified cutoff for classification of QoL in MG patients. It consists of 15 questions selected in 4 dimensions (mobility (8 questions), symptoms (3 questions), general contentment (2 questions), and emotional well-being (2 questions)). Each question is assessed from 0 to 4, and higher score represents more significant impact on quality of life.

The SF-36 questionnaire measures the quality of life associated with a general health condition, by including eight general health dimensions: physical functioning (PF), role functional physical (RP), bodily pain (BP), vitality (VT), social functioning (SF), role functioning emotional (RE), mental health (MH), general health (GH), and total score. The total scores for each of the eight dimensions ranged from 0 to 100 on the scale, were a higher score represents better health.

All patients signed an informed consent for participating in the study.

All data were statistically analyzed by IBM SPSS (version 21) for Windows operative system. Results with $p < 0.05$ were considered statistically significant. Numerical data were presented as means ± SD. The Mann-Whitney test was used to compare continuous variables between the two groups, and the Kruskal-Wallis test was used for comparison of more than two groups. Correlations were assessed using Pearson's correlation coefficients or Spearman's correlation coefficients.

Results

Epidemiological and clinical characteristics of 70 patients are presented in Table 1. The scores obtained by the used questionnaires are presented in Table 2.

Table 1. Demographic and clinical characteristics of MG patients

Variable	Value
Gender (number of patients)	
Male	33 (47.1 %)
Female	37 (52.9 %)
Current age (years) (Mean ± SD)	53.16 ± 15.98
Disease duration (years) (Mean ± SD)	4.45 ± 4.4
Profession (number of patients)	
Physical workers	16 (22.9 %)
Intellectual workers	13 (18.6 %)
Unemployed	13 (18.6 %)
Retired	28 (40.0 %)
First MG symptoms (number of patients)	
<50	35 (50%)
≥ 50	35 (50%)
Thymus pathology (number of patients)	
absent	25 (36%)
present	45 (64%)
Anti nAchR antibodies (number of patients)	
absent	14 (20%)
present	56 (80%)
Current MGFA* (number of patients)	
I	15 (21.4 %)
IIa	11 (15.7 %)
IIb	16 (22.9%)
IIIa	8 (11.5 %)
IIIb	10 (14.4%)
IVa	6 (8.5%)
IVb	4 (5.7%)
QMG[+](Mean score ± SD)	8.16 ±15.67
MGC[#](Mean score ± SD)	7.51± 5.33

* Myasthenia Gravis Foundation of America (MGFA)
[+] Quantitative myasthenia gravis score (QMG)
[#] Myasthenia gravis composite scores (MGC)

Table 2. Scores for all patients obtained on the used questionnaires

PSQI*	6.76 ±4.60
Hamilton scale for depression (Mean score ± SD)	10.29±6.34
Hamilton scale for anxiety (Mean score ± SD)	9.5 ±6.9
MGQOL15r [+]	22.5 ±11.53
SF36 scores [#]	
physical functioning	58.28 ±38.39
role functional physical	43.59 ±49.6
vitality	58.09 ±47.71
mental health	57.43 ±25.23
role functioning emotional	65.37 ±18.9
social functioning	69.65 ±26.28
bodily pain	70.64±26.52
general health	50.28 ±10.03
Sum score	57.5 ±24.7

* Pittsburgh Sleep Quality Index (PSQI)
[+]"Questionnaire of Life Quality Specific for Myasthenia Gravis - 15 items "- revised version (MGQOL15r)
[#]Study Short Form of 36 questions (SF36)

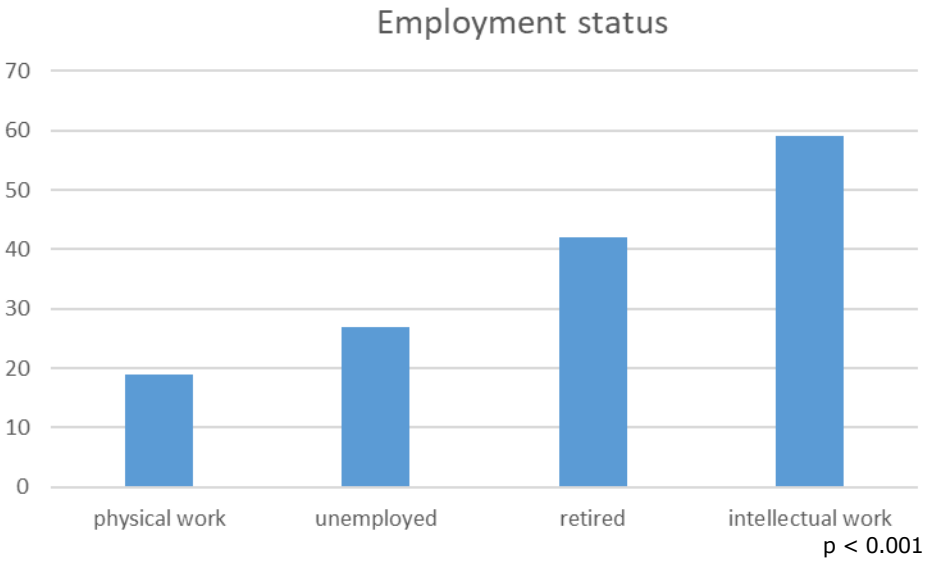
(a) Differences in sleep quality in relation to different socio-demographic and clinical characteristics

In relation to gender, early or late onset of the disease, a statistically significant difference was not found in quality of sleep. Statistically significant difference with regard to quality of sleep exists in patients with pathological changes on the thymus, compared to the group of patients without these changes ($p < 0.001$). There is also a clear statistical difference between the groups with positive anti-nAchr antibodies (lower sleep quality) compared to the

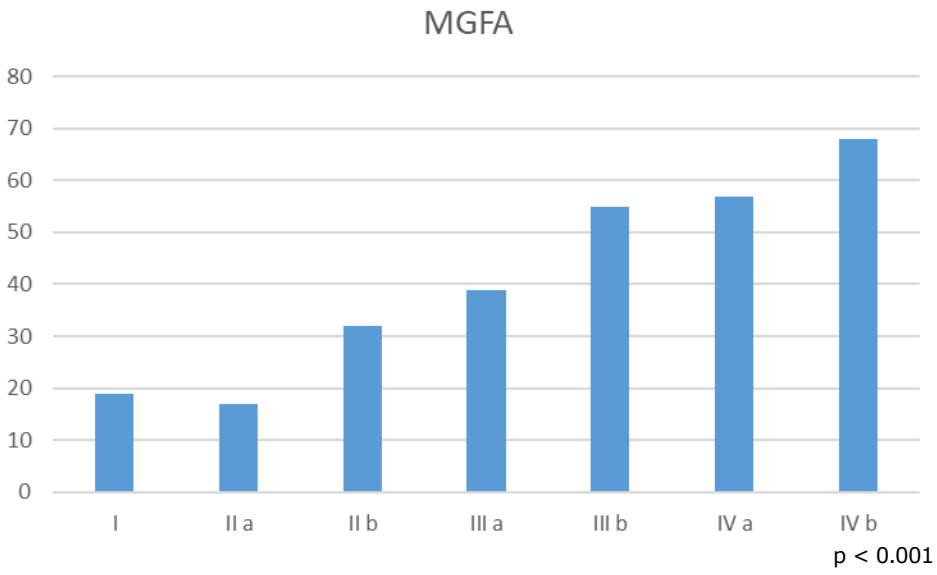
group of seronegative patients (p<0.001). With regard to employment status and the type of work, the worst sleep quality was observed in physical workers, and the best in intellectual or administrative workers, with a statistically significant (p < 0.001) difference between the groups (Graph 1.).

(b) Correlation analysis between the severity of the clinical manifestation and the quality of sleep

In comparison with MGFA classification, patients with severe clinical presentation had the lowest quality of sleep, and this was more pronounced in groups with bulbar symptomatology, with statistically significant difference (Graph 2.).



Graph 1. Mean rank PSQI scores (Kruskal-Wallis test) in relation to employment status



Graph 2. Mean rank PSQI scores (Kruskal-Wallis test) in relation to MGFA classification

In relation to the assessment of the severity of the clinical presentation, there is a clear correlation between more severe clinical manifestation and lower quality of sleep (Table 3.). Age and PSQI showed no clear correlation (p = 0.62), but the correlation was shown between the prolonged duration of disease and lower quality of sleep (p < 0.001).

Comparing other applied scales, a clear correlation was found between estimated lower quality of sleep and lower quality of life, anxiety and depres-

sion (Table 4.). Statistically significant correlation was shown between most of the sub-scales on the SF36 questionnaire and a questionnaire that assess the quality of sleep (for psychical functioning, role functioning emotional and general health $p < 0.001$; and for mental health, social functioning and bodily pain $p < 0.05$).

Table 3. Correlation of sleep quality with duration of disease and severity of clinical picture

	Duration of illness		MGC +		QMC #	
	R	p	R	p	R	p
PSQI*	0.382	<0.001**	0.734	<0.001**	0.694	<0.001**

p < 0.001**

+ Myasthenia gravis composite scores (MGC)
# Quantitative myasthenia gravis score (QMC)
* Pittsburgh Sleep Quality index (PSQI)

Table 4. Correlation of sleep quality with quality of life, depression and anxiety

	MGQOL15r +		SF36 #		Hamilton scale for depression		Hamilton scale for anxiety	
	R	p	R	p	R	p	R	p
PSQI*	0.659	<0.001**	-0.404	<0.001**	0.612	<0.001**	0.596	<0.001**

p < 0.001**

+ "Questionnaire of Life Quality Specific for Myasthnia Gravis - 15 items "- revised version (MGQOL15r)
# Study Short Form of 36 questions (SF36)
* Pittsburgh Sleep Quality Index (PSQI)

Discussion

In accordance with the aim of the study, the results show the correlation between poor quality of sleep and severity of clinical manifestation in MG, prolonged duration of the disease, pathological changes on thymus, the presence of positive anti-nAchr antibodies, poor quality of life and the presence of anxiety and depressive symptomatology in patients with MG.

Although daytime sleepiness and fatigue are common symptoms in neuromuscular diseases, there is not much data on the quality of sleep in these patients (18). Previous studies identified a higher prevalence of daytime sleepiness among MG patients (19, 20), while others found correlation only with the generalized type of the disease (21). Happe et al. (22) have found an association between the severity of clinical manifestations in MG and low quality of sleep, which is in line with the results of our research, while other studies have not established this association (6, 7). Studies that failed to find association between MG and poor quality of sleep had restriction in a form of small statistical samples (21, 23).

The results of our research show a correlation between lower quality of sleep and prolonged duration of the disease, which has never been examined, according to the available literature.

Tascilar et al. (24) found correlation between lower quality of life and PSQI scores only with regard to subjective sleep duration, and the results of our research have determined the correlation between the scores obtained on the PSQI questionnaire and the scores on the questionnaires that assess the quality of life (MG-QOL15). By using the SF-36 questionnaire for assessing the quality of life, Basta et al. (25) found negatively affected mental aspects of SF-36 in patients with MG, while the results of our research determined the correlation between the majority of the sub-scales on the SF-36 questionnaire and the PSQI questionnaire.

Sleep quality disturbances, including difficulty falling asleep or difficulty in maintaining sleep, are very often present in nearly all psychiatric disorders, particularly in depression and anxiety disorders (12). The weakness of skeletal musculature in patients with MG is often accompanied by depressive and anxiety symptomatology (26, 27), as well as impaired quality of life (28). In our research, lower quality of sleep correlated with the intensity of depressive and anxiety symptomatology in patients with MG. Some studies found no correlation between the duration of sleep and depression or anxiety (29), nor the correlation between the severity of MG and anxiety disorders (30).

Our results show association between pathological changes on thymus with poorer quality of sleep. The results also show correlation between

lower quality of sleep and presence of anti-nAchr antibodies, which has not been compared in previous research. Magni et al. (31) have not found association between psychiatric disturbances in MG with pathological changes on thymus, thymectomy, or age of MG onset, and also they did not evaluate the quality of sleep.

Most of these results indicate the significant role of MG in the daily functioning and the fulfillment of social tasks. This is the first study of quality of sleep in patients with MG in Serbia, based on relevant and validated sleep quality questionnaire. Restrictions include a small sample and the fact that we did not perform polysomnography to verify these patient-reported disturbances of sleep.

Conclusion

The results of our study showed a correlation between poor quality of sleep and prolonged duration of the disease. The link between pathological changes on thymus and the presence of positive anti-nAchr antibodies with poor quality of sleep was also demonstrated. Our results confirm the correlation between lower quality of sleep with more severe clinical presentation, poor quality of life and the presence of anxiety and depressive symptomatology in patients with MG. It is our suggestion that scale for assessing the quality of sleep (PSQI) should be used in the daily clinical work, considering that it is easily applied, understandable to patients and provides a clinically useful assessment of various factors that can affect quality of sleep. Considering the lack of literature on the subject, better understanding of the prevalence and severity of sleep disorders in MG requires further research.

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KVALITET SPAVANJA KOD BOLESNIKA SA MIJASTENIJOM GRAVIS

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Miastenija gravis (MG) je hronična neuromišićna bolest koja dovodi do progresivnog zamora, slabosti skeletnih mišića i često je povezana sa psihičkim izmenama, posebno sa lošijim kvalitetom spavanja.

Cilj rada bio je proceniti kvalitet spavanja kod bolesnika koji boluju od MG u odnosu na sociodemografske i kliničke karakteristike oboljenja.

Ukupno 70 odraslih bolesnika klasifikovano je prema klasifikaciji Američke fondacije za MG i podeljeno u grupe u odnosu na starost kada su tegobe počele, pol, zaposlenost i vrstu posla kojom se bave, prisustvo ili odsustvo patoloških promena na timusu i prisustvo ili odsustvo anti-nAchr antitela. Težina kliničkog ispoljavanja procenjena je korišćenjem kvantitativnog MG skora i MG kompozitnog skora. Pored Pitsburgovog upitnika za procenu kvaliteta spavanja, korišćene su i Hamiltonove skale za procenu anksioznosti i depresije, kao i upitnici za procenu kvaliteta života.

Rezultati našeg istraživanja pokazali su korelaciju između lošijeg kvaliteta spavanja i dužeg trajanja bolesti, patoloških promena na timusu i pozitivnih anti-nAchr antitela. Potvrđena je korelacija između lošijeg kvaliteta spavanja i teže kliničke slike, lošeg kvaliteta života, anksioznosti i depresije.

Kvalitet spavanja narušen je kod pacijenata sa MG, posebno kod težeg kliničkog ispoljavanja i dužeg trajanja bolesti. U odsustvu literature, bolje razumevanje prevalencije i ozbiljnosti poremećaja spavanja kod MG zahteva dalja istraživanja.

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Ključne reči: *miastenija gravis, kvalitet spavanja*

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SEXUAL ACTIVITY, KNOWLEDGE AND THE USE OF CONTRACEPTION AMONG HIGH-SCHOOL STUDENTS IN NIŠ AND PREŠEVO

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Adolescents are tomorrow's adults and make up to 16% of the world's entire population. Caring about their reproductive health is both a mission and an obligation for all healthcare institutions and the community as a whole. The aim of this study was to examine sexual behavior, knowledge and the use of contraception, as well as the source of information on the topic, among high school students in Niš and Preševo.

An anonymous survey was conducted among 710 highschool stu-dents in Niš and 215 in Preševo in December 2017. It consisted of 13 questions about sexual activity, knowledge and the use of contraceptive methods.

The average age of the respondents was 17.2 ± 0.5 years. 31.5% of high school students from Niš had sexual intercourse, for the first time with 16.2 ± 1.1 years of age and 23.2 high school students from Preševo with 15.7 ± 1.1 years of age. Male students engaged significantly more often in sexual intercourse in comparison to their female counterparts. Students of both sexes and from both cities most frequently cited love as the reason for having sexual intercourse. The incidence of the regular use of contraception was statistically higher in Niš as opposed to Preševo ($p = 0.007$) while being equal between the sexes ($p = 0.738$). The most used method of contraception during both first and last intercourse in both cities and both sexes was a condom. The majority of respondents from Niš (83.5%) and Preševo (93.2%) did not ask for professional advice. Female students asked for professional advice more often than male students. The Internet was the most frequent tool for gathering information about contraception in both cities (Niš 61.2%, Preševo 55.0%) and with both sexes (male 64.9%, female 51.2%). Two most common reasons for the use of contraception were protection from unwanted pregnancies (Niš 59.6%, Preševo 71.2%) and protection from sexually transmitted diseases (Niš 48.3%, Preševo 16.6%).

It is necessary to plan a long term strategy for sexual education with relevant information in both cities, in a manner appropriate for adolescents, while acknowledging the important role of the Internet in informing adolescent about this topic.

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Key words: *high school students, sexual behavior, contraception, reproductive health*

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Introduction

Adolescents are the adults of tomorrow and they make up approximately 16% of the entire world population, out of whom 85% live in developing countries. Adolescence is a sensitive period in one's development as it represents the transition from childhood to the state of physical, psychological and social maturity. One of the characteristics of modern society is the fact that adolescents start having sexual intercourse very early. More than a quarter of adolescent population from developing countries reported having started having sexual intercourse before the age of 15 (1).

Sexual and reproductive habits can have immediate, but also long-term consequences since they can lead to unwanted pregnancy and sexually transmitted diseases. According to the data issued by World Health Organization, while there is a steady decrease in early pregnancy rates (labour before the age of 15) in the world, the number of labours with older adolescents (labour before 18 years of age)stagnates. Every year, about 2 million girls who

are not yet 15 get pregnant, while there are 21 million of those who get pregnant between 15 and 19 years of age. Within a group of 1000 adolescents, there are 22 of them who become mothers at that age in Serbia. Also, pregnancy and labour complications are considered to be the leading causes of death among girls between 15 and 19 years of age in the world (2).

Sexually transmitted diseases, first of all, AIDS, are the leading cause of death among adolescents in Africa and the second most important in the world. AIDS-related deaths have tripled among adolescents in comparison to the rates of 2000, while their number tends to decline within grownups. Twenty-six adolescents between 15 and 19 years of age get infected with AIDS every hour in the world (220.000 in total), according to the data from 2014 (3). In addition to that, sexually transmitted diseases, especially those caused by Chlamydia trachomatis, Trichomonas vaginalis, HSV, HPV, and Naisseria gonorrhoeaeare more common among adolescents (4).

Reproductive health represents a key element for a healthy life. Numerous factors, such as socioeconomic status, demographic, cultural, religious and individual characteristics of a society and an individual are closely intertwined and influence one's awareness of reproductive health. Taking into consideration that young people are susceptible to different influences coming from the immediate and wider social environment, with the influences affecting their knowledge, attitudes, and behaviour related to sexuality and reproduction to a smaller or larger extent, reproductive healthcare is the job and responsibility of health institutions and the whole society. Education concerning the importance of the use of contraceptives is important in protection against sexually transmitted diseasesas, as well as in the prevention of mortality and morbidity of fertile women caused by abortive procedures (5), especially because the percentage of young people (between the ages of 15 and 19) who use contraceptives is just 21-64% in the world.

The aim of the study

The reasearch has been conducted to examine sexual behaviour, knowledge about and the use of contraceptives among high school students in Niš and Presevo and, also, to serve as a source of information about this topic.

Materials and methods

An anonymous survey was conducted on 215 high school third-grade students from Niš and 215 students from Preševo in December 2017. The survey included 13 questions about sexual activity, the knowledge of the use of contraceptive methods and the source of information regarding this topic.

The data are shown in the form of the arithmetic mean and standard deviation, that is in the form of absolute and relative values. On condition that there is normal distribution of data, continually recorded marks were compared using the t-test for independent samples. If there was no normal distribution, continuous marks were compared with the Mann-Whitney test. Attributive marks between two groups were compared using the chi-squared test or Fisher's exact test (if the absolute frequency was less than 5). The hypothesis was tested with the materiality threshold of $p < 0,05$. Data analysis was done within SPSS 16.0.

Results

The total number of respondents was 710 and those were third-grade high school students from Niš, out of whom 319 female high school students, 119 female secondary vocational school students, 208 male high school students and 64 male secondary vocational school students. The survey was conducted on 215 students from Preševo, 103 of whom were girls attending high schools, 19 girls from secondary vocational schools, 67 boys from high schools and 26 boys from secondary vocational schools. There were 36 invalid questionnaires, that is, those that were filled in incorrectly (3.89%).

The average age of the respondents is 17.2 ± 0.5 years.

As far as Niš is concerned, 79 (46.2%) students from vocational schools engaged in sexual intercourse (of whom 38 (67.9%) male and 41 (35.7%) female students), as well as 133 (26.4%) students from high schools (74 (38.3%) of whom male and 53 (19%) female students). Statistics showed that students from vocational schools engaged in sexual intercourse more often than students from high schools ($p < 0.001$). The number of male students who engaged in sexual intercourse was 112 (45.0%), while there were 100 female respondents (23.5%) (Table 1). Also, the statistics showed that male students engaged themselves in sexual intercourse more often than female students from schools in Niš ($p < 0.001$).

As far as Preševo is concerned, 24 (53.3%) students from vocational school engaged in sexual intercourse (23 (88.5%) of whom were male and 1 (5.3%) female students), as well as 49 (28.8%) students from high school (46 of whom (68.7%) were male and 3 female (2.9%) students). The statistics showed that students from vocational school engaged more often in sexual intercourse in comparison to students from high school ($p = 0.004$). The number of sexually active students was 73, with 69 (74.2%) male students and 4 (3.3%) female students (Table 1). Regarding the schools from Preševo, the statistics showed that, when compared to female students, male students engaged more often in sexual intercourse ($p < 0.001$).

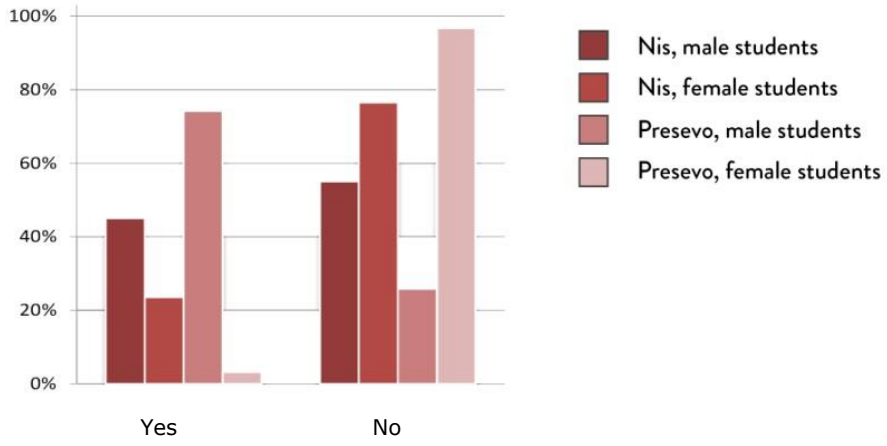
A total of 212 (31.5%) high school students of both sex from Niš and a total of 43 (23.2%) high school students of both sex from Preševo had sexual intercourse (Graph 1). According to the statistics, the students from Niš engaged in sexual intercourse more often than the students from Preševo ($p = 0.038$).

The average age when an adolescent from Niš had his first sexual intercourse was 16.2 ± 1.1 years, and for Preševo that was 15.7 ± 1.1 years. Fe-male students started having sexual intercourse at the age of 16.5 ± 0.90 on average, while male stu-dents had it at the age of 15.7 ± 1.2. The age when they had first sexual intercourse was similar if the criterion for the comparison was the gender (p = 0.137), that is the city (p = 0.156).

The number of sexual partners of the students from Niš is significantly smaller in comparison to the students from Preševo (2 ± 1.4 vs. 4.0 ± 3.0, p < 0.001). Male students had more partners compared to female students (2.8 ± 2.2 vs. 1.5 ± 0.9, p < 0.001).

Table 1. Engagement in sexual intercourse in schools that were involved in the survey

Answer	Niš				Preševo			
	Vocational schools		High schools		Vocational schools		High schools	
	Male	Female	Male	Female	Male	Female	Male	Female
Yes	38 (67.9%)	41 (35.7%)	74 (38.3%)	59 (19.0%)	23 (88.5%)	1 (5.3%)	46 (68.7%)	3 (2.9%)
No	18 (32.1%)	74 (64.3%)	119 (61.7%)	251 (81.0%)	3 (11.5%)	18 (94.7%)	21 (31.3%)	100 (97.1%)



Graph 1. Percentage of students engaged in sexual intercourse

The students of both cities mentioned the love for their partner as the most common reason for engaging in sexual intercourse (Niš - 38.5%, Preševo - 39.4%). The most common reason for engaging in sexual intercourse regarding the gender of the respondents is love for their partner: male students - 33.5%, female students - 45.7% (Table 2). In comparison to other reasons for engaging in sexual intercourse, students mentioned love more often (p = 0.020).

Contraceptive methods were used occasionally or always by 68 (86.1%) students of secondary vocational schools and 113 (84.3%) students of high schools in Niš. Contraceptive methods were used occasionally or always by 97 (86.6%) male and 84 (83.2%) female students (Table 3). No statistically relevant difference was found in terms of the use of contraceptive methods either occasionally or always between vocational schools and high schools (p = 0.922) nor regarding the gender of the re-pondents (p = 0.610).

As far as Preševo is concerned, 24 (87.5%) students from secondary vocational schools and 33 (67.3%) high school students used contraceptive methods occasionally or always (Table 3). It was determined that constant use of contraception and their occasional use were similar in the schools that were surveyed in Preševo (p = 0.119).

The frequency of regular use of contraception is statistically more frequent in Niš than in Preševo (p = 0.007). The use of contraception is similar with reference to the gender (p = 0.738) (Graph 2).

The most common type of contraception du-ring the first sexual encounter in both cities is a con-dom: Niš - 180 students (73.8%), Preševo - 61 stu-dents (83.6%). The most common type of contra-

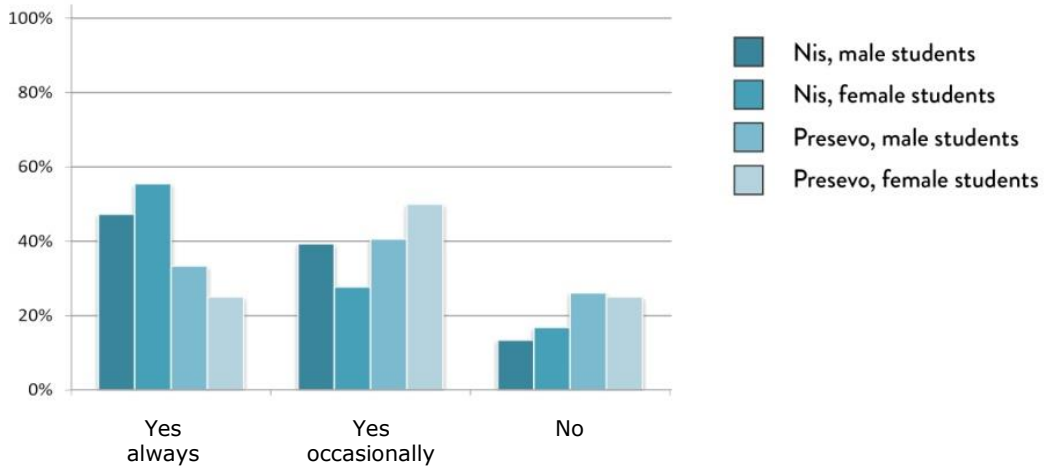
ception during their last sexual encounters in both cities was a condom (Niš - 142 students (64.0%), Preševo - 53 students (72.6%)). Judging from the above-mentioned data, the tendency of decrease in contraception after the first encounter is visible. A condom was the most common contraceptive method during the first sexual intercourse with both males and females: males - 163 (81.5%) and females - 78 (66.7%). Also, the condom was the most common method of contraception during their last sexual intercourse for both genders: males - 127 (70.2%) and females - 68 (59.6%). Statistics has shown that male students use a condom more frequently ($p = 0.004$) than female during their first sexual inter-course (Graph 3).

Table 2. The reason for the first sexual relationship

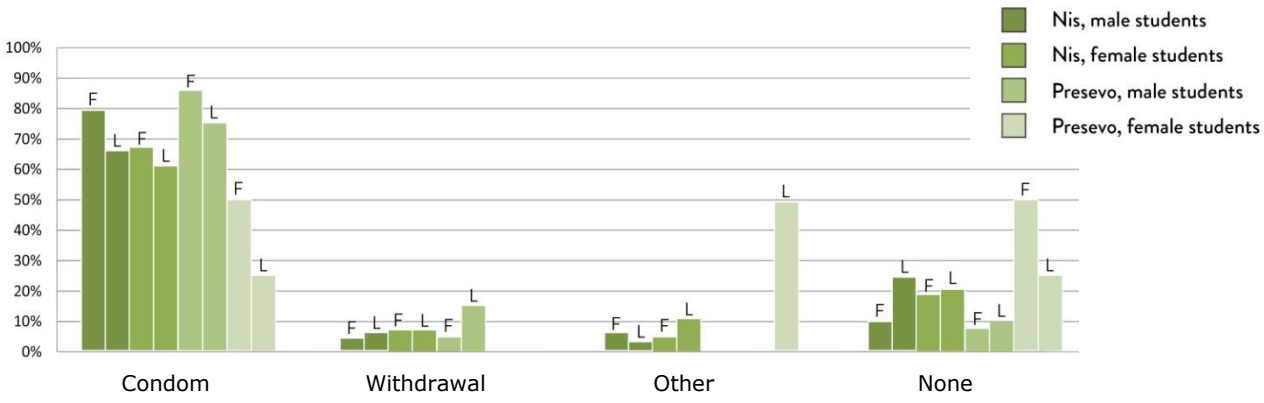
Answer	Niš								Preševo							
	Vocational school				High school				Vocational school				High school			
	M		F		M		F		M		F		M		F	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Love	19	47.4	32	78.0	31	41.9	39	66.1	5	21.7	1	100	19	41.3	3	100
Opportunity	9	31.6	4	9.8	30	40.5	12	20.3	0	0.0	0	0.0	9	19.6	0	0.0
Curiosity	9	15.8	8	19.5	18	24.3	11	18.6	11	47.8	0	0.0	9	19.6	0	0.0
Influence of alcohol or drugs	3	10.5	3	7.3	8	10.8	4	6.8	1	4.3	0	0.0	3	6.5	0	0.0
Readiness	1	10.5	14	34.1	20	27.0	25	42.4	5	21.7	0	0.0	1	2.2	0	0.0
Coercion	0	7.9	4	9.8	1	1.4	1	1.7	0	0.0	0	0.0	0	0.0	0	0.0
Other	3	0.0	0	0.0	2	2.7	3	5.1	1	4.3	0	0.0	3	6.5	0	0.0

Table 3. The use of contraceptive methods

Answer	Niš				Preševo			
	Vocational schools		High schools		Vocational schools		High schools	
	Male	Female	Male	Female	Male	Female	Male	Female
Yes, always	18 (47.4%)	21 (51.2%)	35 (47.3%)	35 (59.3%)	9 (39.1%)	0	14 (30.4%)	1 (33.3%)
Yes, occasionally	17 (44.7%)	12 (29.3%)	27 (36.5%)	16 (27.1%)	11 (47.8%)	1 (100%)	17 (37.0%)	1 (33.3%)
No	3(7.9%)	8 (19.5%)	12 (16.2%)	9 (15.3%)	3 (13.0%)	0	15 (32.6%)	1 (33.3%)



Graph 2. Frequency of contraception use

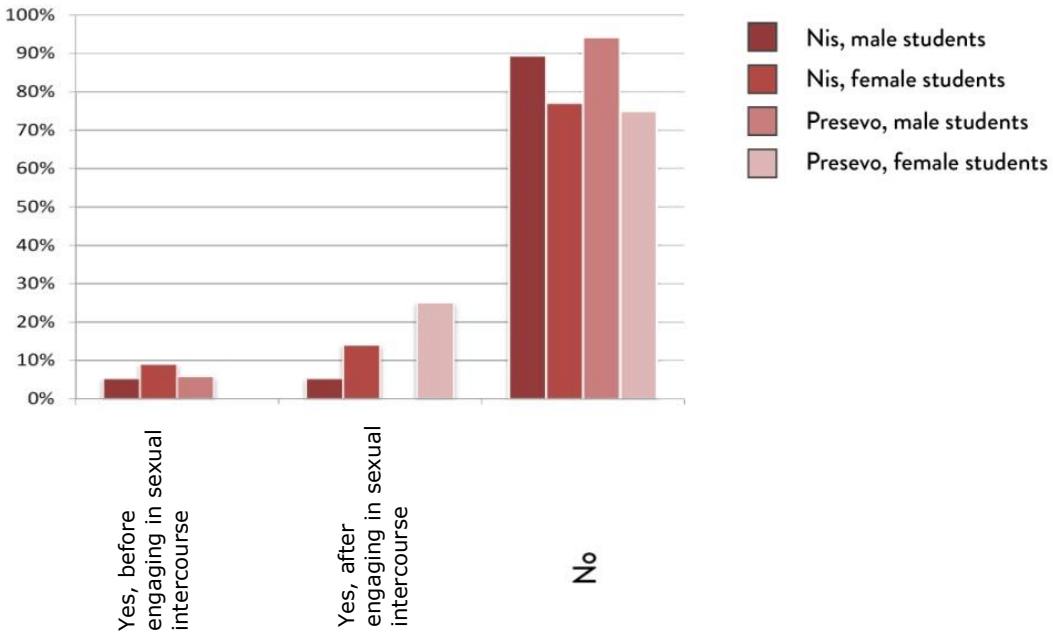


Graph 3. Methods of contraception used during the first and the last sexual intercourse

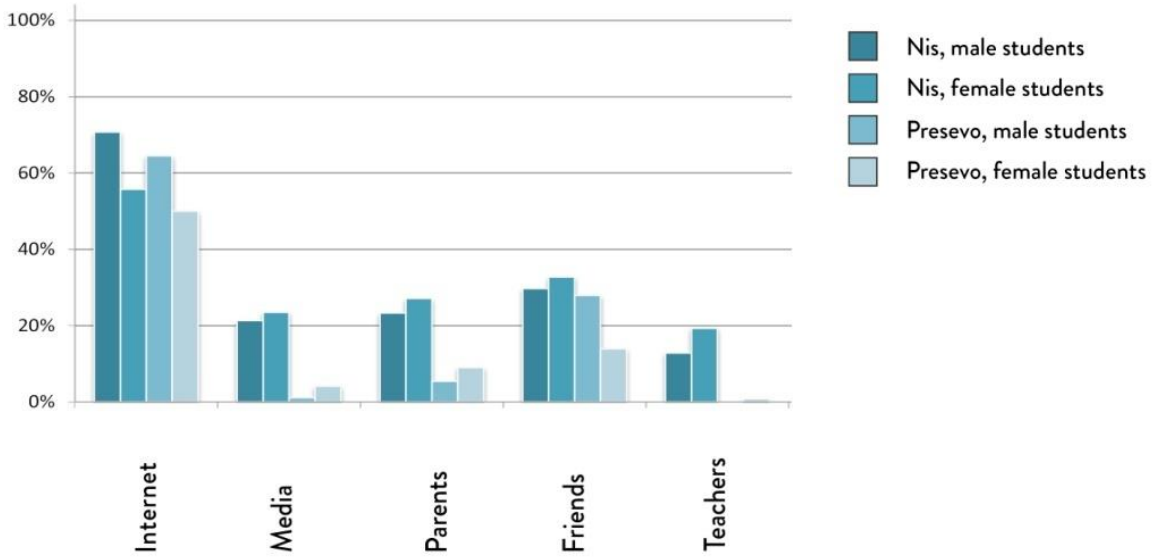
As many as 177 (83.5%) students from Niš and 68 (93.2%) students from Preševo did not seek professional advice on contraception. The Preševo students sought expert advice on contraception less frequently in comparison to the students from Niš ($p = 0.040$). Also, 165 (91.2%) male and 80 (76.9%) female students did not ask for professional help on contraception. Statistics have shown that female students were more likely ($p = 0.002$) to seek professional advice on contraception (Graph 4).

The most common source of information on contraception was the Internet in both cities: Niš - 61.2%, Preševo - 55.0%, as with the gender: males - 64.9%, females - 51.2% were in favour of the Internet (Graph 5).

Two most common reasons for the use of contraception were protection against unwanted pregnancy (Niš - 59.6%, Preševo - 71.2%) and protection against sexually transmitted diseases (Niš - 48.3%, Preševo - 31.5%), so 60.5 % of male and 70.6% of female students considered these reasons to be crucial for the use of contraception (Table 4).



Graph 4. Consulting a healthcare professional about the use of contraception



Graph 5. Ways of informing about contraception

Table 4. Reason for the use of contraception

Answer	Niš				Preševo			
	Vocational schools		High schools		Vocational schools		High schools	
	Male	Female	Male	Female	Male	Female	Male	Female
Protection against unwanted pregnancy	27 (48.2%)	32 (27.8%)	55 (74.3%)	52 (88.1%)	15 (57.7%)	1 (100%)	26 (61.9%)	2 (66.7%)
Protection against STD	22 (39.3%)	22 (19.1%)	52 (70.3%)	38 (64.4%)	8 (30.8%)	0	15 (35.7%)	0
Regulation of menstrual cycle	0	0	0	1 (1.7%)	0	0	0	0
Within other therapy	0	0	0	0	0	0	1 (2.4%)	0

Discussion

Modern societies consider one of their priorities to preserve the reproductive health of the adolescent population. For this reason, we find a significant number of studies dealing with the testing of knowledge and use of contraceptive devices among adolescents.

According to the data obtained in our research, 31.5% of high school students from Niš (45.0% of male and 23.5% of female students) had their first sexual intercourse at the age of 16.2 ± 1.1 years and 23.2 % of high school students from Preševo (74.2% of male and 3.3% of female students) had their first sexual intercourse at the age of 15.7 ± 1.1 years. Similar studies were conducted on the territory of the Republic of Serbia, countries of the region and the world. According to the research of the Institute of Public Health of Serbia from 2013, 33.1% of young people in Serbia between the age of 15 and 19 engaged in sexual intercourse (6). A survey conducted in Indjija in 2015, which included a group of 490 adolescents aged 15-19, (did not show a significant deviation from the results obtained by our survey) of the total number of adolescents that were questioned, 31.22% adolescents had sexual intercourse, of whom 39.57% of boys and 19.80% of girls. The median of the age of the first sexual inter-course for adolescents questioned is 16.33 years, 16.25 years for boys and 16.53 years for girls (7). Data obtained by interviewing high school students in Zaječar in 2012 show that 21.3% of the students had sexual intercourse, with 45.1% of the boys interviewed to have sexual experience and only 10.2% of the girls. The average age of the first sexual intercourse was 16.3 years for girls and 15.5

years for boys (8). According to the studies conducted in 4 large cities in Croatia (Zagreb, Split, Rijeka and Osijek), 39.4% of adolescents had sexual intercourse (51% of boys and 27% of girls), girls at the age of 16, and boys at the age of 15 (9). According to the research conducted in the Tuzla Canton, 13.18% of adolescents were sexually active. The average age to have sex for the first time was 16.5 years for girls and 15.7 years for boys (10). A survey conducted among adolescents in Slovenia showed that 53% of high school students had sexual intercourse, with no statistical significance with reference to the gender as well as to the age they were when they had their first sexual intercourse (11). According to the data obtained through an extensive research of high-risk behaviour among highschool students from 43 federal states in the US, 43.7% of the respondents had sexual relations (12). A study carried out in Sweden in 2014 showed that 21.8% of fifteen-year-old high school students reported having sexual intercourse. Male-female ratio was 3:1, while the average age at which to have first sexual relation was 14.5 ± 0.9 (13). According to the statistics, male students were more likely to have intercourse earlier and more often than female students. According to the available information, the age of the first sexual intercourse does not differ significantly from other cities that were compared in Serbia, countries from the region and the world.

The frequency of regular use of contraceptive methods is statistically more frequent in Niš than in Preševo and it is gender-balanced. The most common method of contraception during the first and last relationship in both cities and for both sexes is a condom, with a reduction in the use of contraception after the first intercourse. The percentage of the use of oral contraceptives, as well as of the double method, is low (7% in Niš, 0% in Preševo). Unlike the results of the research conducted for the purposes of this paper, according to the data from the research Sedlecki et al. 2001, female adolescents in Belgrade most often rely on the coitus interuptus method (54.3%). Results similar to ours were obtained in the above-mentioned research in Indjija (7) and Zaječar (8). The study carried out in Slovenia also shows a reduced use of a condom after the first sexual encounter (from 75 to 50%). However, there is a trend of an increase in the use of oral contraceptives (from 7 to 32%), which is not the case in our research (11). US data show that oral contraceptives are used more frequently (45%) than condoms (38%) among adolescents (14).

Most developed countries, such as Sweden, have health centers where young people can receive adequate information about sexuality, interpersonal relationships, contraception and sexually transmitted diseases (15). However, this type of expert help is not used by the majority of respondents in Serbia, those from Niš (83.5%) and Preševo (93.2%). Instead of professionals, the Internet is the most common source of information about contraception in both cities (Niš 61.2%, Preševo 55.0%) and with both genders (male 64.9%, female 51.2%). The Internet is a creation of civilisation, without which everyday life cannot be imagined in developed countries, especially among the younger population. However, it is also an inexhaustible source of falsehoods and half-truths that carry an enormous risk for both, the physical and psychological state of each individual. The young are particularly sensitive as they are quite susceptible to accept unverified and inaccurate information. Consequently, they are not aware of the risks of sexual activity and disregard the importance of contraceptive method use. On the other hand, if properly used, the Internet can be a powerful tool for learning, creating awareness and a model of behavior. For this reason, the huge potential that the Internet has in providing professional and clear information cannot be ignored, taking into account that in this way one can quickly and easily overcome the barrier of fear, shame, and prejudice that potentially exists when addressing parents or teachers on the subject. Additionally, the fact is that the percentage of the students interviewed who sought information and advice on contraception from parents or teachers is lower than the percentage of respondents who asked their peers for information. Moreover, in comparison to Niš, a fairly smaller number of adolescents in Presevo asked the closest authorities for advice (parents 9%, teachers 1% in Preševo, parents 27%, teachers 19% in Niš). Such results may be explained by cultural and religious characteristics.

The data obtained point to the need for the education system to take more part in health and reproductive education of young people. The education system of a large number of countries in Europe and the USA includes some form of sexual education for schoolchildren, which gives concrete and measurable results in the form of the reduced number of unwanted pregnancies, abortions, sexually transmitted diseases and complications that are included in these conditions (16).

The results of the research that was conducted pointed to a worryingly small degree of participation of the health system in informing young people and promoting reproductive health. The health system can help young people overcome the shame and mistrust that could be the reason for such results by means of the implementation of adolescent sexual education through specifically organized counseling. Such counseling should allow adolescents to develop trust in their doctors and build a friendly relationship so as to get the most accurate information about sexuality, contraception, and diseases that are transmitted through sexual contact.

Conclusion

The age of the first sexual intercourse, as well as the percentage of sexually active adolescents included in our research, does not deviate significantly from other parts of Europe. However, the reduction in condom use after the first intercourse has not been replaced by the use of other contraceptive devices, as is the case in some Western European countries.

The results of the research have shown that adolescents receive most information on this topic from the Internet, then from their peers, and, to a considerably smaller extent, from their parents,

teachers and healthcare workers. However, the information obtained by means of the Internet or from peers may be false, and cannot always be considered relevant and may also threaten their health. This leads us to conclude that there is no need to ignore the great potential of the Internet; instead, we should use it to spread relevant information in a way that is acceptable to adolescents. The use of the Internet reagarding adolescent education on reproductive health should be incorporated into health and education systems, thus making the information that the adolescents receive via the Internet more accurate.

Sexual education inside and outside the school education system, the health system, as well as the increase in the availability of contraceptive devices to adolescents should become integral parts of the strategy for improving the reproductive health of adolescents. The work of the health and education system in the field of reproductive health should essentially be brought closer to the way of thinking of adolescents and the information should be provided in a comprehensible and interesting way, especially through health education lessons in schools and workshops within youth counseling centers at community health centers.

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SEKSUALNA AKTIVNOST, UPOTREBA I POZNAVANJE METODA KONTRACEPCIJE MEĐU SREDNJOŠKOLCIMA NIŠA I PREŠEVA

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Adolescenti su sutrašnji odrasli i čine približno 16% celokupnog svetskog stanovništva. Briga o njihovom reproduktivnom zdravlju zadatak je i obaveza zdravstvenih institucija i društvene zajednice.

Istraživanje je sprovedeno u cilju ispitivanja seksualnog ponašanja, poznavanja i primene kontracepcije među srednjoškolcima Niša i Preševa, kao i izvora informacija o ovoj temi.

Anonimna anketa sprovedena je decembra 2017 godine kod 710 srednjoškolaca Niša i 215 srednjoškolaca Preševa. Anketa je sadržala 13 pitanja o seksualnoj aktivnosti, poznavanju i korišćenju kontraceptivnih metoda, kao i o izvoru informacija o ovoj temi.

Prosek godina života anketiranih je 17,2 godine ± 0,5 godina. U seksualne odnose stupilo je 31,5% srednjoškolaca iz Niša, prvi put sa 16,2 godine ± 1,1 godina i 23,2% srednjoškolaca iz Preševa, prvi put sa 15,7 godina ± 1,1 godina. Učenici su statistički značajno češće i ranije stupali u seksualne odnose u odnosu na učenice. Kao najčešći razlog stupanja u seksualne odnose srednjoškolci oba grada i oba pola naveli su ljubav prema partneru. Učestalost redovnog korišćenja metoda kontracepcije statistički je češća u Nišu u odnosu na Preševo ($p = 0,007$), a ujednačena prema polu ($p = 0,738$). Najčešći vid kontracepcije tokom prvog i poslednjeg odnosa u oba grada i kod oba pola je kondom. Najveći broj anketiranih, iz Niša (83,5%) i Preševa (93,2%), nije tražilo stručnu pomoć o kontracepciji. Učenice su češće tražile stručnu pomoć. Internet je najčešći vid infor-misanja o kontracepciji u oba grada (Niš 61,2%; Preševo 55,0%) i kod oba pola (muški 64,9%; ženski 51,2%). Dva najčešća razloga za primenu kontracepcije su zaštita od neželjene trudnoće (Niš 59,6%; Preševo 71,2%) i zaštita od polno prenosivih bolesti (Niš 48,3%; Preševo 31,5%).

U oba grada potrebno je dugoročno planirati seksualnu edukaciju odgovarajućim informacijama, na način prihvatljiv adolescentima, uz sagledavanje značajne uloge interneta u informisanosti o ovoj temi kod adolescenata.

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Ključne reči: *srednjoškolci, seksualno ponašanje, kontracepcija, reproduktivno zdravlje*

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CLINICAL SIGNIFICANCE OF HISTOCHEMICAL EXPRESSION OF MUCINS IN COLORECTAL ADENOCARCINOMA

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Colorectal carcinoma is the most common malignant tumor of the gastrointestinal tract. In the course of colorectal carcinogenesis, in addition to uncontrolled cell proliferation and accelerated angiogenesis, alterations occur in the structure and/or quantity of epithelial mucins, so the aim of our study was to investigate the histochemical expression of mucins in relation to the clinical characteristics of colorectal carcinoma.

The biopsy material of 75 patients operated from colorectal carcinoma, which was routinely processed and molded into paraffin, was used for the examination. On 3-4 μm thick cuts, routine H&E and histochemical AB-PAS pH 2.5 and HID-AB methods were applied. For the statistical analysis, the statistical software package SPSS (version 13) was used.

Mucin alterations occur in colorectal carcinoma and manifest themselves as a trace to moderate secretions of neutral or fucomucins, moderate to hypersecretions of sialomucins and trace to complete secretions of sulfomucins. The fucomucin and sialomucin secretion is associated with a strong, significant and positive coefficient of correlation with the Astler-Coller classification of the tumor stages, with metastases in the lymph nodes and with distant metastases. Unlike fucomucins, sialomucins are associated with a strong, positive, significant coefficient of correlation with the pathological stage of the tumor. Sulfomucins are associated with the significant, but negative coefficients of correlation with the tumor pathological stage, tumor stages according to the Astler-Coller classification, and metastases in the lymph nodes. The secretions of fucomucins and sialomucins are in a good and significant mutual relationship, only the secretion of sulfomucins is in a negative correlation in comparison to the other mucins.

Histochemical expression of mucins may be a useful prognostic indicator of the progression of colorectal carcinoma.

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Key words: *colorectal carcinoma, epithelial mucins, histochemistry*

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Introduction

The International Agency for Research on Carcinoma (IARC) has classified colorectal carcinoma among the three most frequent malignant neoplasms in human oncology (1). Alarming is the fact that the incidence and mortality in the last three decades have been rising steadily with an average annual growth rate of around 3% or with more than 400,000 newly diseased during a year. A striking increase in the incidence has been recorded in transition countries and in Australia/New Zealand, Europe and North America (2). In Serbia, colorectal carcinoma is the second leading cause of death with a mortality rate of 16.6/100.000, on the basis of which Serbia is ranked among the countries with high mortality (3).

Colorectal carcinoma is a multifactorial disease resulting from the interaction of hereditary factors and environmental factors (4, 5, 6). The risk for the development of colorectal carcinoma rises significantly after 40 years of age, and about 85% of colorectal carcinoma occurs in people older than 50 years of age (7). The risk of colorectal carcinoma has been estimated to be 15 times higher in people over 50 years of age than in people aged 20-49 years (8).

Based on molecular and cytogenetic studies, it has been established that colorectal carcinoma develops in one of two genetic pathways. The first is the pathway of chromosomal instability or the APC/β-catenin pathway, known as the "adenoma-carcinoma" sequence, and the second is the pathway of microsatellite instability or correction of the wrongly paired DNA (9). During colorectal carcinogenesis, in addition to the uncontrolled cell proliferation and accelerated angiogenesis, alterations in the structure and/or quantity of epithelial mucins with subsequent changes in the mucus protective function and cell signal transduction disorders occur (10, 11). These changes in the expression of mucins or glycosylation affect cell growth, differentiation, transformation, adhesion, invasiveness, and immune control of the tumor (12).

Aim of the study

The aim of our study was to examine the histochemical expression of epithelial mucins in relation to the clinical characteristics of colorectal carcinoma.

Materials and methods

Patients and samples

The study included 75 patients with colorectal adenocarcinoma, who underwent the surgical resection of the tumor in the Center for Abdominal Surgery of the Clinical Center of Montenegro (KCCG). In the Institute of Pathology of CCCG, from each operative resection, depending on the size of the tumor, 5-15 biopsies were taken, including 2-3 biopsies of the surrounding healthy tissue of the colon. After the fixation in a 4% buffered formaldehyde solution, the biopsy material was routinely processed, molded into paraffin blocks and archived.

Tumors tissue samples of colorectal carcinoma-made up the study (experimental) group (n = 75). The control group (n = 75) consisted of opera-tive biopsies of adjacent non-tumor tissue of the colon, which were taken from the operative preparation according to the protocol.

This research was carried out in conjunction with the Helsinki Declaration and the World Health Organization's Recommendations for experiments on human material. The consent of the supervisory Ethics Committee exists for the research.

Histopathology and mucin histochemistry

Serial cuts, 3-4 μm thick, have been made from paraffin blocks of all resected tumors and regional lymph nodes, on which routine Hematoxylin-Eosin method, for histopathological verification of lesions, histochemical AB-PAS pH 2.5 (Alcian Blue-Periodic Acid Schiff) method, for the differentiation of neutral from acidic mucins, and histochemical HID-AB pH 2.5 (High Iron Diamine-Alcian Blue) technique, for the differentiation of weak acidic, unsulphated sialomucins from strong acidic, sulphated-sulfomucins, was applied.

Histochemical expression of epithelial mucins has been quantified according to the following scale: - (minus), mucins asecretion; +/- (plus minus), trace secretion of mucins; + (1 plus), moderate secre-tion of mucins; ++ (2 pluses), strongly expressed secretion (hypersecretion) of mucins.

Statistical analysis

For the statistical analysis of the obtained results, the statistical software package SPSS (version 13.0) has been used. The χ^2-test (chi-squared test), Mann-Whitney U test, Kruskal-Wallis test and Student's t-test have been used to analyze the significance of the differences between parametric and non-parametric features between and within the groups. Then, the Kolmogorov-Smirnov test for the normality of distribution, the univariate statistical analysis, the correlation analysis (Spearman's coefficient of rank correlation, Pearson's correlation coefficient for parametric features) have been used. Significance testing has been performed at $p < 0.05$.

Results

In the examined sample of patients, in whom the colorectal carcinoma was removed, there were 45 men (60%) and 30 women (40%). There is no statistically significant difference in the presence of colorectal carcinoma in relation to the gender of the patients, at the adopted level of reliability $p < 0.05$ (χ^2-test = 3.000; $p = 0.083 > 0.05$).

There was also no statistically significant difference between the groups, by the age of the patients (Mann-Whitney U test = 569.500; $p = 0.232$). Male patients had an average age of 65.9 ± 10.8 years and women 60.8 ± 14.5 years, which was not a significant difference (Student's t-test = 1.622; $p = 0.111$).

