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Otto Dix (1891–1969) – The match-box seller (1920); oil and collage on canvas (Staatsgalerie, Stuttgart, Germany).
After the First World War many artists in Germany often presented war cripples in their art works as an example of horrible consequences of the war (see pp. 413–8).

Oto Diks (1891–1969) – Prodavač šibica (1920); ulje i kolaž na platnu (Državna galerija, Štutgart, Nemačka).
Posle Prvog svetskog rata, mnogi umetnici u Nemačkoj često su u svojim delima prikazivali ratne invalide kao primer užasnih posledica tog rata (vidi str. 413–8).

ORIGINAL ARTICLES

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Alternative method for direct measurement of tibial slope

Alternativna metoda za direktno merenje tibijalnog nagiba

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Abstract

Background/Aim. The tibial slope is one of the most frequently cited anatomical causes of anterior cruciate ligament trauma. The aim of this study was to determine the possibility of direct measuring of the tibial slope of the knee without prior soft tissue dissection in cadavers. **Methods.** Measurement was performed on the two groups of samples: osteological and cadaveric. The osteological group consisted of 102 matured tibiae and measurement was performed: indirectly by sagittal photographing of the tibia, and directly by a set of parallel bars. The cadaveric group consisted of 50 cadaveric knees and measurement was performed directly by a set of parallel bars. The difference and correlation between indirect and the direct measurements were observed, which included also measuring of the difference and correlation of the tibial slope on the medial and lateral condyles. **Results.** A statistically significant difference between the direct and indirect method of measuring ($p < 0.01$) of 1° was found for the tibial slope on the medial condyle, which is of no practical importance. Direct measurement of the osteological and cadaveric groups of samples did not show a statistically significant difference regarding the values of the tibial slope on the lateral condyle ($p > 0.05$). However, the slope on the medial condyle, as well as indirect measurement showed a statistically significant difference ($p < 0.01$). **Conclusion.** By the use of a set of parallel bars it is possible to measure the tibial slope directly without removal of the soft tissue. The results of indirect, photographic measurement did not statistically differ from the results of direct measurement of the tibial slope.

Key words:
tibia; anthropometry; photography; anterior cruciate ligament.

Apstrakt

Uvod/Cilj. Tibijalni nagib predstavlja jedan od najčešće navođenih anatomskih parametara koji mogu uticati na rupturu prednje ukrštene veze zgloba kolena. Cilj ove studije bio je određivanje mogućnosti merenja tibijalnog nagiba direktno na tibijama i nepreparisanim kadaverskim kolenima. **Metode.** Merenja su vršena na dve grupe uzoraka: osteološkoj i kadaverskoj. Osteološka grupa se sastojala od 102 golenjače, a merenje je vršeno indirektno, uz pomoć fotografija golenjača u sagitalnoj ravni i direktno, uz pomoć sistema poluga paralelnih pravaca. Kadaverska grupa se sastojala od 50 kadaveričnih kolena a merenje je vršeno direktno uz pomoć sistema poluga paralelnih pravaca. Testirane su razlike i povezanost između indirektnog i direktnog merenja, kao i razlike i povezanost tibijalnog nagiba na unutrašnjem i spoljašnjem kondilu golenjače. **Rezultati:** Utvrđena je statistički značajna razlika od 1° između indirektnog i direktnog načina merenja tibijalnog nagiba na unutrašnjem kondilu ($p < 0.01$) koja nema praktičan klinički značaj. Direktno merenje tibijalnog nagiba na spoljašnjem kondilu golenjače nije pokazalo statistički značajnu razliku između osteološke i kadaverske grupe (0,05). Međutim, za nagib na unutrašnjem kondilu, kao i u slučaju indirektnog merenja utvrđena je statistički značajna razlika ($p < 0.01$). **Zaključak.** Uz pomoć sistema poluga paralelnih pravaca izvodljivo je validno merenje tibijalnog nagiba direktno, bez preparisanja mekih tkiva. Rezultati indirektnog merenja tibijalnog nagiba uz pomoć fotografija ne razlikuju se značajno od rezultata dobijenih direktnim merenjem.

Ključne reči:
tibija; antropometrija; fotografija; ligament, prednji ukršteni.

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Introduction

The tibial slope is one of the anatomical structures most frequently implicated in the injuries of the anterior cruciate ligament (ACL). Even though its role has been extensively studied, results still remain elusive. While some studies support its role in ACL injury, others do not show a correlation between these two parameters [1–6].

The greater the tibial slope, the greater the anterior tibial translation when weight is shifted onto the knee, which leads first to increased stretching and then the rupture of the ACL. Giffin et al. [7] have reported that a small increase in the tibial slope does not influence anterior tibial translation and that it may represent a protective factor in ACL-deficient knees. DeJour and Bonnin [3] have demonstrated in their study that an increase of every 10º in the tibial slope is associated with a 6 mm increase in anterior tibial translation. In their case control study, Stijak et al. [6] divided the tibial slope into the tibial slope on the medial and the tibial slope on the lateral condyle, and demonstrated that an increased slope on the lateral condyle as well as a positive difference between the slope on the lateral and on the medial condyle have an influence on ACL injury. Conversely, Meister et al. [5] did not find a statistical significance in the mean tibial slope between a group with ACL injury (50 knees) and a group with patellofemoral pain syndrome (50 knees).

The tibial slope is defined as the angle between the line perpendicular to the tibial axis and the posterior inclination of the tibial plateau. The direction of weight transmission from the femur to the tibia and on to the foot, i.e. from the tibial condyle onto the trochlea tali, overlaps with the tibial shaft anatomical axis (TSAA), which represents the ideal axis, which is, however, due to its position, difficult to measure. In their studies Brazier et al. [8] and Çullu et al. [9] examined correlations between different anatomical axes of the leg with TSAA and found that the tibial proximal anatomical axis (TPAA) had the biggest correlation with TSAA. One study [10] investigated the divergence of 5 anatomical axes of the leg from the mechanical axis, in relation to the tibial slope. In a study of 90 investigated female knees they have demonstrated that the TPAA has the smallest divergence of the mechanical axis (0.2°).

Taking into consideration many magnetic resonance (MR) studies it can be said that the TPAA is one of the most commonly used axes which can be determined in an isolated tibia, on an X-ray image and a large MR imaging (MRI) but very difficult in intact cadaveric material.

The aim of the study was to research the possibility of direct measurement of the tibial slope of the knee without prior dissection of the soft tissue in cadavers.

Methods

The data used in the study was obtained by measurement of the two groups of samples. The first group consisted of 102 matured tibias from the osteological collection belonging to the Department for Anatomy "Niko Miljanić", Faculty of Medicine, University of Belgrade. There were 47 right and 55 left tibias without gonarthrotic changes, of unknown sex and age. The second group consisted of 50 cadavers from the Institute of Forensic Medicine in Belgrade (32 male and 18 female cadavers, 30 right and 20 left ones) with an intact ACL, without diagnosed gonarthrosis from examinees aged 15–53 (mean 34; SD 11). The tibial slope was measured on the medial and lateral condyles of the tibia. On isolated tibias, measurement was performed by goniometer: directly – with a set of parallel bars – "parallel bars device", and indirectly – on sagittal photographs of the tibia. In order to achieve objectivity, measurements were performed by independent researchers. The values of the angles were expressed with the precision of 1°.

We have designed the parallel bars device for direct measurement of: TPAA, the position of the anterior cruciate ligament in relation to the intercondylar notch and with TPAA. It consists of the "pointer of the tibial axis" and of the "pointer of direction" (Figure 1).

The pointer of the tibial axis consists of two parallel bars (a_1 i a_2) placed at the distal end of the bar "c" at a 100 mm distance from each other. On these two bars, four parallel pointed bars, running through the bars have been placed (two on each bar: b_1, b_2 and b_3, b_4). These pointed bars are per-

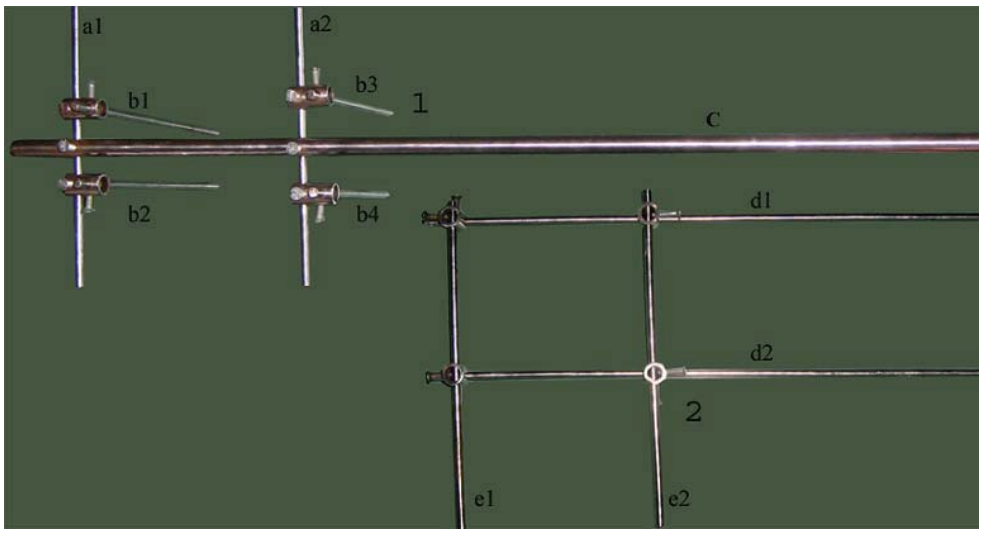

Fig. 1 – Parallel bars device. The pointer of tibial axis (1) and the pointer of direction (2).

pendicular to to direction "a" and can be moved along the bars "a". The bar "c" is vertical to the axis "a" along which it can be moved and placed at an equal distance from the pointed bars "b". The pointer of direction consists of two parallel bars (d_1, d_2) which can be moved along the bars "e" (e_1, e_2). The distance between the "e" bars can be changed accordingly.

For the measurement of the tibial slope on the lateral and medial condyles, on isolated tibias, four pointed bars "b" of the pointer of the tibial axis are placed on the medial side of the tibia perpendicular to the saggital plateau, to be in contact with the anterior and posterior cortices of the tibias at a 50 mm and a 150 mm distance, distal to the tibial tuberosity (Figure 2). By moving the bar "c" along the bars "a", i.e. by placing them at an equal distance from the pointed bars "b", we obtain the direction of the TPAA. By placing the bar "d_1" pointer of direction on the medial or lateral tibial plateau, the "d_2" bar makes with the "c" bar (TPAA) the angle of the tibial slope on the lateral, i. e. medial condyle.

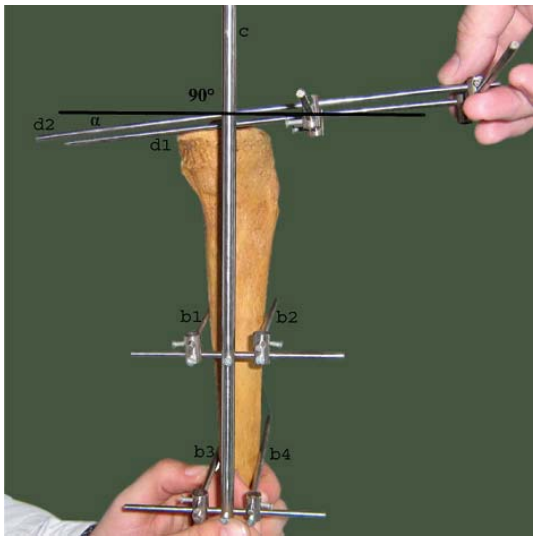

Fig. 2 – Direct measurements of the tibial slope on the medial and lateral condyles of tibia. The angle "α" represents the angle of tibial slope on the medial (lateral) condyle.

For indirect measurement, photographs were taken by a digital camera (with a resolution of 10.0 mega pixels) on the medial and lateral tibial sides (for the medial and the lateral tibial condyles). The digital camera and the upper end of the tibia were positioned in the same plain. The format of the printed photographs was 70 × 140 mm, while the proportion was 1 : 2. Afterwards, with the use of two median points located 50 and 150 mm away from the tibial tuberosity, the proximal tibial anatomical axis was determined (Figure 3). The values of the angles were expressed in relation to the line perpendicular to the TPAA.

Tibial measurement slope on cadaveric knees was performed with the aid of the parallel bars device. The knee joint was accessed from the anterior side (access according to Langebeck). Incision on the skin was performed between the patella and the interior longitudinal retinaculum, from the quadriceps muscle to the insertion of the „pes anserinus".

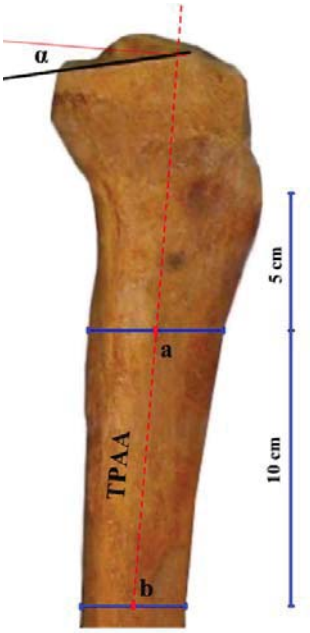

Fig. 3 – Indirect determination of the tibial slope (α). Using two median points ("a" and "b") which were at 5 and consequently 15 cm from the tibial tuberosity, the TPAA was determined, as well the angle of slope of the medial (lateral) condyle of tibia "α".

By cutting the knee joint capsule, and moving the patella outward and by means of a 90° flexion, insight into the anterior portion of the knee joint was obtained. The four pointed bars "b" of the pointer of the tibial axis were placed on the medial side of the leg at a right angle to the sagittal plane by piercing through this plane, so as to touch the anterior and posterior cortices of the tibia at a 50 mm, i.e. 150 mm distance, distally from the tibial tuberosity (Figure 4).

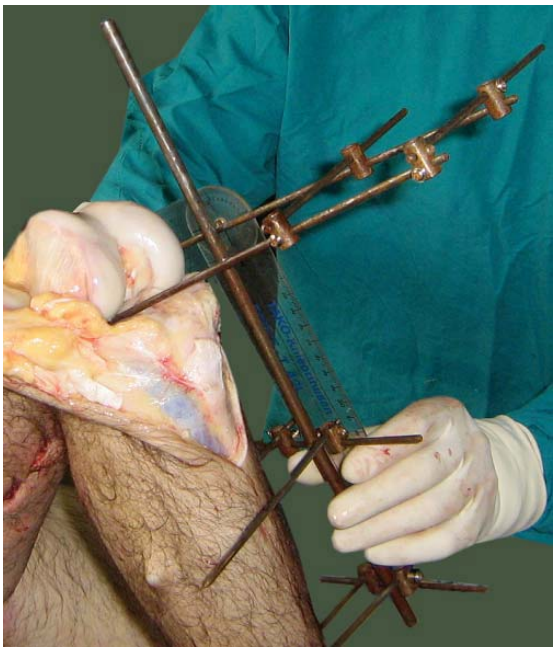

Fig. 4 – Tibial slope determination directly on the knee of cadaver by a pointer of tibial axis and pointer of direction.

By moving the bar "c" along bars "a", i.e. by placing them at an equal distance from the pointed axes "b", we obtained the direction of the TPAA. The "d_1" bar of the pointer of direction was placed below the femoral condyles on the medial or lateral tibial plateau, while the "d_2" bar made with the "c" bar the angle of the posterior tibial slope on the lateral i. e. medial condyle.

We compared the difference and correlation between the two ways of measurement of isolated tibias. In addition, we tested the difference in values of the tibial slope in two groups of samples obtained by means of direct measurement. Also, the difference and correlation of the tibial slope at the lateral and medial condyles, separately for both methods of measurement were compared. The Student's *t*-test for paired pairs and an independent *t*-test at the level $p < 0.05$, as well as the Pearson's correlation coefficient were used.

Results

Although there is a statistically significant difference ($p < 0.01$) between the two different methods of measurement of the tibial slope at the medial condyle, we have estimated that, from the practical point of view, the difference of less than 1° (0.9°), which represents the level of accuracy, has no significance (Table 1). There is no statistically significant difference ($p > 0.05$) between the values of direct and indirect methods of measurement. Both methods of measurements indicate higher values of the tibial slope at the medial than those at lateral condyle ($p < 0.01$), 3° for direct and 2.2° for indirect measurement. The smallest tibial slope measured by direct measurement at the lateral condyle was -5°, while the largest tibial slope at the medial condyle was 21°. The correlation coefficient between the two different methods of measurement was $r = 0.84$ for the lateral and $r = 0.87$ for the medial condyle. In both cases a statistically significant correlation was found ($p < 0.01$). Also, a statistically significant direct connection ($p < 0.01$) of the tibial slope was found at the medial and lateral condyles, both in direct ($r = 0.43$), and in indirect ($r = 0.46$) measurement.

The value of the tibial slope obtained by the direct measurement method on cadaveric knees ranged from 2° to 15° (on the lateral condyle), i.e. from -1° to 16° (on the medial condyle). By direct measurement of the two groups of samples, osteological and cadaveric, no statistically significant difference was obtained with regards to the value of the tibial slope on the lateral condyle ($p > 0.05$, Table 2). However, the slope on the medial condyle, as was the case with indirect measurement, showed a statistically significant difference ($p < 0.01$). Basically, what we have here are the values of the slope on the medial condyle obtained through direct measurement on isolated tibias diverging from all the other measurements of the tibial slope.

Discussion

Direct measurement of the tibial slope on the medial and lateral condyles on cadavers usually depends upon the previous preparation of the leg, i.e. dissection of the soft tissue structures in order to determine the TPAA. Due to these difficulties some authors [11] have chosen the margin of the anterior tibial cortex as a referent axis which exhibits a smaller degree of correlation with the TSAA than the TPAA. The use of markers of the TPAA enables the determining of the TPAA without prior dissection of soft-tissue structures with an anteromedial approach to the knee joint (Figure 4). Sharp, pointed bars (b1-b4) pass through soft-tissue structures, touching the anterior and posterior tibial cortices, while the bar "c" represents the TPAA. By positioning one bar (d_1) of pointer of direction on the tibial condyle through the anteromedial portal of the knee, the other bar (d_2) makes a measurable angle with the bar "c" (TPAA).

The results obtained by direct measurement of isolated tibias on the lateral tibial condyle are almost identical to the results obtained by indirect measurement. Somewhat higher values are obtained by direct measurement on the medial tibial condyle as compared to indirect measurement, however, this difference, although statistically significant, is below the level of accuracy, which is 1°. Based on this, it can

Table 1

Difference between direct and indirect measurement of the tibial slope at medial and lateral condyles isolated tibias (°)

Condyle	Direct measurement ($\bar{x} \pm SD$)	Indirect measurement ($\bar{x} \pm SD$)	*p* (*t*-test)
Medial	10.7 ± 4.5	9.8 ± 4.0	< 0.01
Lateral	7.7 ± 4.8	7.6 ± 3.9	n.s.
p (*t*-test)	< 0.01	< 0.01	----------

$\bar{x}$ – mean; SD – standard deviation; n.s. – non significant.

Table 2

Differences between the values of the tibial slope for both groups of samples obtained by direct measurements (°)

Condyle	Groups of samples		*p* (*t*-test)
	osteological ($\bar{x} \pm SD$)	cadaveric ($\bar{x} \pm SD$)	
Medial	10.7 ± 4.5	7.0 ± 3.7	< 0.01
Lateral	7.7 ± 4.8	6.9 ± 3.0	n.s.
p (*t*-test)	< 0.01	n.s.	----------

$\bar{x}$ – mean; SD – standard deviation; n.s. – non significant.

be said that the results obtained by direct and indirect measurement are "practically" the same. This is also confirmed by the existence of a high correlation between the data obtained by two different methods of measurement on both lateral and medial condyle.

Through direct measurement of the tibial slope of the lateral condyle of cadaveric knees we obtained the values which do not differ from tibial slope values on the lateral condyle of isolated tibias (6.9° : 7.7°). These values are not different from the tibial slope on the medial condyle of the cadaveric group (7.0°). However, there is a difference between the values for the tibial slope obtained by direct measurement performed on the medial condyle of isolated tibias and the medial condyle of cadaveric knees (10.7° : 7.0°). Looking at the values of the tibial slope published in other studies [6, 11–14] both sets of values obtained in our study can be accepted as valid. In fact, one of the main reasons for obtaining different values is the measurement of the tibial slope in two different groups – the osteological and the cadaveric. The similar values obtained by measurement of isolated tibias in two different ways, also speak to that effect.

However, if we ignore the fact that the tibias in question belong to two different groups, we can look for the reason for the differences in the measurement method itself. Namely, on isolated tibias, the pointer of direction was placed on tibial condyles in such a way as to follow the slope but also the antero-posterior direction of the condyle. In the case of the lateral condyle, which is round, the direction of the pointer of direction constructed an approximately right angle (90°) with the pointer of the tibial axis, while in the case of the medial condyle, which is directed from the front towards the back and the inside, this angle had a different value (depending on the direction of the condyle) but was always lesser than 90°. By placing the pointer of direction on cadaveric tibial condyles, we were able to do justice to the lateral condyle and place the pointer of direction at a 90° angle to the pointer of the tibial axis, but due to undissected structures of the knee we could not be completely certain that the direction of the pointer of direction was matching the anteroposterior axis of the medial condyle of the tibia. However, if we were to analyze the measuring technique in this way, we could then also criticize all studies performed on X-ray, MR and computed tomography (CT) images, with the exception of studies with a controlled position of leg rotation.

Chiu et al. [11] used the line of the anterior tibial cortex to determine the tibial slope on previously photographed tibias and obtained the values of 14.8º for the medial and 11.8º for the lateral tibial condyle. This study, as well as our study on isolated tibias, speaks in favor of a greater tibial slope on the medial than on the lateral plateau. On the other hand, the causes of substantially greater values of both the medial and the lateral plateau are multiple. One of them is the use of the anterior line of the tibial cortex as a starting axis in measurement by the cited authors. The other reason could be the difference between the Chinese and the European population. Both studies are the same in that they used (the cited study and our own), from the clinical point of view, tibias from the population without data on possible ACL rupture. As the incidence of ACL injuries in the total population is relatively low, it can be assumed that in both cases tibias did not have ACL rupture. This data is substantiated by a greater tibial slope on the medial than on the lateral condyle. The difference between the tibial slope on the lateral and on the medial condyle has been established by Stijak et al. [6] in their case-control study on 66 examinees. The tibial slope was determined on MRI and TPAA with the use of X ray images of the leg. The patients with ACL rupture had a greater angle on the lateral (7.5°) than on the medial (5.3°) condyle, while the patients without a rupture had a ratio of the tibial slope on the medial and the lateral condyle similar to the one in our study; the angle on the medial condyle (6.6°) was greater than on the lateral condyle (4.4°). Somewhat smaller values obtained in the cited study could be attributed to the difference in the methodology.

In their study performed on MRI of patients without ACL rupture, Matsuda et al. [14] have shown a greater tibial slope on the medial than on the lateral condyle both in "normal" (lateral 6.0º; medial 9.9º) and in varus knees (lateral 7.2º; medial 10.7º). These values, although obtained by a different methodology are the closest to our results. Also, a recent radiographic study performed on 100 healthy patients has determined a greater tibial slope on the medial than on the lateral condyle (9.2º : 4.8º) [13].

In their study on 50 osteoarthritic knees, Kuwano et al. [12] obtained values of 9º for medial and 8.1º on lateral condyle. Taking into account osteoarthritic changes and different methodology (3D CT scanner), these results are in accordance with the results obtained in both of our groups. Two different indirect methods for determining the tibial slope on MRI and radiographic images were performed by Hudek et al. [15] and demonstrated a significant correlation between these two measurements. The values of the tibial slope on the medial condyle showed in their study, were 4.8º on MRI and 8.2º on radiographic images. Taking into account that the authors used the images of patients with and without ACL rupture, it can be said that the results obtained in our study speak in favor of the values showed on their radiographic images.

Conclusion

Based on the data obtained in this study it can be concluded that two methods of determining the tibial slope, direct and indirect, do not significantly differ and they exhibit correlation. With the use of the parallel bars device it is possible to determine the tibial slope directly on isolated tibias without significant differences as compared to the indirect measurement. Also, with the help of this apparatus it is possible to determine the tibial slope on cadaveric knees, and future studies should perfect measurement techniques and confirm the validity of data obtained by using a parallel bars device.

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Heidelberg Retina Tomography II parameters in evaluating high- and normal-pressure glaucoma progression

Parametri Hajdelberg tomografije II mrežnjače u proceni progresije glaukoma pri visokom i normalnom očnom pritisku

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Abstract

Background/Aim. Heidelberg retina tomography II (HRT II) haves been employed to quantitatively assess the topography of optic discs in eyes with high-pressure glaucoma (HPG) and normal-pressure glaucoma (NPG), in order to determine which of global and segmental optic disc parameters will prove to be most suitable for monitoring the progression of these two conditions. **Methods.** The results of 73 eyes of 73 patients with HPG and NPG were analyzed in relation to age, refractive error, quality of HRT images and stereometric parameters. **Results.** A statistically significant difference ($p < 0.05$) between the global baseline and follow-up results was found in: rim volume, maximum cup depth and cup shape measure (in the HPG group), and C/D ratio, cup volume, rim volume and cup shape measure (in the NPG group). The baseline and follow-up results of the retinal nerve fiber layer in the temporal and inferotemporal sectors show a significant difference in both groups. **Conclusion.** Several HRT stereometric parameters are useful for monitoring the progression of changes of the optic disc and local retina in eyes with HPG and NPG. Both segmental and global scanning is of importance in glaucoma progression analysis.

Key words:
glaucoma; retina; tomography; disease progression.

Apstrakt

Uvod/Cilj. *Heidelberg retina tomography* (HRT II) koristi se za kvantitativnu procenu topografije optičkog diska očiju sa glaukomom kod povećanog intraokularnog pritiska (*high-pressure glaucoma* – HPG) i kod normalnog intraokularnog pritiska (*normal-pressure glaucoma* – NPG) da bi se utvrdilo koji od globalnih i segmentnih parametara optičkog diska mogu biti najpodesniji za praćenje progresije ova dva poremećaja. **Metode.** Urađena je analiza rezultata dobijenih za 73 oka 73 bolesnika sa HPG i NPG u odnosu na starost bolesnika, refraktivnu grešku, kvalitet HRT snimaka i stereometrijske parametre. **Rezultati.** Utvrđena je statistički značajna razlika ($p < 0,05$) između globalnih početnih vrednosti i rezultata praćenja u zapremini oboda, maksimalnoj dubini ekskavacije i parametrima trećeg momenta (grupa HPG) i u odnosu C/D, zapremini ekskavacije, zapremini oboda i parametrima trećeg momenta (grupa NPG). Početne vrednosti i rezultati praćenja retinalnog sloja nervnih vlakana u temporalnom i inferotemporalonom sektoru pokazali su statistički značajnu razliku u obe grupe. **Zaključak.** Nekoliko HRT II stereometrijskih parametara korisno je za praćenje progresije promena na optičkom disku i okolnoj mrežnjači kod bolesnika sa HPG i NPG. Za analizu progresije glaukoma korisno je, prema tome, praćenje i segmentnih i globalnih parametara optičkog diska.

Ključne reči:
glaukom; mrežnjača; tomografija; bolest, progresija.

Introduction

Accurate and prompt detection of optic nerve damage is of tremendous importance in early diagnosis and prevention of blindness from glaucoma. Primary open-angle glaucoma (POAG) is a disorder that demonstrates typical structural changes in the optic disc along with visual field defects. Depending on whether these changes are accompanied by increased intraocular pressure or not, POAG is divided into two subgroups: normal-pressure glaucoma (NPG) and high pressure glaucoma (HPG). Assessment of the optic disc is included in the standard examination of patients with suspected or manifest glaucoma. It is difficult to detect early changes of glaucoma with standard procedures because

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nerve fiber degeneration and loss of visual field do not progress in parallel in the early stage of glaucoma. A number of studies have shown that visual field abnormalities are detected only after 20−50% of the retinal ganglion cells have been lost [1–3]. The early changes in patients with POAG are those in the thickness of the nerve fiber layer and in the morphology of the optic disc [4, 5]. All these data speak in favor of how it is important to know the real principles of early detection and precise monitoring of progression of glaucoma, as well as diagnostic procedures that make use of it. To assess and follow these changes, modern instruments based on laser confocal and other systems have been developed [6–9]. Heidelberg retina tomograph II (HRT II, Heidelberg Engineering, GmbH, Heidelberg, Germany) uses confocal scanning laser ophthalmoscopy to evaluate quantitatively the three-dimensional surface topography of the optic nerve head and the surrounding nerve fiber layer [10–13]. HRT II is an instrument we used in our study for collecting data. The aim of this study was to determine which stereometrical HRT parameters are most suitable for monitoring progression of glaucoma in both HPG and NPG group, as well as to find out which of the 6 sectors of neuroretinal rim significantly changed over time in both groups, and how the damaged zone of the neuroretinal rim area is changed over time.

Methods

This retrospective study included 73 eyes of 73 patients from the Ophtalmological Institute of Faculty of Medicine, University of Belgrade. The research followed the Declaration of Helsinki and was approved by the Regional Ethical Review Board. We investigated stereometric parameters of 50 eyes in 50 patients with HPG and 23 eyes in 23 patients with NPG (Table 1). The patients diagnosis was assessed according to the rules of the European Glaucoma Society [14]. The average period of monitoring the patients in the HPG group was 26 months, while in the NPG group it was 23 months, for which period at least three HRT examinations for each patient were done. The restriction of the study to the one eye of each patient for each group was to facilitate statistical analysis. Those eyes with excessive refractive error of more than + 6 diopters or less than – 6 diopters, cataracts, diabetic retinopathy or with any history of surgical treatment or eye trauma were excluded. We used HRT II in our study to get a series of photographies of the cross section of the optical nerve head of different deepness. After 3D reconstruction it produces topographical photographies of the papilla and peripapillar retina [15]. To quantify morphometric rim and cup parameters in optic disc topography, a reference plane is defined, which is stable over each examination, so that the parameters change only when true structural changes in the optic disc occur. The retinal surface located above the reference plane is defined as a rim, and below the reference level as a cup. In order to verify the quality of topographic images we used images with standard deviation less than 40 μm. Ten stereometric parameters [(disc area in mm^2, cup area in mm^2, rim area in mm^2, cup-to-disc area ratio (C/D ratio), cup volume in mm^3, rim volume in mm^3, height variation contour in mm, mean cup depth in mm and maximum cup depth in mm and cup shape measure in mm)] of baseline and follow up examinations has been taken into consideration in this study. We also investigated baseline and follow-up data for the mean retinal nerve fiber layer (mRNFL) thickness in each of the 6 sectors to which neuroretinal rim was divided. Moorfields regression analysis (MRA) is a part of HRT programe, representing the method for analyzing regression logarithmic of the global and 6 sectoral rim areas (temporal, inferotemporal, superotemporal, nasal, superonasal, inferonasal) to the matching disc areas and compares the results to a normative database. It defines these areas as damaged, borderline and normal based in the 95% and 99.9% confidence intervals. In our study we examined which of the studied HRT parameters was statistically most suitable for monitoring the progression in both groups, as well which of the 6 sectors of neuroretinal rim showed the greatest change in the mRNFL.

We analyzed the basic demographic characteristics (age, gender), also a refractive error and standard deviation of HRT images and examined stereometrical parametres of the optical disc of both groups, with the aim to establish the existance of a statistically significant difference between the same parametres in baseline and follow-up examinations (statistically significant difference in t test is when $p < 0.05$).

Results

Table 1 shows the basic statistics relating to sex, age, size of refractive error in patients eyes and standard deviation of topographic HRT images. We examined the difference between the aforementioned parameters between the HPG and NPG groups and found that there were no significant differences in refractive error and standard deviation of topographic HRT images among the two groups. There was a statistically significant difference in age between the patients

Table 1

Characteristics of the patients in both studied groups

Parameters	HPG	NPG
Number of eyes, n	50	23
Male/Female, n	20/30	6/17
Age (years), $\bar{x} \pm SD$	60.38 ± 9.41	51.04 ± 6.30
Refractive error (years), $\bar{x} \pm SD$	-0.5 ± 1.5	-0.8 ± 2.5
Topographic standard deviation, $\bar{x} \pm SD$	25.3 ± 6.9	27.2 ± 5.6

HPG – high – pressure glaucoma; NPG – normal – pressure glaucoma

with HPG and NPG. The patients with NPG were significantly younger than the patients with HPG. Tables 2, 3 and 4 show basic statistically summarized results of HRT parameters measurements in both groups (descriptive statistics). Examining the significance of differences among parameters between baseline and the last follow-up examination in the HPG and NPG groups we found different results (statistically significant difference is when $p < 0.05$). In the HPG group stereometrical parameters of follow-up examinations which showed a significant difference from baseline examinations were rim volume, maximum cup depth and cup shape measure (Table 5).