In the examined sample of patients, the most frequent localisation of the carcinoma was in the rectum. The frequency of this localisation with 65.3% of the cases was highly statistically significant (χ^2-test = 7.053; $p = 0.008$), compared to all other localisations in the colon.

There was no statistically significant difference in the distribution of colorectal carcinomas with respect to the macroscopic type of the tumor (from 26.7% to 37.3%), but it was noticed that the most commonly occurring type was the ulceroinfil-trative type (37.2% of the cases).

The greatest number of the examined cases (74.7%) of colorectal adenocarcinoma were diagnosed in the third stage according to the pathological classification (pT3), which significantly differed from the number of cases diagnosed as stage pT2 (16%). Colorectal carcinoma rarely occurs in the lowest and highest pT stage (2.7% and 6.7% of the cases).

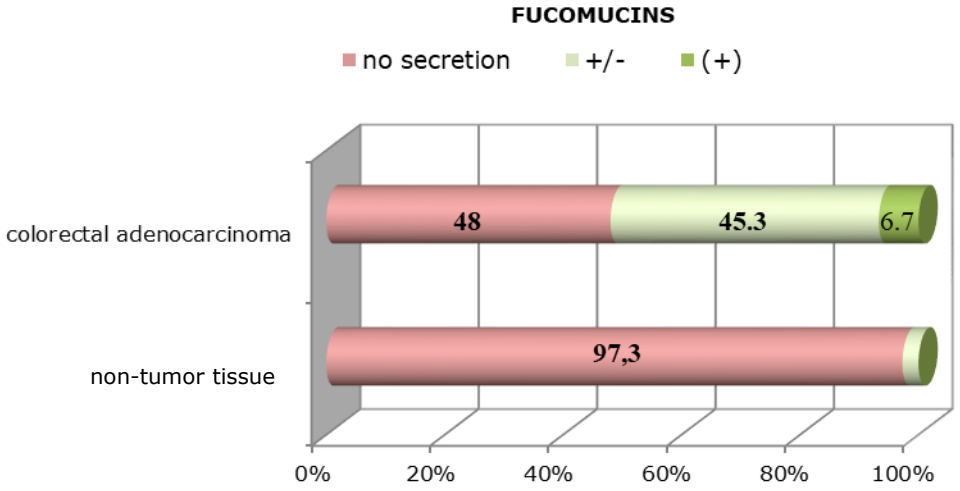
The distribution of the examined colorectal carcinoma in relation to the Astler-Coller classification showed the lowest frequency of tumors in the C1 stage (one case, 1.3%) and the D stage (7 patients, 9.3%). This frequency was followed by the

B1 stage (11 cases, 14.7%). The highest frequency of patients was in the B2 stage (25 patients, 33.3%) and the C2 stage (31 cases, 41.3%).

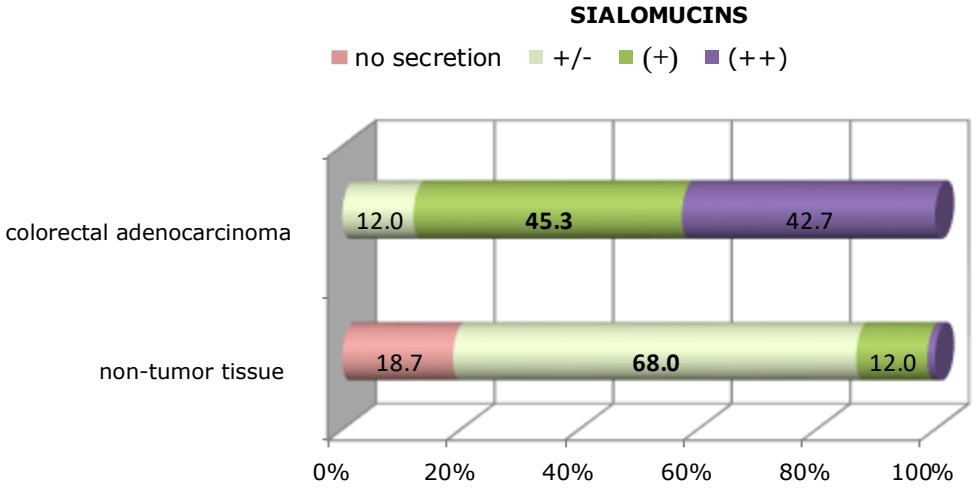
Metastases in lymph nodes were found in 39 patients (52%), while in 36 patients (48%) metastases were not found in the lymph nodes. Metastatic deposits in 1-3 lymph nodes were found in 22 patients (29.3%), deposits in 4-6 lymph nodes were found in 8 patients (10.7%), and in 9 patients (12%) deposits were present in more than 7 lymph nodes (Graph 2). Distant metastases were found in 10.7% of the patients.

By examining the histochemical expression of neutral mucins (fucomucins) in the non-tumor tissue, in 97.3% of the cases, their complete asecretion was noticed, which was an obvious significance that did not need to be statistically proven. In the colorectal adenocarcinoma tissue of 48% of the cases, the fucomucine asecretion was verified, in 45.3% of the cases there was a trace secretion present, and in 6.7% of the cases, a moderate secretion of neutral mucins was found (Graph 1).

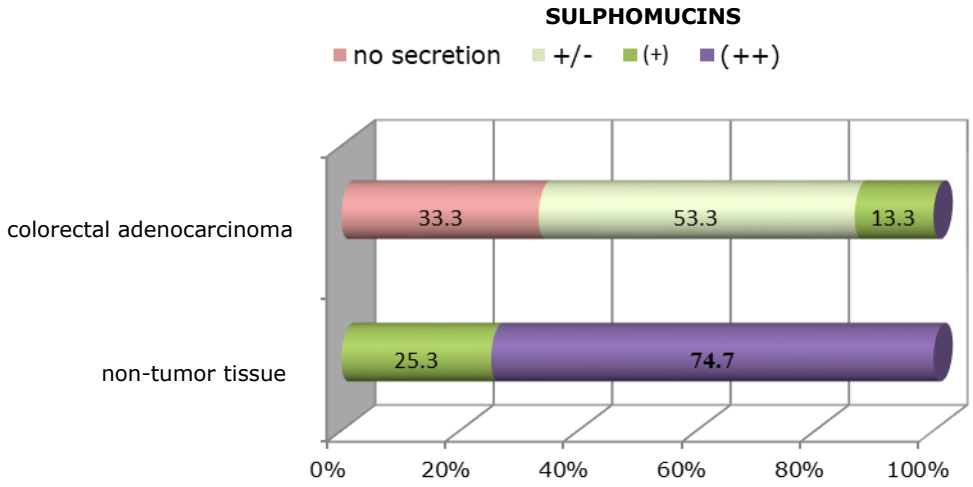
In the control group (non-tumor tissue), in a significant number of cases, the most common finding was the trace secretion of sialomucins (68%; χ^2-test = 9.720; p = 0.002). The frequency of asecretion (18.7%) or moderate secretion (12%) of sialomucins in this group, was significantly less common and without significant statistical difference. The occurrence of sialomucin hypersecretion in non-tumor surrounding tissue was extremely rare (1.3% of the cases). In contrast to this distribution, in colorectal adenocarcinoma of 42.7% of the cases hypersecretion of sialomucins was found (Figure 1), moderate secretion in 45.3%, trace secretion in 12% of the cases, and in no case the asecretion of sialomucins was found (Graph 2).



Graph 1. Distribution of neutral – fucomucins in colorectal carcinoma and adjacentnon-tumoral tissue



Graph 2. Distribution of poorly acidic – sialomucins in colorectal carcinoma and adjacent non-tumor tissue



Graph 3. Distribution of highly acidic – sulfomucins in colorectal carcinoma and adjacent non-tumor tissue

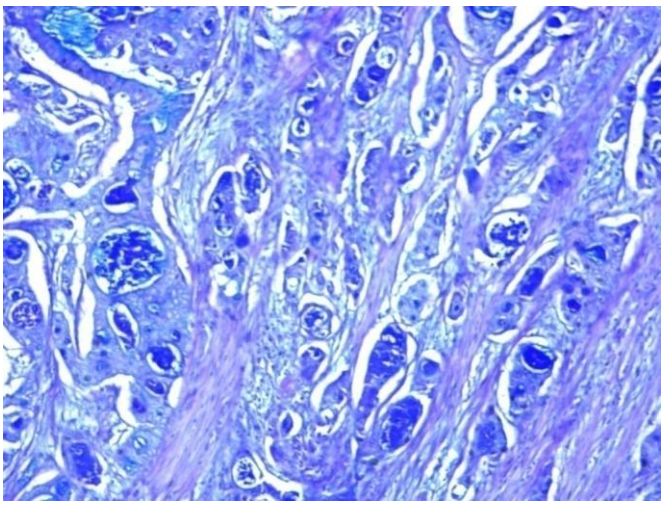


Figure 1. Pronounced histochemical expression of sialomucins in colorectal carcinoma

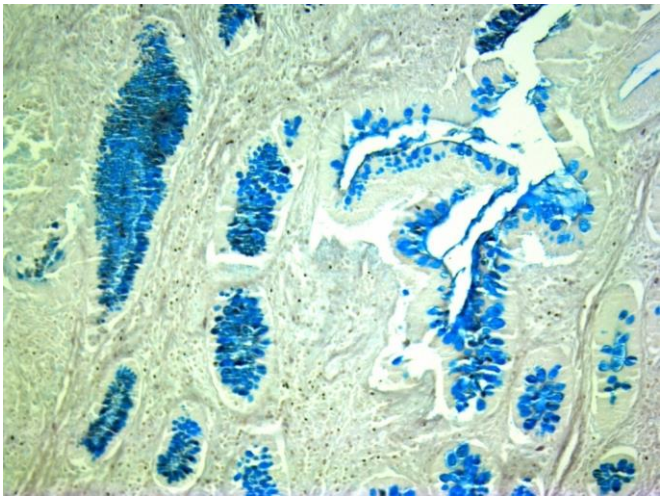


Figure2. Excessive sulfomucins reduction (black) and hypersecretion of sialomucins in colorectal carcinoma (HID-AB x200).

By analyzing the non-tumor tissue, significant hypersecretion (74.7%) of strong acidic sulphomucins was observed, which is a statistical significance that should not be specifically proven. Only in about 25.3% of the cases a moderate secretion was found, while trace secretion and asecretion of sulphomucins were not detected in this group of subjects.Contrary to this finding, in the colorectal adenocarcinoma group, hypersecretion of sulphomu-cins was not identified in any case, and moderate secretion was a significantly rare occurrence (13.3%) compared to the remaining cases. In most of the remaining cases, the trace secretion of sulphomucins (53.3% of the cases) (Figure 2), or the complete asecretion of mucins (33.3% of the cases) was found (Graph 3).

The connection of the mucin secretion with the clinical parameters of colorectal carcinoma

By examining the mucin secretion in relation to the gender and age of patients and by testing the significance (Mann-Whitney U test), no statistically significant differences were found in either parameter (fuco-, sialo-, and sulphomucins) between the comparable gender and age groups.

No statistically significant difference in the expression of mucins (fuco-, sial- and sulphomucins) (Kruskal-Wallis test; $p = 0.212$ to $p = 0.833$) was found compared to the macroscopic type of tumor.

Mucin expression in relation to colorectal carcinoma localisation also did not show statistically significant differences (Kruskal-Wallis test; $p = 0.212$ to $p = 0.833$).

The distribution of all the analysed mucins in relation to the pathological stage of the tumor (pT) showed a change in the secretion from the second (pT2) to the third (pT3) pathological stage in the following manner: the secretion of neutral-fucomucins and weak acidic sialomucins increased significantly ($p = 0.008$ and $p = 0.001$) and of strong acidic-sulphomucins decreased significantly ($p = 0.003$). The described mucin secretion trend was also observed in the highest pathological stage of the carcinoma, but due to the low number of data (5 cases), was not the subject of statistical comparison (Table 1).

Mucin expression in relation to the Astler-Coller tumor stage is shown in Table 2. It was noticed that mucin secretion, picturesquely divided the stages according to the Astler Coller classification into two parts, wherein the mucine secretion parameters were arranged in a similar manner in stages B1 and B2 versus stages C1 to D. It was also observed that the expressions of mucins within the B1 and B2 groups, but also within the C1 to D groups, were statistically indifferent (Mann-Whitney U test for B1-B2; $p = 0.074$ to $p = 0.839$ and C1 to D; $p = 0.053$ to $p = 0.658$). However, when the whole group B1-B2 was compared with the C1-D group, a highly significant difference in the expression of mucins ($p < 0.0001$) was obtained.

The trend of the distribution of neutral-fucomucins was such that at the B1 and B2 stage the frequency of the cases with increased secretion increased, but it was still similar. However, it was noticed that there was no significant difference between the high frequency of the cases without the secretion of neutral mucins in stage B1 (81.8%) and 64% of the cases in stage B2. A significant increase in trace fucomucins occured in C2 stage (67.7%) and continued in stage D with the occurrence of cases with moderate secretion of this mucin (42.9%). The trend of the increase of the secretion of neutral mucins followed an increase in the Astler Coller stage (χ^2-test = 5.444; $p = 0.020$).

By examining the expression of weak acidic-sialomucins in relation to the Astler Coller Stages, it was observed that the trace presence of this mucin in the B1 and B2 stage (36.4% and 12%) linearly increasesed by the intensity of the secretion up to the hypersecretion that prevailed in the C2 stage and especially in the D stage (61.3% and 71.4% of the cases, respectively), while C2 and D stage trace secretion was extremely rare (6.5% and 0% respectively).

Asecretion of the strong acidic-sulphomucins was a rare occurrence in stages B1 and B2 (0% and 16%), as opposed to C2 and D stages, where there was no secretion of this mucins in 58.1% and 42.9% of the cases. The trace secretion of sulphomucins did not differ significantly relative to the tumor stage (χ^2-test = 0.900; $p = 0.343$). Moderate secretion of strong acidic mucins was a more frequent occurrence in stage B1 (45.5%), while it was significantly rarer in higher stages (16% in B2, 0% in C2 and 14.3% in D). (χ^2-test = 11.560; $p = 0.001$).

Examination of mucin secretion in relation to the occurrence of metastases in the lymph nodes and in relation to the number of affected lymph nodes showed that groups, classified by metastases in the lymph nodes, were significantly different only in relation to the cases without metastases (Table 3).

A group of the cases without metastatic deposits was significantly different in the distribution of mucins in relation to the each individual group with a presence of deposits in the lymph nodes. (Kruskal-Wallis test; $p < 0.001$ to $p < 0.007$). When the distribution of mucin secretion was considered within the groups without metastases, it was noticed that the absence of deposits in the lymph nodes was approaching the distribution of mucins according to the characteristics of normal colorectal tissue because it did not deviate much from the normal tissue.

The occurrence of metastases in the lymph nodes significantly changed the secretion of the mucins, increasing the intensity of fucomucins and sialomucins secretion with the increase in the number of affected nodes, while the secretion of sulphomucins was decreasing. However, these changes in the secretion of mucins did not show a significant difference between the groups with deposits. That is, the groups of lymph nodes with metastatic deposites did not significantly differ between themselves in relation to mucin expression (Kruskal-Wallis test; $p = 0.119$ to $p = 0.755$).

Table 1. Distribution of mucin secretion in relation to the pathological stage of the tumor

Parameters	**Pathological stage of the tumor**							
	Stage I		Stage II		Stage III		Stage IV	
FUCOMUCINS	n	%	n	%	n	%	n	%
No secretion	1	50.0	10	**83.3**	23	41.1	2	40.0
+/-	0	0.0	2	16.7	29	51.8	3	60.0
(+)	1	50.0	0	0.0	4	7.1	0	0.0
SIALOMUCINS	n	%	n	%	n	%	n	%
+/-	0	0.0	4	33.3	5	8.9	0	0.0
(+)	1	50.0	8	**66.7**	23	41.1	2	40.0
(++)	1	50.0	0	0.0	28	50.0	3	60.0
SULFOMUCINS	n	%	n	%	n	%	n	%
No secretion	0	0.0	0	0.0	21	37.5	4	80.0
+/-	1	50.0	8	**66.7**	30	53.6	1	20.0
(+)	1	50.0	4	33.3	5	8.9	0	0.0

Table 2. Distribution of mucin secretion according to Astler-Coller tumor classification

Parameters	**Astler-Collerstage of the tumor**									
	B1		**B2**		**C1**		**C2**		**D**	
FUCOMUCINS	n	%	n	%	n	%	n	%	n	%
No secretion	9	**81.8**	16	**64.0**	0	0.0	10	32.3	1	14.3
+/-	1	9.1	8	32.0	1	100.0	21	**67.7**	3	**42.9**
(+)	1	9.1	1	4.0	0	0.0	0	0.0	3	**42.9**
SIALOMUCINS	n	%	n	%	n	%	n	%	n	%
+/-	4	**36.4**	3	**12.0**	0	0.0	2	6.5	0	0.0
(+)	6	**54.5**	16	**64.0**	0	0.0	10	**32.3**	2	**28.6**
(++)	1	9.1	6	24.0	1	100.0	19	**61.3**	5	**71.4**
SULFOMUCINS	n	%	n	%	n	%	n	%	n	%
No secretion	0	0.0	4	16.0	0	0.0	18	**58.1**	3	**42.9**
+/-	6	**54.5**	17	**68.0**	1	100.0	13	**41.9**	3	**42.9**
(+)	5	**45.5**	4	**16.0**	0	0.0	0	0.0	1	14.3

Table 3. Distribution of mucin secretion in relation to metastases in the lymph nodes

Parameters	**Metastases in the lymph nodes**							
	Without deposits in lymph nodes		Deposits in 1-3 lymph nodes		Deposits in 4-6 lymph nodes		Deposits in more than 7 lymph nodes	
FUCOMUCINS	n	%	n	%	n	%	n	%
No secretion	25	69.4	8	36.4	1	12.5	2	22.2
+/-	9	25.0	12	54.5	6	75.0	7	77.8
(+)	2	5.6	2	9.1	1	12.5	0	0.0
SIALOMUCINS	n	%	n	%	n	%	n	%
+/-	7	19.4	2	9.1	0	0.0	0	0.0
(+)	22	61.1	5	22.7	4	50.0	3	33.3
(++)	7	19.4	15	68.2	4	50.0	6	66.7
SULFOMUCINS	n	%	n	%	n	%	n	%
No secretion	4	11.1	11	50.0	3	37.5	7	77.8
+/-	23	63.9	11	50.0	4	50.0	2	22.2
(+)	9	25.0	0	0.0	1	12.5	0	0.0

Table 4. Correlation analysis - interdependence of parameters - significance and degree of dependence

		Fucomucins	Sialomucins	Sulfomucins	pT stage	AC stage	Metastases in the lymph nodes	Distant metastases
Fucomucins	cc	1.00	**0.37***	**-0.34***	0.22	**0.43***	**0.39***	**0.33***
	p	.	0.00	0.00	0.06	0.00	0.00	0.00
Sialomucins	cc	**0.37***	1.00	-**0.44***	0.35	**0.47***	**0.42***	0.23
	p	0.00	.	0.00	0.00	0.00	0.00	0.04
Sulfomucins	cc	**-0.34***	**-0.44***	1.00	**-0.45***	**-0.50***	**-0.50***	-0.10
	p	0.00	0.00	.	0.00	0.00	0.00	0.39
pT stage	cc	0.22	**0.35***	**-0.45***	1.00	**0.60***	**0.44***	0.17
	p	0.06	0.00	0.00	.	0.00	0.00	0.13
AC stage	cc	**0.43***	**0.47***	**-0.50***	**0.60***	1.00	**0.84***	**0.54***
	p	0.00	0.00	0.00	0.00	.	0.00	0.00
Metastases in the lymph nodes	cc	**0.39***	**0.42***	**-0.50***	**0.44***	**0.84***	1.00	0.23
	p	0.00	0.00	0.00	0.00	0.00	.	0.05
Distant metastases	cc	**0.33***	**0.23***	-0.10	0.17	**0.54***	0.23	1.00
	p	0.00	0.04	0.39	0.13	0.00	0.05	.

* Significant $p < 0.05$, cc - Spearman's correlation coefficient

Mucin secretion significantly differed only in relation to the metastases in the lymph nodes compared to the cases without the metastases, and a significant difference in the secretion of mucins between the cases with metastatic deposits and the cases without deposits was observed.

In a preliminary analysis, the cross-examination of the tested parameters indicated a high significance relationships in certain interrelated relationships. The correct measure of their connection was shown by a correlation analysis where the significance of that relationship was proved by the significance of the correlation coefficient, and the strength of the connection by its size. In Table 4, all the parameters that were the subject of the analysis in this study are shown through the correlation coefficient (cc) with its statistical significance (p).

A group of the cases without metastatic deposits was significantly different in the distribution of mucins in relation to the each individual group with a presence of deposits in the lymph nodes. (Kruskal-Wallis test; $p < 0.001$ to $p < 0.007$). When the distribution of mucin secretion was considered within the groups without metastases, it was noticed that the absence of deposits in the lymph nodes was approaching the distribution of mucins according to the characteristics of normal colorectal tissue because it did not deviate much from the normal tissue.

The occurrence of metastases in the lymph nodes significantly changed the secretion of the mucins, increasing the intensity of fucomucins and sialomucins secretion with the increase in the number of affected nodes, while the secretion of sulphomucins was decreasing. However, these changes in the secretion of mucins did not show a significant difference between the groups with deposits. That is, the groups of lymph nodes with metastatic deposites did not significantly differ between themselves in relation to mucin expression (Kruskal-Wallis test; $p = 0.119$ to $p = 0.755$).

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Fucomucins were in a good, positive, and significant correlation with the Astler-Coller tumor stage (cc = 0.43), with the metastases in the lymph nodes (cc = 0.39) and with the distant metastases (cc = 0.33).

Sialomucins were in an even stronger, significant relationship with the Astler-Coller tumor stage (cc = 0.47), with metastases in the lymph nodes (cc = 0.42) and with the distant metastases (cc = 0.23). In contrast to neutral mucins, sialomucins were with the good, positive and significant correlation coefficient (cc = 0,35) associated with the pathological stage of the tumor.

Sulphomucins were with the good, significant, but negative correlation coefficients associated with

the pathological stage of the tumor (cc =-0.45), with the Astler-Coller tumor stage classification (cc =-0.50), and with lymph node metastases (cc =-0.50).

The secretions of neutral and weak acidic mucins were in a good and significant mutual relationship (cc = 0.37), only the secretion of sulphomucins was in the negative correlation with the other mucins (cc =-0.34, cc =-0.44).

Discussion

Mucus (phlegm) is a viscous colloidal substance produced by specialised goblet cells, and mucocytes of mucous or mixed seromucous glands. In the form of a thin layer, the water-insoluble gel covers the epithelium of the tubular organs and separates it from the lumen content. The mucus covers the mucous membranes that directly or indirectly come into contact with the outer environment, where it primarily has a protective role (13). Mucus contains water, antiseptic enzymes, immunoglobulins, inorganic salts, proteins and glycoprotein macromolecules-mucins (14). The most present component of mucus are the mucins that are responsible for the biochemical and biophysical properties of the mucus. They are divided into two large groups: membrane and secretory mucins (15).

Mucins represent a selective molecular barrier on the epithelial surface, provide protection of the cell surface and participate in morphogenetic cell transduction (16, 17). Based on the histochemical characteristics, they are divided into neutral fucomucins, weak acidic or sialomucins and strong acidicsulphomucins. Fucomucins contain N-acetyl derivates of hexosamine, D-galactose and L-fructose. Fukomucins do not have acid groups, so they are stained using PAS (Periodic Acid Schiff) method (12). Sialomucins contain hexosamine, glucuronic and sialic acid, and sulfomucins besides hexosamine and uronic acid also contain sulphate groups (11, 18, 19). Specific molecular structure contributes to the specialised role of membrane mucins through which the information about the conditions in the external environment is transferred into the epithelial cells, and so they serve as receptors and sensors on the cell surface. They carry signals to extreme stimuli, thus leading to a coordinated cell response that includes differentiation, apoptosis, and secretion of specific products (11, 17).

The secretion of mucus glycoproteins is a slow but continuous process, which maintains a thin layer on the surface of the mucous membrane. Accelerated secretion occurs under the influence of various stimuli, physical or chemical factors. Mucins that are released from the cytoplasmic vesicles bound themselves to water and in the form of gel coat cover the mucous membrane (20).

In healthy colon mucosa the secretion of strong acidic sulphated mucins dominates, while neutral and weak acidic-sialomucins are mostly present in traces (21). In accordance with this, we have verified the pronounced and moderate secretion of strong acidic sulphomucins and a complete absence or trace secretion of neutral and weak acidicsialomucins in the non-tumor tissue, in the vicinity of the carcinomas of all of our patients.

The first significant aberrations of the mucin secretion have been observed in the second and third pT tumor stages, when the secretion of neutral and weak acidic mucins significantly increases, and the secretion of strong acidic sulphomucins significantly decreases. In relation to the Astler-Coller classification, a slight increase in the secretion of neutral mucins is observed already in B1 to B2 stages, while a significant increase in the secretion of fucomucins occurs in the C2 stage and continues in D stage with the occurence of moderate secretion. A slight increase in the secretion of weak acidic mucins has also been observed in the stages B1-B2, and then the secretion intensity increases linearly all the way to the hypesecretion of sialomucins, which dominates in the C2 stage, and especially in the D stage. In the C2 and D stages, a high significant absence of secretion (asecretion) or a trace secretion of strong acidicsulphomucins is observed. It has been observed in the literature that an increased quantity of sialomucins, besides in the tissue of the colorectal carcinoma, is also found in the transitional (transient) zone that surrounds the primary carcinoma. The same authors have claimed that the occurrence of sialomucins hypersecretion in this zone has been associated with poor prognosis and that the morphological and mucin components of the transitional zone are the prognostic factors for the progression of colorectal carcinoma (22).

In our research, we have observed that with the occurrence of metastases in the lymph nodes and the increase in the number of affected lymph nodes the secretion of neutral fucomucins and weak acidicsialomucins significantly increases, while the secretion of strong acidic-sulphomucins decreases. It is thought that increased sialinisation stimulates the migration of tumor cells through the extracellular matrix (23, 24), and it has been emphasized in numerous studies that the expression of sialomucins affects the processes of invasion and metastasis of the tumor (25).

Hypersecretion of sialomucins with the reduction of sulfomucins has been observed in adenoma-carcinoma sequences in humans (26) and in the "aberrant cryptal focus" (ACF) in rats (27). If ACF is known to be the earliest known precancerous lesion, then it is clear that the occurrence of the hypersecretion of sialomucins is an early event in colorectal carcinogenesis.

Aberrations in the secretion of mucins simultaneously with the disorder of the protective function of mucus in the intestines also cause a disturbance of the signal transduction in which the mucin molecules are involved. This triggers the inflammatory processes in the intestinal mucous membrane and predisposes the development of the carcinoma. Chronic inflammation in the intestines leads to a change in glycosylation and causes deregulation of the mucins, and it is therefore thought that precisely this aberrant mucin expression represents a link between inflammation and carcinoma (28).

The mechanism by which the mucins participate in the pathogenesis of the carcinoma is not fully

clear, but there is a presumption that the tumor growth area is hypoxic, acidic and full of proteases and other biologically active substances, and so the tumor tissue likely uses mucins for the configuration of the microenvironment during invasion, metastasis and growth under unfavorable conditions (18). Increased concentrations of mucin glycoproteins in serum are in correlation with an increased risk for tumor formation and poor prognosis, and therefore it is thought that excessive expression, inadequate expression, or the expression of aberrant mucin forms contributes to the pathogenesis of the carcinoma (14, 29).

In this study it has been noticed that mucin secretion does not depend on the demographic characteristics of the patients, on the localisation and on the macroscopic type of the tumor. The secretion of weak acidic sialomucins is associated with strong, significant and positive correlation coefficients with the pathological stage of the tumor, with the stages of the tumor according to the Astler-Coller classification and with metastases in the lymph nodes. Sulphomucins are associated with significant, but negative correlation coefficients with the pathological stage of the tumor, with the stages of the tumor according to the Astler-Coller classification and with metastases in the lymph nodes.

Conclusion

In colorectal carcinoma, numerous alterations in the secretion of epithelial mucins occur, which are primarily manifested as a moderate to hypersecretion of sialomucins, trace or a moderate secretion of fucomucins and a trace or a complete asecretion of sulphomucins. Changes in the quality and quantity of mucins depend on the pathological stage of the tumor, on the Astler-Coller classification of the tumor stage, on the metastases in the lymph nodes and distant metastases, which indicates that the histochemical expression of mucins may be a useful prognostic indicator of colorectal carcinoma progression.

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KLINIČKI ZNAČAJ HISTOHEMIJSKE EKSPRESIJE MUCINA U KOLOREKTALNOM ADENOKARCINOMU

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Kolorektalni karcinom je najučestaliji maligni tumor gastrointestinalnog trakta. Tokom kolorektalne karcinogeneze, osim nekontrolisane proliferacije ćelija i ubrzane angiogeneze nastaju i alteracije u strukturi i/ili količini epitelnih mucina, pa je cilj našeg rada ispitivanje histohemijske ekspresije mucina u odnosu na klinčke karakteristike kolorektalnog karcinoma.

Za ispitivanje je korišćen biopsijski material 75 bolesnika operisanih od kolorektalnog karcinoma, koji je rutinski obrađivan i kalupljen u parafin. Na rezovima debljine od 3 μm do 4 μm, primenjene su rutinska H&E i histohemijske AB-PAS pH 2,5 i HID-AB metode. Za statističku analizu korišćen je statistički programski paket SPSS (verzija 13).

U kolorektalnom karcinomu nastaju alteracije mucina koje se manifestuju kao sekrecija u tragu do umerene sekrecije fukomucina, umerene do hipersekrecije sijalomucina i sekrecije u tragu do potpune asekrecije sulfomucina. Sekrecija fukomucina i sijalomucina je jakim, signifikantnim i pozitivnim koeficijentima korelacije povezana sa stadijumima tumora po Astler-Coller klasifikaciji, sa metastazama u limfnim žlezdama i sa udaljenim metastazama. Za razliku od fukomucina, sijalomucini su jakim, pozitivnim, značajnim korelacionim koeficijentom povezani i sa patološkim stadijumom tumora. Sulfomucini su signifikantnim, malim negativnim koeficijentima korelacije povezani sa patološkim stadijumom tumora, sa stadijumima tumora po Astler-Coller klasifikacijii i sa metastazama u limfnim žlezdama. Sekrecije fukomucina i sijalomucina u dobroj su i značanoj uzajamnoj povezanosti, jedino je sekrecija sulfomucina u negativnoj korelaciji prema ostalim mucinima.

Histohemijska ekspresija mucina može biti koristan prognostički pokazatelj progresije kolorektaknog karcinoma.

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Ključne reči: *kolorektalni karcinom, epitelni mucini, histohemija*

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SELECTION OF TREATMENT METHODS FOR BLEEDING ANEURYSMS

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Whether to treat bleeding aneurysms surgically or by endovascular occlusion is a frequent subject of debate between neurosurgeons and radiologists.

The aim was to take a general position on when to apply which method through the overview of the results that have been achieved so far. However, each patient must be seen as an individual in order for the right decision to be made.

A prospective study of 336 patients who were treated at the Clinic of Neurosurgery in Niš for a ruptured aneurysm in the period between January 2007 and December 2010 has been conducted. Hunt-Hess grading system was used and the patients who were treated were those with grade I, II and III.

Out of 336 bleeding aneurysms, embolization was used to treat 154 of them, whereas 282 patients underwent operative treatment.

Embolization was a method of choice when dealing with aneurysms in the basilar flow, as well as in the initial segment of the carotid flow.

Better results were obtained in patients treated, either surgically or endovascularly, 72 hours after the bleeding.

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Key words: *bleeding aneurysm, endovascular occlusion, clipping aneurysm, embolization, basilar artery, middle cerebral artery*

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Introduction

A sudden headache followed by nausea and vomiting is usually caused by subarachnoid hemorrhage (SAH) caused by aneurysm rupture on the blood vessels of the brain. Intracranial aneurysms represent localized extensions of structurally altered walls of blood vessels of the brain that, with their rupture, lead to blood flowing into the subarachnoidal area. It may be accompanied by minor hemorrhage "warning laic" or severe intracranial hemorrhage, which is accompanied by loss of conscience and neurological deficits.

CT findings on the brain can show the presence of blood in the subarachnoidal area, intracerebral or intraventricular hemorrhage. The definitive confirmation of the presence of an aneurysmal change in the blood vessels of the brain is made by a CT-angio, MR-angio scan and digital subtraction angiography.

Treatment of ruptured intracranial aneurysms continues to be a challenge and requires top technique and working precision of the neurosurgeon.

In addition to operative treatment of aneurysms, the endovascular solution to these changes has also been used recently. The choice of the method of treatment depends on the clinical condition of the patient, CT findings, form and localization of an aneurysm, as well as on the condition on the carrying blood vessel of an aneurysm.

After examining the clinical status of the patient, CT findings of the brain as well as localization of an aneurysm on the blood vessel, a decision is made on how to treat the patient. The use of surgical treatment of bleeding aneurysms or endovascular occlusion is a common topic of discussion between a radiologist and a neurosurgeon. The decision on the method of treatment is made by a neurosurgeon and a neuroradiologist after looking at all the parameters for each patient individually.

Patients and methods

A prospective study of the 336 patients treated at the Clinic for Neurosurgery in Niš, where aneurysm rupture occurred, ran from January 2015 to December 2017.

All of the treated patients were diagnosed with SAH, after using brain CT, in 324 cases standard DSA was used, only in 12 cases, CTA was a main diagnostic tool for the diagnosing the location of the bleeding the aneurysm.

In 66 cases (19.64%) multiple aneurysms were found, but only in 31 (46.99%) cases we were able to manage operatively or to embolism each of the patient's aneurysms.

According to initial clinical status, we could grade patients using Hunt-Hess scale, and brain CT scan findings determined their Fisher scale.

Patients were in all of the cases initially postoperatively or postembolisation treated in Intensive care unit. All of the patients received antiedematous therapy, analgesics before and after operation or intervention. Embolised patients were on a combination of two antiplatelet medications, after the intervention, as neuroradiologist indicated.

In all of the patients, a CT brain scan control was done according to the protocol of the Clinic. In twelve of them, control DSA was done, due to the clinical signs of the vasospasm or when CTA was not much of the informative significance.

Data were presented in mean ± standard deviation, and statistical significance was defined as $p < 0.001$, in all comparisons. Statistical significance was analyzed with chi-square test for categorical variables and two sample t-tests for continuous variables. Results were presented in tables as numeric value and percentage.

Results

Out of 336 hemorrhaging aneurysms, 154 were treated with embolization, while in 182 patients were operated. There were 211 (62.80 %) female patients and 135 male (37.20%). The average age of the treated patients was 52.14 ± 16.14.

The existence of highly statistically significant differences in the method of the treatment of an aneurysm by localization was confirmed ($\chi^2 = 164.648$; $p < 0.001$). It was found that the aneurysms on the carotid artery and the basilar artery were embolized significantly more often than operated on ($\chi^2 = 52.927$; $p < 0.001$ and $\chi^2 = 61.405$; $p < 0.001$, respectively) (Table 1).

There was a significant difference in patient gradation according to the Hunt-Hesse scale relative to the method of treatment of an aneurysm ($\chi^2 = 13.51$; $p < 0.001$). Patients of the I stage were embolized more often ($\chi^2 = 14.287$; $p < 0.001$) while the patients of the III stage were operated on more often ($\chi^2 = 21.850$; $p < 0.001$) (Table 2).

A significant difference in patient gradation according to the Fisher scale and the method of treatment was determined ($\chi^2 = 69.771$; $p < 0.001$). Significantly higher number of patients who were in stage II were embolized ($\chi^2 = 72.479$; $p < 0.001$), while patients in stage III were operated on more often ($\chi^2 = 76.576$; $p < 0.001$) (Table 3).

There was no difference in treatment dynamics when performing operation when taking into account the localization of an aneurysm ($\chi^2 = 5.380$; $p = 0.250$) (Table 4).

There was no difference in treatment dynamics when performing embolization when taking into account the localization of an aneurysm ($\chi^2 = 7.758$; $p = 0.170$) (Table 5).

The outcome of the patient's treatment showed a significant difference when taking into account the dynamics of the operation ($\chi^2 = 9.980$; $p = 0.018$). A good outcome had a significantly greater number of patients who were operated on after 72 hours ($\chi^2 = 6.808$; $p = 0.009$), while the neurological deficit was more present in patients who were operated on within 72 hours ($\chi^2 = 24.809$; $p < 0.001$) (Table 6).

Regarding the outcome of the patient's treatment, it significantly differed in relation to the duration of the implementation of the embolization ($\chi^2 = 36.416$; $p < 0.001$). A good outcome had a significantly larger number of patients who were embolized after 72 hours ($\chi^2 = 32.046$; $p < 0.001$), while the neurological deficit was more present in patients embolized within 72 hours ($\chi^2 = 9.394$; $p = 0.002$) (Table 7).

Table 1. Localization and method disposing of aneurysm

Localization of aneurysm	Operatively treated	Embolized	χ^2	p
A. carotis internal	27	80	164.648	< 0.001
A. comunicans posterior	10	4		
A. cerebri media	88	9		
A. comunicans anterior	53	11		
A. pericalosa	4	5		
A. basilaris	0	45		
Total	182	154		

Table 2. Gradation of patients according to the Hant-Hesse scale

Gradus	Operated	Embolized	χ^2	p
I	77	97	27.021	< 0.001
II	73	54		
III	32	3		
IV	0	0		
V	0	0		
Total	182	154		

Table 3. Gradation of patients according to the Fissher scale

Gradus	Operated	Embolized	χ^2	p
I	0	0	69.771	< 0.001
II	111	152		
III	68	2		
IV	3	0		
Total	182	154		

Table 4. Time of operational performance

Localization aneurysm	Operated within 72h	Operated after 72h	Total	χ^2	p
A. carotis internal	10	17	27	5.380	0.250
A. comunicans posterior	7	3	10		
A. cerebri media	49	39	88		
A. comunicans anterior	28	25	53		
A. pericalosa	1	3	4		
A. basilaris	0	0	0		
Total	95	87	182		

Table 5. Time of embolization performance

Localization aneurysm	Embolization within 72h	Embolization after 72h	Total	χ^2	p
A. carotis internal	23	57	80	7.758	0.170
A. comunicans posterior	1	3	4		
A. cerebri media	2	7	9		
A. comunicans anterior	4	7	11		
A. pericalosa	0	5	5		
A. basilaris	5	40	45		
Total	35	119	154		

Tabela 6. Outcome of operatively treated patients

Outcome of the patient	Operated within 72h	Operated after 72h	Total	χ^2	p
Good	71	78	149	9.980	0.018
Neurological deficit	10	7	17		
Vegetative state	5	0	5		
Deadly	9	2	11		

Table 7. Outcome of embolized patients

Outcome of the patient	Embolized within 72h	Embolized after 72h	Total	χ^2	p
Good	21	114	135	36.416	< 0.001
Neurological deficit	7	5	12		
Vegetative state	2	0	2		
Deadly	2	0	5		
Total	35				

Discussion

The annual incidence of the hemorrhage is 6-7/100,000; during the lifetime, 15-20% of aneurysms bleed and half of the patients are below 55 years of age (1, 2, 3, 4). Recently, two methods for the treatment of hemorrhaging have been used: operational, i.e. clipping of an aneurysm or an endovascular treatment where a detachable coil (5) is applied. The method of treatment depends on the localization of an aneurysm, the CT findings of the brain and angiography, also the general condition of the patients (6).

In our study of a total of 336 bleeding aneurysms, 154 were treated with embolization, while 182 patients were operated on (Table 1). The existence of highly statistically significant differences in the method of treatment of an aneurysm by locaization was found (χ^2 = 164.648; p < 0.001). Aneurysms on the carotid and basilar arteries were embolized more often (χ^2 = 52.927; p < 0.001 and χ^2 = 61.405; p < 0.001, respectably). Many years of experience have shown that the aneurysms on the carotid artery, especially in the initial part, as well as on the basilar artery, are difficult to reach through surgery. Also, aneurysms solved by surgery, especially in the basilar portion of Wilson's hexagon, had a poor postoperative course. The introduction of endovascular techniques in the treatment of these aneurysms gave far better results. Molyneux et al. showed similar results in their studies (7, 8).

The choice of the method of treatment was influenced by the condition of the patient gradated by the Hunt-Hesse scale (Table 2), as well as the CT findings of the brain classified by using the Fisher scale (Table 3). The importance of clinical status, management, and monitoring of patients with subarachnoidal hemorrhage were demonstrated by Sodhi in his study (9). Namely, there is a significant difference in patient gradation according to the Hunt -Hesse scale in relation to the method of treatment of an aneurysm (χ^2 = 13.51; p <0.001). Patients in the stage I were often embolized (χ^2 = 14.287; p < 0.001) while patients in the stage III were operated more often (χ^2 = 21.850; p < 0.001). The general condition of the patients depends in large part on the CT findings of the brain, thus determining that there is a significant influence of the Fisher scale in relation to the method of treatment of an aneurysm (χ^2 = 69.771; p < 0.001). A significantly larger number of patients who were in the stage II were embolized (χ^2 = 72.479; p < 0.001), while stage III patients are more frequently operated on (χ^2 = 76.576; p < 0.001). Subgroup analysis in the study of Li et al (5) showed that embolization yielded better results in patients who had a good clinical status prior to the intervention. In the stage III of the Fisher scale, intracerebral hematoma was present, which could only be extracted through surgery, which explains the choice of the applied treatment method (10).

The duration of treatment of bleeding depends on the general condition of the patient, the CT findings of the brain and the angiographic findings as well. The duration of treatment of the patients largely depends on the presence of vasospasm in

the blood vessels. Certain authors divide the first week after the hemorrhage into two separate periods: from day zero (the day of hemorrhage) to 2 days (48 hours after the hemorrhage) and a period of 3 to 7 days when the vasospasm is observed (11, 12, 13, 14). Angiographically verified vasospasm is rare in the first two days after the hemorrhage, and its incidence rises after the fourth day (15).

In our study, no significant statistical difference was found when comparing the dynamics of treatment of the aneurysms and its localization (Table 4 and 5), unlike its comparation with outcome. In both the embolized patients and those operated on, much better results were achieved with interventions done after 72 hours.

A good outcome had a significantly greater number of patients who were operated on after 72 hours (χ^2 = 6.808; p = 0.009), while the neurological deficit was more present in patients who were operated on within 72 hours (χ^2 = 24.809; p < 0.001) (Table 6). In embolised patients, those who were coiled after 72h have had good outcomes (χ^2 = 32.046; p < 0.001), while the neurological deficit was more present in patients operated on within 72 hours (χ^2 = 9.394; p = 0.002)(Table 7).

Some authors consider that it is best for the intervention to be done within 72h of the bleeding because there are no spasms of blood vessels. Although accumulated evidence suggests that (16) arterial narrowing is not the only cause of clinical deterioration (17), but just a part of a comprehensive change and the impact of multiple factors (18). Our study also confirms that a better result is achieved in people treated after 72h from the primary act of hemorrhage. These facts should lead to research for a more comprehensive and more adequate theory that will not only explain the observed disagreements but also lead to the development of a specific and effective treatment strategy.

A higher percentage of fatal outcomes (9.09%) in patients treated with surgery is explained by the fact that patients treated this way were in the 3rd and 4th stage on the Fisher scale, which means that their preoperative condition was even worse than those of patients in the 1st and 2nd stages.

Conclusion

Patients whose CT findings of the brain belonged to the 3rd and 4th stage by Fisher's scale were treated using an operative method.

The decision on the treatment method is based on the clinical condition of the patient, CT findings of the brain, angiography findings, and localization of aneurysmal changes.

The application of endovascular occlusion has shown to be the method of choice for the treatment of aneurysms in the basilar basin as well as those of the initial segment of the carotid.

Better results were obtained in patients treated either surgically or endovascularly, 72 hours after the bleeding.

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IZBOR METODE LEČENJA KRVAREĆIH ANEURIZMI

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Primena operativnog lečenja krvarećih aneurizmi ili endovaskularana okluzija, česta je tema diskusije između radiologa i neurohirurga.

Cilj rada bio je da se sagledavanjem dosadašnjih rezultata okvirno zauzme stav kada koju metodu treba primeniti. Mada, uvek je bitno svakog bolesnika sagledati ponaosob u cilju donošenja prave odluke.

Vršena je prospektivna studija bolesnika lečenih na NHK u Nišu, kod kojih je došlo do rupture aneurizme, u periodu od januara 2015. do decembra 2017. godine. Korišćena je skala gradacije po Hant-Hessu i tretirani su bolesnici iz I, II i III gradusa.

Od ukupno 336 krvarećih aneurizmi, kod 154 pristupilo se embolizaciji, dok je kod 182 bolesnika primenjeno operativno lečenje.

Primena embolizacije pokazala se kao metoda izbora pri rešavanju aneurizmi u bazilarnom slivu, kao i na početnom segmentu karotidnog sliva.

Bolji rezultati dobijeni su kod bolesnika tretiranih bilo operativno ili endovaskularno, nakon 72h od hemoragije.

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Ključne reči: *krvareće aneurizme, endovaskularna okluzija, klipovanje aneurizme, bazilarna arterija, srednja cerebralna arterija*

Original article

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DIFFERENT CLINICAL PRESENTATIONS OF RECURRENT EPISODE OF MAJOR DEPRESSIVE DISORDER WITH OR WITHOUT POSTTRAUMATIC STRESS DISORDER

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The aim of this study was comparison of the severity of the recurrent episode in the group of subjects suffering from Major Depressive Disorder with comorbid Posttraumatic Stress Disorder and the group of subjects suffering only from Major Depressive Disorder. A total of 120 subjects were assessed and divided into two groups. Group D/PTSD consisted of subjects who fulfilled diagnostic criteria for recurrent episode of Major Depressive Disorder and comorbid Posttraumatic Stress Disorder. Group D/only consisted of subjects who fulfilled diagnostic criteria for Recurrent episode of Major Depressive Disorder. Assessments were performed using the following instruments: Montgomery-Asberg Depression Rating Scale, Hamilton Depression Rating Scale and Quick Inventory for Depressive Symptomatology Self -Report Version. Statistical analyses were performed using chi square and independent-samples t-test. Results suggest that recurrent episode of Major Depressive Disorder occurring in comorbidity with Posttraumatic stress disorder is more severe than the recurrent episode in the category of patients suffering from recurrent episode of Major Depressive Disorder only and that recurrent episode occurring in comorbidity with Posttraumatic stress disorder might represent a significant clinical entity.

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Key words: *stress disorders; post-traumatic; depressive disorder, major*

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Introduction

A large number of individuals are exposed to traumatic events: death, the threat of death, or serious injury to the person and others. Previously without manifested psychopathology, a number of individuals develop symptoms. The manifested group of symptoms corresponds, most often, to the diagnosis of posttraumatic stress disorder (PTSD), but also Major depressive disorder (MDD), and these two disorders were often present in comorbidity (1).

MDD is a well-known clinical entity with clearly defined therapeutic guidelines. However, in the comorbidity with PTSD, MDD becomes a confusing entity. Symptoms of MDD are clearly present, but do not respond to treatment with antidepressants and psychotherapeutic interventions, do not have the usual episodic flow and are accompanied by a significant decrease in functioning and suicidality (2, 3). Comorbid altered patterns of cognitive, affective, and vegetative responses related to PTSD make the clinical presentation of patients difficult and disturbing (4, 5). Faced with such a complex presentation, the clinician has a dilemma whether the diagnosis of MDD is reliable. Literature data show that variations in causal factors of MDD are associated with variations in the clinical picture, that individuals with comorbidity of MDD and PTSD have different neurobiological profiles than those with PTSD only (6-8).

The Aim

This study was designed to compare clinical presentations of recurrent episode of MDD, with and without posttraumatic stress disorder by using the self-rating and clinician-rated instruments for measuring depression severity.

Materials and methods

Study design

This cross-sectional study was performed at The Clinic for Mental Health, Clinical Center Niš, Serbia. The subjects, treatment-seeking, were initially diagnosed as suffering from recurrent episode of the Major Depressive Disorder (MDD) with or without PTSD, using The Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I, modified) (9). The study was performed according to Declaration of Helsinki and all procedures were approved by the Institutional Review Boards at Clinical Center Niš.

Participants

A total of 120, consecutively admitted subjects, all males, aged between 25 and 65, were included in the analysis. The subjects were included in the study if they met diagnostic criteria for recurrent episode of MDD with or without PTSD, according to Diagnostic and Statistical Manual of Mental Disorders – DSM-IV (9). The exclusion criteria were: lifetime history of psychotic disorder, severe somatic illness, alcohol dependence disorder, and epilepsy. The subjects were divided into two groups: Group D/PTSD consisted of the subjects suffering from recurrent Episode of MDD and PTSD. Group D/only consisted of the subjects suffering from a recurrent episode of MDD only. Each group consisted of 60 subjects. The assessments of the severity of the recurrent episodes were performed within 14 days upon admission.

Procedures

After having been informed, the participants completed the study procedures. Sociodemographic characteristics were assessed using the semi-structured clinical interview. The severity of the recurrent episode of MDD was assessed using clinician-rated instruments Montgomery-Asberg Depression Rating Scale (MADRS) (10) and Hamilton Depression Rating Scale (HDRS-17) (11). Self-rating was performed using Quick Inventory for Depressive Symptomatology Self -Report Version (QUIDS-SR) (12).

Measures

Montgomery-Asberg Depression Rating Scale, as a clinician- rated instrument was used to measure the presence and the severity of the MDD. The scale consists of 10 items. Each item yields score 0 to 6 on the Likert scale. The overall score determines the severity of the disorder. The higher numerical rating determines more severe disorder. The items assess somatic, cognitive, vegetative and anxious symptoms. MADRS score higher or equal to 20 is considered significant in diagnosing single episode of MDD (10).

Hamilton Depression Rating Scale, as a clinician-rated instrument was used to measure the presence and the severity of depressive symptoms. The scale consists of 17 items. Each item is scored from 0 to 2 or from 0 to 4 on the Likert scale; total scores can range from 0 to 52. The higher numerical rating determines more severe disorder. The scale predominantly assesses cognitive and vegetative symptoms, with relatively few items related to anxiety, social, motor, and mood factors (11).

Quick Inventory for Depressive Symptomatology Self-Report Version is a self-rating instrument designed for screening of depressive symptoms. It consists of 16 questions that include all Diagnostic and Statistical Manual for Mental Disorders-Fourth Edition (DSM-IV) criterion items required to diagnose MDD. The QIDS-SR utilizes equivalent weightings for each symptom item as well as clearly stated anchors for each response. The QIDS-SR 16 scoring system converts each of the 16 response items into the nine symptom criteria domains of MDD (i.e. sad mood, concentration, self-criticism, suicidal ideation, interest, energy/fatigue, sleep disturbance, decrease /increase in appetite/weight, and psychomotor agitation/retardation) and provides a total score ranging from 0 to 27 (12).

Statistical significance between groups was defined as a two-sided p value of 0.05 for all analyses, which were carried out using the SPSS software package, v15.0 (13).

Results

The analysis of demographic data showed that the average age of subjects in the Group D/PTSD was 43.05 years (SD = 7.15), and in 44.62 years (SD = 9.79) in the Group D/only. The sample was 100% Caucasian. In D/PTSD group 80% of subjects were married, 5% were single, 15% were divorced. In D/only group 61.67% of subjects were married, 15% were single, and 18.33% were divorced. In D/PTSD group, 11.67% of subjects completed elementary school, 85% completed secondary school, and 3.33% had earned master's degree. In D/only group 25% of subjects completed elementary school, 60% completed secondary school, and 15% had earned master's degree. All were employed at the time of the interview. A chi-square test reported significant difference in the educational structures of the investigated groups ($p = .007$). There were no significant differences on participant characteristics regarding age and marital status between two groups.

The independentsamples t-tests examined potential group differences on the MDRS and HDRS scores. The D/PTSD group reported higher MDRS and HDRS-17 total scores ($p < .001$) (Table 1). The total scores for D/PTSD group were also higher with respect to QIDS-SR scale ($p < .001$). (Table 2).

Table 1. MADRS and HDRS total scores for D/PTSD and D/only group

	D/PTSP					D/only					p
	X	SD	Cv	95%	CI	X	SD	Cv	95%	CI	
MADRS_sum	33.17	6.47	19.50	31.50	34.84	23.40	4.23	18.07	22.31	24.49	***.000
HDRS_sum	28.48	5.75	20.18	27.00	29.97	22.87	4.86	21.23	21.61	24.12	***.000

*** p < .001

Table 2. The values of the total scores of QIDS instrument

	D/PTSD					D/only					p
	X	SD	Cv	95%	CI	X	SD	Cv	95%	CI	
QIDS_sum	18.38	4.52	0.58	17.22	19.55	15.68	3.94	0.51	14.66	16.70	***.000

*** p < .001

Discussion

This study compared the clinical presentation of individuals with recurrent episode of Major depressive disorder, with and without PTSD. D/PTSD group was rated as having more severe depression measured by the MADRS, HDRS-17 and QIDS-SR total score.