Table 2

Values of baseline (B) and the last follow-up (F) examination of the studied Heidelberg retina tomography stereometrical parameters in the high-pressure glaucoma (HPG) and the normal-pressure glaucoma (NGP) groups

Sterometrical parameters	HPG		NPG	
	B ($\bar{x} \pm SD$)	F ($\bar{x} \pm SD$)	B ($\bar{x} \pm SD$)	F ($\bar{x} \pm SD$)
Disc area (mm^2)	2.480 ± 0.501	2.480 ± 0.501	2.715 ± 0.631	2.715 ± 0.631
Cup area (mm^2)	0.971 ± 0.590	0.979 ± 0.598	1.087 ± 0.456	1.043 ± 0.453
Rim area (mm^2)	1.508 ± 0.542	1.501 ± 0.540	1.628 ± 0.358	1.671 ± 0.340
Cup/disc area ratio	0.382 ± 0.209	0.385 ± 0.211	0.388 ± 0.106	0.369 ± 0.111
Cup volume (mm^3)	0.319 ± 0.337	0.349 ± 0.300	0.351 ± 0.240	0.312 ± 0.237
Rim volume (mm^3)	0.347 ± 0.204	0.156 ± 0.205	0.390 ± 0.153	0.411 ± 0.133
Mean cup depth (mm)	0.276 ± 0.130	0.283 ± 0.130	0.304 ± 0.118	0.296 ± 0.126
Maximum cup depth (mm)	0.678 ± 0.238	0.850 ± 0.236	0.783 ± 0.225	0.956 ± 0.225
Height variation contour (mm)	0.400 ± 0.201	0.584 ± 0.176	0.386 ± 0.126	0.384 ± 0.107
Cup shape measure (mm)	0.138 ± 0.083	0.299 ± 0.020	0.167 ± 0.060	0.159 ± 0.051

Table 3

Values of baseline and the last follow-up examination of the mean retinal nerve filter layer (mRNFL) in each of the 6 sectors in the high-pressure glaucoma group

Rim area sectors	Baseline mRNFL ($\bar{x} \pm SD$)	Follow-up mRNFL ($\bar{x} \pm SD$)	Baseline *vs* follow-up (values of *t*-test)
Temporal	0.069 ± 0.035	0.039 ± 0.018	2.292*
Temporal superior	0.212 ± 0.115	0.216 ± 0.136	0.162
Temporal inferior	0208 ± 0.128	0.140 ± 0.069	2.681*
Nasal	0.201 ± 0.129	0.198 ± 0.139	0.137
Nasal superior	0.256 ± 0.147	0.254 ± 0.148	0.085
Nasal inferior	0.268 ± 0.148	0.263 ± 0.158	0.165

***p < 0.05 (statistically significant difference).**

Table 4

Values of baseline and the last follow-up examination of the mean retinal nerve filter layer (mRNFL) in each of the 6 sectors in the normal-pressure glaucoma group

Rim area sectors	Baseline mRNFL ($\bar{x} \pm SD$)	Follow-up mRNFL ($\bar{x} \pm SD$)	Baseline *vs* follow-up (values of *t*-test)
Temporal	0.084 ± 0.029	0.036 ± 0.015	2.435*
Temporal-superior	0.306 ± 0.091	0.312 ± 0.079	0.229
Temporal-inferior	0.217 ± 0.079	0.156 ± 0.098	2.461*
Nasal	0.268 ± 0.102	0.259 ± 0.116	0.262
Nasal-superior	0.359 ± 0.082	0.358 ± 0.094	0.037
Nasal-inferior	0.315 ± 0.106	0.315 ± 0.095	0.007

***p < 0.05 (statistically significant difference).**

Table 5

Testing significance of the differences between the baseline and follow-up results in both groups

HPG	Baseline *vs* follow-up (*t*-test)	NPG	Baseline *vs* follow-up (values of *t*-test)
Disc area	0.00	Disc area	0.00
Cup area	0.063	Cup area	0.33
Rim area	0.067	Rim area	0.423
Cup/disc area ratio	0.062	Cup/disc area ratio	2.592*
Cup volume	0.184	Cup volume	2.545*
Rim volume	2.286*	Rim volume	2.498*
Mean cup depth	0.282	Mean cup depth	0.202
Maximum cup depth	2.345*	Maximum Cup depth	0.785
Height variation contour	0.123	Height variation Contour	0.086
Cup shape measure	2.005*	Cup shape measure	2.455*
m RNFL	0.445	m RNFL	0.096

HPG – high-pressure glaucoma; NPG – normal pressure glaucoma; mean retinal nerve filter layer (m RNFL);
***p < 0.05 (statistically significant difference).**

In the NPG group stereometrical parameters of follow-up examinations which showed a significant difference from baseline examinations were C/D ratio, cup volume, rim volume and cup shape measure (Table 5). Analyzing the progression of mRNFL damage in each of the 6 sectors of the neuroretinal rim in the HPG group, we found statistically significant differences between baseline and follow-up examinations in the temporal and inferotemporal sector (Table 3). The same sectors showed a statistically significant difference between baseline and follow-up examinations in the NPG group (Table 4). Reading of the MRA findings of both groups, showed that the size of the damage (in percent) of the neuroretinal rim was higher in the group with NPG (10.5%), than in the group with HPG (7.5%), observing only baseline examinations. Also, observing only baseline MRA examinations we found that in the group with HPG the most often clasified as damaged was nasal segment while the least one was temporal, also in the group with NPG the most often clasified as damaged was the nasal segment, and the least one was the temporal (Table 6).

Table 6

Moorfields regression analysis (MRA) results: Damaged sectors distribution by the baseline and follow-up results in both groups [(50 eyes in the high-pressure glaucoma (HPG) and 23 eyes in the normal pressure glaucoma (NPG) group]

Groups	MRA tmp (n)	MRA tmp/sup (n)	MRA tmp/inf (n)	MRA nsl (n)	MRA nsl/sup (n)	MRA nsl/inf (n)
HPG						
baseline	3	8	5	10	9	5
follow-up	9	9	10	12	10	8
NPG	MRA tmp	MRA tmp/sup	MRA tmp/inf	MRA nsl	MRA nsl/sup	MRA nsl/inf
baseline	1	2	2	4	3	2
follow-up	5	2	9	4	3	2

tmp – temporal; sup – superior; inf – inferior; nsl – nasal.

Discussion

Structural alterations of the optic disc nerve fiber layer complex provide the earliest reliable signs of damage from glaucoma [15–17]. Accurate and objective quantitative measurements of the optic nerve head and nerve fiber layer are required to improve our ability to regularly recognize early glaucomatous progression. The quest for more accurate and objective methods has caused several qualitative and quantitative systems to be proposed to detect optic disc changes. The reproducibility and effectiveness of confocal scanning lasers using HRT II, as in our study, has already been reported [16–19]. According to our HRT results, the mRNFL was most vulnerable in the temporal and temporal inferior segments of the optic disc. The same results were found in both studied groups. According to the authors, the most vulnerable segment was the nasal inferior, and the second most vulnerable segment was the temporal segment or superotemporal segment [20]. Similar results were published by Marjanovic et al. [21]. The most sensitive parameters in tracking HPG progression in our study were for the three parameters of global optic disc: rim volume, maximum cup depth and cup shape measure. The most sensitive parameters in tracking NPG progression in our study were four parameters of global optic disc: C/D ratio, cup volume, rim volume and cup shape measure. Similar results can be found in other authors [22–24]. According to Uchida et al. [25] the parameters that best defined the presence of glaucomatous damage were those which analyze the cup, followed by those that analyze the neuroretinal ring, and finally those that are dependent on RNFL measurements. The parameters with highest diagnostic value were cup shape measure and the C/D ratio [25]. Other studies show that the rim area is reproducible and potentially useful as a marker of progression. These features can be expected in standard reference plane analysis of HRT II images and should be considered when evaluating glaucoma progression [24]. There seems to be great variability in the appearance and progression of initial glaucomatous optic disk and nerve fiber layer abnormalities in patients with glaucoma. Our study indicate that segmental as well as global analysis of optic disc images are required to detect a glaucomatous change, and suggest that HRT may be able to detect a change in the mRNFL in areas such as the temporal and inferotemporal segments, and also in a few global parameters in both studied groups, which may not be detected clinically.

Conclusion

Retinal nerve fiber layer progression in our study is mostly represented in the temporal and inferotemporal segments of the optic disc, and this applies to both high preasure glaucoma (HPG) and normal-pressure glaucoma (NPG) group. Considering the global parameters, the most frequently stricken in the HPG group were rim volume, maximum cup depth and cup shape measure, and in the NPG group C/D ratio, cup volume, rim volume and cup shape measure. Based on the baseline MRA results in both groups the most often clasified as damaged were the nasal segment, and the least often the temporaline. Thus, both segmental and global scanning are of importance in HPG and NPG progression analysis.

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When do the symptoms of autonomic nervous system malfunction appear in patients with Parkinson's disease?

Kada se pojavljuju simptomi oštećenja autonomnog nervnog sistema kod obolelih od Parkinsonove bolesti?

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Abstract

Background/Aim. Dysautonomia appears in almost all patients with Parkinson's disease (PD) in a certain stage of their condition. The aim of our study was to detect the development and type of autonomic disorders, find out the factors affecting their manifestation by analyzing the potential association with demographic variables related to clinical presentation, as well as the symptoms of the disease in a PD patient cohort. **Methods.** The patients with PD treated at the Clinic of Neurology in Belgrade during a 2-year period, divided into 3 groups were studied: 25 *de novo* patients, 25 patients already treated and had no long-term levodopa therapy-related complications and 22 patients treated with levodopa who manifested levodopa-induced motor complications. Simultaneously, 35 healthy control subjects, matched by age and sex, were also analyzed. **Results.** Autonomic nervous system malfunction was defined by Ewing diagnostic criteria. The tests, indicators of sympathetic and parasympathetic nervous systems, were significantly different in the PD patients as compared with the controls, suggesting the failure of both systems. However, it was shown, in the selected groups of patients, that the malfunction of both systems was present in two treated groups of PD patients, while *de novo* group manifested only sympathetic dysfunction. For this reason, the complete autonomic neuropathy was diagnosed only in the treated PD patients, while *de novo* patients were defined as those with the isolated sympathetic dysfunction. The patients with the complete autonomic neuropathy differed from the subjects without such neuropathy in higher cumulative and motor unified Parkinson's disease rating score (UPDRS) ($p < 0.01$), activities of daily living scores ($p < 0.05$), Schwab-England scale ($p < 0.001$) and Hoehn-Yahr scale. There was no difference between the patients in other clinical-demographic characteristics (sex, age at the time of diagnosis, actual age, duration of disease, involved side of the body, pain and freezing), but mini mental status (MMS) score and Hamilton depression and anxiety rating scale were significantly lower ($p < 0.05$). **Conclusion.** Our results confirm a high prevalence of autonomic nervous system disturbances among PD patients from the near onset of disease, with a predominant sympathetic nervous system involvement. The patients who developed complete autonomic neuropathy (both sympathetic and parasympathetic) were individuals with considerable level of functional failure, more severe clinical presentation and the existing anxiety and depression.

Key words:
parkinson disease; autonomic nervous system.

Apstrakt

Uvod/Cilj. Poremećaji autonomnog nervnog sistema javljaju se kod gotovo svih obolelih od Parkinsonove bolesti (PB) u nekom stadijumu njihove bolesti. Cilj našeg istraživanja bio je da se utvrde pojava i tip autonomnih poremećaja, kao i faktori koji utiču na njihovu pojavu ispitivanjem potencijalne povezanosti sa demografskim varijablama vezanim za kliničku prezentaciju i simptome bolesti u grupi bolesnika sa PB. **Metode.** Ispitivali smo obolele od PB koji su lečeni u Klinici za neurologiju u Beogradu u dvogodišnjem periodu, podeljene u tri grupe: 25 *de novo* bolesnika, 25 obolelih koji su lečeni i nisu imali nikakve komplikacije vezane za dugotrajnu primenu levodope i 22 bolesnika na levodopi kod kojih su se pojavile motorne komplikacije izazvane levodopom. Istovremeno je analizirano 35 zdravih (kontrolnih) ispitanika, uparenih prema uzrastu i polu. **Rezultati.** Disfunkcija autonomnog nervnog sistema definisana je prema Ewing-ovim dijagnostičkim kriterijumima. Testovi, pokazatelji ispada simpatičkog i parasimpatičkog nervnog sistema, bili su statistički značajno različiti kod obolelih od PB u poredjenju sa kontrolom, što je sugerisalo poremećaj oba sistema. Kada su izdvojene pojedine

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grupe obolelih, međutim, pokazano je da disfunkcija oba sistema postoji kod dve grupe lečenih od PB, dok je kod *de novo*, nelečenih bolesnika, uočen samo poremećaj simpatičkog nervnog sistema. Zbog toga je kompletna autonomna neuropatija dijagnostikovana samo kod lečenih od PB, dok su *de novo* oboleli definisani kao oboleli sa izolovanom simpatičkom disfunkcijom. Osobe sa kompletnom autonomnom neuropatijom razlikovale su se od osoba bez autonomne neuropatije po višem kumulativnom i motornom unifikovanom skoru za Parkinsonovu bolest ($p < 0,01$), skoru aktivnosti svakodnevnog života ($p < 0,05$), Schwab-England skali ($p < 0,001$) i procenjenoj težini bolesti prema Hoehn-Yahr-u. Bolesnici se nisu razlikovali prema drugim kliničkodemografskim karakteristikama (pol, uzrast u vreme postavljanja dijagnoze, aktuelnom uzrastu, trajanju bolesti, zahvaćenoj strani, bolu i „*freezing*-u"), ali su imali statistički značajno niži mini mentalni skor (MMS), kao i skor na Hamiltonovoj skali depresivnosti i anksioznosti ($p < 0,05$). **Zaključak.** Rezultati ove studije potvrđuju visoku prevalenciju poremećaja autonomnog nervnog sistema u ranoj fazi bolesti kod obolelih od Parkinsonove bolesti, sa predominantnom disfunkcijom simpatikusa. Bolesnici kod kojih se razvila kompletna autonomna neuropatija (simpatička i parasimpatička) imali su izraženu funkcionalnu slabost, teže kliničke simptome, uz prisustvo anksioznosti i depresije.

Ključne reči:
parkinsonova bolest; nervni sistem, autonomni.

Introduction

Autonomic nervous system dysfunctions have long been the "cornerstone" of Parkinson's disease (PD) and multiple systemic atrophy (MSA), and even currently accepted criteria of diagnosis and recognition of PD have exactly emphasized, as an exclusion symptom, the presence of severe autonomic neuropathy [1]. Nevertheless, autonomic dysfunction is being recorded in almost all PD patients in a certain stage of their disease [2–4], and a Sydney study has shown that 71% of patients develop, after 15 years of their condition, autonomic disorders, such as sphincter control dysfunction [5]. On the other hand, the presence of multiple autonomic disorders has been verified even before development of motor failures, when they have been accepted as non-motor manifestations of already existing disease [6], and perennial duration of individual non-motor signs has made them possible prodromal symptom of the disease [7].

Autonomic symptoms are consistent with the results of neuropathological studies and the presence of changes in hypothalamus, brainstem, intermediolateral cell column, autonomic ganglia, and myenteric plexus; the theory of Braak et al. [8] justifies and explains their appearance early in the course of disease, given the assumption of the ascending spread of disease in which the involvement of dorsal nuclei of the vagus nerve is one of the earliest stages of disease [8].

The aim of our study was to detect the occurrence and type of autonomic nervous system symptoms, to find out factors influencing their presence, by assessing the relation with demographic, disease-related and clinical variables in cohort of PD patients.

Methods

The patients with PD, diagnosed according to the Brain Bank Criteria [1], treated at the Institute of Neurology, Clinical Center of Serbia in the period from 2004 to 2006, and age-matched controls without PD, were evaluated. Three groups of PD patients were tested: 25 *de novo* patients (group I), 25 levodopa treated patients without levodopa-induced motor complications (group II), and 22 levodopa treated patients with levodopa-induced motor complications (group III), as well as 35 age-matched controls (group IV). Among the PD patients, as well as among the control group, neither diabetes mellitus nor other vascular risk factors were detected (hypertension, hypercholesterolemia, heart disease and peripheral nerve diseases).

After obtaining the informed consent and the approval of the Ethics Committee of the Faculty of Medicine, University of Belgrade (No. 440/XII-2), all the patients were tested in Neurocardiology Unit, Clinical Center "Bežanijska kosa", Belgrade. All the tests were performed under standardized conditions, in climate-controlled rooms (temperature 23°C), in the morning, after a period of relaxation. Tobacco, alcohol, caffeine, and medications were not allowed before the test. Autonomic nervous system dysfunction was diagnosed by means of cardiovascular reflex tests according to Ewing [9], and was considered to exist if, at least, two tests were positive. Vagal dysfunction was diagnosed using 3 tests: Valsalva maneuver, deep breathing test and heart rate response to standing. Sympathetic dysfunction was assessed with 2 tests: blood pressure response to standing, and handgrip test. A patient is diagnosed as one with autonomic neuropathy (AN) after having two pathological tests (two sympathetic or two parasympathetic tests) and as complete autonomic neuropathy (CAN) in case of confirmed both parasympathetic and sympathetic denervation.

The results of each test were expressed as normal (0), borderline (1) or abnormal (2), as in reference values according to Ewing [9]. Maximal possible cumulative score was 10 (i.e. if all five tests had pathological findings). A cumulative score of 0 or 1 was considered normal, while the score of 2 or 3 was interpreted as mild autonomic dysfunction. The patients with scores between 4 and 6 were diagnosed with moderate dysfunction and those who scored 7 or higher were considered to have severe autonomic dysfunction.

The Mini Mental State Examination (MMSE) [10], the 21-item Hamilton Depression Rating Scale (HDRS) [11], as well as Hamilton Anxiety Rating Scale (HARS) [12] were conducted by the same trained interviewer (MS).

When data collection was completed, the differences between arithmetic means were assessed by Student's *t*-test and between proportions by $\chi 2$ test.

Results

A total of 72 patients as well as 35 age-matched controls agreed to participate in the study by signing the informed consent.

Clinical and demographic data of our PD patients and the control subjects are shown in Table 1.

Autonomic function tests

Tests for the sympathetic nervous system (SNS) evaluation were significantly different [hand grip – $p < 0.0001$; orthostatic hypotension (OH) – $p < 0.0001$) in comparison to the controls, suggesting the sympathetic failure in parkinsonian patients ($p < 0.0001$). The same was proved for the parasympathetic nervous system functions (Valsalva maneuver – $p < 0.0001$; deep breathing – $p < 0.05$; standing test – $p < 0.001$) (Table 2).

However, when the SNS and parasympathetic nervous system (PNS) were compared in *de novo* patients and the control group, the significance was found in both tests for SNS (hand grip – $p < 0.0001$; orthostatic hypotension – $p < 0.01$), while the significance was irrelevant for PNS (Valsalva maneuver – $p < 0.05$; deep breathing – NS; standing – non significant). The patients with good therapeutical response, as well as those with developed complications of levodopa treatment differed from the controls in both SNS ($p < 0.0001$) and PNS dysfunction ($p < 0.0001$).

Autonomic neuropathy was established in 44% of the *de novo* patients, 88% of the patients with good response to the therapy and in all the patients with complications of levodopa treatment (on average, 76% of the PD patients). Complete autonomic neuropathy was not established in the group I, while 36% and 50% in the groups II and III, respectively, had the dysfunction of both SNS and PNS (on average, 28% of the PD patients) (Figure 1). The presence of autonomic neuropathy ($p < 0.0001$) as well as CAN ($p < 0.0001$) differed significantly between the groups.

Table 1

Clinical and demographic characteristics of Parkinson's disease patients

Parameters	Group I	Group II	Group III	*p*
Sex (F:M)	9/16	13/12	9/13	NS
Age at onset (years)	56.9 ± 7.6	57.2 ± 8.2	55.3 ± 6.9	NS
Actual age (years)	57.1 ± 7.2	60.6 ± 8.8	63.0 ± 5.0	NS
Disease duration (months)	4.6 ± 9.7	41.6 ± 39.5	102.2 ± 60.3	< 0.0001
Schwab-England scale	84.4 ± 6.5	70.0 ± 11.2	58.6 ± 11.7	< 0.0001
Hoehn&Yahr scale	1.5	2	3	< 0.0001
UPDRS	26.3 ± 12.8	40.3 ± 17.2	53.2 ± 17.5	< 0.0001
MMS	27.7 ± 1.6	26.5 ± 2.6	25.6 ± 3.4	NS
Hamilton A	5.5 ± 3.3	6.9 ± 3.4	10.1 ± 4.3	< 0.0001
Hamilton D	6.4 ± 3.7	8.4 ± 4.1	10.9 ± 4.4	< 0.0001

Note: results are given as n or $\bar{x}$ ± SD; UPRDS – Unified Parkinson's Disease Rating Score; MMS – mini mental state; NS – non significant; F – female; M – male; group I – *de novo* patients; group II – levodopa treated patients with no levodopa-induced motor complications; group III – levodopa treated patients with levodopa-induced motor complications.

Table 2

The tests for evaluation of the sympathetic (SNS) and parasympathetic nervous system (PNS) symptoms in the Parkinson's disease (PD) and control groups

Parameters	Points	PD (n)	Control (n)	*p*
SNS				
hand grip	0	3	12	< 0.0001
	1	5	15	
	2	64	8	
orthostatic hypertension	0	34	34	< 0.0001
	1	11	1	
	2	27	0	
DS	0	7	27	< 0.0001
	1	65	8	
PNS				
Valsalva maneuver	0	16	15	< 0.0001
	1	25	18	
	2	31	2	
deep breathing	0	41	28	< 0.05
	1	15	6	
	2	16	1	
standing	0	19	21	< 0.001
	1	15	7	
	2	38	7	
DPS	0	17	25	< 0.0001
	1	55	10	

0 – normal; 1 – borderline; 2 – pathological; DS – denervation of the sympathetic nervous system; DPS – denervation of the parasympathetic nervous system.

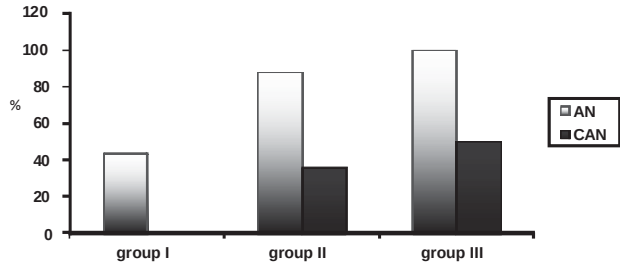

Fig. 1 – The percentage of patients with autonomic neuropathy (AN) and complete autonomic neuropathy (CAN).

group I – *de novo* patients; group II – levodopa treated patients with no levodopa-induced motor complications; group III – levodopa treated patients with levodopa-induced motor complications.

Patients with and without complete autonomic neuropathy

The patients with determined complete autonomic neuropathy (CAN) significantly differed from those without CAN, according to Schwab-England ($p < 0.001$) and Hoehn-Yahr scale ($p < 0.01$), while there was no difference in other examined clinical characteristics (sex, age at diagnosis, actual age, duration of disease, affected side, pain and "freezing"). A statistical difference was found in cumulative and motor UPDRS ($p < 0.01$), as well as in daily activities score ($p < 0.05$), when compared the patients with and without CAN. All the performed mental tests (MMSE, HDRS and HARS) showed a statistical difference ($p < 0.05$) between the patients who developed CAN and those who did not (Table 3).

saliva is continually draining from the mouth….") [13]. The conflicting conclusions were reported in the following decades. Many researchers stated that there was no direct connection between the autonomic failure and PD [14], contrary to others who estimated that 80–90% of all parkinsonian patients suffered from one or another type of dysautonomia [15]. Besides conflicting conclusions regarding the presence and frequency, an important issue was the onset of autonomic symptoms.

Our results support the view that dysfunction of autonomic nervous system represents important clinical feature of PD, even in its early phase. It is intriguing that not all parts of autonomic system were involved equally. In *de novo* patients, the tests for sympathetic evaluation showed significant difference, while the results for PNS were not statistically different among the groups, thus defining *de novo* patients as those manifesting only sympathetic dysfunction.

Shibata et al. [16] and Buob et al. [17] also found considerable dysfunction of the SNS, even at the initial phase of the disease, which was confirmed by recent results obtained by Gaenslen et al. [6]. They demonstrated dysregulation of the heart sympathetic noradrenergic innervation (PET scan using 6-[^{18}F] fluorodopamine) even before clinical diagnosis of Parkinson's disease. Post-mortem studies of the accidental Lewy bodies in patients without the clinical features of Parkinson's disease showed a decrease in tyrosine hydroxylase immunoreactivity in the epicardial nervous tissue, which also pointed to early noradrenergic denervation of the heart [18]. Okada et al. [19] showed the existence of Lewy body formation in the sinoatrial ganglion in 33% of patients with idiopathic PD.

Table 3

Clinical and demographic characteristics and (complete) autonomic neuropathy

Scores and demographic characteristics	Autonomic neuropathy	Complete autonomic neuropathy	*p*
UPDRS (cumulative)	39.7 ± 21.6	53.7 ± 24.1	< 0.01
UPDRS (mental)	3.5 ± 2.2	4.0 ± 2.3	
UPDRS (daily activities)	13.2 ± 7.5	17.7 ± 7.5	< 0.05
UPDRS (motor)	22.9 ± 13.8	32.0 ± 15.6	< 0.01
UPDRS (complications)	4.1 ± 3.6	5.5 ± 3.9	
Schwab-England	71.4 ± 15.8	58.9 ± 16.9	< 0.001
Hoehn-Yahr	2	3	< 0.01
MMS	26.9 ± 2.5	25.5 ± 3.2	< 0.05
HARS	7.3 ± 4.0	9.7 ± 5.1	< 0.05
HDRS	8.4 ± 4.4	11.0 ± 5.9	< 0.05
Sex (M/F)	39/19	15/14	
Age at onset	59.1 ± 8.1	62.4 ± 8.7	
Duration of disease (months)	42.4 ± 53.6	59.8 ± 58.8	
Affected side (left/right/both)	2/39/17	7/19/3	
Pain (yes/no)	25/33	14/15	
Freezing (yes/no)	18/39	14/14	

Note: results are given as n or $\bar{x}$ ± SD; UPDRS – Unified Parkinson's Disease Rating Score; MMS – mini mental state; HARS – Hamilton Anxiety Rating Scale; HDRS – Hamilton Depression Rating Scale; M – male; F – female.

Discussion

In 1817 James Parkinson described that other than classical symptomatology, the disease includes dysautonomia as well ("… the bowels demand stimulating medicines of very considerable power, … the urine passed involuntarily, … the

In attempt to stress an early manifestation of the autonomic dysfunction, Barbic et al. [20] compared a control group and PD individuals with and without orthostatic hypotension (OH), revealing certain autonomic impairment in non-OH patients, therefore suggesting that parkinsonian patients without dysautonomia were somewhere between healthy in-

dividuals and PD subjects with developed OH. Mihci et al. [21] reached similar conclusions, emphasizing the need for autonomic assessment, even in the absence of autonomic symptoms. Nowadays, it is a possibile to early detect postganglionic sympathetic dysfunction using I-125-MIBG-SPECT [22].

There are assumptions that the autonomic disturbances may be prodromal signs of Parkinson's disease [23]. It is also considered that constipation is an early manifestation of Parkinson's disease and that even men who reported having less than one bowel movement daily had a risk of developing PD 2.7 times higher than men who had bowel movements on regular daily basis [24]. It is even believed that the presence of constipation with the abnormal sense of smell and REM sleep behavior disorder already represent a defined type of PD [25].

Interestingly, however, there are people with PD who have a normal finding of the heart suggesting that the autonomic disturbances (loss of cardiac uptake of 123-I-MIBG) and nigrostriatal degeneration can exist independently in patients with PD [26].

Contrary to previous findings, some researchers [13, 16] suggest a simultaneous deficit of both divisions of ANS, while Siddiqui et al. [27], as well as Buob et al. [17], claim that the initial dysfunction is of parasympathetic nature, whereas both SNS and PNS become involved with the disease evolution.

If we accept the theory of Braak et al. [8], then a huge percentage of patients with the autonomic disturbances in *de novo* group can be explained by their hypothesis. According to their proposition of six stages of pathological process, after the initial degeneration in *bulbus olfactorius* and anterior olfactory nucleus, this degeneration, in the next stage, spreads to the brain stem, the region that supposedly represents the key role in mediating autonomic phenomena.

An early appearance of autonomic disorders is the reason why sometimes it is not possible to differentiate, on the basis of positive tests, the multiple system atrophy and Parkinson's disease [28].

The issue of risk factors associated with CAN is also interesting, because an association between the presence of autonomic nervous system and disease severity, age at onset and drug intake is not clear [29].

The Schwab-England and Hoehn-Yahr scales differ significantly parkinsonian patients with complete autonomic neuropathy from those without it. This characterizes individuals with developed CAN as patients with higher degree of functional impairment and severe clinical status. CAN patients vary according to the number of UPDRS motor scale items especially according to postural instability and bradykinesia. This is consistent with the finding that autonomic disorders are more common problem in people with PIGD form of the disease [3]. Interestingly, albeit negative correlation between parkinsonian symptoms and autonomic failure indicates that patients with more prominent disorders of gait and stability have more severe dysautonomia, this connection is lost among our patients when daily levodopa therapy is taken into consideration.

CAN patients also vary from non-CAN group by the presence of anxiety and depression. Other clinical and demographic characteristics did not vary between these two groups of patients. Verbaan et al. [30] found that the severity of the autonomic phenomena was higher in people with the severe motor disorders, depressive symptoms, cognitive impoverishment, psychiatric complications, sleep disorders and excessive day sleepiness.

It was found that the most frequent symptoms in patients with the autonomic dysfunction are the orthostatic hypotension, bladder dysfunction, constipation and erectile disturbances [31].

In our study, the patients with CAN were not different from the patients without CAN by age. Although aging changes the function of the autonomic nervous system, according to many studies, an autonomic failure becomes apparent just after the age of 75 years [32]. In addition, our patients did not differ by the application and length of dopaminomimetic treatment (data not shown), though it has been described that levodopa aggravates the impairment of the autonomic control of BP and HR [29]. In our group of patients, impaired cardiovascular autonomic control was detected early in the course of PD, when patients were drug naïve. Contrary, in some reports, the use of levodopa agonists produced lessening of detrusor hyperreflexia, whereas in others it provided improvement of voiding difficulty [31]. A large study of Sakakibara and coworkers [33] showed that patients taking levodopa and dopamine agonists had voiding phase disorder more frequently than those taking levodopa only. Lucetti et al. [34] offered an interesting concept after 5-year follow-up of PD patients with and without autonomic dysfunction. The patients with dysautonomia required earlier introduction of dopaminergic therapy, which indirectly suggested their rapid deterioration.

Conclusion

Our results confirm a high prevalence of autonomic nervous system disturbances among Parkinson's disease patients from the near onset of disease, with a predominant sympathetic nervous system involvement. The patients who developed complete autonomic neuropathy (both sympathetic and parasympathetic) were individuals with considerable level of functional failure, more severe clinical presentation and the existing anxiety and depression.

Conflict of interest

The authors had no conflict of interest regarding the material and information presented in the article.

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Natural autoantibodies in healthy neonatals recognizing a peptide derived from the second conserved region of HIV-1 gp120

Prirodna antitela prisutna kod zdrave novorođenčadi koja prepoznaju peptid poreklom iz drugog konzerviranog regiona HIV-1 gp120

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Abstract

Background/Aim. High sera reactivity with a peptide derived from human immunodeficiency virus HIV-1 envelope protein gp120, NTM1, correlate with non-progressive HIV-1 infection and also may have protective role in breast and prostate cancer. We also detected a low NTM1 reactive antibodies titer in healthy HIV negative sera and showed that antibody levels can be significantly increased with vigorous physical activity. However, the immune system seems to be unresponsive or tolerant to this peptide, implicating that the NTM1 sequence encompasses or overlaps a certain innate immune epitope. The aim of this study was to present evidences that NTM1 – binding antibodies – are components of innate immune humoral response, by confirming their presence in sera of newborn babies. For this purpose we collected a set of 225 innate antigen sequences reported in the literature and screened it for candidate antigens with the highest sequence and spectral similarity to NTM1 derived from HIV-1 gp120. **Methods.** Sera from 18 newborns were tested using ELISA, with peptide NTM1. Sequences from innate antigen database were aligned by an EMBOSS Water bioinformatics tool. **Results.** We identified NTM1 reactive antibodies in sera of HIV negative newborn babies. Further, in order to identify which of already known innate antigens are the most similar to NTM1 peptide we screened innate immune antigen sequence database collected from the literature. This screening revealed that the most similar sequence are ribonucleoproteins RO60, in addition to previously identified N-terminus of vasoactive intestinal peptide. **Conclusion**. The results of this study confirm the hypothesis that NTM1 recognizing antibodies are a part of humoral innate immune response. Further, computational similarity screening revealed a vasoactive intestinal peptide and RO60 as the most similar sequences and the strongest candidate antigens. In the light of the presented results, it is appealing that testing blood reactivity at birth, with specific innate antigens, particularly a vasoactive intestinal peptide, can reveal the potential to develop or boost protective immune response in breast and prostate cancer and HIV infection later in life.

Key words:
immunity, innate; infant, newborn; infant, premature; antibodies; vasoactive intestinal peptide; hiv envelope protein gp120.