Comparison of the severity of the recurrent episode of MDD, measured by MADRS, HDRS-17 and QIDS-SR, showed that the severity of the recurrent episode was higher in the group of subjects with PTSD than in the group of subjects suffering from MDD only as demonstrated in other studies (14). Also, from the clinical perspective, results suggest that the recurrent episode of MDD occurring in comorbidity with PTSD represents a significant clinical entity (15). High values on scales measuring the severity of a recurrent episode of MDD suggest that the clinical diagnosis of MDD in comorbidity with PTSD is adequate (16, 17).

The recurrent episode of MDD occurring in comorbidity with PTSD often remains underdiagnosed. The main reason is the overlap of the symptoms of these entities (18). Our approach suggests that the overlap of symptomatology should not be taken as a criterion that excludes, but as a criterion that confirms another diagnosis. The nature of psychopathology is essentially complex and subject to change, and what is now conceptualized as a simultaneous event of multiple disorders can be better reformulated as the complexity of more psychiatric conditions, and as the fact that the increase in complexity is an obvious predictor of more severe disorder, of disability and of use of the health services (8).

Furthermore, the reason for underdiagnoses of the recurrent episode of MDD comorbid to PTSD may be that depressive symptomatology is often overshadowed with the flamboyant symptomatology of posttraumatic stress disorder and therefore remains unrecognized. The nature of intrusive symptomatology implies that the traumatic contents persistently intrude into the consciousness of individuals. Intrusive thoughts, features of nightmares, intrusions of morbid images and episodes of reexperiencing of events, increased psychological and physiological reactivity, high tension, irritability, insomnia, cognitive dysfunction, symptoms of avoidance and affective restriction, manifested in detachment, alienation, strangeness and the sense of foreshortened future, all suggest that the dominant complaints of the person seeking treatment relate to the symptoms of posttraumatic stress disorder. The disturbing nature of a real-life traumatic event associated with simultaneous existence of intrusive cognitive contents and high vegetative hyperarousal implies the absence of thinking activity related to the analysis of the experience, of reflecting, perceiving and analyzing thoughts and affects (19, 20).

Also, the nature of the altered affective reactivity in the posttraumatic stress disorder is that it interferes with the reporting of depressive symptoms by patients. In posttraumatic stress disorder, deverbalization, dissociation, and resomatization of affects occur (21). Predominant feature is the loss of the signaling function of emotions (22). The emotions lose the capacity to serve as signals and no longer lead to organized and adaptive action, but lead to disorganized patterns of response, to flight or fight reactions. (23).

Finally, limitations of the current study should be addressed. First, we recruited a modest number of participants for this study. Second, due to the nature of the PTSD screening, the severity of PTSD was unable to be determined, hindering examination of how PTSD severity affects MDD severity. With these limitations in mind, the study has several strengths. Specifically, structured clinical interviews (i.e., the MADRS, the HDRS-17, and QIDS-SR) were utilized in the assessment of recurrent episode of MDD. Additionally, all participants in the sample met diagnostic criteria for MDD providing a homogeneous sample for the comparison of a subject with and without PTSD.

Conclusion

The obtained results suggest the validity of the diagnostic construct of comorbidity of MDD and PTSD. Our data on the severity of current recurrent episodes of MDD in subjects suffering from PTSD indicate the importance of MDD diagnostics and the necessity of determining different pharmacological and psychotherapeutic treatment approaches.

Competing interests

The authors declare no competing interests.

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KLINIČKA PREZENTACIJA REKURENTNE EPIZODE VELIKOG DEPRESIVNOG POREMEĆAJA SA POSTTRAUMATSKIM STRESNIM POREMEĆAJEM ILI BEZ NJEGA

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Cilj rada je upoređivanje težine aktuelne rekurentne epizode u grupi subjekata koji pate od velikog depresivnog poremećaja i komorbidnog posttraumatskog stresnog poremećaja sa grupom subjekata koji pate samo od velikog depresivnog poremećaja. Ukupno 120 subjekata sagledano je i raspoređeno u dve grupe. U grupu D/PTSP raspoređeni su subjekti koji su ispunjavali dijagnostičke kriterijume za rekurentnu epizodu velikog depresivnog poremećaja i posttraumatski stresni poremećaj. U grupu D/only raspoređeni su subjekti koji su ispunjavali dijagnostičke kriterijume za rekurentnu epizodu velikog depresivnog poremećaja. Procena težine rekurentne epizode vršena je korišćenjem instrumenata: Montgomery-Asberg skale za depresiju, Hamiltonove skale za depresiju i inventara depresivne simptomatologije. Podaci su analizirani Studentovim t-testom nezavisnih uzoraka. Rezultati su pokazali da je težina aktuelne rekurentne epizode velikog depresivnog poremećaja bila veća kod subjekata koji su patili od komorbidnog posttraumatskog stresnog poremećaja u odnosu na subjekte koji su patili samo od rekurentne epizode velikog depresivnog poremećaja i potvrđuju da predstavlja značajan klinički entitet.

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Ključne reči: *stresni poremećaj, posttraumatski poremećaj, veliki depresivni poremećaj*

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PREDICTORS OF THERAPY RESPONSE AND EARLY RECURRENCE IN PATIENTS WITH POTENTIALLY RESECTABLE COLORECTAL LIVER METASTASES TREATED WITH BEVACIZUMAB AND FOLFOX4 AS A CONVERSION THERAPY

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The aim of this study was to define predictive factors for the therapy response and early recurrence after hepatectomy in patients that received conversion therapy FOLFOX4 and bevacizumab for colorectal liver metastases.

This observational retrospective single center analysis included sixty-five patients treated with bevacizumab and FOLFOX4 regimen for potentially resectable colorectal liver metastases. Patients were divided in groups based on objective therapeutic response. Groups with early (≤ 3 months) and late recurrence (≥ 12 months) after hepatectomy were selected. Disease characteristics among groups were compared as well as univariate and multivariate analysis.

Independent risk factor for the lack of therapy response was rectal localization (OR 3.86 [95% CI 1.31-11.34]; p = 0.014). Left colon cancer was independent protective factor for the response absence (OR 0.205 [95% CI 0.05-0.80]; p = 0.022). Independent predictive factors for early recurrence were synchronous liver disease (OR 18 [95%CI 2.47-131.28]; p = 0.004) and the number of metastases (OR 2.42 [95% CI 1.14-5.01]; p = 0.021). In multivariate model only synchronous liver metastases had statistical significance (OR 13.79 [95% CI 1.54-123.77]; p = 0.019).

Left colon cancer was predictor of response to therapy with bevacizumab and FOLFOX4 and rectal localization was indicative of response absence. Independent risk factors for early recurrence were the number of metastases and synchronous liver involvement.

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Key words: *colorectal cancer, metastases, liver, predictors*

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Introduction

Liver metastases are present in 20-25% of patients at the time of colorectal cancer (CRC) diagnosis and in up to 50% of patients during the disease course (1, 2), making this organ the most common site for CRC dissemination. The aim in planning treatment of colorectal cancer liver metastases (CRLM) should be hepatectomy, which in combination with chemotherapy provides 5-year overall survival (OS) in up to 67% of patients and the chance for long term disease free survival and even cure (3-5). Despite curative (R0) metastatic liver resection, up to 70% of the patients will have a recurrence, in most cases in the first 2 years and predominantly in the liver (6-8).

The fact that in 85% of patients CRLM are not resectable at the presentation (9) sets the stage for conversion therapy, with the goal of downsizing metastatic deposits so that resection could be performed. Application of conversion therapy with subsequent surgery provides better long term outcome than systemic therapy only (10). In order to achieve the best response and resectability, the most active therapeutic regimen should be used (chemotherapeutic doublet or even triplet, with the addition of targeted agent, bevacizumab or cetuximab in KRAS wild type) (11). Response to preoperative therapy correlates with recurrence free survival (12) and overall survival (14) in patients with initially resecta-

ble CRLM. In the setting of unresectable liver disease, response to chemotherapy strongly correlates with resection rates (13-15). However, this response is hard to predict.

Long-term survival in patients with surgically removed CRLM is slightly shorter for patients that undergo conversion therapy comparing to those with initially resectable metastases (16). These patients are at higher risk of early recurrence, in case of which the purpose of surgical treatment might be questioned. There were several attempts to define predictive models for the outcome in patients with resected CRLM, but they were not applicable because of mutual diversity (17). For patients with initially unresectable liver metastases there is a strong need for predictors of efficacy of applied therapeutic modalities in order to tailor medical and surgical treatment accordingly.

The aim of this retrospective single center analysis was to access clinicopathological disease characteristics in patients receiving conversion therapy FOLFOX4 and bevacizumab for unresectable CRLM and define predictive factors for therapy response and early recurrence after hepatectomy.

Materials and methods

From 141 patients medically treated for CRLM at the Clinic of Oncology, Clinical Center Niš, Serbia, in the period from 2010 to 2015, we selected patients with initially unresectable liver-only disease with potential resectability, which have received conversion chemotherapy. Patients with treatment complications leading to discontinuation of therapy, incomplete liver resection (R2 resection) or a two-stage hepatectomy, operative mortality (non-cancer-related 90-day mortality), follow up shorter than 6 months and incomplete medical record did not participate in the analysis. A total number of included patients was 65.

Performance status of patients according to Eastern Cooperative Oncology Group (ECOG) was 0 – 1. Liver-only metastatic disease was diagnosed by contrast enhanced multislice computed tomography (MSCT) examination of thorax and abdomen. Potential resectability was defined after MSCT scan analysis by an experienced hepatobilliary surgical team. The criteria for unresectability were: not possible upfront R0/R1 resection of all hepatic lesions, < 30% estimated residual liver volume after resection or metastases in contact with major vessels of the remaining liver. After the regular initial laboratory tests and clinical examination, all patients received chemotherapy (FOLFOX4: oxaliplatin 85 mg/m^2 on day 1, leucovorin 200 mg/m^2, 5-FU 400 mg/m^2 bolus and 600 mg/m^2 22-hour continuous intravenous infusion on days 1 and 2; repeated every 2 weeks) and bevacizumab (5mg/m^2; repeated every 2 weeks). Assessment of therapeutic response according to Response Evaluation Criteria in Solid Tumours (RECIST) and resectability were performed after 4 cycles of chemotherapy on abdominal MSCT scan. Patients with achieved resectability were subjected to surgery and closely followed afterwards.

The following data from the medical records of patients were selected: age, gender, localization and histopathology reports of primary tumor, synchronous (defined as liver involvement at the time of diagnosis or at the surgery of the primary tumor) or metachronous liver disease, number of metastases, number of received chemotherapy cycles, response to therapy and progression free survival (PFS) after hepatectomy.

The group with therapeutic response included patients with complete (CR) or partial response (PR), and the group without response consisted of patients with stable (SD) or progressive (PD) disease. According to the disease free survival after liver resection we selected patients with early recurrence (PFS ≤ 3 months) and late recurrence (PFS ≥ 12 months). Disease characteristics among defined groups were compared.

Statistical analysis was performed using SPSS statistical software, version 16.0 for Windows. Descriptive statistics was used for qualitative and quantitative assessment of the results: absolute numbers, relative numbers (%), mean value ( ), standard deviation (SD), and median value. The distribution pattern was assessed with Kolmogorov Smirnov test. Independent two-sided t-test (t), nonparametric Mann-Whitney U test, Kruskall Wallace or chi-square (χ^2) test were applied to compare variables between the groups, where appropriate. Univariate and multivariate logistic regression analysis was used to identify predictors of therapy response and early recurrence. P values of < 0.05 were considered statistically significant.

Results

From total number of 65 patients, 41 (63.1%) were men and 24 (36.9%) women. The average age of the patients was 59.27 ± 9.69 years, the youngest, aged 29, and the oldest, aged 81. The age difference between men and women was not statistically significant (59.31 ± 11.17 vs. 59.21 ± 6.86; p = 0.969).

Primary tumour was localized in colon in 31 (47.7%) patients: ascending colon/hepatic flexure in 9 (29%) patients, transversal colon in 3 (9.6%) patients and lienal flexure/descending/sigmoid colon in 19 (61.4%) patients; 34 (52.3%) patients had rectal primary. Mucinous adenocarcinoma was diagnosed in 7 (10.8%) patients.

Synchronous and metachronous metastatic liver disease was present in 37 (56.9%) and 28 (43.1%) patients, respectively. In 27 (41.5%) patients 4 or more metastases were registered.

The number of received chemotherapy cycles was ranging from 4 to 12, mean cycle number was 5.49 ± 2.24. Objective therapeutic response (CR/PR) was present in 40 (61.5%) patients, while absent in 25 (38.5%) patients (PD/SD). Resectability was achieved in 33 (50.8%) patients. Postoperative chemotherapy was applied to 10 (30.3%) patients.

In the group of patients with liver resection, early recurrence was verified in 14 (42.4%), and late recurrence in 12 (36.4%) patients. Seven

patients (21.2%) had progression free interval from 4 - 11 months. Median progression free survival was 6.5 [95% CI 7.49-13.71] months, ranging from 3 to 38 months. Liver was the site of relapse in 23 (69.7%) patients.

Predictors of therapeutic response

Groups with and without therapy response were balanced in terms of age (p = 0.389) and gender (p = 0.902). The number of received chemotherapy cycles did not differ between groups (p = 0.670) (Table 1).

Significant difference between patients with and without therapeutic response was found in localization of primary tumour (p = 0.027). Independant risk factor for the lack of objective response was rectal localisation of the primary (OR 3.86 [95% CI 1.31-11.34]; p = 0.014). Left colon cancer (lienal flexure, descending and sigmoid colon) was protective factor for response absence (OR 0.205 [95% CI 0.05-0.80]; p = 0.022) meaning that it was predictive of therapy response. Multivariate analysis was performed with variables which proved to be statistically independent factors. Neither of two variables brought statistically significant contribution (Table 1).

Predictors of early recurrence

In the group with early recurrence synchronous liver disease was significantly more frequent (p = 0.002) and these patients had significantly more metastases (p = 0.013) compared to the late recurrence group. Independent risk factors for early relapse were synchronous CRLM (OR 18 [95% CI 2.47-131.28]; p = 0.004) and the number of metastases (OR 2.42 [95% CI 1.14-5.01]; p = 0.021) (Table 2).

Multivariate model included variables that appeared to be statistically independent factors. Unique statistically significant contribution to the model was provided only by synchronous CRLM (OR 13.79 [95% CI 1.54-123.77]; p = 0.019) (Table 2).

Table 1. Demographic and clinicopathologic factors according to objective therapeutic response

		N = 40	N = 25	p	Univariate analysis		Multivariate analysis	
Factor		CR/PR	SD/PD		OR(95%CI)	p	OR(95%CI	p
Gender n (%)	Men	25 (62.5)	16 (64.0)	0.902				
	Women	15 (37.5)	9 (36.0)					
Age $\bar{x}$±SD		60.07 ± 8.85	57.87 ± 11.06	0.389				
Localization n (%)	Ascending colon and hepatic fl.	5 (12.5)	4 (16.0)	0.027	1.33 (0.32-5.52)	0.629		
	Transversal colon	3 (7.5)	0 (0.0)		0.001 (0.01-0.02)	0.999		
	Lienal fl., descending and sigmoid colon	16 (40.0)	3 (12.0)		0.205 (0.05-0.80)	0.022	0.37 (0.07-2.1)	0.264
	Rectum	16 (40.0)	18 (72.0)		3.86 (1.31-11.34)	0.014	2.25 (0.57-8.91)	0.248
Time of detection n (%)	Synchronous	22 (55.0)	15 (60.0)	0.692				
	Metachronous	18 (45.0)	10 (40.0)					
Number of metastases $\bar{x}$±SD		3.22 ± 1,46	3.40 ± 1.58	0.705				
Mucinous histology n (%)		3 (7.5)	4 (16.0)	0.282				
Number of cycles $\bar{x}$±SD		5.55 ± 2.07	5.40 ± 2.50	0.670				

Table 2. Demographic and clinicopathologic factors according to time to recurrence

Factor		N = 14 PFS≤3 months	N = 12 PFS≥12 months	p	Univariate analysis OR(95%CI)	Univariate analysis p	Multivariate analysis OR(95%CI)	Multivariate analysis p
Gender n (%)	Men	6 (42.8)	7 (58.3)	0.578				
	Women	8 (57.2)	5 (41.7)					
Age $\bar{x}$±SD		58.14 ± 9.24	63.77 ± 8.92	0.12				
Localization n (%)	Ascending colon and hepatic fl.	3 (21.4)	0 (0.0)	0.276				
	Transversal colon	0 (0.0)	1 (8.3)					
	Splenic fl., descending and sigmoid colon	5 (35.7)	5 (41.7)					
	Rectum	6 (42.9)	6 (50.0)					
Time of detection n (%)	Synchronous	12 (85.7)	3 (25.0)	0.002	18 (2.47-131.28)	0.004	13.79 (1.54-123.77)	0.019
	Metachronous	2 (14.3)	9 (75.0)					
Number of metastases $\bar{x}$±SD		3.78 ± 1,36	2.33 ± 1.15	0.013	2.42 (1.14-5.01)	0.021	2.17 (0.89-5.31)	0.088
Mucinous histology n (%)		2 (14.3)	0 (0.0)	0.173				
Number of cycles $\bar{x}$±SD		5.86 ± 2.35	5.41 ± 2.10	0.347				

Discussion

CRC is a heterogeneous disease which is reflected by different response to therapy and variable outcomes. It has recently been reported that localization of the primary tumour is independent prognostic factor in metastatic CRC (18). Patients with metastatic right colon cancer (RCC) have significantly lower OS compared to the patents with localization of the primary tumour in left colon (LCC) (18-21). Additionally, different effects of chemotherapy regimens and targeted agents have been noticed between those two entities. LCC patients have more benefit, in terms of PFS and OS, when treated with combination of irinothecan and 5-fluorouracyl compared to RCC patients, while the combination of irinotecan and oxaliplatin has similar effects for both localizations (21). It is widely reported that LCC patients benefit more from cetuximab (22-23) and bevacizumab (24, 25) therapy.

Significant differences at the molecular level exist between RCC and LCC (26). According to recent consensus, four biologically homogenous subtypes of CRC were defined, based on molecular and genetic characteristics: MSI immune (hypermutated, microsatellite unstable, with strong immune activation); Canonical (epithelial, chromosomally unstable, with marked WNT and MYC signalling); Metabolic (epithelial, with evident metabolic dysregulation); and Mesenchymal (prominent TGF-β activation, stromal invasion and angiogenesis) (27).

In present study, LCC was identified as predictor of objective therapy response, since it lowers the risk for PD/SD (OR 0.205 [95% CI 0.05-0.80]; p = 0.022). Conversely, rectal primary presents negative predictive factor for response to bevacizumab and FOLFOX4 therapy, since it represents a risk factor for PD/SD (OR 3.86 [95% CI 1.31-11.34]; p = 0.014). It is probable that some molecular features of the tumor that interfere with therapy response can be linked to tumor localization, which may be the reason for all these apparent differences. For the confirmation of this hypothesis, further molecular studies should be conducted. For the time being, simple information about localization of the primary might be helpful when it comes to treatment. However, neither of these factors showed statistical significance in multivariate analysis.

Mucinous adenocarcinoma accounts for 10-15% of colorectal cancers (28). Prognostic effects of this specific histopathological type are not clearly stated, and the results are ambiguous. In comparison to adenocarcinoma, mucinous type is associated with young age, advance tumor stage, females, as

well as with MSI ad activating mutations of BRAF gene (29). These genetic features are predictive of poor outcome. In this study, 10.8% of patients had mucinous adenocarcinoma, and we could not associate this factor with the response to bevacizumab and FOLFOX4 therapy (p = 0.282).

With the development of powerful chemotherapeutics and new surgical techniques, indications for hepatectomy in CRLM are rising (30). Thus, a problem with early recurrence is emerging. In case of early relapse, the purpose of hepatectomy is questioned, and this standard approach might be challenged. It is widely reported that early recurrence after CRLM resection is a poor prognostic factor of survival (30-32).

Two recent large observational studies considered predictive factors for early recurrence in patients with surgical resection of CRLM. Imai et al. (30) defined early recurrence within 8 months, based on the minimum p value for survival after initial recurrence. In the subset of patients who received preoperative chemotherapy, 43% had initially unresectable liver disease. Early recurrence was present in 45% of patients, and independent predictive factors were age ≤ 57 years, more than 1 chemotherapy line, progression on last line of chemotherapy and CA 19-9 levels > 60 U/ml. In the study of Vigano et al. (32), relapse within 6 months after liver resection was defined as early. Independent risk factors for early recurrence in the subset of patients that received preoperative chemotherapy were T3-4 primary tumor, associated radiofrequent ablation, no objective response to chemotherapy and the lack of postoperative chemotherapy. Both studies identified number of metastases as a predictor of early relapse, the first with more than 3 metastases at the diagnosis (32), the later with more than 3 metastases at hepatectomy (30). Synchronous CRLM was independent predictive factor for early relapse for the whole study population (32), but not in the preoperatively treated patients (30, 32). However, neither of these two studies separately analyzed the predictors of early relapse for the patients with initially unresectable CRLM.

In the present study, the limit for the early recurrence has been set to be 3 months while for these patients surgery seems to be of minimal benefit. Independent predictive factors for early recurrence compared to late recurrence were the number of metastases in univariate (OR 2.42 [95% CI 1.14-5.01]; p = 0.021), and synchronous liver disease in univariate (OR 18 [95% CI 2.47-131.28]; p = 0.004) as well as multivariate analysis (OR 13.79 [95%CI 1.54-123.77]; p = 0.019). Patients with synchronous CRLM and large tumor burden have very aggressive disease, so early recurrence after surgery is more common. In such cases, liver resection has negligible contribution to PFS, and if not avoided, should be supported with different means of systemic treatment.

The shortcomings of this study were low number of patients, retrospective nature and lesser number of examined factors. However, this is, according to our knowledge, the first report of predictive factors in the setting of initially unresectable CRLM, and predictors of chemotherapy response were also assessed as an important part of these patients' treatment.

When it comes to treatment of unresectable CRLM, assessing the predictive factors for response to conversion therapy may be helpful in choosing the most potent combination therapy for CRLM downsizing or redefining the goal of treatment (palliative or curative). Knowing predictors of early relapse might be of assistance in selecting the patients with real benefit from liver surgery and adapting postoperative chemotherapy accordingly. The cooperation between medical and surgical oncologists is essential in this patient subgroup in order to get the most of the multimodality treatment.

Conclusion

This study identified localization of primary tumor in the rectum as an independent risk factor for the lack of response, and left colon cancer as a predictor of response to therapy with bevacizumab and FOLFOX4 in univariate analysis. Independent risk factors for early recurrence were the number of metastases in univariate and synchronous CRLM in univariate and multivariate analysis.

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PREDIKTORI TERAPIJSKOG ODGOVORA I RANOG RELAPSA KOD PACIJENATA SA POTENCIJALNO RESEKTABILNIM METASTAZAMA KOLOREKTALNOG KARCINOMA U JETRI LEČENIH KONVERZIONOM TERAPIJOM BEVACIZUMAB I FOLFOX4

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Cilj rada je definisati prediktivne faktore za terapijski odgovor i rani relaps nakon resekcije jetre kod bolesnika koji su primili preoperativnu konverzionu terapiju FOLFOX4 i bevacizumab zbog metastaza kolorektalnog karcinoma u jetri.

Ova opservaciona retrospektivna analiza uključila je šezdeset pet bolesnika koji su lečeni FOLFOX4 i bevacizumab režimom, usled potencijalno resektabilnih metastaza kolorektalnog karcinoma u jetri. Bolesnici su podeljeni u grupe na osnovu objektivnog terapijskog odgovora. Izdvojene su grupe sa ranim (≤ 3 meseca) i kasnim (≥ 12 meseci) relapsom nakon resekcije jetre. Među ovim grupama analizirane su karakteristike bolesti i rađene su univarijantna i multivarijantna analiza.

Nezavisni faktor rizika za izostanak terapijskog odgovora bila je lokalizacija tumora na rektumu (OR 3,86 [95% CI 1,31-11,34]; p = 0,014). Lokalizacija tumora na levom kolonu bila je nezavisni protektivni faktor za izostanak terapijskog odgovora (OR 0,205 [95% CI 0,05-0,80]; p = 0,022). Nezavisni prediktori za rani relaps bili su sinhrone jetrene metastaze (OR 18 [95% CI 2,47-131,28]; p = 0,004) i broj metastaza (OR 2,42 [95 % CI 1,14-5,01]; p = 0,021). U multivarijantnom modelu statističku značajnost imala je samo sinhrona bolest jetre (OR 13,79 [95% CI 1,54-123,77]; p = 0,019).

Lokalizacija primarnog tumora na levom kolonu prediktor je odgovora na terapiju, a karcinom rektuma povezan je sa odsustvom odgovora na terapiju bevacizumab i FOLFOX4. Nezavisni faktori rizika za rani relaps nakon resekcije jetre su veći broj metastaza i sinhrono zahvatanje jetre.

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Ključne reči: *kolorektalni karcinom, metastaze, jetra, prediktori*

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PAROTID GLAND METASTASES OF OTHER PRIMARY TUMORS - A 10 YEAR RETROSPECTIVE STUDY

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Metastatic cancers of the parotid are very rear and hard to diagnose neoplasms. They can be classified into two major groups: tumors with supraclavicular and inflaclavicular primary localization. Squamous cell carcinoma of the skin is the most common histological type found; it is followed by melanoma, Merkel cell carcinoma and adenocarcinoma of the lungs. We analyzed 420 patients that were diagnosed with parotid gland tumors at the department of Maxillofacial surgery, Clinic of Dentistry in Niš, from 2007 to 2017. In 121 patients pathohistological findings showed malignant tumors out of which 21 were metastatic. Radical parotidectomy with adjuvant radiotherapy of the head and neck region was the most common type of treatment. Neck dissection was performed in all of the patients but the extent of it depended on the clinical findings. Lymph nodes were negative for the metastatic disease in only 4 patients. Survival rate is very different among patients ranging from 8 to 138 months.

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Key words: *parotid tumors, metastases, therapy*

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Introduction

Salivary gland tumors occur in about 3% of all head and neck tumors. The most common of them are parotid gland tumors; they appear in almost 80% (1). Parotid cancers are uncommon and they develop in less than 20% of all parotid tumors, but they are very, histologically and biologically, diverse group of neoplasms (1, 2). There are two major groups of parotid gland cancers; primary salivary gland tumors and metastatic cancers. Metastatic cancers can develop by direct extension through neighboring tissues, lymphatic channels, or less commonly, through haematogenous spread (3). The site of the primary tumor is usually the skin of the head and neck, thus being squamous cell carcinoma or melanoma (3). Infraclavicular or noncutaneous cancers of the head and neck are far less often found (4, 5).

Clinical findings in patients with metastatic tumors of parotid gland may vary from severe where facial nerve is also infiltrated, to mild, benign-like tumors.

The aim of this study was to show clinical and pathohistological (ph) findings in patients with metastatic cancers of the parotid as well as therapeutic procedures used and the survival rate in these patients treated in our clinic.

The study analyzed 420 parotidectomies performed at the Department of Maxillofacial surgery, Clinic of Dentistry in Niš during the period from 2007 to 2017. All patient data were obtained by hand searching of patient records. Data were maintained in an anonymised database and analyzed using a statistical software package. Medical information that was analyzed consisted of sex and age of patients, primary localization of tumors, treatment, pathohistological findings and treatment of metastases.

Results

Total number of parotidectomies was 420. Malignant tumors were confirmed in 121 (28.8%) cases. Including criteria: metastatic tumor of the parotid gland. Excluding criteria: primary cancer of parotid, lymphoma, metastatic tumors with an unknown origin. These criteria were met by 21 (5%) cases, 10 male and 11 female patients, 55 to 87 years of age.

Most commonly, primary tumors were located in the head and neck (95.24%). Squamous cell carcinoma (SCC) was seen in 13 cases, five patients had melanoma and Merkel cell carcinoma was re-

corded in two cases. There was only one tumor that was primary located below clavicle, it was adenocarcinoma of the lungs. (Table 1)

In patients with SCC, there were 4 cases where the pathohistological examination of the neck lymph nodes showed no traces of the disease in the neck which is why these patients received radiotherapy only in the parotid region. Surgical treatment and adjuvant radiotherapy are shown in Tables 2 and 3.

Survival rate is very diverse, ranging from 8 to 135 months (Table 4).

Table 1. Sites of tumors that metastasized to the parotid gland

Site	No. of cases	(%)
Cutaneous, head and neck	**20**	**95.24%**
• Squamous cell carcinoma (SCC)	13	(61.9%)
• Melanoma	5	(23.8%)
• Merkel cell carcinoma (MCC)	2	(9.54%)
Infraclavicular	**1**	**4.76%**
• Lung, adenocarcinoma	1	(4.76%)

Table 2. Extent of surgery for the Parotid and the Neck

	Parotidectomy		**Neck dissection**	**Facial nerve resection**	
	Radical	*Superficial*		*Partial*	*Complete*
SCC	9	4	13	2	2
Melanoma	5	0	5	0	0
MCC	2	0	2	0	0
Adenocarcinoma	1	0	1	0	0
Total	**18 (85.7%)**	**4 (14.3%)**	**21 (100%)**	**2 (9.5%)**	**2 (9.5%)**

Table 3. Adjuvant radiotherapy

	Parotid region only	**Parotid region with the neck**
SCC	4	9
Melanoma	0	5
MCC	0	2
Adenocarcinoma	0	1
Total	**4 (19.05%)**	**17 (80.95%)**

Table 4. Survival rate of patients with metastatic tumors of the parotid (in months)

Type of the cancer	Min	Max	Alive
SCC	8	135	4 (30 month follow up)
Melanoma	8	76	2 (31 month follow up)
MCC	10	20	1 (20 month follow up)
Adenocarcinoma	13	13	0

Discussion

The majority of tumors metastasizing into the parotid gland originate from the skin of the head and neck (6). In our study, that is the case in 95.24 percent. It is no surprise that this happens considering the lymph drainage paths of the skin of the face, scalp and neck. The literature shows small number of cases where the primary tumor was located below clavicle (5-7), which is the same in our study, where only one infraclavicular localization was found. Squamous cell carcinoma is the most common histological type (2, 3, 8) and that is the case in our study in 61.9%. In therapeutic terms, there is no difference whether metastases are located in the parotid parenchyma or in the parotid lymph nodes (9). Supraclavicular tumors metastasize in the parotid region usually through lymphogenous routes or per continuitatem, depending on the localization unlike the infraclavicular tumors which usually spread through haematogenous routes. There are even discussions about metastases in the parotid lymph nodes being an expression of lymphogenous metastases whereas parenchymal metastasis is an indication for haematogenous metastasis (10).

Before considering treatment for patients with parotid cancers, it is very important to make a clear distinction between primary and metastatic disease. Parotid metastases in some cases are the first clinical manifestation of a previously unknown tumor (10). Primary tumors can be treated less offensive, with or without neck dissection or adjuvant radiotherapy whereas metastatic cancers demand radical approach (11). In our study, radical parotidectomy was made in 80.95% and superficial parotidectomy (SP) in 19.05%. We agree with the opinion of Malata (12) that SP is an adequate solution when cancer has spread only in the superficial lobe of the gland. McKean et al. (13) investigated parotid glands on cadavers and they have come to conclusion that 2-22 lymph nodes of the parotid are located in the superficial lobe whereas only 0-4 are located in the deep lobe. The reason for 80.95% of radical parotidectomies in our case is that the patients have reached out to us when the cancer has already spread tremendously. All of the patients had surgical treatments that included neck dissection, in five cases it was selective neck dissection (SND) and the rest was modified radical neck dissection (MRND). SND was performed in patients that had no clinical signs of enlarged lymph nodes (we had no possibility of making CT and NMR scans routinely). Our decision to do the neck dissection in patients whose ultrasound of the lymph nodes showed no evidence of the disease is in compliance with Jackson and Ballantine (14) as well as Marks et al. (15) They have made confirmation of the metastases in the neck after ph examination in 24% of the cases that had no clinical signs of the disease. We did SND in five patients with no clinical signs of the disease and for one patient (20%) the ph confirmation came back positive. In 2 cases or 9.5% facial nerve had to be sacrificed and in the same number of cases we had to make a partial nerve resection. Studies have shown that adjuvant radiotherapy to the parotid region is given to 78% of patients (10). All of our patients received postoperative radiotherapy, in 19.05% it was just in the parotid region, but the majority (80.95%) received radiotherapy in the parotid region as well as the neck because of the positive lymph nodes.

Survival rate depends on the number of factors among which are primary location and the staging of the tumor, general condition of the patient, histological type etc., but the best prognosis is for the patients with the SCC that is caught early.

High mortality rate shows just how aggressive these tumors are and is in compliance with the literature (2, 6, 10).

Conclusion

Metastatic tumors of the parotid were confirmed in 5% of all parotid tumors. In 61.9% it was squamous cell carcinoma originating from the skin of the head and neck. There was only one case of non-cutaneous and infraclavicular localization, adenocarcinoma of the lungs, that metastasized to the parotid. Radical parotidectomy was performed in 85.7%, but neck dissection and adjuvant radiotherapy were part of the treatment in every patient. In 81% of patients, complete preservation of the facial nerve was achieved.

Establishing the right diagnosis is the essential part of managing this type of disease. Radical approach is necessary when dealing with metastatic cancers, but we should always keep in mind that we do not treat tumors, we treat patients. Palliative care is sometimes the only possible treatment that we can provide and it should also be considered when proposing therapy to our patients.

Conflict of interest

Authors declare no conflict of interest.

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METASTATSKI TUMORI PAROTIDNE ŽLEZDE – DESETOGODIŠNJA RETROSPEKTIVNA STUDIJA

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Maligni tumori parotidne žlezde se mogu razviti kao primarni tumori ili nastati metastaziranjem malignih neoplazmi u limfne noduse parotidne regije. Prema poreklu primarne lezije, metastatski tumori parotidne žlezde se dele na one koji nastaju od tumora lokalizovanih supraklavikularno i infraklavikularno. Supraklavikularni tumori dominiraju kao uzročnici metastaza u parotidnoj žlezdi, a patohistološki je najčešći planocelularni karcinom kože glave i vrata, zatim melanom, karcinom Merkelovih ćelija, dok infraklavikularno najčešće metastazira adenokarcinom pluća. U radu je analizirano 420 bolesnika koji su lečeni od tumora parotidne žlezde u Službi za maksilofacijalnu hirurgiju Klinike za stomatologiju u Nišu u periodu od 2007. godine do 2017. godine. Kod 121 bolesnika je potvrđen maligni karakter bolesti, a za potrebe studije je analizirano njih 21, kod kojih je postavljena dijagnoza metastatske maligne bolesti. Najveći broj bolesnika je lečen radikalnom parotidektomijom, a nakon toga i zračnom terapijom. Kod svih bolesnika je rađena disekcija vrata, ali se obim disekcije razlikovao u zavisnosti od kliničke slike. Kod samo četiri bolesnika nije dokazano postojanje metastatske bolesti u limfnim nodusima. Vreme preživljavanja se kreće u opsegu od 8 meseci do 138 meseci.

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Ključne reči: *tumori parotidne žlezde, metastaze, terapija*

Original article

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MULTIMODAL IMAGING IN PATIENTS WITH MULTIFOCAL CHOROIDITIS WITH OUTER RETINAL/CHORIOCAPILLARIS-BASED PATHOLOGY

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The purpose of this study was to present different multimodal imaging technics in patients with multifocal choroiditis, punctate inner choroidopathy (PIC), birdshot chorioretinopathy (BCR), acute posterior multifocal placid pigment epitheliopathy (AMPPE), multiple evanescent white dot syndrome (MEWDS) and serpinginous choroiditis (SPC) and to estimate their diagnostic and prognostic value.

The study was performed at the Clinic for Eye Disease, Clinical Centre Niš, Serbia. During the period of six years, ten patients with diagnosed multifocal chorioretinitis were examined. Standard ophthalmological examination in all patients included: visual acuity evaluation, slit lamp biomicroscopy, applanation tonometry, indirect ophthalmoscopy, photodocumentation and fluorescein angiography, optical coherence tomography (OCT) and ultrasonography in indicated cases. Standard laboratory examination, immunological examination and HLA typing were performed as well.

Visual acuity was well preserved in all cases except in SPC. The disease was bilateral, in cases of SPC, AMPPE and PIC, and recurrence was present in cases of BCR and PIC. SPC and PIC were characterized by chronic evolution.

The common features of all presented cases of multifocal choroiditis entities were: minimal lesions at the initial stage of disease, multifocal or white yellow multiple lesions, during the progression of disease and consequent degeneration of retinal tissue and choroid proven by OCT and FA.

OCT is important for diagnose, differential diagnose and follow-up.

The obtained results are comparable with the existing studies that follow individual response variability, which has not yet been fully elucidated.

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Key words: *chorioretinitis, diagnosis, imaging*

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Introduction

Multifocal chorioretinitis presents a group of rare disorders which primary pathologic process occurs at or near the level of retinal pigment epithelium (RPE) with or without photoreceptor outer segment and choriocapillaris involvement (1, 2, 3). The pathophysiology presumes either vasculitic obstruction of the choriocapillaris with secondary infarction of the overlying RPE or, possible, immunologic response on RPE itself (5, 6, 7). Many clinical features of these individual entities overlap, causing confusion (8, 9).

The aim of this study was to present different multimodal imaging technics such as, color fundus photography (FF), fluorescein angiography (FA), high definition ocular tomography HD OCT, ultrasonography in patients with multifocal choroiditis with outer retinal/choroidal based pathology-punctate inner choroidopathy (PIC), birdshot chorioretinopathy (BCR), acute posterior multifocal placid pigment epitheliopathy (AMPPE), multiple evanescent white dot syndrome (MEWDS) and serpinginous choroiditis (SPC) and to estimate their diagnostic and prognostic value.

Materials and methods

During the period of six years, ten patients were diagnosed with multifocal choroiditis at the Clinic for Eye Disease, Clinical Center Niš. All patients gave standard written consent for all diagnostic procedures before the examination. Standard ophthalmological examination was performed in all patients: visual acuity, slit lamp biomicroscopy, applanation tonometry, indirect ophthalmoscopy, photodocumentation and fluorescein angiography (FA) (Carl Zeiss Digital Fundus Camera Visucam Lite), high definition, standard domain, optical coherence tomography (SD OCT Cirrus and RTVue 100) and ultrasonography B scan (USG Tomey) in indicated cases. The imaging methods, FA and SD OCT were done at the same condition and repeated during the treatment of disease. Standard laboratory examination, immunological examination and HLA typing were performed as well.

Results

Of 20 eyes examined during our study, 6 were diagnosed with AMPPE, 2 MEWDS, 4 PIC, 4

SCP and 1 BCR. Clinical and multimodal imaging results of examined patients are presented in Table 1. and Figures 1 to 6.

In case with PIC, acute lesions appeared as nodular collections under the retinal pigment epithelium, and presented as solid inflammatory retinal pigment detachment (PED) on OCT (Figure 1). These solid PED appeared to be ruptured leading to inflammatory infiltration of the subretinal space and outer retina, often with a widespread loss of the outer retinal architecture beyond the area of the inflammatory exudate. During the treatment with corticosteroids, in patients with PIC, rapid regression of PED was noted, with a slower resolution of the abnormalities of outer retinal architecture. FA revealed more lesions that appeared as early hyperfluorescent lesions and late staining (Figure 1).

Anterior segment examination presented with mild inflammation, and mild vitritis in patients with BCR (Figure 2) (Table 1). On OCT focal disruption of the photoreceptors, inner and outer (IS/OS) focal or generalized were present (Figure 3). In macula, cystoid macular edema could be diagnosed. FA revealed prolonged arteriovenous transit time, optic disc hyperfluorescence, and vascular leaking (Figure 3). The delay of transit time is a phenomenon known as "quenching" and is pathognomonic for BCR.

Diagnosis of AMPPE was established in 3 patients and clinical signs were present in both eyes in different stages of disease (Table 1). On OCT hyperreflectivity of the outer retinal layers, inflammatory PED with presence of inflammatory cells were present (Figure 4). As the lesions resolved, hyperreflectivity of outer layer decreased. The IS/OS disruption, and atrophy of RPE can persist (Figure 4). FA demonstrated lesions with early hypofluorescence followed by the subsequent hyperfluorescence in the late venous phase (Figure4).

Table 1. Clinical and multimodal imaging findings in MPC

Entity	Birdshot	AMPPE	SPC	PIC	MEWDS
Anterior segment	Without inflammation	Without inflammation	With mild cellular elements	Without inflammation	Without inflammation
Posterior segment	Vitritis, creamy yellow, oval, diameter ¼ to 1 disc diameter subretinal infiltrates from optic disc to periphery	Vitritis white or creamy yellow placoid lesions/differ during phase	Vitritis creamy yellow subretinal infiltrates intermaculopapillary and macula and progress to periphery	Small, well defined yellow white lesions CNV and scars	yellow white lesions ill-defined in perimacular area
FA	Optic disc hyperfluorescence with vascular leaking	Early hypofluorescence with late hyperfluorescence	Early hypofluorescence with late hyperfluorescence old lesions are hypofluorescent	Hypofluorescence with hyperfluorescence on margin	Hyperfluorescence with pinpoint lesions
OCT	Disruption of IS/OS junction, tinning of Sattler's layer, atrophy of choroid	Hyperreflectivity of outer layers; RPE atrophy in late stage	Thickening of outer retina with disruption of IS/OS junction and with increased reflectivity of choroid	Focal hyperreflectivity and subsequent focal atrophy of RPE	Accumulation of hyperreflective material on RPE

A and C Photofundus of the right eye
B and D Photofundus of the left eye
E and F FA of the right and left eye
G- OCT active lesion
H- OCT inactive lesion

Figure1. PIC multimodal imaging

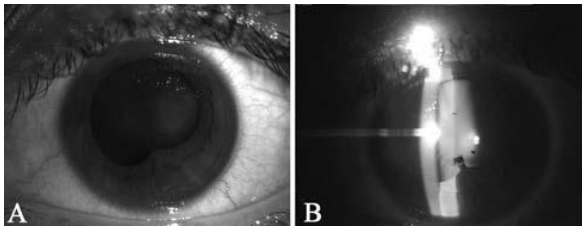
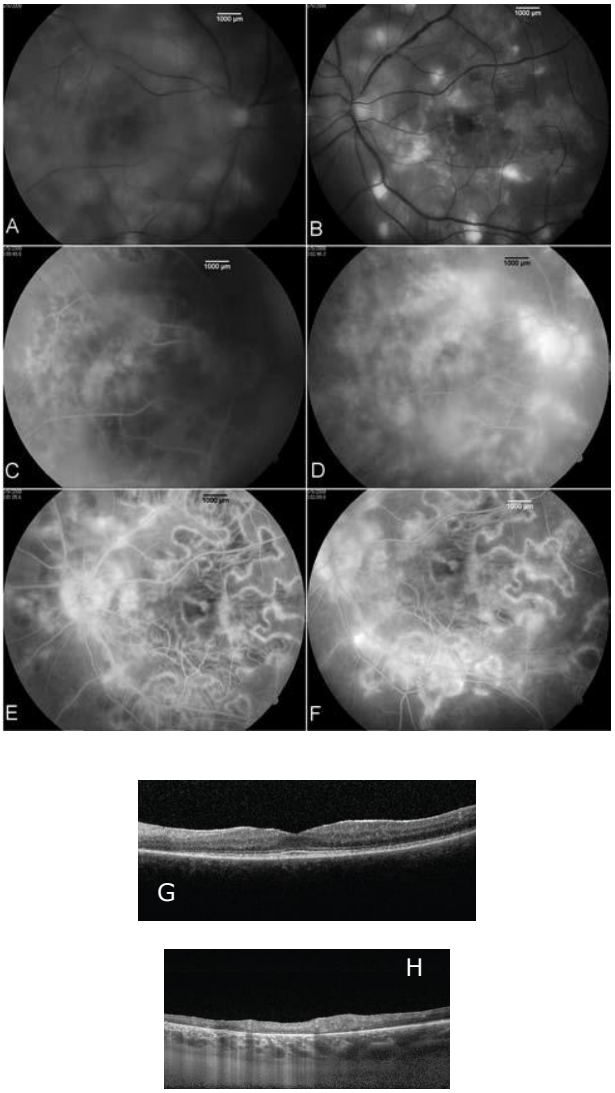
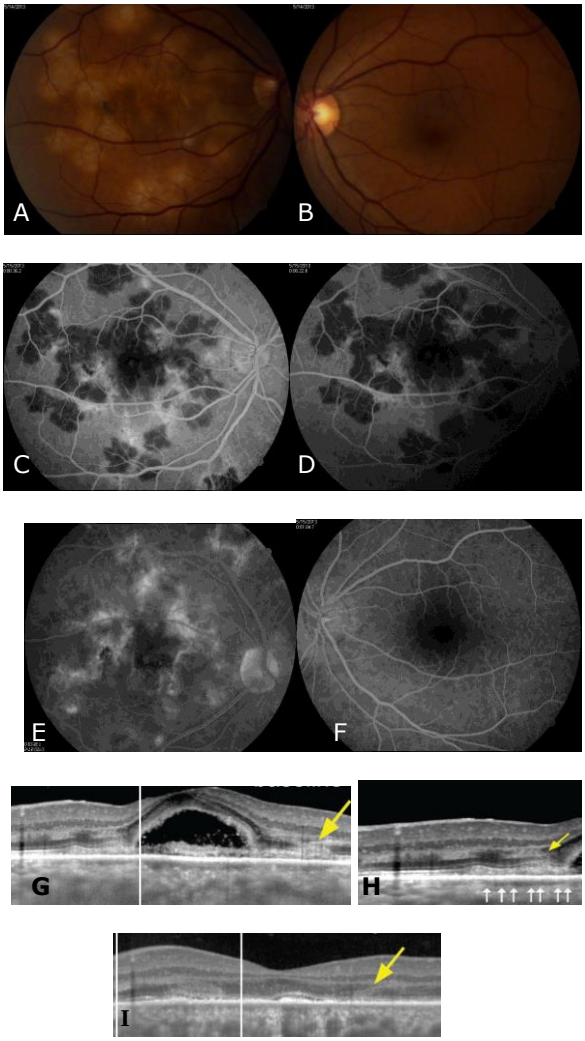


Figure 2. A and B Anterior segment in patient with SPC



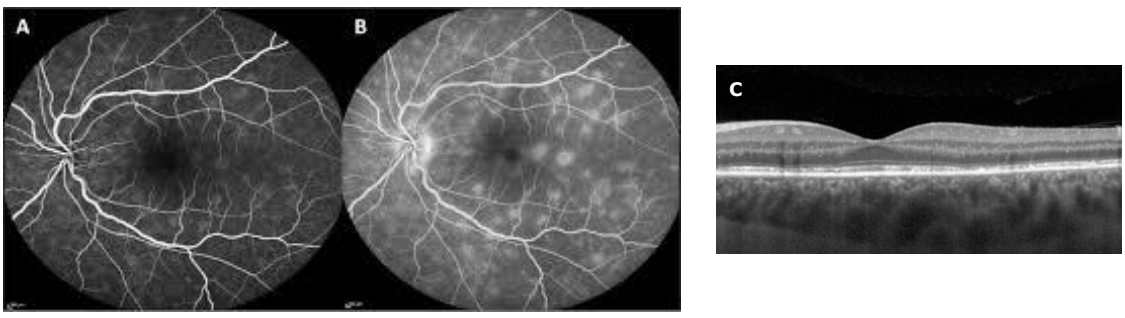
A and B Photography
C, D, E, F -FA
G- OCT active lesion
H- OCT inactive lesion

Figure 3. SPC multimodal imaging



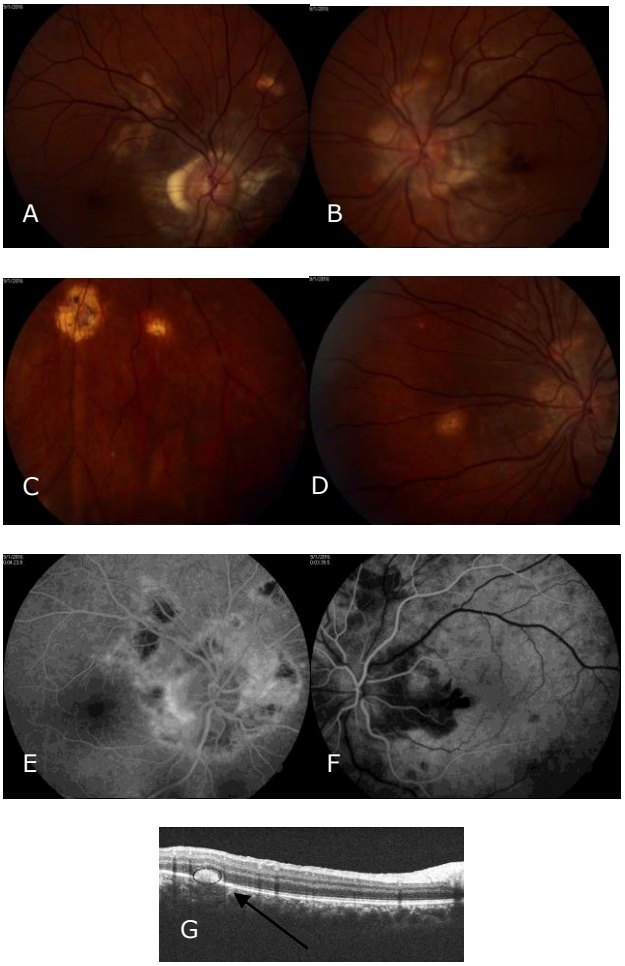
A- Color photography of the right eye
B- Color photography of the right eye
C, D ,E ,F - FA
G, H- OCT active lesion
I- OCT inactive lesion

Figure 4. AMPPE multimodal imaging



A- Photography, B- FA, C- OCT

Figure 5. MEWDS multimodal imaging



A, B, C, D- Color photography
E, F- FA and G- OCT active lesion

Figure 6. BRC multimodal imaging

Accumulation of hyperreflective material that rested on RPE, and extended through the interdigital zone, ellipsoid zone, and outer nuclear layer (ONL) was observed by OCT in patients with MEWDS (Figure 5). The abnormalities of retinal architecture restored spontaneously. During FA in these patients, multiple lesions with early hyperfluourescence in a wreath-like configuration were detected. The lesions were presented in mid retina and hyperfluorescence persisted in late phase of FA (Figure5).

During the active phase in patients with SPC, hyperreflective lesions and thickening of the outer retina were noted (Figure 6). Hyperreflective inflammatory PED, similar to PIC, was present as well (Figure 6). The reflectance of choroid was increased causing the so called waterfall effect (Figure 6). The inner and outer photoreceptor layers as well as their junction were interrupted. Active lesions on FA showed early hypofluorescence and late hyperfluorescence of the border (Figure 6). Old lesions were presented by window defect and late staining on FA (Figure 6).

Discussion

Multifocal chorioretinitis presents separate group of posterior uveitis with similar clinical findings. Multimodal imaging such as color photofundus, FA, OCT, and ultrasonography can help in diagnosis, differential diagnosis and follow-up of disease course (10, 11, 12, 13). Authors also proposed fundus autofluorescence (FAF) and angio OCT as methods of choice for diagnosis and follow-up of MPC (13).

Anterior segment inflammation was present in patients with SPC. In our patient, in the color photography, findings differed in size, color or localization of lesions (Table 1). Atrophic lesions were present in patients with BRC and SPC; pigmented lesions were present in patients with AMPPE. Similar results are present in the literature (1, 7, 14, 15, 16). Retinal architecture restored spontaneously in patients with MEWDS. BRC, SPC and PIC had a chronic course. These entities need to be monitored closely in order to detect the development of choroidal neovascularization (CNV) (13). During our study, we did

not notice CNV in patients. New imaging technic, angio OCT, is more sensitive in detection of CNV (13).

Imaging technics are important in differential diagnosis of MPC for infectious uveitis as well as for primary or secondary neoplastic changes (6, 7, 16).

Limitations of FA imaging are opacification of anterior segment intensive vitreous haze or hemorrhage in vitreous. It is an invasive method of examination, and practitioner should be cautious about patients with allergic reactions, heart, liver and kidney disease.

OCT examination is easy to perform and non-invasive method. However, some limitations of OCT examination are present. Corneal opacification, dense cataract, significant vitreous haze or hemorrhage in the eyes are limiting factors for OCT examination. Eye tracking system overcomes acquisition errors and enables a high intra-observer reproducibility allowing determination of minimal changes that may occur between successive examinations (7). Shadowing of the underlying retinal structures by hyperreflective lesions such as exudates, hemorrhages and major retinal vessels can lead to the loss of detail of underlying morphology.

Conclusion

Multimodal imaging has allowed us to better understand morphology, activity and stage of lesions. The common features of all presented cases of multifocal chorioiditis entities are: minimal lesions at the initial stage of disease, multifocal white, or white/yellow multiple lesions, during the progression of disease degeneration of retinal tissue and choroid confirmed by OCT and FA, similar clinical presentation. OCT is important in diagnosis, differential diagnosis and follow-up.