Apstrakt

Uvod/Cilj. Visoka reaktivnost seruma na peptid NTM1, poreklom iz proteina omotača gp120 virusa humane imunodeficijencije, HIV-1, koreliše sa neprogresivnom HIV-1 infekcijom, a, takođe, može imati zaštitnu ulogu u kanceru dojke i prostate. Nedavno smo detektovali nizak titar antitela reaktivnih na NTM1 i u zdravim HIV-negativnim serumima i pokazali smo da se nivo ovih antitela može značajno povećati energičnom fizičkom aktivnošću. Međutim, postoje dokazi da je ovaj peptid neimunogen ili da je nevidljiv za imuni sistem čoveka, čime se sugeriše da NTM1 sekvenca obuhvata ili se preklapa sa određenim epitopom urođene imunosti. Cilj ove studije bio je da se dokumentuje da su vezivna antitela, NTMI, sastavni delovi urođenog humoralnog imunog odgovora potvđivanjem njihovog prisustva u serumu novorođenčadi. U tu svrhu, sakupili smo ukupno 225 urođenih sekvenci antigena prikazanih u literaturi i testirali ih za kandidate antigena sa najvećom sličnošću sekvence i spektra NTM1 nastalih iz HIV-1 gp120. **Metode.** Serumi 18 novorođenčadi testirani su ELISA probama sa NTM1 peptidom vezanim za ploču. Poravnavanje sekvenci urođenih antigena vršeno je bioinformatičkim alatom EMBOSS Water. **Rezultati.** U serumima HIV negativnih novorođenčadi identifikovali smo antitela reaktivna na NTM1. Dalje, da bi utvrdili koji od već poznatih antigena urođene imunosti je najsličniji peptidu NTM1, pretražili smo bazu sekvenci antigena urođene imunosti formirane na osnovu literaturih

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podataka. Ovo pretraživanje pokazalo je da, pored predhodno identifikovanog N-terminusa vazoaktivnog intestinalnog peptida, najsličnija je NTM1 peptidu sekvenca ribonukleoproteina RO60. **Zaključak.** Ovom studijom potvrdili smo hipotezu da su antitela koja prepoznaju NTM1 deo urođenog humoralnog imunog odgovora. Dalje, kompjuterskim pretraživanjem sličnosti utvrdili smo da su vazoaktivni intestinalni peptid i RO60 sekvence najsličnije NTM1 i, tako, najjači kandidati za antigene. U svetlu predstavljenih rezultata, privlačna je ideja da testiranje reaktivnosti krvi sa specifičnim urođenim antigenima, pre svega vazoaktivnim intestinalnim peptidom na rođenju, može otkriti potencijal za razvoj protektivnog imunog odgovora kasnije u životu i to ne samo za određene autoimune bolesti, već i za kancer dojke i prostate, kao i HIV infekcije.

Ključne reči:
imunitet, prirodni; novorođenče; novorođenče, prevremeno; antitela; vazoaktivni intestinalni peptid; protein omotača gp120 virusa hiv.

Introduction

In spite tremendous progress that has been made by introducing highly active antiretroviral therapy (HAART) in inhibiting HIV-1 virus through disrupting reverse transcription, integration or proteolytic processing of viral proteins, some important problems as persistent viral reservoirs, drug resistance and toxicities still remain. HAART can effectively keep the viral replication at an undetectable level, prolonging the life expectancy of the infected and reducing the viral transmission. However, several host and virus factors can slow down and block the disease progression [1–4] and also, various immunological factors [5, 6] might play an important role. NTM1 peptide, derived from the C-terminus of the second conserved region of HIV-1 envelope protein gp120, and anti-NTM1 antibodies have been suggested to be important in controlling HIV disease. These antibodies have been significantly prevalent in sera of long-term nonprogressors (LTNP), the HIV positives that are capable of keeping viral load bellow 10 000 copies/mL without any retroviral therapy for more than 10 years. The same is observed in patients in asymptomatic phase of the HIV-1 disease [7]. NTM1 binding antibodies are reported in a small number of healthy individuals and extremely high titer values are detected in elite athletes [8]. Although the immune system seems to be unresponsive or tolerant to this peptide [9–11], the titer NTM1 recognizing antibodies can be increased by continuous and vigorous exercising [12]. Taken together these data implicate that NTM1 peptide encompasses or overlaps the innate immune epitope sequence involved in cellular pathway activated by physical activity. Based on the sequence similarity and *in vitro* cross-reactivity we previously hypothesized that the candidate binding antigen for these was vasoactive intestinal peptide (VIP), the pleiotropic extracellular molecule important in many physiologic functions, including glucose homeostasis, neuroprotection, memory, gut function, modulation of the immune system and circadian function. Due to its important immunomodulatory and neuromodulatory activities, circulating levels of VIP are under tight control. Natural anti-VIP autoantibodies are potent modifiers of its biological actions and important regulators of its circulating level [13–17].

Natural autoantibodies (NAbs) are an important component of the immune system, existing in all vertebrates and demonstrating a non-pathogenic anti-self reactivity [18]. NAbs are reactive with only a restricted and specific set of proteins [19]. The function of these antibodies, although not fully elucidated, is to provide early innate immune protection against certain pathogens, as well as the removal of possible autoantigens through scavenging dead or apoptotic cellular debris through the lifetime (for review see Lutz et al. [20]). In general, mediators of innate humoral immunity are low-affinity polyreactive antibodies with an ability to bind to diverse, similar epitopes.

The aim of this study was to present evidences that NTM1-binding antibodies are components of innate immune humoral response, by confirming their presence in sera of newborn babies. For this purpose we collected a set of 225 innate antigen sequences reported in the literature and screened it for candidate antigens with the highest sequence and spectral similarity to NTM1 derived from HIV-1 gp120. Computational similarity screening revealed VIP and a ribonucleoprotein, RO60, as the most similar sequences and the strongest candidate antigens, although other highly similar sequences were also identified. These results imply that testing blood reactivity with specific innate antigens at birth can reveal potential to develop or boost protective immune response in breast and prostate cancer and HIV infection.

Methods

Sequences

The HIV1 gp120 NTM1 sequence was as in paper of Djordjevic et al. [21] Innate immune antigen sequences are listed in Addendum 1.

Sequence alignments

Sequence alignments are calculated by an EMBOSS Water, a tool that uses the Smith-Waterman algorithm to calculate the local alignment of two sequences [22]. Sequences with scores higher than 20 (scores were from 7 to 23) are selected as candidate antigens.

Human subjects

Sera were collected from 9 preterm and 9 term newborns within the first 7 days after birth. The preterm infants were born between the weeks 27–34 of gestation, with the weight 950–2,200 g. The term newborns were born after 37 weeks of gestation, with the weight more than 2,500 g. After centrifugation, sera were collected and stored at -20°C. The sample material used in this study was what remained of serum used for standard biochemical testing. Blood specimens were not collected specifically for these experiments. The study was approved by the Ethics Committee of the Institute for Neonatology, Belgrade, Republic of Serbia.

ELISA

ELISA was performed with peptide (NTM1)4-SOC4 by the following procedure: polystyrene microtiter plates (Greiner, Germany) were incubated overnight at 4°C with 100 µl of peptides (1.25 µg/well) diluted in carbonate buffer, pH 9.6. Plates were washed with phosphate-buffered saline (PBS)–0.05% Tween and non-specific sites were blocked with 200 µl PBS containing 5% bovine serum albumin (BSA) for 2 h at room temperature. After 6 washings, serum specimens were added to the wells (100 µl /well). Sera were diluted 1 : 20 in 5% BSA in PBS. Plates were incubated for 4h at room temperature. After 6 washings with PBS–0.05% Tween, 100 µl of goat anti-human IgM alkaline phosphatase-conjugated antibodies (Sigma), diluted 1 : 50 were added and the plates were incubated for 30 minutes at room temperature. After 6 washings, p-nitrophenyl phosphate (pNPP) substrate was added and the absorbance optical density (OD) measured at 405–620 nm after 15 minutes. Each sample was tested twice. As a control we used antigen from Serion ELISA classic Cytomegalovirus IgM (Institute Virion\Serion GmbH).

Statistical analysis

The significance of the differences in O.D. values for preterm and term infants was calculated by Student's *t*-test, as the sample sizes were relatively small. The 2 groups were unpaired with uneven variance and therefore they were considered as part of a two-tailed, heteroscedastic matrix.

Results

In order to show that NTM1 binding antibodies are innate natural self-binding antibodies we determined their presence in sera of HIV-1 negative neonates. Our previous studies showed the presence of NTM1 antibodies of IgG subclass in HIV positive LTNPs, as well as in elite athletes and in small percentage of healthy HIV- adult population [7, 23], but here NTM1 binding of IgM subclass of antibodies specific to neonatal population were measured. Maternal antibodies of IgG subclass actively transfer across the placenta and therefore can be related to her adaptive immune response [24]. Reactivity of sera in a small cohort of 18 HIV-1 negative new born babies (Table 1) was determined by the ELISA immunoassay (Figure 1). Statistical analysis revealed that this reactivity in sera from preterm neonates was significantly higher in comparison with the reactivity of term neonates ($p < 0.001$).

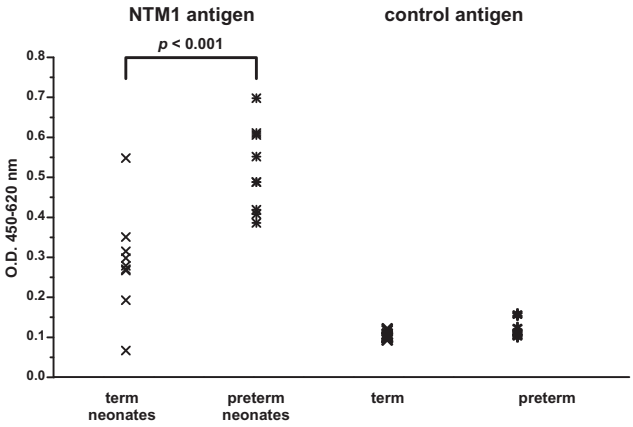

Fig. 1 – ELISA results of sera reactivity in the preterm and the term neonates. Antibodies recognizing peptide NTM1 were significantly more prevalent in serum samples from the preterm neonates compared to the term neonates ($p < 0.001$).

In search for innate epitopes similar to NTM1 we screened literature data and identified 225 protein sequences reported to encompass innate immune epitopes (Addendum 1) [20, 25–47]. Considering the ability of natural antibodies to bind to diverse epitopes we selected the set candidate antigens as described in Methods section (Table 2).

Discussion

Certain types of autoreactive immune cells and antibodies are common in healthy individuals and play an important role in body homeostasis. Several functions have been assigned to NAbs: neutralization of microbes and microbial toxins as natural first-line defence against infection, removal of senescent/altered self molecules and cells and immunomodulation and immunosignaling. New and unexpected insights into the functional roles of NAbs are still emerging, especially regarding their protective functions. NAbs are encoded by V(D)J genes in germline configuration and belong to IgG, IgM and IgA subclasses.

Several studies which investigated humoral immune response associated with the control of HIV-1 disease progression showed that the antibodies recognizing the peptide derived from C-terminus of the second conserved region of HIV-1 gp120, NTM1, correlate with non-progressive HIV-1 infection. Recently, the potential protective role of NTM1 antibodies has also been shown in breast and prostate cancer patients. Due to the fact that the peptide NTM1 is not immunogenic in

Table 1

Characteristics of infants at birth and the mean NTM1 ELISA absorbance values

Characteristics of infants	Preterm (n = 9)	Term (n = 9)
Gestational age at birth (weeks)	< 34	> 37
Birth weight (grams)	< 2500	> 2500
Sampling days	< 7	< 7
Absorbance values for NTM antigen, $\bar{x} \pm SD$	0.517 ± 0.107	0.287 ± 0.128
Absorbance values for control antigens, $\bar{x} \pm SD$	0.120 ± 0.022	0.106 ± 0.009

Table 2

Candidate antigens for NTM1 natural autoantibodies

Protein name	AC No	Score	Pairwise sequence alignment
VIP	P01282	23	NTM1 1 FTDN 4 |||| VIP_HUMAN 130 FTDN 133
RO60 ribonucleoprotein	P10155	23	NTM1 1 FTDN 4 |||| RO60-HUMAN 467 FTDN 470
ATP synthase subunit alpha	P25705	22	NTM1 1 FTDNAK 6 |.||.| ATPA_HUMAN 300 FRDNGK 305
Histone H2A 2C	Q16777	21	NTM1 3 DNAKT 7 ||.|| H2A2C_HUMAN 73 DNKKT 77
Serum albumin	P02768	21	NTM1 1 FTDNAKT 7 |.||.:| ALBU_HUMAN 151 FHDNEET 157
Hsp70	P08107	21	NTM1 1 FTDNAKTI 8 |||..:.| HSP71_HUMAN 44 FTDTERLI 51
Hsp71	P11142	21	NTM1 1 FTDNAKTI 8 |||..:.| HSP7C_HUMAN 44 FTDTERLI 51
Myeloperoxidase	P05164	21	NTM1 1 FTDNAKTI 8 |.||.:.: PERM_HUMAN 369 FQDNGRAL 376
Fibronectin	P02751	21	NTM1 1 FTDNAKT 7 ||.|.:| FINC_HUMAN 364 FTYNGRT 370
Thyroglobulin	P01266	21	NTM1 1 FTDNAKTI 8 ||.|.|.: THYG_HUMAN 460 FTTNPKRL 467

VIP – vasoactive intestinal peptide.

humans, it has been suggested that the antibodies recognizing this peptide represent natural autoantibodies. Thus, we have confirmed this hypothesis by detecting NTM1 antibodies in newborn babies. Sera samples of a small cohort of 18 newborns not older than 7 days tested in ELISA experiments showed a significant binding to NTM1 peptide. The difference between preterm and term serum titers are in line with already observed major differences of functional immune components between these two categories of neonates as reviewed in Sharma et al. [48]. This may be explained by specific innate immune defense pathways in protecting preterm infants against infection, or by deregulated innate immune responses which play a major role in the etiology of certain preterm neonatal complications later in life. Further, assuming that NTM1 recognizing antibodies belong to the majority of NAbs displaying rather low affinity and polyreactivity for a range of ligands [49] we scanned the set of innate antigen sequences in order to identify those that are most similar to NTM1. The collection of the 225 tested protein sequences from literature data represents significant, but far from exhaustive list of innate immune antigens (Addendum 1). Based on this inquiry we selected 10 most similar sequences as potential binding antigens for NTM1 recognizing antibodies (Table 2). The two prominent candidate antigens which show the highest similarity score to NTM1 are extracellular VIP, whose sera titer is tightly regulated by NAbs and RO60, which is an antigen for anti-RO60 antibodies involved in the clearance of apoptic debris [50]. These findings are in perfect agreement with major functional roles attributed to the natural IgM antibodies in the maintenance of tissue homeostasis and immune regulation [51–53]. However, we suggest that more probably VIP serves as the most important self-binding molecule for these antibodies for the following reasons: it was identified as one of the most important nodes in IgM antigen network conserved in both, mothers and babies [54], NTM recognizing IgG antibodies are cross-reactive with VIP in sera of HIV positives and in

healthy adults [55], and the levels of circulating VIP in newborns are significantly prevalent in preterm neonates compared to term born babies [56] which could explain the statistically significant prevalence of NTM1 recognizing antibodies ($p < 0.001$) in our study.

Conclusion

The information about the presence of NTM1 recognizing natural antibodies at birth may be of significant importance later in life due to the fact that these antibodies might have protective roles in HIV-1 disease and in breast or prostate cancer. Previous findings that these NAbs are retained throughout life with potential of sera titer to be significantly increased by physical exercise emphasize the need for future exploration of NTM1 recognizing antibodies as personalized therapeutic strategy based on the natural antibodies repertoire. In the light of the presented results, it is appealing that testing blood reactivity at birth, with specific innate antigens, particularly vasoactive intestinal pephole (VIP), can reveal the potential to develop or boost protective immune response against certain life threatening diseases.

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Addendum 1

Innate immune antigen sequences

Protein name	SwissProt ID	Reference
Alpha-2-macroglobulin precursor - Homo sapiens (Human)	[P01023]	24
Acetylcholinesterase precursor - Homo sapiens (Human)	[P22303]	24
Serum albumin precursor - Homo sapiens (Human)	[P02768]	24
Fructose-bisphosphate aldolase A - Homo sapiens (Human)	[P04075]	24
Fructose-bisphosphate aldolase B - Homo sapiens (Human)	[P05062]	24
Fructose-bisphosphate aldolase C - Homo sapiens (Human)	[P09972]	24
Amyloid beta A4 protein-Homo sapiens (Human)	[P05067]	25
Anion exchange protein 3-Homo sapiens (Human)	[P48751]	26
Atrial natriuretic factor precursor - Homo sapiens (Human)	[P01160]	24
Natriuretic peptides B precursor [Contains: Gamma-brain natriuretic peptide; Brain natriuretic peptide 32 - Homo sapiens (Human)	[P16860]	24
Annexin A10 - Homo sapiens (Human)	[Q9UJ72]	24
Annexin A11 - Homo sapiens (Human)	[P50995]	24
Annexin A13 - Homo sapiens (Human)	[P27216]	24
Annexin A1 - Homo sapiens (Human)	[P04083]	24
Annexin A2 - Homo sapiens (Human)	[P07355]	24
Annexin A3 - Homo sapiens (Human)	[P12429]	24
Annexin A4 - Homo sapiens (Human)	[P09525]	24
Annexin A5 - Homo sapiens (Human)	[P08758]	24
Annexin A6 - Homo sapiens (Human)	[P08133]	24
Annexin A7 - Homo sapiens (Human)	[P20073]	24
Annexin A8 - Homo sapiens (Human)	[P13928]	24
Annexin A9 - Homo sapiens (Human)	[O76027]	24
ATP synthase subunit alpha, mitochondrial-Homo sapiens (Human)	[P25705]	27
ATP synthase subunit beta, mitochondrial-Homo sapiens (Human)	[P06576]	27
Beta-2-glycoprotein 1 precursor - Homo sapiens (Human)	[P02749]	24
Beta-2-microglobulin precursor [Contains: Beta-2-microglobulin form pI 5.3] - Homo sapiens (Human)	[P61769]	24
Complement C1q subcomponent subunit A precursor - Homo sapiens (Human)	[P02745]	24
Complement C1q subcomponent subunit B precursor - Homo sapiens (Human)	[P02746]	24
Complement C1q subcomponent subunit C precursor - Homo sapiens (Human)	[P02747]	24
Calcitonin precursor [Contains: Calcitonin; Katacalcin - Homo sapiens (Human)	[P01258]	24
Caspase-3 precursor - Homo sapiens (Human)	[P42574]	24
Caspase-8 precursor - Homo sapiens (Human)	[Q14790]	24
Catalase - Homo sapiens (Human)	[P04040]	24
C-C chemokine receptor type 5- Homo sapiens (Human)	[P51681]	28, 29
C-X-C chemokine receptor type 4- Homo sapiens (Human)	[P61073]	28
Citrate synthase, mitochondrial- Homo sapiens (Human)	[O75390]	30
T-cell surface glycoprotein CD4- Homo sapiens (Human)	[P01730]	28, 31, 32
Cyclin-A1 - Homo sapiens (Human)	[P78396]	24
Cyclin-A2 - Homo sapiens (Human)	[P20248]	24
Choriogonadotropin subunit beta precursor - Homo sapiens (Human)	[P01233]	24
60 kDa heat shock protein, mitochondrial precursor - Homo sapiens (Human)	[P10809]	24
Coagulation factor VIII Homo sapiens (Human)	[P00451]	33, 34
Collagen alpha-1(I) chain precursor - Homo sapiens (Human)	[P02452]	24
Collagen alpha-2(I) chain precursor - Homo sapiens (Human)	[P08123]	24
Complement component C9 precursor [Contains: Complement component C9a; Complement component C9b] - Homo sapiens (Human)	[P02748]	24
Collagen alpha-1(X) chain precursor - Homo sapiens (Human)	[Q03692]	24
Corticotropin-lipotropin precursor - Homo sapiens (Human)	[P01189]	24
Corticoliberin precursor - Homo sapiens (Human)	[P06850]	24
C-reactive protein precursor [Contains: C-reactive protein(1-205)] - Homo sapiens (Human)	[P02741]	24
Cytotoxic T-lymphocyte protein 4 precursor - Homo sapiens (Human)	[P16410]	24
Glutamate decarboxylase 1 - Homo sapiens (Human)	[Q99259]	24
Glutamate decarboxylase 2 - Homo sapiens (Human)	[Q05329]	24
Granulocyte-macrophage colony-stimulating factor- Homo sapiens (Human)	[P04141]	35
DnaJ homolog subfamily B member 1 - Homo sapiens (Human)	[P25685]	24
Endoplasmin/ gp96 homolog- Homo sapiens (Human)	[P14625]	27
Endothelin-1 precursor - Homo sapiens (Human)	[P05305]	24
Endothelin-2 precursor - Homo sapiens (Human)	[P20800]	24
Alpha-enolase- Homo sapiens (Human)	[P06733]	27
Pro-epidermal growth factor precursor - Homo sapiens (Human)	[P01133]	24

Leukocyte elastase precursor - Homo sapiens (Human)	[P08246]	24
Coagulation factor X precursor - Homo sapiens (Human)	[P00742]	24
Coagulation factor VII precursor - Homo sapiens (Human)	[P08709]	24
Alpha-2-HS-glycoprotein precursor - Homo sapiens (Human)	[P02765]	24
Fetuin-B precursor - Homo sapiens (Human)	[Q9UGM5]	24
Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A] - Homo sapiens (Human)	[P02671]	24
Fibrinogen beta chain precursor [Contains: Fibrinopeptide B] - Homo sapiens (Human)	[P02675]	24
Fibrinogen gamma chain precursor - Homo sapiens (Human)	[P02679]	24
Fibronectin precursor - Homo sapiens (Human)	[P02751]	24
Glyceraldehyde-3-phosphate dehydrogenase - Homo sapiens (Human)	[P04406]	24, 27
Glyceraldehyde-3-phosphate dehydrogenase, testis-specific - Homo sapiens (Human)	[O14556]	24
Gastrin precursor [Contains: Gastrin-71 - Homo sapiens (Human)	[P01350]	24
Gelsolin precursor - Homo sapiens (Human)	[P06396]	24
Glycoprotein hormones alpha chain precursor - Homo sapiens (Human)	[P01215]	24
Glucagon precursor [Contains: Glicentin; Glicentin-related polypeptide - Homo sapiens (Human)	[P01275]	24
Progonadoliberin-1 precursor - Homo sapiens (Human)	[P01148]	24
Stress-70 protein, mitochondrial precursor - Homo sapiens (Human)	[P38646]	24
Glutathione S-transferase A1 - Homo sapiens (Human)	[P08263]	24
Glutathione S-transferase A2 - Homo sapiens (Human)	[P09210]	24
Glutathione S-transferase A3 - Homo sapiens (Human)	[Q16772]	24
Glutathione S-transferase A4 - Homo sapiens (Human)	[O15217]	24
Glutathione S-transferase A5 - Homo sapiens (Human)	[Q7RTV2]	24
Glutathione S-transferase kappa 1 - Homo sapiens (Human)	[Q9Y2Q3]	24
Glutathione S-transferase Mu 1 - Homo sapiens (Human)	[P09488]	24
Glutathione S-transferase Mu 2 - Homo sapiens (Human)	[P28161]	24
Glutathione S-transferase Mu 3 - Homo sapiens (Human)	[P21266]	24
Glutathione S-transferase Mu 4 - Homo sapiens (Human)	[Q03013]	24
Glutathione S-transferase Mu 5 - Homo sapiens (Human)	[P46439]	24
Glutathione transferase omega-1 - Homo sapiens (Human)	[P78417]	24
Glutathione transferase omega-2 - Homo sapiens (Human)	[Q9H4Y5]	24
Glutathione S-transferase P - Homo sapiens (Human)	[P09211]	24
Glutathione S-transferase theta-1 - Homo sapiens (Human)	[P30711]	24
Glutathione S-transferase theta-2 - Homo sapiens (Human)	[P30712]	24
High affinity immunoglobulin epsilon receptor subunit alpha- Homo sapiens (Human)	[P12319]	36
Histone H2A type 2-C - Homo sapiens (Human)	[Q16777]	24
Hemoglobin subunit alpha - Homo sapiens (Human)	[P69905]	24
Hemoglobin subunit theta-1 - Homo sapiens (Human)	[P09105]	24
Hemoglobin subunit zeta - Homo sapiens (Human)	[P02008]	24
Hemoglobin subunit beta - Homo sapiens (Human)	[P68871]	24
Hemoglobin subunit delta - Homo sapiens (Human)	[P02042]	24
Hemoglobin subunit epsilon - Homo sapiens (Human)	[P02100]	24
Hemoglobin subunit gamma-1 - Homo sapiens (Human)	[P69891]	24
Hemoglobin subunit gamma-2 - Homo sapiens (Human)	[P69892]	24
Hemoglobin subunit mu - Homo sapiens (Human)	[Q6B0K9]	24
Sperm protamine-P1 - Homo sapiens (Human)	[P04553]	24
Heat shock 70 kDa protein 1 - Homo sapiens (Human)	[P08107]	24
Heat shock 70 kDa protein 4 - Homo sapiens (Human)	[P34932]	24, 27
Heat shock 70 kDa protein 6 - Homo sapiens (Human)	[P17066]	24
Putative heat shock 70 kDa protein 7 - Homo sapiens (Human)	[P48741]	24
Heat shock cognate 71 kDa protein - Homo sapiens (Human)	[P11142]	24, 27
Heat shock 70 kDa protein 14 - Homo sapiens (Human)	[Q0VDF9]	24
Heat shock protein HSP 90-beta- Homo sapiens (Human)	[P08238]	27
Heat shock protein beta-1 - Homo sapiens (Human)	[P04792]	24
Islet amyloid polypeptide precursor - Homo sapiens (Human)	[P10997]	24
Interferon gamma- Homo sapiens (Human)	[P01579]	35
Interleukin-1 alpha- Homo sapiens (Human)	[P01583]	35
Interleukin-10 precursor - Homo sapiens (Human)	[P22301]	24
Interleukin-12 subunit alpha precursor - Homo sapiens (Human)	[P29459]	24
Interleukin-12 subunit beta precursor - Homo sapiens (Human)	[P29460]	24
Interleukin-15 precursor - Homo sapiens (Human)	[P40933]	24
Interleukin-2 precursor - Homo sapiens (Human)	[P60568]	24
Interleukin-21 precursor - Homo sapiens (Human)	[Q9HBE4]	24
Interleukin-2 receptor alpha chain precursor - Homo sapiens (Human)	[P01589]	24
Interleukin-2 receptor subunit beta precursor - Homo sapiens (Human)	[P14784]	24
Interleukin-4 precursor - Homo sapiens (Human)	[P05112]	24
Interleukin-5 precursor - Homo sapiens (Human)	[P05113]	24
Interleukin-6 precursor - Homo sapiens (Human)	[P05231]	24, 37

Interleukin-8 precursor - Homo sapiens (Human)	[P10145]	24
Insulin precursor [Contains: Insulin B chain; Insulin A chain] - Homo sapiens (Human)	[P01308]	24
Keratin, type I cytoskeletal 18 - Homo sapiens (Human)	[P05783]	24
Keratin, type II cytoskeletal 8 - Homo sapiens (Human)	[P05787]	24
Laminin subunit alpha-1 precursor - Homo sapiens (Human)	[P25391]	24
Laminin subunit alpha-2 precursor - Homo sapiens (Human)	[P24043]	24
Laminin subunit alpha-3 precursor - Homo sapiens (Human)	[Q16787]	24
Laminin subunit alpha-4 precursor - Homo sapiens (Human)	[Q16363]	24
Laminin subunit alpha-5 precursor - Homo sapiens (Human)	[O15230]	24
Laminin subunit beta-1 precursor - Homo sapiens (Human)	[P07942]	24
Laminin subunit beta-2 precursor - Homo sapiens (Human)	[P55268]	24
Laminin subunit beta-3 precursor - Homo sapiens (Human)	[Q13751]	24
Laminin subunit gamma-1 precursor - Homo sapiens (Human)	[P11047]	24
Laminin subunit gamma-2 precursor - Homo sapiens (Human)	[Q13753]	24
Laminin subunit gamma-3 precursor - Homo sapiens (Human)	[Q9Y6N6]	24
Low-density lipoprotein receptor precursor - Homo sapiens (Human)	[P01130]	24
Lupus La protein- Homo sapiens (Human)	[P05455]	38
Galectin-1 - Homo sapiens (Human)	[P09382]	24
Galectin-3 - Homo sapiens (Human)	[P17931]	24
Malate dehydrogenase, mitochondrial- Homo sapiens (Human)	[P40926]	30
Melanoma-associated antigen E1 - Homo sapiens (Human)	[Q9HCI5]	24
Melanoma antigen recognized by T-cells 1 - Homo sapiens (Human)	[Q16655]	24
Pro-MCH-like protein 1 - Homo sapiens (Human)	[Q16048]	24
Pro-MCH-like protein 2 - Homo sapiens (Human)	[Q9BQD1]	24
Macrophage migration inhibitory factor - Homo sapiens (Human)	[P14174]	24
Interstitial collagenase precursor - Homo sapiens (Human)	[P03956]	24
72 kDa type IV collagenase precursor - Homo sapiens (Human)	[P08253]	24
Stromelysin-1 precursor - Homo sapiens (Human)	[P08254]	24
Matrix metalloproteinase-9 precursor - Homo sapiens (Human)	[P14780]	24
Myelin-associated oligodendrocyte basic protein - Homo sapiens (Human)	[Q13875]	24
Myelin-oligodendrocyte glycoprotein precursor - Homo sapiens (Human)	[Q16653]	24
Mucin-1 precursor - Homo sapiens (Human)	[P15941]	24
Myc proto-oncogene protein - Homo sapiens (Human)	[P01106]	24
Myoglobin - Homo sapiens (Human)	[P02144]	24, 39
Myelin proteolipid protein - Homo sapiens (Human)	[P60201]	24
Oxytocin-neurophysin 1 precursor - Homo sapiens (Human)	[P01178]	24
Neurotensin/neuromedin N precursor [Contains: Large neuromedin N - Homo sapiens (Human)	[P30990]	24
Neuropeptide Y precursor [Contains: Neuropeptide Y - Homo sapiens (Human)	[P01303]	24
Myeloperoxidase precursor - Homo sapiens (Human)	[P05164]	24, 40
Phosphatidylinositol-glycan-specific phospholipase D precursor - Homo sapiens (Human)	[P80108]	24
Phosphoglycerate mutase 1- Homo sapiens (Human)	[P18669]	27
Plasminogen precursor - Homo sapiens (Human)	[P00747]	24
Protein disulfide-isomerase/Prolyl 4-hydroxylase subunit beta- Homo sapiens (Human)	[P07237]	27
Protein disulfide-isomerase A5- Homo sapiens (Human)	[Q14554]	27
Protein disulfide-isomerase A3- Homo sapiens (Human)	[P30101]	27
Proteolipid protein 2 - Homo sapiens (Human)	[Q04941]	24
Prostatic acid phosphatase precursor - Homo sapiens (Human)	[P15309]	24
Protamine-2 - Homo sapiens (Human)	[P04554]	24, 41
Protamine-3 - Homo sapiens (Human)	[Q9NNZ6]	24
Parathyroid hormone-related protein precursor - Homo sapiens (Human)	[P12272]	24
Parathyroid hormone/parathyroid hormone-related peptide receptor precursor - Homo sapiens (Human)	[Q03431]	24
Parathyroid hormone precursor - Homo sapiens (Human)	[P01270]	24
Serpin H1 precursor - Homo sapiens (Human)	[P50454]	24
Serum amyloid A protein- Homo sapiens (Human)	[P02735]	42
Sialic acid-binding Ig-like lectin 8/Siglec 8- Homo sapiens (Human)	[Q9NYZ4]	43
Sialic acid-binding Ig-like lectin 9/Siglec 9- Homo sapiens (Human)	[Q9Y336]	31
Small nuclear ribonucleoprotein Sm D1- Homo sapiens (Human)	[P62314]	38
Small nuclear ribonucleoprotein Sm D2- Homo sapiens (Human)	[P62316]	38
Small nuclear ribonucleoprotein Sm D3- Homo sapiens (Human)	[P62318]	38
Somatoliberin precursor - Homo sapiens (Human)	[P01286]	24
Somatostatin precursor - Homo sapiens (Human)	[P61278]	24
60 kDa SS-A/Ro ribonucleoprotein- Homo sapiens (Human)	[P10155]	38
Superoxide dismutase [Cu-Zn] - Homo sapiens (Human)	[P00441]	24
Extracellular superoxide dismutase [Cu-Zn] precursor - Homo sapiens (Human)	[P08294]	24
Superoxide dismutase [Mn], mitochondrial precursor - Homo sapiens (Human)	[P04179]	24

Spectrin alpha chain, erythrocyte - Homo sapiens (Human)	[P02549]	24
Spectrin beta chain, brain 1 - Homo sapiens (Human)	[Q01082]	24
Alpha-synuclein - Homo sapiens (Human)	[P37840]	20, 24
Transforming growth factor beta-1 precursor - Homo sapiens (Human)	[P01137]	24
Transforming growth factor beta-2 precursor - Homo sapiens (Human)	[P61812]	24
Transforming growth factor beta-3 precursor - Homo sapiens (Human)	[P10600]	24
Protein-glutamine gamma-glutamyltransferase K - Homo sapiens (Human)	[P22735]	24
Protein-glutamine gamma-glutamyltransferase 2 - Homo sapiens (Human)	[P21980]	24
Protein-glutamine gamma-glutamyltransferase E precursor - Homo sapiens (Human)	[Q08188]	24
Protein-glutamine gamma-glutamyltransferase 6 - Homo sapiens (Human)	[O95932]	24
Protein-glutamine gamma-glutamyltransferase 4 - Homo sapiens (Human)	[P49221]	24
Protein-glutamine gamma-glutamyltransferase 5 - Homo sapiens (Human)	[O43548]	24
Protein-glutamine gamma-glutamyltransferase Z - Homo sapiens (Human)	[Q96PF1]	24
Prothrombin precursor - Homo sapiens (Human)	[P00734]	24
Talin-1- Homo sapiens (Human)	[Q9Y490]	44
Talin-2- Homo sapiens (Human)	[Q9Y4G6]	44
Thy-1 membrane glycoprotein- Homo sapiens (Human)	[P04216]	45
Thyroglobulin precursor - Homo sapiens (Human)	[P01266]	24
Protachykinin 1 precursor - Homo sapiens (Human)	[P20366]	24
Tubulin beta-4B chain/ Tubulin beta-2 chain- Homo sapiens (Human)	[P68371]	27
Tumor necrosis factor precursor - Homo sapiens (Human)	[P01375]	24
Tumor necrosis factor receptor superfamily member 6/Apo-1 antigen/FASLG receptor- Homo sapiens (Human)	[P25445]	46
Trafficking kinesin-binding protein 1- Homo sapiens (Human)	[Q9UPV9]	27
Tropomyosin alpha-1 chain - Homo sapiens (Human)	[P09493]	24
Tropomyosin beta chain - Homo sapiens (Human)	[P07951]	24
Tropomyosin alpha-3 chain - Homo sapiens (Human)	[P06753]	24
Tropomyosin alpha-4 chain - Homo sapiens (Human)	[P67936]	24
Serotransferrin precursor - Homo sapiens (Human)	[P02787]	24, 39
Lactotransferrin precursor - Homo sapiens (Human)	[P02788]	24
Ubiquitin - Homo sapiens (Human)	[P62988]	24
Vimentin- Homo sapiens (Human)	[P08670]	27
Vinculin	[P18206]	44
VIP peptides precursor [Contains: Intestinal peptide PHV-42; Intestinal peptide PHM-27 - Homo sapiens (Human)	[P01282]	24
Vitronectin precursor - Homo sapiens (Human)	[P04004]	24

ORIGINAL ARTICLE

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Apolipoprotein E gene polymorphisms as risk factors for carotid atherosclerosis

Polimorfizmi u genu za apolipoprotein E kao faktori rizika od ateroskleroze karotidnih arterija

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Abstract

Background/Aim. Atherosclerosis is still the leading cause of death in Western world. Development of atherosclerotic plaque involves accumulation of inflammatory cells, lipids, smooth muscle cells and extracellular matrix proteins in the intima of the vascular wall. Apolipoprotein E participates in the transport of exogenous cholesterol, endogenously synthesized lipids and triglycerides in the organism. Apolipoprotein E gene has been identified as one of the candidate genes for atherosclerosis. Previous studies in different populations have clearly implicated apolipoprotein E genetic variation (ε polymorphisms) as a major modulator of low density lipoprotein cholesterol levels. Data considering apolipoprotein E polymorphisms in relation to carotid atherosclerosis gave results that are not in full compliance. The aim of present study was to investigate the apolipoprotein E polymorphisms in association with carotid plaque presence, apolipoprotein E and lipid serum levels in patients with carotid atherosclerosis from Serbia. **Methods.** The study group enrolled 495 participants: 285 controls and 210 consecutive patients with carotid atherosclerosis who underwent carotid endarterectomy. Genotyping of apolipoprotein E polymorphisms were done using polymerase chain reaction and restriction fragment length polymorphism methods. **Results.** Patients had significantly decreased frequency of the ε2 allele compared to controls. Patients who carry at least one ε2 allele had a significantly higher level of serum apolipoprotein E and significantly lower low density lipoprotein cholesterol levels compared to those who do not carry this allele. **Conclusion.** Our results suggest protective effect of apolipoprotein E ε2 allele on susceptibility for carotid plaque presence as well as low density lipoprotein cholesterol lowering effect in Serbian patients with carotid atherosclerosis. Further research of multiple gene and environmental factors that contribute to the appearance and the progression of atherosclerosis should be continued with respect to different populations.