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No conflict of interests

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MULTIMODALNE TEHNIKE VIZUELNOG PRIKAZA PACIJENATA SA MULTIFOKALNIM HOROIDITISOM SA PATOLOGIJOM NA NIVOU SPOLJAŠNJE HORIO – RETINALNE BARIJERE

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Cilj naše studije je prikaz različitih tehnika vizuelnog prikaza bolesnika sa multifokalnim horoiditisom i to: punktiformnom unutrašnjom horoidopatijom (PIC), Birdshot horioretinopatijom (BRC), akutnom multifokalnom pigmentom plakoidnom epiteliopatijom (AMPPE), sindromom multiplih nestajućih belih tačkica (MEWDS) i serpenginoznim horoiditisom (SPC); i utvrditi njihov dijagnostički prognostički značaj.

Studija je sprovedena na Klinici za očne bolesti Kliničkog Centra u Nišu. U periodu od šest godina kod deset bolesnika postavljena je dijagnoza mulifokalnog horioretinitisa. Standardni oftalmološki pregled podrazumevao je: određivanje subjektivne vidne oštrine, pregled prednjeg segmenta biomikroskopom, aplanacionu tonometriju, indirektnu oftalmoskopiju, fotodokumentaciju, flureseinsku angiografiju (FA), optičku koherentnu tomografiju (OCT) i pregled ultrazvukom. Sprovedeno je i standardno laboratorijsko ispitivanje, imunološko ispitivanje i sprovedena je HLA tipizacija.

Vidna oštrina bila je očuvana kod svih bolesnika osim kod pacijenta sa dijagnozom SPC. Bolest je bila bilateralna kod bolesnika sa AMPPE, SPC I PIC. Hronični tok bolesti bio je prisutan kod bolesnika sa dijagnozom SPC i PIC.

Zajedničke karakteristike kod svih bolesnika sa multifokalnim horoiditom bile su: minimalna oštećenja u početnom stadijumu bolesti, brojne beličaste ili žutobeličaste lezije, kao i to da napredovanje oboljenja dolazi od degenerativnog oštećenja tkiva. Promene su dokazane OCT i FA metodom.

OCT je značajan za diferencijalnu dijagnozu i praćenje.

Dobijeni rezultati u skladu su sa rezultatima sprovedenih studija, a različitost nalaza u pojedinim oboljenjima predmet je daljeg istraživanja.

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Ključne reči: *horioretinitis, dijagnoza, vizuelni prikaz*

Case report

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COLON-COLONIC INVAGINATION CAUSED BY CECUM CANCER – A CASE REPORT

Saša Dragović

Invagination (intussusception) represents telescoping of the proximal segment of the intestine (intususceptum) into the distal segment of the intestine (intususcepiens). We report the case of a patient with colon-colonic invagination caused by adenocarcinoma of the cecum as one of the rare causes of colonic invagination.

Multidetector computerized tomography of the abdomen and lesser pelvis is a diagnostic method of choice and intestine resection according to the principle of oncological resection is a treatment of choice in adults with malignant etiology of the disease.

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Key words: *invagination (intussusception), multidetector computerized tomography, colon, surgical treatment, resection*

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Introduction

Invagination (intussusception) was first mentioned in 1674 by Barbette of Amsterdam (1) and later, in 1789, it presented in more detail in a report by John Hunter as intussusception - telescoping of the proximal segment of the intestine (intususceptum) into the distal segment of the intestine (intususcepiens) (2). The first invagination operation was performed by Ser Jonathan Hutchinson in 1871 (3). The highest percentage of invagination is present in infants and children, whereas it is present in as much as 5% of adults (4).

Contrary to the younger population where the cause of intussusception is unknown, the most frequent cause in adults are malignant processes in the intestines, primarily in the colon.

The clinical picture of intussusception in infants and children is dominated by a sudden onset of the disease followed by cramps, vomiting and bloody mucus in stool, while in adults it is followed by subacute and chronic nonspecific problems (constipation, diarrhea, etc.) (5-7).

Case Presentation

The patient M. V., aged 82, was admitted with pain located paraumbilically to the right. The pain had lasted for three days and the patient had nausea without the urge to vomit. During the last three days, the stool had liquid consistency without blood and mucus. The patient denied febrility and dysuric problems. She was treated for hypothyreosis (she was taking Letrox) and denied other chronic significant diseases. She had pollen allergy and denied allergies to medications. Family history was not significant.

On admission, the patient was conscious, oriented in space, time and person, communicative. The skin and visible mucosa had normal color and hemodynamic compensation. She was afebrile, eupneic, normotensive, the tongue was moist and uncoated. The head, neck and thorax had no specific findings. The abdomen was in line with the thorax, soft on palpation, painful on deep palpation paraumbilically to the right where a 5x5 cm movable tumefaction could be palpated. Abdominal musculature was without defense and without peritoneal irritation on palpation. On auscultation, gurgling sound was heard above the abdomen. No abdominal herniation was present. The extremities were not swollen or deformed.

Laboratory findings were normal.

Multidetector computerized tomography (MD-CT) of the abdomen and lesser pelvis showed intussusception of the cecum with ileocecal valve into the

ascending colon. Cecal fundus was located in the ascending colon towards the hepatic flexure. Mesenteric pedicle of the ileocecal segment protruding into colon lumen is observed. Signs of subocclusion were observed in the small intestine.

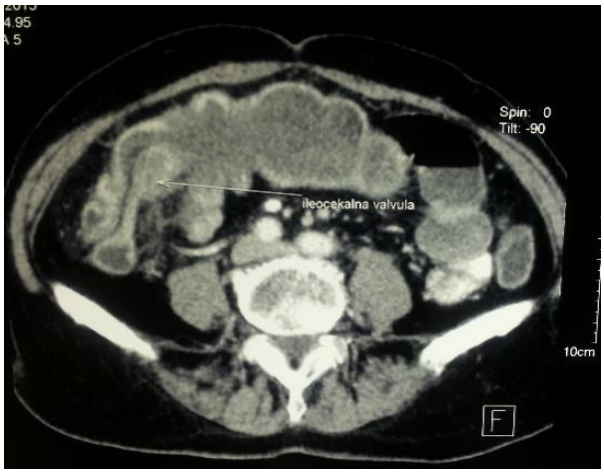

Figure 1

After the diagnosis, the following surgical intervention was indicated and performed: laparotomia explorativa. Hemicolectomia dex. cum ileotransversocolo anastomosis termino-lateralis.

Intraoperative findings: No free fluid present in the abdomen. Parietal and visceral peritoneum showed no changes in the form of secondary deposits. Adhesions were present between the omentum and anterior abdominal wall. Intussusception of the cecum with ileocecal valve in the ascending colon was found. Cecotomy was performed. A 5x5 cm infiltrate of hard consistency was found which involved all layers of the wall, did not perforate the serous membrane and partially occluded the lumen of the intestines. Figures 2 and 3.

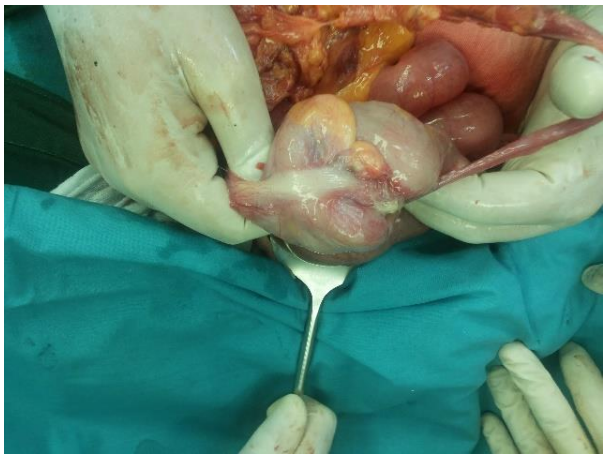

Figure 2. Invagination caused by tumor

Figure 3. Invagination caused by tumor

Pathohistological examination of the preparation: macroscopically – the resection was the final part of the small intestine, 190 mm long, the cecum without appendix, the ascending and part of transversal colon 250 mm long, with associated fat tissue. The cecum was completely filled with lobulated tumor mass, pink and with softer consistency. Microscopically – polypoidmucinous adenocarcinoma, well -differentiated, spreading infiltratively, without venous and lymphatic invasion, with absent metastases in lymphatic nodes (0/24) and with no tumor on the edges of the resection. Final histopathological findings: Adenocarcinoma mucinosuminvasivum intestine crassi HG1 in stadio B secundum Dukes ((B3 secundum Astler Coller) pT4b N0(0/24) Mx secundum AJCC/UICC TNM L0V0.

Postoperative course was regular. Bowel transit was established. Nasogastric probe was taken out on the third day. The drain was removed on the fifth day. Sutures were removed on the tenth postoperative day. Medical advisory board for malignant diseases of the digestive tract: regular checkups and monitoring were indicated.

The first control checkup with a surgeon one month later: findings were regular.

Discussion

Invagination (intussusception) in adults is clinically manifested by nonspecific, chronic problems which point to a possible bowel obstruction. The most common symptom is abdominal pain followed by vomiting and nausea. The exact mechanism of development is unknown but it is believed that a lesion in the intestinal wall or presence of an irritant in lumen initiates invagination by changing the normal intestinal peristalsis. The presence of palpable masses in the abdomen is present in 24% to 42% of examined patients. Invagination may be indicated by the presence of a movable mass in the abdomen along with the above mentioned symptoms (4, 5).

The dominant locations of intussusception are the places where movable segments of the colon go into the less movable segment (8).

Invagination is classified into four groups according to the place of origin:

1) entero-enteric, limited to the small intestine,

2) colon-colonic, which includes only the segments of the colon,

3) ileo-colonic, prolapse of the terminal ileum into the colon and

4) ileo-cecal, the main initiator of invagination is ileocecal valve (6, 9, 10).

The diagnosis is established based on history data, clinical picture and diagnostic procedures (native RTG graph of the abdomen, abdominal ultrasound, MDCT of the abdomen, colonoscopy).

Native RTG graph of the abdomen is usually the first diagnostic procedure which shows the signs of bowel obstruction and provides initial information about the location of the obstruction (11).

Ultrasound diagnostics as an easily available and noninvasive procedure has its application in the diagnosis of intussusception with signs of pseudokidney but it also has drawbacks due to the masking of the visual field by gas collections in the intestines (12).

Colonoscopy can visually determine the exact cause of the obstruction, do the biopsy of the change and its location and it is an appropriate method particularly in nonspecific, chronic problems (13).

In the last several years, MDCT scanner has become the leading diagnostic procedure which shows the exact location of intussusception and helps to plan surgical intervention (14).

Invagination in infants and children can be resolved with barium irigography or air insufflation into the colon. The treatment of invagination in adults is in most cases finalized surgically. Oncological principles of large intestine resection are performed due to malignant processes which are dominant in the etiology (15, 16).

Medial laparotomy has mostly been used so far as an approach in surgical treatment and recently, laparoscopic resection of the small and large intestine has been introduced into practice in recent years observing the oncologic principles of resection (17, 18).

Conclusion

Intestinal invagination in adults is rare and the main cause is a malignant process. MDCT scan of the abdomen is the most specific and the most sensitive preoperative diagnostic method. The treatment of invagination of intestines in adults is, as a rule, surgical. The principles of surgical treatment include oncological resection of the intestines by the use of classic or laparoscopic approach.

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Prikaz bolesnika

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KOLONO-KOLONIČNA INVAGINACIJA IZAZVANA KARCINOMOM CEKUMA - PRIKAZ SLUČAJA

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Invaginacija (intususcepcija) predstavlja uvlačenje proksimalnog segmenta creva (intususceptum) u distalni segment creva (intususcepiens).

Studija daje prikaz bolesnika sa kolono-koloničnom invaginacijom uzrokovanom adenokarcinomom cekuma, kao jednim od ređih uzroka kolonične invaginacije.

Multidetektorska kompjuterizovana tomografija trbuha i male karlice dijagnostika je izbora, a resekcija creva, po principu onkološke resekcije terapija je izbora invaginacije u odraslih osoba kod maligne etiologije bolesti.

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Ključne reči: *invaginacija (intususcepcija), multidetektorska kompjuterizovana tomografija, kolon, hirurško lečenje, resekcija*

Case report

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GLOMUS TYMPANICUM PARAGANGLIOMA IN A 63-YEAR-OLD MALE - A RARE ENTITY IN THE MIDDLE EAR: A CASE REPORT

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Glomus tympanicum paraganglioma arise from the glomus bodies that run with the tympanic branch of the glossopharyngeal nerve (Jacobson nerve). Although *glomus tympanicum* tumours are the most common primary neoplasms of the middle ear, these tumours are the rarest of head and neck paragangliomas. In most cases these benign tumours grow slowly, but can be locally aggressive. We present the case of small *glomus tympanicum* in a 63-year-old male patient with one year long history of pulsatile tinnitus and ear fullness as well as decreased hearing. On otoscopy, a pulsatile reddish mass was seen behind the tympanic membrane and Brown sign was elicited. Imaging revealed middle ear cavity mass on the cochlear promontory with strong post contrast enhancement. The knowledge of the clinical presentation, the imaging features and the differential diagnosis of the middle ear masses is necessary to establish the correct diagnosis. This case illustrates small size *glomus tympanicum* paraganglioma with typical clinical and imaging findings. Imaging and imaging based classification play most important roles in diagnostic and treatment planning in patient with *glomus tympanicum* paragangliomas.

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Key words: *glomus tympanicum paraganglioma, imaging, classification*

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Introduction

Head and neck paragangliomas (also commonly referred to as glomus tumours or chemodectoma) are highly vascular lesions originating from the paraganglionic tissue, located at four typical locations: the carotid bifurcation (carotid body tumours), along the vagus nerve (vagal paragangliomas), and in the jugular fossa and tympanic cavity (jugular and tympanic paragangliomas) (1, 2, 3). These tumours originate from the chief cells of the paraganglia, or glomus bodies, located within the wall (adventitia) of the jugular bulb, and can be associated with either the auricular branch of the vagus nerve (Arnold nerve) or the tympanic branch of the glossopharyngeal nerve (Jacobson nerve). Paraganglia develop from the neural crest and are believed to function as chemoreceptors (4).

Paragangliomas account for 0.6% of all neoplasmas of the head and neck and 0.03% of all neoplasms (5).

These lesions are histologically similar to the pheochromocytomas that may develop in the adrenal medulla, but unlike pheochromocytomas, head and neck paragangliomas rarely secrete cathecholamines (6).

In most cases they are benign neoplasms; overall less than 10% of all paragangliomas have been cited to be malignant (5).

Tumours in the area of the middle ear cavity are called glomus tympanicum paragangliomas; they arise from the glomus bodies that run with the tympanic branch of the glossopharyngeal nerve (Jacobson nerve). Although glomus tympanicum tumours are the most common primary neoplasms of the middle ear, these tumours are the rarest of head and neck paragangliomas (7, 8, 9, 10).

Case report

We present a 63 year old male patient with one year long history of pulsatile tinnitus in the right ear. The tinnitus was accompanied by ipsilateral ear fullness. Last few months patient noted decreased hearing on the right side. There was no history of

headache, vertigo and facial weakness, as well as otorrhea and ear pain. No other comorbidities were present.

On otoscopy, a pulsatile reddish mass was seen behind the tympanic membrane and Brown sign was elicited. Audiometry revealed conductive hearing loss in the right ear. The left ear had normal audiometric finding.

The patient came to our department after clinical examination which was made few days before imaging. The first diagnostic method in our department was computed tomography (CT).

CT demonstrated oval middle ear cavity mass on the cochlear promontory. The diameter of the mass was 8 mm. There were no ossicular chain destruction and no evidence of bony erosion. The vestibule, cochlea and semicircular canals had normal morphology. The carotid canal had normal configuration and it was intact. There was no evidence of dehiscence of the internal jugular vein bulb. No abnormalities were seen along the course of the facial nerve canal. The postcontrast CT study resulted in intense enhancement of the cochlear promontory mass, which was well-defined and slightly bulged the tympanic membrane.

The next diagnostic tool was magnetic resonance imaging (MRI). The mass demonstrated low T1 weighted and high T2 weighted signal intensity on MR images. It presented intense enhancement after contrast administration.

According to the CT and MR presentation of the middle ear cavity mass, as well as clinical presentation, otoscopic examination and audiometric findings, we made the diagnosis of glomus tympanicum paraganglioma.

The patient underwent excision of the glomus tumour via a transcanal approach. Follow up few months after surgery showed the absence of tinnitus and improvement of hearing.

Histopathological examination showed branching vascular canals separated by stroma. In the stroma, grouped glomus cells surrounding blood vessels were seen. Nuclei of these cells were small and showed low mitotic activity. Final histopathology was consistent with glomus tumour.

Discussion

Glomus tumours have an annual incidence of 1 per 1.3 million people (11).These tumours are commonly seen in 5th and 6th decade of life, with a female predominance (12).

Our patient is male in the 7th decade of life, with right sided glomus tympanicum paraganglioma.

Paragangliomas are typically described as benign and slow growing. Glomus tympanicum may be confined to the middle ear or may spread along the paths of least resistance to the mastoid or even up to the nasopharynx. In our patient the tumour was on cochlear promontory in the middle ear cavity, without spread to surrounding structures.

Glomus tympanicum may present with pulsatile tinnitus and hearing loss, and our patient had these symptoms. Other symptoms may include headache, vertigo and facial weakness, as well as otorrhea and ear pain. Tachycardia, hypertension, tremors and headaches can indicate the possibility of a functional tumour. None of these symptoms were present in our patient.

On otoscopy, a pulsatile reddish mass was seen behind the tympanic membrane and Brown sign was elicited. Brown's sign is the pulsations elicited by pneumatic compression of the tumour. All these signs were present in our patient.

Imaging is the primary modality of investigation for glomus tympanicum tumours. A combination of contrast enhanced computed tomography, magnetic resonance imaging and angiography is ideal for the diagnosis and localisation of glomus tumours.

Strongly enhancing mass at its site of origin is typical in the diagnosis of glomus tympanicum tumours (13).

CT is best for evaluation of bony erosion and destruction which is demonstrated in larger glomus tympanicum. Similar to CT, contrast enhanced MRI shows strongly enhancing soft tissue mass, which is typical for diagnosis, at characteristic locations (13, 14).

Multiple areas of high and low signal intensity on MRI, the so-called "salt and pepper appearance", originally described by Olsen et al. (15) can be seen within the larger lesion on T1 weighted and T2 weighted images. The "pepper" component represents the multiple areas of signal void interspersed with the "salt" seen as hyperintense foci (due to slow flow or hemorrhage) on both T1 weighted and T2 weighted images (15, 16). This feature is especially seen in larger paragangliomas, and was not seen in our patient, who had small glomus tympanicum tumour.

Angiography identifies the primary feeding vessels to the lesion, helps in detecting multicentric tumours and allows for possible preoperative embolisation. Angiography is imaging procedure which can confirm the diagnosis of a head and neck paraganglioma and some authors report that it is still the gold standard in detection of small paragangliomas (15, 17, 18). Angiography shows the specific vascular supply of the paraganglioma (19). The ascending pharyngeal artery can be considered "the artery of the paraganglioma", because its branches can supply tympanic, jugular, vagal, carotid, and even laryngeal paragangliomas (20). Embolisation can be performed with angiography for preoperative devascularisation of the large tumour.

This diagnostic procedure was not performed in our case.

Surgical resection is the treatment of choice especially in small paragangliomas. The surgical approach depends on the size and extent of the tumour. Preoperative image based classification of glomus tympanicum paragangliomas is essential, since the operative approach will be chosen depending on the tumour stage (21).

The tympanic paragangliomas present usually like a small mass at the cochlear promontory, ossicular destruction is not common and is especially present in larger lesions. The extension of temporal bone destruction is important to classify those tu-

mours. The two established classifications of temporal bone paraganglioma are based mainly on tumour size with special emphasis on intracranial extension as a decisive factor for resectability. Paragangliomas of the temporal bone, as well as the glomus tympanicum paragangliomas are usually staged according to the classification by Fisch (22) and Glasscock and Jackson (23).

In the present case, the tumour was limited on the cochlear promontory, without any destruction of the ossicular chain and surrounding bone, and it was classified as Fisch type A and Glasscock – Jackson type 1, with recommendation for surgical treatment. Type 1 glomus tympanicum tumours are generally approached by transcanal tympanotomy (24).

Surgery remains the treatment of choice in an otherwise healthy patient who desires the immediate cure of disease provided by a total resection. Preoperative embolisation is necessary in patients especially when a tumour diameter is larger than 3 cm (4). It can reduce intraoperative blood loss significantly.

Paragangliomas may be treated with either surgery or radiotherapy. The goal of any treatment is to control tumour growth and prevent further neurological compromise and it is important to select the intervention with the greatest chance of tumour control and the lowest risk of complications (25).

However, radiotherapy is often used for larger slow growing tumours with an aim to restrict growth in unresectable tumours. Preoperative evaluation with CT, MRI and angiography has been proposed as essential for optimal planning (26).

The differential diagnosis considerations for the middle ear mass include benign neoplasms and diseases (adenoma, endolymphatic sac tumours, choristoma, cholesteatoma, cholesterol granulomas) and malignant neoplasms (squamous cell carcinoma, adenocarcinoma, sarcoma). Adenomatous tumours (mixed pattern type) of the middle ear manifest as soft-tissue masses in the tympanic cavity, usually without destruction of surrounding osseous structures. These tumours may enhance intensely following the intravenous administration of contrast material (27). Endolymphatic sac tumours of the temporal bone arise from the region of vestibular aqueduct. Although they may closely resemble glomus tumours, their origin from the vestibular aqueduct is a useful discriminating characteristic. They are frequently quite large at the time of presentation, demonstrate lytic changes in the temporal bone and characteristically have regions of hyperintensity on T1-weighted sequences on MRI(28).

Cholesterol granuloma occurs secondary to nonspecific chronic inflammatory changes and it can be seen in the middle ear cavity. Hemorrhage, cholesterol, and granulation tissue are the hallmarks of the lesion and account for its appearance on MRI (29). Patients most often present with conductive hearing loss and a vascular appearing retrotympanic mass on physical examination. On CT, the imaging appearance is nonspecific with a soft tissue middle ear mass. Findings on MRI are more characteristic, demonstrating a nonenhancing middle ear mass which is hyperintense on both T1 and T2 sequences secondary to hemorrhagic blood products (30). Care must be taken to compare pre and postcontrast T1 sequences, since the lesions are hyperintense on both which may be mistaken for enhancement. With adjacent inflammation, there may be thin peripheral enhancement.

Cholesteatoma also can be found in the middle ear cavity, but on the MRI it exhibit specific findings with restriction of diffusion and does not demonstrate the postcontrast enhancement. Cholesterol granuloma and cholesteatoma usually are not located at cochlear promontory.

Glomus tympanicum tumours are usually described as arising on the promontory (31).

Cavernous hemangioma is a rare benign neoplasm of the middle ear, which can rarely cause tinnitus and mimic a glomus tympanicum clinically, radiographically and otoscopically (32). Middle ear hemangiomas should be differentiated from the more common geniculate ganglion hemangiomas (33), which usually present with facial nerve palsy (34) but may rarely cause pulsatile tinnitus (35, 36). The diagnosis is often possible only at surgery. The literature review revealed one case of hemangioma localized on the promontory of the middle ear confirmed by histopathological study as capillary hemangioma (37).

In our case the diagnosis is made after clinical examination and imaging, and confirmed after histopatological examination.

Conclusion

In conclusion, we report the case of a rare entity - glomus tympanicum paraganglioma in a 63 old male patient with one year long history of pulsatile tinnitus and ear fullness as well as decreased hearing. Imaging revealed middle ear cavity mass on the cochlear promontory with no ossicular chain destruction and no evidence of bony erosion. On otoscopy, a pulsatile reddish mass was seen behind the tympanic membrane and Brown sign was elicited. The imaging features demonstrated strongly enhancing mass. All these signs are important and can help to make the diagnosis of the glomus tympanicum tumour. The knowledge of the clinical presentation, the imaging features and the differential diagnosis of the middle ear masses is necessary to establish the correct diagnosis. Imaging based classification is essential in management of the treatment choice.

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GLOMUS TYMPANICUM PARAGANGLIOM KOD 63 GODINE STAROG MUŠKARCA – REDAK ENTITET SREDNJEG UVA: PRIKAZ SLUČAJA

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Glomus tympanicum paragangliomi potiču od glomus tela lokalizovanih duž timpanične grane glosofaringealnog nerva (Jacobsonov nerv). Iako su *glomus tympanicum* tumori najčešće primarne neoplazme srednjeg uva, ovi tumori najređi su paragangliomi regije glave i vrata. U najvećem broju slučajeva ovi benigni tumori imaju spor rast, ali mogu biti lokalno agresivni. Prezentujemo slučaj malog *glomus tympanicum* paraganglioma kod 63 godine starog bolesnika muškog pola, sa jednogodišnjom istorijom puslatilnog tinitusa i osećajem punoće u uvu, kao i sa oslabljenjem sluha. Otoskopija je pokazala puslatilnu crvenu masu iza timpanične membrane kao i Brownov znak. Imidžing je pokazao masu srednjeg uva na kohlearnom promontorijumu sa izraženim postkontrastnim pojačanjem. Poznavanje kliničke prezentacije, imidžing karakteristika, kao i diferencijalne dijagnoze masa srednjeg uva, neophodni su za postovljanje tačne dijagnoze. Ovaj slučaj pokazuje *glomus tympanicum* paragangliom malih dimenzija sa tipičnim kliničkim i imidžing karakteristikama. Imidžing i imidžing klasifikacije igraju najvažniju ulogu u dijagnostičkom i terapijskom planiranju kod bolesnika sa *glomus tympanicum* paragangliomima.

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Ključne reči: *glomus tympanicum paragangliom, imidžing, klasifikacija*

Review article

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TUMORS OF PARATHYROID GLANDS

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Parathyroid glands are small endocrine glands found in the neck which secrete parathyroid hormone or parathormone (PTH) which has a combined role together with calcitonin and vitamin D in regulating levels of calcium and phosphate concentrations in humans.

The most common disease of parathyroid glands is increased and uncontrolled secretion of PTH which is defined as primary hyperthyroidism, if it is offset as a result of intensified function of one or more parathyroid glands, or as secondary hyperparathyroidism which is mostly caused by chronic kidney insufficiency or by vitamin D deficiency.

Carcinoma of parathyroid glands is a rare disease and one of the rarest malignant endocrine tumors.

Frequency of carcinoma in patients with primary hyperparathyroidism is less than 1%.

Best treatment for patients with this primary lesion is intraoperative recognition of the tumor by surgeons, adequate resections with removal of primary lesion, and histopathological verification.

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Key words: *parathyroid glands, neoplasm, surgery, surgical procedures, hyperparathyroidism*

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Introduction

Malignant neoplasms – disease of modern times

Modern man lives surrounded by carcinogens because everything is contaminated, the water, the air and the food. It is established that in only one cigarette there are 30 different chemical carcinogens, and then there are many types of radiation, cesium, strontium, different types of medicaments, especially sedatives and hormones, etc. Although, nowadays we know a lot about oncogenes, we still don't have an explanation for molecular basis of carcinogenesis. It has been discovered that in the genome within the human cell, there are genes in a latent state which, when activated may cause malignant transformation, i.e. cancer.

In various stages of man's evolution and development of the society, there were frequent and lethal dangers for mankind caused by various infections and food shortages. This is explained by the fact that man was firstly a hunter and used spears, bows and arrows, until he evolved into higher forms of social organizations, concluding with present day. Along the way, tools for production were improved and socio-economic, cultural and political relations were also changed. In all of those specific stages of evolution of the society, the human environment also changed, all of the living organisms, and also various forms of human pathology were replaced by others thus giving importance to the medical science and its protective role.

It is believed that mankind could have been and actually was prone to malignant diseases from his very origin. This is confirmed by scientific research in the past decades, as well as by the numerous documents that we have inherited from ancient cultures and civilizations. Thus, cancer and malignant neoplasms are not new, unknown diseases to mankind and other living organisms. There is no doubt that better knowledge of human anatomical structure and the functions of certain organs and tissues and especially fundamental discoveries, such as microscopes and such, contributed enormously to our discovery that malignant diseases exist indeed.

Although in the beginning it was only the physicians who were interested in researching this field of work, malignant diseases, nowadays scientists from other fields of work joined in, so today it is

almost impossible to do research projects without the full involvement of physics, molecular biology, chemistry and related sciences.

Beginnings in cancer research are usually associated with an article published by an English doctor P. Pott. Namely he noticed that cancer in chimney sweepers was more frequent than in the rest of the population, probably as he himself states, because of the toxic effects of smoke and soot with which they came into contact on daily basis. At the same time, other doctors noticed that tars, lubricants and oils could be the cause of skin cancer. Some doctors have, for example, succeeded in causing cancer in various organs of animals that they were experimenting on, by treating them with petrol and tar derivatives. Later, these carcinogenic properties of tar have been confirmed by many scientists through their numerous research undertakings which involved a wider spectrum of carcinogenic substances thus a new scientific discipline called experimental oncology was formed.

Oncology, as the science of cancer, has a significant place in modern medicine, especially in prevention and protection of human health.

Endocrine oncology deals with etiology and pathogenesis, clinical pictures and tumor therapy of the endocrine system. Thanks to the application of the latest diagnostic procedures which determine the clinical endocrinology, especially neuroendocrinology and psychoneuroendocrinology, it is now possible to detect tumors of endocrine glands in time, thus enabling a significant number of patients to be cured. It is a well known fact that malignancies are constantly increasing and that they are one of the most common diseases with fatal outcome in modern times. Undoubtedly, many factors influence this enormous increase of malignant diseases. Long-term monitoring of the epidemiological study indicates that there are many carcinogenic agents. Some scientists pay special attention to the profession-related carcinogenic factors, while the majority of competent experts divide all carcinogenic substances in chemical, physical and biological categories, regardless of their etiology. It has been observed that many profession-related agents are connected to industrial professions, although the environmental influence, the way of life, nutrition, customs, habits (smoking, alcoholism, drug-addiction), previous illnesses, etc., all have a significant influence as well.

Inorganic matter has a significant etiological share in the development of malignant diseases. It was noticed that in many industrial spheres there is a higher mortality rate among workers working in plants with aluminum, asbestos, chrome, nickel, cadmium, arsenic, in various metalworking combines for iron processing, etc.

Organic matters are also of great significance when we speak of epidemiology and etiopathogenesis of primary neoplasms.

Because of the enormous dangers employees are exposed to all over the world, necessary measures, based on suggestions from scientific institutions, are undertaken in order to prevent possible health hazards.

A specific malignant potential arises from profession-related pneumoconiosis (dust).

Employees involved in the production of coal and petrol derivates (various types of coal, coke, bitumen, tar) are probably the most vulnerable. This is also the case with employees in agriculture, mill industry, chemical industry, and paint and varnish industry. There is also danger for employees in the rubber industry, for uranium miners, and especially for the personnel exposed to x-ray radiation in laboratories for the preparation of radioisotope, in cases of damage to nuclear power plants, using of atomic weapons.

The goal

When we speak of malignant diseases in humans, it is important to be aware that we can reduce the number of sick individuals and consequently increase the number of cured ones only through knowledge. Humans have conquered many impediments and they are on a good path to discover the very essence of cancer.

Hypoparathyroidism

Hypoparathyroidism occurs as a consequence of lack of parathyroid tissue whether the cause is developmental disorder, or the tissue was destroyed by a pathological process or due to an error in removal of parathyroid glands during thyroidectomy. The most common cause of hypoparathyroidism is surgical removal of parathyroid glands, but nowadays the complications of thyroidectomy result in less than 1% of cases. One of the rarest conditions is congenital absence or aplasia of parathyroid glands.

Idiopathic hypoparathyroidism can be an inherited disease or a sporadic congenital disease and is often associated with thymic aplasia. It may occur during the first year of life, but it is usually manifested in childhood and it occurs at a higher rate in females (2:1). In some patients we can find circulating anti-parathyroid antibodies, as well as in members of their family but without a manifestation of the disease, which suggests the participation of autoimmune mechanism in the genesis of the disease. Similar antibodies can also be found on adrenal and thyroid tissue in patients with idiopathic hypoparathyroidism, although they do not show clinical signs of Addison's disease or hypoparathyroidism. It is interesting that idiotic hypoparathyroidism can sometimes be united with adrenocortical insufficiency, chronic lymphoid thyroiditis, insufficiency of islets of Langerhans and diabetes, chronic hepatitis, atrophy of gastric mucosa and pernicious anemia.

Patients with hypoparathyroidism usually manifest episodes of tetany, epilepsy, mental disorders or some other neurological signs, and there are also certain morphologic changes which can assist in giving a precise diagnosis. Patients often have epidermal lesions with rough and scaly skin, nails are deformed and fragile, hair and body hair are thin, rough and dry. There are cases of lenticular cataract

also. At the beginning of the disease, dental aplasia and hypoplasia occur on teeth which are developing, so the roots of the molars are flattened. Lamina dura thickens, and there is an occurrence of bilateral spotted calcifications in basal ganglia and cerebellum, which probably results in epileptic seizure. As a rule hypocalcemia and hypocalciuria are manifested along with hyperphosphatemia and hypophosphaturia. A disorder in mineral levels requires a substitution therapy with parathyroid hormones.

Some authors state that they have found fat tissue in place of parathyroid glands, while others state that during autopsy they did not find any parathyroid glands. There was a case of a two-and-a-half year old girl who had treatment for primary hypoparathyroidism, suprarenal gland insufficiency, hepatic cirrhosis, and was sent for autopsy because of rapid progression of lethal outcome. At the autopsy, serial intersection of both lobes and the isthmus of thyroid glands was performed but no tissue of parathyroid gland was found, neither macroscopically nor histologically (1).

Hyperparathyroidism

Hyperplasia of parathyroid glands

1. Primary hyperplasia

For a long time every nodus in parathyroid glands which was not cancer was considered to be adenoma. In 1958 the existence of primary hyperplasia within major cells of parathyroid glands was proven, and the fact that even though it affects all four glands, it may be more distinct in one or more glands, simulating adenoma in such a way. Since that time, a lot of studies have appeared which indicate the importance of primary hyperplasia of major cells as the cause of hyperparathyroidism, while, nowadays, some experts even consider that to be the most important cause of primary hyperparathyroidism. However, some other publications still show a greater frequency of adenoma. The results of 557 studies of primary hyperparathyroidism have shown that in over 80% of cases of primary hyperparathyroidism is caused by adenoma (2).

Primary hyperplasia of the major cells occurs in about 15% of patients suffering from primary hyperparathyroidism. It appears more often in the younger population, it can be present in the whole family, and in one sixth of the patients with primary hyperplasia other endocrine glands are also effected (multiple endocrine neoplasia).

The line viewed macroscopically in the earliest stage of hyperplasia shows rounding of the already flattened gland, which progressively turns into a spheroid shape. The glands can be normal size, with just a little bit of increase in size or they can increase significantly in size. A single hyperplastic gland can be only 100mg in weight while the combined weight of parathyroid glands may weigh between 1-25gm. Although all the glands are usually hyperplastic, the lower pair is usually bigger, and it is not rare to find one gland bigger than the others. In such cases macroscopic differentiation of adenoma is very difficult. In practice every gland that weighs more than 60mg is suspected as abnormal, although the surgeon can not specify in situ a minimal enlargement along these lines. Hyperplasia is usually homogenous and diffused but in long-term cases there is a tendency towards modularity. Hyperplastic nodes are clearly limited and in time they become contoured by fibrous septa and they show a uniform cytological image, but we can also encounter nodes which are constituted of several cytological types. Big nodes can be haemorrhagic or cystic.

There is a rare syndrome where the medullar cancer of the thyroid gland and pheochromocytoma are joined. In such situations parathyroid glands change in appearance, and that change usually results in primary nodular hyperplasia. This syndrome is called multiple endocrine neoplasia type 2 or Sipple syndrome.

2. Secondary hyperplasia

The hyperplasia of parathyroid glands is almost a regular find in chronic insufficiency of kidneys with azotemia, in hyperphosphatemia, and hypocalcemia. It can also be found in patients with renal acidosis, and with disorder of metabolism of vitamin D. In all of these cases hyperplasia is physiologic and reflects an excessive production of parathormones caused by long-term hypocalcemia. A long-term hypocalcemia leads to a continuous growth of para-thyroid glands.

3. Terciary hyperparathyroidism

The term hyperparathyroidism is introduced in order to explain the development of autoimmune hyperparathyroidism which occurs after a long-term secondary hyperparathyroidism. These changes occur in patients with chronic kidney diseases or in patients with intestinal malabsorption, in whom there is a development of parathyroid adenoma, and the patients become normocalcemic or hypercalcemic.

Tumors

1. Adenoma

Parathyroid adenoma is the most frequent cause of hyperparathyroidism and it is considered that it is in 80% of the cases responsible for primary hyperparathyroidism (3). We find adenoma in four out of five patients with primary hyperparathyroidism, while we encounter cancer in only 3% of cases (4). Parathyroid adenoma is practically always solitary and the finding of another tumor is very rare. Adenoma of parathyroid glands appears in a very wide range, but most frequently in the fourth decade of someone's life. It occurs much more frequently in women than in men, in relation 2.5:1 (5).

The size of adenoma varies within wide limits. They are usually small masses and their weight can be from 25mg to over 50g.

There is a correlation between parathyroid adenoma, values of the circulating parathormone and the level of hypercalcemia. It is stated that patients with changes on their bones tend to have bigger tumors, shorter duration of illness, and higher levels of calcium in the serum, while patients with renal calculosis on the average have smaller tumors, a longer history of illness and lower levels of calcium in serum. The difference be-tween those two groups may result from the differences in how fast the tumor grows, and differences in how fast the hormones are secreted. Macroscopically parathyroid adenomas are small, soft, smooth, oval in shape, dark red tumors, usually darker in color than normal parathyroid glands. The tumors are well encompassed by a fragile capsule beside which can be found various remains of preserved compromised parathyroid tissue. Dissection of tumors shows tissues of homogenous appearance of dark orange or dark red color. There are often found cystic spaces filled with clear, yellowish or haemorrhagic fluid, as well as bleeding parts or necrosis. Bleeding may be the result of surgical trauma caused by the rupture of the fragile walls of blood vessels.

2. Carcinomas

Carcinomas of parathyroid glands may develop within normal glands, in glands with primary hyperplasia within the major cells, or, and this occurs vary rarely, within the adenoma.

Carcinoma of parathyroid glands is very rarely the cause of primary hyperparathyroidism-that is only in 4% of all cases (6).

Unlike adenoma which develops in fourth and fifth decades of someone's life carcinoma appears in a time span between adolescence and very old age. Contrary to adenoma, carcinoma appears somewhat more frequently in male population than female. Most of the parathyroid carcinoma is endocrinologically active and associated with very high-level calcium in serum and with bone changes such as osteitis fibrosa cystica. Similarly to other endocrine tumors it is believed that there is also dysfunctional carcinoma of parathyroid kind, but it is very difficult to differentiate diagnostically carcinoma of thyroid gland and thymus.

As far as diagnosing the malignancy, the only certain criteria is, as is also the case with other endocrine tumors, invasion of adjacent structures (thyroid, trachea, esophagus) and spreading of metastasis.

Carcinoma of parathyroid glands varies in size from 2-40gm.

Macroscopically it can appear to resemble adenoma, but it can also appear as solid and irregular multinodular mass. The capsule is generally well defined and thick, and wide fibrous bands intersect the tumor tissue. At intersections the tissue is whitish, brownish-grey or brown in color. Metastases are generally found in regional lymph glands, with the lungs being affected along with the liver and the bones as well.

Surgical excision of parathyroid carcinoma is very difficult and therefore recurrences are very common. A lethal outcome is usually the result of a very serious hypercalcemia.

Surgery of parathyroid glands

Indications for surgery

Materials and methods

Primary hyperparathyroidism-regardless of whether the predominant signs are hypercalcemia syndrome, bone-joints, urological, digestive or other disturbances, all the patients with primary hyperparathyroidism (HTP) who have clinical disturbances have an indication for surgical treatment, unless there are transparent contraindications against surgery (7).

Special importance is attributed to patients with hypercalcemia crisis. For such patients there is an enormous risk during surgery, including general anesthesia, because of dehydration and hypercalcemia, so it is important that the patients undertake a number of conservative preparations before surgery such as infusion of physiologic solution, diuretics, and administration of phosphates. In cases of renal insufficiency, dialysis should be used as part of preparation for surgery, or possibly peritoneal dialysis.

One hundred and forty-seven patients from such a group were monitored and 20% of them were operated because their diseases progressed. Recent findings have affirmed that there is a risk of so called asymptomatic HPT (8).

Thirty-four percentage of patients who were monitored at least five years have developed very serious complications (ulcus, kidney insufficiency, hypercalcemia crisis) which were the cause of lethal outcome in four patients (9). That is why nowadays it is considered that the patient with asymptomatic HPT should undergo surgery, because the danger of further evolution of the disease is much greater than morbidity and mortality of cervical exploration.

Patients with normocalcemia primary HPT who have relapses of urinary lithiasis are also candidates for cervical exploration, but only after a very careful investigation which, if required, should be repeated many times.

Secondary and tertiary HPT – patients from this group are rarely considered for surgery, only if pharmacological treatment is ineffective. They often have changes in the bones, deposition of calcium in soft tissue, or unbearable pruritus.

Surgical strategy

There are several key elements which are responsible for successful surgery of HPT. They imply that the surgeon:

Must have enough experience so that he can, without great difficulty, find all four parathyroid glands during the operation. If one or more of them are missing, the surgeon must know where the most frequent aberrant locations are.

He should be able to differentiate between normal and pathologic parathyroid, and be sure to check his impression by intraoperative histological analysis (biopsy "ex tempore").

He must stand by the principle that the essence of surgical intervention is removal of diseased and saving of healthy parathyroid glands. The extent of resection is planned around the nature of pathologic changes, because the primary operation is the best opportunity for the cause of HPT to be definitely eliminated.

In most cases, viewed macroscopically, an experienced surgeon knows how to recognize the diseased gland-hyperplastic parathyroid is, as a rule, bigger, oval or round, hyperemic and dark red in color. Adenoma are usually solitary, dark in color, ranging from a few millimeters to a few centimeters in diameter, while at the same time the other glands are suppressed, paler, flattened and soft. Carcinoma is bigger in size, hard and adherent, and if there are at the same time metastasis in the regional lymphatic glands then intraoperative identification is not hard. In an early stage and when there is no infiltration of the surrounding organs, it is hard to differentiate carcinoma from adenoma of parathyroid, and even sometimes it is hard to differentiate adenoma from hyperplastic glands.

Numerous processes have been suggested in order to help differentiation of healthy from diseased parathyroid. Intravital imaging through infusion of toluidine blue was abandoned because of the toxic effects, while the methylene blue which mostly results in coloring the changed parathyroid glands is rarely used in practice.

Another, a newer test which is more widely used is the test of density (specific weight). The test is based on the fact that cells of a normal parathyroid gland contain much more intracellular fat than in the cases of hyperplasia or tumor, so thanks to that in solutions of mannitol of certain density (1.049-1.069) pathologically changed glands sink, and the healthy ones float on the surface. This test can be used for differentiation between adenoma and hyperplasia (10).

Nowadays the surgeons mostly rely on intraoperative histological analysis, which requires a well trained pathologist in this specific field of work. However, we must keep in mind that a competent pathologist has limited possibilities in diagnosing of adenoma, carcinoma and hyperplasia when using frozen cutouts. The surgeon expects reliable information whether a healthy or a pathologically changed parathyroid gland is in question, and that kind of conclusion is safest if it is based on the content of intracellular fats (11).

Technical principals – the surgeon should be relaxed and concentrated on this operation, without making plans for any other activities, and it should always be scheduled the first thing in the morning.

The patient should be well-situated on the operating table with the neck in hyperextended position. A low collar incision from one outer edge to the other outer edge of m. sternocleidomastoideus should provide fairly wide access. Meticulous hemo-

is applied in order to enable recognition of fine mic structures, such as parathyroid glands and n. recurrens, which is difficult and impossible when bleeding occurs. During the operation the patient should not lose more than a few cubic centimeters of blood.

In order to discover the parathyroid glands, a lobe of the thyroid gland should be freed and mobilized in front and inside, which will enable access into tracheoesophagus space. A. thyroidea inferior and n. recurrens must be found; as the basic anatomic marks they help us discover the position of parathyroid glands. Biopsy of the parathyroid gland should be cautiously executed on the antihilar side so that there is no damage of vascularization. If a. thyroidea superior is missing you should look for it in retropharyngeal, tracheoesophageal sulcus and retroesophageal space as well as intrathyroidally. If a. thyroidea inferior is not present in the expected space you should execute thymectomy through the existing cervical access (12).

Volume of resection-the volume of resection depends on the cause of the disease. The most common cause is adenoma, sometimes hyperplasia, and exceptionally rarely carcinoma (13).

Table 1. The causes of primary HPT (26)

Author	Number of operated	Adenoma	Hyperplasia	Carcinoma
Russell	500	79%	15%	0.4%
Cady	104	87.5%	11.5%	1%
Headman	839	87%	12.8%	0.2%

Table 2. Number of enlarged parathyroid glands in a 100 patients with primary HPT (27)

Number of enlarged glands	Number of patients
1	65
2	15
3	10
4	10

It is logical that the volume of resection is planned in a way that allows us to remove the diseased glands and save the healthy ones. When only one gland is contaminated, as is characteristic for adenoma, it is extirpated. The remaining glands in uniglandular form of disease macroscopically appear normal, or they are atrophic, so in that case it is enough to do biopsy on one of them so that the pathologist can intraoperatively confirm its normal structure.

In cases of parathyroid carcinoma the tumor is removed together with an appropriate thyroid lobe, accompanied by regional dissection of lymphatic nodes. It is important that in the course of preparation the tumor capsule is not opened, because the tumor cells are capable of local implantation which can lead to relapse (14). It is also best to surgically remove the distant metastasis if they are approach-able because they are hormonally active. These patients are directly threatened by hypercalcemia and the possibility of pharmacological treatment is very limited.

It is undoubtedly the hardest task to choose the right type of operation for patients with hyperplasia of all four parathyroid glands, whether we are talking about hyperplasia of major cells or bright cells. By executing the classical subtotal 31/2 parathyroidectomy we leave 40-60mg of well vascularized tissue in situ, and we can achieve normocalcemia in majority of patients, but relapses are not rare. Permanent hypoparathyroidism also appears. In order to reduce the risk of hypoparathyroidism in cases of subtotal parathyroidectomy to a minimum, it is necessary that after the identification of all four glands and histological verification first of all we recess the part of the gland which is intended to remain. Only when we are sure that its vitality has been preserved we can perform extirpation of the rest. If the removed tissue is subjected to deep freezing it can be saved 9 to 12 months and if the need arises it can be used for autotransplantation (15).

An alternative cure for hyperplasia of all parathyroid glands is total parathyroidectomy and autotransplantation 40-60 mg in the muscle. This method is particularly recommended when the whole of the family is effected and in cases of secondary hyperparathyroidism.

The technique in transplantation of parathyroid tissue-the technique used in transplantation is not complicated. Removed parathyroid gland is immediately after extirpation placed in freezing physiologic solution or in the solution for tissue culture. In the freezing physiologic solution, apart from the fact that the metabolism slows down to a minimum, the gland also achieves the necessary firmness, so that it can be easily cut into fragments of 1x1x2mm in diameter. After being previously histologically confirmed that it is parathyroid tissue indeed, every fragment is transplanted into the muscle tissue, in a special pouch formed by blunt separation of muscle fibers. At the same time bleeding within the muscle must not occur. In the beginning the transplant is nourished by diffusion and it takes several weeks for it to start functioning. The most common places for transplantation are m. sternocleidomastoideus in the neck region, and the brachioradialis muscle of the forearm of non-dominant arm. If no rough technical omissions occur, the transplant definitely becomes accepted. Up till now, many statements about relapses of HPT caused by hyperplasia in the transplant have been published. In such cases surgical excision is performed in local anesthesia because of the transplanted tissue (16).

Results

Effects of primary operations and the results of treatment

According to data from famous institutions worldwide which are specialized in dealing with this problem, in primary operation the surgeon is capable of finding the cause of hypercalcemia and correcting it successfully in 85-95% of patients with primary HPT (17).

By doing extirpation of the diseased parathyroid tissue, normalization of calcium in plasma is achieved very quickly. After a successful operation relapse may occur. Unlike in cases of persisting hypercalcemia, only cases in which the patient has been without hypercalcemia at least six months after the primary operation are marked as relapses.

Definitely the optimum results of surgical treatment of primary HPT are achieved in parathyroid adenoma. Extirpation of adenoma which has developed in only one parathyroid gland has registered definite healing in over 99% of patients (18).

Out of 3204 patients in whom the disease was caused by only one gland, relapse occurred in 24 patients (0.7%) at a later stage (19).

With parathyroid carcinoma relapse is quite common, although parathyroid carcinoma is very rare.

Reoperation

The need for reoperation occurs when in the primary operation the cause (persisting HPT) is not found and eliminated or because of relapse.

The major characteristic of reoperation is that it is performed in the scar and in altered anatomic relations. The possibility of finding parathyroid glands in repeated surgery is lesser than in primary operation, including more risks and complications caused by surgery.

After reoperation, hypercalcemia persisted in about 21% of patients, in 24% of them permanent hypoparathyroidism was developed, in one case a permanent tracheotomy was done because of bilateral recurrent nerve lesions, while 2 out of 35 of operated patients died because of intraoperative lesions of the larger blood vessels in the neck (20). Stated data illustrates how exceptionally delicate reoperations on parathyroid glands are, and demands caution when deciding on surgery, in the sense that: HPT diagnosis should be reviewed again. To patients with mild forms of disease and light hypercalcemia surgery is usually not recommended, only occasional thorough check-ups.

For patients where surgery is necessary one must do a detailed study of operative findings from the previous surgery and re-check pathohistological slides.

Reoperation is based on the same principles of operative techniques as the primary operation. Cervical approach is used with excision of the scar as opposed to the previous incision.

The side, on which the cause of the disease is probably to be found, is the side that is always firstly explored. When there are significant changes on the scar, exploration is usually done through the space between carotid space and thyroid glands. During surgery in over 50% of the cases parathyroid adenoma is found in the place which corresponds to the normal anatomic location (21). In these cases the localization of the diseased gland is subnormal or ectopic and is usually found in anterior or posterior mediastinum, intrathyroidally or in the carotid membrane. After a detailed exploration of the neck and transcervical thymectomy is done, and the cause of HPT not is found, we can begin with exploring mediastinum through longitudinal sternotomy. Sometimes reoperation begins with mediastinotomy, primarily if all four parathyroid glands in the neck were identified in the previous surgery, or if by using topographic diagnosis the presence of adenoma in the mediastinum was proven. Longitudinal sternotomy is used in about 20% of patients who undergo reoperation, although that percentage could be reduced (22). Namely, for some adenoma localized in the front mediastinum and extirpated through sternotomy, only when the tumor is found it becomes clear that it was available for extirpation by transcervical access (23).

Most of the ectopically localized adenoma in the frontal mediastinum is in close anatomic relation with thymus, so thymectomy is usually an integral part of mediastinum exploration. Adenoma localized in the posterior mediastinum can, by rule, be removed through the neck. If the cause cannot be found either in the neck or in the mediastinum, resection of one or both of thyroid lobes is considered because of possible intrathyroidal location.

The success of reoperation in treating primary hyperparathyroidism of HPT is rather smaller than the success in primary operations.

Hypercalcemia is corrected in 63-91% (24).

Hypercalcemia remains in 9-27% of patients who are reoperated, and it requires new exploration with higher risk of complications, or the disease progresses further and usually ends up with renal insufficiency.

Second significant deficiency of reoperative therapy of HPT is permanent hypoparathyroidism, which is registered in highly specialized institutions in 3-24% (25).

Chances that the patient may lose the parathyroid gland after reoperation are increased for two main reasons. First of all in primary surgery often normal parathyroid glands are removed as well but the adenoma which was not detected remains, and secondly because during reoperation there is a higher level of devascularization and traumatic damage of normal parathyroid tissue.

This problem is nowadays successfully overcome by the possibility of using direct autotransplantation and preservation of part of the parathyroid tissue by deep-freezing it, which can, at a later stage if necessary, be used for transplantation.

Discussion

Carcinoma of parathyroid glands is very rare, and in the structure of all malignant tumor diseases it shows in only 0.003%.

Clinical picture of carcinoma in parathyroid glands is very variable and depends on the fact if the tumor is functional or non-functional.

Surgical complications range from n. reccurens to hypoparathyroidism.

Conclusion

Because it is not very common, carcinoma of parathyroid glands is an insufficiently researched malignant tumor for whose treatment there are no clear instructions. Surgery remains the major form of treatment.

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TUMORI PARATIROIDNIH ŽLEZDA

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Paratiroidne žlezde su male endokrine žlezde u vratu čoveka koje sekretuju paratiroidni hormon ili parathormon (PTH), koji zajedno sa kalcitoninom i D-vitaminom ima primarnu ulogu u regulaciji koncentracije kalcijuma i fosfata u organizmu.

Najčešće oboljenje paratiroidnih žlezda predstavlja povećano i nekontrolisano lučenje PTH koje se može definisati kao primarni hiperparatiroidizam, ako nastaje kao posledica pojačane funkcije jedne ili više paratiroidnih žlezdi ili kao sekundarni hiprparatiroidizam koji se javlja najviše u hroničnoj bubrežnoj insuficijenciji ili kao posledica deficita vitamina D.

Karcinom paratiroidnih žlezdi je veoma retko oboljenje i predstavlja najređi maligni endokrini tumor.

Učestalost karcinoma kod bolesnika sa primarnim hiperparatiroidizmom je manja od 1%.

Intraoperativno preoznavanje ovog tumora od strane hirurga i adekvatna resekcija sa odstranjivanjem primarne lezije, uz patohistološku verifikaciju su najbolji tretman za bolesnike sa ovom primarnom lezijom.

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Ključne reči: *paratiroidne žlezde, neoplazme, hirurgija,operativne procedure, hiperparatiroidizam*

Review article

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THE IMPORTANCE OF THE PRECHTL METHOD FOR ULTRA-EARLY PREDICTION OF NEUROLOGICAL ABNORMALITIES IN NEWBORNS AND INFANTS

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Early detection of cerebral palsy and minimal neurological deficit symptoms in newborns and infants is of crucial importance since it enables timely inclusion of children in neurodevelopmental treatment which contributes to the improvement of motor functional status at a later age, taking into account a biological phenomenon known as "brain plasticity". Conventional methods of neurological assessment of newborns and infants include neurological examination, quantitative assessment of motor functions using scales and tests, and utilization of additional diagnostic neuroimaging procedures as well. All the aforementioned methods are absolutely necessary for wider use in clinical practice as well. They enable fast diagnosis of neurological disorders and fast detection of central nervous system impairments, but they also share a common defect - inability of making adequate prognosis of neurological deficits. Thanks to Prechtl's Method based on qualitative assessment of spontaneous motor activity, specific neurological patterns that are excellent predictors of a child's neurological development can be defined even in the prenatal stage, as well as after birth in preterm and term newborns. The aim of the paper is to review literature data on the possibilities and importance of Prechtl's method in comparison to other conventional methods for ultra-early identification of newborns and infants at risk of the development of permanent neurological deficit – cerebral palsy and/or minimal neurological dysfunction.

Prechtl's method is the most sensitive and specific clinical diagnostic procedure for the assessment of future neurological outcomes in high-risk newborns and infants.

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Key words: *newborn, infant, spontaneous motor activity, early diagnosis, cerebral palsy, Prechtl's method*

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Introduction

Motor development of newborns and infants is completely spontaneous according to the natural laws. It directly correlates with central nervous system (CNS) status - that is with its anatomical and functional adequacy. Motor development in a healthy child is determined by genetically established developmental patterns and environmental stimuli at the same time (1). Understanding normal motor development in newborns and infants and its clinical manifestation regarding motor activities is necessary for the detection and interpretation of certain developmental deviations (2). Developmental deviations are more common in newborns at neurorisk. A newborn at neurorisk is a baby born after a variety of risks during pregnancy. About 10% of newborns are considered to be at neurorisk (2).

Complications in newborns with neurorisk may be short- and long-term ones. Short-term complications are related to respiratory, cardiac, and gastrointenstinal complications. Long-term complications of the newborns, such as minor neurological dysfunction (MND) and cerebral palsy (CP) are particularly distinguished. Neurological assessment of newborns in everyday clinical practice includes classical neurological examination, scales and tests for discrimination, detection, elimination and evaluation of neurological deficits, as well as advanced diagnostic procedures, such as endocranial ultrasound (US) and mag-

netic resonance imaging (MRI). All the aforementioned methods are of great importance in everyday clinical practice. They allow rapid detection of the CNS damage and rapid establishment of the diagnosis as well, but they share a common defect, which is the inability to adequately predict neurological deficit.

Prechtl's method–qualitative assessment of spontaneous motor activity in newborns and infants

Heinz Prechtl, an Austrian neurophysiologist and professor, developed a standardized method for early identification of neurological anomalies in newborns and infants after several decades of research. This method is based on qualitative assessment of spontaneous motor activity (SMA). All the present studies have confirmed that Prechtl's method has high sensitivity and specificity in detecting neurological anomalies in newborns and infants, especially for MND and CP.

Prechtl started his research in the 1960's. He believed that motor activities of newborns and infants are rhythmic and cyclic, both in the intrauterine period and following birth, as confirmed by later studies. He also concluded that rhythmic locomotion patterns are generated at low levels of the CNS, in the bipolar cells of the spinal cord, known as central pattern generator (CPG). When activated, CPG produces patterns of rhythmic activity without receiving any sensory stimulus or descending stimulation from higher control centers. Examples of CPGs include central mechanisms for breathing, sucking, swallowing, and for locomotion crawling, walking and swimming.

Prechtl's studies have advanced rapidly since the introduction of ultrasound diagnostics and its intensive application in clinical practice. It has revealed important facts regarding intrauterine fetal motor activity. Prechtl identified whole-body movements in the period of 9^{th} and 10^{th} gestational week (they can clearly be seen with a transvaginal probe) and called them 'general movements' (GMs) (3).

General movements (gms)

Apart from the fact that general movements are endogenously generated, it is important to note that they are present from early foetal life and persist until 5 or 6 months after term. GMs are complex, they involve all parts of the body in a variable sequence of all the segments of upper and lower extremities, head, neck, and trunk movements. Their intensity, force and speed, wax and wane, show that there is a sequence of involved body parts movements and gradual beginning and end. Changes in the direction of movement make them look variable, complex and elegant. Up to term age GMs are called foetal or preterm movements by Prechtl. At term age until about 6 (sometimes 9) weeks post-term age he named emerging GMs "writhing movements" (wriggling, twisting, stretching). These movements are characterized by moderate amplitude and moderate speed. Their form is elliptical, giving the impression of having a writhing characteristic. At 6 to 9 weeks post-term age, writhing movements gradually disappear and new GMs emerge, called by Prechtl "fidgety" movements (restless, uneasy). "Fidgety" movements are small movements of moderate speed and variable acceleration of the head, neck, trunk, and limbs, primarily involving their distal parts (radiocarpal and talocrural joint). They are manifested continually in the awake infant, except during crying. These movements can be seen as early as 6 weeks post-term, but they usually occur around 9 weeks and are present until 20 weeks or a few weeks longer. After that, voluntary and antigravity movements occur and dominate (3).

Pathological patterns of general movements

When it comes to newborns and infants with CNS damage, their SMA patterns change in comparison to infants with an intact brain. The studies conducted on SMA in foetuses with CNS damage reported that the character of SMA changes towards abnormal patterns and various forms of the CNS damage were confirmed after birth in these subjects. These studies show that SMA assessment may be a window into the CNS integrity. In infants with compromised CNS, the quantity of movements does not change in comparison to healthy population, but the quality of movements does. If the nervous system is impaired, SMA loses the characteristic complexity and variability. Prechtl classified abnormal writhing movements into three groups:

1. Poor repertoire writhing movements (PR) (4),
2. Cramped-synchronized writhing movements (CS) (5),
3. Chaotic writhing movements (Ch).

Prechtl described abnormal fidgety movements as:

1. Absent fidgety movements (F-) (6),
2. Abnormal fidgety movements (Ab F).

Poor repertoire writhing movements (PR) occur during preterm, term age and early post-term period. This model of writhing movements is characterized by less variability and the sequence of movement components that are monotonous and do not occur in a complex way, unlike their presentation in normal writhing movements. A newborn starts a movement, but does not complete it, giving the impression of an interrupted sequence. PR is more common in children with brain abnormalities. The predictive value of these movements is low. Later in life they can be followed by either normal or pathological motion patterns.

Cramped synchronized writhing movements (CS) occur from preterm period onwards. This type of pathological writhing movements is characterized by simultaneous contraction and relaxation of the trunk and upper and/or lower limb muscles. If these abnormal movements are carefully observed and if they exist over several weeks, they may be of great importance in predicting definitive neurological outcome in a newborn (5). These movements are of high predictive value for the development of CP, especially if they persist even after 9 weeks PMA.

Chaotic writhing movements (Ch) are abrupt, large amplitude movements of upper and lower

limbs that are in chaotic order without any fluency or regularity. Chaotic movements can be observed in preterm, term, and early post-term period. Newborns with chaotic movements often develop cramped synchronized movements a few weeks later (3).

The absence of fidgety movements (F-) has high predictive value for later development of neurological impairments (6). In case fidgety movements do not occur in the period from 9-20 weeks post-term, there is a high probability of spastic and dyskinetic cerebral palsy development at a later age. If cramped synchronized movements persist during 3rd and 4th month or longer, fidgety movements do not occur.

Abnormal fidgety movements (AbF) resemble normal fidgety movements, but their amplitude and speed are moderately or greatly exaggerated. Abnormal fidgety movements are rare and their predictive value is low.

Assessment of spontaneous motor activity in newborns and infants

The qualitative assessment of SMA in newborns and infants can only be performed by a certified examiner for this examination technique. Training courses for the qualitative assessment of SMA are educational programmes conducted by licensed tutors. After the verified completion of the training for the application of this method of evaluation, the interobserver agreement on pattern recognition of SMA in newborns and infants is 89-93% (3).

The simplest way of observation and assessment of SMA is by direct naked eye observation of the movements. Of course, higher reliability of the assessment is achieved when SMAs are evaluated by means of video recording, since it enables multiple evaluations during playback of the video recording. Adequate behavioural state of a newborn required for proper SMA observation includes eyes open, absence of crying, irregular respirations and general movements present (8).

Conclusion

The usability of the Prechtl Method is also proved by the fact that no studies have confirmed so far that normal general movements are followed by CP as a neurological outcome, on condition there were no serious interval complications. There are no cases with false negative findings. A study observing SMA in children with Rett's syndrome showed that PR may be indicative of later development of both MND and CP (9). The occurrence of CS "writhing" movements during preterm and early post-term period, as well as the absence of 'fidgety' movements in infants is predictive of spastic cerebral palsy, while abnormal "fidgety" movements are not predictive of CP, but they are associated with MND later in life (10).

Thanks to the Prechtl Method it is possible to predict normal neurodevelopmental outcome with high probability by using observation and detection of SMA normal patterns in newborns and infants. Also, by observing and detecting pathological patterns of movement, it is possible to recognize specific neurological symptoms in prenatal period (US diagnostics), or after birth in preterm and term neonates, which are excellent indicators of later development of CP (11, 12, 13, 14). Early detection of MND and CP symptoms has enormous clinical significance since it enables timely inclusion of children into neurodevelopmental treatment, thus contributing to the improvement of motor functional status at a later age, taking into account a biological phenomenon known as "brain plasticity".

Since there are only 6 medical doctors in Serbia with a certificate of reliability in assessing SMA using the Prechtl Method, the aim of this paper was also to point out the necessity of including more physicians in the field of pediatric pathology into educational programme for early recognition and timely treatment of neurological abnormalities in children.

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ZNAČAJ PREHTLOVE METODE U ULTRARANOJ PREDIKCIJI NEUROLOŠKIH ABNORMALNOSTI KOD NOVOROĐENČADI I ODOJČADI

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Rana detekcija simptoma cerebralne paralize i minimalnog neurološkog deficita kod novorođenčadi i odojčadi od krucijalnog je značaja, jer omogućava pravovremeno uključivanje dece u neurorazvojni tretman, što doprinosi poboljšanju funkcijskog motoričkog statusa u kasnijem uzrastu, računajući na biološki fenomen "plasticiteta mozga". Konvencionalne metode neurološke procene novorođenčeta i odojčeta podrazumevaju neurološki pregled, kvantitativnu procenu motoričkih sposobnosti pomoću skala i testova, kao i korišćenje dopunskih dijagnostičkih neuroimidžing procedura. Sve nabrojane metode svakako su neophodne i u širokoj su kliničkoj primeni i služe za brzo postavljanje dijagnoze neuroloških oboljenja ili detekciju oštećenja centralnog nervnog sistema, ali imaju jedan zajednički nedostatak - nemogućnost adekvatne prognoze neurološkog deficita. Zahvaljujući Prehtlovoj metodi, koja se zasniva na kvalitativnoj proceni spontane motoričke aktivnosti, moguće je definisati specifične neurološke obrasce, čak i prenatalno kod fetusa, kao i postnatalno kod preterminskog ili terminskog novorođenčeta, koji su odlični pokazatelji kasnijeg neurološkog razvoja deteta. Cilj rada je revijski prikaz podataka iz literature o značaju i mogućnostima Prehtlove metode naspram drugih konvencionalnih metoda u ultraranom prepoznavanju rizične grupe novorođenčadi i odojčadi za razvoj trajnog neurološkog deficita – cerebralne paralize i/ili minimalne neurološke disfunkcije.

Prehtlov metod spada u najsenzitivniju i najspecifičniju kliničku dijagnostičku proceduru u proceni budućeg neurološkog ishoda rizične grupe novorođenčadi i odojčadi.

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Ključne reči: *novorođenče, odojče, spontana motorička aktivnost, rana dijagnoza, cerebralna paraliza, Prehtlov metod*

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PROSTATE-SPECIFIC ANTIGEN DYNAMICS IN DIAGNOSIS OF PROSTATE CANCER

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Prostate-specific antigen (PSA) is now globally applied as the best serum marker for diagnosis and monitoring of prostate cancer (CaP), but with certain limitations in terms of specificity and sensitivity. The upper limit for a normal PSA level of 4 ng/ml was suggested in 1986. In the PCPT study, PSA sensitivity (for a 4 ng/ml limit) was 21% for all types of CaP, or 51% for high grade carcinoma, with only one third of patients with high PSA value having CaP. However, it has been shown that there is a continuum of risk, in which patients with higher PSA values have a higher risk for CaP. In order to increase sensitivity (increase in the number of diagnosed CaP) and specificity (reduce the number of unnecessary biopsies), there was a need for development of other parameters: PSA doubling time, PSA velocity, f/t PSA, PSA density, Prostate Health Index-PHI, 4K score test. Also, in order to optimize patients for CaPscreening, so-called nomograms and risk calculators have been created. It is still questionable whether PSA screening has an impact on patients' survival, and two of the largest, randomized, prospective studies (ERSPC and PLCO) could not resolve this question. While ERSPC showed a 27% reduction in mortality after 13 years of follow-up, PLCO study did not show the benefit of screening on tumor-specific mortality.

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Key words: *prostate-specific antigen, prostate cancer, screening*

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Introduction

In order to successfully treat patients with prostate cancer, it is necessary to implement early diagnosis, adequate risk assessment and optimal treatment. In recent decades, great effort was made to find reliable and easily measurable tumor marker that could be used for early detection, staging and monitoring of the disease on a large scale. Prostate-specific antigen (PSA) is now applied globally as the best serum marker for diagnosis and monitoring of prostate cancer, but with certain limitations in terms of specificity and sensitivity (1). Elevated serum PSA, except in prostate cancer, can often be registered in benign prostatic hyperplasia and inflammatory conditions. On the other hand, significant number of patients with prostate cancer may have normal PSA value (2). It is not often possible to make a difference between indolent and aggressive prostate cancers by measuring PSA level (3).

History of psa

There is a lot of controversy related to the discovery of this marker, and there is no consensus about who came to its invention first. The scientist who first conducted experiments on prostate tissue antigens was Rubin Flocks (1960.) (4). Hara et al. discovered the prostate-specific protein in the seminal fluid and named it gamma-seminoprotein (1966.) (5). However, most of the scientific community believes that the first discoverer of PSA was American scientist Richard Ablin in 1970. He isolated the antigen exclusively localized in prostatic tissue (normal, hyperplastic or malignant) which was immune and histochemical different from prostatic acid phosphatase, which was used as a diagnostic marker for prostate cancer at that time (6). The presence of PSA in serum was first registered by Papsidero et al. in 1980, which proved that the value of PSA in serum and prostatic tissue is identical (7). Thomas Stamey and colleagues came to a revolutionary discovery in 1987, by proving that the level of serum PSA correlates with prostate cancer stage and tumor size (8). That same year, the PSA was introduced into clinical practice and approved by the FDA (Food

and Drug Administration) as a marker for prostate cancer monitoring. PSA have been also used as a marker for prostate cancer screening since 1994. (9).

Initial psa as diagnostic parameter

Cut-off for normal PSA value with 4ng/ml was suggested in 1986, after a study on a small group of men (472) who did not have CaP (10). Cooner et al. in a study of 1807 men over the age of 50 years, concluded that PSA > 4ng/ml in presence of abnormal DR finding may be a predictive parameter for CaP (11). The same results were published in two additional studies by Catalone et al. and Brawer et al. in 1991 and 1992. This conclusion was also published in two large studies in 1992 (12, 13). PSA was approved by FDA as a screening marker for CaP after Catalone et al. suggested the cut-off value of 4ng/ml for all age groups (14). Studies have shown that initial PSA is significant, independent, diagnostic parameter for CaP.

However, it was registered that CaP was not a rare case in patients with PSA < 4 ng/ml. In PCPT study (Prostate Cancer Prevention Trial) 5519 men older than 55 years, which had PSA ≤ 3 ng/ml and normal DR finding, 7-year surveillance was conducted, whereby PSA value and DR examination were conducted annually. In case of abnormal DR finding and PSA ≥ 4ng/ml prostate biopsy was conducted, while in patients in whom CaP was not diagnosed at the end of the study, biopsy was done after a 7-year surveillance period. Biopsy was positive in 15% of men who had PSA ≤ 4ng/ml, while 15% of them had high-grade cancer (Gleason score ≥ 8). Sensitivity (for a cut-off value of 4 ng/ml) was 21% for all CaP types, and 51% for high-grade CaP. Specificity was 91%, and positive predictive value about 30%, which means that every third male with PSA ≥ 4ng/ml had CaP (15). It is evident that there is no PSA threshold below which we can be absolutely certain that the patient does not have CaP. However, it was shown that there is a risk continuum, in which patients with higher PSA levels have a higher risk for developing CaP (16) (Table 1).

Increasing sensitivity and specificity of psa

Since the initial use of PSA in the diagnosis of CaP showed some limitations, there is a need for other parameters that could possibly increase the sensitivity (increased number of diagnosed cancer) and specificity (reduce the number of unnecessary biopsies). One way to improve the specificity of PSA is setting the cut-off value in relation to age, given that the value of PSA increases with age. Therefore reference cut-off values are recommended for specific age-groups and racial affiliation (17) (Table 2).

Table 1. Continuum of risk for prostate cancer also exists at low PSA levels

PSA value	No of patients (N = 2950)	No of patients with CaP (N = 449) No (%)	No of patients with high grade CaP (N = 67) No of high grade CaP/No of all CaP (%)
≤ 0.5 ng/ml	486	32 (6.6)	4/32 (12.5)
0.6 – 1.0 ng/ml	791	80 (10.1)	8/80 (10.0)
1.1 – 2.0 ng/ml	998	170 (17.0)	20/170 (11.8)
2.1 – 3.0 ng/ml	482	115 (23.9)	22/115 (19.1)
3.1 – 4.0 ng/ml	193	52 (26.9)	13/52 (25.0)

Table 2. PSA reference values by age groups and racial background

Age groups (years)	Americans of Asian origin (ng/ml)	African Americans (ng/ml)	Caucasian race (ng/ml)
40 – 49	0 – 2.0	0 – 2.0	0 – 2.5
50 – 59	0 – 3.0	0 – 4.0	0 – 3.5
60 – 69	0 – 4.0	0 – 4.5	0 – 4.5
70 - 79	0 – 5.0	0 – 5.5	0 – 6.5

Change of PSA value in time can serve as a diagnostic parameter. PSA Doubling Time (PSADT) is defined as the time period for which the PSA value is doubled compared to the initial value (18). It was shown that this parameter has poor diagnostic, but more significant prognostic value, particularly in patients with biochemical recurrent CaP (19). PSA velocity (PSAV) is an absolute annual increase in serum PSA expressed in ng/ml/year. Studies have shown that an increase in PSA greater than 0.75 ng/ml/year increases the risk of CaP in subjects whose initial PSA values were between 4-10 ng/ml (20). Some authors suggest that the cut-off value of PSAV should be complied with age. The proposed cut-off values of PSAV for the age group between 40-59 years was 0.25 ng/ml, 0.5 ng/ml for the age-group between 60-69 years, and 0.75 ng/ml for men over 70 years (21). However, unlike the initial PSA value, PSAV is not significant, independent diagnostic parameter for CaP (22, 23).

The ratio of free and total PSA (f/t PSA) is a very important parameter in the differential diagnosis between BPH and CaP, in the case where the value of PSA is in so called "gray zone" (4-10 ng/ml). CAP patients have smaller concentrations of free PSA compared to those with BPH. CaP was diagnosed in 56% of patients with f/t PSA < 0.1, and only in 8% of those with f/t PSA > 0.25. It is believed that the f/t ratio has no diagnostic significance if the PSA value is > 10 ng/ml (24).

The ratio between prostate volume and PSA has a certain diagnostic value and can be calculated when the value of PSA is divided by the total prostate volume (PSA density, PSAD) or by a volume of prostate transitional zone (transitional zone PSA density, TZPSAD), measured using a transrectal ultrasound or magnetic resonance imaging. Given that increased prostate volume (benign or malignant) causes a progressive increase in PSA level, using this parameter specificity of PSA test can be increased and number of unnecessary biopsies can be reduced. It was proposed that value of PSAD > 0.15 may be predictive for CaP (25). It is proved that PSAD has a higher diagnostic value if serum PSA is < 4 ng/ml, while when PSA value is between 4-10 ng/ml f/t PSA is more important diagnostic parameter (26). Chen et al. found that PSAD is superior to f/t PSA, when it comes to reducing the number of unnecessary repeated biopsy in patients with PSA levels of 4-10 ng/ml (27).

Newer studies on free PSA fraction, a marker that has significantly increased specificity of PSA test, led to the realization that he can exist in at least 3 different forms: benign PSA, intact PSA and the proPSA (28). Studies have shown that proPSA is one of the forms that could be of great importance in the diagnosis of CaP, especially its most stable serum isoform - p2PSA. This isoform is primarily present in the peripheral zone of prostate, and slightly in the transition zone of the prostate. In addition, serum p2PSA levels were significantly higher in patients with CaP than in patients without cancer (29). In order to increase the sensitivity and specificity of the test, a mathematical algorithm called Prostate Health Index (PHI) was developed, which incorporates tPSA, fPSA and p2PSA values, and is defined by the formula: PHI = (p2PSA / fPSA) x (tPSA) ½ (30). Compared to standard markers for the detection of CaP which showed a lot of uncertainty, p2PSA and PHI were imposed as a potentially better and more specific for the detection of CaP, particularly in PSA levels of 2-10 ng/ml (31).

4K test score is one of the newest diagnostic tests, in which by using four individual kallikreins (total, free, intact PSA and kallikrein-related peptidase 2) the risk of aggressive CaP is determined. This test combines test results with data such as age, DR finding and previous prostate biopsy finding. Large prospective studies showed significant predictive value of this test for poorly differentiated CaP (Gleason score ≥ 7) (32).

Advantages and disadvantages of global application of psa screening CaP

Screening for prostate cancer has an opportunistic character, which means non-systematic testing in men who themselves appear to urological examination. There is no doubt that the PSA era has led to increased detection of CaP, especially in the earlier stages, as well as a significant reduction in metastatic disease (33). When it comes to the impact on survival, two largest, randomized, prospective studies could not answer the question if massive use of PSA is justified or not: ERSPC (European Randomized Study for Prostate Cancer) and PLCO (Prostate, Lung, Colorectal and Ovary trial).

While ERSPC after 13 years of follow-up showed a reduction in mortality of 27%, the PLCO study showed no benefit of screening in terms of tumor-specific mortality. However, there are certain differences in methodological approach between these two studies that should be noted.

PLCO study involved 76,693 respondents aged between 55-74 years, which had PSA test and DR examination carried out once a year. ERSPC study, in which the results of several small studies were summarized, included 162, 243 respondents aged between 55-69 years, while the measurement of PSA was done mainly on a 4 year-interval. Almost half of the men before pulling into the PLCO study underwent PSA testing, unlike ERSPC study, where subjects were not previously screened. The PLCO study also recorded slightly lower compliance of patients in the screening group, in terms of responding to urological examination and prostate biopsy, and also a higher percentage of respondent contamination in the control group (52%). However, the major methodological diversity between two studies was PSA threshold taken as a trigger for prostate biopsy. PSA > 4 ng/ml or abnormal DR finding were an indication for biopsy in the PLCO study, while in ERSPC study, PSA value of 3 ng/ml was used in most cases as a threshold. Regardless of this fact, two studies demonstrated an increased incidence of CaP in a screening group compared to the control group. The largest percentage of these cancers was localized, well-differentiated cancers (34, 35). The fact that a large number of these indolent cancers were subjected to some form of active treatment is indicative of so-called overdiagnosis and overtreatment problem. In other words, due to discovery of a

large number of clinically insignificant CaP (overdiagnose), a significant number of these patients are subjected to treatment that does not cause prognostic benefit and post-treatment complications can often reduce the quality of life of patients (overtreatment) (36). It is estimated that it is necessary to implement screening of 781 men, and actively treat 27 men diagnosed with CaP, in order to directly prevent one death from CaP (34).

In order to optimize patients for CaP screening, there was a need for the creation of so-called risk calculators and nomograms. They represent a special scheme which contains parameters such as: age, DR finding, race, family history, previous prostate biopsies, tPSA and fPSA, and based on these data, calculate the risk for CaP and its aggressive form. In accordance with these findings, recommendations are given for further follow-up, or if there is an indication, prostate biopsy is considered. The ultimate goal is to reduce the overdiagnosis problem, but simultaneously to reduce the tumor-specific mortality by aggressive and poorly-differentiated tumors being diagnosed at an early stage (37). Several nomograms have been presented in current practice but none of them shown superiority in comparison to others (38). Memorial Sloan Kettering Cancer Center has recently outlined the scheme for CaP screening: begin screening at age 45; if PSA is < 1 ng/ml repeat testing in 6-10 years; if PSA is ≥ 1 and < 3 ng/mL repeat testing in 2-4 years; if the PSA ≥ 3 ng/ml prostate biopsy is considered. In making decisions for prostate biopsy, we should consider: risk factors (family history, racial origin), previous prostate biopsies, PSA dynamics and whether there is an inflammatory component as the cause of PSA elevation (antibiotic prophylaxis). Screening should be discontinued at age 60 if PSA value is ≤ 1ng/ml, or at age 75 if the PSA level in the normal range (39).

Conclusion

The widespread use of PSA has led to the discovery of a large number of indolent cancers, which led to the problem of overdiagnosis and overtreatment. On the other hand, CaP in its aggressive form continues to cause significant morbidity and mortality in the male population. In the absence of hard evidence about the benefits of global screening, but with due caution when taking into account the positive effects of PSA testing, opportunistic screening should be conducted. Therefore, it is necessary to stratify patients in groups on the basis of initial PSA and other parameters, and based on that, to propose a scheme for determining PSA individually.

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DINAMIKA SPECIFIČNOG ANTIGENA PROSTATE U DIJAGNOSTICI KARCINOMA PROSTATE

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Prostata specifični antigen (PSA) danas se globalno primenjuje kao najbolji serumski marker za dijagnostiku i praćenje karcinoma prostate (CaP), ali sa određenim ograničenjima, u smislu specifičnosti i senzitivnosti. Gornja granica za normalne vrednosti PSA od 4 ng/ml predložena je 1986. godine. U PCPT studiji senzitivnost PSA (za graničnu vrednost od 4 ng/ml) bila je 21% za sve tipove CaP, odnosno 51% za karcinome visokog gradusa, pri čemu je tek svaki treći muškarac sa visokim vrednostima PSA imao CaP. Ipak, pokazano je da postoji kontinuum rizika po kojem bolesnici sa većim vrednostima PSA imaju veći rizik od oboljevanje od CaP. U cilju povećanja senzitivnosti (povećanja broja dijagnostikovanih karcinoma) i specifičnosti (smanjenja broja nepotrebnih biopsija) javila se potreba i za drugim parametrima: PSA doubling time, PSA velocity, f/t PSA, PSA density, Prostate Health Index – PHI, 4K score test. Takođe se, u cilju optimizacije bolesnika za skrining CaP izrađuju i tzv. nomogrami i kalkulatori rizika. Kada je reč o uticaju skrininga PSA na preživljavanje, pitanje opravdanosti masovne upotrebe PSA nisu uspele da razreše ni dve najveće, randomizovane, prospektivne studije: ERSPC studija i PLCO studija. Dok je ERSPC nakon 13 godina praćenja pokazala redukciju mortaliteta za 27%, studija PLCO nije pokazala korist skrininga u pogledu tumor specifičnog mortaliteta.

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Review article

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HYPERSENSITIVITY POTENTIAL OF GYNECOLOGICAL DEVICES

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Medical devices encompass an extremely wide range of products used in variety of settings for the diagnosis, prevention, monitoring or treatment of illness or disability. Development of medicine and technology causes constantly increasing number of different medical devices with characteristics corresponding to biomaterials and whose application can lead to development of hypersensitivity reactions. Despite the fact that gynecology is a wide field for biomaterials applications, there are no summarized data about hypersensitivity reactions to gynecological devices. This paper gives an overview of hypersensitivity potential and common clinical manifestations of medical devices that are specifically used in gynecology. Summarizing these data is very important for improvement of current medical practice and also for designing and creating new medical devices.

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Introduction

Medical devices encompass an extremely wide range of products used in variety of settings for diagnosis, prevention, monitoring or treatment of illness or disability (1). Development of medicine and technology causes constantly increasing number of different medical devices, with characteristics corresponding to biomaterials. They are most commonly used in orthopedics, maxillofacial surgery, dentistry and vascular surgery as artificial joints, bone plates, bone cement, artificial ligaments and tendons, dental implants, dental filings, heart valves, vascular grafts, pacemaker leads, catheters, drug delivery systems etc. (2). Developments in medical devices are enhanced rapidly by technological advances in diverse fields, such as biomaterials science, bioengineering, electronics, software and IT.

Gynecology and obstetrics are dedicated to women's health and also represent a wide field of biomaterials application. Depending on application, biomaterials used in gynecological practice include: biomaterials used in contraception, biomaterials used for prevention and treatment of infertility, biomaterials that are used during labor and delivery, and biomaterials used in gynecologic surgery (3).

Since biomaterials represent foreign bodies, they can lead to various desirable and undesirable reactions when put in contact with a human organism. One group of adverse and undesired reactions to medical devices represents hypersensitivity reactions.

Despite the fact that gynecology is a wide field for biomaterials applications, there are no summarized data about hypersensitivity potential and hypersensitivity reactions to gynecological devices.

The aim of this paper is to give an overview of hypersensitivity potential and common clinical manifestations of medical devices that are specifically used in gynecology. Summarizing these data is very important for improving current medical practice and also for designing and creating new medical devices.

Biomaterials and hypersensitivity reactions

According to Second Consensus Conference on Definitions of Biomaterials, biomaterials represent a wide group of natural or artificial "materials that are intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function in the body" (4).

In the preclinical safety biomaterials evaluation, examination of biocompatibility is the first and

the most important step in biomaterials testing. It includes physico-chemical characterization of biomaterials, evaluation of physiological environment effects on materials and effects of materials on the environment through different aspects. Biodegradability, reactions between the tissue and biomaterials, cytotoxicity, genotoxicity, mutagenicity etc. can be predicted trough different tests validated *in vitro* and *in vivo* (5, 6, 7, 8), while the problem of hypersensitivity prediction in pre-clinical phase of biomaterials testing still persists.

Hypersensitivity reactions are very common and extensive health problems, to which physicians from almost every field of medicine face in everyday practice. They are considered as excessive and inappropriate immune responses to presence of an antigen (9). A precondition for developing hypersensitivity reaction is previous sensitization of organism to a specific antigen. Manifestation of the hypersensitivity reaction occurs after re-contact of the organism to the antigen to which it is sensitized.

Clinical manifestations of hypersensitivity reactions are very diverse and many of them are mild, while others can be severe and life-threatening. They can be confined to a small area of the body, or may affect the entire body (10).

The first step towards successful treatment of allergic reactions is determining allergen that provoked hypersensitivity reaction. If the allergen remains undetected, the therapy is symptomatic, and this could further lead to recurrence or persistence of symptoms and further impairment of health and quality of life.

Depending on generated effectors, molecules and mechanisms of their action, four types of hypersensitivity reactions have been clearly defined so far (Type I—IgE mediated hypersensitivity, Type II—cytotoxic—IgG/IgM mediated hypersensitivity, Type III—immune complex mediated—IgG/IgM immune complex and Type IV—delayed hypersensitivity or cell mediated hypersensitivity) (11), while the fifth type is still a subject of speculations (12). Type I and Type IV are the only two types of hypersensitivity reactions which are described as an undesirable response to biomaterials.

For confirming a diagnosis of hypersensitivity reactions, several validated *in vitro* and *in vivo* tests are used (skin test prick, *in vitro* measurement of specific IgE antibodies, cutaneous patch testing, lymphocyte transformation tests etc.) (13, 14, 15, 16, 17). However, there are no validated *in vitro* or *in vivo* methods for screening sensitizing potential in the pre-clinical phase of biomaterials testing so far. The problem of immunologically-based hypersensitivity reactions non-predictability is related to lack of appropriate experimental models, because beside biomaterial composition, individual physiological characteristics of the host organism are of primary importance for development of hypersensitivity reactions (18).

Predicting hypersensitive potential in pre-clinical phase of biomaterials testing is currently based on clinical experience and previously published data about confirmed hypersensitivity reactions to specific components of medical devices.

Gynecological devices

In 2015, 64 percent (%) of married women or common-law wives in reproductive age worldwide were using some kind of contraception (UN 2015) (19). Globally, 22.8% of women using contraception use intrauterine devices (IUDs) (20). Its application includes introducing device into uterus, where it persists several years and where its mechanical and/or chemical action provokes contraceptive effect. Today, two types of IUDs are in use: copper-releasing devices and hormone-releasing devices (21). Both types of IUDs consist of different metallic and polymeric components with different hypersensitivity potential (22).

Nickel, cobalt and chromium are the three most common metals that elicit both cutaneous and extracutaneous allergic reactions from chronic internal exposure (23). In copper releasing devices, approximately 99% of metal components represent copper, while other metal components include nickel, silver and gold (24, 25). Sensitizing capacity of copper sulphate is very low (26), but contact dermatitis and urticaria (27, 28) and endometritis and urticaria-angioedema syndrome in women wearing a copper-containing IUDs have been reported (29). It is interesting that allergy to copper sulphate is usually not monovalent, and is commonly associated with other metal allergies, especially with nickel and cobalt sensitization (22); in these reports, only monosenzitisation to copper was confirmed. Besides the fact that nickel in IUDs is usually present in very small quantity, and that there are still no reports on hypersensitivity to this component of IUDs, clinical experience suggests that its vast hypersensitivity potential should not be ignored (23).

Hormone releasing IUDs are made of a polymer frame with a central reservoir containing levonorgestrel. Levonorgestrel, a highly potent second generation progestin, thickens cervical mucus and suppresses endometrial proliferation (preventing decidualization of the stroma). This creates a hostile environment for sperm survival, inhibiting motility and capacitation with the net effect combining to prevent fertilization (30). Polymeric components of hormone releasing IUDs often include polydimethylsiloxane, polyethylene, polypropylene and colloid silica, while metal components often include barium sulphate, iron oxide, silver and copper (24, 25). Chen et al. 2014 (31), reported a case of acute urticaria associated with Mirena® implantation, while both Pereira and Coker 2003, (32) and Karry et al. 2006 (33) reported cases of acute dermatitis related to application of Mirena®. No one of the authors analyzed sensitization to specific components of this type of IUDs, but according to the literature data all components of Mirena® possess hypersensitivity potential (34, 35, 36, 37).

Permanent tubal sterilization is a method for irreversible contraception and involves laparoscopic tubal ligation or permanent obstruction of fallopian tubes using tubal devices. For permanent obstruction of fallopian tubes, there are two types of devices with different composition commonly used: inserts composed of polyethylene terephthalate, stainless steel and nickel titanium alloy, and inserts com-

posed of cured silicone (38). Application of both types of intrauterine devices is accompanied with numerous desirable and undesirable, but frequently present, side effects. There are several reports on hypersensitivity reaction to nickel in women after permanent tubal sterilization by Essure® (39, 40). On the other side, there are no publications about adverse effects to inserts composed of cured silicone in terms of hypersensitivity reactions, which suggests lower hypersensitivity potential compared to the previous type of inserts.

Today, one of the options for long lasting reversible contraception is application of subdermal implantable devices. All currently available implantable contraceptive devices are based on the same principle: progestogen hormone is released from one or more biologically inert tubes that are placed in subdermal layer of upper inner aspect of the woman's non-dominant arm. Biocompatible polymers or copolymers of polydimethyl/ polymethyl vinyl-siloxanes or ethylvinylacetate are used for making biologically inert tubes, to hold the steroid crystals and control the rate of progestogen hormone release (41). Up to date, there are several reports of hypersensitivity reactions to Nexplanon® (42, 43, 44) in the form of erythaema, oedema and local itching at the site of insertion. Nexplanon® consists of etonogestrel, ethylene vinyl acetate copolymer and barium sulfate, and authors have associated described hypersensitivity reactions to barium sulfate. Hypersensitivity to barium sulphate is extremely rare (2 per million) (43). It is interesting that in most of these cases, before the use of Nexplanon, women used other contraceptive devices (Implanon, Mirena ...). Some authors confirmed specific hypersensitivity reactions to barium-sulphate, while others did not, and at the same time they did not evaluate possibility of hypersensitivity to other components of this implantable device (45).

Induction of labor is artificial initiation of labor before its spontaneous beginning for the purpose of delivery of the feto-placental unit. The rate of labor induction varies by location and institution, but appears to be increasing. If the cervix is unfavorable, cervical ripening is warranted prior to labor induction. Some of current mechanical methods of cervical ripening include application of hygroscopic dilators (e.g. Laminaria, Dilapan-S®, Lamicel etc.). Laminaria is natural dilator made from dried seaweed, while Dilapan-S® and Lamicel, are produced from synthetic hygroscopic material. So far, there have been several reports about severe anaphylactic reactions to laminaria (45, 46, 47) in patients with at least one previous pregnancy terminated with laminaria. The main hypothesis is that the allergen responsible for IgE reactivity with anaphylactic potential was the carbohydrate component of this plant, called laminarin (47). On the other hand, hypersensitivity reactions to hygroscopic dilators were not described in the literature.

Over past decades, the use of synthetic biocompatible materials has become more common in gynecologic surgery. The most common procedures involving use of synthetic meshes are the abdominal sacrocolpopexy, suburethral sling, retropubic urethropexy, adhesion prevention and pelvic floor hernias treatment (49). Chemical components of synthetic meshes are commonly polypropylene, polytetrafluoroethylene (PTFE), expanded PTFE, polyethylene terephthalate, polyglycolic acid, polyglactin 910 (49), oxidized regenerated cellulose, chemically modified sodium hyaluronate and carboxymethylcellulose (50). Some components of synthetic meshes possess hypersensitivity potential, and it is not surprising that there have been reported cases of localized allergic reactions to meshes (51), oxidized regenerated cellulose (52) and systemic reaction to midurethral sling (53). It is interesting that carboxymethylcellulose is widely used as an additive in non-pharmaceutical and pharmaceutical industry as a disintegrant for capsules, tablets and granules (54, 55), and also as a component of synthetic meshes used in gynecological surgery. While there are no records of hypersensitivity to carboxymethylcellulose as a component in devices used in gynecological surgery, there are several reported cases of severe hypersensitivity reaction to carboxymethylcelluse as a component of other medical devices, which suggests it has a vast hypersensitivity potential (54, 55).

Conclusion

Gynecology represents a wide field for medical devices application. Whether or not, in what way and to what extent the host will respond to presence of devices, depends on composition of biomaterials applied, the site of application, and also it greatly depends on the physiological characteristics of the host organism. Since it is proved that great number of medical devices components possess hypersensitivity potential, physicians should always be careful when planning application of new medical devices. It is necessary to counsel patients about their personal history of hypersensitivity reactions and previous use of medical devices. According to this information and composition of available medical devices for specific use, physicians should make plans about further treatment. Also, in case of hypersensitivity reaction where personal anamnesis and/or medical documentation reveals use of any type of medical devices, hypersensitivity to biomaterials should be always considered as potential cause.

Conflict of interest

The authors report no conflict of interest.

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HIPERSENZITIVNI POTENCIJAL GINEKOLOŠKIH MEDICINSKIH SREDSTAVA

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Medicinska sredstva obuhvataju izuzetno širok spektar proizvoda koji se koriste u cilju postavljanja dijagnoze, prevencije, praćenja i lečenja bolesti ili invaliditeta. Razvoj medicine i tehnologije uzrokuje konstantno povećanje broja i upotrebe različitih medicinskih sredstava čije karakteristike odgovaraju biomaterijalima i koja istovremeno mogu da dovedu do razvoja hipersenzitivnih reakcija. Uprkos činjenici da je ginekologija široko polje primene biomaterijala, nema sumiranih podataka o hipersenzitivnim reakcijama na ginekološka medicinska sredstva. Ovaj rad daje pregled hipersenzitivnog potencijala i uobičajenih kliničkih manifestacija hipersenzitivnih reakcija na medicinska sredstva koja se specifično koriste u ginekologiji. Sumiranje ovih podataka veoma je važno kako za unapređenje trenutne medicinske prakse, tako i za poboljšanje karakteristika postojećih i dizajniranje novih medicinskih sredstava.

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Ključne reči: *medicinska sredstva, biomaterijali, ginekologija, hipersenzitivne reakcije*

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APPLICATIONS OF ARTIFICIAL INTELLIGENCE IN MEDICINE AND PHARMACY - ETHICAL ASPECTS

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In the last 30 years the development of artificial intelligence (AI) that can be applied in all areas of science has brought numerous benefits. Many researchers have explored potential applications of intelligent techniques in every field of medicine. The importance of AI is reflected in the possibility of proper decision-making, without subjectivity, fatigue, with unlimited possibilities of remembering and making conclusions. This is important in medicine, for the prevention and diagnosis of various diseases, as well as therapy monitoring. Numerous studies have shown that AI will soon replace medical staff in numerous activities, as results obtained by AI are better and more precise. A number of applications have been developed to simplify patient adherence to therapy, which ultimately affect the therapy. The application of AI is present in the pharmaceutical industry, in the design of new drugs. This precludes preclinical tests, which are extremely long and expensive. AI makes conclusions based on the available data, so the validity of data must be taken into account because extremely important algorithms are based on them. An important aspect is the protection of patient data since the possibility of data becoming public is a major ethical problem. Computers and AI are the cause why many people are replaced at their jobs, and the tendency is that such a trend continues. The question arises whether the machines should replace people in areas such as medicine, where feelings, empathy, and warmth are very important factors.

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Introduction

For centuries, there was a distinction between man and the rest of the natural world as well as human domination over the rest of the living world. This distinction is seen in the capability of learning, concluding and making decisions. The logic and ability of inductive thinking make the human brain superior to the most advanced computer. As a result of man's desire to produce software that, like the human brain, would be able to solve complex, sophisticated problems in an intelligent way, artificial intelligence (AI) was created (1).

The importance of intelligent agents nowadays

AI is the main issue of the contemporary world. Every day people can hear about its achievements in the most diverse areas of science, technology, even art, and entertainment. AI considers thinking, making decisions, navigating in new situations for computers or machines. Computers can run 24 hours a day, without tiredness, loss of concentration, without prejudice, and the amount of information that can be processed per unit of time is disproportionately greater (the computer that defeated Chess Grandmaster Kasparov had the ability to estimate 200 million moves per second). Also, they do not die; their knowledge remains forever, unlike the knowledge of a human that disappears after their death.

The idea of an ideal machine that will have the capability of thinking comes from the ancient time, but the conception of AI was introduced by John McCarthy in 1956 (2). Great progress in this area has been remarkable in the last 60 years. It was conditioned by discoveries in the field of neuroscience, cognitive psychology, information technology, statistics, mathematics, cybernetics, and logic. There are tendencies to secure progress in the future. AI refers to artificial neural networks (ANN), fuzzy ex-

pert systems (FES), evolutionary calculations (EC), and hybrid intelligent systems (HIS) (3).

The "thinking" of a computer is based on algorithms, which represent the steps in that process, similar to solving a puzzle. Very often, people use intuition to solve the problem, and views on the effectiveness of the use of intuition are varied and often conflicted (3).

Potential applications of AI in solving some of the global health problems

The health and well-being of every human being is a priority of the modern world. There is a constant need for the promotion of health and disease prevention, of both chronic and acute conditions. There are diseases that medicine has eradicated, for example through the usage of vaccines, but on the other hand, there are new diseases, new infectious agents; the number of cancer patients and patients with metabolic diseases is on the rise. Data from the World Health Organization on the number of people with diabetes: in 2016, there were 422 million people affected, while in 2013 it declined to 381 million.4 Data collected in the period from 2008 to 2012 on the incidence of cancer: 454.8 patients per 100,000 inhabitants and mortality: 171.2 deaths per 100,000 inhabitants per year (5).

The number of HIV-infected people is 36.7 million, of which 2.1 million children under the age of 15, according to data from the end of 2016 (6). There are diseases that can be controlled by adequate drug therapy; life expectancy with adequate therapy is prolonged, but the quality of life of patients must also be considered. The achievements of modern medicine and pharmacy from the invention of aspirin and penicillin until today are high, but further progress is also necessary.

From the middle of the last century, researchers have explored the potential applications of intelligent techniques in every field of medicine.

The application of AI technology in the field of surgery was first applied by Gunn in 1976, for diagnosing the cause of acute abdominal pain by computer analysis (7). The last two decades have seen a growing interest in the medical application of AI. Modern medicine faces the challenge of analyzing and applying a large amount of acquired knowledge in order to solve complex clinical problems. The development of medical AI was linked to the development of AI programs to help the doctor make a diagnosis, make therapeutic decisions, and predict the outcome. They are designed to ease the daily work of health care workers, specifically in tasks that rely on manipulation of data and knowledge, because empathy, support, and human warmth cannot be replaced by artificial systems (8).

Artificial neural networks

Artificial neural networks (ANNs) are computer programs designed to simulate some of the functions of the human brain such as learning, the ability of generalization and concluding based on prior experiences (1).

Based on the number of published papers in the field of application of AI in medicine, it is easy to conclude that the most important is ANN. ANNs are designed to solve various problems where classical methods do not yield satisfactory results. The basic principle taken from nature is that the nervous system processes the information processing with the help of simple process units - neurons. The basic principle from nature is that the nervous system processes information through simple process units neurons. Another blueprint taken from nature is the system's ability to learn. ANNs are not programmed, they learn by observing a number of examples of solved problems. Using different learning algorithms, they detect links and templates within a group of data presented to them, thus gaining the ability to anticipate responses to new experimental conditions (1).

It is the ability of neural networks to generalize the problem, learn from memorized examples, analyze non-linear data, handle imprecise information by enabling the application of independent data model that makes them a very attractive analytical tool in the field of medicine (1, 8).

For the successful application of ANN, it is necessary to collect as much data as possible. ANN are networks driven by data so that the quality of the model depends on the amount of data; therefore, they are suitable for data fusion. They learn "coaching" in an adequate, created environment.

ANN already have a wide range of application in the real world. Their ability to classify and identify patterns makes them almost ideal in solving many clinical problems. As we know the diagnosis, treatment and outcome prediction in many clinical cases depend on the complex interaction of many clinical, biological and pathological variables, so there is a growing need for analytical tools such as ANN that can perceive the complex relationships between these variables and use these perceptions to get to the correct conclusion (8).

Fuzzy expert system

Expert systems represent intelligent computer programs that contain "expert" knowledge, that is, knowledge equivalent to that of an expert in the field (8).

Expert systems place knowledge in a knowledge base that is used through illation mechanisms. The obvious advantage of expert systems is that knowledge from various specific areas of study becomes more accessible via computer programs. They cannot completely replace expert people, especially in terms of creativity, and the use of general knowledge (from ordinary life and other fields), but they also have some of the advantages over people, because human knowledge can be lost over time, especially if it is not often used (8).

Fuzzy logic is the field of expert systems. Unlike the formal logic in which the reasoning is done

with two values (true-false, 0-1), fuzzy logic uses numbers from the interval [0, 1], which is much closer to reality, to human thinking and expression. Many phenomena in nature are difficult to describe with only two states that are mutually exclusive. Fuzzy logic allows describing such "imprecise" systems, which is extremely important in medicine because very often only two values cannot be used to describe the condition, quality, but it is necessary to use more of the value (8).

Fuzzy controllers have been designed and implemented to administer vasodilators during surgery, as well as anesthetics (9, 10).

Evolutionary computation

Evolutionary computation (EC) is a general concept for several computer techniques based on natural evolution, that is, the mechanism of natural selection and the survival of the most suitable. Therefore, evolutionary computation can be used to solve real problems. The most common use of EC in medicine is "Genetic Algorithms". The principle is to suggest more solutions to the problem. One set of solutions will remain and "be transferred to a new generation, evolve" and advance, to solutions that will be optimal, and inadequate solutions will be eliminated. Most medical decisions can be formulated as data searches in a very large and complex area. For example, a cytologist who analyzes a cytology sample to decide whether they are malignant or not examines all possible cellular features for a set of functions that allow him to make a clear diagnosis. Genetic algorithms use a natural evolution mechanism to effectively search in a given area. They are applied to perform several types of tasks such as diagnosis and prognosis, medical image and signal processing.

Hybrid intelligent systems

All listed systems have their advantages and disadvantages so that new artificial systems use more mechanisms, which increases their potential. Their synergy allows a hybrid system that can get clear information from raw data, use human mechanisms of reasoning, can work with imprecise information and learn to adapt to a changing and unknown environment (8).

Application of AI in Medicine

Diagnostics

An analysis of disease status involves grading the degree of disease or progression of the disease. The purpose is to improve the accuracy of the treatment decisions and forecasting the prognosis. In order to ensure reproducibility, disease analysis is performed using clear, verbalized observations. Skilled doctors can collect data from patients who do not cooperate adequately. The problem is human inconsistency caused by tiredness (11, 12) or blood sugar level (13, 14). However, this is not the case with AI.

Bakst was one of the first researchers to explore the clinical potentials of ANN (15, 16). He has developed a neural network model that accurately diagnoses acute myocardial infarction. Since then, ANN has been applied in almost every field of medicine. ANNs were used in clinical diagnosis, in the interplay of radiographs, MRI-magnetic resonance imaging, CT, ultrasound, and wave analysis, electrocardiogram (ECG), electroencephalograms (EEG), interpretation of data in the placement intensive care (8).

Stamei et al. have developed a classification algorithm using neural networks called Prost Asure Index that can classify changes to the prostate as benign or malignant (17). This model was subsequently validated in prospective studies with a diagnostic accuracy of 90%, with a sensitivity of 81% and a specificity of 92% (18).

Some of the other relevant ANN applications in diagnosis include abdominal pain and appendicitis (19) retained stone particles in the bile ducts (20) glaucomas (21) and back pain (22).

Fuzzy logic is a data processing methodology that allows ambiguity and is therefore particularly suitable for the application in medicine. Zadeh wrote in 1969 that "the most probable field of application of this theory lies in medical diagnostics and, to a lesser degree, in the description of biological systems (23).

Schneider et al. have shown that fuzzy logic was more precise than multiple logistic regression analysis in lung cancer diagnosis using tumor markers (24). It is also used in the diagnosis of leukemia, cancer, for the characterization of breast ultrasound, MRI brain.

EC and the principles of genetic algorithms are used for the diagnosis of lung cancer (25), computerized analysis of mammographic micro-calcifications (26) for MRI segmentation of brain tumor to measure the efficacy of treatment strategies (27) and to analyze computerized 2-D images for the diagnosis of malignant melanoma (28).

Deep learning, a branch of the developing field of machine learning, has advanced over the past several years. In 2012, a deep convergent neural network, AlecNet (29) showed increased accuracy in the classification of high-resolution images and in 2015, similar versions, including Google's deep neural network GoogLeNetResNet's (30) and deep neural network (31) have exceeded the human limit of image recognition accuracy.

Takahashi et al. in 2017, evaluated an ANN system for disease analysis, which assesses the stage of diabetic retinopathy, and the retina areas that cannot be visualized on the fundoscopy, as well as another system that directly suggests treatments and determines prognoses (32).

Esteve et al. published a paper in 2017 on the classification of skin cancer using deep neural networks. Skin cancer is diagnosed visually. Using AI, processing a series of images in its databases (specifically 129,450 images), more precise classification of skin lesions can be made that represents skin cancer in different stages. Further development in-

volves the creation of an application that will allow dermatologists to work beyond their specialist offices, which is of great importance in the early screening of skin cancer (33).