Key words:
genetic predisposition to disease; carotid artery disease; polymorphism, genetic; apolipoproteins e.

Apstrakt

Uvod/Cilj. Ateroskleroza je vodeći uzročnik smrtnosti u zapadnom svetu. Proces formiranja aterosklerotskog plaka podrazumeva akumulaciju ćelija inflamatornog odgovora, lipida, glatkih mišićnih ćelija i proteina vanćelijskog matriksa u intimi zida krvnog suda. Apolipoprotein E učestvuje u transportu egzogenog holesterola, endogeno sintetisanih lipida i triglicerida u organizmu. Gen koji kodira apolipoprotein E smatra se jednim od gena kandidata u izučavanju ateroskleroze. Rezultati prethodnih studija, urađenih na različitim populacijama, jasno ukazuju da su varijacije u genu za apolipoprotein E glavni modulatori nivoa lipoproteina male gustine u serumu. Do sada objavljeni rezultati koji se tiču povezanosti polimorfizama u genu za apolipoprotein E sa aterosklerozom karotidnih arterija su kontradiktorni. Zato je cilj ove studije bio da se ispita povezanost polimorfizama u genu za apolipoprotein E sa rizikom od nastanka aterosklerotskog plaka u karotidnim arterijama kao i sa nivoom proteina apolipoprotein E i lipida u serumu bolesnika sa aterosklerozom karotidnih arterija iz Srbije. **Metode.** Ovom studijom bilo je obuhvaćeno ukupno 495 ispitanika: 285 zdravih ispitanika u kontrolnoj grupi i 210 bolesnika sa aterosklerozom karotidnih arterija koji su bili upućeni na endarte-

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rektomiju. Genotipizacija polimorfizama u genu za apolipoprotein E je urađena metodama lančane reakcije polimeraze i analizom fragmenata dobijenih digestijom sa restrikcionim enzimima. **Rezultati.** Bolesnici su imali statistički značajno manju frekvenciju alela ε2 u odnosu na kontrolnu grupu. Ispitanici koji su bili nosioci genotipa sa bar jednom kopijom alela ε2 imali su statistički značajno viši nivo proteina apolipoprotein E i statistički značajno niži nivo lipoproteina male gustine u serumu u odnosu na bolesnike koji su bili nosioci genotipova bez prisustva alela ε2. **Zaključak.** Rezultati ove studije ukazuju na zaštitni efekat alela ε2 od nastanka aterosklerotskog plaka u karotidnim arterijama, kao i povezanost sa nižim nivoom lipoproteina male gustine u serumu bolesnika sa njihovom aterosklerozom. Neophodna su dalja istraživanja gena i faktora rizika iz spoljašnje sredine uključenih u nastanak ateroskleroze u različitim populacijama.

Ključne reči:
bolest, genetska, predispozicija; a.carotis, bolesti; polimorfizam, genetički; apolipoproteini e.

Introduction

As a multifactorial vascular disease, atherosclerosis is one of the most frequently occurring illnesses of the modern world. According to the theory given by Ross and Glomset [1], which is still among the most common, atherosclerosis is initiated by a blood vessel injury accompanied by inflammatory processes. Plaque, an accumulation of cells with different content (including lipids), forming on the inside of blood vessels in the area of injury can lead to a large lumen narrowing (stenosis) of the vessel [2]. The consequences of atherosclerotic lesions rupture may be heart attack or stroke, which eventually could lead to death.

Taking into account the causes and consequences associated with the formation of atherosclerotic plaques, effective prevention of this disease including genetic data and epidemiological studies could provide the only permanent solution to combat this disease. Many genes can be considered as candidates whose polymorphisms in interaction with certain risk factors such as: dyslipidemia, hypertension, obesity, stress, smoking, diabetes and physical inactivity, can lead to the development and/or progression of atherosclerosis [3–5].

Apolipoprotein E gene (APOE) has been identified as one of the candidate genes for atherosclerosis. Apolipoprotein E (apoE) is apolipoprotein which plays a major role in regulating the metabolism of chylomicrons, very low density lipoproteins (VLDL), and high density lipoproteins (HDL) *via* the apoE receptor and by the low density lipoprotein (LDL) receptors [6]. It is responsible, in part, for uptake of dietary cholesterol in the form of chylomicron remnants, clearence of VLDL remnants, and removal of the excess cholesterol from peripheral tissues through hepatic clearance of HDL containing apoE [6]. Thus, ApoE participates in the transport of exogenous cholesterol, endogenously synthesized lipids and triglycerides (TG) in the organism [7]. This protein has two domains: the C-terminal, which is associated with lipoprotein particles and the N-terminal, which binds to the lipoprotein receptor [8, 9].

Polymorphisms in the APOE analyzed in this study are located in a region that encodes the N-terminal domain of apoE protein. Depending on whether the Arg or Cys are present in two polymorphic positions (codons 112 and 158), the possible alleles are: ε2 (112 Cys, 158 Cys), ε3 (112 Cys, 158 Arg) and ε4 (112 Arg, 158 Arg) [10]. Previous studies in different populations have clearly implicated APOE genetic variation as a major modulator of LDL cholesterol levels [11–13]. The ε2 allele carriers have decreased, whereas ε4 carriers have increased the level of cholesterol compared to the ε3 allele carriers [14]. A meta analysis by Dallongeville et al. [15] indicated that subjects with the ε2 and ε4 alleles had higher triglyceride levels than subjects with the ε3 allele. Blood lipid level has been recognized as risk factor for carotid artery plaque formation, so variation at APOE locus could be a major determinant of atherosclerosis risk in the general population.

Previously published studies, which investigated APOE polymorphisms in relation to carotid atherosclerosis, gave results that are not in full compliance. The ε2 allele was associated with a reduced risk for carotid plaque presence, compared with the presence of allele ε3 [16]. In other study the same was suggested for the carriers of E4 allele [17].

Since the formation of a plaque *via* lipid accumulation on the inside of the vessel is an important factor for the initiation of atherosclerosis, and there is no clear conclusion about which of the APOE alleles or genotypes are associated with the carotid plaque (CP) presence the present study examined the APOE polymorphisms in association with CP presence, apoE and lipid serum levels in carotid atherosclerosis (CA) patients from Serbia who underwent carotid endarterectomy.

Methods

Subjects

The study group enrolled 495 participants: 285 controls and 210 consecutive patients with CA who underwent carotid endarterectomy. All the participants were Caucasians of European descendent from Serbia. From all of them medical history was collected including smoking and drinking habits, presence of diabetes, peripheral arterial occlusive disease, coronary artery disease and drug treatment. Exclusion criteria for all patients were carotid kinking, carotid aneurism, history of previous carotid endarterectomy (possible restenosis), tumors, autoimmune disease, chronic inflammatory diseases or renal failure. The patients already diagnosed with diabetes mellitus, having a fasting glucose $\geq$ 7.0 mmol/L, or taking insulin or hypoglycemic drugs were classified as having diabetes mellitus. Those with previous myocardial infraction or stable angina pectoris evaluated by selective coronography that confirmed coronary artery disease were classified as having coronary heart disease. Peripheral artery disease was diagnosed in those with an ankle–brachial index < 0.90. Hypertension was defined as systolic blood pressure

≥ 140 mmHg, a diastolic blood pressure ≥ 90 mmHg, or current treatment with antihypertensive drugs [18]. From the individuals undergoing annual medical check-up at Occupational Medical Center, Belgrade, Serbia, who underwent clinical and ECG examination, 285 that were without evidence of CA, cerebrovascular or cardiovascular disease, any chronic inflammatory disease, renal failure or diabetes mellitus were recruited as controls [19].

A total of 210 patients were recruited from individuals consecutively admitted for carotid endarterectomy to the Clinic for Vascular and Endovascular Surgery, Clinical Center of Serbia, Belgrade, Serbia, during 2008 with evidence of carotid plaque in the internal carotid artery (ICA) or common carotid artery (CCA). Ultrasound assessment of the bilateral carotid arteries was performed as previously described [18.]

Atherosclerotic plaques were defined as focal widening relative to adjacent segments as evidenced by protrusion into the lumen and/or localized roughness with increased echogenicity. For ultrasound carotid measurements, intraclass correlation coefficients for inter-rater and intra-rater reliability were 0.916 and 0.968, respectively. Carotid atherosclerosis was defined as the presence of atherosclerotic plaques in internal or common carotid artery [18].

All biochemical analyses were performed at the hospital laboratory by standard procedure.

The study was approved by Ethic Committee of the participating medical center with written informed consent given by each participant in this study.

Genetic analysis

Genomic DNA was isolated from the whole blood samples collected with ethylenediamine tetraacetic acid (EDTA) by standardized BloodPrep® DNA Chemistry isolation kit (Applied Biosystems, Forester City, CA) on the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Forester City, CA). Genotyping of APOE polymorphisms was done by polymerase chain reaction (PCR) using the following primers: forward 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3' and reverse 5'-ACAGAATTCGCCCCGGCCT GGTACAC-3', designed according to the previously described protocol [20]. PCR mixture component concentrations were as follows: 1X PCR buffer (10X), 1.5 mM $MgCl_2$, 1% DMSO, 0.2 mM dNTP, 0.45 mM of each primer, 0.25 U/μl Taq polymerase. Temperature conditions of the PCR reaction were as follows: 95 °C 7 min., and 33 cycles (95 °C 40s, 65 °C 30s, 72 °C 60s) without a final extension step. After PCR, the synthesized fragment was 244 bp in length. Restriction analysis of synthesized DNA fragments (RFLP) was carried out by a Hin6I-mediated digestion of amplified PCR fragments. The lengths of the fragments obtained by digestion with the enzyme Hin6I are as follows: E2: 36bp, 16bp, 91bp, 18bp, 83bp; E3: 36bp, 16bp, 91bp, 18bp, 48bp, 35bp; E4: 36bp, 16bp, 19bp, 72bp, 18bp, 48bp, 35bp. The digestion products were loaded on an 8% polyacrilamide gel for genotyping and run for 2 h in electric field of 12 V/cm. Gels were stained with silver nitrate and visualized using a GDS8000 gel documentation system (Ultra Violet Products Inc, Upland, USA).

Statistical analysis

The allelic frequencies and genotype distribution were estimated by gene counting method. Differences in allele frequencies and genotype distribution between the cases and controls as well as deviation from Hardy-Weinberg equilibrium were estimated by χ^2. Normal distribution of continuous variables was tested by Kolmogorov-Smirnov test with Lilliefors's correction. The influence of genotype on the variability of biochemical parameters was analyzed using ANOVA and appropriate *post-hoc* test or Kruskal Waliss ANOVA as a nonparametric test. The results are presented as mean ± standard deviation (SD). Statistical analysis was performed using Statistica Version 8, software package (StatSoft Inc, 2008). In all tests, differences with two-tailed alpha–probability $p < 0.05$ were considered significant.

Results

This study examined the association of APOE polymorphisms with the carotid atherosclerotic plaque presence. The study included 285 subjects in the control (healthy) population and 210 patients with CA. None of the genders was significantly prevalent in any of the groups (49.64% of women and 50.36% of men in the control population and 42.58% women and 57.42% men in the atherosclerotic population, $p > 0.05$). However, the group of patients was significantly older than the control population (age of the control population was 51.50 ± 9.30 years and of the patients' group it was 60.02 ± 10.08 years, $p < 0.01$). The relative frequencies of APOE genotypes did not deviate significantly from Hardy-Weinberg equilibrium and differences in their distribution between the studied groups were not statistically significant (Table 1).

Table 1

Relative frequencies of apolipoprotein E (APOE) genotypes in the controls and the group of patients with carotid atherosclerosis

Genotype	Control group	Patient group	*p*
	n (%)	n (%)	
E2/2	6 (2.11)	3 (1.43)	
E2/3	44 (15.44)	17 (8.10)	
E2/4	5 (1.75)	0 (0.00)	
E3/3	188 (65.96)	156 (74.29)	NS
E3/4	39 (13.68)	32 (15.24)	
E4/4	3 (1.05)	2 (0.95)	
Total	285 (100)	210 (100)	

***p* – Pearson χ^2 test; NS – not statistically significant.**

Allele frequency distribution was significantly different between the patients and the controls (Table 2). The ε2 allele had significantly decreased frequency in the patient group compared to the controls ($\chi^2 = 11.5$, df = 1, $p < 0.01$).

Table 2
Relative frequencies of apolipoprotein (APOE) alleles in the controls and the patients with carotid atherosclerosis

Alelle	Controls (%)	Patients (%)	*p**
ε2	0.11	0.05	
ε3	0.80	0.86	< 0.05
ε4	0.09	0.09	

***Pearson χ^2 test.**

We also measured concentrations of apoE in the serum of patients with carotid atherosclerosis. Concentrations of apoE, represented as mean ± SD, were not significantly different according to the APOE genotypes (Table 3).

Table 3
Mean serum concentration of apolipoprotein E (apoE) with the reference to apolipoprotein E (APOE) genotypes

Genotype	n	ApoE (mg/L)
		mean ± SD
E2/2	1	103.20 ± 0.00
E2/3	11	69.14 ± 36.37
E2/4	0	0.00 ± 0.00
E3/3	76	46.98 ± 11.54
E3/4	15	42.75 ± 10.75
E4/4	2	35.30 ± 15.13
Total	105	49.01 ± 18.01

Nevertheless, when we grouped APOE genotypes in those containing ε2 allele (E2/E2 + E2/E3), ε4 allele (E3/E4 + E4/E4) and homozygotes for the ε3 allele (E3/E3) (not including the genotype E2/4), Kruskal-Wallis ANOVA test showed a statistically significant difference ($p < 0.01$) in serum apoE levels among these groups of genotypes. The patients who carried at least one ε2 allele (E2/E2 + E2/E3) had a significantly higher level of serum apoE compared to those with E3/3 and E3/E4 + E4/4 genotypes (Table 4).

Table 4
Mean serum concentrations of apolipoprotein E (apoE) in the serum of patients by the grouped genotype

Genotype	n	ApoE (mg/L)	
		mean ± SD	*p**
E2/2 + E2/3	12	71.98 ± 36.05	< 0.01
E3/3	76	46.98 ± 11.54	
E3/E4 + E4/4	17	41.88 ± 11.03	
Total	105	49.01 ± 18.01	

***Kruskal-Wallis ANOVA.**

We also examined values of lipid parameters measured in the serum of patients with atherosclerosis according to the grouped apoE genotypes, excluding E2/E4 genotype (Table 5). ANOVA LSD *post-hoc* test revealed that the carriers of at least one ε2 allele had significantly lower LDL cholesterol levels compared to those who did not carry this allele. The concentrations of HDL cholesterol and triglyceride levels were not significantly different ($p > 0.05$) according to the grouped genotypes.

Discussion

This study investigated a possible association of APOE polymorphisms with the occurrence of carotid plaque as well as with apoE and lipid serum values in Serbian patients with CA. There were no statistically significant differences in the frequencies of APOE genotypes between the two groups, but there was a statistically significant decrease in the frequency of allele ε2 in the group of patients compared to the controls. This result indicates the protective effect of the ε2 allele for carotid plaque presence and is consistent with previous studies [16, 21]. Some studies have shown that the ε2 allele contributes to the reduced risk of atherosclerosis occurrence only if subjects are of normal weight and younger than 80 [22]. The presence of ε2 allele has also been shown to lead to the smallest intima media thickness [16, 23].

This study, however did not demonstrate compliance with some of the results from previous studies in respect to the ε4 allele. It was characterized as atherogenic in French and Thai population [21, 24], but not in the Caucasians from The Rotterdam study [16]. Concordantly with our results, they did not find any significant association of the ε4 allele with carotid plaques presence in a large sample of more than 4,000 Caucasians [16]. The majority of studies showed contradictory results regarding the impact of different APOE alleles on the development of atherosclerosis [16, 17, 21, 23–25]. Potential reasons for the conflicting results in these studies may be: a non-representative sample, analysis of different age groups of persons and/or different stages of the illness, different environmental conditions, and different ethnic origin. Frequency of the ε4 allele in the Serbian population was in concordance with the frequency obtained from Italian population and the study group of Caucasians investigated in The Rotterdam study [14, 16].

The obtained values of serum apoE concentrations indicate a declining trend in the direction: E2/2 > E2/3 > E3/3 > E3/4 > E4/4. After grouping of the genotypes, it was found

Table 5
Mean serum values of lipid parameters according to the grouped apolipoprotein E (APOE) genotypes

Genotype	n	HDLC (mmol/L)	LDLC (mmol/L)	TG (mmol/L)
		mean ± SD	mean ± SD	mean ± SD
E2/E2 + E2/E3	16	1.09 ± 0.37	3.09 ± 1.17*	2.08 ± 0.92
E3/E3	126	1.17 ± 0.35	3.79 ± 1.06	1.80 ± 0.78
E3/E4 + E4/E4	26	1.20 ± 0.32	3.88 ± 1.07	1.58 ± 0.68
Total	168	1.17 ± 0.35	3.74 ± 1.09	1.79 ± 0.78

***$p < 0.05$; [ANOVA, Fisher's Least Significant Difference (LSD)] *post hoc* test;**
HDLC- HDL cholesterol; LDLC- LDL cholesterol; TG – triglycerides.

that carriers of at least one ε2 allele had significantly increased, and the carriers of at least one ε4 allele significantly decreased apoE levels in serum, compared to the carriers of genotype E3/3. These results are consistent with previous studies [16, 26]. According to Weisgraber et al. [8], ApoE3 and ApoE4 isoforms have normal receptor-binding activity because arginine is at the position 158 in the protein, unlike ApoE2 isoforms, which at this position contains cysteine and binds to the receptor with reduced efficiency.

Among patients with CA, the carriers of at least one ε2 allele showed a significant reduction in LDL cholesterol levels compared to the carriers of any other genotype, while no significant differences in the level of HDL cholesterol was noted between genotypes. Previously published results for the random population from Serbia [27] have shown the same pattern. The results we got for the LDL cholesterol levels correspond to previous studies [16, 28, 29]. From the obtained results it can be concluded that the ε2 allele has atheroprotective function, but the exact mechanism of ApoE2 and ApoE4 isoforms' influences on LDL cholesterol remains unknown.

This study found no association of APOE genotypes and HDL cholesterol levels, which is consistent with the results of some previous studies [30] but differs from others [31]. HDL participates in reverse cholesterol transport from peripheral tissues to the liver, thereby performing the atheroprotective role. Also, our study showed no statistically significant association of APOE genotype with serum triglyceride levels. Analyzing the results from numerous studies there is no evidence of a consistent relationship between the APOE genotypes and triglyceride or HDL levels [32]. Thus, we can assume that the effect APOE genotypes could have on variability of HDL or triglycerides is rather small or population specific.

Conclusion

This study suggests a protective effect of APO ε2 allele on carotid plaque presence as well as LDL cholesterol lowering effect. A considerable number of contradictory results regarding the exact role of APOE polymorphisms in the development and progression of atherosclerosis are a consequence of the very nature of atherosclerosis as a disease. The development of atherosclerosis depends on a large number of both genetic (other apolipoprotein isoforms, polymorphisms in genes for proteins involved in inflammatory processes), and environmental factors (lifestyle, therapy, diet). In order to get the most reliable results on the genetic causes of atherosclerosis, it is certainly necessary to do analysis of multiple genes in a representative sample of healthy and diseased individuals and to estimate the risk accurately by adjusting for confounding factors. Also, it is possible that ApoE performs a dual function in apoE-related transport of lipids in the body and so the balance between processes that it performs determines its atherogenic or atheroprotective role in atherosclerosis. Further research of multiple gene and environmental factors that contribute to the appearance and the progression of atherosclerosis should be continued with respect to different populations.

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Chromosomal instability in patients with Fanconi anemia from Serbia

Hromozomska nestabilnost kod bolesnika sa Fankonijevom anemijom u Srbiji

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Abstract

Background/Aim. Fanconi anemia (FA) is a rare hereditary disease in a heterogeneous group of syndromes, so-called chromosome breakage disorders. Specific hypersensitivity of its cells to chemical agents, such as diepoxybutane (DEB), was used as a part of screening among patients with clinical suspicion of FA. The aim of this study was to determine chromosomal instability in patients with FA symptoms in Serbia. **Methods.** A total of 70 patients with phenotypic symptoms of FA, diagnosed at the Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić", Belgrade and University Children's Hospital, Belgrade from February 2004 to September 2011, were included in this study. Cytogenetic instability analysis was performed on untreated and DEB-treated 72 h-cultures of peripheral blood. **Results.** Ten patients in the group of 70 suspected of FA, showed increased DEB induced chromosome breakage and were classified into the FA group. The range of DEB induced aberrant cells percentages in the FA group was from 32% to 82%. DEB sensitivity of 58 tested patients were bellow FA values (range: 0–6%) (non-FA group), with no overlapping. The remaining two patients showed borderline sensitivity (borderline FA group – FA*), comparing to the healthy controls. **Conclusion.** This study revealed 10 patients with FA on the basis of cytogenetic analysis of DEB induced chromosome aberrations. Our results are in consistency with those from the literature. Early and precise diagnosis of FA is very important in further treatment of these patients, considering its cancer prone and lethal effects.

Key words:
fanconi anemia; diagnosis, differential; cytogenetics; chromosome aberrations; chromosome disorders.

Apstrakt

Uvod/Cilj. Fankonijeva anemija (FA) je retka nasledna bolest hromozomske nestabilnosti sa specifičnom hipersenzitivnošću ćelija na dejstvo DNK-unakrsno-vezujućih agenasa kao što je diepoksibutan (DEB) i mitomicin C. Cilj rada bio je da se utvrdi hromozomska nestabilnost kod bolesnika sa simptomima FA u Srbiji. **Metode.** Hromozomska senzitivnost na DEB ispitivana je kod 70 bolesnika klinički suspektnih na FA, koji su dijagnostikovani u Institutu za zdravstvenu zaštitu majke i deteta Srbije „Dr Vukan Čupić", Beograd i Univerzitetskoj dečijoj klinici, Beograd u periodu 2004–2011. godine. Analiza je sprovedena na netretiranim i DEB-om tretiranim 72-časovnim kulturama limfocita periferne krvi bolesnika i zdravih osoba. **Rezultati.** Kod 10 bolesnika je uočen povećan broj DEB-om indukovanih hromozomskih prekida (FA grupa), kako u odnosu na ostale pacijente (ne-FA grupa), tako i u odnosu na zdrave kontrole. Procenat aberantnih ćelija u FA grupi kretao se u rasponu 32–82%, dok je raspon u grupi od 58 ne-FA bolesnika iznosio 0–6%, a u kontrolnoj grupi 0–8%. Preostala dva bolesnika pokazala su granični odgovor na DEB (FA* grupa) u poređenju sa zdravim kontrolama. **Zaključak.** Na osnovu citogenetske analize DEB-om indukovane hromozomske nestabilnosti u limfocitima periferne krvi bolesnika suspektnih na FA, dijagnoza bolesti je postavljena kod njih 10. Postavljanje rane i precizne dijagnoze FA je od velikog značaja za dalje lečenje ovih bolesnika, s obzirom na to da se radi o veoma teškom oboljenju sa letalnim ishodom.

Ključne reči:
anemija, fankoni; dijagnoza, diferencijalna; citogenetika; hromosomi, aberacije; hromosomi, anomalije.

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Introduction

Chromosomal instability diseases are a heterogeneous group of inherited diseases which is determined with the growth and development disorders, defects of the immune system and bone marrow function, as well as increased predisposition to cancer [1, 2]. These conditions occur as a result of mutations in genes involved in the process of repairing and maintaining genome stability. Beside Fanconi anemia (FA) and Nijmegen Brekage syndrome (NBS), this group includes Ataxia-telangiectasia, Bloom syndrome, Xeroderma pigmentosum, Cockayne syndrome, Werner syndrome, Trichothiodystrophia, Rothmund-Thompson syndrome, and immunodeficiency syndrome with centromeric heterochromatin instability (ICF) [1]. The common characteristic of these diseases is the increased spontaneous chromosome breakage in the cells of affected patients, as a result of mutations in genes responsible for DNA repair [1]. However, the percentage of spontaneous chromosomal aberrations is very variable and nonspecific. For the purpose of accurate differential diagnosis, the induction of chromosomal aberrations with the number of specific mutagenic agents such as mitomycin C, mephalan or diepoxybutane (DEB) for FA or bleomycin for NBS, etc., is used to increase the baseline sensitivity [2, 3].

There are no available data in the literature about the frequency of affected with chromosome instability syndromes in Serbia. However, the most of the childhood patients referred for the genetic testing to the Laboratory for Medical Genetics Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić" in Belgrade had clinical signs of two such disease: NBS and FA. FA is a rare autosomal recessive and X-linked polygenic disease characterized by pancytopenia, aplastic anemia (AA) with progressive bone marrow failure, short stature, developmental abnormalities, increased susceptibility to cancer development and cellular hypersensitivity to DNA cross-linking agents such as DEB [4, 5].

The aim of this study was to determine chromosomal instability in FA patients using DEB sensitivity test in a large group of patients from Serbia, with clinical suspicion of FA.

Methods

From February 2004 to September 2011, 70 children with FA symptoms, such as pancytopenia, AA, congenital anomalies and other, were treated at the Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić" and University Children's Hospital in Belgrade. They all underwent DEB test in the laboratory for medical genetics, in order to establish the differential diagnosis of FA.

Chromosome fragility tests on blood samples from the patients with clinical suspicion of FA and controls (healthy family members) were performed as described by Auerbach [3], with minor modification [2]. Two cultures from each patient and a healthy individual (control) were treated with DEB in the final concentration of 0.1 μg/mL for the last 24 h, and the remaining two cultures were left for the evaluation of spontaneous chromosome fragility [2]. After 70 hours of cultivation, colcemid (2.5 μg/mL) was added and cytogenetic analysis was performed according to standard protocol [2]. A total of 100 metaphases from each culture were analyzed, using G banding, for the presence of chromosome/chromatid breaks and other aberrations [6]. Chromatid and chromosome breaks, and acentric fragments were counted as one break, while dicentrics, ring chromosomes and radial structures are counted as two breaks [2]. The parameters of chromosomal instability evaluation were: the percentage of aberrant cells, the number of breaks per cell and the number of breaks per aberrant cell [2, 3].

Statistical analysis

Chi-square test was used to determine a level of differences between two groups of aberrant cells of each patient and its control counterpart [2].

The obtained values of cytogenetic sensitivity to DEB divided patients into two main subgroups: FA and non-FA patients. Statistical analysis of differences between these groups included Mann-Whitney test, which revealed that there were no overlapping ranges of values among them, and with the third one – the subgroup of patients with borderline sensitivity.

Results

Clinical features of patients with suspecion of FA treated at the Mother and Child Health Care Institure of Serbia "Dr Vukan Čupić and University Childrens Hospital, Belgrade, from February 2004 to Septembre 2011, are shown in Table 1.

In the sample of 70 children, 10 patients with a positive response to DEB and a significant difference comparing individually to their control counterparts (Chi-square test: $p < 0.05$), were classified into the FA group. No significant level of chromosomal response was found in 58 patients and they were classified as non-FA group. The remaining of two patients revealed chromosomal breakage values between FA

Table 1

Clinical features of 70 patients with suspicion of Fanconi anemia

Clinical features	Patients n (%)
Haematologic abnormalities (aplastic anemia, pancytopenia, trombocytopenia, myelodisplasy, etc)	6.7 (95.71%)
Physical abnormalities (congenital anomalies, short stature, etc)	4 (5.71%)
Malignacy (tumor, ALL, lymphoma, etc)	6 (8.57%)

ALL – acute lymphoblastic leukemia.

and non-FA ranges (FA* group). The results of spontaneous and DEB-induced chromosomal instability analysis for all the groups of patients, including the control group, are presented in Table 2.

The percentage of DEB-induced aberrant cells in the FA group, ranged from 32% to 82%, and the mean value was 52.52%, which is about 40 times more than in the control group (range 0–8% ; mean: 1.30%), and about 32 times

Table 2

Spontaneous and diepoxybutane (DEB) chromosome breakage findings in Fanconi anemia (FA), borderline-FA (FA*), non-FA and control groups

Disease (n)	Chromosome instability ($\bar{x} \pm SD$, x_{min}-x_{max})					
	Break/cell		Aberrant cells (%)		Breaks/aberrant cell	
	S	DEB	S	DEB	S	DEB
FA (10)	0.14 ± 0.13	1.47 ± 1.16	10.50 ± 9.17	52.52 ± 18.50^{+}	1.11 ± 0.43	2.58 ± 0.17
	0.00–0.39	0.48–4.39	0.00–29.00	32.00–82.00	0.00–1.50	1.20–5.35
FA* (2)	0.06 ± 0.01	0.23 ± 0.04	6.00 ± 1.41	17.00 ± 7.07	1.07 ± 0.14	1.43 ± 0.35
	0.06–0.07	0.20–0.26	5.00–7.00	12.00–22.00	1.00–1.20	1.18–1.67
Non-FA (58)	0.01 ± 0.01	0.02 ± 0.02	0.81 ± 1.22	1.60 ± 1.50	0.55 ± 0.61	0.86 ± 0.51
	0.00–0.06	0.00–0.08	0.00–5.00	0.00–6.00	0.00–2.00	0.00–2.67
Control	0.00 ± 0.01	0.02 ± 0.02	0.34 ± 0.64	1.30 ± 1.54	0.24 ± 0.44	0.69 ± 0.76
(healthy) (97)	0.00–0.03	0.00–0.17	0.00–3.00	0.00–8.00	0.00–1.50	0.00–3.40

n – number of respondents; S – spontaneous chromosome instability; DEB – DEB-induced chromosome instability; $\bar{x}$ – mean value; SD – standard deviation. Notes: (+) Mann-Whitney test revealed a statistically significant difference between the groups FA and non-FA ($p < 0.001$).