Combining of such algorithms with the knowledge and experience of physicians is important. Participants of the International Symposium on Biomedical Imaging have created computer systems for detection of metastatic breast cancer with the help of data obtained via biopsy of the lymph nodes. The winner's algorithm successfully detected cancer with an accuracy of 92.5%. When the pathologist independently examined the same images, the success rate was 96.6%. Combining the prediction of a deep learning system with pathologist diagnostics increased the pathologist's success rate to 99.5%, that is approximately an 85% reduction of the human error rate (34).

IBM has developed an AI system called Watson. The supercomputer has the ability to quickly review a patient's genetic data to diagnose the disease, which would normally take several weeks. The IBM System Watson has diagnosed a woman who was unsuccessfully treated for acute myeloid leukemia, a rare type of leukemia by comparing the patient's genetic testing and oncological data from over 20 million cases to which it had access (35).

A new project called Deep Mind has a goal to create tools for improving the radiotherapeutic treatment of head and neck cancer. Medicine specialists need 4 hours to inspect the obtained video in detail. Bearing in mind that there are parts of vital importance in that area, it is extremely important that the healthy cells remain intact. By reviewing a large database and applying adequate algorithms, this process would take Deep Mind about an hour to finish, which leaves the physician more time to work with patients (36).

Prevention of disease

Prevention of disease is an extremely important branch of medicine. With the help of neural networks, patients who have been predisposed to a condition can be identified, and it is possible to focus on them in order to prevent the progress of the disease. It is also possible to predict the survival of patients with breast cancer and colorectal carcinoma (37, 38).

When predicting the outcome of treatment for patients in intensive care units, ANN showed better prediction power compared to APACHE IItest for the severity of patients' condition (39).

In a search for a reliable sign that a patient with mild cognitive impairment would suffer from Alzheimer's, experts initiated neuroimaging of brain structures of people with Alzheimer's desease. They have pulled the data from global tests, in which they collected recordings and clinical assessment of patients with mild cognitive impairment. A group of researchers then used AI and big data to develop an algorithm that would recognize dementia two years before the first symptoms. The data they used to teach AI to recognize dementia are amyloid positron emission tomography (PET) of the brain of patients who are prone to developing Alzheimer's disease. After analyzing thousands of PET patient records, the algorithm has managed to recognize which of them are susceptible to dementia with 84% accuracy (40).

Applications for physicians and patients

A study conducted in the UK has shown that thousands of patients die in acute renal failure or sepsis due to insufficiently rapid diagnosis. That's why Deep Mind has developed an application that generates patient information and notifies doctors about changes in vital signs, biochemical parameters, without the need for medical staff to review the documentation. This application saves 2 hours of working time to medical staff daily (41).

An application has been developed to monitor patients using antidepressant therapy. It is known that the effects of therapy need to be prolonged for a longer period of time, but with the help of this application, monitoring of the effects of therapy is enabled. The condition of the patient is evaluated more often and in a more efficient manner, which speeds up their recovery. The effects of the therapy can be monitored daily. The test works on smartphones, tablets and any web browser. Patients complete the test within 10-15 minutes, which includes recognizing facial expressions and emotions, and answering a range of health issues, which determine whether there is a shift in therapy (42).

The American Heart Association and the American Society for Infarction of the brain sponsored a web environment for the monitoring of cardiac patients called the Heart360 Cardiovascular Wellness Center. Heart360 allows patients to monitor their blood pressure, blood glucose levels, cholesterol, weight, diet, and physical activity, and on the basis of these data, they receive advice and information specific to their condition. More precisely, patients can collect and record these parameters, set goals, and monitor their progress, review their data in charts that they can print and share with others involved in their family health, receive news and articles of potential interest based on their health information (43).

More than 20% of the time of the medical staff is spent on data entry. Considering that physicians are overloaded with technical tasks, such as electronic health records, instructions, orders, they have less time for patients, research, mastering new technologies and improving their skills. Radical productivity improvements are needed, in order to maintain the current health standards and to make progress. The combination of human expertise and automated functionality creates a model of "perfect" physician (44).

Immortality is the idea of alchemists since ancient times. Company Insilico medicine has created an algorithm, which can calculate the biological age of any patient based on parameters of their blood. This is the way in which AI can help people, by calculating biological age, to prolong life. This artificial

neural network is trained by hundreds of thousands of patient data. After the acquired values, a person can change the lifestyle, include certain medications, supplements in the therapy. In a study published in 2018, the accuracy of the acquired values was tested with this application, and the results showed that blood parameters can be used reliably in determining biological age (45).

The modern world is unimaginable without social networks, where two billion images are published each day. After a study showing that blind people feel outcast and frustrated about the inability to access such content, Facebook has decided to help these people with the help of AI. There are 39 million blind people in the world, and even 246 million have vision disorders. Using AI, which, with the help of algorithms, recognizes visual representation, blind people are enabled to recognize the content of the picture. It is planned that detailed and precise image descriptions will be written in the upper right corner of the photo, which will then be translated into an audio signal, also using the application (46).

Another social network focused on the research of users who potentially have depression. With the help of machine learning and the correlation between the colors of the published images and the mental health of an individual examined, a significant correlation was observed. In addition to colors, the use of contrasts and ace recognition were taken into account, and it was concluded that people with depression do not have a lot of pictures with other people on the profile, which is an indicator of sociality, as well as the number of likes and comments. The researchers have shown that the success of this algorithm is 70%, which is a higher percentage compared to general practitioners' (47).

A new application that is used on the software platform for data analysis processes information from the sensor. This software platform relies on the original algorithm for identifying hand-to-mouth gestures that characterize smoking cigarettes. Observation of smokers while smoking and immediately notification of smokers through the SmokeBeat application led to a reduction in smoking and showed a significant change in the study group regarding the number of cigarettes per day, while the control group did not make any significant changes (48).

Application of AI in Pharmacy

The application of AI is not only seen in the diagnosis, monitoring, prediction of the disease, but also in the development of new drugs. The use of machine learning, in preliminary (early stages) drug discovery, starts from the initial screening of drugs to the predicted rate of success.

Clinical trials

Clinical trials of drugs are long-lasting and costly, and machine learning has several useful potential applications in helping to organize clinical trials. The application of an advanced, predictive analysis in identifying candidates for clinical trials, finding the best size sample for increased efficiency, adjusting the differences in patient recruitment sites and using electronic medical records to reduce data errors can lead to more efficient and more cost-effective testing.

Machine learning can also be used for remote monitoring and access to real-time data for increased security; for example, monitoring biological and other signals for any sign of injury or death of the participants (49).

Drug design

The way in which AI is used in the design of new drugs is based on monitoring the interaction of the 3D models of molecules and target sites (receptors, enzymes,...) which can represent possible therapy. This is achieved by the application of deep learning based on the existing behavioral history of the molecules. In other words, just as AI manages to learn how to recognize images - by inspecting thousands of examples of images, it generates potential drugs based on the behavior of the molecules in its base. Different programming companies, in collaboration with scientists from natural sciences, have created an algorithm that detects an interaction between drugs and a broad biological system, which then narrows into smaller groups of activities. It also points out that the testing of tens of millions of cases makes extremely accurate and quick predictions. And that is precisely the key to ensure success in the development of drugs, primarily because AI can scan all possible combinations more quickly and narrow down possible actions. In addition, instead of average 12 years and $2.9 billion for drug development, scan results can be obtained in a few weeks with a significant reduction in costs (50).

Last year, a virtual search was launched for safe, existing drugs that could be redesigned to treat the Ebola virus. Two drugs were found through the AI technology of the company that can significantly reduce the Ebola virulence. This analysis, which usually lasts for months or years, was completed in less than one day (51).

Epidemiology

By using machine learning and AI, the history of the epidemic can be studied, the activity of social media analyzed, and it can be predicted where and when the epidemic can occur with considerable accuracy. The AIME-AI Project in Medical Epidemiology and their platform make it possible to predict the exact geographical location and the date of outbreaks of infectious diseases such as tropical fever or zyka for three months in advance, with a precision of 86.37%. It has been found that 270 changeable factors affect the outbreak of epidemics. An autonomous system was developed so that every 23 seconds it autonomously automatically goes through 270 variables. Some of them are the speed and direction of the wind, the temperature, the movement of the population (52).

Formulation of Pharmaceutical Preparations

The application of neural networks as one of the AI technologies is a modern approach to solve the complex problems of formulating pharmaceutical preparations. The application of ANN represents a new dimension in the formulation of pharmaceutical preparations due to unique advantages, such as nonlinearity, the ability to model and optimize with a small number of experiments. ANN have been successfully applied in designing compositions of pharmaceutical preparations, optimizing production processes, providing and controlling quality, predicting the stability of pharmaceutical preparations, in vitro testing the rate of release of the active substance from the pharmaceutical form and *in vitro / in vivo* correlation. With the development of new, powerful software packages adapted to the user, the application of ANN in the design and development of new pharmaceutical preparations is also foreseen, as well as for the quick and simple assessment of their stability, safety, and efficiency while at the same time significantly reducing costs (1).

Adherence

The development of medicine is aimed at treating the disease, but without an adequate way and frequency of taking drugs, the success of therapy is, almost certainly, impossible. New technologies in medicine have found application in monitoring of therapy. Chronic therapy adherence is important because chronic diseases such as diabetes, hypertension, have no clear symptoms, so patients can often skip the dose. Studies suggest that 33-50% of patients do not take medication correctly, which contributes to nearly 100,000 premature deaths each year. Inadequate adherence is the main cause of uncontrolled hypertension, which is the main cause of stroke, coronary heart disease, cardiac insufficiency, and mortality.

To cope with these challenges, researchers have applied AI to develop an adherence model that automatically adjusts the communication of text messages to the individual needs of patients. An adherence monitoring study was conducted in two groups of patients. One group was control and respondents applied a drug from the bottle that had the ability to record the date and time of the opening. The second group was examined and respondents used the same bottle and SMS reminder system. SMS had a motivational content, but content and frequency of messages would automatically be adjusted using AI algorithms. Adjustment of the content is done due to differences in the use/non-use of drugs. It is based on the principle of rewards and punishments, and further content and frequency of messages depend on it. The results showed that the adherence of the investigated group was significantly improved over 3 months, compared to the control group (53).

Application of AI in Genetics

Genome sequencing is known to be a large, long-lasting venture, which is believed to allow scientists to inspect etiology of numerous diseases. However, no one predicted the enormous amount of data they will receive and which someone will have to process to get useful information. Today, machine learning and hardware help to get to data with computer support, which further accelerates research. It is now known that the causes of various diseases do not lie in the mutation of a single gene, but involve multiple genes, as well as their interaction. The sequencing of the genome that earlier lasted for days has now been shortened for a few minutes, and the information can be used concretely. Genome sequencing is extremely important in early detection of disease, infertility, non-invasive prenatal tests, mental health tests and prediction of genetic therapy, in infectious diseases, and cluster redistribution of Cluster (Clustered Regularly Interspaced Short Palindromic Repeats) genome modification.

Ethical Aspects of applications of AI in Medicine

The difference between man and machines and other beings is the ability to think. But, is this still true today? With the development of AI and intelligent agents, it may no longer be the case. Is the man still a "superior" race, and if so, for how long? What is the distinction between man and machine now? Today we can say that these are emotions, conscience, empathy. Again, questions arise, until when? As we could not imagine an agent who will be able to think independently, make decisions, so it is incredible to us today that it is possible that they will have emotions.

It is believed that automation and AI will take over, and over the next 5 years, 7.1 million jobs will be extinguished, mainly in the area of administration and industry. There are various workplaces that are dangerous to people, such as mines, quarries, so replacing them with robots is an exceptional benefit. Also, in different jobs where there is a continuous repetition of actions, due to loss of concentration, injuries, disabilities, even deaths are possible.

The administration is an inevitable obligation for professionals in all areas, but with AI, it is possible to shorten the time spent in performing the bureaucratic chore. This applies, first of all, to medical staff, who need to be dedicated to patients. Such changes would certainly influence the improvement of the quality of health care.

How smart is it to give so much power to agents? It's one thing that they have a picture of the environment themselves, and quite another about the circumstances, aspects of the society in which they occur. The question arises as to whether they will turn against us, whether it will have some devastating tendencies. Such dilemmas have surfaced

since the time of the invention of dynamite, then the invention of nuclear energy, since they were later used for the purpose of killing people, i.e. warfare. Is a human being a humane being? A man has made various disasters through history, started wars in which millions have dyed, developed new infectious agents, etc.

The drone, which transmits defibrillator to patients with myocardial infarction, was previously mentioned. What happens if there are two simultaneous calls? Is the drone, despite AI, capable of making a decision to help patients? Can we let it make such important decisions? This is just an example, but the ethical question of leaving the decision to intelligent agents pervades all other areas of AI.

When it comes to AI, it is about collecting and procesfourquestions: PAPA (privacy, accuracy, property, accessibility).

Privacy: What information should a person disclose to others, and under what conditions? What can people keep for themselves and not be forced to disclose to others?

Accuracy: Who is responsible for the authenticity, loyalty, and accuracy of the information? Similarly, who will be responsible for information errors?

Ownership: Who owns information? What is the fair "price" for information exchange? Who owns the channels through which information is transmitted?

Accessibility: What information does a person or organization have the right or privilege to obtain, under what conditions and with which safeguards?

Who collects this information, where, in what way? This process is extremely important and responsible because based on this data, the setting of an algorithm will play an important role.

Our moral imperative is clear. We must ensure that the information technology and the information they handle are used to improve the dignity of mankind. In order to achieve these goals, we need to formulate a new social contract, which ensures everyone the right to fulfill their human potential (54).

The data collected must be adequately guarded, as the Data Protection Act requires this. The Deep Mind project is working to analyze 1.6 million patients annually in three hospitals in London in order to analyze and improve radiological healing of the head and neck. Information management has signed a special agreement with a precise data protection plan, regulations, methods of implementation and control, and the possibility for patients to veto the use of their data. There are always concerns about possible hacking incursions or the sale of information.

Google, which is the maker of the Deep Mind project, is one of the most powerful corporations in the world, and it is undoubted that with the development of these projects in healthcare, you will earn huge amounts of money. If this improves health care and helps save lives and treatments, then this is not a moral issue, because human health is imperative about which there is no dilemma. The real question is not to allow hospitals to become dependent on the technology of one of the most powerful companies in the world.

Use of AI is impossible without complex softwares, hardwares, cameras and other sensory systems, or smartphones. Such devices are not available in all countries of the world due to lack of money. Is it necessary to develop new technologies, while in some parts of the world there is still a shortage of food, water, basic livelihoods, where there is a high percentage of illiterate people suffering from a wide range of illnesses? The difference in economic status and the way of life of people in different parts of the world has always been a big gap, and there are chances that it will be even greater, using AI.

Social networks have possibilities to investigate people’s interests. Based on searched products, which sometimes can be drugs, psychoactive substances, plants, herbal products, people’s interests can be analyzed. Collected data are important for marketing of companies which produce those products. Advertisings are presented to other people in, for example, the same social network groups. Social groups usually consist of people of similar age, sex, interests, but they are not the same health status. If advertised products are health products, it is dangerous, because the same products are not safe for every person. This can lead to many adverse effects and intoxications.

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PRIMENA VEŠTAČKE INTELIGENCIJE U MEDICINI I FARMACIJI – ETIČKI ASPEKTI

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Poslednjih 30 godina zabeležen je razvoj veštačke inteligencije, čije se dobrobiti mogu primeniti u skoro svim oblastima nauke i života. Od sredine prošlog veka, istraživači su otkrili potencijalne primene (tehnika veštačke inteligencije) u svakom polju medicine. Važnost veštačke inteligencije ogleda se u mogućnosti pravilnog odlučivanja, bez subjektivnosti, bez umora, sa neograničenim mogućnostima upoređivanja, pamćenja i zaključivanja. Ovo je veoma važno u medicini, za prevenciju i dijagnostiku različitih oboljenja, kao i za praćenje efekata terapije. Brojne studije pokazale su da će uskoro veštačka inteligencija zameniti medicinske radnike u brojnim aktivnostima, jer su rezultati dobijeni primenom veštačke inteligencije bolji i precizniji. Razvijene su brojne aplikacije koje bolesnicima pojednostavljuju pridržavanje terapije, čime se poboljšava adherenca i u konačnome efekat terapije. Primena veštačke inteligencije zastupljena je i u farmaceutskoj industriji, u dizajnu novih lekova. Ovim se skraćuju pretklinička ispitivanja, koja su izuzetno duga i skupa. Veštačka inteligencija donosi zaključke na osnovu podataka koji su joj dati, pa se mora voditi računa o validnosti tih podataka, jer se na osnovu njih razvijaju izuzetno bitni algoritmi. Važan je aspekt zaštita podataka o bolesnicima, jer je mogućnost objavljivanja tih podataka veliki etički problem. Zbog računara i veštačke inteligencije već mnogo ljudi gubi posao širom sveta, a postoji tendencija nastavka ovakvog trenda. Pitanje je da li je potrebno da mašine zamene ljude u oblasti kao što je medicina, gde su osećanja, empatija i toplina vrlo važni faktori.

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Ključne reči: *veštačka inteligencija, medicina, farmacija, primena, etika*

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EMBRYONIC STEM CELLS: WHERE DO WE STAND AT THE MOMENT?

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Stem cells are functionally defined as cells that have the ability to self-replicate and generate differentiated cells. They can generate multiple differentiated cell types (multipotent or pluripotent) or produce one type of differentiated cells (unipotent) depending on the replication capacity and potency of stem cells. Today stem cells might have potential application in regenerative medicine, transplantation, treatment of autoimmune, chronic and progressive diseases, as well as disease modelling. Now we are faced with a dilemma between the two types of stem cells, which are more suitable for research and therapeutic use, which will be Embryonic Stem Cells (ESC) or Induced Pluripotent Stem Cells (iPSC)? ESCs represent the gold standard of pluripotency *in vitro*, which compares all other types of stem cells, but iPSC are more convenient in autologous transplants because of the avoidance of tissue rejection and without ethical concerns. The aim of this paper is presenting the most important characteristics of the ESCs which have therapeutic significance.

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Key words: *embryonic stem cells, legislation, therapy*

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Introduction

Stem cells are non-specialized cells of the organism at an early stage of development with large proliferative capacity which in normal conditions can be differentiated into different types of functionally specialized mature cells (1, 2). The basic characteristics of stem cells are self-renewal, clonogenicity and potential. Self-renewal is the ability of a cell to divide into at least one cell of plenty cells which can retain the capacity of self-renewal and differentiation. Division can be symmetric (the stem cell divides into two identical cells, both of them retain the ability to self-renewal and differentiation) and asymmetric (one daughter cell retains the stem cell characteristics, the other being the progenitor cell which is the intermedia cell between the stem cell and the target tissue cell whose division is limited). Clonogenicity is the ability to create clones, or cells with identical genetic material, while the potential of stem cells is the ability to differentiate into different types of specialized cells (1, 2).

Classification of stem cells

Fertilization of an egg results in a diploid zygote that is dividing rapidly into 16-cells that form so called morula. The cells continue dividing and generating blastocyst by multiple mitotic cell divisions during early embryogenesis occurs. The blastocyst consists of an outer layer of cells called trophoblast (trophectoderm) and an inner layer of cells called the embryoblast. The trophectoderm also known as the outer cell mass forms the extraembryonic tissue that might give rise to placenta, chorion and the umbilical cord. The embryoblast also known as the inner cell mass (ICM) develops into the embryo (3, 4). Totipotent embryonic stem cells have the potential to develop into any type of cells or to form the entire organism. The definition of totipotency is based on the capacity of a single cell to contribute to all lineages (5). The only cells which own that ability are the cells generating during early embryogenesis so called blastomeres that form morula (1, 2, 5). According to the capacity for differentiation, stem cells can be classified into several types: pluripotent, multipotent and unipotent stem cells (1, 2). Pluripotent stem cells have the ability to differentiate into almost all cell types, giving different types of

cells that develop from three germ layers (mesoderm, endoderm and ectoderm) from which all the cells in the body are formed. The sources of human pluripotent stem cells were isolated from are early human embryos, i.e. from the internal cell mass of the blastocyst (embryo 4 - 5 days old, which has 50 - 150 cells) - so called embryonic stem cells, and from the fetal tissue that was predetermined to be part of the gonads, embryonic germinal cells. The ectoderm develops: skin, hair, nails, skin glands, eyes, mouth, nose, pigment cells, nerve cells, spinal cord, brain and others. The mesoderm develops: muscles, connective tissue, skeleton, heart, blood vessels, blood cells, urogenital system, gonads, fat cells and others. The endoderm develops: throat, extremities, salivary glands, pancreas, liver, lungs, thymus, thyroid gland, paralytic gland and others. They can give rise to all tissue types, but they cannot give rise to an entire organism (1, 2). Multipotent stem cells have the ability to differentiate into cells which come from one germ layer or single tis-sue cells. An example of multipotent stem cells are hematopoietic stem cells or mesenchymal stem cells from the bone marrow that can differentiate into bone, cartilage and fat tissue cells. They are pro-genitor cells that give rise to a limited range of cells within a tissue type (1, 2). Unipotent stem cells have the potential to differentiate into only one type of cells. They came from multipotent cells and have limited potential for differentiation, but retain the ability to self-renewal. An example of unipotent stem cells are limbal cells (enabling the regeneration of the eye tissue and the ocular nerve, they are found along the length of the iris) and epithelial cells. They are precursor cells (1, 2). In relation to the function of the stem cells in organism, they might be clas-sified into normal and cancerous stem cells. Cancer-ous stem cells (CSCs) have been identified in many tumors, some of the cluster of differentiation (CD) that identifies cell surface antigens were also found in CSCs (2).

Bonnet 1997 (6) identified CD34+/CD38- in acute myeloid leukemia. *Al-Hajj 2003* (7) identified CD44+/CD24- in breast cancer, in the same year *Singh* (8) identified CD133+ in brain tumor. *Matsui 2004* (9) identified CD138+ in multiple myeloma. In 2007, *O'Brien* (10) and *Ma* (11) identified same surface antigen CD133+ in colon cancer and liver cancer, in the same year Li (12) identified CD44+/CD24+ in pancreatic cancer. In 2008 *Eramo* (13) identified CD133+ in lung cancer. Many other surface antigens such as CD133+/CD44+ in prostate cancer by *Maitland* (14), CD44+/CD117+ in ovary cancer by *Zhang* (15), and CD4+/CD25+/FoxP3+ in melanoma by *Schatton* (16) were identified in the same year. In 2009, Takaishi (17) identified CD44+ in gastric cancer. Compared to the source of isolation stem cells might be classified into embryonic, fetal, adult and induced pluripotent stem cells (1, 2, 18). Embryonic stem cells (ESCs) are isolated from the inner cell mass of the embryo before its implantation (19). Human Embrionic Stem Cells (hESCs) have the ability to generate cells from all three embryonic germ layers and also represent great candidate cells source for therapeutic uses (1, 2, 18, 20). Fetal stem cells (FSCs) are isolated from the fetus after abortion or umbilical cord blood (e.g. hematopoietic stem cells), (1, 2, 18). Adult stem cells (ASCs) are undifferentiated cells, found in different tissues and organs, between differentiated cells. These stem cells could be of different origin - e.g., endodermal origin are gastrointestinal tract stem cells, pulmonary epithelial stem cells, hepatic oval cells, pancreatic stem cells, ovarian and testicular stem cells, mammary and prostatic gland stem cells; mesodermal origin are mesenchymal stroma stem cells, mesenchymal precursor stem cells, mesenchymal stem cells, hematopoietic stem cells, cardiac stem cells, multipotent adult progenitor cells, bone marrow stem cells, fetal somatic stem cells, unrestricted somatic stem cells, satellite cells of muscle; ectodermal origin are skin stem cells, neural stem cells, ocular stem cells. They have the capacity for self-renewal and multiline differentiation; they differentiate into the tissue cells in which they are found. The primary role of adult stem cells is to repair damaged tissue (1, 2, 18). Induced pluripotent stem cells (iPSCs) are generated by reprogramming terminally differentiated cell genome to the stage of pluripotent stem cell in vitro induction and by the forced expression of certain gene and factors that are important for the maintenance of pluripotency (Sox2, Oct3.4, c- Myc, KLF4). These stem cells have common features with Embryonic Stem Cells in terms of morphology, surface antigen expression, proliferation, gene expression, and telomerase activity. They can be differentiated into the cells of all three germ layers (1, 2, 18).

Morphological characteristics of embryonic stem cells

In vitro embryonic stem cells (ESCs) grow in flat colonies with clear boundaries of cells in the monolayer of culture. They produce small, round colonies, and at the cellular level show a high ratio of nucleotide-cytoplasm with one or more prominent nucleolus and a characteristic spacing between the cells. Embryonic stem cells are identified by the expression of surface cell antigens as stage specific embryonic antigen (SSEA 3 and 4) and tumor rejection antigen (TRA-1-60 and TRA-1-81) used as markers for undifferentiated ESCs (21). These globular glycolipids are recognized by monoclonal antibodies[1]. During the differentiation of ESC, the poor regulation of SSEA-3 and SSEA-4 is observed. ESCs are characterized by high activity of telomerase and alkaline phosphatase, as well as the activity of genes necessary for the maintenance of cells pluripotency. All cell lines of ESCs express high levels of telomerase, an enzyme that helps maintain telomeres that protect the ends of the chromosomes. Telomerase activity and long telomeres are features of proliferative cells in embryonic tissues and germ

[1]Monoclonal antibodies are homogeneous populations of identical antibody molecules that are derived from an antibody-producing cell with specificity for a particular epitope. Epitopes are antigenic determinants that recognize the antibody and bind to antibody.

cells. Somatic cells do not show telomerase activity and their telomers are significantly shorter. In contrast to ESCs, differentiated somatic cells are not shared in culture - the phenomenon of replication aging. Embryonic stem cells are also defined by the presence of various transcription factors and surface proteins. Specific transcription factors (pluripotent stem cells markers) such as octamer-binding transcription factor 3/4 (OCT3/4), Homeobox protein NANOG (NANOG), SRY (sex determining region Y)-box2 (SOX2), Myelocytomatosis cancers (c-Myc), Kruppel-like factors (Klf4), Growth and differentiation factor 3 (GDF3), Reduced expression 1 (REX1), Fibroblast growth factor 4 (FGF4), Embryonic cell-specific gene 1 (ESG1), Developmental pluripotency-associated 2 (DPPA2), DPPA4 and Telomerase reverse transcriptase (TERT) play a role in retaining pluripotence and inducing genes that lead to differentiation. Oct4 is a classic proto-oncogene whose abnormal expression extends to the dysplastic growth and formation of various types of tumors, and affects the malignancy of ES cells *in vitro*. Sox2 is also proto-oncogene. Klf4 was also identified as a potential oncogene and as a tumor suppressor. c-Myc is proto-oncogene, increased expression is observed in more than 70% of various human tumors, making it one of the most commonly detected tumor markers (1, 2, 18, 21, 22).

Functional characteristics of embryonic stem cells

The functional characteristics of the ESCs include the following criteria: embryonic stem cells can be isolated from the inner cell mass of the blastocyst; they are capable of prolonged proliferation in culture without differentiation; ESCs are pluripotent, and can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers, both in teratomas after grafting or in vitro under appropriate conditions; embryonic stem cells do not show X-chromosome inactivation; they can maintain a normal diploid cariotype *in vitro*; embryonic stem cells can be cloned, i.e. they have the ability to form a homogeneous cell line with all the characteristics of the parent ESCs; ESCs can remain in the S-phase of the cell cycle most of their lifetime; they can express a high level of Oct4 a transcription factor known to be involved in their self-renewal process; embryonic stem cells may be induced to differentiate after continuous cultivation in an undifferentiated state (1, 2, 23, 24).

Derivation of embryonic stem cells

Three different methods can be used for derivation of ESCs, depending on the morphology of the blastocyst and the appearance of internal cell mass (ICM). There is a strong relationship between the shape and size of the ICM of blastocyst and its viability. Immunosurgical method is used for blastocysts with large and distinct ICMs. The partial-embryo culture method is used for expanded blastocysts with distinct but smaller ICMs. The whole-embryo culture method is used in cases in which the blastocyst exhibited an indistinguishable ICM and trophoectoderm. Isolation of the ESCs line is a relatively simple procedure with a success of 30-50% (1, 25, 26). *Solter and Knowles* (1975) (27) developed a procedure known as immunosurgery for selective isolation within the cellular mass of blastocysts to investigate early embryonic development. This method of selective isolation of the intracellular mass of blastocysts was the basis of the first ESCs line derived from the mouse blastocyst. The process involves several stages. Initially, the glycoprotein external layer of the pelucide zone is dissolved by Tyrode's solution or by enzyme pronasa. The exposed embryo is then incubated for about 30 minutes in antihuman-whole-serum antibodies. The penetration of antibodies into the blastocyst is prevented by cellular connections within the outer layer of trophoblasts, leaving the intracellular mass of blastocysts intact. After washing the residuals of the antibody, the blastocyst is transferred to the medium and incubated once until the cell line of the trophoectoderm is reached. Since the pelucide zone allows the antibody to penetrate, the complement of proteins formed after the lysis can be alternatively removed. After selective removal of trophoectoderm, the intact intracellular mass is further cultured on mitotic-inactivated MEFs (Mouse Embryo Fibroblast) (1, 25, 27). Partial-embryo culture method usually used forblastocysts with smaller ICM and higher risk of losing their ICMs when processed via immunosurgery. ESCs lines can be isolated directly from cultured blastocysts by mechanical dissection and partially removing the trophoblast layer using a 27G needle or by drawing Pasteur pipette onto mitotically inactivated embryonic fibroblasts. When the trophoblasts are only partially removed or unglazed, embryos attach to the nutrient layer and align, allowing continuous growth within the cell mass, with the remaining surrounding single-layer trophoblasts. When the intra-cell mass reaches the required size, it is selectively removed and propagated. This method has a significantly lower success rate compared to immunosurgery due to tendencies of intra-cellular mass to differentiate (1, 25). Whole-embryo culture method is used for blastocysts with no visible ICM, carried out by seeding whole blastocysts without the pelucid zone directly on the STO-feeder layer. The excessive growth of trophoectoderms tends to prevent the spread of ICMs during the initial steps of the ESCs derivation. There-fore, a partial-embryo culture method for the pro-duction of ESCs from blastocysts with a smaller ICM was developed (25).

Cultivation of embrionic stem cells

The cultivation of ESCs is done in a few steps. The pluripotent stem cells are isolated from the inner cell mass of the blastocyst, by the before mentioned methods (e.g., immunosurgery) and then cultured on mitotic-inactivated embryonic fibroblasts of mice (MEFs); MEFs are usually inactivated by mitomycin-C or γ-radiation to prevent its replication. Although ESCs require precise care, they can be cultivated in large numbers and frozen and defrosted with a good survival rate. The ESCs cultures have the ability to grow in serum-free conditions; they

can use human "feeder" layers as substitutes for MEFs and can be maintained in an undifferentiated state in food-free conditions. During cultivation of ESCs in MEFs with Fetal Bovine Serum (FBS) enriched medium, there is a risk of cells exposure to retroviruses or other pathogens. One of the solutions to this problem is the isolation and cultivation of hESCs in an environment that is completely without animal origin. In the culture medium, FBS was replaced by the addition of 20% human serum, and MEFs was replaced by the human "feeder" layer. However, many hESCs lines, such as NTERA2 lines, do not depend on the "feeder" layers (1, 22, 28). Pluripotent stem cells that were isolated from inner cell mass of human blastocysts belong to one of the five hESCs lines - H1, H7, H9, H13 and H14. After 9 to 15 days, the inner cell mass is dividing, mechanical grouping and replating some of the cells under conditions of the same culture and that result in creation of different cells lines. The hESCs lines, which grew in the culture of high-density suspension, will form embryonic bodies. Embryonic bodies are groups of cells that form when the hESCs are cultivated on plates or in the medium and do not occur under natural conditions. Embryonic bodies contain undifferentiated and partially differentiated cells (19). However, the hESCs remain undifferentiated when cultivated in high density. The hESCs can be differentiated into the cells of all three germ layers *in vitro* and they can form teratomas *in vivo* (tumors built from undifferentiated and differentiated cells) after injection into immunodeficient mice. The hESCs lines carry genetic variations related to tumor genes that limit their differentiation capacity) (1, 22, 28). *In vivo*, after injection into immunocompromised mice, hESCs differentiate into bone, cartilage, squamous epithelium, nerve cells, glandular epithelium, striated muscles and also teratomas (1, 19, 22, 28).

The importance of "Feeder" cells, growth factors and serum-free media for cultivation of ESCS

In the 1970s, pluripotent teratocarcinoma (stem cells) cell lines were obtained after the introduction of a "feeder" cells layer. "Feeder" cells (boost cells) were useful in the study of teratocarcinoma (stem cell) and embryonic carcinoma (EC) cells. "Feeder" cells are usually inactivated by mitomycin-C or γ-radiation. Although the "feeder" cells live, they do not replicate. The "feeder" layer has a dual role: first, mouse embryonic fibroblasts (MEFs) support hESCs growth; second, MEFs prevent spontaneous differentiation of hESCs during cultivation. MEFs maintain pluripotency of the hESCs, a secreting factor identified as leukemia inhibitory factor (LIF), also known as differentiation inhibiting activity (DIA). ESCs colonies are cultured on "feeder" cells in the presence of serum and growth factor cocktails, such as leukemia inhibitory factor, stem cells factor (SCF) and basic fibroblast growth factor (bFGF). Without the "feeder" layer, cultured embryonic stem cells will not remain pluripotent, suggesting that fibroblasts promote either self-renewal or suppress differentiation or both. LIF is sufficient to guarantee self-renewal ESCs in a serum enrichment environment. However, when the ESCs are cultured in a serum-free medium containing LIF, they tend to spontaneously undergo neural differentiation. This indicates the existence of one or more growth factors contained in the Fetal Calf Serum (FCS), which together with LIF participates in the maintenance of pluripotence of the ESCs. Ying and associates (29) identified Bone morphogenetic protein 4 (BMP4) as a growth factor that works in combination with LIF to maintain self-renewal, as well as to prevent/block neural differentiation of ESCs in a culture without serum. The activity of BMPs was first identified in the 1960s (30), the proteins responsible for bone induction remained unknown until the purification and sequence of bovine BMP3 (osteogenin) and cloning of human BMP2 and BMP4 in the late 1980's (31-33). LIF is a member of the interleukin-6 family of cytokines, including IL-6, oncostatin-M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (ST-1). IL-6 cytokine families are structurally and functionally linked. They act on different cells, actually, they are pleiotropic and can mediate proliferation or differentiation or both, according to target cell types (1, 22, 28, 29, 34-37).

Why ESCs?

Because they have not passed the aging period as an adult stem cell, and therefore have a higher potential of potency, clonogenicity and self-renewal; they are isolated from excess embryos and the method is not invasive or painful; there is no reprogramming, so the transcription of genes has not been impaired, the epigenetic landscape, the potential of differentiation and mutational loads are considerably lower than in the application of iPSCs. And because the ESCs are the gold standard of pluripotency *in vitro*, comparable to all other types of stem cells (38-40).

Legislation

Research with hESCs and their use is more controversial than the involving adult stem cells. Usually, hESCs come from embryos that develop from fertilized eggs *in vitro*. Most of the public opinion is convinced that the embryo has a moral status, which is the main cause of the dilemma about hESCs research (41, 42). The hESCs can be differentiated into any type of cell of all three germ layers (21). Human embryonic cells pass through physical changes for implantation in the uterine wall about 5-7 days after the fertilization. If these cells are isolated and grown in laboratory conditions in the Petri dish, the human embryonic stem cells continue to divide and remain stem cells that can produce all cell types. This type of cells can only be obtained by the destruction of human embryos, which is the reason of the discussion, since the respondents have opposing attitudes regarding whether or not the embryo has a moral status (41, 43). Some people believe that embryos or blastocysts are just a bunch of cells that can be used without any limitation. Therefore, they support research with the hESCs provided there is scientific justification, careful supervision and

consent of a woman or em-bryo research donor (42, 44, 45). Ethical, religious, national attitudes and insufficient public opinion about research and implementation of hESCs are one of the leading problems in numerous studies. The study of human embryonic stem cells is ethically and politically controversial because it involves the destruction of human embryos. It is not disputed that embryos have the potential to develop into a human being, but only if they are implanted in the uterus of a woman at the appropriate hormonal stage, they can develop into a fetus and become a live baby. However, a large percentage of public opinion is of the conviction that the embryo has the same moral status as an adult or a live-born child, and therefore has interests and rights that must be respected. From this perspective, taking a blastocyst and removing the inner cell mass to obtain human embryonic stem cell lines is similar to murder (41, 42, 44, 46). In many countries including Germany, the Embryo Protection Act is forbidding the creation of supermodern embryos, donation of eggs and embryos; the Stem Cell Act, which came into force in 2002, regulates the study of human stem cells. IVF couples, like most physicians, have voted to legalize the donation and production of embryonic stem cells from embryo surplus (47). In Italy, the legal pro-tection of the embryo has been regulated by the Law on Medical Aid in Reproduction (Law 40/2004) since 2004. Manipulation of embryos is only permitted if used for therapeutic or diagnostic purposes and when there is no alternative. This law does not specify any rule regarding the fate of excess embryos obtained from in vitro fertilization procedures before the law is passed and actually stored in different Reproductive Technology Centers or for potential studies whereby embryonic stem cells are imported from abroad. Autologous storage of embryonic stem cells for personal use is permitted only in case of serious illness, in the case of families at high risk of genetic diseases (48). Very liberal models occur in countries such as Spain, Belgium, Sweden, the United Kingdom, the Czech Republic, Switzerland and Greece where the embryo acquisition/use is allowed for research purposes under the strict supervision of the authorized institutions. In the United Kingdom, the Law on Human Fertilization and Embryology of 2001 encourages the use of embryos for research and therapeutic cloning. In Belgium, Greece and Sweden, embryos are allowed until 14 days after fertilization, and in the Czech Republic up to 7 days after fertilization, but embryo cloning is prohibited. The Court of Justice of the European Union has confirmed that the destruction of embryos should be avoided and it has been ruled that medicines derived from research with human embryonic cells cannot be patented (48).

Examples of clinical studies for potential ESCs use

In addition to the potential use of stem cells in regenerative medicine, transplantation, dentistry, disease modeling, gene therapy, it should be noted that there are certain risks in the occurrence of point mutations that are numerous in tumor cell genes. Therefore, in the use of human induced pluripotent stem cells, it is necessary to check and eliminate the possibility of any mutations on the tumor suppressor genes, as tumorigenesis may occur after implantation in the patient. Human embryonic stem cells can also cause tumorigenesis, as well as transplant rejection (21, 43). Stem cells ability to develop into any cell type, maintain tissue and organ health, make them a potential treatment to assist organ and tissue repair after invasive procedures such as surgery. Hepatic administration of autologous hematopoietic stem cells (HSCs) has shown feasibility and safety based on nine published trials reviewed by Stutchfield and colleagues (2010) (49), at the end they suggest further research specifically on the use of ESCs for hepatic engraftment and hepatocyte differentiation (50). ESCs may have potential in facial nerve repair and regeneration based on eight animal trials. The review by Euler De Souza Lucena and colleagues (2014) (51) did not identify any human trials in this area, but it gives us hope and motivation to continue further research. There are promises for stem cell treatment for spinal and brain injury, the review by Tator (2006) (52) identifies pre-clinical research on the use of human ESCs. There are some researches and reviews that noted the potential of ESCs *in vitro* for tissue-engineering. Trachea and laryngeal tissue were engineered using stem cells (53). Regeneration of tissue-engineered tracheal airway was trialled in one human study with 15 months follow-up and showed complete epitheliali-sation on endoscopy (54). Several reviews by Greeta Shroff and colleagues (55-60) noted the potential use of ESCs treatments. In the case of a 65-year-old male patient with Parkinson's disease (PD) treated with human embryonic stem cell (hESC) significant improvement was noted in the reduction of tremor, bradykinesia, muscle rigidity, pain and stiffness in the neck, shoulders and back, as well as improvement in the range of neck movement. The patient was able to maintain balance while walking, had a stiffness reduction in his left hand and both legs. He could speak louder and improve his writing skills. No adverse effects or teratomas were observed (55). Stem cells therapy has shown positive results in three diabetic (diabetes mellitus-DM) patients. Patients were treated with human embryonic stem cells and that resulted in reduced blood glucose levels. Improved vision, endurance, mental focus and muscle strength were observed. After treatment, undesirable effects and teratoma development have not been reported (56). Research on treatment options for spinal cord injuries (SCI) has been shifted to cellular therapies. The use of human embryonic stem cells (hESCs) in five patients who were paraplegics or quadriplegics showed a significant improvement in sedation, control and sensation of the intestine and bladder, strength and movement of the limbs. Unwanted effects have not been reported (57). Human embryonic stem cells therapy (hESCs) is a potential therapeutic option for treating patients with aplastic anemia (AA). The study showed remarkable improvement in patients with AA after hESCs administration. There were no side effects in patients (58). Empirical therapy with hESCs resulted in the improvement and reduction of symptoms in a patient with pulmonary emphysema (59). It has not been

established that pharmacological treatment is effective in the treatment of spinocerebellar ataxia. hESCs based therapy appears as a promising therapeutic option for the treatment of spinocerebellar ataxia. Three patients with spinocerebellar ataxia were treated with human embryonic stem cells. After treatment, all patients showed significant changes in their state of health, improved eye coordination, stroke patterns, the ability to stand without support, muscle strength in all limbs, the ability to walk and swing while standing without support, clarity of speech, good energy levels, reduction in muscle twitching, durability and coordination (60). Recent studies have been followed by two open-label phase 1/2 studies two open I/II phases involving 18 patients with age-related macular degeneration (AMD), suggesting that it is possible to safely implant RPE (hESC-RPE) derived from human embryonic stem cells in an attempt to save photoreceptor and visual function. Anatomical and functional results were encouraging with more than half of treated patients experiencing permanent visual acuity enhancements. However, any conclusion remains relative for a short period of time, a lack of a formal control group, a weak initial visual acuity, and a small number of patients. In addition to the case of postoperative infectious endophthalmitis, no adverse effects associated with cell therapy, such as hyperproliferation, tumorigenesis or inflammation associated with rejection, have been observed in this initial 18-patient group. All studies describing the potential therapeutic use of hESCs showed a beneficial effect in the treatment of various diseases, but future clinical trials are needed to gather more evidence to support the findings and enable wider application of these new therapeutic modalities. On the other hand, since they were first isolated more than three decades ago, embryonic stem cells have been proposed as a source of cells in regenerative medicine, but their plasticity and unlimited capacity for self-renewal cause concern over the safety of their use, as there is a risk of tumor formation (teratoma), potential immunological rejection of cells and tissues, and the risk of differentiating into unwanted cell types (61, 62).

Conclusion

The potential use of embryonic stem cells could be a very attractive and important therapy in medicine and certainly belongs to the therapeutic options of the future. However, it has been addressed with various legislative problems and inadequate education of health personnel. Future research should be directed towards education of health personnel and public opinion, change of legislation, which could reflect the increased use of this research and potential therapy with less scepticism.

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EMBRIONALNE MATIČNE ĆELIJE: GDE SMO TRENUTNO?

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Matične ćelije su funkcionalno definisane kao ćelije koje imaju sposobnost da se replikuju i generišu diferencirane ćelije. One mogu generisati višestruko diferencirane tipove ćelija (multipotentne ili pluripotentne) ili mogu proizvesti jedan tip diferenciranih ćelija (unipotentne) u zavisnosti od kapaciteta replikacije i potencijala matičnih ćelija. Danas, matične ćelije mogu imati potencijalnu primenu u regenerativnoj medicini, transplantaciji, lečenju autoimunih, hroničnih i progresivnih bolesti, kao i u modeliranju bolesti. Sada se suočavamo sa dilemom između dva tipa matičnih ćelija koje su pogodnije za istraživanje i terapijsku upotrebu, embrionalne matične ćelije (ESC) i indukovane pluripotentne matične ćelije (iPSC)? ESC predstavljaju zlatni standard pluripotencije u *in vitro* uslovima, koji upoređuje sve ostale tipove matičnih ćelija, dok je iPSC pogodniji za autologne transplantacije zbog izbegavanja odbacivanja tkiva i zbog odsustva etičkih dilema. Cilj ovog rada bio je predstaviti najvažnije karakteristike ESC koje imaju terapijski značaj.

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Ključne reči: *emrionalne matične ćelije, regulativa, terapija*

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TOTAL SURGICAL RESECTION OF SOLITARY BONE PLASMACYTOMA OF THE SPINAL COLUMN

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Solitary plasmacytomas are a hematological disorder. They can take the form of solitary bone plasmacytoma (SBP) and the form of solitary extramedular plasmacytoma (SEP). These types tend to become multiple myeloma (MM). Treatment is predominately hematologic and radiation therapy. In most cases, SBP is detected after the destruction of the vertebrae and the occurrence of neurological deficits. It is very important to indicate the appropriate and timely surgical approach before applying further therapeutic procedures.

Eight patients in whom it was decided to apply surgical procedure of total tumor resection prior to the application of chemo- and radiation therapy were subjected to analysis. The surgical approach involved the complete removal of altered vertebral bodies with anterior and posterior reconstruction of the spinal column. The primary preoperative and post-operative neurological status was monitored.

Removing the corpus which compromises the width of the spinal canal and the removal of the spinal cord pressure allows neurological recovery or the prevention of severe neurological damage resulting from the destabilization of the spinal column. In all cases there was significant neurological improvement. In addition, in cases of SBP of vertebrae, long-term remission or healing is often achieved.

Complete removal of solitary plasmacytomes of the vertebrae allows the patient to actively continue with further treatment and the prevention of severe neurological damage. A complete 3D reconstruction of the spinal column and the achievement of its mechanical stability is a prerequisite for achieving neurological stability. A timely surgical intervention is conditioned by quality preoperative diagnostic approach and early detection.

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Key words: *solitary bone plasmacytoma, spine, multiple myeloma*

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Introduction

Solitary plasmacytomas (SP) are a rare condition of plasma cells and comprise 5% of all plasma cell neoplasms (1-4). There are two entities depending on the localization: solitary bone plasmacytoma (SBP) and extramedullary plasmacytoma (EMP) (5). They are basically defined as a proliferation of monoclonal plasma cells without evidence of significant infiltration of the bone marrow with plasma cells. The spinal column is the most common site of the solitary plasmacytoma.

According to the new criteria of the International Myeloma Working Group (IMWG), the patient may have solitary bone myeloma if there is no M protein in the serum or in the urine (a small amount of M protein may be present). In addition, it is necessary to define the existence of only one area of bone destruction associated with the presence of plasma cells by means of the MRI finding of the spine and pelvis (6). Recommendations for the treatment of these changes are generally obtained from retrospective studies due to the small number of patients observed in the studies (1, 4, 7, 8). The most common approach to treating these changes is the application of radiation therapy (RT) to which plasmacytoma is highly sensitive. However, bone plasmacytoma is known to progress and grow into multiple myeloma (MM) despite the applied radiotherapies (9). There is no information in the literature on the optimum RT for SP. Most centers use between 30 and 50 Gy or more to treat SP (2, 10, 11).

In the latest solitary bone plasmacytoma (SBP) treatment protocols, the British Society for Hematology (12) recommends surgical removal of SBP, followed by radiochemotherapy. In addition, a large number of papers suggest that surgical intervention is useful for eliminating tumors, correcting pathological fractures and preventing neurological deficits (12). A number of papers have indicated that surgery contributed to better local control and survival length (3). The Guidelines from the National Comprehensive Cancer Network (NCCN) (NCCN, Version 4, 2014) recommend the use of surgery or a combination of surgery and radiotherapy.

Materials and methods

Eight patients were subjected to the surgical treatment for solitary plasmacytoma of the spinal column vertebrae in the period from 2014 to 2018. Surgeries were performed at the Neurosurgical Clinic of the Clinical Center in Niš. The localization of solitary plasmacytomas was as follows: two were found in the cervical spine, 4 in the craniocervical passage and 2 in the thoracic part of the spine.

The selection of patients was done after the performed MRI of the spine due to the development of neurological deficits of varying degrees. The occurrence of neurological damage was on average related to a short period before MRI or after innocuous injuries.

In all patients, complete blood chemistry testing was carried out after the discovery of solitary, destructive changes on the vertebral bodies of the spinal column. Because of the suspicion of plasmacytoma, based on the MRI features, the level of M protein in the urine and blood was measured and MSCT of lungs or abdomen and RT of the pelvis and the skull were performed in all patients.

The surgical approach consisted of 4 basic principles:

- 1st surgical principle: the principle of complete removal of the pathological process and replacement of the bone structures by auto- or allomaterial
- 2nd surgical principle: enabling complete decompression of the nerve elements in the affected part of the spinal canal
- *3rd* surgical principle: achieving adequate correction of the cervicothoracic curve while preserving the cervical lordosis
- 4th surgical principle: good stabilization without the possibility of postoperative static disorders of the spine

By applying this surgical technique, 3D reconstruction of the spinal column was obtained, which enabled complete mechanical and then neurological stability.

Results

Due to the localization of vertebral destruction, one of the neurological deficits was registered in all patients. Preoperative muscular strength and its immediate postoperative recovery score after 2 months were observed – BMRC (Table 1).

Surgical intervention implied the complete removal of the spinal column vertebra that was affected by the process. The applied technique of complete reconstruction of the spinal column had its own surgical requirements depending on the localization of destruction of the vertebral body.

In the cervical spine area, the anterior approach was first applied to completely remove the vertebral body altered by the destruction with releasing of the spinal canal and pressure on the spinal cord and replacing the removed vertebral body with an artificial corpus. For stability reasons, the anterior stabilization was always done with a titanium plate. This was followed by applying the posterior approach and stabilization of the posterior bone systems, in order to achieve the necessary stability of the spinal column (Figure 1).

In the cervicothoracic area, two approaches were applied due to the complex anatomical localization:

1. Posterior bilateral approach with resection of the ribs and lateral-posterior bilateral removal of the corpus, and then inserting an artificial corpus with the application of the posterior cervicothoracic stabilization and
2. Combined posterior approach with removal of the corpus and anterior stabilization, followed by the use of the posterior cervicothoracic stabilization (Figure 2).