The main criteria for the determination of chromosome fragility were as follows: the percentage of aberrant cells, the number of breaks per cell and the number of breaks per aberrant cell. Ten patients (14.3%) revealed an increased number of induced chromosome and chromatid breaks, and other chromosome aberrations (Figures 1 and 2).

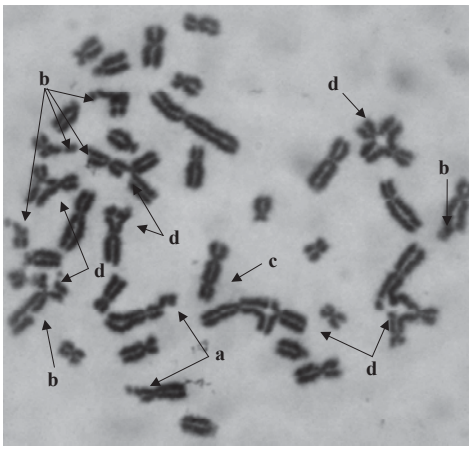

Fig. 1 – Diepoxybutane induced chromosome aberrations in patient with Fanconi anemia: a – chromosome break, b – chromatid break, c – dicentric, d – chromatid exchanges.

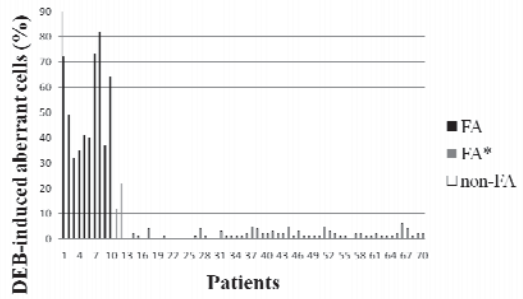

Fig. 2 – Diepoxybutanol – induced chromosomal breakage in Fanconi anemia (FA), borderline FA* and non-FA groups of patients.

higher than in non-FA group (range 0–6%; mean: 1.60%). The patient No. 8 reached a maximum percentage (82%) of cells with aberrations induced by DEB, while patient No. 3 had the minimum value of 32% for the same parameter (Table 3).

The mean value of DEB-induced breaks per cell in the FA group (mean: 1.47 breaks/cell, range of values: 0.48 to 4.39 break/cell) was 73 times higher than the mean value in the non-FA group (mean: 0.02 break/cell, range of value 0.00–0.08 break/cell). patient no. 8 reached the maximum number of break/cell (4.39) and the minimum value was found in the patient no. 6 (0.48 breaks/cell) (Table 3). Statistical analysis showed a significant difference between the FA and non-FA groups (Mann-Whitney test: $p < 0.001$) with ranges of values that were not overlapped (Table 2).

However, two patients, according to DEB test, were classified in the FA* group showing borderline sensitivity for DEB-induced percentage of aberrant cells (range: 12–22%; mean 17%), about 10 times higher than in the non-FA group and three times lower than in FA group, as well as for break/cell findings (range: 0.20–0.26 breaks/cell; mean: 0.23 breaks/cell) (Table 2).

Spontaneous chromosomal instability values (percentage of aberrant cells and the number of breaks/cell) for 10 FA patients are partially overlapped with the values in the groups non-FA and FA-borderline (Table 2). Nevertheless, the mean percentage of spontaneously aberrant cells in the FA group was 12 times higher (mean 10%; range 0–29%) comparing to those from the non-FA group (mean: 0.81%; range 0–6%) (Table 2). Baseline chromosomal instability of the borderline FA* patients was also in the ranges of values for other groups of examinees (non-FA group ranges: 0.06–0.07 breaks/cell and 5–7% of aberrant cells vs. control group ranges: 0.00–0.03 breaks/cell and 0–3% of aberrant cells) (Table 2).

Table 3

Spontaneous and diepoxybutane induced chromosome instability in 10 patients with Fanconi anemia (FA)

N° of FA patient	Chromosome instability					
	Break/cell (n)		Aberrant cells (%)		Breaks/aberrant cell (n)	
	S	DEB	S	DEB	S	DEB
1.	0.01	2.15	1.00	72.22	1.00	2.97
2.	0.07	1.50	5.00	49.00	1.40	3.06
3.	0.00	0.95	0.00	32.00	0.00	2.97
4.	0.08	0.68	8.00	35.00	1.00	1.94
5.	0.27	0.58	18.00	41.00	1.50	1.41
6.	0.12	0.48	9.00	40.00	1.33	1.20
7.	0.18	1.75	15.00	73.00	1.20	2.40
8.	0.39	4.39	29.00	82.00	1.34	5.35
9.	0.03	0.91	3.00	37.00	1.00	2.46
10.	0.22	1.31	17.00	64.00	1.29	2.05
Total ($\bar{x}$ ± SD)	0.14 ± 0.13	1.47 ± 1.16	10.50 ± 9.17	52.52 ± 18.50	1.11 ± 1.16	2.58 ± 1.17

n – number; S – spontaneous chromosome instability; DEB – DEB-induced chromosome instability; $\bar{x}$ – mean value; SD – standard deviation.

Discussion

FA is a rare autosomal recessive disease that occurs with a frequency of about 2.5 : 100,000, depending on the population [7]. This disease is both clinically and genetically heterogeneous [8–11]. FA diagnosis based on clinical indications is difficult because of variations in phenotypic expression, which significantly reduces the possibility of distinguishing FA from other clinically similar disorders (patients with AA and other signs of bone marrow failure) [5, 12]. However, FA patients are characterized clinically from other patients in specific hypersensitive response to DNA cross-linking agents such as DEB or MMC, which results in the presence of a large number of chromosome and chromatid breaks in the cells of these patients. This characteristic of FA cells is now widely used as a differential diagnostic test tool in the screening of FA patients.

In this paper we presented the results of DEB test as a screening method for FA patients in the group of patients from Serbia with clinical suspicion of FA. The frequency of FA in the group of children with AA and other signs of bone marrow failure from Serbia was lower (14.3%) as compared to the published ones (25–30%), which could be explained with the fact that not all such patients were referred to DEB test [4].

In this study, parameter values of baseline chromosomal instability (% aberrant cells and breaks/cell) in the FA group overlapped with the corresponding values in the non-FA group (Table 2), meaning that these two groups could not be distinguished on the basis of spontaneous chromosomal breaks. Our results are consistent with the same results of a study published in the International Registry of Fanconi anemia (Fanconi's Anemia International Registry – IFAR) [13] as well as in similar works [12, 14].

Based on hypersensitive response to DEB, the mean values of chromosomal breakage parameters were much higher in the FA than in the non-FA group (32 times higher percentage of aberrant cells and 73 times higher breaks/cell), which confirmed the ability of DEB test to differ FA affected from other patients with similar symptoms, and corresponded to previously published studies [12, 14].

It should be noted that the number of DEB-induced aberrant cells in the group of 10 FA patients varied much ranging from 32% to 82%. Also, there were some difficulties in classifying the rest of two patients with borderline sensitivity to DEB, and possible somatic mosaicism pointing to FA phenotype. These variations and deviations are probably caused by the presence of patients with the mosaic form of FA in our group of patients in which the values of induced aberrant cells are generally lower (< 60%) compared to non-mosaic forms [12, 14–17]. Specifically, somatic mosaicism is a phenomenon of FA mutations reversion in certain hematopoietic cells, so that in the blood sample of the same patient can be found two clones of cells: FA clone and clone with a normal (insensitive to DEB) cells with reverted mutation [17–19]. According to previously published studies, FA patients with values of aberrant cells < 40% are classified as mosaic FA, while those with values of aberrant cells ranging from 40% to 60% are considered potential mosaics; FA patients with aberrant cells values ≥ 60% are considered complete, non-mosaic type of FA [20]. Our data indicated that FA patients with values: aberrant cells < 50%, may be considered as a form of mosaic FA, while those with non-mosaic form of FA had values greater than > 60%. For the remaining six patients with FA values of aberrant cells between 32% and 49%, further evaluation in order to confirm/exclude mosaicism is required. The presence of borderline FA patients and the variation of DEB-induced aberrations in non-FA patients (0–6%) can also be the consequence of a limiting sensitivity of patients with mosaic form of FA to DEB. Based on these results and the fact that there are some non-FA patients with increased sensitivity (up to 16%) to DEB and mitomycin C, which cannot be explained, identifying of mosaic FA forms is more complicated and could be done with less certainty [20].

The main characteristic of FA cellular phenotype is high sensitivity to DNA intercalating agents, so that in the case of mosaicism it is difficult to make accurate diagnosis of FA. The study of Castella et al. [20] proposed a new index of chromosome fragility that includes both % of aberrant cells and breaks/multiaberrant cell, and allows unambiguous diag-

nostic differentiation of mosaic and non-mosaic patients with FA, as well mosaic forms of FA, compared to non-FA patients. This approach could be applied to our group of patients in order to more accurately distinguish these groups of affected patients.

Early and precise diagnosis of this disease is very important for further treatment of the patients and, also, for providing accurate information, concerning genetic counseling of families with affected members [21].

Conclusion

The diepoxybutane test proved to be very effective and relatively simple diagnostic tool in the process of screening among Fanconi anemia patients with aplastic anemia and other symptoms of bone marrow failure.

Molecular testing and identification of complementation groups of Fanconi anemia for each Fanconi anemia patient sensitive to diepoxybutane and further clarification of borderline sensitivity, examining the cellular functionality of other tissue are perhaps the next steps in establishing the final diagnosis of Fanconi anemia.

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Development and initial validation of a scale to measure attitudes and beliefs of pharmacists toward their work with patients

Razvoj i inicijalna validacija skale za ispitivanje opštih stavova i uverenja farmaceuta o sopstvenom radu sa pacijentima

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Abstract

Background/Aim. Studies on physicians and other health care professionals indicate that attitudes towards and beliefs in their work with patients, can affect the quality of health care, and patients' behaviour and compliance, thus an instrument is needed to survey pharmacists as healthcare providers. The aim of this study was to describe the development and psychometric validation of a survey instrument to assess attitudes and beliefs of pharmacists toward their work with patients (Pharmacists' Attitudes and Beliefs Scale, PABS). The aim of this research was to determine the reliability, validity and factor structure of a newly constructed instrument – PABS. **Methods.** The statements from the cognitive, affective, and behavioral areas were identified by literature review and selected to cover the behavior of pharmacists in providing pharmaceutical care at community settings. The initial 5-point Likert type scale of 30 items was constructed and after initial validation its revised form developed. The reliability, construct validity and factor structure of the scale were established. **Results.** The reliability of the scale was determined by the method of internal consistency, on a convenient sample of 123 community pharmacists. The Cronbach's alpha coefficient was 0.67. Factor analysis of principal components was performed and 7 factors with latent roots greater than 1 were extracted, explaining 64.92% of total variance, a single 30.84%, 8.20%, 6.55%, 5.63%, 5.01%, 4.68% and 4.01%. Based on the results of factor analysis in the development of the scale, some items in the scale were excluded (totally 7), so that the revised form of the PABS contained a total of 23 items. **Conclusion.** The initial PABS scale did not meet theoretical statistical criteria for reliability (Cronbach's alpha coefficient was < 0.7), but the findings indicated its potentially acceptable construct validity. The results support its use as a research tool to assess the behavior of pharmacists in daily practice, and provide its use as an indicator of quality in delivering pharmaceutical care.

Key words:
pharmacists; patients; serbia; questionnaires; sensitivity and specificity; patient satisfaction.

Apstrakt

Uvod/Cilj. Istraživanja na populaciji lekara i drugih zdravstvenih radnika pokazuju da stavovi i uverenja koja imaju o svom radu sa pacijentima, mogu uticati na kvalitet pružene zdravstvene zaštite, te se stoga nameće potreba da se ovo istraži i kod farmaceuta. Cilj ove studije bio je da se razvije skala za ispitivanje opštih stavova i uverenja farmaceuta o sopstvenom radu sa pacijentima (SOSUF) i da se ispitaju metrijske karakteristike ovog instrumenta. **Metode.** Izdvojene su tvrdnje iz kognitivne, afektivne i bihevioralne oblasti kojima je obuhvaćeno ponašanje farmaceuta prilikom pružanja farmaceutske zdravstvene zaštite u javnoj apoteci. Razvijena je inicijalna skala (SOSUF-i) kao 5-ostepena skala Likertovog tipa od 30 tvrdnji. Izvršena je validacija inicijalnog instrumenta utvrđivanjem pouzdanosti, validnosti i faktorske strukture skale i predložena nova razvijena verzija skale SOSUF. **Rezultati.** Na uzorku od 123 farmaceuta zaposlena u javnim apotekama sprovedena je validacija SOSUF-a. Pouzdanost je određena primenom metode unutrašnje konzistencije, Kronbah-ov koeficijent alfa iznosio je 0,67. Izvršena je faktorska analiza glavnih komponenti i dobijeno je sedam faktora sa latentnim korenima većim od 1, koji objašnjavaju 64,92% ukupne varijanse, a pojedinačno 30,84%, 8,20%, 6,55%, 5,63%, 5,01%, 4,68% i 4,01%. Na osnovu rezultata faktorske analize, a u sklopu razvoja skale, neke tvrdnje u skali izuzete su (ukupno 7), tako da razvijena verzija SOSUF-a sada sadrži ukupno 23 tvrdnje. **Zaključak.** Inicijalna skala SOSUF-i ne zadovoljava teorijske statističke kriterijume pouzdanosti (Kronbach-ov alfa koeficijent < 0,7), ali na osnovu rezultata može se pretpostaviti da efektivno meri stavove i uverenja farmaceuta o sopstvenom radu sa pacijentima. Rezultati merenja ukazuju na ponašanje farmaceuta u svakodnevnoj praksi kojim utiču na ponašanje pacijenata, i mogli bi se koristiti kao pokazatelj kvaliteta rada u pružanju farmaceutske zdravstvene zaštite na primarnom nivou.

Ključne reči:
farmaceuti; bolesnici; srbija; upitnici; osetljivost i specifičnost; bolesnik, zadovoljstvo.

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Introduction

Effective pharmaceutical care about patients sequires a high level of knowledge, communication skills for delivery and self-observation. When pharmacists interact with patients they should consider of any patients' reactions during the assessment process, when talking to the patients as well as counseling or reviewing patients' medication and clinical records [1–4]. For all health care professionals the delivery of health care is focused on the patients' therapeutic needs and should be supplemented by the behavior assessment process and quality assessment process. The behavior assessment process involves health professionals' assessment of patients' behavior, as well as their own behavior and attitudes in prevention, health promotion, improvement of pharmacotherapeutic measures and procedures in the rational use of medicines and certain types of medical devices [2]. Studies with health care professionals indicate that attitudes and beliefs they have about their work with patients, can affect the quality of health care which may result with better clinical/social/economic outcomes for health care consumers [5–13]. However, there is evidence that interaction with patients could result with problems that may put patients at risk produced by inadequate professional behavior [14–16]. To our knowledge, this has been very little explored in community pharmacy practice, and some published results with health care students [5] and professionals [5, 6, 8] show the necessity to construct specific instruments to assess attitudes and beliefs in each health care practice.

Development of scales measuring attitudes

Attitude is an important concept that is often used to understand and predict people's reaction to an object or change and how behavior can be influenced [17, 18]. It is a mental and neural state of readiness, organized through experience, exerting a directive or dynamic influence upon the individual's response to all objects and situations it relates to.

Three generally accepted components of attitude are: cognitive component (knowledge, belief, opinion, information that anyone has about the subject of observation); affective component (like or dislike, expectations) and action component (expectation of future conduct) [17, 18].

Attitudes can be measured toward self or others, and it is easier to measure attitude than behavior [19]. Attitude scales attempt to determine individuals' believes, perceives or feels [20]. An attitude scale is a direct technique that consists of a series of affirmative or negative evaluative statements about the object position, in the form of claims. A total measure of a scale, that is the intensity of the paragraph, is a composite of responses to individual statements [18].

There are several types of scales that have been developed to measure attitudes and beliefs: generic ones including important forms of behavior in one area; specific ones including certain forms of behavior, which means that they are highly sensitive and specific for detecting characteristics and comparison of certain types of behaviors of different social or professional groups [21]; discriminatory which determine differences among subjects regarding certain forms of behavior; predictive that classify individuals in a particular category in relation to certain attitudes and behaviors [22, 23]. The Likert-type scale is the most widely used instrument for measuring attitude and it falls within the ordinal level of measurements. Categories range from completely negative attitudes, through neutral, to completely positive attitudes (agreements) in each individual item (usually 3 or 5 categories). Responses to all items are added and a total score is formed as a composite indicator that measures properties (summated scale) [21]. The most commonly reported psychometric properties of the scale as an instrument are reliability and validity which are the minimum requirements to be completed [24–28].

Although several scales [29, 30] have been developed to measure attitudes and beliefs of pharmacists and other healthcare workers towards specific groups of patients or specific subjects, less attention has been paid to the development of measures of their general attitudes and beliefs in everyday healthcare practice. To our knowledge, no scale exists currently to assess general attitudes and beliefs with regards to pharmacists own work as a whole. Furthermore, given the potential for negative attitudes, measures are needed to capture negative beliefs as well as professional behavior. To date, relatively little is known about the impact of behavior on the health care system, including how it may influence pharmaceutical care and health care. The development of a scale measuring attitudes and beliefs will facilitate studies investigating health care outcomes, patient reported outcomes and quality of health care provided, including the contribution of this type of research to behavioral aspects of delivering health care and pharmaceutical care in Serbia.

The overall objective of the research was to assess the attitudes and beliefs of pharmacists about their own work with patients in community pharmacies in Serbia. Specific research objectives were: construction of a new specific instrument for assessment of attitudes and beliefs of pharmacists towards their work as an of attitudes scale (PABS) and examination the PABS' psychometric properties *ie* reliability, construct validity and factor structure.

Official permission was to develop and test the instrument given from the Pharmaceutical Chamber of Serbia and all the pharmacists who participated were given a full explanation of the study and were garanteed anonymity. No financial compensation was given to any of the participants. The Ethics Committeee for Clinical Research of the University of Belgrade Faculty of Pharmacy approved the study as well.

Methods

This study was a part of an ongoing exploratory research project on social and behavioral insights into pharmacy practice under community settings in Serbia, which started in March 2010. This article reported the first stage process of that project (March 2010 – December 2011) documenting the development and initial validation of a new instrument (PABS) designed to assess attitudes and beliefs of pharmacists with regards to their own work with patients in everyday community practice in Serbia. The research was divided into two phases: scale development and initial vali-

dation. The development process began by reviewing the literature to generate items which refer to design, development and standardization of the scale for the assessment and monitoring of health workers' attitudes towards their own work with patients. Afterwards, the process of making the scale was conducted through the 3 groups of activities: defining criteria for scale structure and selection of appropriate measurement scale; determination of adequate sample of items within each of the content areas of specified domains and creation of the initial items pool; technical design of scale and way of its administration by the participants. The scale was multidimensional with each dimension representing a specific aspect of pharmacist's personal interaction with patients. For ease of construction and acceptable reliability each item of the summated (Likert) rating scale was used to represent each dimension. Item format was that traditionally used to measure attitudes and beliefs, constructed as statements of opinion with multiple response options to an agree/disagree continuum. Several points were considered under the construction process of the PABS: to cover a wide range of face to face interactions between pharmacists and patients within pharmacy service within primary health care; to make it suitable for self-administration and short in order not to be easy to answer; the items should be constructed so to increase the accuracy of responses (*ie*, to describe a specific conduct or attitude, rather than categories of events); to recognize individual differences in the perception of attitudes and beliefs of pharmacists through the inclusion of subjective reactions to the instrument; to avoid the position of arbitrator in determining the reality of events [31, 32].

In designing PABS' items the following criteria were taken into account: items should be formulated in terms most commonly used by respondents – pharmacists in primary health care system (pretesting was done); items should be derived from everyday situations and events from practice; sufficient level of items should be maintained in order to minimize subjectivity in response. Items should contain personal *vs* general referent, that is, they would focus on personal experience rather than on experience of people in general. For example, the item: "I believe that patients need to follow my instruction for usage of drug" was used instead of: "I believe that patients need to follow the instruction of pharmacist for usage of drug".

All claims were formulated as beliefs in certain aspects of pharmacist's own work with patients, with no terms that refer to emotional states. For each of the 30 items (affirmative or negative evaluative statements) respondents gave answers using the 5-degree Likert-type scale, ranging from "I do not agree at all" (1) to "I agree completely" (5). The survey instrument was pretested by 7 experienced pharmacy practice members to ensure that all the questions were understandable and then revised based on their comments. Those respondents did not participate in the study further on. A convenient sample of 250 pharmacists was included in the initial investigation of the psychometric properties of the instrument. The reliability of the scale was obtained by internal consistency and expressed with Cronbach's alpha coefficient. Internal consistency reliability defines the consistency of the results delivered in a test, ensuring that the various items measuring the different constructs deliver consistent scores. This type of reliability is obtained by a single usage application of the measuring instrument (PABS). Although in this case there is no data on temporal stability of the scale (PABS), there is data on homogenity and meaning of the internal consistency is probably the closest to the basic idea of reliability [32].

To determine the number and type of factors that underlie the scale items, principal component analysis and factor analysis was conducted.

Data collection was performed from October to December 2011. Respondents were asked to express their own views and to indicate in the scale the degrees to which they personally agreed or disagreed to the items. A total score for the scale was obtained by summing individual responses to the items so that the results could range from 30 to 150, with a higher score meaning a greater perceived advantage in working with patients. Sociodemographic questions were included in the PABS for collecting the information about age, gender, experience, location of work in terms of Pharmaceutical Chamber of Serbia Branch (only registered members of the Pharmaceutical Chamber of Serbia).

Retrieved and useable survey instruments were coded and the data were entered into a database. Statistical analysis was performed using the SPSS program (SPSS 18.0 for Windows, Inc., Chicago, IL, USA).

Results

The survey achieved a response rate of 49.2% (123/250). Of 123 pharmacists who completely filled questionnaire, the majority, 107 (87%), were females, at the beginning of their professional career, 6–10 years of professional practice (47.2%). Nearly half of the respondents were in big cities, 65 (52.8%), and almost equally in small towns, 58 (47.2%). Descriptive characteristics of the sample are presented in Table 1.

Table 1

Demographic characteristics of the study participants

Parameters	n (%)
Gender	
male	16 (13.0)
female	107 (87.0)
total	123 (100.0)
Age (years)	
> 30	22 (17.9)
31–40	58 (47.2)
41–50	26 (21.1)
51–60	14 (11.4)
< 60	3 (2.4)
Years of pharmacy service	
> 5	27 (22.0)
6–10	53 (43.1)
11–20	32 (26.0)
< 20	11 (8.9)
Total	123 (100.0)

After applying the PABS (the initial version given in Appendix 1), the reliability was determined by the method of internal consistency, Cronbach's alpha coefficient was 0.67.

To determine the number and type of factors that underlie the scale items, the factor analysis was conducted using principal components analysis. The validity of the scale items was determined by an overall score derived from the initial scale.

The matrix of variables intercorrelations was first analyzed using the principal components. Based on the number of latent roots (eigenvalue) which is grater than 1, it was determined that it can be explained by 7 factors with latent roots greater than 1, explaining respectively 30.84%, 8.20%, 6.55%, 5.63%, 5.01%, 4.68% and 4.01% of total variance, as shown in Table 2. The components from 8 to 30 explain less than 3% of the total variance.

To achieve a simple structure in which each variable should be as saturated as possible with a single factor, these 7 factors were then rotated for one of the methods of orthogonal rotation of factors, so called varimax rotation proposed by Kaiser [33]. Table 3 summarises the results of varimax rotation of the first seven factors. For each factor, high loadings (correlations) resulted in a few variables; the rest was near zero. Each factor has a small number of large loadings and a large number of zero (or small) loadings [34].

The results showed that the first factor (pharmacists' interaction with patients) consisted of the following items: education, anxious patients, reliance, motivation, demanding patients, lack of understanding. These items had the highest loading (saturation) of the factor.

Concerning the interpretation of factors, some items could also be of interest: errors, praise for the help, discontinuation of therapy.

Table 2

The total variance explained by principal component analysis

	Component initial eigenvalues of the scale			Extraction sums of squared loadings			Rotation sums of squared loadings		
	Total (%)	Variance	Cumulative (%)	Total (%)	Variance	Cumulative (%)	Total (%)	Variance	Cumulative (%)
1	9.253	30.843	30.843	9.253	30.843	30.843	4.010	13.366	13.366
2	2.459	8.196	39.039	2.459	8.196	39.039	3.193	10.644	24.010
3	1.966	6.552	45.591	1.966	6.552	45.591	2.872	9.574	33.585
4	1.688	5.626	51.217	1.688	5.626	51.217	2.818	9.394	42.979
5	1.503	5.010	56.226	1.503	5.010	56.226	2.545	8.485	51.464
6	1.403	4.678	60.905	1.403	4.678	60.905	2.306	7.686	59.149
7	1.204	4.013	64.918	1.204	4.013	64.918	1.731	5.768	64.918

Extraction method: Principal component analysis.

Table 3

Varimax solution for 7 principal components factors

Items in the scale	Featured factors						
	factor 1	factor 2	factor 3	factor 4	factor 5	factor 6	factor 7
Devoting time	0.010	0.366	0.432	0.394	-0.052	-0.128	-0.368
Courtesy	0.021	0.266	0.661	0.386	-0.150	-0.077	-0.289
Attentiveness	0.197	0.354	0.421	0.582	-0.012	-0.090	-0.106
Love for work	0.162	0.139	0.087	0.796	-0.219	-0.055	0.078
Information	0.315	0.180	0.101	0.614	-0.074	-0.329	0.122
Critique of patients	-0.164	-0.109	0.079	-0.378	0.361	0.474	0.196
Lack of understanding	-0.553	-0.187	-0.256	0.200	0.399	0.212	0.035
Praise	0.189	0.676	-0.005	0.114	-0.022	0.294	-0.197
Advice	0.076	0.776	0.146	0.151	0.114	-0.101	-0.026
Explanation	0.170	0.644	0.102	0.167	-0.197	-0.113	0.294
Instructions	0.288	0.512	0.355	0.137	-0.262	-0.176	0.320
Demanding patients	-0.580	-0.088	-0.098	-0.109	0.355	0.189	0.108
Understanding of patients	-0.020	-0.298	-0.157	-0.059	0.724	0.079	-0.013
Lack of understanding of drug	-0.363	0.009	-0.206	-0.168	0.563	0.320	0.064
Cooperation	0.384	0.277	0.652	-0.040	-0.010	-0.076	0.020
Discontinuation of therapy	-0.404	-0.438	0.100	-0.241	0.214	0.134	0.267
Satisfaction with service	0.257	0.487	0.327	0.051	-0.134	-0.087	0.316
Aggressive patients	-0.314	0.200	0.213	-0.185	0.684	-0.101	0.101
Anxious patients	-0.696	-0.055	0.052	-0.051	0.036	-0.129	0.103
Respect	0.014	0.130	-0.027	0.031	0.205	-0.034	0.750
Praise for the help	0.428	0.347	0.085	0.338	-0.036	-0.166	0.183
Motivation	0.614	0.268	0.460	0.168	0.079	-0.131	0.168
Reliance	0.674	0.178	0.276	0.327	-0.166	-0.206	0.088
Education	0.785	0.158	0.170	0.207	-0.078	0.026	0.074
Valuable time	0.156	0.181	-0.207	-0.409	0.329	-0.211	-0.546
Conflicts	0.020	-0.010	-0.008	-0.171	-0.077	0.799	-0.026
Non-compliance with advice	-0.195	-0.064	-0.214	-0.019	0.330	0.765	-0.007
Misunderstandings	0.083	-0.010	-0.461	-0.085	0.493	0.406	0.073
Compliance with the instructions	-0.151	0.044	-0.704	-0.121	0.069	-0.004	-0.188
Errors	0.456	0.282	0.189	0.494	0.053	-0.173	0.163
Scare of	30.843	8.196	6.552	5.626	5.010	4.678	4.013

The second factor (patient advised by pharmacists) consisted of the following items: advice, praise, explanation and instructions, and items that could also be of interest were: satisfaction with service and discontinuation of therapy.

The highest saturation of the third factor (kind and polite behavior) had the following items: compliance with the instructions, courtesy and cooperation, and of some importance may be the motivation.

The highest loading of the fourth factor (love/no love for the work) included the items: love for work, information, attentiveness, and of some importance may be the errors and valuable time.

The highest saturation of the fifth factor (understanding of patients) included the items: understanding of patients, aggressive patients and lack of understanding of drug and of a substantial nature may be misunderstandings.

The greatest saturation of the sixth factor (conflicts and misunderstandings with patients) was with the items: conflicts and non compliance with the advice and substantial nature may be critique of patients and misunderstandings.

The highest saturation of the seventh factor (pharmacists respect for their patients) had the variables: respect and valuable time. There were many correlations among extracted factors (Table 4).

Table 4

Intercorrelations of the extracted factors *,†

Extraction factors	1	2	3	4	5	6	7
1	0.541	0.440	0.409	0.413	-0.306	-0.283	0.046
2	0.027	0.512	0.142	0.026	0.657	0.433	0.313
3	-0.739	0.020	0.472	0.254	0.056	-0.362	0.178
4	-0.214	0.447	-0.050	0.008	0.087	-0.008	-0.863
5	-0.119	0.000	-0.587	0.794	0.019	0.071	0.072
6	0.159	0.101	-0.339	-0.177	0.464	-0.772	0.085
7	0.273	-0.577	0.356	0.321	0.500	0.014	-0.335

***Extraction method: Principal component analysis.**
†Rotation method: Varimax with Kaiser normalization.

Based on the results of factor analysis in the development of the scale, some claims were excluded in the scale (total 7), so that the final revised version of PABS contained a total of 23 claims. Items that were excluded from the initial version of the PABS are: 1) I'm not mistaken in working with patients; 2) Patients criticize me about working with them; 3) I have noticed that patients discontinue the therapy they had been prescribed; 4) The patients showed satisfaction with the service received from the pharmacist at the pharmacy; 5) I get compliments from patients about the received treatment; 6) In the process of interaction and patient misunderstandings arise related to the drug; 7) Patients spend a lot of time in work.

Discussion

To the best of our knowledge, this study is the first one to assess pharmacists' general attitudes and beliefs towards their work with patents using the self-completion scale constructed for pharmacists. There are, however, a great number of scales which measure pharmacists and other health care professionals' attitudes and beliefs towards patients, concordance and pharmaceutical care [10–12, 16, 35–39]. Our intention was to describe the process of development of the new instrument, whose potentional usefulness will be further tested and reported elsewere.

Reliability is one of the basic metric characteristics of testing or measuring instruments in general, and refers to the accuracy of measurements regardless of what is measured [27, 39–41]. When testing the reliability by using Cronbach's alpha coefficient, one should consider the statistical criteria of satisfactory and acceptable level of reliability. Reliability coefficient should be statistically significant at the 0.01 level. The statistical definition of the reliability coefficient indicates that a measurement error increases its value if it departs from the value 1.00 and *vice versa*. The coefficient of internal consistency is obtained on the basis of the intercorrelation of the items and it is interpreted as the coefficient of reliability. The size of this coefficient depends on the number of items and their correlation. It is a generally accepted standard that instruments (questionnaires, scales or tests) having Cronbach's alpha coefficient greater than 0.9 are considered very highly reliable, those with Cronbach's alpha coefficient above 0.8 are considered highly reliable, and above 0.7 have satisfactory reliability [35, 42, 43]. Since Cronbach's alpha coefficient was 0.67, we can say that the PABS did not meet the criteria for statistical reliability [35, 44].

The results of some studies on validation tools indicated unsatisfactory reliability of instruments whose Cronbach's alpha coefficient was < 0.7. These instruments were used either as an additional tool for the evaluation of phenomena, or as a part of the battery with the other scales [45–48].

The PABS was multidimensional scale with each dimension representing a specific aspect of pharmacist's personal interaction with patients, and built from items that were causal indicators. Therefore, it is unlikely that a high homogeneity could be achieved, because the content of items was different, and to different extent contribute to comprehensiveness of the phenomenon that is measured.

The scale contained several items which had low saturation factors, which reduced the average correlation between the items. Removing these items from the scale, was expected to increase Cronbah alpha coefficient for the developed version of the PABS.

Factor analysis of the results allows us to identify a small number of latent variables or factors that explain a set of correlations within existing group of manifest variables, which is one way to determine the construct validity of the scale (factor validity). That is equal to the proportion of the

factors that participate in the variance of the test results, that is equal to the saturation factor of the test individual or the individual psychological latent variable [35, 49].