Table 1. Degrade of damage of most affected muscle group before and two months after surgery

Localization SBP	**C3**	**C6**	**Th1**	**Th2**	**Th6**	**Th7**	**Th8**	**Th8**
MSG before surgery	3	2	3	3	4	3	3	4
MSG two months after surgery	5	4	5	5	5	4	5	5

*Muscular Strength Graded (MSG) according to the Royal Medical Research Council of Great Britain

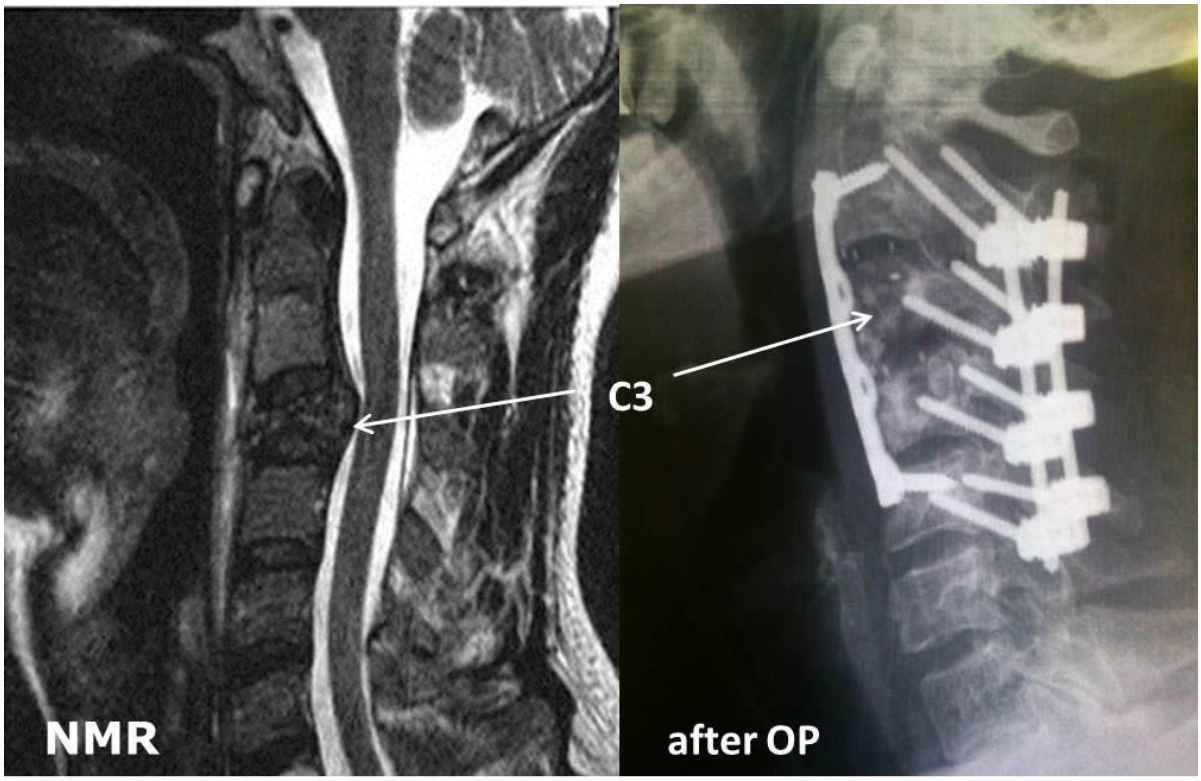

Figure 1. Reconstructive surgery of the cervical spine

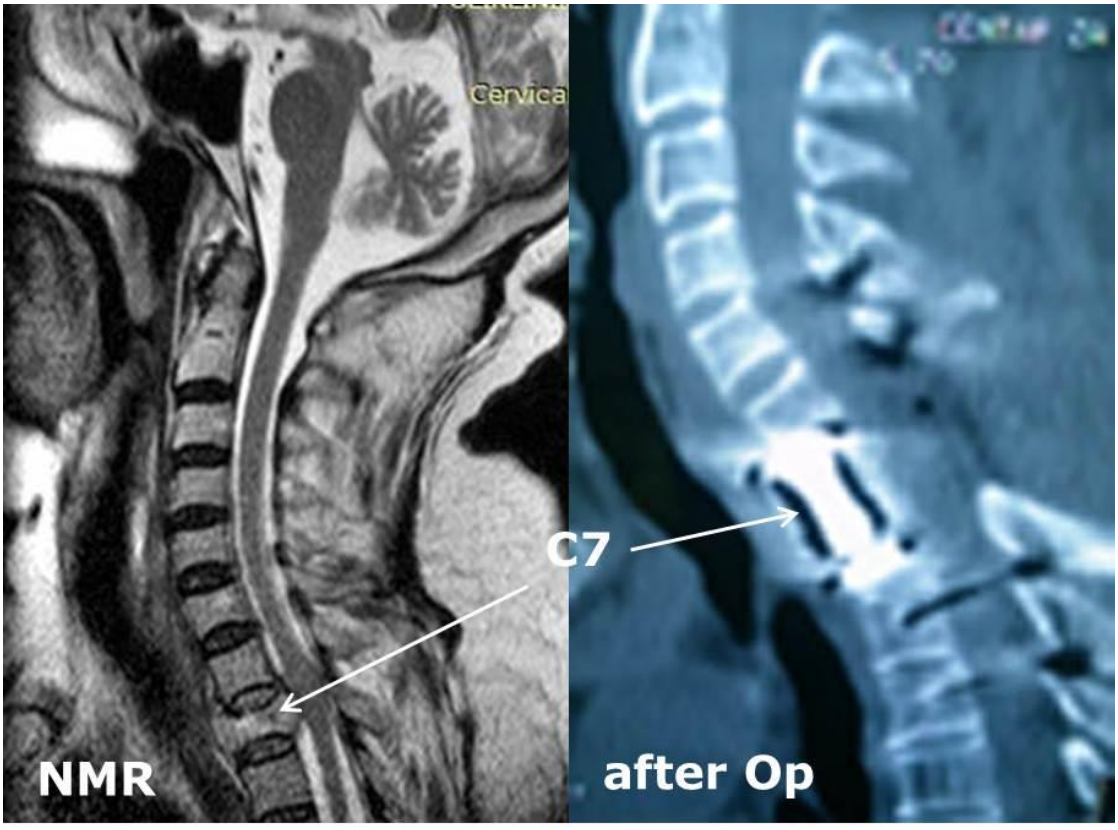

Figure 2. Reconstructive surgery of the cervicothorascic segment of the spine

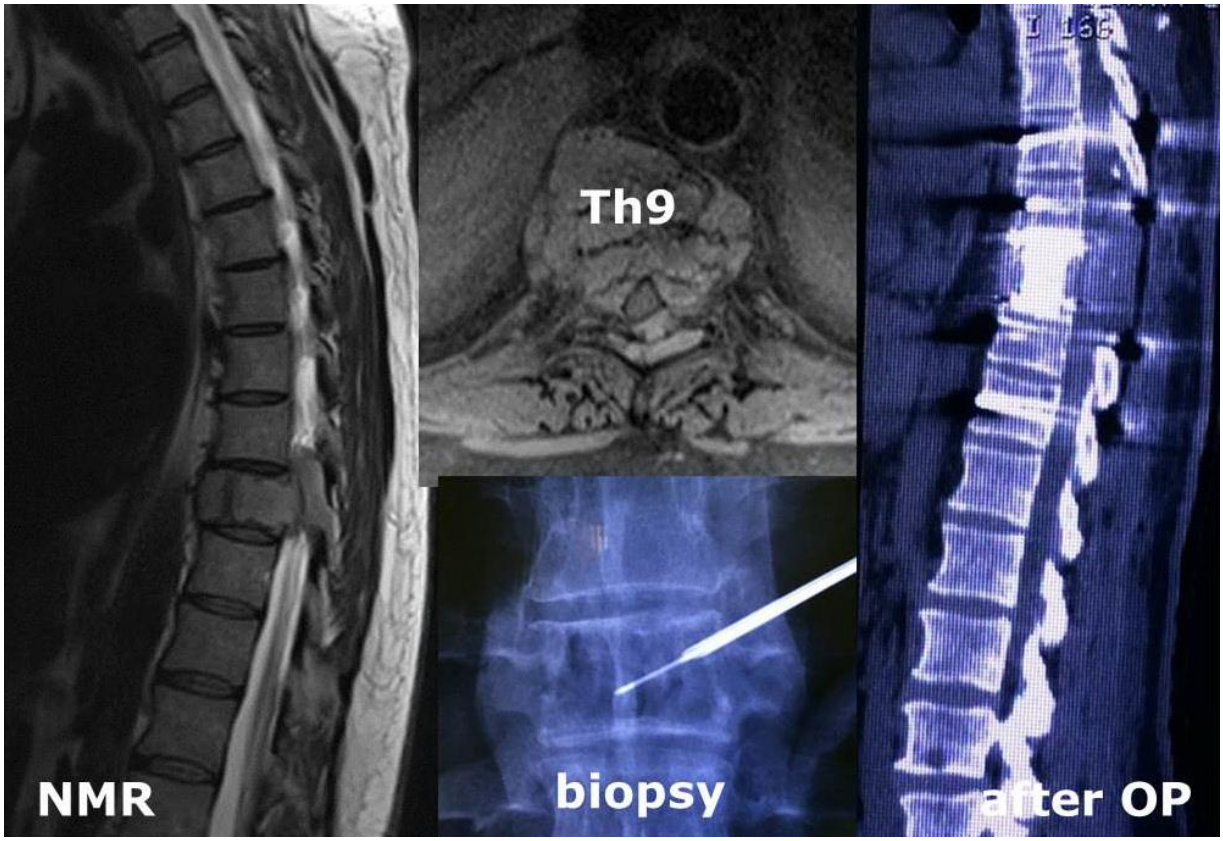

Figure 3. Reconstructive surgery of the thoracic segment of the spine

In the thoracic part of the spinal column, a lateral approach was applied through the thoracic duct, with resection of the rib and collapse of the left lung. The removal of the corpus was complete with the implantation of the artificial corpus. This was always followed by the posterior thoracic transpeduncular stabilization (Figure 3).

Discussion

The application of the principle of complete surgical removal of solitary bone plasmacytoma has been emphasized in many papers (12, 13). The length of survival and the disease-free period are considerably longer. Due to the lack of complete multicentric studies and inadequate post-operative control of the patient, data on the length of survival are not unique (3, 13). In addition, the surgical techniques used to remove the tumor processes have not been harmonized (14). The radical elimination of solitary plasmacytomas of the spinal column can significantly affect the prolongation of the remission of the disease (15).

The question arises of the need for a combined procedure, which would involve the use of radiotherapy, and even chemotherapy, after surgery. The protocols have not been clearly harmonized.

Within this small series, it is not possible to give clear and accurate guidelines for the treatment of solitary bone plasmacytoma. Still, there are data which should be taken into consideration, namely, that radiation therapy was not used in 2 patients after surgery; in 2 patients only radiation therapy was used, and in 4 both radiation therapy and chemotherapy were used.

In patients undergoing surgery alone, chemotherapy was introduced after 14 and 18 months due to the deterioration of the general biohumoral status and the increase in the level of M protein in the urine. Subsequently, due to the progression of the disease towards multiple myeloma (MM) disease, bone marrow transplantation was also performed in one of the patients.

In the group of patients in whom only radiation therapy was used, 2 patients are still without progression of the disease, and in 2 patients an additional chemotherapeutic approach was applied.

In the group of patients receiving radiochemotherapy, one patient was in remission and under control, whereas no data was available for the other patient (after regular surgical controls and general well-being, he did not show up for his follow-up appointments).

From this small analysis of the treatment outcomes, the application of radiotherapy may also be suggested although no high levels of M proteins in the blood and urine were recorded after the surgical removal of solitary bone plasmacytomas. Chemotherapy should already be considered as well in patients with M protein levels at a 20g/l limit. There is

also an opinion that the approach for treating solitary bone plasmacytomas should be the same as in multiple myeloma (16, 17).

Conclusion

By using partial reduction surgical techniques, which are directed only to the decompression of the spinal canal, most of the solitary bone plasmacytoma remain, which extremely increases the risk of further progression of the disease despite the application of other treatment procedures. Likewise, partial reduction of the tumor does not lead to the achievement of the mechanics of stability of the spinal column, and patients are often bed-ridden.

Complete surgical removal of solitary plasmacytomas of vertebral bodies of the spinal column results in a very rapid neurological recovery. With the application of the 3D reconstruction technique and complete implementation of the artificial corpus, and by achieving the necessary mechanical stability of the spinal column, the patient can be fully involved in further treatment procedures. The patient becomes active without the risk of developing neurological diseases.

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TOTALNA HIRURŠKA RESEKCIJA SOLITARNOG PLAZMOCITOMA U VRATNOM I TORAKALNOM DELU KIČMENOG STUBA – ANALIZA SLUČAJEVA

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Solitarni plazmocitomi su hematološko oboljenje, koje može poprimiti formu solitarnih koštanih plazmocitoma (SBP) i formu solitarnih ekstramedularnih plazmocitoma (SEP). Ove vrste imaju tendenciju prerastanja u multiple mijelome (MM).Pristup lečenju je, dominanto, hematološkom i zračnom terpijom. U većini sličajeva do otkrivanja SBP dolazi nakon destrukcije kičmenih prišljenova i pojave neuroloških deficita. Veoma je značajno pravovremeno indikovati odgovarajući hirurški pristup pre primene daljih terapijskih procedura.

Analiziranju je podvrgnuto 8 bolesnika kod kojih je odlučeno da se pristupi hirurškoj proceduri totalne resekcije tumora pre primene hemioterapije i zračne terpije. Hirurški pristup je podrazumevao kompletno uklanjanje izmenjenih kičmenih pršljenskih tela sa prednjom i zadnjom rekonstukcijom kičmenog stuba. Praćeni su primarni preoperativni i post operativni neurološki statusi.

Uklanjanjem korpusa, kojim je kompromitovana širina kičmenog kanala i uklanjanjem pritiska na kičmenu moždinu, omogućuje se neurološki oporavak ili prevencija teških neuroloških oštećenja zbog destabilizacije kičmenog stuba. U svim slučajevima, došlo je do značajnog neurološkog poboljšanja. Pored toga, u slučajevima solitarnih plazmocitoma pršljenskih tela, često dolazi do postizanja dugovremenih remisija i/ili izlečenja.

Kompletno uklanjanje solitarnih plazmocitoma kičmenih pršljenova omogućava da bolesnik aktivno nastavi sa daljim lečenjem uz preventiranje teških neuroloških oštećenja. Kompletna 3D rekonstrukcija kičmenog stuba i postizanje njegove mehančke stabilnosti bitan je preduslov za postizanje i neurološke stabilnosti. Pravovremena hirurška intervencija je uslovljena i kvalitetnim preoperativnim dijagnostičkim pristupom i ranim otkrivanjem.

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Ključne reči: *solitarni koštani plazmocitom, kičma, multipli mijelom*

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COMPARATIVE ANALYSIS OF DIFFERENT METHODS FOR BODY FAT ASSESSMENT IN ADOLESCENTS

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In order to determine the differences between results obtained by using two different methods for estimating the body fat percentage in adolescents, a comparative analysis was performed with bioelectrical impedance method and a traditional method for assessing body composition by skinfold measurement. The sample of this study consisted of 86 seventh grade students of elementary school (42 girls and 44 boys). Body fat percentage was estimated using electronic scale through bioelectrical impedance for assessing the body composition "OMRON BF-511, Japan" and traditionally by measuring skinfolds with caliper and further calculations using equations according to Slaughter (1988). After analyzing the obtained results, it was found that no statistically significant differences were present between body fat percentage obtained by the method of bioelectrical impedance and the method of skinfold measurement of triceps and subscapular ($p = 0.711$) and triceps and calf ($p = 0.850$) in girls, while statistically significant differences were found between the results of these two methods ($p = 0.001$; $p = 0.009$) in boys. Comparison of two most commonly used methods for assessing body fat percentage shown similar results in girls, while in boys, significant differences were present between measurements of these two methods.

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Key words: *body fat, BIA, skinfolds, comparison, adolescents*

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Introduction

There is an increasing demand for body composition analysis in personal use or homecare to monitor weight status, weight loss therapy, or outcome of strength or endurance exercise (1) and the most important fact to monitor general health status of children and adolescents because of presence of obesity prevalence among them.

The body composition represents different tissues within human body, as well as their mutual relationship. According to the American Association for Health, Physical Education, Recreation and Dance (2), the body composition represents the ratio of fat, muscle and bone tissue in the overall body mass. Body composition is defined as a fitness component that is closely related to the relative values of muscle, fat, water, bones, and other vital parts of the human body (3, 4). In practice, the most commonly used methods for determining the composition of the body are based on a two-compartment model where total body mass consists of two parts: fat and fat free mass (5). Currently, there is a range of methods for measuring body composition. They vary depending on instrumental or personal needs, as well as in how accurately the observed values are determined (6).

For the evaluation of fat tissue, direct and indirect methods are used. In practice, methods that have been shown to be much more accurate and valid are direct methods such as dual-energy X-ray absorptiometry (DEXA), hydrodensitometry, plethysmography (BOD POD), ultrasonic method, magnetic resonance and the most accessible, but not as accurate as previously mentioned, method of bioelectrical impedance (BIA). Less accurate are indirect methods where results are obtained by measuring a number of anthropometric characteristics to determine the body fat percentage. The anthropometric method involves measuring body weight, body height, body circumference, skinfolds and diameters at reference points from which individual parts of the

body composition can be calculated by using equations (7). The most famous and most commonly used traditional method which is based on a two-component model of body composition is skinfold thickness measurement. Such methods require trained gauges and knowledge of anatomy of the body. According to the measurement protocol, measurements are taken at least twice, so that the final results can be traced, as opposed to the modern method for assessing the body composition by bioelectrical impedance where the procedure is fairly simple, fast and allows almost instantaneous results (8, 9).

The bioelectrical impedance method (BIA) is a non-invasive, fast, simple and reliable method which evaluates body composition by emission of a safe low-frequency current through the body structure and measures the impedance (resistance) of various tissues. The current flows through the body fluid that contains electrolytes without excessive resistance. In addition, the fat tissue contains only small amounts of water, so current will not easily pass through fat tissue. Unlike body fat component, a fat free component, which contains large amounts of body fluid and, therefore, electrolytes, is a better conductor of the current. The latest generations of BIA devices allow precise calculations of body fat, body cell mass, extracellular mass, intracellular water and extracellular water as well as several other derivatives (10).

The aim

The aim of this study was to determine body fat percent of elementary school students using indirect (two equations for skinfold thickness measurement) and direct (BIA) field methods for assessing body fat percentage and to find out the differences among these methods.

Material and methods

The sample of this study consisted of 86 - 7th grade elementary school students, age 13 years ± 6 months. The sample was divided into two subsamples: girls (n = 42; BMI = 19.97) and boys (n = 44; BMI = 20.59). The sample includes every student who volunteered to participate in the research with the consent of their parents. An additional requirement was that students during the testing were clinically healthy. Subjects were familiar with basic methods, procedures and research objectives.

For assessment of body fat percent by indirect method, anthropometric measurements of three skinfolds were performed: triceps (TRI), subscapular (SUB) and calf (CA). Measurement of skinfold thickness was carried out according to the methodology recommended by the International Biological Program (11) using the GPM GmbH GPM (GPM GmbH Switzerland) with a measurement accuracy of 0.2 mm. The pressure of the instrument clamps on the skin and subcutaneous tissue was standard (10gr/mm²). The measurement result was read 2 seconds after the grip had been caught on the skinfold. The results of the fat tissue from indirect method were calculated using the following equations according to Slaughter (12):

For girls:

1.) $\mathbf{BF\%_{TRI+CA}}$ = .610 (**TRI+CA**) + 5.1

2.) $\mathbf{BF\%_{TRI+SUB}}$ = 1.33 (**TRI+SUB**) - .013 (**TRI+SUB**)2 - 2

For boys:

1.) $\mathbf{BF\%_{TRI+CA}}$ = .735 (**TRI+CA**) + 1.0

2.) $\mathbf{BF\%_{TRI+SUB}}$ =1.21 (**TRI+SUB**) - .008 (**TRI+ SUB**)2 - 3.4

For assessment of body fat percent by direct method, bioelectrical impedance analysis (BIA) method was used with single frequency tetrapolar hand-to-foot electronic scale OMRON-BF511 (Omron, Japan), which, according to the technical specifications of the device, gives results with a precision of 0.1%. Before measurements, the previously obtained body height data (anthropometer by Martin), age and sex of the subjects were entered using the device key. Subjects were measured barefoot in light underwear following manufacturer's instructions. The single frequency hand-to-foot BIA devices provides estimated values for BF% by passing an low frequency (50hz) alternating current through the subject than calculate impedance (ohms) of different body tissues.

Statistical analysis

In order to determine the differences in results between the indirect and the direct method for estimating body fat percent, a t-test for dependent samples (paired t-test) was applied at the level of statistical significance $p < 0.05$.

For raw data processing and statistical analysis, statistical software - STATISTICA 8.0 for Windows (StatSoft, Inc., Tulsa, OK), was used.

Results

The results of t-test paired samples analyses of repeated measurements and comparison of two methods for estimating body fat percentage for girls were showed in tables 1-2. T-test analysis (Table 1.) showed no significant differences between body fat percentage measured from triceps and calf skinfolds equation (BF% = 22.66) and body fat percentage measured from Omron BF-511 bioelectrical impedance scale (BF% = 22.80). Also, t-test analysis (Table 2.) showed no significant differences between body fat percentage measured from triceps and subscapular skinfolds equation (BF% = 22.59) and body fat percentage measured from Omron BF-511 bioelectrical impedance scale (BF% = 22.80).

The results of t-test paired samples analyses of repeated measurements and comparison of two methods for estimating body fat percentage for boys were showed in tables 3-4. T-test analysis showed significant differences ($p = 0.009$) between body fat percentage measured with triceps and calf skinfolds equation (BF% = 19.56) and body fat percentage measured from Omron BF-511 bioelectrical impedance scale (BF% = 17.65).

Table 1. T-test results of body fat percentage measured by triceps and calf skinfolds equation and bioelectrical impedance analysis for girls

Variable	N	Mean	Std. Dev.	t	p
BF%$_{TRI+CA}$	42	22.66	5.681	-0.191	0.850
BF%$_{BIA}$	42	22.80	7.556		

TRI+CA – body fat percent from triceps and calf skinfold equation; BIA – body fat percent from bioelectrical impedance analyzer Omron BF-511

Table 2. T-test results of body fat percentage measured by triceps and subscapular skinfolds equation and bioelectrical impedance analysis for girls

Variable	N	Mean	Std. Dev.	t	p
BF%$_{TRI+SUB}$	42	22.59	5.145	-0.374	0.711
BF%$_{BIA}$	42	22.80	7.556		

TRI+SUB – body fat percent from triceps and subscapular skinfold equation; BIA – body fat percent from bioelectrical impedance analyzer Omron BF-511

T-test analysis showed significant differences (p= 0.001) between body fat percentage measured with triceps and subscapular skinfolds equation (BF%= 19.88) and body fat percentage measured from Omron BF-511 bioelectrical impedance scale (BF%= 17.65).

Table 3. T-test results of body fat percentage measured by triceps and calf skinfolds equation and bioelectrical impedance analysis for boys

Variable	N	Mean	Std. Dev.	t	p
BF%$_{TRI+CA}$	44	19.56	6.662	2.739	0.009*
BF%$_{BIA}$	44	17.65	7.357		

TRI+CA – body fat percent from triceps and calf skinfold equation; BIA – body fat percent from bioelectrical impedance analyzer Omron BF-511;* - significant at $p < 0.01$

Table 4. T-test results of body fat percentage measured by triceps and subscapular skinfolds equation and bioelectrical impedance analysis for boys

Variable	N	Mean	Std. Dev.	t	p
BF%$_{TRI+SUB}$	44	19.88	6.289	3.645	0.001*
BF%$_{BIA}$	44	17.65	7.357		

TRI+SUB – body fat percent from triceps and subscapular skinfold equation; BIA – body fat percent from bioelectrical impedance analyzer Omron BF-511; * - significant at $p < 0.01$

Discussion

The purpose of this study was to determine body fat percent of elementary school students using two field methods for assessing body fat percentage and to find out the differences among these methods. Skinfold measurements and bio-electrical impedance analysis (BIA) are popular and most commonly used methods for assessing body composition in children and adolescents due to their cost efficiency and ease of use.

Measurement of some skinfolds requires the

removal of clothing, which can be awkward in some testing situations (13). Compared to other choices of skinfold sites, measuring the calf and triceps skinfolds and the subsequent prediction of percent body fat (% BF) (12) can be used to assess body composition of children in public settings while remaining sensitive to issues of privacy that have become increasingly prevalent (14). Another good thing from practical point of view is measurement speed when using only two skinfold measurement, it is as fast as measuring with BIA.

Bioelectrical impedance analysis (BIA) also represents a simple, inexpensive and noninvasive method for assessing body composition that has broad application in research laboratories, hospitals, private clinics, health centers and schools (15). The human factor plays an important part when measurements are carried out using calipers. It is important for the person conducting the measurements to be sufficiently experienced. Measurements based on BIA methods bring some other factors that significantly influence accuracy of these measurements, such as body hydration, abnormalities in body composition, underweight or overweight. Therefore, when using BIA methods, the only results considered valid are those of individuals with BMI ranging between 18.5 and 34 kg/m^2 (6, 16-19) which is in accordance with BMI values from population in this study (BMI=19.97 for girls and BMI=20.59 for boys).

The results from this study showed no significant differences between skinfold and BIA methods in girls. Triceps and calf skinfold equation (BF% = 22.66) gave closer values to BIA (BF%bia= 22.80), than triceps and subscapular skinfold equation (BF% = 22.59) in girls. The results in boys showed that body fat percent from both skinfold equations statistically differ from BIA values. Triceps and calf skinfold equation (BF% = 19.56) and triceps and subscapular skinfold equation (BF% = 19.88), gave higher values than BIA (BF% = 17.56). In accordance with these results, some studies reported that the triceps and calf skinfold equation overestimated BF% in boys (14), as well as triceps and subscapular skinfold equation (20). In their study, Čokorilo et al. reported no significant differences between skinfold method and BIA method results in adult females (8). Some researchers reported opposite results, that four different skinfold prediction equations underestimated BF% 8 to 12 year old boys and girls (21). Also, Parker et al., reported that the sum of the triceps and subscapular skinfolds underestimated of BF% in 10 to 14 year old boys (22). Janz et al., reported that the triceps and calf skinfolds equation delivered by Slaughter (12), overestimated of BF% in girls, and the total error increased in males with higher levels of maturation (23). On another side, the investigation of the accuracy of single-frequency BIA provided inconsistent results, with some studies showing a good accuracy (1, 24) and others reporting only a poor agreement between BIA and reference methods (25).

The majority of mentioned authors have reference method like DEXA for comparison of agreement between methods, so it is very difficult to claim from results of this study which method is more accurate. In the absence of reference method as a gold standard, it is impossible to know whether any of the methods investigated here is providing a "true" measure of body fat percentage (26). Also, we must consider that equations derived more than 30 years ago (12), possibly no longer represent the body fat characteristics of children today because of increasing prevalence of overweight and obesity in adolescents (14).

Conclusion

In summary, the present study results showed that two methods are interchangeable only for girls, while for boys there are significant differences between methods. From practical point of view, most suitable method for body fat assessment for girls is triceps and calf skinfold equation, it is fast and good for public use because do not require the removal of clothing. Based on the results of this study it is impossible to suggest which method is more accurate for body fat assessment in girls or boys because of the absence of reference method for comparison. It can be concluded that these methods are more appropriate for assessing body fat of large population rather than an accurate measurement of individuals. According to many studies these methods are population sensitive, so we must imply that obtained results refers only to population from this study. Furthermore, future studies should aim to replicate results on different population with the reference method, also to aim at the development and validity of new equations for adolescents.

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KOMPARATIVNA ANALIZA RAZLIČITIH METODA ZA PROCENU MASNOG TKIVA KOD ADOLESCENATA

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Sa ciljem da se utvrde razlike između rezultata dobijenih primenom različitih metoda za procenu količine masnog tkiva u organizmu kod adolescenata, izvršena je komparativna analiza metode bioelektrične impedanse i tradicionalne metode za procenu telesne kompozicije merenjem kožnih nabora. Uzorak ispitanika činilo je 86 učenika sedmog razreda osnovne škole (42 devojčice i 44 dečaka). Količina masnog tkiva procenjivana je pomoću vage, koja koristi bioelektričnu impedansu za procenu telesne kompozicije, "OMRON BF-511, Japan" i tradicionalno, pomoću merenja kožnih nabora kaliperom i daljim izračunavanjem pomoću matematičkih formula prema Slaughteru (1988). Nakon analize dobijenih rezultata, utvrđeno je da ne postoje statistički značajne razlike između procenta masnog tkiva dobijenog metodom bioelektrične impedanse i metodom merenja kožnih nabora tricepsa i leđa ($p = 0,711$) i tricepsa i potkolenice ($p = 0,850$) kod devojčica, dok su kod dečaka utvrđene statistički značajne razlike između rezultata dveju metoda ($p = 0,001$; $p = 0,009$). Obe metode, kao najzastupljenije i praktično primenljive, pokazale su slične rezultate kod devojčica, dok isti slučaj nije kod dečaka.

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Ključne reči: *masno tkivo, bioelektrična impedansa, kožni nabori, komparacija, adolescenti*

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RESEARCH THE COGNITIVE AND PATHOLOGIC CONATIVE CHARACTERISTICS OF HANDBALL PLAYERS FROM DIFFERENT COMPETITION LEVEL

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The research was conducted on a sample of 180 handball players classified into 3 subsamples of 60 athletes who are competing in I, II and republic handball Serbian league, with the application of 15 variables (3 cognitive and 12 conative). The aim was to estimate if there are significant differences between athletes from different competition levels. Data processing was conducted using canonical discriminative analysis, and the results showed that statistically significant difference exist between handball players from different competition levels in all systems (multivariate) estimated by cognitive and conative variables. In cognitive area 91.73% of the significant interclass differences between I league handball players and handball players from other two levels of competition were estimated by efficiency parallel area S1 test. In cognitive area, the correlations were 0.58 and 0.31, which is 82.82% and 17.11% of variance. In the I league handball players, from all measured parametres, there were only phatological symptoms which refer to depression, hypersensitivity and paranoia, which could be a consequence of greater engagement of cognitive processes and the fear of not fulfilling the expectations set before them, and the complexity of the task, and whether they will continue to pursue their career with even greater success, which is contrary to the results obtained in athletes of the lower ranking, where the pathological concurrent factors are present to a larger extent, indicating a very high level of their somatic anxiety and a higher loading of defense mechanisms and regulators of organic functions.

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Key words: *intelligence, personality, cognitive characteristics, conative characteristics, handball*

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Introduction

There is no sport, no matter how simple, in which cognitive and conative dimensions are not included in the equation that specifies that particular sport. The reason for the increased number of studies which deal with various aspects of the personality of athletes should mostly be sought in the characteristics of the sports activity itself, which place exceptionally strenuous and varied demands on the players, not only in the field of motor abilities, but also in other domains of functioning of an individual. Therefore, the assumption that active and successful participation in certain sports, and therefore in handball, requires a specific group of personality dimensions is justified. These dimensions are the ones best suited for these sports, or at least are groups of personality dimensions suitable for participation in sports as such, and not in other types of activities. Stanković, namely, considers that individuals with the optimal cognitive abilities needed for success in sport achieve success in other activities as well, and that factors such as economic and social status determine orientation towards a particular activity (1).

It is for that reason that increasing the body of knowledge of the structure of cognitive abilities and conative characteristics of athletes is of special importance for the planning and reorganization of work and the predictions of success in sport. A cybernetic model of cognitive abilities (2) is based on the assumption of Luria regarding the cortical functions, and is similar to the model of Das, Kirby & Jarman (3). As part of this study, intelligence was operationally defined as the effectiveness of the in-

formation processing system in situations when intelligent responses were needed, which takes place through the following seven functional units of the system for information processing: 1) the receptor system; 2) the processor for decoding, structuring and analyzing input which in interaction with other processors, gives effects which can be interpreted as perceptive abilities; 3) units of short-term memory, whose purpose is to temporarily store information which has passed through the input processers, or is being treated by the other processors; 4) units of long-term memory whose basic function is to permanently store already processed units of information; 5) a processor for the serial analysis of information, responsible for sequential cognitive processes, sequential search processes in short-term and long-term memory, and the analysis of information which has been transformed into a symbolic code; 6) a processor for parallel (simultaneous) analysis of information (P) needed for the simultaneous processing of an increasingly larger amount of information flow and the parallel search processes in short-term and long-term memory and 7) a central processor (G) whose basic function is the programming, regulating and control of the work of the other processors, and the integration of the results obtained in these processors. In this model the central processor is also the main one needed to make decisions and control their realization.

Handball represents a structurally complex sports activity from all three aspects of the cognitive functioning of sports activities which were mentioned by Horga: the structural system, which represents all of the requirements, cooperation between players, certain rules and specially defined connections between all the parts of the group, movement (a group of movements which are performed during the activity) and the system of required motor abilities which enable athletes to learn and realize movement structures which are part of a particular sport with above-average success. Also, the more complex the sport based on the first two systems of characteristics, structural and movement, the richer the structure of the required motor abilities, and thus greater the cognitive load of the athletes. In other words, the structural elements will be acquired much faster and organized into a structure more easily by cognitively more efficient athletes. Mejovsek claims that if sports tasks resemble motor tasks with elements of information complexity, any deviations from the usual types of movement, rhythm and equal use of both sides of the body, then we can expect the greater involvement of cognitive potential of the athlete when solving problem situations in the game (4). Therefore, if the structure of the required motor skills belonging to some sport is dominated by abilities such as coordination, precision, rhythmic realization of movement, and balance, the greater the cognitive requirements of that sport.

Athletes during sports games build up a cognitive structure made up of all the elements of the game. Various strategies and tactical variants are organized in an optimal and systematic manner, and a structure formed in this way provides a faster, and compared to the less experienced players, more effective evaluation of the current situation in the game. Allard, Graham & Paarsalu studied the perception of structural and non-structural basketball situations among experienced and inexperienced female basketball players and determined that there are significant differences among them in terms of memory and reproduction of structural situations, while the greater use of knowledge of the structure of the game among experienced female players could not help them memorize and reproduce non-structured situations (5). The authors concluded that higher quality players have a greater ability for involuntary learning since they code information on a deeper level.

No athlete is able to perceive all the elements of a situation, and instead must be able to recognize what is important and maintain focus on those important signs in accordance with his own cognitive and motor limitations, the amount of information, and the available time. Tenenbaum and Bar–Eli consider that differences in terms of ex-perience are also more prominently expressed in making complex decisions, i.e. that more expe-rienced athletes are more skilled since their ability for compiling information is of a higher quality (es-pecially visual surveys of the field), as is their ability to focus on what is in the given moment most important (6). A similar conclusion was reached by Helsen & Starks, that elite athletes, compared to those less experienced ones, manage to, based on smaller amounts of information and over a shorter period of time, recognize the structure of movement, the intention of the opponent, and respond accordingly (7).

However, effectiveness in performing any kind of human activity is not independent of the features which regulate the modalities of behavior. Namely, it is known that some features from conative space directly limit effectiveness in various activities directly, and in others indirectly, for example due to the contaminating effects on some other anthropological features, abilities or characteristics (8). This does not exclude the possibility of the same conative features of some activities representing a restrictor, especially in situations which take part in the successful performance of certain activities. Accordingly, the rule that there are no two subjects with identical structures of any dimensions, even conative ones, irrespective of their final number, has been proven. At the same time, the conative regulatory mechanisms which have the task of neutralizing the effects of various interferences (that is, to produce adaptable behavior or emotional stability), can themselves become an impediment the moment when they cross a certain threshold of regulation.

For these reasons, knowledge of the complexities of an activity, which includes the space of conative characteristics, is an important assumption of the operationalization of the goals of every activity, including handball, which is of itself a demanding contact, team sport, whose structure contains elementary movements and types of motion such as running, sprinting, jumping, swinging, hitting, blocking and pushing, along with interactive

contact with the opponent during the game (9). According to some authors (10-12), the equation of the specification of sports results includes as many as 13 factors (morphological, motor, functional, conative, cognitive, motivational, sociological, health, etc.).

Conative characteristics are responsible for the modalities of human behavior and are determined as the latent structures on which the modalities of considered responses, other people and society as a whole depend, as do the characteristic modalities of emotional reactions, and determine the manner, form, and stability, but not the intensity of the reaction. What is known of conative characteristics is that they are connected with the reduction or increase in adaptive power, and that they cause disorders in personality integration, which disrupts the balance between the processes of stimulation and inhibition (13, 14), and that they diminish the adaptational and active effectiveness of the individual. The shared characteristic of both types of conative dimensions (normal and pathological) is that they determine the types of human behavior and reactions, and thus influence its effectiveness and success.

That is why the aim of the research is the analysis of the differences between conative and cognitive abilities of the handball players of various rankings, as indicators of their sports success.

Materials and methods

Sample of participants

A sample of 180 handball players was divided into 3 subsamples of 60 participants each, members of the I, II and national handball league, on which a system of 3 cognitive and 12 conative variables was applied.

Measuring instruments

To evaluate the cognitive abilities, the following variables were applied from KOG 3 battery (15):

1. test IT-1 for the evaluation of the effectiveness of the perceptive processor – taken from MFBT. It was designed with the intention of measuring perceptive ability, which represents a synthesis of the group of perceptive abilities of analysis, structuring and identification. It consists of 39 multiple choice tasks. The participant is required to evaluate which of the 4 test-images is identical to the criterion. The duration of the test is limited to 4 minutes;

2. test AL-4 for the evaluation of the effectiveness of the serial processor – a test of synonyms and antonyms taken from the revised form of the ALFA battery of F.L. Wells and meant to evaluate verbal understanding. It consists of 40 tasks, each made up of a pair of words. The participants are required to determine whether the words in the pair have the same or opposite meaning. The test belongs to the group of speed tests. The duration of the test was limited to 2 minutes;

3. test S-1 for the evaluation of the effectiveness of the parallel processor – is a constituent part of the SVPN-1 battery. The intentional subject matter of the measurement is visual specialization. It numbers 30 multiple-choice tasks. Each consists of a drawing of a three-dimensionally arranged group of bricks, as well as 4 transversal projections of that group. The task of the participant is to find that transversal projection which is suited to the group of bricks viewed from the given direction. It belongs to the category of tests of strength. The duration of the test was limited to 8 minutes.

The pathological conative characteristics were operationalized through the scores on selected scales of the test of pathological conative characteristics (16), C.I.-N4:

- the effectiveness of the system for the regulation and control of organic functions (HI) - 1. cardiovascular conversion (K10), 2. gastrointestinal conversion (G11), 3. inhibitory conversion (I7), 4. hypochondria (H13),
- the effectiveness of the system for the regulation and control of the defense reactions (ALPHA) - 5. anxiety (A1), 6. obsession (O3), 7. hypersensitivity (S5), 8. phobias (F2),
- the effectiveness of the system for the regulation and control of the attack reactions (SIGMA) - 9. impulsivity (N14), 10. aggression (T15) and
- the effectiveness of the system for the coordination of regulatory functions (DELTA) - 11. paranoia (P18), 12. depression (D6).

Methods of data processing

With the aim of finding the differences in the cognitive and conative functioning of handball players of various rankings, the data were analyzed using the canonical discriminant analysis from the statistical package STATISTICA for Windows 8.0 (StatSoft, Inc., Tulsa, OK).

Results

The results of the discriminant analysis of the cognitive variables indicate that the tested athletes differ significantly in relation to their ranking. By condensing the variables in the cognitive space, we isolated two discriminant variables which separate groups of athletes based on the discriminant coefficients of which only one is statistically significant and whose canonical correlation has a value of .38. The significance of this discrimination was tested using Wilks' test and Bartlett's χ^2 test (Table 1).

The first discriminant function explains the differences with 91.73 % of the intergroup variability in the cognitive space of the applied discriminant variables. By analyzing Table 2, we noted that the first discriminant function separates athletes based on the S-1 test which in terms of its basic subject matter of measurement is meant to evaluate the effectiveness of spatial relations. This factor is, actually, superior to the mechanism responsible for determining relations among the elements of a structure and the necessary characteristics of such struc-

tures in solving those problems in which the processes of determining and reconstructing are independent of the previously acquired amount of information (this is the widely known mechanism for parallel processing). Based on the value and sign of the centroid projections on the first discriminant function, we can conclude that the handball players of the Serbian First League of Handball have the most developed abilities for determining the relations among elements of some structure and necessary characteristics of such structures, and then the handball players of the Serbian Second League and finally the Handball League of Serbia (Table 3).

Table 1. Canonical discriminant funtion of cognitive and conative variables

Cognitive Variables							
Fcn	Eigen V.	% of Var	Can Corr	Wilks L	χ^2	df	p
1	0.17	91.73	0.38	0.83	31.84	4	0.00*
2	0.01	8.27	0.12	0.98	2.82	1	0.09
Conativne Variables							
1	0.52	82.89	0.58	0.58	91.06	20	0.00*
2	0.10	17.11	0.31	0.90	17.86	9	0.03*

* - statistical significance at p<0.05 level

Table 2. Matrix of structure of cognitive and conative variables

Variable	Cognitive		Variable	Conative	
	D1	D2		D1	D2
S-1	.95*	.31	A1	.76*	-.22
IT-1	-.22	.97*	O3	.58*	.01
AL-4	.17	.17	F2	.58*	.19
			T15	.47*	.01
			H13	.40*	-.12
			K10	.19*	-.17
			D6	.24	-.47*
			S5	.31	-.41*
			P18	.11	-.31*
			I7	.23	.27*
			G11	.17	.27*
			N14	-.16	.17*

D1, D2 – discriminant function;
* - statistically significant correlation coefficients with a discriminant functions

Table 3. Centroid of groups in cognitive and conative variables

Group	Cognitive		Conative	
	D1	D2	D1	D2
G-1	.39	.13	-.02	-.46
G-2	.18	-.16	-.87	.24
G-3	-.58	.03	.89	.22

G–1, G-2, G-3 – centroid of groups;
D1, D2 – discriminant function

The results of the discriminant analysis in the conative space are shown in Tables 1-3, and a careful analysis can be used to determine that the two obtained significant canonical correlations (0.58 and 0.31) which explain 82.89% that is 17.11% of the valid variance of the overall system of the evaluated space.

The first discriminant function is defined by anxiety, obsession and phobias – and was used to evaluate the mechanism for the regulation and control of defense reactions; Aggression – for the evaluation of mechanisms for the regulation and control of attack reactions, hypochondria and cardiovascular conversion which is used to evaluate the effective-

ness of the system for the regulation and control of organic functions.

Based on the size and sign of the centroid of the first discriminant function, we can note that the handball players of the Serbian First League of Handball have the ability to adequately model tonic stimuli based on the programs transferred by genetic code or formed under the influence of learning, which are located in the centers for the regulation and control of defense and attack reactions. The handball players of the Serbian First League of Handball are able to coordinate functionally and hierarchically different subsystems and to effectively make a bridge between the subcortical regulatory functions of organic systems and cortical systems which perform their regulation and control. The handball players of the Handball League of Serbia are characterized by lowered excitation in the higher centers for coordination and control. It is clear that the asthenic syndrome is the dimension which decreases adaptation in general, and thus in sport as well, since it deactivates precisely those structures of the nervous system which are responsible for adaptational reactions. The handball players of this ranking are overly sensitive, have great performance anxiety, struggle with effort and their training ability is decreased compared to the handball players of higher ranking. The second function is defined by depression, hypersensitivity, paranoia, inhibitory conversion, gastrointestinal conversion and im-pulsivity. Based on the size and sign of the centroids, for the second discriminant function it can be noted that the handball players of the Serbian First League of Handball are able to adequately model the excitatory-inhibitory processes which achieve better results compared to the handball players of lower ranking. The handball players of the Serbian Second League of Handball and the Handball League of Serbia are characterized by a disorder of the mechanism for the regulation of the functions of the vegetative system, where the primary central disorder of the nervous system is manifested in the work of certain organs or functions of organs.

Discussion

In the cognitive space, based on the value and sign of the centroid projections on the first discriminant function, we can conclude that the handball players of the Serbian First League of Handball have the most developed abilities for determining the relations among the elements of a structure and the necessary characteristics of such structures, followed by the handball players of the Serbian Second League of Handball and finally the Handball League of Serbia. That is why we can conclude that visual spatialization is directly related to the quality of the performance and ranking in handball. Namely, handball is a sports game in which the result depends not only on the adopted techniques and abilities, primarily motor and functional, and also on the solution of complex tactical tasks, and the obtained results of the discriminant analysis are probably the consequence of the selection of handball players, but also their sports experience. It was Momirović, Gredelj & Hošek (17) who determined that what is most responsible for success in sport is the better adaptation of cognitive abilities, especially among elite athletes. The second discriminant function exhausts the smaller variance and cannot sensibly be interpreted, since it is not statistically significant.

Table 4. Comparison of the results of athletes from different sports on KOG 3

	IT1		AL4		S1	
	M	SD	M	SD	M	SD
handball players from I league (N = 60)	25.00	5.56	35.53	5.46	26.30	3.53
handball players from II league (N = 60)	23.38	6.45	34.70	3.81	24.96	4.78
handball players from republic league (N = 60)	25.78	7.30	34.41	3.77	21.86	5.40
football players from I league (N = 136) - Malacko (30)	20.79	4.92	27.83	5.67	16.22	5.58
boxers from different categories (N = 92) - Blažević (31)	33.25	3.30	35.97	2.55	22.11	3.03
handball players from I and II league (N = 50) - Ilić (24)	23.02	6.61	29.42	7.41	20.62	6.57
Volleyball players from I and II league (N = 50) - Ilić (24)	25.00	5.72	31.78	6.19	22.92	5.84
basketball players from I and II league (N = 50) - Ilić (24)	24.34	5.61	31.32	6.21	20.74	5.90
football players from I and II league (N = 50) - Ilić (24)	21.20	5.18	28.54	6.07	19.94	5.87

Table 5. Comparison of conative characteristics of athletes

	Handball players I league (N = 60)		Handball players II league (N = 60)		Handball players republic league (N = 60)		Young volleyball players (N = 126) of various rankings – Ivanović M, Milosavljević and Ivanović U (32)	
	M	SD	M	SD	M	SD	M	SD
Cardiovascular conversion	2.55	2.36	0.98	0.77	3.61	2.25	13.01	8.61
Gastrointestinal conversion	0.48	0.79	0.18	0.39	1.05	1.17	0.85	1.33
Inhibitory conversion	1.06	1.31	0.46	0.50	1.06	1.20	1.29	1.30
Hypochondria	0.40	0.92	0.03	0.18	0.33	0.68	0.23	0.80
Anxiety	0.26	0.82	0.10	0.30	0.46	0.76	0.19	0.68
Obsession	0.36	0.68	0.36	0.51	0.63	0.75	0.30	0.90
Hypersensitivity	0.16	0.52	0.20	0.40	0.40	0.96	0.39	0.90
Phobias	1.36	1.71	0.56	0.67	1.88	1.72	1.24	1.50
Impusiveness	0.50	0.89	0.10	0.35	0.95	1.04	0.60	0.90
Agression	0.38	0.69	0.55	0.50	0.36	0.68	0.49	0.84
Paranoia	0.90	1.39	0.45	0.67	1.41	1.25	1.51	1.49
Depression	1.25	0.93	1.01	0.59	1.16	0.64	1.17	0.70

But, if we bear in mind that in addition to the findings obtained in some previous studies, regarding the fact that athletes achieved higher scores on the g factor compared to non-athletes (18, 19), the result itself, achieved on tests which measure the g factor, does not suffice for the precise prediction of the success in sport as such, and even less so for a particular type of sport, and especially not for the evaluation of the success of a specific position or discipline. Lazarević claims that various situations in sport also require developed specific abilities such as perception of space, differentiation of the schedule and the position of other players, anticipation of their movement, making decisions, etc., which should also be studied, along with the general ability which combines them and which influences the successful performance of each of the cited individual abilities (20). That is why the results of the importance of the abilities of visual spatialization for the differentiation of handball players of various levels of success were obtained in this study agree with his results as well, but also the assumptions of authors such as Rushall (21), Bushan & Agarwal (22) and Straub (23), that athletes, based on their cognitive abilities and personality features, differentiate not so much based on the types of sport, as based on their success in it.

However, comparing the results obtained on the KOG 3 in various sports also indicates that these authors were not completely right when they claimed that athletes of various sports do not differ to a great extent in terms of cognitive abilities. Ilić in his study determined that the highest scores on the tests of the KOG 3 battery are achieved by volleyball players, then basketball players, handball players, and the lowest scores by football players (24). When comparing the handball players of all three rankings of competition with athletes from other sports (Table 4), we can see that on all three tests, the lowest scores are achieved by the football players, while handball players competing in the Serbian First League of Handball achieve significantly higher scores than all the other groups on the test of visual spatialization S1. The results of all three categories of handball players are quite even on the test of verbal comprehension AL 4, meant for the evaluation of the effectiveness of the serial processor, and are in agreement with the scores achieved by boxers. On the other hand, boxers achieve by far the highest scores on the IT–1 test for the evaluation of effectiveness of the perceptive processor, that is, the perceptive ability of analysis, structuring and identification.

All this speaks in favor of the results which were obtained by numerous authors (25-28) that athletes who compete in higher rankings of competitions achieve significantly better results on tests of cognitive abilities, compared to athletes who compete at lower rankings of competition.

In the case of the factor structure, in our research, we determined that the cognitive structure of the handball players was explained to the greatest extent by the test of strength S-1 which is essentially meant to evaluate the effectiveness of spatial relations. This factor is actually superior to the mechanism responsible for the determination of the relations among the elements of a structure and the necessary characteristics of such structures in solving of problems in which the processes of determination and reconstruction is independent of the previously acquired amount of information (this is a well-known mechanism for parallel processing). Ilić

determined that the first discriminant function separates athletes involved in team sports to the greatest extent based on the IT-1 test which was meant to evaluate perceptive reasoning (24), while Nešić, Fratarić, Banić and Goranović also determined that among kumite karatekas (N = 100, various rankings) there is one general cognitive factor which to the greatest extent is explained by the AL-4 test of synonyms and antonyms for measuring the effectiveness of the serial processor, which belongs to the group of speed tests (29). The obtained findings speak in favor of the hypothesis that athletes who come from various sports still differ more in terms of the quality than the quantity of their cognitive abilities, which is directly conditioned by the requirements of the sports they are involved in.

The results obtained in the space of pathological conative variables are such that the differences which are obtained between the handball players of various rankings of competition were also explained by two discriminant functions. The first discriminant function is defined by anxiety (to the greatest extent), obsession and phobias – which were used to evaluate the mechanism for the regulation and control of defense reactions, aggression – for the evaluation of the mechanisms for the regulation and control of attacks, hypochondria and cardiovascular convulsions which are used to evaluate the effectiveness of the system for the regulation and control of organ functions. The second function, which explains the remainder of the variance is defined by the absence of depression, hypersensitivity and paranoia, with a mild presence of inhibitory gastrointestinal conversion and impulsivity.

The handball players of the Serbian First League of Handball do not show to any great extent symptoms of anxiety, obsession, phobia, aggression, hypochondria, cardiovascular conversion, inhibitory and gastrointestinal conversion and impulsivity, but in their case we also find the presence of indicators of depression, hypersensitivity and paranoia. Unlike them, the handball players of the Serbian Second League of Handball are characterized by an almost complete absence of symptoms of anxiety, obsession, phobias, aggression, hypochondria, cardiovascular conversion, but also depression, hypersensitivity and paranoia, while we find the presence of symptoms of inhibitory and gastrointestinal conversion and impulsivity. The handball players of the Handball League of Serbia are characterized, unlike the handball players of higher ranking competitions, by a very pronounced presence of symptoms of anxiety, obsession, phobia, aggression, hypochondria, and cardiovascular conversion, which results in the disorder of the mechanisms for the regulation and control of defense reactions, for the regulation and control of attack reactions, and the functions of the vegetative system where the primary central disorders of the nervous system are manifested by a disorder in the functions of certain organs or functions of organs. At the same time, even in their case we have a pronounced presence of depression, hypersensitivity, and paranoia.

In the case of handball players of this ranking of competition, among whom this warm type of behavior is more pronounced, who are more polite, attentive towards others, there is an increase in the scores for depression, as in the case of those who expressed more pronounced liveliness and impulsivity and expressivity. The handball players who more often manifest dominant, competitive, assertive or even aggressive behavior are more prone to anxiety. Those who are more morally inclined and who have more respect for the rules are more prone to hypersensitivity, and those who are fearless and more adventurous have higher scores for obsession. Tense, energetic, impatient handball players often develop gastrointestinal conversion, hypochondria, and phobias.

Stanković cites that in his research of handball players of the Serbian First League of Handball, he determined that the factor of gastrointestinal conversion functions as a separate entity, while on the sample of handball players of the Handball League of Serbia, this is characteristic of the factor of impulsivity, and among the handball players of the Serbian Second League of Handball, there is a high correlation between depression and obsession (28).

Conclusion

Handball is a sports game in which the result does not depend only on the adopted technique and abilities, primarily motor and functional ones, but also on the solution of complex tactical tasks.

Based on these results, we can conclude that the differences in terms of pathological conative variables are far bigger in terms of quality than in terms of intensity when it comes to ranking, unlike cognitive abilities. In the case of handball players who play in the most elite league, of all the measured parameters we only found the pathological symptoms of depression, hypersensitivity and paranoia, which speaks of the greater engagement of cognitive processes, the fear of whether they will fulfill the expectations before them and live up to the challenge, or whether they will be able to pursue their career in a more successful foreign club or national team. These findings are quite opposite to the results obtained for the lowest ranked handball players, where the pathologically conative factors are present to a great extent, with the absence of depression, hypersensitivity and paranoia, which indicates a very high level of their somatic anxiety and greater load on the defense mechanisms and regulators in charge of the optimal functioning of organic functions. The handball players of the Serbian Second League of Handball, unlike the two other groups, to a great extent are characterized by impulsivity when making decisions and a propensity for converting psychological difficulties into physiological ones, which could be suited to the development of psychosomatic conditions, if the regulatory mechanisms in charge of their regulation are exposed to excessive work of a prolonged duration. Somatic and cognitive anxiety in this ranking of competition are not of decisive importance for the development of pathological conative functions.

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ISPITIVANJE KOGNITIVNIH I PATOLOŠKIH KONATIVNIH KARAKTERISTIKA RUKOMETAŠA RAZLIČITOG RANGA TAKMIČENJA

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Na uzorku od 180 rukometaša, podeljenih u 3 subuzorka po 60 ispitanika, koji pripadaju I, II i Republičkoj rukometnoj ligi Srbije, bio je primenjen sistem od 15 varijabli (3 kognitivne i 12 konativnih) sa ciljem da se utvrdi da li postoje statistički značajne razlike između sportista koji pripadaju različitim rangovima takmičenja. Obradom podataka pomoću kanoničke diskriminativne analize, dobijeni rezultati pokazali su da između rukometaša različitih rangova takmičenja u celom sistemu (multivarijantno) primenjenih kognitivnih i konativnih varijabli postoji statistički značajna razlika. U kognitivnom prostoru 91,73% intergrupne razlike pokazalo je da test za procenu efikasnosti paralelnog procesora S1 statistički značajno izdvaja rukometaše I lige od rukometaša druga dva ranga takmičenja. Na konativnom planu korelacije su 0,58 i 0,31 što je 82,82%, tj. 17,11% varijanse. Kod rukometaša I lige, od svih merenih parametara, prisutni su samo patološki simptomi depresivnosti, hipersenzitivnosti i paranoidnosti, koji govore o većoj angažovanosti kognitivnih procesa i strahu od toga da li će ispuniti pred njih postavljena očekivanja, biti na visini zadatka i da li će nastaviti karijeru sa još većim uspehom. Ovi rezultati suprotni su rezultatima dobijenim kod rukometaša najnižeg ranga takmičenja, gde su patološki konativni faktori prisutni u velikoj meri, što ukazuje na veoma visok nivo njihove somatske anksioznosti i veće opterećenje odbrambenih mehanizama i regulatora organskih funkcija.