We presented the significant variance between the factors (intercorrelations between the factors). This confirms the view that assertive, calm and polite behavior in dealing with patients improves and increases the patients' motivation, compliance and adherence. If pharmacists feel that patients do not take their precious time, they would adequately advise them so that patients would respect the pharmacists' information and advice and would probably not interrupt the ongoing therapy. If pharmacists love their job, it is more likely that working with patients will not create an impression that patients "take precious time", having more understanding for patients. Thus, fewer patients would be perceived as aggressive and pharmacists would not enter into conflict with them.

The findings of our research were similar to other studies conducted among health care workers. Scales designed to measure attitudes of health professionals according to different phenomena, in order to achieve adequate health care have shown adequate validity and reliability [50, 51].

A systematic review of 32 articles published from 1980 by 2008 dealing with validity and reliability of epidemiological questionnaires for measuring psychosocial and organizational factors at healthcare working practice among nurses, red to a conclusion that most questionnaires have good psychometric properties, but data are lacking on the predictive validity of these instruments [52, 53].

A study on creation of scales to measure attitudes of people in primary health care to dementia, showed satisfactory validity (Cronbach's alpha coefficient 0.83), and pointed to the possibility of using these instruments in study on attitudes of health professionals [47, 54]. The new developed psychometric scale to assess moral development and ethics for pharmacists in Australia [55] showed satisfactory validity according to the Cronbach's alpha coefficient of 0.75. Testing the level of job satisfaction on a sample of 1,600 physicians in Norway carried out by the Likert-type scale, showed a satisfactory reliability [56]. Factor analysis of an instrument to measure job satisfaction of health workers in providing health care, conducted in the USA on a sample of 328 respondents, identified three factors (reliability amounted to 0.74) [57].

Several limitations together with suggestions for future studies should also be noted. Due to a relatively small sample the research results might not be generalized to the entire population of pharmacists in primary care. For this purpose it is recommended to conduct research on a larger sample.

Because attitudes and beliefs are not always a steady state but sometimes are changeable psychological traits, retest was not performed in this research. So test-retest reliability remains unknown for this scale. It is suggested that test-retest reliability test be assessed in future studies to prove robustness of the scale (we suggest relatively short interval of no more than two weeks). However, the main purpose of the study was to develop a scale for further testing and this goal was achieved. Further study on a larges sample is suggested to confirm the robustness and to improve this instrument. Additionally, we consider that the limitations of the study, do not question the usefulness of this new instrument. The current version of the scale may at least be used as a prototype for further development of a similar scale to be used for other health care professionals, as well.

Conclusion

The findings of our study demonstrate the reliability and validity of the PABS, supporting its use as a research tool and to identify the factors associated with pharmacists, which could serve as potential predictors for assessing the quality of services provided by pharmacists when evaluating primary level health care services. Further research with a finally revised version of PABS (23-items scale) is needed concerning internal validity and reliability. Additionally, this instrument could be developed on a larger and heterogeneous sample of pharmacists.

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Appendix 1

Scale to measure pharmacists' attitudes and beliefs toward their work with patients (PABS)

Gender:

a) male

b) female

What is your age?

a) to 30

b) from 31 to 40

c) from 41 to 50

d) from 51 to 60

e) over 60

Your professional experience at pharmacy service is up to:

a) to 5 years

b) from 6 to 10 years

c) from 11 to 20 years

d) over 20 years

Which Branch of the Pharmaceutical Chamber of Serbia you belong to? _____

Dear fellow pharmacist,

The Instrument (Scale) in front of you is a part of a Research project on Social and Behavioral Insights of Pharmacy Practice in Community Settings in Serbia.

Your responses will be kept confidential and the findings will only be reported as group data in publications from the study. Your name will never be matched to your answers. The Instrument (Scale) takes 15 minutes to complete. Please respond to each item and do not skip any of the items. What is important in answering to this instrument is that you openly express your own views in term of agreement at the 5 point scale by circling the number offered: 1- not at all disagree, 2-mostly disagree, 3-disagree, 4-mostly agree, 5- I completely agree.

	do not agree at all	mostly disagree	disagree	mostly agree	I agree completely
I devote a lot of time in working with patients.	1	2	3	4	5
I am kind with patients.	1	2	3	4	5
While trying to be forthcoming in working with patients, they do not know how to appreciate it.	1	2	3	4	5
Although I love my job, I often find my work with patients very embarrassing.	1	2	3	4	5
Information provided to patients are very important for therapy.	1	2	3	4	5
Patients criticize me about working with them.	1	2	3	4	5
Patients do not understand what I say.	1	2	3	4	5
Every day I get compliments from patients related to my work with them.	1	2	3	4	5
Every day I offer an advice to patients.	1	2	3	4	5
When issuing a medicine I always provide instructions to patients on the drug therapy administration.	1	2	3	4	5
Patients understand my instructions regarding the routes of drug therapy administration.	1	2	3	4	5
Patients may be embarrassing.	1	2	3	4	5
I think I'm less understandable for patients.	1	2	3	4	5
Patients do not understand what I refer to regarding their drug application.	1	2	3	4	5
Patients co-operate with me regarding the treatment they were prescribed.	1	2	3	4	5
I have noticed that patients discontinue the therapy they were prescribed.	1	2	3	4	5
Patients are satisfied with service received from the pharmacist stuff.	1	2	3	4	5
I think that patients are more and more aggressive.	1	2	3	4	5
Patients are often impatient.	1	2	3	4	5
Patients refer unrespectfully to me.	1	2	3	4	5
I get compliments from patients about the treatment received.	1	2	3	4	5
I think that my ways of interaction with patients may affect their motivation.	1	2	3	4	5
Patients are increasingly relying on pharmacists regarding drug use.	1	2	3	4	5
Patients are interested to be well educated regarding medicines they use.	1	2	3	4	5
Patients take my precious time that I could use in a better way.	1	2	3	4	5
I'm daily engaged in conflicts with patients.	1	2	3	4	5
I think that patients do not want to listen to the advice I gave them.	1	2	3	4	5
In the process of interaction with patient misunderstandings arise related to drug use.	1	2	3	4	5
I believe that patients need to follow my drug instructions.	1	2	3	4	5
I'm not mistaken in working with patients.	1	2	3	4	5

Thank you for taking part in this Study!

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The impact of anabolic androgenic steroids abuse and type of training on left ventricular remodeling and function in competitive athletes

Uticaj zloupotrebe androgenih anaboličkih steroida i tipa treninga na remodelovanje i funkciju leve komore kod elitnih sportista

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Abstract

Background/Aim. Long-term intensive training is associated with distinctive cardiac adaptations which are known as athlete's heart. The aim of this study was to determine whether the use of anabolic androgenic steroids (AAS) could affect echocardiographic parameters of left ventricular (LV) morphology and function in elite strength and endurance athletes. **Methods.** A total of 20 elite strength athletes (10 AAS users and 10 non-users) were compared to 12 steroid-free endurance athletes. All the subjects underwent comprehensive standard echocardiography and tissue Doppler imaging. **Results.** After being indexed for body surface area, both left atrium (LA) and LV end-diastolic diameter (LVEDD) were significantly higher in the endurance than strength athletes, regardless of AAS use ($p < 0.05$, for both). A significant correlation was found between LA diameter and LVEDD in the steroid-free endurance athletes, showing that 75% of LA size variability depends on variability of LVEDD ($p < 0.001$). No significant differences in ejection fraction and cardiac output were observed among the groups, although mildly reduced LV ejection fraction was seen only in the AAS users. The AAS-using strength athletes had higher A-peak velocity when compared to steroid-free athletes, regardless of training type ($p < 0.05$ for both). Both AAS-using and AAS-free strength athletes had lower e' peak velocity and higher E/e' ratio than endurance athletes ($p < 0.05$, for all). **Conclusions.** There is no evidence that LV ejection fraction in elite athletes is altered by either type of training or AAS misuse. Long-term endurance training is associated with preferable effects on LV diastolic function compared to strength training, particularly when the latter is combined with AAS abuse.

Key words:
athletes; substance-related disorders; androgens; ventricular remodeling; risk assessment; echocardiography.

Apstrakt

Uvod/Cilj. Dugotrajni intenzivni trening povezan je sa adaptivnim promenama srčanog mišića poznatim kao sportsko srce. Cilj rada bio je da se utvrdi uticaj primene anaboličkih androgenih steroida (AAS) na ehokardiografske parametre morfologije i funkcije leve komore (LV) kod elitnih sportista koji se bave sportovima snage i izdržljivosti. **Metode.** Dvadeset elitnih sportista snage (10 korisnika AAS i 10 onih koji ne koriste AAS) upoređeni su sa 12 sportista izdržljivosti koji ne koriste AAS. Svi ispitanici bili su podvrgnuti standardnom ehokardiografskom pregledu sa tkivnim Dopler-om. **Rezultati.** Nakon indeksiranja prema telesnoj površini, leva pretkomora (LA) i end-dijastolni prečnik leve komore (LVEDD) bili su značajno veći kod sportista izdržljivosti nego kod sportista snage, bez obzira na uzimanje AAS ($p < 0,05$, za oba). Nađena je značajna korelacija između veličine LA i LVEDD kod sportista izdržljivosti koji ne uzimaju AAS, koja pokazuje da 75% varijablnosti veličine LA zavisi od varijabilnosti LVEDD ($p < 0,001$). Nije pokazana značajna razlika u ejekcionoj frakciji (EF) LV (LVEF) i minutnom volumenu između grupa, mada je blago snižena LVEF viđena samo kod sportista koji koriste AAS. Sportisti snage koji koriste AAS imali su veću vrednost pika A-talasa u poređenju sa sportistima koji ne koriste AAS, bez obzira na tip treninga ($p < 0,05$ za oba). Sportisti snage, bez obzira na primenu AAS, imali su niže vrednosti brzine e' talasa i veći E/e' odnos u poređenju sa sportistima izdržljivosti ($p < 0,05$ za sve). **Zaključak.** Nema dokaza da je primena AAS povezana sa promenom LVEF, bez obzira na tip treninga. Dugoročni trening izdržljivosti povezan je sa povoljnim efektima na dijastolnu funkciju LV u poređenju sa treningom snage, pogotovu ako je trening snage povezan sa zloupotrebom AAS.

Ključne reči:
sportisti; zloupotreba supstanci; androgeni; srce, remodelovanje; rizik, procena; ehokardiografija.

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Introduction

Long-term intensive training is associated with distinctive cardiac adaptations which are known as athlete's heart [1]. Although a certain relationship between the type of training (endurance *versus* strength exercise) and cardiac remodeling has been documented, the nature and magnitude of training-induced changes are still the subject of debate.

Anabolic androgenic steroids (AAS) have been abused by both professional and recreational athletes to increase muscle mass and improve performance [2]. The use of AAS is particularly prevalent among powerlifters and bodybuilders – as many as 55% of elite powerlifters admitted using these agents [3, 4]. In contrast to numerous documented toxic and hormonal effects of AAS, their impact on left ventricular (LV) structure and function was not been yet completely understood. In animal model, AAS have been shown to induce cardiac renin-angiotensin system, increase cardiac collagen content and impair the beneficial effects of training [5]. In competitive athletes, self-administration of AAS has been linked to serious cardiac adverse events, including sudden cardiac death [6, 7], although reports on their impact on cardiac morphology and function varied [8].

We hypothesized that there would be the differences in echocardiographic parameters of LV morphology and function between strength and endurance athletes and that the magnitude of these differences would be affected by AAS abuse. To test this hypothesis, we compared elite strength athletes using or not using AAS to steroid-free endurance athletes.

Methods

A total of 22 elite male athletes, aged 22–40 years, were recruited from the national power-lifting, bodybuilding, wrestling and running clubs. All the subjects gave written informed consent and were divided into three groups.

The group I consisted of 10 strength athletes (6 powerlifters and 4 bodybuilders) who reported both past and current self-administration of AAS. All the subjects used the combination of oral and injectable substances (methandienone, stanozolol, nandrolone decanoate and testosterone) for at least 3 years, in cycles lasting between 7 and 14 weeks. The group II consisted of 10 strength athletes (4 bodybuilders and 6 wrestlers) who denied taking AAS. They were all negative on several doping tests during and out of competition. The group III consisted of 12 endurance athletes (long-distance runners) who did not use AAS. They were also negative on doping tests performed during professional career. None of the subjects in either group had a history of cardiovascular or any other organic system disorder and were not taking any medications.

Anthropometric measurements

Body mass and height were measured using a balance beam scale and a height gauge, respectively. Lean body mass was calculated according to the formula provided by Hallynck et al. [9], whereas body surface area was calculated using the Mosteller formula [10].

Electrocardiography and blood pressure measurement

Twelve-channel electrocardiography (ECG) recording was done prior to blood pressure measurement and echocardiographic examination.

Blood pressure measurements were done in sitting position, using a cuff adjusted to upper arm circumference. The mean value of two measurements on both arms, 10 min apart, was recorded.

Echocardiographic examination

All examinations were done in supine left decubitus position using a Hewlett–Packard Sonos 2500 machine (Andover, MA, USA), with a 2.5 MHz transducer. Echocardiograms consisted of two-dimensional, M-mode, Doppler flow measurements and tissue Doppler imaging (TDI) from standard parasternal and apical positions. All measurements were made by a single experienced observer (VD) who was blinded to the subjects' data.

M-mode measurements were performed for the assessment of LV diastolic and systolic diameters, according to the most recent guidelines [11] and presented both as raw data and adjusted for body surface area (BSA) when appropriate. Measurements obtained with this method served for calculation of LV mass, using the Devereux et al. formula [12]. Relative wall thickness (RWT) was calculated when the sum of interventricular septal wall (IVS) thickness and posterior wall (PW) thickness was divided by LV end-diastolic diameter (LVEDD).

For the assessment of systolic function LV volumes were measured by tracing the endocardial border in apical four- and two-chamber view. The ejection fraction was estimated using the Simpson's biplanar method [11]. Cardiac output was determined by calculating the product of stroke volume and heart rate that was obtained from the final loop of each study.

Pulsed-Doppler LV inflow recordings were made in the apical four-chamber view, with the sample volume placed at the tips level of the mitral valve. Early (E) and atrial (A) peak velocities, E-wave deceleration time and isovolumetric relaxation time were measured. TDI recordings were performed in apical four-chamber view, with the pulse-wave Doppler sample volume placed at the septal and lateral side of mitral annulus. Longitudinal tissue Doppler velocities of a systolic wave (S) and 2 diastolic waves – early (e') and atrial (a') – were reported as the mean of 3 consecutive cardiac cycles. Most recent guidelines on the chamber quantification and the assessment of LV diastolic function were used to define a reference range for all echocardiographic parameters [11, 13].

Statistical analysis

Data are expressed as mean ± standard deviation. Comparison between the groups was performed using the analysis of variance or a Kruskal–Wallis test, with Bonferroni correction for multiple comparisons. The relations between selected measures were calculated by the linear regression analysis and correlation analysis using the Pearson or Spearman's method. A p-value of < 0.05 was considered significant.

Results

The athletes from the 3 groups were comparable for age, body mass, BSA, lean body mass and duration of training (Table1).

After being indexed for BSA, both left atrium (LA) dimension and LVEDD were higher in the endurance than AAS-using strength athletes. Further, a significant correlation between LA diameter and LVEDD was found, but only in the endurance athletes, in whom more than 75% of LA

Table 1

Mean clinical characteristics of the study participants

Characteristics	Athletes type		
	AAS-using strength athletes (n = 10), x̄ ± SD	AAS-free strength athletes (n = 10), x̄ ± SD	AAS-free endurance athletes (n = 12), x̄ ± SD
Age (yrs)	27 ± 6	29 ± 6	27 ± 4
Height (cm)	181 ± 5	179 ± 4	190 ± 11*†
Body mass (kg)	100 ± 19	85 ± 18	87 ± 18
Body surface area (m^2)	2.2 ± 0.2	2.1 ± 0.2	2.1 ± 0.3
Lean body mass (kg)	69 ± 8	64 ± 8	69 ± 12
Heart rate (beats/min)	77 ± 16	68 ± 15	56 ± 8*
PR interval (ms)	150 ± 22	161 ± 25	174 ± 19*
Systolic BP (mmHg)	133 ± 23	128 ± 15	118 ± 12
Diastolic BP (mmHg)	88 ± 14	80 ± 11	71 ± 8*
Duration of training (yrs)	10.1 ± 3.0	13.4 ± 4.2	13.6 ± 2.8
Intensity of training (hrs/week)	11.6 ± 3.0	10.1 ± 3.4	20.8 ± 15.7†

AAS – anabolic androgenic steroids; BP – blood pressure;
***significantly different from AAS-using strength athletes ($p < 0.05$);**
†significantly different from AAS-free strength athletes ($p < 0.05$).

Resting heart rate and diastolic blood pressure were significantly lower in the endurance than the AAS-using strength athletes, with no significant difference between ASS-free athletes.

Standard echocardiographic parameters

The standard echocardiographic parameters are shown in Table 2. No significant differences in wall thickness were found among the 3 groups.

size variability ($R^2 = 0.761$) depended on variability of LVEDD (Figure 1A). It was also shown for this group that each increase of 1 mm in LVEDD was associated with approximately 0.7 mm increase in LA diameter (95% confidence interval (CI) 0.44 to 1.01, $p < 0.001$). A trend towards a significant correlation between LVEDD and LA diameter was noted in the AAS-free strength athletes (Figure 1B), while such correlation was not observed in the AAS-using strength athletes (Figure 1C).

Table 2

M-mode and two-dimensional echocardiographic measurements in the study participants

Parameter	Athletes type		
	AAS-using strength athletes (n = 10), x̄ ± SD	AAS-free strength athletes (n = 10), x̄ ± SD	AAS-free endurance athletes (n = 12), x̄ ± SD
LVEDD (mm)	49.9 ± 4.8	52.2 ± 3.2	57.0 ± 3.9*†
LVEDD per unit BSA (mm/m^2)	22.5 ± 3.0	25.7 ± 2.3	27.0 ± 3.3*
LA (mm)	34.2 ± 5.2	34.9 ± 1.9	39.3 ± 4.56*
LA per unit BSA (mm/m^2)	15.3 ± 2.3	17.2 ± 1.9	18.6 ± 2.7*
LV mass (g)	194 ± 44	180 ± 43	239 ± 58*†
LV mass per unit BSA (g/m^2)	87 ± 16	88 ± 21	114 ± 33*
IVS thickness (mm)	10.7 ± 2.2	9.7 ± 1.2	10.7 ± 1.7
PW thickness (mm)	10.3 ± 2.1	8.9 ± 1.3	9.7 ± 1.4
Relative wall thickness	0.41 ± 0.12	0.35 ± 0.04	0.36 ± 0.06

AAS – anabolic androgenic steroids; BSA – body surface area; IVS – interventricular septum; LA – left atrium; LV – left ventricle; LVEDD – left ventricular end-diastolic diameter; PW – posterior wall; *significantly different from AAS-using strength athletes ($p < 0.05$); †significantly different from AAS-free strength athletes ($p < 0.05$).

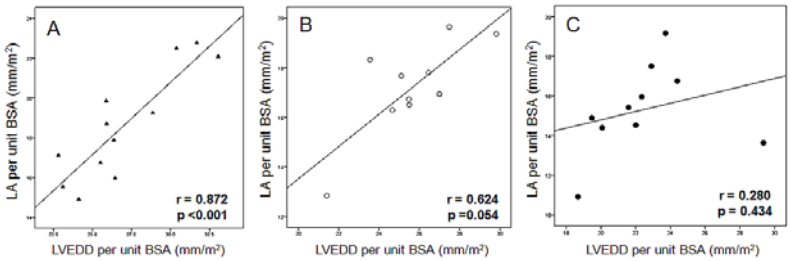

Fig. 1 – Correlation between the left ventricular end-diastolic diameter (LVEDD) and left atrial size (LA), both indexed for body surface area (BSA).

Left ventricular systolic function

No significant differences in ejection fraction, cardiac output and cardiac index were observed among the groups (Table 3). However, 3 of the 10 AAS users had LV ejection fraction below 55%, while all AAS-free athletes had normal LV ejection fraction (≥ 55%). Peak systolic velocity (S) at septal level was significantly higher in the endurance than AAS-free strength athletes.

and higher E/e' ratio than the endurance athletes, when measurements were done at lateral wall level (Table 3). Peak e' velocities at lateral wall level were within reference range in all endurance athletes, while in 30% of AAS-using strength athletes laid outside the normal range.

The 95% confidence itervals (CI) for the peak lateral e' velocity in the endurance steroid-free and steroid-using strength afthletes, compared to the reference range for different age groups are shown in Figure 2.

Table 3

Echocardiographic data on the left ventricular systolic and diastolic function

Parameter	Athletes type		
	AAS-using strength athletes (n = 10), $\bar{x}$ ± SD	AAS-free strength athletes (n = 10), $\bar{x}$ ± SD	AAS-free endurance athletes (n = 12), $\bar{x}$ ± SD
Ejection fraction (%)	57 ± 5	59 ± 4	59 ± 4
Stroke volume (mL)	107 ± 24	112 ± 15	129 ± 17*
Cardiac output (L/min)	8.1 ± 1.7	7.6 ± 1.7	7.2 ± 1.3
Cardiac index (L/min/m^2)	3.6 ± 0.9	3.4 ± 0.7	3.4 ± 0.4
Transmitral Doppler			
peak E velocity (cm/s)	84 ± 9	72 ± 15	74 ± 12
peak A velocity (cm/s)	52 ± 10$^{\Delta\square}$	40 ± 10	39 ± 12
peak E/A ratio	1.7 ± 0.4	1.9 ± 0.7	2.1 ± 0.5
e wave DT (ms)	218.1 ± 36.3	233.4 ± 35.1	244.3 ± 32.2
IVRT (ms)	80 ± 8	78 ± 9	78 ± 6
TDI – septal mitral annulus			
s peak (cm/s)	8.3 ± 1.3	7.9 ± 0.64	9.3 ± 1.3†
e' peak (cm/s)	12.1 ± 2.6	12.4 ± 2.7	13.4 ± 2.4
a' peak (cm/s)	8.8 ± 1.4	8.6 ± 2.0	8.4 ± 1.9
E/a' ratio	9.8 ± 1.9	8.9 ± 2.8	9.2 ± 2.5
E/e' ratio	7.2 ± 1.4	6.0 ± 1.8	5.7 ± 0.9*
TDI – lateral mitral annulus			
s peak (cm/s)	10.8 ± 2.0	9.9 ± 1.9	10.6 ± 3.5
e' peak (cm/s)	14.8 ± 2.6	16.4 ± 2.2	19.2 ± 2.6*†
a' peak (cm/s)	8.7 ± 1.8	9.0 ± 1.8	8.4 ± 2.6
E/a' ratio	10.0 ± 2.2	8.8 ± 3.6	9.4 ± 3.1
E/e' ratio	5.2 ± 0.7	5.0 ± 1.0	3.9 ± 0.8*†

AAS – anabolic androgenic steroids; DT – deceleration time; *significantly different from the AAS-using strength athletes ($p < 0.05$); †significantly different from the AAS-free strength athletes ($p < 0.05$); $^{\Delta}$significantly different from the AAS-free endurance athletes ($p < 0.05$); $^{\square}$significantly different from the AAS-free strength athletes ($p < 0.05$).

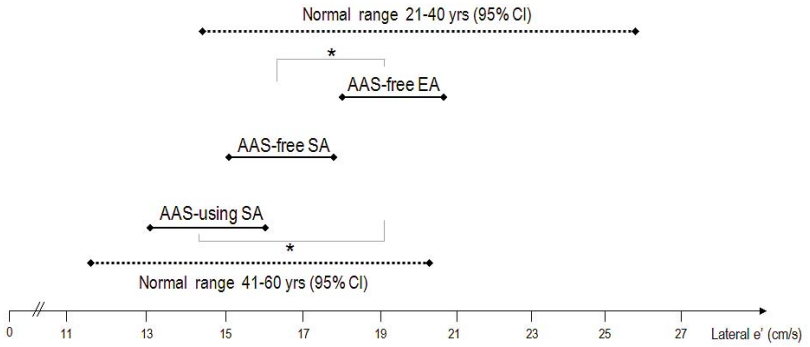

Fig. 2 – The 95% confidence intervals (CI) for the peak lateral e' velocity in the endurance, steroid-free and steroid-using strength athletes, compared to the reference range for different age groups. Normal values (given as 95% CI) are adopted from the most recent guidelines 13. AAS – anabolic androgenic steroids; EA – endurance athletes; SA – strength athletes; *denotes p < 0.05.

Transmitral Doppler velocities and tissue Doppler Imaging data

The AAS-using strength athletes had higher peak A-wave velocity when compared to both endurance and AAS-free strength athletes (Table 3). Regardless of AAS misuse, the strength athletes had significantly lower e' peak velocity

Discussion

Our data indicate that both type of training and AAS abuse may affect LV diastolic function. Although paradoxically associated with increased LA size, it appears that a long-term AAS-free endurance training may have preferable effects on LV filling and relaxation parameters, compared to

strength training, particularly in the presence of AAS abuse. No significant differences between the elite strength and endurance athletes were found for systolic function indices, although mildly reduced LV ejection fraction was seen only in AAS users.

Parameters of LV diastolic function

Reflecting the LA-LV pressure gradient during late diastole, mitral A-wave velocity is affected by LV compliance and LA contractile function [13]. In line with this, a significantly higher peak A-velocity in AAS abusers, regardless the type of training, might indicate a relationship between AAS misuse and decreased LV compliance.

On the other hand, peak e' velocity, a parameter of LV relaxation, did not significantly differ between strength athletes with respect to AAS abuse. However, abnormally low values of this parameter were observed only in the AAS abusers – in 30% of AAS-using strength athletes (aged 27–31 years), e' velocities values were as low as they were measured in individuals aged between 41–60 years (Figure 2).

Although all the AAS-free athletes had normal e' velocity and E/e' ratio values, significant differences related to the type of training were observed. The peak e' velocity was higher and E/e' ratio lower in the endurance than the strength athletes, suggesting that strength training may not produce equally favorable effects on diastolic function as endurance exercise.

Mechanisms responsible for the possible alterations of LV diastolic function with AAS abuse are poorly understood. The transient increase in blood pressure, also observed among the AAS users in this study, may negatively alter LV diastolic function, but it is usually mild and its clinical significance remains most likely modest [14].

On the other hand, since no increase in LV wall thickness was found, AAS-mediated changes in myocardial intrinsic properties might be responsible for the differences in LV diastolic function.

Hence, *in vitro* and histological studies have shown that an increase in myocardial collagen content might occur as a repair mechanism against AAS-induced myocardial damage [15], and also that chronic administration of 17a-methyltestosterone, frequently used anabolic steroid, may reduce LV compliance [16].

Data from previous (small-scale) studies are widely inconsistent, showing either negative [17–20] or no effect [21–23] of AAS on LV diastolic function. The inconsistency could be explained by methodological differences (pulsed-wave *vs* tissue Doppler imaging) and by the lack of power to detect true affects of AAS.

Relationship between LA remodeling and LV diastolic function

In non-athletic population, dilatation of LA reflects the cumulative effects of LV filling pressures and is an independent predictor of death, heart failure, atrial fibrillation and stroke [24]. Our data support the belief that LA enlargement in athletes should be regarded as a physiological adaptation to exercise conditioning [25], particularly in endurance athletes. We demonstrated that the variability of LA size was predominantly influenced by LV dimension, but only in the absence of AAS misuse. The correlation between the LVEDD and LA dimension was statistically significant in AAS-free endurance athletes and borderline significant in the group of AAS-free strength athletes. A lack of correlation between LA size and LVEDD in AAS-using strength athletes may therefore be reflective of detrimental effects of AAS on LV diastolic function, regardless of training type. In line with this, the endurance athletes had the largest LA dimension but the lowest E/e' ratio, suggesting that LA enlargement should not be considered pathological in these athletes. Conversely, when LA enlargement occurs in strength athletes, particularly in the presence of AAS abuse, it should not be entirely ascribed to a long-term strength training, as it could also reflect the disturbances of LV diastolic function.

Left ventricular remodeling, type of training and AAS abuse

LV end-diastolic dimensions and LV mass, after being indexed for BSA, were higher in the endurance than in the AAS-using athletes, with no differences between the strength athletes with respect to AAS abuse. Our data are consistent with previous echocardiographic and magnetic resonance imaging studies showing that long-term endurance training has the strongest impact on LV cavity size, mass and thickness while strength training does not necessarily induce wall thickening [18, 26, 27].

A significant increase in LV mass related to AAS administration was observed in some studies [20], but not confirmed by others [21–23].

Left verticular systolic function

Even though we did not observe a significant difference in LVEF among the 3 groups, a mild reduction of LVEF was detected only in the AAS users. Results from recent studies suggest that systolic dysfunction associated with AAS abuse might be subclinical and advanced echo techniques are needed for its detection [28, 29]. It has been shown that chronic misuse of AAS is associated with reduced peak systolic strain, and strongly correlated with mean dosage and duration of AAS use [28]. However, it has been recently reported that, on top of reduced peak strain values, long-term AAS use might even be associated with a clinically relevant reduction in LV ejection fraction [29].

Study limitations

Our study has some important limitations. First, like most previous studies, we did not perform plasma or urine assessment for drug levels and the history of AAS use was self-reported by the athletes included in the study. Although the results should be interpreted cautiously, we believe that the observed differences in athletes' clinical characteristic support the accuracy of athletes' statements regarding AAS use.

Both resting heart rate and diastolic blood pressure, which increase had been previously linked to AAS abuse [30], were highest in the AAS-using athletes, with no difference

between the AAS-denying endurance and strength athletes. The elevation of blood pressure is usually transient, returning to basal levels several weeks or months after drug discontinuation [31] which might explain why the AAS users had not been diagnosed of having hypertension during regular physical examinations.

Second, since AAS abuse is a very sensitive matter in professional sports, particularly among elite athletes, we recruited a small number of subjects. However, this limitation is more likely to produce type II errors (false-negative results) than type I errors (false-positive results) due to a reduced statistical power. On the other hand, type II errors might explain why several nonsignificant trends were observed – there were striking differences in mean values among the 3 groups for several clinical and echocardiographic variables, but with large variance. Therefore, further studies with adequate power are required.

Conclusion

There is no evidence that LV ejection fraction in elite athletes is altered by either type of training or AAS misuse. Long-term endurance training is associated with preferable effects on LV diastolic function compared to strength training, particularly when the latter is combined with AAS abuse.

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Difficulties in proving medical errors – Where do we stand?

Teškoće u dokazivanju medicinskih grešaka – Gde smo trenutno?

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Key words:
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Ključne reči:
etika, medicinska; lekar-bolesnik odnosi; medicinske greške; pravna nauka; zakonodavstvo; srbija.

Introduction

Developements in medicine, better informed patients and more complicated medical procedures have increased public interest in regard to errors in treatment. Heightened public awareness is also reflected in the increased number of judicial proceedings related to medical malpractice in many countries worldwide [1]. Approaching European standards dealing with protection of patient's rights further magnifies the significance of this subject. Awareness of contemporary medical achievements and developement of pharmaceutical technology often leads patients to form unrealistic expectations of doctor's and medicine's capabilities, and the perception that all ills must be cured. The sensitivity of this subject heightens the importance of health and the perceived impact that actions or omissions on the part of medical practioners may have on the lives of individuals. The constitutions of many countries address this as well [2].

According to the report of Institute of Medicine, between 44,000 and 98,000 deaths *per* year were caused by errors in treatment in the U.S. health system alone [3]. One of the report's main conclusions was that the majority of medical errors do not result from the recklessness of one individual. More commonly such errors are caused by faulty systems, processes, and conditions that lead people to make mistakes or fail to prevent them. Ten years later, with little transparency and no public reporting (except in jurisdictions where hard fought state laws now require public reporting of hospital infections), scarce data does not paint a picture of real progress [3].

Medical ethics on infallibility leads to an atmosphere in which errors are seen as an individual problem of the one who treats, for which the doctor punishes him and feels ashamed, instead of seeking the root of the problem and searching for a solution to improve the health system. On the other hand, proving guilt often becomes a long process which can be traumatic for patients, members of their family, and also for the doctor who, even if acquitted, has no impression that they gained something, but only that they have lost less.

Medical error – Error in treatment – Medical malpractice

Error in treatment is not synonymous with neglect, negligence and medical malpractice.

Medical error – error in treatment – medical malpractice – these terms are not identical in their meaning or legal and other consequences. Medical error and error in treatment are terms derived from the medical profession with numerous ethical and deontological implications. Medical malpractice is a term arising from the legal standard prescribed by law as the basis of doctors' liability.

According to The Joint Commission (TJC), a Sentinel Event or "Never Event" is defined as an unforseen event in a health system resulting in death of a patient or serious phisi-

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cal or mental health damage, non-related to natural course of patient's illness [4–6].

The criminal offense of medical malpractice, as defined in the Criminal Law of Republic of Serbia [7], occurs when a doctor, in providing medical help, uses an obviously inadequate remedy or obviously inappropriate treatment or does not apply appropriate hygiene measures at all, or obviously acts unconscionably, thereby causing deterioration of a person's health.

In order to determine a crime of medical malpractice, it is necessary to establish a causal relationship between a doctor's unconscionable action and a patient's deteriorating health [2]. That means that the aggrieved person can be both healthy and sick. What is important is that their health deteriorated due to medical malpractice. On the other hand, if a doctor did act unconscionably but there was no health deterioration, a criminal offence does not exist. Serbia and neighboring former Yugoslav republics have been and remain among the few countries whose criminal justice specifically exculpates unconscionable doctors [2, 8].

Penalties in the case of medical malpractice resulting in a patient's deteriorating health are different in different legislatures – a fine or two years imprisonment (Penal Code of Republic of Croatia) or imprisonment from three months to three years (Penal Codes of Republic of Serbia, Republic of Montenegro, Serb Republic, and Federation of Bosnia and Herzegovina [2].

In all previously mentioned legislatures, more lenient punishment is provided if it is proven that a doctor's medical malpractice occurred out of negligence – a fine or imprisonment up to one year may be imposed.

Every legal system mentioned above also provides for more serious cases of this criminal offence, as determined by more serious outcomes (such as severe injuries or death of a patient). Therefore, the more serious case of this criminal offence is a separate article of the Criminal Law in Serbian legal system. Serious offenses against public health provide for punishment from one to eight years of imprisonment (in case of severe injury) or two to twelve years (in case of death of a patient). For the same criminal offence, neighboring countries stipulate similar punishments [2].

Serbia statistics

The crime of medical malpractice is one of the rarest offenses in Serbian judiciary for years [9]. A disproportionately small number of cases of medical malpractice was observed and described in works dating from the mid-nineties [10].

From 2006 to 2010, in Serbia from 4,052 to 4,895 charges *per* year against adult persons for a crime against public health were documented. This is an average of 4 to 5.5% of total charges for all crimes. Looking at individual offenses in 2010, there are 4,052 charges with crimes against public health. Of these, 47 cases were based on negligence in providing medical care, and 4 cases on not providing medical help. The total number of convictions in 2010 for crimes against public health was 2,564, but of that number only 3 cases were based on negligence in providing medical aid, while not a single person has been convicted of not providing medical help. In all 3 cases there was a guilty verdict in whom they received probation sentences.

In order to ilustrate the everyday practice in Serbia, we analized twelve years data from the Municipal Court in Kragujevac. According to the current organization of the courts, the Municipal Court in Kragujevac covers the territory of city of Kragujevac, municipalities Aranđelovac, Batočina, Rača and Topola. Data on the number of all initiated proceedings filed against doctors and other health workers, and the number and gender of the accused persons for the period from 2000 to 2011 were collected and processed (Table 1).

The total number of prosecutions and other prosecution acts during this twelve years period was 18,732, an average of (mean ± standard deviation) 1,561 ± 352.3 *per* year. In the analyzed period there were six charges for the crime of medical malpractice, which makes 1 crime *per* two years.

The situation is similar in Croatia. For the five-year period (2005–2009) there were 10 reports for medical malpractice, but were all rejected, so there were no convictions [11].

Difficulties in proving medical errors

Any medical procedure that was performed according to the rules of medical profession, with the consent of an informed patient and performed by a qualified person does not

Table 1

Number of cases of medical malpractice in Kragujevac Court

Year	Total number of charges and other acts of prosecution	Number of cases of medical malpractice
2000	1,147	1
2001	1,170	0
2002	1,227	0
2003	1,382	0
2004	1,147	0
2005	1,674	0
2006	1,612	0
2007	1,478	1
2008	1,890	1
2009	2,064	0
2010	2,031	1
2011	1,910	2
Total	18,732	6

come within the scope of criminal behavior even if a harmful outcome for life and health of a patient does occur. On the other hand, even if just one condition is not fulfilled, a medical procedure may result in criminal responsibility.

In order to even initiate a judicial proceeding, it is necessary that the injured patient file a charge. Next, a preliminary proceeding will be conducted to determine whether there are grounds for suspicion of a criminal offense. Of course, charges may also be filed by any other party, especially by the doctors who are familiar with the questionable medical treatment. The prerequisite is that a patient is informed about his rights and the procedure.

To prove that a medical error does exist is problematic *per se*. It is an imprecise standard that refers to the legal standard "manifestly unconscionable (inappropriate)," creating the main problem of proving that such crimes were committed. The next question is whether there is obvious inappropriateness in a general sense or just in the narrow professional sense. If obvious, what defences may be raised by the accused?

An obvious unconscionable/inappropriate act should be seen as a striking fault, which is beyond the scope of medical tolerance [9]. The specificity of the medical profession allows for a certain amount of tolerance, i.e. from the criminal justice/legal standpoint, all monetary losses that are not clearly related and/or obviously ineligible are considered irrelevant [7].

Where does that dose of tolerance towards medical profession come from?

The concept of a reasonable dose of tolerance primarily comes from the fact that while medicine is a relatively exact science, causes of illness and death are not nearly as clear as their consequences [12]. It cannot be predicted with absolute certainity how a person reacts to a disease. Therefore the selection of a particular means of treatment is always relative. Another reason is that medicine is not a complete and closed knowledge system but, on the contrary, medical knowledge is enriched on a daily basis, so questionable treatment will generally be resolved in the health sector within professional circles.

In every specific situation, a doctor's ignorance manifested during the treatment of a patient is a necessary (requisite), but not a sufficient condition for examination of the doctor's responsibility. Obviously using clearly inappropriate means or methods of treatment constitute a more drastic and serious deviation from the rules of science and the medical profession, i.e. reflecting gross medical ignorance [7].

The question of knowledge, i.e. minimum standards of knowledge, skill and expertise reasonably expected of a qualified doctor to act in every circumstance, is the measure of that tolerance that recognizes the possibility of acceptable variations in treatment while on the other hand does not create suspicion for alleged medical profession privilege. From that comes the obligation for doctors to engage in continuous vocational training and to adopt new practices resulting from the rapid progress of medical science and technology.

The law also recognizes other risk factors in medicine. Every patient's individual reaction to illness, their specific response to therapy, even when applying known and trusted treatment methods, may be variable. This requires that in doctors' everyday practice they must take into account that a certain procedure or therapy may not be successful, i.e. may have a negative effect on the patient [7]. Factors that cannot be predicted or avoided must be recognized as objective risks of treatment that, with all the knowledge, skill, conscientiousness and technical equipment cannot be avoided. Objective risk in medicine may be considered as the equivalent to force majeure that excludes responsibility of a doctor in case of unfavorable outcome of a treatment.

Subjective risk is one that could and should be avoided if a doctor is more professional, careful, and conscientious. In contrast, objective risk is a potential risk factor beyond the control of the doctor which should be disclosed to each individual patient with consent being obtained and understanding confirmed. Subjective risk (insufficient knowledge and competence of a doctor) directly leads to a doctor's responsibility in terms of medical malpractice if proved that it was crucial to harmful effects to the patient's health. Special and delicate discussion is required if attempting to present subjective risk as inevitable, that is, objective risk during treatment, in order to avoid responsibility for the harm caused the patient.

Advantages and disadvantages of team work

One more aspect of the medical profession is teamwork. This implies that several doctors of different specialities are included in the treatment of a common patient. This approach provides the highest quality treatment for each patient, but in case of serious harm to health of a patient, the question of responsibility is raised. There is no doubt that even in a team, every individual is primarily responsible for their own work, and teamwork is based on mutual trust where team members complement and help each other in knowledge and action. Thus there is the potential of causing a mutual impact in which one's error becomes an error in the work of another team member (accumulation of errors), but also the team concept can create the possibility of averting error of one by corrective action of another team member. In each individual case it is assessed whether and to what extent there is a duty/obligation to remove an error of another team member who had treated the patient. In this case also, these issues are resolved by hiring forensic experts.

Expert selection

According to the rules of a proceeding, expertise is determined in the investigation. Bearing in mind that the actions involved in committing the offense may be debatable or unclear, expertise is crucial in determining whether the doctor committed a crime or not. As judges do not have sufficient expertise in medical issues that would shed light on relevant circumstances of the offence, medical evaluation, implementation or lack of implementation of proper medical procedures are entrusted to experts of medical profession by the judges.

When determining the expertise required, all relevant circumstances pertaining to the particular case should be considered before delegating to an expert, an expert commission, an expert institute or other institution. The main objective is to provide appropriate and non-biased professional expertise. Proper selection of experts requires that judges also have a broad enough education and knowledge in those fields to assess which experts are required in each particular case.

In any particular case of medical malpractice, it must be taken into account that experts of the respective specialty are present, or whether it is necessary that several specialists in different fields be involved. The decision also depends on the institution where the offender works.

After that assessment, the judge or president of the panel decides the composition of the commission or institution to which the expertise will be delegated. Due to the very few specialized institutions available to provide expertise and the narrow scope of their work, the process of selection may be unjustifiably long. This can be affected by the administrative part of a proceeding – correspondence and paying the costs of expertise.

Practice has shown that judges often hire the same experts. This is usually a consequence of a judge's trust and confidence and the need for quick and competent expertise, rather than, as sometimes negatively portrayed, that some experts are privileged.

In the judicial process itself, of great importance is to what extent the clinical environment in which a doctor or other health care provider operated may have impacted the circumstances (i.e. conditions in the institution, equipment, number of patients, level of training of other staff). Also relevant are the problems of collecting valid documentation related to a particular patient. Proving guilt therefore often becomes a long process which can be traumatic for patients, members of their family, and also for the doctor who, even if acquitted, has no impression that they gained something, but only that they have lost less than would otherwise have been the case.

Final remarks

On behalf of patients

The prerequisite is that a patient is informed about his rights and the procedure. Patient safety may be enhanced by better information, which depends primarily on the doctor's competence, the conditions provided by the institution, the time the doctor can devote to patient, and also on the interest of the doctor to provide services and information to the patient.

On behalf of doctors

Doctor's safety would be influenced by protocols used, good teamwork, trained "support staff", better working conditions, continuous education of doctors, financial satisfaction, and anything else that could improve medical knowledge and provide the basis for the provision of better medical care. Regulating error reporting in medicine is also very important in reducing potentially fatal medical errors. In the future, medical associations should be open to discussion of these issues also.

On behalf of low

Bearing in mind that the actions involved in committing the offense may be debatable or unclear, expertise is crucial in determining whether the doctor committed a crime or not.

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In each individual case it is assessed whether and to what extent there is a duty/obligation to remove an error of another team member who had treated the patient.

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Conclusion

Errors do happen. Like every human being, doctors and other medical workers are not infallible. As the errors in the course of treatment can be rightly expected, the system must be adjusted so that it prevents errors and resolves them.

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CASE REPORTS

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Congenital thrombocytopenia with nephritis – The first case of MYH9 related disorder in Serbia

Kongenitalna trombocitopenija sa nefritisom – prvi bolesnik sa MYH9 poremećajem u Srbiji

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Abstract

Introduction. The group of autosomal dominant disorders – Epstein syndrome, Sebastian syndrome, Fechthner syndrome and May-Hegglin anomaly – are characterised by thrombocytopenia with giant platelets, inclusion bodies in granulocytes and variable levels of deafness, disturbances of vision and renal function impairment. A common genetic background of these disorders are mutations in MYH9 gene, coding for the nonmuscle myosin heavy chain IIA. Differential diagnosis is important for the adequate treatment strategy. The aim of this case report was to present a patient with MYH9 disorder in Serbia. **Case report.** A 16-year-old boy was referred to our hospital with the diagnosis of resistant immune thrombocytopenia for splenectomy. Thrombocytopenia was incidentally discovered at the age of five. The treatment with corticosteroids on several occasions was unsuccessful. Although the platelet count was below 10×10^9/L, there were no bleeding symptoms. Besides thrombocytopenia with giant platelets, on admission the patient also suffered sensorineuronal hearing loss and proteinuria. The diagnosis was confirmed with immunofluorescence and genetic analyses. **Conclusion.** Early recognition of MYH9-related diseases is essential to avoid unnecessary and potentially harmful treatments for misdiagnosed immune thrombocytopenia, and also for timely and proper therapy in attempt to delay end-stage renal failure and improve quality of life.

Key words:
thrombocytopenia; nephritis hereditary; myosin heavy chains; diagnosis, serbia.

Apstrakt

Uvod. Grupu autozomno dominantnih poremećaja – Epsteinov sindrom, Sebastianov sindrom, Fechthnerov sindrom i May-Hegglinovu anomaliju – odlikuju trombocitopenija sa džinovskim trombocitima, inkluzije u granulocitima, kao i različita zastupljenost gluvoće, poremećaja vida i funkcije bubrega. Genetska osnova ovih sindroma su mutacije u genu za teški lanac nemišićnog miozina IIA, a za ovu grupu sindroma predložen je naziv bolesti vezane za MYH9. Diferencijalna dijagnoza prema trombocitopenijama druge etiologije je značajna zbog pravilnog izbora terapijskih postupaka. Cilj rada bio je prikaz bolesnika sa MYH9 poremećajem u Srbiji. **Prikaz bolesnika.** Bolesnik, star 16 godina, sa dijagnozom rezistentne imunske trombocitopenije upućen je radi daljeg lečenja splenektomijom. Trombocitopenija je otkrivena u petoj godini života rutinskim pregledom krvne slike. U više navrata bolesnik je lečen kortikosteroidima ali bez povoljnog terapijskog odgovora. Iako je broj trombocita najčešće bio manji od 10×10^9/L, nisu se javljali simptomi krvarenja. Pored trombocitopenije sa džinovskim trombocitima, na prijemu su nađeni senzorineuralna gluvoća kao i proteinurija. Dijagnoza je potvrđena imunofluorescentnim nalazom i genetskom analizom. **Zaključak.** Pravovremeno prepoznavanje poremećaja mutacije MYH9 neophodno je kako bi se izbegli neadekvatni i potencijalno opasni načini lečenja koji se primenjuju kod imunske trombocitopenije. Takođe, odgovarajućom terapijom odlaže se razvoj terminalne bubrežne insuficijencije i poboljšava kvalitet života.

Ključne reči:
trombocitopenija; nefritis, nasledni; miozin, teški lanci; dijagnoza; srbija.

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Introduction

The group of autosomal dominant disorders formerly called Epstein (OMIM # 153650), Fechtner (OMIM # 153640), and Sebastian syndrome (OMIM # 605249) and May-Hegglin anomaly (OMIM # 155100) is characterized by thrombocytopenia with giant platelets and Döhle-like inclusion bodies in granulocytes. The diagnosis was established on the clinical grounds, assessing the involvement of kidney, inner ear or eye [1, 2]. A decade ago, it was recognized that these different entities have unique genetic background, with variable clinical expression, varying from mild macrothrombocytopenia with leukocyte inclusion bodies to a severe form complicated by hearing loss, cataract and renal failure [3–5]. Since all clinical features are the consequence of different mutations in MYH9 gene, the new term "MYH9 disorders" or "MYH9-related disease" (MYH9-RD) was proposed [4]. MYH9 is the gene encoding for the nonmuscle myosin heavy chain IIA (NMMHC-IIA), which is localized on chromosome 22q11-13 [5].

In this paper we presented the clinical and laboratory findings, and the course of the disease in a 16-year-old boy suffering from MYH9-RD, with clinical features previously classified as Epstein syndrome [1], which was misdiagnosed as immune thrombocytopenia in early childhood. To the best of our knowledge this is the first report on MYH9-RD in Serbia, as well as in the region of Southeastern Europe.

Case report

The boy was admitted to our hospital at the Department of Hematology for the first time at the age of 16 years for evaluation of thrombocytopenia and for assessment for splenectomy. Thrombocytopenia was discovered incidentally at the age of five, and he was treated with prednisolone on several occasions, with no response. Although the platelet count was most of the time below 10×10^9/L, the patient was almost free of bleeding symptoms. Surgical correction of hypospadia at the age of seven years took an uneventful course, without unexpected bleeding. At the time of admission, full blood count showed a very low platelets count of 4×10^9/L, with giant platelets on blood smear (Figure 1A), hemoglobin (Hgb) was 138 g/L, red blood cells (RBC) 4.08×10^{12}/L, mean corpusculer volume (MCV) 98.8 fl and white blood cells (WBC) 7.0×10^9/L. Bone marrow aspirate revealed normal cellularity, with small, hypolobulated megakaryocytes. Platelet kinetics, investigated with indium-111 labelled autologous platelets, showed significantly decreased platelet production whose life span was shortened to 3.6 days.

Urine analysis revealed microscopic hematuria and nephrotic range proteinuria, with 24-hour protein excretion of 5.5 g. Blood chemistry showed normal levels of serum protein, albumin and cholesterol. Serum urea and creatinine were 5.4 mmol/L and 77 μmol/L, respectively, and the estimated glomerular filtration rate (eGFR) was 134.4 mL/min per 1.73 m^2. Owing the risk of bleeding due to thrombocytopenia, renal biopsy was not done.

Findings of urine abnormalities prompted further investigations and a more detailed family history. Both patient parents were healthy; father's blood pressure, platelet count, GFR, urine sediment and 24-hour protein excretion all were normal. Audiometry showed high tone sensorineural deafness with hearing defects of more than 70 db in the range of high tone frequencies (2,000 and 4,000 Hz). Ophthalmologic examination gave normal findings. On the basis of thrombocytopenia with giant platelets, hearing defect and renal abnormalities, the clinical diagnosis of Epstein syndrome was made [2, 3].To further substantiate the Epstein syndrome diagnosis, peripheral blood smears were sent to a specialized laboratory [6]. Immunocytochemistry for NMMHC-IIA organization in neutrophils was reported as normal, and therefore, any further investigations were considered unnecessary. Treatment with an angiotensin-converting enzyme (ACE) inhibitor was started and hearing amplification was prescribed. At follow up appointment, two months later, 24-hour protein excretion was reduced to 3.5 g. Unfortunately, the patient stopped taking ACE inhibitor after two months and refused to use hearing amplifications. At the last regular follow-up visit, at his 16 years and 9 months of age (8 months after the first referral) his blood pressure (BP) was 140/80 mmHg, serum urea 4.6 mmol/L, creatinine 89 μmol/L (1.0 mg/dL), the estimated GFR 91.9 mL/min *per* 1.73 m^2, and proteinuria 4.37 g/24hr. The patient did not come to follow-up visits during the next three years. When we finally succeeded to contact him, he was 19 years and 8 months old, deaf and with end-stage renal disease (ESRD); eGFR was 11.2 mL/min *per* 1.73 m^2, serum urea and creatinine levels were 23 mmol/L and 726 μmol/L, respectively. Urinalysis confirmed hematuria and nephrotic range proteinuria (7g/24h). His platelet count remained low (4×10^9/L).

These findings convinced us more deeply in the diagnosis of Epstein syndrome. Reanalysis of our patient's peripheral blood smear was done at the Clinical Research Center of Nagoya Medical Center, Japan. Although granulocyte inclusion bodies were invisible on peripheral blood [7] smears (Figure 1A), immunofluorescence analysis revealed abnormal NMMHC-IIA localization in neutrophils (Figure 1B). After extraction of DNA from the remaining peripheral blood smear, MYH9 sequence analysis disclosed p.R702C missense mutation, finally confirming our clinical diagnosis (Figure 1C).

Discussion

The main manifestations of MYH9-RD, i.e. thrombocytopenia, giant platelets, and granulocyte inclusion bodies, are present at birth. In most cases thrombocytopenia is found incidentally, because the associated bleeding tendency is mild. Glomerulonephritis develops in 30–70% of patients with MYH9-RD, usually at mean age of 23 years and ESRD develops before the fourth decade of life in the majority of patients [8]. More than half of patients have bilateral or unilateral sensorineural hearing loss for high tones. Presenile cataract has the lowest incidence of all clinical MYH9-RD manifestations [9].

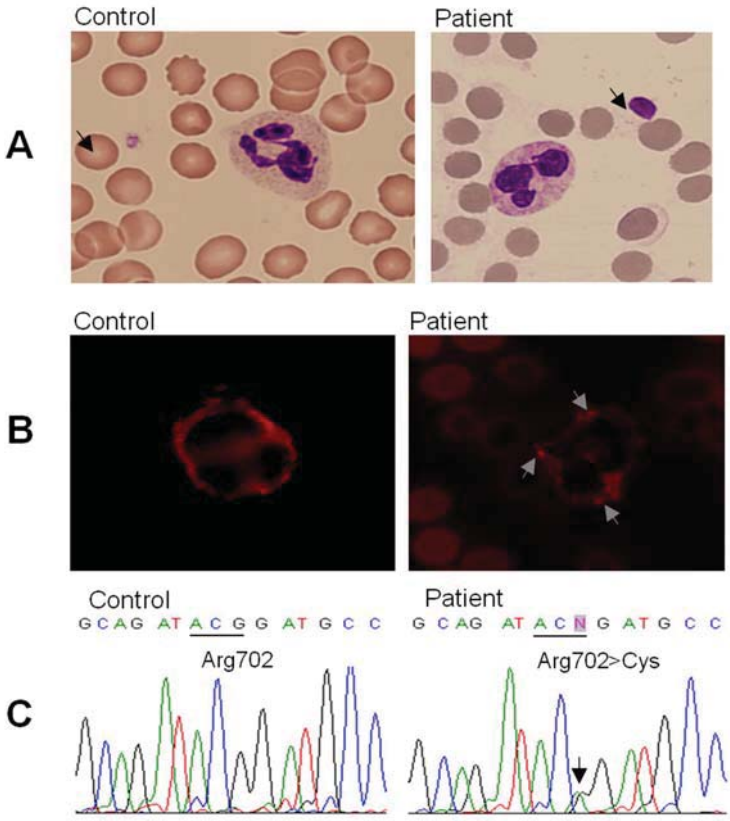

Fig. 1 – A. Giant platelet in the patient compared to that from the normal control; B. Abnormal NMMHC-IIA aggregations in the cytoplasm of neutrophils are indicated by arrows; C. Sequence electrophoregram showing c.2104C > T substitution resulting in p.R702C in the patient.

Up to date, more than 40 different mutations in the MYH9 gene have been identified. Most affected individuals have missense, nonsense or frame shift mutations in only 6 exon locations that have been found in 80% of affected families [9].

There is a genotype – phenotype correlation regarding the onset and severity of disease. Mutations in the motor domain of MYH9 are frequently associated with the development of nephritis and deafness, whereas mutations in the tail domain are associated with a lower risk (10%) of developing such impairments. Mutations at Arg702 produce glomerulopathy and deafness at a juvenile age [10]. Association of the p.R702C missense mutation with earlier progression to end-stage renal disease (ESRD) explains rapid deterioration of renal function in our patient as it was reported previously [9, 11, 12]. Sekine et al. [12] found that patients with this mutation over 15 years old developed ESRD between 15 and 20 years, each of them progressed to ESRD shortly after serum creatinine level exceeded 1.0 mg/dL. The above presented data show that our patient's clinical course followed this pattern: he reached ESRD within 3 years after his serum creatinine level rose to 1.0 mg/dL.

Although the clinical diagnosis of Epstein syndrome in our patient was made on initial examination, the molecular genetic confirmation was delayed because the initial immunocytochemistry diagnostics of granulocyte NMMHC-IIA localization, conducted by an outside research organization was negative [6]. We reevaluated its localization by the immunofluorescence analysis and found abnormal accumulation/aggregation [7] (Figure 1B). Subsequent DNA analysis revealed MYH9 p.R702C missense mutation in the patient. Although faint staining of inclusion bodies using May-Grünwald-Giemsa stains has the potential to hamper the diagnosis in patients with MYH9 head domain mutations such as at Ser96 and Arg702, immunofluorescence analysis can detect abnormal NMMHC-IIA localization. Thus, immunofluorescence analysis but not immunocytochemistry analysis facilitates the diagnosis of MYH9-RD.

The absence of clinical and laboratory abnormalities in the parents suggests *de novo* origin of the mutation in our patient. MYH9-RD is typically of autosomal dominant inheritance, but more than 30% show *de novo* mutations [13].

Because of the risk of bleeding, renal biopsy was not done in most cases with nephritis caused by MYH9 gene mutations. In less than 20 patients in whom the biopsy data were reported main histological findings were mesangial expansion or proliferation in the earlier stage and focal segmental or global glomerulosclerosis (FSGS) in the late stage of renal disease. Electron microscopy (EM) showed thickening, splitting or attenuation of glomerular basement membrane (GBM) or focal podocyte foot process effacement [11, 14].

The pathogenesis of glomerulopathy in MYH9 disorders remains uncertain. NMMHC-IIA is expressed in podocytes, mesangial cells, in certain endothelial cells, and most tubular cells [15]. NMMHC-IIA is a major component of actin myosin contractile apparatus in the podocyte foot processes and has a role in maintaining and disassembling the slit diaphragm [16]. The mutated NMMHC-IIA, by altering the podocyte cyto-

skeleton, impairs the function and structure of the slit diaphragm resulting in proteinuria and the development of FSGS [11, 14]. It is unknown whether mesangial hypercellularity is due to direct effect of abnormal MYH9 protein on mesangial cells [16]. GBM abnormalities, often found in patients with MYH9-RD, raise the possibility that MYH9 mutations disrupt the ability of the podocyte to produce extracellular matrix proteins with the appropriate amount and stoichiometry or the ability to regulate the incorporation of these proteins into GBM in the process of physiologic remodeling [14].

Renin-angiotensin system blockade may be effective in reducing proteinuria of patients with progressive nephropathy caused by MYH9 mutations [14, 17]. The efficacy of ACE inhibitors in reducing proteinuria was observed in our patient, the long-standing effect could not be confirmed because of preterm therapy termination. Early recognition of MYH9-RD also offers a better quality of life to these patients [18].

Conclusion

Although MYH9-RD is a well-defined entity with precise diagnostic work-up recommendations available, the diagnosis is sometimes delayed due to misinterpretation of laboratory data, especially in the patients with *de novo* mutations and a negative family history. Reviewing peripheral blood smear to assess the platelet size should be mandatory. A high index of suspicion and understanding the pathophysiology of MYH9-RD is a clue to correct diagnosis. Screening for both nephritis and hearing impairment has to be considered in all patients with macrothrombocytopenia. Although there is no effective treatment for progressive renal disease and deafness, potentially harmful treatment of patients with thrombocytopenia caused by MYH9 mutations, such as intravenous immunoglobulin, corticosteroids or splenectomy could be avoided.

Acknowledgements

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CASE REPORT

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Isolated hepatic sarcoidosis

Izolovana sarkoidoza jetre

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Abstract

Introduction. Sarcoidosis is a multisystem granulomatous disease of unknown etiology. Although hepatic granulomas occur in 50–65% of patients with systemic sarcoidosis, isolated liver sarcoidosis is rare. Clinical presentation varies from asymptomatic to manifest. The diagnosis is based on a characteristic histopathological finding of liver biopsy. **Case report.** We reported a 69-year old man was admitted due to abdominal swelling and abdominal pain. Laboratory studies detected: cholestasis, pancytopenia and elevaton of angiotensin-converting enzyme. Abdominal imaging techniques showed liver cirrhosis, splenomegaly and ascites. The diagnosis of the hepatic sarcoidosis was confirmed by histopathological examination of liver biopsy. The patient was treated with corticosteroids. After 18 months the patient was without any subjective symptoms, and with biochemical and clinical improvement. **Conclusion.** Isolated hepatic sarcoidosis should be considered in the differential diagnosis of asymptomatic or simptomatic patients with hepatosplenomegaly and changes in liver functional tests. Only the timely diagnosis and proper treatment can lead to subjective and objective improvement of patients.

Key words:
sarcoidosis; liver cirrhosis; splenomegaly; ascites; diagnosis; histological techniques; treatment outcome.

Apstrakt

Uvod. Sarkoidoza je multisistemsko granulomatozno oboljenje nepoznate etiologije. Hepatični granulomi nalaze se kod 50–65% bolesnika sa sistemskom sarkoidozom, ali je izolovana hepatična sarkoidoza retka. Klinička slika varira od asimptomatske do manifestne. Dijagnoza se postavlja na osnovu karakterističnog patohistološkog nalaza. **Prikaz bolesnika.** Prikazan je muškarac, star 69 godina, primljen zbog oticanja trbuha i abdominalnih bolova. U laboratorijskim analizama imao je povišenje enzima holestaze, pancitopeniju i povišenje angiotenzin-konvertirajućeg enzima. Ultrasonografija i magnetna rezonanca abdomena ukazali su na cirozu jetre, splenomegaliju i ascites. Dijagnoza hepatične sarkoidoze potvrđena je patohistološkim pregledom bioptata jetre. Bolesnik je lečen kortikosteroidima. Nakon 18 meseci, bolesnik je bio bez tegoba, sa laboratorijskim i kliničkim poboljšanjem. **Zaključak.** Kod bolesnika sa hepatosplenomegalijom i poremećajima u hepatogramu, i kod onih bez simptoma, i onih sa simptomima oboljenja, u diferencijalnoj dijagnozi treba razmotriti izolovanu hepatičku sarkoidozu. Jedino pravovremena dijagnoza i adekvatna terapija mogu dovesti do subjektivnog i objektivnog poboljšanja.

Ključne reči:
sarkoidoza; jetra, ciroza; splenomegalija; ascit; dijagnoza; histološke tehnike; lečenje, ishod.

Introduction

Sarcoidosis is a multisystem granulomatous disease of unknown etiology [1]. The main characteristic of this disease is the presence of uncaseous epitheloid granuloma in the tissue. In 80–95% of patients, the changes affect the lungs and hilar lymph nodes [2, 3]. Extrapulmonal disease is often seen as part of systemic sarcoidosis and all the organs can be affected, particullary: skin, eyes, liver, spleen, lymph nodes and bone marrow [2, 4]. Although hepatic granulomas occur in 50–65% of patients with systemic sarcoidosis, isolated liver sarcoidosis is rare [4–8]. Hepatic events may be the first and only clinical sign of extrapulmonal sarcoidosis [9]. The clinical presentation varies from asymptomatic to symptoms and signs such as abdominal pain, nausea, vomiting, hepatosplenomegaly, clinical signs of cirrhosis and liver failure [7, 9, 10]. The diagnosis is based on

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clinical, laboratory and radiological findings with a characteristic histopathological finding of liver biopsy, if previously excluded other causes of hepatic granulomas [1].

We reported a patient with isolated liver sarcoidosis which was presented as decompensated liver cirrhosis.

Case report

A 69-year-old man was admitted to the Clinic for Gastroenterology, Clinical Center of Serbia, with abdominal swelling and abdominal pain located mostly in the left but also in the right hypohondrium. Symptoms lasted for two months before admission. The previous 6 years the patient had occasional mutual hypohondrial pain, but he did not find it significant. He denied other diseases, surgeries, and allergies. The family history was positive for cardiovascular diseases, but not for granulomatous or autoimmune disorders or any relevant disease of the gastrointestinal tract. Physical examination showed tenderness in the left upper quadrant. The liver was not enlarged, and the lower spleen border palpated 3 cm below the left costal margin. There were clinical signs of ascites.

Laboratory studies detected the elevation of cholestasis enzymes, with normal transaminase values. There were also signs of pancytopenia, prerenal azotemia, hypoproteinemia with hypoalbuminemia, sideropenia, elevated inflammatory markers and angiotensin converting enzyme (ACE) (Table 1). Other causes of liver diseases were excluded (no history of alcohol consumption, negative viral markers, autoantibodies and laboratory tests for metabolic liver diseases). Urine sample was normal. Stool sample was positive for muscle fibers and digested starch. The purified protein derivative test (PPD) was negative.

Abdominal ultrasonography and magnetic resonance imaging (MRI) showed the normal sized, inhomogeneous, macronodular liver with wavy edges and regular hypoechogenic nodules, surrounded by a thin hyperechoic bands (Figures 1 and 2). The gallbladder had no intraluminal content,

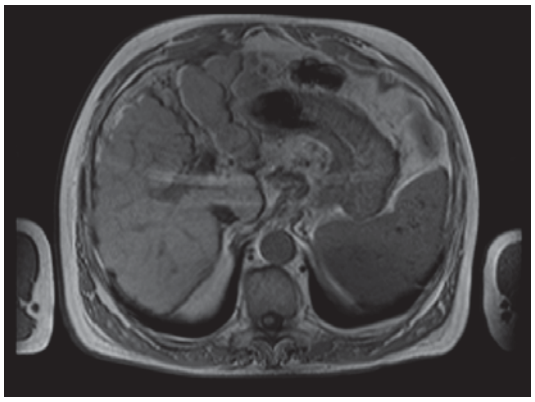


Fig. 1 – Abdominal magnetic resonance imaging showed normal sized, inhomogeneous, macronodular liver.

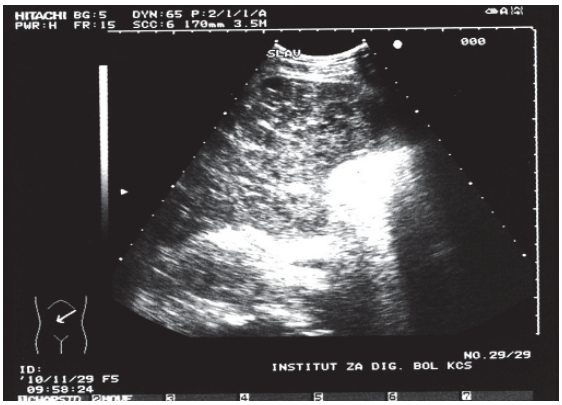


Fig. 2 – Abdominal ultrasound revealed the inhomogeneous liver with wavy edges and regular hypoechogenic nodules, surrounded by a thin hyperechoic bands.