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Ključne reči: *inteligencija, ličnost, kognitivne karakteristike, konativne karakteristike, rukomet*

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COMPARISON OF SCHWARTZ EGFR-CR WITH GFR MEASURED BY TC-99M-DTPA CLEARANCE IN HEALTHY CHILDREN AND IN CHILDREN WITH URINARY TRACT INFECTION WITH AND WITHOUT VESICOURETERAL REFLUX

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The aim of this study was to assess the diagnostic reliability of the estimated glomerular filtration rate based on serum creatinine and body height (eGFR-Cr Schwartz) as compared to the glomerular filtration rate (GFR) measured by a radioisotope method using Tc-99m-DTPA clearance in healthy children and in children with urinary tract infections (UTI) with and without a vesicoureteral reflux (VUR) using Bland-Altman analysis.

The retrospective study enrolled 451 paediatric patients (104 male and 347 female, aged 7.07 ± 3.02, range of 2-15) from a single-institution database. Groups of participants were formed according to their diagnosis: the control group (CG, n = 64), the group with UTI with no documented VUR (UTI, n = 299), and the group with UTI and VUR (VUR, n = 88). The GFR was measured by the Tc-99m-DTPA clearance from a single blood sample drawn 180 minutes after administering the radiopharmaceutical. The eGFR-Cr was determined from an equation which included body height and the serum creatinine level.

When compared to Tc-99m-DTPA GFR, the eGFR-Cr yielded the following mean deviation values in the examined groups: CG: 1.937 ml/min/1.73m^2 (95% limits of agreement [LOA]: -36.759–40.633 ml/min/1.73m^2), UTI: -3.010 ml/min/1.73m^2 (LOA: -57.292–51.272 ml/min/1.73m^2) and VUR: 2.183 ml/min/1.73m^2 (LOA: -64.019–68.385 ml/min/1.73m^2). eGFR-Cr demonstrated comparable accuracy to Tc-99m-DTPA GFR in the CG and UTI groups with 95% and 82% of values within 30% of the Tc-99m-DTPA GFR. The eGFR-Cr demonstrated a lower measurement reliability in the VUR group, amounting to only 68% of the values within 30% of the Tc-99m-DTPA clearance.

The results proved eGFR-Cr to be a reliable alternative to the radioisotope method in children with preserved renal function and children with a urinary tract infection with no reflux, but a less reliable method in children with both VUR and renal impairment.

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Key words: *estimated glomerular filtration rate, Tc-99m-DTPA clearance, children, vesicoureteral reflux, urinary tract infection*

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Introduction

The glomerular filtration rate (GFR) is the most reliable quantitative indicator of renal function. It is of essential significance in diagnosing and assessing acute and chronic renal impairment in children and adults (1). It is a parameter that designates the volume of plasma filtered by nephrons until forming urine in a unit of time (2, 3). Determining the GFR precisely is especially significant in childhood during and after treating nephro-urological disorders with the aim of preventing renal impairment. In addition, the precise measurement of the glomerular filtration rate is of vital importance in paediatric oncology before administering cytostatic drugs with a nephrotoxic effect because the dosage of the cytotoxic drugs must be adjusted to the GFR value, i.e., reduced

renal function demands a reduction in the cytostatic dosage.

The gold standard for determining the GFR is measuring the clearance of inulin, an exogenous marker which is excreted exclusively through glomerular filtration. As inulin does not undergo tubular secretion and reabsorption and does not depend on metabolism, it presents an ideal GFR marker.

The inulin clearance is a reliable marker of the glomerular filtration rate in all ages, even with prematurely born babies (4). However, the performance of this method is time-consuming and demanding, requires continuous infusion, and the extraction of a large number of blood samples with the simultaneous measurement of the concentration of inulin in urine in order to calculate this compound's clearance (3). These drawbacks make this method of determining GFR unsuitable for use in everyday clinical practice, especially when it comes to paediatric patients, and limit it to rare cases in the area of scientific research, primarily for testing the reliability of new, simpler methods for determining the GFR.

Standing out from the alternative methods of determining the GFR based on measuring clearance using non-ionising means, there is a method that uses the radiological contrast medium Iohexol, which has a very low degree of toxicity due to its low osmolarity and negligibly low concentration of free iodine (5-7). Another method applied is determining the clearance of endogenous creatinine if gathering urine is possible, but this method is less present in the paediatric population not only due to the inability to collect urine during 24 hours, but also due to the fact that the production of creatinine depends on muscle mass, which increases with growth and is dependent on the gender and age of the child (4).

When endogenous creatinine production is constant, its plasmatic concentration depicts the glomerular filtration so that the decline of renal function leads to the increase in the value of this marker. The last few years saw the publication of a large number of mathematic equations based on the value of endogenous creatinine (8-10), cystatin C (11-14), or combined markers (15) which consider all the variables that can influence its value, primarily the height, weight, and the gender of the child.

The calculation of the GFR by radionuclide methods most often uses Cr-51-ethylenediamine-tetraacetate (51Cr-EDTA) and Tc-99m diethylene-triamine-pentaacetate (Tc-99m-DTPA), and in some centers I-125-iothalamate. These methods proved to be extremely reliable and reproducible in comparison to the method of measuring inulin clearance (16). The clearance is determined by measuring the plasma sample activity after an intravenous application of one of the radiopharmaceuticals which is characterized by secretion through glomerular filtration. Due to a high correlation with the "gold standard", radioisotope methods of determining clearance are today considered reference methods and are used for precisely determining the GFR, as well as testing simplified equations for determining the GFR.

The last few years witnessed an increase in non-invasive, harmless, and simple methods of measuring GFR with children based on anthropometric variables and the concentration of serum creatinine or cystatin C. Out of the mentioned methods for "measuring GFR" that yield a quick insight into the renal function, the one that is applied most often is the Schwartz method, which current guidelines, the Improving Global Outcomes (KDIGO) 2012 Clinical Practice Guideline (CPG) for the Evaluation and Management of Chronic Kidney Disease, recommend for measuring GFR with children (17-19). Therefore, the goal of this paper was to test the values determined by the Schwartz Cr method in comparison to the clearance acquired by radioisotope measurement using Tc-99m-DTPA with children with urinary tract infections with and without proven vesicoureteral reflux by applying a Bland-Altman analysis.

Materials and methods

Participants and grouping

The retrospective analysis encompasses charts of 451 consecutive paediatric patients (104 male and 347 female, aged 2-15, mean age 7.07 ± 3.02) referred to the Centre of Nuclear Medicine, Clinical Centre of Niš in order to evaluate their kidney and bladder function. The data were analysed from the database of the Centre of Nuclear Medicine.

Based on the final diagnosis, the patients were divided into the following groups:

- The control group (CG) of children (n = 64) constituted of 31 boys and 33 girls aged between 2 and 13, without any symptoms of lower urinary tract dysfunction. These were children extensively investigated in our department owing to unclear abdominal symptoms. Final biochemical and imaging test results, including dynamic renal scintigraphy, confirmed a normal function and anatomy of the urinary system.
- Children with recidivate urinary tract infections with no documented VUR (UTI, n = 299)
- Children with urinary tract infections and confirmed vesicoureteral reflux (VUR, n = 88)

Parents had to sign a written consent before the radionuclide investigation after being carefully informed about the procedures.

Special groups were made in regard to GFR values according to the KDIGO classification of renal function based on the glomerular filtration rate (18):

-CDK 1: Normal or increased GFR (> 90 ml/min per 1.73 m^2)

-CDK 2: Mild reduction in the GFR (60 to 89 ml/min per 1.73 m^2)

-CDK 3: Moderate reduction in the GFR (30 to 59 ml/min per 1.73 m^2)

-CDK 4: Severe reduction in the GFR (15 to 29 ml/min per 1.73 m^2)

-CDK 5: Kidney failure (GFR < 15 ml/min per 1.73 m^2 or dialysis)

Methodology

Determining the Tc-99m-DTPA clearance from a single blood sample

Parents were advised to additionally hydrate their children with a volume of 10 ml/kgbw using water, tea, or non-carbonated juice at least an hour before applying the radiopharmaceutical. The radiopharmaceutical Tc-99m-DTPA (The Radioisotope Laboratory of the Nuclear Science Institute "Vinča", Serbia) was prepared by adding a fresh eluate of Technetium-99m (Tc-99m) from a Mo-99/Tc-99m generator to a lyophilized chelate compound of DTPA, according to the manufacturer instructions. After determining the dosage activities in relation to the children's body weight (1.85 MBq/kgbw), the syringe volume was filled up with physiological saline to a volume of 1 ml. The syringe activity was measured in a scintillation counter before and after applying the radiopharmaceutical in order to determine the total activity of the applied radiopharmaceutical.

The distribution volume was determined from a blood sample taken 180 minutes after administering the radiopharmaceutical from the arm contralateral to the one the radiopharmaceutical was applicated to.

Determining the GFR is based on the empirical relation between the distribution volume of the radiopharmaceutical (ECV) and the clearance determined from several blood samples (20), and this relation was represented by a regression equation:

$$y = -0.0128x2 + 3.077x - 30.3$$

where y = GFR, x = ECV

The measured plasma activity was corrected for body surface area (BS) with the aim of acquiring the current body surface area (fBS) according to the following equation (21, 22):

$$ECV = (ID/CxfBS)$$

(ID = the administered dosage activity, C = plasma sample activity)

The correction factor for the body surface, fBS, was determined according to the following equation (21, 22):

$$fBS = 1.73/BS$$

(BS = body surface area)

The body surface area is determined according to Haycock's method (23) using the equation:

$$BS = 0.024265 \times BH^{0.3964} \times BW^{0.5378}$$

(BH = body height in m, BW = body weight in kg)

Determining the Schwartz eGFR-Cr

The estimated GFR using the Schwartz method was calculated in accordance with the following equation:

$$eGFR\text{-}Cr(ml/min/1.73m^2) = 41.3xBH(m)/sCr\ (mg/dl)$$

Serum creatinine was determined by an enzymatic method using an Olympus AU400 analyser from Beckman Coulter Inc.

Statistical methodology

Statistical analysis was performed in the IBM SPSS program, version 20.0. A Bland-Altman analysis that compares the results of the two measurements was performed to determine whether the new, simpler method could replace a measurement technique that is more complicated but proven reliable (24).

The Bland-Altman method was used to determine the means of the deviation of the Schwartz eGFR-Cr in comparison to the means of the GFR determined by the Tc-99m-DTPA clearance for every participant group. The limits of agreement were determined (LOA, deviation mean ± 1.96xSD). Reliability was determined as the percentage of participants whose Schwartz eGFR-Cr value was within 30% of the value gained for GFR using Tc-99m-DTPA.

The ANOVA and Student's paired t-test were used to compare the means of the numeric variables within the examined groups. The frequency analysis was performed using a χ^2 test.

The differences were statistically significant for the values of $p < 0.05$.

Results

Table 1 shows the clinical characteristics of children within the defined groups.

Patients from the control group, as well as children with urinary tract infections with no vesicoureteral reflux, were significantly older than the group of children with reflux. Consequently, the UTI group with no vesicoureteral reflux had a significantly larger body height compared to the children with vesicoureteral reflux because the age difference between these two groups was the greatest. However, the differences in body high and age cannot affect the GFR values in children with healthy kidneys because there are no oscillations of GFR values found after the age of two, when the process of nephron maturation is completed (17). While the number of male and female children in the control group was equal, females significantly exceeded the number of males in the UTI and VUR groups (UTI: χ^2 140.555 $p < 0.001$; VUR: χ^2 16.409 $p < 0.001$).

The GFR mean values obtained from the Tc-99m-DTPA clearance in children with reflux were significantly lower in relation to the values established in the control group of healthy children and children with a urinary tract infection with no reflux (Table 2). When comparing the Schwartz eGFR-sCr means, a significantly lower value was found in children with UTI without reflux as compared to the control group. However, after comparing the GFR means by applying a paired t-test between the participants of the same groups, there was no significant difference

(CG: t 0.773 p = 0.443; UTI: t -1.812 p = 0.071; VUR: t 0.574 p = 0.567). The values of serum creatinine were within the normal range in all studied children without statistical differences between the study groups.

Table 3 shows the comparative results of studying the renal function using the two different methods. Children from the CG exhibited the same eGFR values in 97% of the participants for the clearance value > 90 ml/min/1.73m^2, while 3% of the children yielded a value lower than the reference one, which classified the children into the CKD2 group. When it comes to the group of children with VUR, the eGFR value placed an additional 2 children within the CKD2 group and 3 children into the CKD3 group. The largest deviation was noticed in the UTI group, so 16 more children (6%) were classified into the CKD2 group and 4 children were placed into the CKD3 group.

Table 1. The clinical characteristics of the patients

Clinical characteristics		**CG**	**UTI**	**VUR**
Age (years)	Mean±SD	6.60±3.40 [a]	7.4±3.02	6.09±3.20[a]
	Median, IQR	6.50(3.2-10)	7.0(5-10)	6.0(3-8)
Gender	(male/female)	32/32	47/252	25/63
Height (meters)	Mean±SD	1.21±0.18	1.26±0.18[b]	1.19±0.19
	Median, IQR	1.21(1.06-1.36)	1.27(1.12-1.36)	1.17(1.01-1.30)

[a] vs UTI $p < 0.001$, [b] vs VUR $p < 0.005$

Table 2. Parameters of renal function

Parameters		**CG**	**UTI**	**VUR**
sCr(mg/dl)	Mean±SD	0.66±0.05	0.72±0.10	0.76±0.19
	Median, IQR	0.64(0.62-0.69)	0.72(0.64-0.80)	0.69(0.62-0.81)
Tc-99m-DTPA GFR (ml/min/1.73m^2)	Mean±SD	130.87±5.87	123.02±14.5	122.18±22.91[a]
	Median, IQR	130(129-135)	126.00(112-135)	128.5(110.25-140)
eGFR-sCr Schwartz (ml/min/1.73m^2)	Mean±SD	132.84±19.6	119.32±22.67[b]	124.37±31.53
	Median, IQR	133.98 (118.71-147.48)	118.35 (102.05-136.02)	126.53 (103.88-150.60)

sCr-serum creatinine, [a] vs CG and UTI $p < 0.001$, [b] vs CG $p < 0.001$

Table 3. Comparative studying of renal function

CKD stage	GFR values	Groups		
		CG	UTI	VUR
		Number (%)		
CKD 1	GFR	64 (100%)	292 (98%)	78 (89%)
	eGFR	62 (97%)	272 (91%)	77 (88%)
CKD 2	GFR	-	7 (2%)	10 (11%)
	eGFR	2 (3%)	23 (8%)	8 (9%)
CKD 3-5	GFR	-	-	-
	eGFR	-	4 (1%)	3 (3%)

CKD stage: CKD1, >90 ml/min/1.73 m^2; CKD2, 60–89 ml/min/1.73 m^2; CKD3–5, <60 ml/min/1.73 m^2

A Bland-Altman analysis (Figure 1) was used to determine the measurement deviation as a mean value of the difference between the GFR acquired using Tc-99m-DTPA and Schwartz eGFR-Cr, while the limits of agreement (LOA) were determined as a deviation mean value of ± 1.96xSD. Reliability was determined as a percentage of patients whose Schwartz eGFR-sCr values were within 30% of the Tc-99m-DTPA clearance value (Table 4).

Table 4. The diagnostic reliability of the Schwartz eGFR-Cr method

eGFR-Cr	Mean deviation, (ml/min/1.73 m^2)	Measurement reliability within 30%,(%)
CG	1.937 ml/min/1.73m^2	95
UTI	-3.010 ml/min/1.73m^2	82
VUR	2.183 ml/min/1.73m^2	68[a]

[a] vs CG, UTI $p < 0.01$

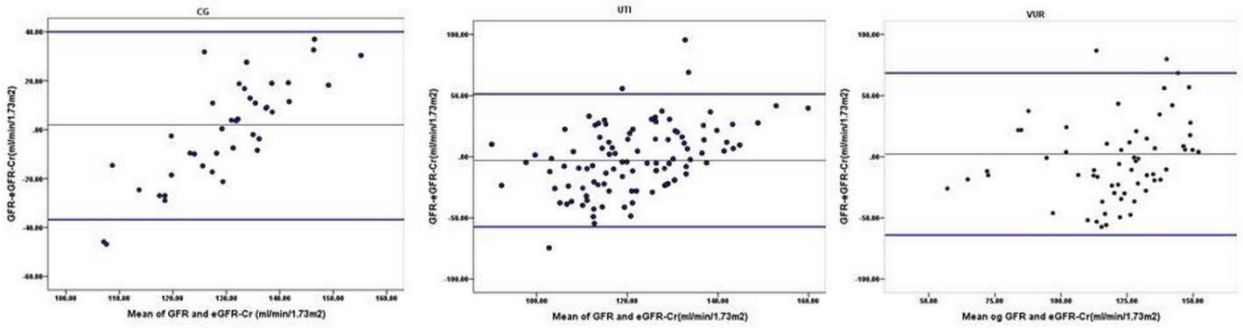

Figure 1. The Bland-Altman plot of Schwartz GFR-Cr against Tc-99m DTPA GFR in children from the CG, UTI, and VUR groups, where the light-grey line shows the mean of the Schwartz GFR-Cr deviation, while the dark-grey lines designate the 95% limits of agreement (deviation mean of ±1.96xSD)

The Bland-Altman charts show a dispersion of the Schwartz eGFR-sCr values below and above the deviation mean value within all three examined groups. The mean deviation value was 1.937 ml/min/1.73m^2 (95% LOA: -36.759 to 40.633 ml/min/1.73m^2) for the CG participants, -3.010 ml/min/1.73m^2 (95% LOA: -57.292 to 51.272 ml/min/1.73m^2) for the UTI participants, and 2.183ml/min/1.73m^2 (95% LOA: -64.019 to 68.385 ml/min/1.73m^2) for the VUR participants. The greatest Schwartz eGFR-Cr value dispersion was in the group of children with VUR.

The diagnostic reliability of the eGFR-Cr measurement was the best for children in the control group, amounting to 95%, and acceptable with children with urinary infections with no reflux, where 82% of the eGFR values were within 30% of the reference GFR measurement method. However, it was determined that with children with UTI and reflux only 68% of the measured values were within the limits of 30% of the value gained from the Tc-99m-DTPA clearance, which was significantly lower in contrast to the reliability of both the control group and the UTI group.

Discussion

The renal clearance of a substance can be carried out via glomerular filtration or tubular secretion, but of these two processes, glomerular filtration has proven to most likely be the most representative parameter of renal function due to its invariability under standard circumstances and its independence of urine flow. The passive GFR process can be quantified by measuring the clearance of a substance from plasma only provided that the substance fulfils certain conditions, which are that it is exclusively filtered by glomerular filtration and that it does not undergo the processes of tubular secretion or reabsorption. It is also necessary that the substance whose clearance is determined doesn't bind to plasma proteins and that it is not excreted from the organism via alternative routes. Further-more, the substance needs to be metabolically inert and exert no influence upon the renal function (25). These criteria have to be met because determining a substance's clearance is based on measuring the decline of its plasmatic concentration under the assumption that the same matter is excreted exclusively through the kidneys. 51Cr-EDTA and Tc-99m-DTPA (25) are most commonly used when the GFR is measured using radionuclide methods (2). Both of the compounds have the same pharmacokinetics, so there is no difference between calculations after one of the models is used to determine the clearance. Clinical practice shows that Tc-99m-DTPA is used more often because, apart from calculating the absolute renal function, a single application of this radiopharmaceutical allows the subsequent visualization of the entire urinary system, and the activity curves above the regions of interest can determine numerous semiquantitative function indicators.

After a single application of the marked filterable substance in the bolus, the disappearance curve is used to determine the clearance as the relation between the applied activity and surface under the curve using a biexponential model (26). However, even though this method is considered a "gold standard" due to its high reliability, it is not suitable for

everyday clinical practice, especially with children because it requires taking a large number of blood samples over time. This is why two simplified radiopharmaceutical clearance calculation models are advised with children, and they include a) taking two blood samples (the slope-intercept model) and b) a volume distribution model requiring the taking of one blood sample (27-29). This paper calculated the Tc-99m-DTPA clearance in all the children using the theoretical volume of distribution model, which includes taking one blood sample within 180 minutes from applying a radiopharmaceutical (20-22). When compared to the inulin clearance, the method including a single blood sample is reliable for clearance values up to 30 ml/min/1.73m^2 (19). Contraindications to calculating the GFR using one blood sample that can invalidate the results exist in patients with edema and hyperhydration, which cause a significant delay of the mixing of the radiopharmaceutical throughout the distribution volume, resulting in an overestimation of the GFR (19). The participants in our paper included neither children with a severe reduction in renal function nor children with oedema, so this method could be considered reliable with all the participants, regardless of the group. Moreover, when compared with the inulin clearance in healthy children in the age range of 2.7-11.6 of 127.1 ± 13.5 ml/min published previously (1), our results for the Tc-99m DTPA clearance in the control group of healthy children showed a very close correlation.

However, even though determining the clearance from a single blood sample using a radioisotope method is a simple and reliable method, the application of ionizing radiation is the main factor that excludes this method from recommended routine practices in the paediatric population. During the previous years, there was a large number of alternative eGFR calculation methods from anthropometric parameters and serum values of endogenous creatinine and cystatin C (8-14, 17). In time, the mathematic equations changed, mostly due to changes in the correctional factors for gender, age, height, weight, and body surface area of children (30-32). Newer eGFR measuring methods recommend an enzymatic method of determining creatinine (isotope-dilution mass spectrometry) as superior to the immunochemical method (33). Comparing the eGFR values is obligatory with reference, "gold standard" methods. The paper by Blufpand et al. tested new mathematical equations with 152 paediatric participants of different pathologies regarding the inulin clearance values (33). It was determined that the Potella eGFR equation (31), which disregards the height of the child, and the Schwartz eGFR are comparable to the values of the inulin clearance reference method with respectively 77% and 76% of measurements within 30% of the inulin clearance values (33).

The present paper performed the testing of the most frequently used eGFR method based on determining serum creatinine in comparison to the method using the Tc-99m-DTPA radioisotope clearance. The results clearly showed a flaw in the traditional statistical procedures of testing the clearance means acquired by different methods. Namely, means acquired in participant groups within the control group of healthy children, children with UTI with no reflux, and children with UTI and vesicoureteral reflux did not show significant differences. However, the application of the Bland-Altman analysis graphically showed a deviation of individual values from the mean value of the difference between the two measurements, as well as the dispersion rate of individual values within groups.

The greatest Schwartz eGFR-Cr value bias was in the group of children with VUR. The diagnostic reliability of the eGFR measurement was the best for children in the control group, amounting to 95%, and acceptable with children with urinary infections and no reflux, where 82% of the eGFR was within 30% of the referential GFR measurement method. However, it was determined that with children with reflux only 68% of the measured values were within the limits of 30% of the value gained from the Tc-99m-DTPA clearance, which was significantly lower in contrast to the reliability of both the control group and the UTI group. The comparative results of studying the renal function in our paper showed a high degree of agreement in the children from the control group, which was 97% for the clearance value > 90 ml/min/1.73 m^2, while only 3% of the children showed a value lower than the reference one, placing the children into the CKD2 group.

Considering the group of children with UTI, the eGFR value placed 16 more children (6%) within the CKD2 and 4 into the CKD3 group, while in the VUR group 2% of children were placed into the CK2 group, and 3% into the CK3 group, resulting in more underestimated eGFR clearance results, especially within the group with vesicoureteral reflux. Although the Schwartz method of GFR estimation has come to life in clinical practice as an excellent screening tool, this paper has shown that renal function may have been underestimated for a number of children, especially in those with possible impaired, decreased renal function. It is therefore essential that during the treatment of diseases in which a fall or recovery of renal function is expected, a reliable method of measuring absolute renal function should be selected.

Conclusion

An accurate and precise monitoring of renal function in children is of great importance in clinical practice during the treatment of nephro-urological diseases in children and especially when prescribing fluids, antibiotics, and chemotherapeutic medication. As the most accurate methods of measuring kidney function using inulin clearance are not suitable for routine practice, more simplified and harmless methods chosen for that purpose should be reliable and accurate. Our results proved Schwartz eGFR-Cr to be a reliable alternative to the radioisotope method in children with preserved renal function and children with a urinary tract infection without reflux, but a less reliable method in children with both vesicoureteral reflux and renal impairment and suggest that other more accurate methods for glomerular filtration rate measurement should be applied in this group of children.

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Conflict of interest

The authors declare that they have no conflict of interest.

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KOMPARACIJA JAČINE GLOMERULSKE FILTRACIJE DOBIJENE METODOM SCHWARTZ- EGFR-CR I KLIRENSA TC-99M-DTPA KOD ZDRAVE DECE I KOD DECE SA INFEKCIJOM URINARNOG TRAKTA SA VEZIKOURETERALNIM REFLUKSOM I BEZ NJEGA

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Cilj ovoga rada bio je utvrditi pouzdanost metode određivanja jačine glomerulske filtracije (GFR) bazirane na serumskom kreatininu i telesnoj visini (eGFR-Cr Schwartz) u odnosu na GFR određenu pomoću klirensa 99mTc-DTPA kod dece bez nefrouroloških bolesti, kod dece sa infekcijom urinarnog trakta bez UTI i kod dece sa vezikoureteralnim refluksom (VUR), pomoću Bland-Altmanove analize.

Retrospektivna studija obuhvata 451 pedijatrijskog pacijenta (104 dečaka i 347 devojčica, prosečne starosti 7,07 godina ± 3,02 godine, ranga od 2 godine do 15 godina) iz baze podataka Centra za nuklearnu medicinu Kliničkog centra u Nišu. U odnosu na dijagnozu, formirane su grupe ispitanika i to: kontrolna grupa (CG, n = 64), grupa sa UTI bez VUR (UTI, n = 299) i grupa sa UTI i VUR (VUR, n = 88). GFR je određivana iz klirensa Tc-99m-DTPA vađenjem jednog uzorka krvi 180 minuta nakon aplikacije radiofarmaka. Određivana je eGFR iz formule pomoću vrednosti telesne visine i serumskog kreatinina.

Komparacijom sa GFR 99mTc-DTPA dobijene su sledeće srednje vrednosti odstupanja eGFR-Cr u ispitivanim grupama:
- CG: 1,937 ml/min/1,73m^2 (95% granice podudaranja [LOA]: -36,759 to 40,633 ml/min/ 1,73m^2);
- UTI: -3.010 ml/min/1,73m^2 (LOA: -57,292 do 51,272 ml/min/1,73m^2) i
- VUR: 2,183 mL/min/1,73m^2 (LOA:-64,019 do 68,385 mL/min/1,73m^2).

Pouzdanost eGFR-Cr u odnosu na GFR 99mTc-DTPA nađena je za CG i UTI grupe ispitanika, sa 95% i 82% vrednosti unutar 30% GFR 99mTc-DTPA. Niža pouzdanost merenja eGFR dobijena je za decu iz grupe VUR, iznoseći samo 68% vrednosti unutar 30% klirensa 99mTc-DTPA. Naši rezultati pokazali su da je eGFR pouzdana alternativa radioizotopskoj metodi kod dece sa očuvanom bubrežnom funkcijom kao i kod dece sa infekcijom urinarnog trakta bez refluksa, ali da je manje pouzdana kod dece sa vezikoureteralnim refluksom i oštećenjem bubrežne funkcije.

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Ključne reči: *jačina glomerulske filtracije, 99mTc-DTPA, deca, infekcija urinarnog trakta*

Case report

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POSSIBILITY OF RADIOLOGICAL DIAGNOSTICS OF SPLENIC ARTERY PSEUDOANEURYSM - A CASE REPORT AND REVIEW OF LITERATURE

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Splenic artery pseudoaneurysm (SAP) is extremely rare, but potentially fatal medical condition. In the literature to date, pseudoaneurysms of the splenic artery were described in less than 250 cases. The most frequent cause of this rare condition is pancreatitis. Patients with splenic artery pseudoaneurysms are almost always presented by severe clinical symptoms. Ruptured pseudoaneurysms can cause fatal complications. The aim of this study was to present radiological possibilities in diagnostics of SAP in a patient suffering from chronic pancreatitis. Patient was initially advised for Ultrasound and Color Dopller sonography where lesion suspicion for SAP was made. Further, CT angiography and DSA were performed to provide certain diagnosis. The patient was surgically treated and histopathological examination definitely confirmed diagnose of SAP.

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Key words: *splenic artery pseudoaneurysm, radiological diagnostics, ultrasound, CT, DSA*

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Introduction

Visceral artery aneurysms are a rare but potentially life-threatening form of vascular disease and represent 0.1-0.2% of all aneurysms (1). Splenic artery aneurysms (SAA) are the most common visceral aneurysms with a rate of about 60% (2). Also, splenic artery is the third most common loca-tion of intraabdominal aneurysms, after aneurysms of the abdominal aorta and the iliac arteries, with prevalence from 0.2% to 10.4% (3). They are more common in females with 4:1 predominance (4). The vast majority of the splenic artery aneurysms are discovered incidentally, with more widespread use of advanced imaging. Splenic artery pseudoaneurysm (SAP) is extremely rare condition, quite the opposite of the true aneurysms of splenic artery (5). In the literature to date, SAPs were described in less than 250 cases (6). Unlike SAA, SAPs nearly always present with symptoms (3). The aim of this article is a case report with literature review of the clinical features, radiological appearance and management of SAP.

Case presentation

We present a 49-year-old patient with history of chronic pancreatitis and alcohol abuse. In the period of last two months, he complained about the occasional pulsating pain in the upper abdomen so he was hospitalized with suspicion of SAP.

After a clinical examination, the patient was advised for abdominal ultrasound (US) and color Doppler sonography (CDS) examination which was performed with CH 6- 2 abdominal probe on Siemens Acuson Antares ultrasound machine.

In the region of the pancreas body, US revealed an oval, well defined, multilayered, thick walled, cystic lesion, dimension 6 x 5.5 cm (Figure 1) with CDS registered turbulent blood flow (Figure 2). The lesion is with short and narrow neck associated with splenic artery. In the neck of the lesion it was registered characteristic bidirectional high-speed flows by the type so called "to and fro" with peak systolic velocity (PSV) up to 250 cm/s (Figure 3).

In a continuation of diagnostics, Computed Tomography (CT) angiography of the abdomen was performed on Toshiba Aquilion 64 CT scanner. The examination was done with intravenously administered contrast medium in the arterial, early and late venous phase.

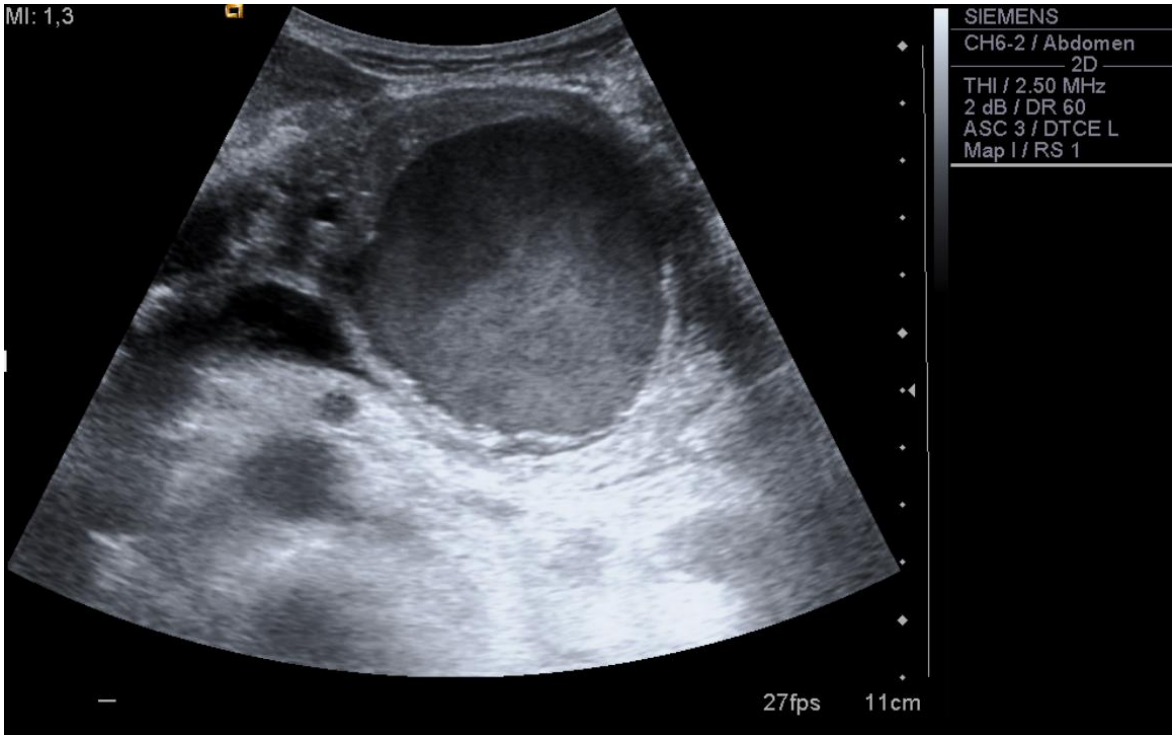

Figure 1. Ultrasound image of SAP

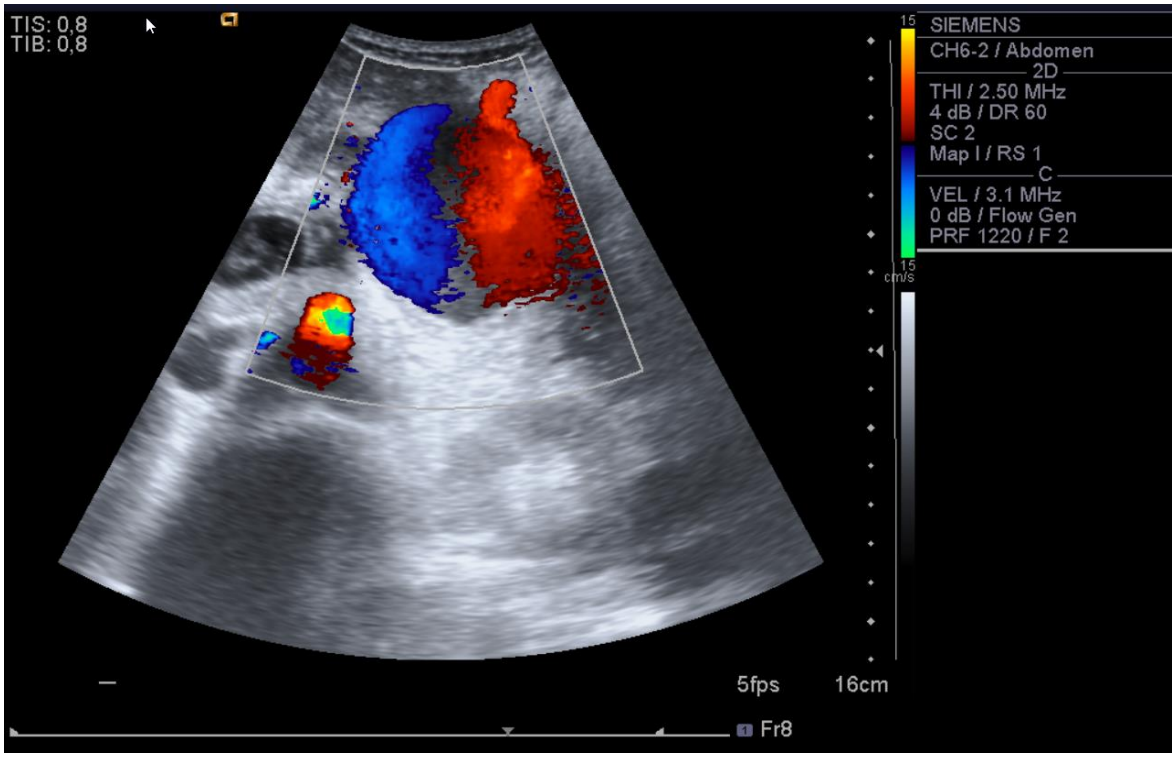

Figure 2. CDS registered turbulent blood flow

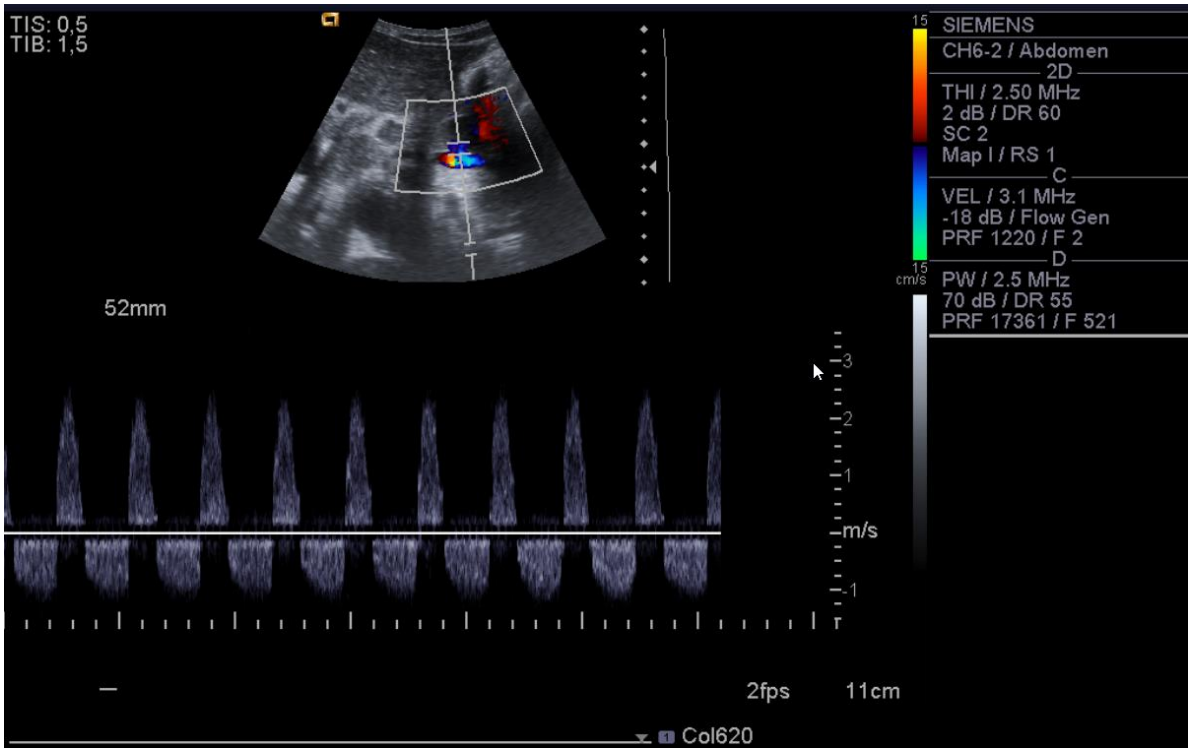

Figure 3. Characteristic bidirectional high- speed flows in the neck of SAP

An examination of the arterial phase presents the common hepatic artery, a splenic artery, and an upper mesenteric artery with its branches. In the proximal third of the splenic artery, retropancreatically, it is presented as an oval, smoothly marginated formation with thicker walls, measuring about 5 x 5 cm and showing gradual enhancement, with maximum densities reaching in the late venous phase probably because of the extremely thin SAP neck (Figure 4). The aforementioned formation could be differentiated as pseudoaneurysm of the splenic artery with thin neck.

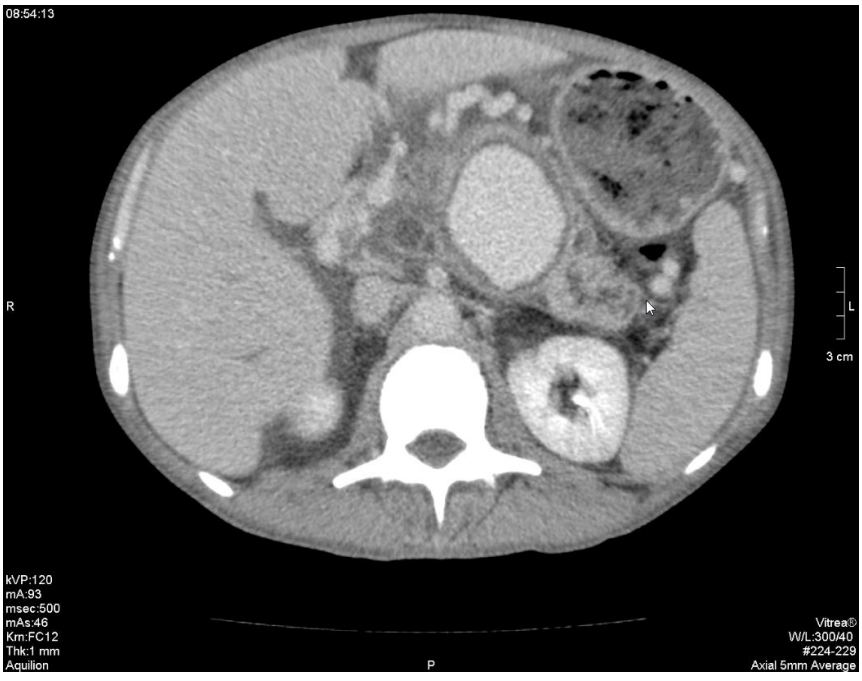

Figure 4. CT axial image of SAP, late venous phase

Figure 5. DSA shows extravasation of the contrast medium in SAP

For the purpose of additional diagnostics, digital subtractional angiography (DSA) of the celiac trunk was indicated and implemented.

At the level of the proximal third of the splenic artery, it is observed extravasation of the contrast medium in the previously described lesion, which corresponds to the pseudoaneurysm of the splenic artery (Figure 5).

After appropriate radiological diagnostics, patient was treated surgically by radical splenic artery ligation and splenectomy with distal pancreatectomy. Histopathological verification of previously observed and resected lesion confirmed that it was a pseudoaneurysm of splenic artery.

Discussion

An insight into available literature shows that a typical patient with SAP is about 52 years old (range, 35–78 years), suffers from chronic pancreatitis and has a history of alcohol abuse as the case of our patient was (7).

The most common etiology of SAP is pancreatitis, precisely recurrent acute or chronic form, even though, less common cause is the first episode of acute pancreatitis (5). A large proportion of patients have a significant history of alcohol intake (7). It is estimated that up to 21% of patients with chronic pancreatitis and 10% patients with chronic pancreatitis and pseudocyst develop SAP as additional complication (8, 9). Blunt and penetrating trauma as well as iatrogenic trauma or peptic ulcer disease are less common causes (10). In a small number of SAP, there is no identifiable cause (7).

Pseudoaneurysm based on pancreatitis is caused by at least three different mechanisms:

1. enzymatic digestion of the adjacent artery wall by peripancreatic fluid within a pseudocyst, forming necrotising arteritis with fragmentation of elastic tissues
2. local compression of the blood vessel wall by longstanding pseudocyst, which can cause vascular erosion and weakness of the vessel wall architecture or
3. aneurysmal rupture into pseudocyst leading to pseudoaneurysm (5, 8). Splenic artery is the most frequently affected blood vessel (30-50%) (5).

Normal diameter of the splenic artery is in range from 0.43 cm to 0.49 cm. The splenic artery is aneurysmal dilatated when a focal diameter is greater than 50% compared to the normal vessel lumen. True aneurysms involve all 3 layers (intima, media and adventitia), while pseudoaneurysms forming a false lumen, so that consist of only one or two layers (9).

Retrospective case series reported different size of splenic artery pseudoaneurysms, from 2 cm to 17 cm (mean diameter 4.8 cm), similar to our measurements 5.5 x 6 cm on US examination and 5 x 5 cm on CT angiography respectively (7). Thus, we could suspect SAP based only on the history of the disease and US and CDS examination results. Further, we were able to conclude that there was no

significant difference in the dimensions of the lesion, either measured by the ultrasound or CT examination.

The literature data leads to the conclusion that the size of SAP is not a predictor of rupture (9). Pseudoaneurysms, independent of their size, are more prone to rupture because of lack a true wall unlike true splenic aneurysms with all three intact layers wall. Larger pseudoaneurysms probably originate from rupture into a pseudocyst (8).

The most accurate term for pseudoaneurysm is pulsatile hematoma, which describes both disease and the high risk of rupture without treatment (11). Clinical diagnosis of SAP is challenge because its presentation often varies from an incidental finding to hemodynamic collapse from sudden rupture and bleeding (6). Patients with SAP are almost always presented by severe clinical symptoms such as an episode of abdominal pain - 29.5%, melena and tarry stool - 26.2%, hematochezia (vomiting blood) - 14.8% or ischemic symptoms of affected organ which is consequence of intermittent embolism. Acute and massive hemorrhage with the loss of large amounts of blood can cause hemorrhagic shock (5, 12). Ruptured pseudoaneurysms can cause fatal complications due to massive gastrointestinal bleeding in the pancreatic duct, termed "hemosuccus pancreaticus", which is the main cause of hemobilia, hematemesis, hematochezia or melena. Rupture can also occur into the nearby organs, peritoneum, retroperitoneum or form a pseudocyst (9). It is estimated that the risk of rupture of a SAP is about 37%, with the mortality rate of 90% for the untreated ones (3). Except early rupture complications of pseudoaneurysms also include, compression of nearby structures and a high frequency of aneurysm infection (11).

The US with CDS, is low cost, wide available, real time and very informative hence is usually the first diagnostic modality for abdomen and pelvic pathologies. It can be useful for detecting SAP, as well as belonging neck. Accurate and reliable diagnosis, in fact, requires additional diagnostics methods such as CT angiography and DSA. A CT is the most commonly used diagnostic tool for evaluation of all visceral pseudoaneurysms.

On US imaging, pseudoaneurysm commonly appears as multilayered lesion with central or peripheral anechoic area continuous with arterial lumen, showing continuous arterial pulsation on real time ultrasonography if SAP is from the splenic artery. Continuous arterial pulsation is not visualized if pseudoaneurysm arising from the small artery or when only small part of lesion is continuous with arterial lumen. Sonographic appearance of pseudoaneurysm depends on the size of the lesion, patency of vessel and aneurysmal lumen, presence of hemorrhage and thrombus, and level of calcification (6). This technique is depending on the surgeon and also may be limited by obesity, shadowing from bowel gas or massive arteriosclerosis which can interfere with retroperitoneal and mesenteric vessels obscuring visualisation. Limited spatial resolution may cause missing small lesions on sonography (3). On CDS examination, because of the turbulent forward and backward flow, there may be seen characteristic "yin-yang" sign, while a "to and fro" sign may be seen with pulsed wave Color Doppler.

A CT angiography is very useful method for confirming the diagnosis as well as presenting the anatomy, size and extent of the lesion, character and continuation of pseudoaneurysm with parent vessel, detecting extent of thrombus, hemorrhage and status of adjacent organs. On CT, pseudoaneurysm appears during the arterial phase, which is most important for identifying these lesions. A SAP is at imaging similar as aneurysms with the more irregular margin, presented as hypodense or multilayered lesion which is continuous with the parent artery and typically surrounded by a hematoma (3, 5, 11). If acute hemorrhage is present, it will be demonstrated as hyperdense area. The presence of gas suggests a gastrointestinal fistula, and the lack of very high-density oral contrast suggests that the lesion does not communicate with the gastrointestinal tract (6). New generation scanners with a large number of detector rows with multiplanar reconstruction (MPR) capabilities as well as three-dimensional renderings (3D rendering) allow identification of aneurysms and pseudoaneurysms, better separation of smaller aneurysms from tortuous blood vessel or hyperattenuating islet cell tumor of the pancreas. Multiplanar reconstruction (MPR) and 3D rendering of the lesion is useful for a better evaluation of the whole condition (3). Relative contraindications to CT angiography are renal insufficiency, poor intravenous access, and allergy to contrast agents.

Magnetic resonance imaging (MRI) and MRI angiography are useful diagnostic modalities in patients with chronic renal failure, because of the use of potentially less nephrotoxic contrast agents, although MRI can detect vascular flow without applying contrast material (11). Contraindication for MRI includes patients with pacemakers or aneurysm clips, while relative contraindications include patients suffering from claustrophobia, or unable to hold their breath (3).

In hemodynamically stable patients, preoperative DSA helps confirming the diagnosis, define the character of the lesion and allows therapeutic planning. In a high-risk patient, with bleeding vessel, preoperative DSA might gain temporary control of hemorrhage by performing transcatheter embolization, thus providing a time window for operation under optimum clinical conditions (5). DSA is the most specific diagnostics, but also may be a therapeutic method, so it is the gold standard for identification of artery aneurysms and pseudoaneurysms (8). In carefully selected patients, endovascular visceral aneurysm artery reconstruction with stent graft can be a reasonable method of treatment, as a safer option and an alternative to open surgical repair (13).

Almost all visceral pseudoaneurysms require treatment because of the tendency of continuous enlargement, great risk of rupture, and consequently significant mortality (11). In the past, the most commonly used treatment of SAP was radical splenic artery ligation, splenectomy with or without pancreatectomy (distal, partial or total) (14). Nowadays, endovascular, less invasive approach, for uncomplicated pseudoaneurysms in hemodynamically stable

patients, is increasingly in use with better outcomes and less morbidity (9). The treatment of choice is coil embolization or stent-graft placement across the lesion when the anatomy allows (11). Endovascular procedures have less severe complications such as splenic infarction or postembolization syndrome (12). If surgical intervention and arterial embolization are not possible or not feasible, percutaneous US or CT-guided application of thrombin can be performed for the first-line treatment (8).

Conclusion

SAP is a potentially life-threatening condition that can be difficult to diagnose. It is important to make the distinction between visceral aneurysm and pseudoaneurysm for further treatment and prognostic opportunities. SAP can be diagnosed by clinical approach, US with CDS examination, CT angiography, MRI angiography and DSA. The CT is proven to be quite valuable method, but DSA is considered to be the gold standard due to better spatial resolution and potentially successful treatment method.

Death rate in untreated pseudoaneurysms is not neglectable, so that, radiologists, who identify these vascular pathologies, have to recognize the clinical significance and understand the management because prompt and well-timed treatment depends directly on fast and accurate radiological diagnosis.

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Prikaz bolesnika

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MOGUĆNOSTI RADIOLOŠKE DIJAGNOSTIKE PSEUDOANEURIZMI LIJENALNE ARTERIJE – PRIKAZ SLUČAJA I PREGLED LITERATURE

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Pseudoaneurizme lijenalne arterije predstavljaju ekstremno retko, ali potencijalno fatalno medicinsko stanje. U literaturi je do sada opisano ukupno manje od 250 slučajeva. Najčešći uzročnik ovog retkog stanja je pankreatitis. Bolesnici sa pseudoaneurizmama lijenalne arterije skoro uvek imaju ozbiljne kliničke simptome. Ruptura pseudoaneurizme može izazvati komplikacije sa fatalnim posledicama. Rad ima za cilj da prikaže mogućnosti radiološke dijagnostike pseudoaneurizme lijenalne arterije kod bolesnika sa hroničnim pankreatitisom. Bolesniku su inicijalno urađene ultrazvučna i kolor dopler dijagnostika, pomoću kojih je otkrivena promena suspektna na pseudoaneurizmu lijenalne arterije. Nakon ultrazvučnog pregleda sa kolor doplerom, urađene su CT angiografija i digitalna subtrakciona angiografija, kojima je potvrđena inicijalna dijagnoza. Bolesnik je lečen hirurški, a histopatološkim nalazom definitivno je postavljena dijagnoza pseudoaneurizme lijenalne arterije.

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Ključne reči: *pseudoaneurizma lijenalne arterije, radiološka dijagnostika, ultrazvuk, CT, DSA*

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The first page of a research article must contain: a) article title b) full name of author(s) c) full name(s) of institutions and/or address(es) of department(s) where either reasearch was conducted or research article written d) following signs *, **, ***, #, ##, ### signifying author(s) and institutions e) full address, phone number and e-mail of a corresponding author.

The second page should contain only research article title, abstract and key words without names of author(s) and institution(s). Abstract for research and professional articles, review articles and meta-analyses should have up to 350 words while abstract for all other types of publications should consist of 250 words. Key Words section should have up to 5 key words or phrases related to a submitted article. It is desirable that authors use corresponding descriptors from Medical Subject Heading (MeSH) that can be found on Index Medicus list for key words. The first and the second page should not be numbered.

Body of a Research Article – Research and professional articles, as well as general surveys and meta-analyses should not exceed 11 pages altogether; current topics - 6 pages, casuistics - 6 pages and proceding statements - 5 pages, history of medicine articles - 3 pages while conference reports and book reviews - 2 pages. Research and professional articles should comprise the following mandatory chapters: introduction, aim(s), material and method(s), result(s), discussion and conclusion(s). Result(s) and discussion can be comprized into one chapter. Acknowledgments of any kind in a submitted article should be written at the end of the paper after "Conclusion(s)". It is necessary to clearly mark a place for additions in the text.

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