Table 1

Laboratory analyses on admission and on control examination

Variables	On admission	After 18 monts	Reference range
Red blood cells (1×10^{12}/L)	3.7	4.1	4.3–5.7
Hemoglobin (g/L)	109.7	123	138–175
Hematocrit (L/L)	0.32	0.36	0.41–0.53
MCV (fL)	87.2	88	83–97.2
White blood cells (1×10^{9}/L)	2.3	4.6	3.4–9.7
Lymphocites (1×10^{9}/L)	0.4	0.7	2.1–6.5
Platelets (1×10^{9}/L)	74	64	158–424
Glucose (mmol/L)	6.0	10.5	4.2–6.1
Urea (mmol/L)	12.9	4.3	3.2–7.1
Creatinine (μmol/L)	97	73	62–133
Protein (g/L)	60	71	63–82
Albumin (g/L)	35	44	39–50
Iron (μmol/L)	4.7	11.1	8.8–32.4
TIBC (μmol/L)	45.2	60.5	44.8–80.6
Aspartate aminotransferase (U/L)	38	27	14–50
Alanine aminotransferase (U/L)	31	28	21–72
Alkaline phosphatase (U/L)	254	109	38–126
γ -glutamyl transferase (U/L)	157	83	8–78
Erythrocyte sedimentation rate (mm/h)	30	20	2–10
Fibrinogen (g/L)	4.7	4.2	2–4
Angiotensin-converting enzyme (U/L)	95	64	8–65
Hemoglobin A1c (%)		7.7	3.9–6.1

MCV – mean corpuscular volume, TIBC – total iron-binding capacity.

but its wall was irregularly thickened (5–13 mm). The spleen was enlarged, homogeneous, 180 mm in craniocaudal diameter (Figure 3). The portal (15 mm) and lienal vein (13.6 mm)

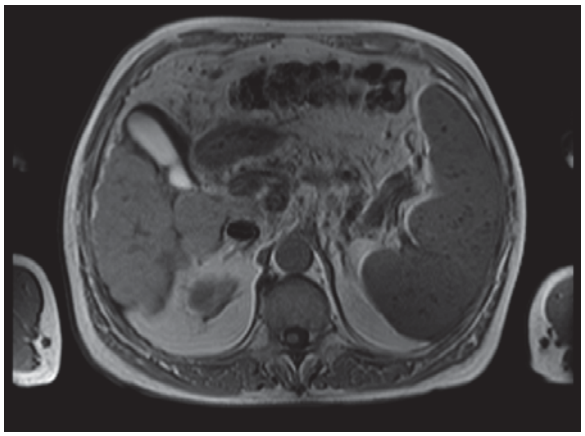
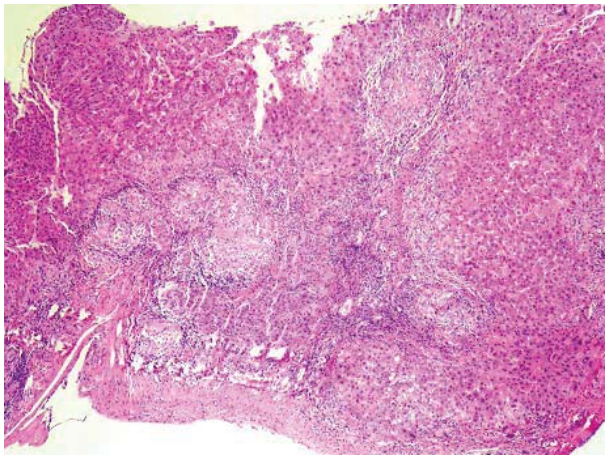


Fig. 3 –Abdominal magnetic resonance imaging showed splenomegaly.

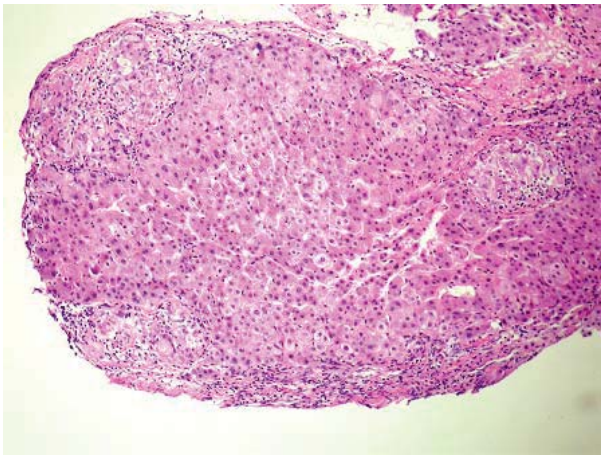
were dilated. No intraabdominal lymphadenopathy was detected, but there was a reasonable amount of ascites. Transient elastography showed the signs of liver cirrhosis (Stiffness 20.6 kPa). During diagnostic laparoscopy regeneratory nodules with several centimeters in diameter were seen in the liver, with smooth capsule, and in some places covered with whitish planes. Biopsies were taken. Histopathological examination indicated chronic granulomatous hepatitis accompanied by an irregular sinusoidal dilation, but without other elements of Budd-Chiari syndrome. In some slices, the structure of the liver was changed showing regenerative nodules and cirrhotic changes parenchyma (Figure 4a). The dominant finding were numerous portal and periportal non-necrotizing granulomas, sarcoid type, consisting of epithelioid cells, macrophages, multinuclear giant cells with a few lymphocytes in the periphery and rarely partially fibrosing (Figure 4b). Staining with PAS (Periodic acid-Schiff) and Ziehl-Neelsen revealed no acid fast bacillus, nor other microorganisms. Esophagogastroduodenoscopy revealed varices of the oesophagus gradus III with "cherry red spots", varices of the cardia and portal hypertensive gastropathy. Chest X-ray and thoracic multi-detector computed tomography (MDCT) were normal. Histopathology finding of bone marrow did not show signs of granulomatous inflamation, but only reactive changes. The patient was treated with intravenous prednisolon (30 mg/day). On the day 6 of corticosteroid therapy hyperglycemia occurred, which was up to 32.6 mmol/L, so we started the therapy with metformine, long-lasting and short-lasting insulin. The dose of prednisolone was reduced to 20 mg a day. This therapy continued for the next 3 months and then corticotsteroid therapy gradually reduced to the maintenance dose of 5 mg a day. The patient was also treated with proton pump inhibitors, non-selective beta blocker, diuretic and supportive care. After 18 months, the patient was without any subjective symptoms, control laboratory tests were better than on admision, ACE was also normal (Table 1). Control abdominal MRI was without progression, the amount of ascites was reduced. Control chest radiography did not show signs of sarcoidosis/granulomas in the lung and hilar lymph glands. The maintenance therapy with prednisolone (5 mg a day) along with proton pump inhibitors, non-selective beta blocker and diuretic was continued.

Discussion

Sarcoidosis is a chronic multisystem disease. The incidence of this disease is 1–40 *per* 100,000 population [9, 11]. The highest incidence is in Scandinavian countries, the USA and Japan and the lowest in Asia and North America [12]. Most commonly it affects adults aged 20–40 years [12]. Sarcoidosis occurs equally in both genders, but sarcoid-related liver disease is more common in men [13]. The etiology of the disease is unknown but is expected to be multifactorial and includes genetic, environmental factors and infectious agents [14]. The clinical presentation of liver sarcoidosis can vary from asymptomatic to manifest. The dominant clinical manifestation of liver sarcoidosis is hepatomegaly, which occurs in 10–40% of patients [7, 9, 10], while splenomegaly is found in



a)



b)

Fig. 4 – Liver histopathology: a) Regenerative nodules and cirrhotic changes of parenchyma. In some slices, the structure of the liver is changed and shows regenerative nodules and cirrhotic of changes parenchyma (HE, ×13); b) Numerous portal and periportal non-necrotizing granulomas, sarcoid type, consisting of epithelioid cells, macrophages, multinuclear giant cells with a few lymphocytes in the periphery (HE, ×52).

10–30% of patients [9]. Non-specific symptoms are: weight loss, abdominal pain, night sweats, erythema nodosum, fever and arthralgia [10]. Fever and arthralgias are more frequently seen in patients with liver sarcoidosis than in those with sarcoidosis without liver involvement [15]. The patient presented with nonspecific symptoms such as abdominal swelling and pain mostly in left hypohondrium which probably was caused by splenomegaly and ascites. Eventually decompensated liver cirrhosis with portal hypertension was diagnosed.

Liver cirrhosis occurs in 6–8% of patients with hepatic sarcoidosis [8, 16] mostly in advanced cases and may be accompanied by all its complications (portal hypertension, variceal bleeding, ascites, hepatic failure, etc.). Portal hypertension is a rare complication of liver sarcoidosis in patients with or without cirrhosis [8]. It occurs as a consequence of chronic hyperdynamic circulation in the portal system caused by arteriovenous shunts and/or compromited portal vein flow in the regions with the presence of granuloma, fibrosis and hyalinization of the portal triad which leads to the presinusoidal block [6]. Ascites occurs as transudate (portal hypertension, deterioration of right heart function) or exudate (peritoneal infiltration with sarcoid nodules) [17]. Rare clinical presentation of liver sarcoidosis are jaundice and Budd-Chiari syndrome [18]. Jaundice occurs as a result of direct affection of the biliary system (intrahepatic cholestasis, "vanishing" bile duct and ductopenia) [18]. If hepatic granulomas or enlarged lymph nodules compress the main biliary ducts, it may develop jaundice which corresponds to the differential diagnosis of cholangiocelular carcinoima [6, 19–21]. Budd-Chiari sindrome is caused by compromited hepatic veins blood flow and consequent development of thrombosis [18]. Blood analysis in patients with sarcoidosis can show leucopenia, lymphopenia, eosinophilia and monocytosis. ACE is elevated in around 70% of patients with hepatic sarcoidosis [6]. Elevation of alkaline phosphatase and γ-glutamyl transferase is found in 20–40% of patients [6, 10, 18, 22]. Transaminases may be normal or slightly elevated. Inflammatory markers (erythrocyte sedimentation rate, C-reactive protein) are normal or slightly elevated. Hypercalcemia may be present, as well as the increase in γ-globulins. Our patient had the elevation of alkaline phosphatase and γ-glutamyl transaminase, with normal transferase values . Regarding tha fact that we excluded alternate etiology of liver lesion (alcoholic, viral, autoimmune, cholestatic and metabolic liver disease), and a patient had an increase in ACE, we suspected on liver sarcoidosis. Abdominal imaging techniques [ultrasound, computed tomography (CT) and MRI] are used in setting the diagnose of liver sarcoidosis. Liver ultrasonography may indicate hepatomegaly, hyperechoic, inhomogeneous liver with or without nodules, denticulate edges and focal calcification [6, 23]. CT detect hepatic granulomas in less than 5%, since they are usually microscopic [18]. It can detect enlarged, homogeneous liver with diffuse, hypodense, various sized regenerative nodules, without contrast gain [6]. These nodules are low density in T2 sequences of MRI examination [6, 7]. Both imaging techniques can detect intraabdominal lymphadenopathy. The diagnosis of hepatic sarcoidosis is confirmed by pathological finding of uncaseous epithelioid granuloma, which are predominantly found in the portal and periportal areas [18]. Regarding the fact that granulomas can be found in other liver diseases, it is necessary to exclude other causes before the diagnosis of sarcoidosis. Epitheloid liver granulomas are with or without necrosis. Uncaseous granulomas without necrosis occur as a result of non-infectious diseases (sarcoidosis, primary biliary cirrhosis, drug-induced liver lesion) while granulomas without necrosis occur as a result of infectious genesis [24, 25]. In Western Europe and the USA sarcoidosis is in the second place (after primary biliary cirrhosis) as a cause of hepatic granulomas, accounting for 12–36% [12, 26, 27]. In the Middle East, the dominant cause of hepatic granulomas are infections diseases. In Saudi Arabia it is schistosomiasis (54%) [12], and in Iran the major cause is tuberculosis (52.8%) [28]. In presenting case imaging techniques indicated a modified cirrhotic liver, ascites and splenomegaly. Since the presence of ascites made percutaneous liver biopsy less desirable, we decided to go for laparoscopic liver biopsy. The diagnosis of liver sarcoidosis in our patient was confirmed based on histological findings of epithelioid granuloma in the portal and periportal areas, with the prior exclusion of other diseases that might cause similar changes. Normal finding of chest radiography and thoracic multislice computed tomografy (MDCT) excluded the diagnose of pulmonary sarcoidosis. Treatment of hepatic sarcoidosis depends on the clinical and laboratory presentations. Patients with histopathological finding of liver sarcoidosis, who are asymptomatic and had referent liver enzyms values need no therapy. Asymptomatic patients with mild elevation of liver enzymes but without clinical and laboratory sings of systemic sarcoidosis are suggested clinical and laboratory monitoring, without medication treatment with the possibility of a spontaneous resolution [6, 18, 29, 30]. In patients with refractory changes in liver enzyms values, clinical signs of cholestasis, cirrhosis, and systemic signs of sarcoidosis, corticosteroids are the treatment of choice. Most patients achieve satisfactory results with the low-dose prednisolone (10–15 mg/day), while in patients with jaundice and itching higher doses (40–60 mg/day) are used [18, 30]. Corticosteroids can not prevent disease progression including the development of portal hypertension, biliary duct depletion and fibrosis [8, 18]. Described cases of cholestasis enzymes normalization in patients with liver sarcoidosis responded to ursodeoxycholic acid therapy [8, 31]. Along the corticosteroid therapy immunomodulators (azathioprine, methotrexate, hydroxychloroquine, and infliximab) have a positive effect on symptoms, disorders of liver enzyms and hepatomegaly, but do not prevent disease progression [30]. The use of chloroquine, cyclosporine, cyclophosphamide, thalidomide and pentoxyphiline is also described but the strict guidelines do not exist [18]. In patients with advanced disease who do not respond to medical therapy, the only modality of treatment is liver transplantation [16]. Our patient was treated with prednisolone, which lowered the intensity of symptoms and improved laboratory findings. We started with diuretic therapy due to ascites, and propranolol due to portal hypertension with esophageal varices and gastric fornix varices. Clinical course was complicated by iatrogenic diabetes which was controlled by diet, oral antidiabetics and insulin.

Conclusion

Isolated hepatic sarcoidosis is a rare disease of unknown etiology which can lead to cirrhosis with all its complications. It should be considered in the making of differential diagnosis in both asymptomatic and symptomatic patients with hepatosplenomegaly and changes in the level of liver enzyms. Early diagnosis and appropriate therapy lead to subjective and objective improvement with a questionable effect on the progression of the disease.

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CASE REPORT

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Aggravation of symptomatic occipital epilepsy of childhood by carbamazepine

Pogoršanje simptomatske okcipitalne dečje epilepsije izazvano karbamazepinom

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Abstract

Introduction. Carbamazepine can lead to aggravation of epileptic seizures in generalized epilepsies (primary or secondary) with clinical manifestations of absence (typical or atypical) and/or myoclonic seizures. However, some focal epilepsies can be also aggravated by the introduction of carbamazepine. **Case report.** We presented a 10-year-old boy born after a complicated and prolonged delivery completed by vacuum extraction, of early psychomotor development within normal limits. At the age of 8 years he had the first epileptic seizure of simple occipital type with generalization and urination. Brain magnetic resonance imaging (MRI) showed focal cortical reductions in the left parietal and occipital regions. Interictal EEG recorded slowed basic activities above the posterior regions of the left hemisphere, with intermittent occurrence of occipital sharp waves and bioccipital sharp and slow-wave complexes. Initially, treatment with valproate was administered; however, the addition of carbamazepine into therapy induced aggravation of seizures and EEG findings, changed behavior and poor performance at school. By withdrawal of carbamazepine the condition improved both clinically and in EEG findings. **Conclusion.** Childhood occipital epilepsy lesions show deterioration due to carbamazepine, which if administered induces aggravation of seizures, behavior changes, cognition with occurrence of long-term bilateral discharges, and posterior sharp and slow-wave high amplitude complexes recorded by EEG.

Key words:
epilepsies, partial; seizures; valproate acid; carbamazepine; chilld; treatment outcome; drug incompatibility.

Apstrakt

Uvod. Karbamazepin može dovesti do pogoršanja epileptičnih napada kod generalizovanih epilepsija (primarnih ili sekundarnih), koje u kliničkoj slici imaju apsanse (tipične ili atipične) i/ili mioklonusne napade. I neke fokalne epilepsije, međutim, mogu se pogoršati uvođenjem karbamazepina. **Prikaz bolesnika.** Prikazali smo dečaka, starog 10 godina, rođenog posle komplikovanog i produženog porođaja koji je završen vakuum ekstrakcijom, urednog ranog psihomotornog razvoja. U 8 godini imao je prvi epileptični napad, tipa jednostavnog okcipitalnog, sa generalizacijom i umokravanjem. Nalaz snimanja magnetnom rezonancom (MRI) mozga ukazao je na fokalne kortikalne reduktivne promene parietookcipitalno, levo. Interiktusni EEG je beležio usporenu osnovnu aktivnost iznad zadnjih regiona leve hemisfere, sa povremenim izbijanjem okcipitalnih oštrih talasa, i biokcipitalnih oštar talas-spori talas kompleksa. U početku dečak je lečen valproatom, a kada je u terapiju uveden i karbamazepin, došlo je do pogoršanja napada, EEG nalaza, promene ponašanja i lošeg uspeha u školi. Isključenjem karbamazepina stanje se popravilo, kako klinički, tako i u EEG nalazu. **Zaključak.** Lezione okcipitalne epilepsije u detinjstvu pokazuju pogoršanje sa karbamazepinom, jer posle njegovog uvođenja dolazi do pogoršanja napada, promena ponašanja, kognicije sa pojavom dugotrajnih bilateralnih pražnjenja i posteriornih oštar talas-spori talas kompleksa visoke amplitude u EEG.

Ključne reči:
epilepsije, parcijalne; konvulzije; valproinska kiselina; karbamazepin; deca; lečenje, ishod; lek, inkompatibilnost.

Introduction

The basic goal of antiepileptic therapy (AET) is to fully control seizures with a satisfactory quality of life of the patient. AET is primarily aimed at the suppression of clinical manifestation of seizures, while the expected normalization of epileptiform EEG abnormalities is a reasonable therapeutic goal. The basic precondition of successful treatment is making the correct diagnosis of epilepsy. The type of seizures, the type of epileptic syndrome and etiology of epilepsy should be de-

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termined as soon as possible, as this will influence the choice of medication, mode of treatment and prognosis [1]. Today AET is still the first-line method in the treatment of most epilepsies, while the choice of drugs is in constant increase [2].

Carbamazepine (CBZ) is an antiepileptic with a wide clinical application in treatment of focal and secondary generalised tonic-clonic (GTC) seizures. Idiopathic generalized epilepsies (IGE), such as juvenile absence epilepsy, juvenile myoclonic epilepsy and epilepsy with GTC seizures on awaking feature seizure aggravation when CBZ is introduced into therapy [3, 4]. Epilepsies with clinical manifestations involving absences and/or myoclonic seizures, and EEG recording with bilateral generalized 4–6 Hz spike-and-wave complex discharges, show clinical and EEG worsening by introduction of CBZ [4]. CBZ-induced aggravation of focal idiopathic epilepsies is atypical form of childhood benign epilepsies with centrotemporal spikes (atypical BECTS) [5], atypical cases of Panayiotopoulos syndrome [6], and cases of late idiopathic occipital epilepsies of childhood (Gaustat type) [7]. Focal symptomatic epilepsies presenting aggravation by CBZ are occipital epilepsies of childhood with unilateral or bilateral occipital sharp-wave and slow-wave complexes recorded by EEG [5]. CBZ is also contraindicated in the following syndromes: Dravet, Dosse, Lennox-Gastaut, Angelman, Landau-Kleffner and epileptic syndrome with bilateral continuous spike-and-wave activity during slow-wave sleep,…[7–11]. Besides, their EEGs demonstrate bilateral discharges of spike-multiple spike and slow-wave complexes at 1–2.5 Hz, which clinically often correlates with atypical absences and myoclonic seizures. Misinterpretation of focal discharges as recorded by EEG in idiopathic generalized epilepsies and consecutive inclusion of CBZ, the medication for focal seizures, leads to the paradoxical aggravation of seizures [12].

Case report

We reported a 10-year-old boy, body weight (BW) 63.5 kg, body height (BH) 148 cm, born of the first term pregnancy after a complicated and prolonged delivery completed by vacuum extraction, with normal early psychomotor development, normal neurological and psychiatric status, negative heredity for epilepsy, febrile attacks, inflammatory and traumatic brain disorders. At the age of 8, one morning after breakfast, the boy developed the first epileptic seizure, which, according to the patient's case history and the parents initiated with optical images of bright colored circles, headache, version of the eyes and head to the left, tonic-clonic convulsions right-sided at onset and later of the whole body, loss of consciousness and urination. Seizures occurred every 1–2 months, during the day only. Standard EEG demonstrated a slow basic activity above the posterior region of the left hemisphere with occasional focal sharp spike-slow-wave complexes or slow-waves at 4–5 Hz in the left parietal-occipital and temporal-occipital regions (Figure 1). Short bioccipital sharp and slow-wave complexes at 1.5 Hz, amplitude exceeding 100 μV with left-sided predomination were also recorded. In view of such EEG findings in correlation with cortical MRI reductions in the left parietal-occipital region it was implicitly concluded that this was indicative of a symptomatic epileptic lesion of the occipital lobe with secondary generalization. Valproat (VAL) in the dose of 30 mg/kg/day was included into therapy, which resulted in a complete control of secondary GTC seizures over a 6-month period. As the patient still complained of occasional visual problems followed by headaches, CBZ in dose of 10 mg/kg/day was also included into therapy. After including of CBZ, GTC seizures relapsed, which led the neurologist to increase the dose of CBZ to 30 mg/kg/day, without previously performing a check-up EEG. Beside the aggravation of seizures, the parents noticed changes in the child's behavior: he seemed withdrawn, often aggressive and performed poorly at school. Being dissatisfied with the developed situation, the parents turned to another neurologist. EEG was performed showing the presence of long-term bilateral discharges of a posterior sharp wave-slow-wave complex at 1–1.5 Hz, amplitude exceeding 300 μV (Figure 2), which are seen in aggravation by carbamazepine. On check-up, blood antiepileptic (VAL and CBZ) levels were within the therapeutic levels. Based on the deterioration of clinical features and EEG findings, it was concluded that CBZ was probably the cause of the developed condition. By withdrawal of CBZ, secondary GTC seizures ceased, as well as the long-standing

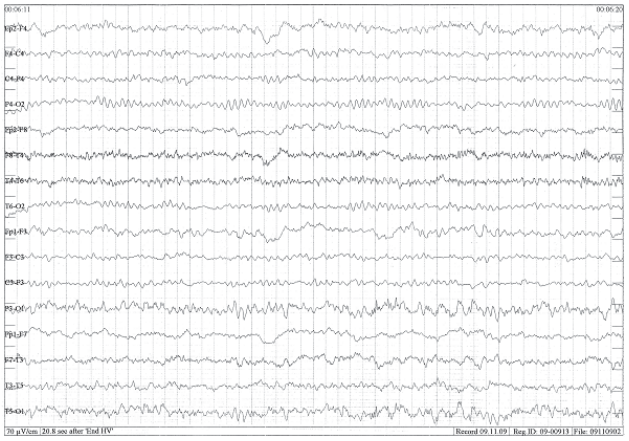

Fig. 1 – EEG before introduction of carbamazepine (CBZ) into therapy.

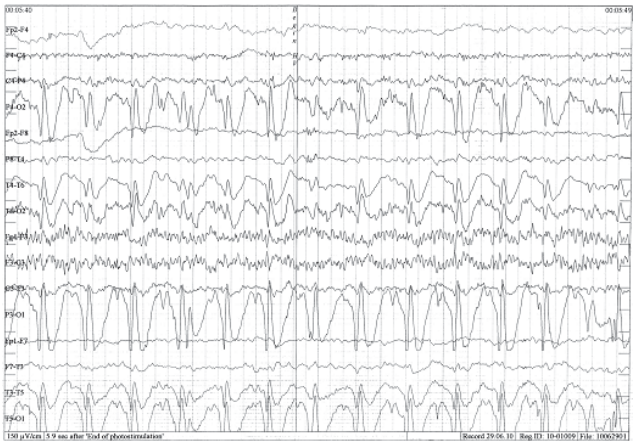

Fig. 2 – Long-term discharges of bilateral posterior high-voltage sharp-wave and slow-wave complex.

bilateral discharges of the high-voltage posterior sharp wave-slow-wave complex (Figure 3). Carbamazepine was replaced with lamotrigine, but having caused skin rash it had to be withdrawn. By a daily dose of 500 + 0 + 500 mg valproate and 25 + 0 + 25 mg topiramate, we achieved a full control even of simple occipital seizures. The patient has been without seizures for a year now, cognitive and affective disorders withdrew and his performance at school also improved.

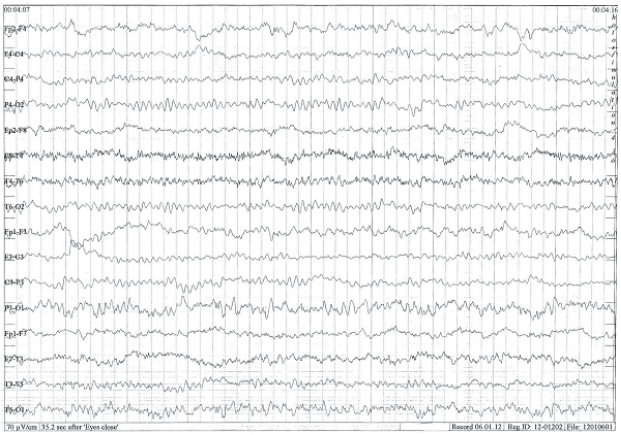

Fig. 3 – Discontinuation of bilateral posterior high-voltage sharp-wave and slow-wave complex discharge after withdrawal of carbamazepine (CBZ) from therapy.

Discussion

Occipital lobe epilepsies are not frequently seen in the clinical practice, and have a prevalence ranging from 5–10% of all epilepsies. They can be symptomatic, i.e. of cryptogenic etiology occurring at any age or idiopathic which as a rule occur in childhood [13]. In symptomatic occipital lobe epilepsies about 50% of children have MRI ischemic lesions of type porencephalia, periventricular leukomalacia or cerebral infarction [14]. The clinical semiology of seizures involves visual and oculomotor symptoms. Seizures are of simple focal type, while the occurrence of complex focal seizures is the sign of extra-occipital propagation of discharges in the parietal, temporal or frontal lobes. The visual symptomatology is predominated by elementary, quite rare and complex visual hallucinations, illusions and palinopsia. They are often in the form of small colorful circles moving across the visual field or rarely flashing and flickering lights [15]. Elementary visual hallucinations are often followed by oculomotor symptoms with tonic deviation of the eyes and head, epileptic nystagmus or eye blinking. Visual hallucinations are the key symptoms suggesting an occipital focus. If visual symptoms are not marked, the semiology of seizures and standard EEG can be a frequent cause of incorrect diagnosis, as they often reflect the condition of discharges propagation and less their initiation [16]. Interictal EEG shows occipital spikes and/or sharp wave slow-wave complexes in 57% of cases, unilaterally or bilaterally [17]. Biocipital discharges of sharp wave and slow wave complexes (spike and slow-wave complex) occur in epilepsies of symptomatic and idiopathic etiology [18]. Prognosis is favorable in idiopathic and unfavorable in symptomatic epilepsy when it essentially depends on the type and size of the causal lesion. Pharmacoresistance is seen in most cases with disorders of cortical development or tumor of the occipital lobe when surgical treatment is the method of choice [17]. By the example of our patient the deterioration of seizures occurred when, beside VAL, CBZ was introduced into therapy in order to control simple occipital seizures followed by headaches. Despite the parents' suggestion that seizures worsened after the introduction of CBZ, the neurologist increased CBZ dose without performing a check-up EEG; i.e. therapeutic effects of CBZ were not under concurrent clinical and EEG follow-up. CBZ can induce unusual electroclinical manifestations that can be explained by synchronization and increased bilateral EEG discharges [19]. It is well known that CBZ can increase present or activate new bilateral EEG discharges of spike-polyspike and spike-wave complex and sharp wave-slow wave complex, and that it is contraindicated in such cases [11, 20]. Oxcarbazepine, vigabatrin, tiagabine and gabapentin are also contraindicated [5, 9, 21]. Increased bilateral EEG discharges often result in exacerbation of seizures, as well as the occurrence of other type of seizures [6]. It should be pointed out that it is impossible to predict the effect of an antiepileptic on the EEG of a person with epilepsy and that some patients respond to antiepileptics on individual for a group unpredictable way [22]. In our patient, the deterioration of EEG findings induced by CBZ correlated with the aggravation of seizures, change of behavior and cognition. Studies have shown that long-term bilateral interictal discharges of spike-wave complex or sharp wave-slow wave complex, particularly in childhood, can be followed by transitory cognitive and behavioral disorders [20, 23]. In children and adults with epilepsy, bilateral interictal EEG discharges of spike-polyspike and spike-wave complex or sharp wave-slow wave complex are a stabile predictor of aggravation by carbamazepine [11, 12]. Our example shows that symptomatic lesions of occipital lobe epilepsy, which have unilateral and/or bilateral discharges of sharp-and-slow-wave complex in interictal EEG, can also manifest aggravation by CBZ. In such cases it is possible that there is also present an additional (probably genetic) factor that enables the reaction of aggravation by CBZ. Although epilepsy is primarily a clinical diagnosis, the correlation of epileptic seizures and EEG changes is necessary, both for diagnostics, as well as for the follow-up of antiepileptic therapy effects [1].

Conclusion

Epilepsy treatment with drugs inadequate for the given type of seizures has a deteriorating effect on epilepsy, and when the drug of choice causes clinical worsening correlating with the activation of new and increase of already present EEG discharges, aggravation by an antiepileptic should be taken into consideration. Lesional occipital epilepsies of childhood show aggravation by carbamazepine, which, if administered, induces aggravation of seizures, behavior changes, cognition with the occurrence of long-term bilateral discharges, and posterior sharp-wave and slow-wave high amplitude complexes recorded by EEG.

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CASE REPORT

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Bilateral giant angiomyolipomas revealed after massive retroperitoneal hemorrhage – A case report

Veliki bilateralni angiomiolipomi otkriveni posle masivne retroperitonealne hemoragije

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Abstract

Introduction. Angiomyolipomas (AML) are benign neoplasms composed of fat, smooth muscle and thick-walled blood vessels in varying proportions. These tumors have a significant female predominance. **Case report.** We reported a 61-year-old man with spontaneous rupture of AML. Computerized tomography revealed a change in morphology of both kidneys. Multiple lesions of fat density with dilated blood vessels were found in the left kidney. The right retroperitoneum was obliterated with a giant heterogeneous mass originating from the right kidney with a massive hemorrhage, active extravasations, compression of inferior the vena cava and intraperitoneal collections. After radical nephrectomy, histological examination revealed that the tumor was composed of relative proportions of fat, smooth muscle and blood vessels. We incidentally found small renal adenoma. **Conclusion.** The true nature of AML is unclear, but they are usually classified as hamartomas. Angiomyolipomas are generally benign lesions, although the epithelioid angiomyolipoma, a subtype that occurs in about 3% of cases, can behavior aggressively.

Key words:
kidney neoplasms; angiomyolipoma; diagnosis, differential.

Apstrakt

Uvod. Angiomiolipomi (AML) su benigni tumori, izgrađeni od masnog i glatkomišićnog tkiva i krvnih sudova u različitim odnosima. Ovi tumori češći su u ženskoj populaciji. **Prikaz bolesnika.** Predstavljen je bolesnik, star 61 godinu, sa spontanom rupturom AML. Kompjuterizovanom tomografijom uočena je izmenjena morfologija oba bubrega. U levom bubregu viđene su multiple lezije u masnom tkivu sa dilatiranim krvnim sudovima. Na desnom bubregu uočena je velika heterogena masa koja je ispunjavala desni retroperitonealni prostor, sa masivnim krvarenjem, aktivnom ekstrvazacijom, kompresijom donje šuplje vene i intraperitonealna kolekcija. Posle radikalne nefrektomije, histološkom analizom utvrđeno je da tumor čini nejednak odnos masnog i glatko-mišićnog tkiva i krvnih sudova. Mali bubrežni adenom bio je uzgredan nalaz. **Zaključak.** Pravo poreklo ovih AML nije u potpunosti razjašnjeno, ali često se svrstavaju u hamartome. To su uglavnom benigni tumori, dok su epiteloidni angiomiolipomi varijanta koja se sreće kod oko 3% slučajeva, mogu da imaju agresivni tok.

Ključne reči:
bubreg, neoplazme; angiomiolipom; dijagnoza, diferencijalna.

Introduction

Renal angiomyolipoma (AML) stands for mostly benign tumors originating from mesenchymal elements of the kidney [1]. They occur with an incidence of 0.3–3%, indicating that such lesions are present in more than 10 million people worldwide [1, 2].

AML may appear associated with tuberous sclerosis or as an isolated lesion with frequency of symptoms and risk of bleeding increasing with the size of the lesion [3–9].

Ultrasonography (US), computered tomography (CT) or magnetic resonance imaging (MRI) are usually sufficient for the diagnosis, so histological confirmation with biopsy is rarely needed [10].

The main mortality from AML is spontaneous life-threatening hemorrhage 11.

Herein we reported a case with bilateral multifocal renal angiomyolipomas and massive retroperitoneal hemorrhage resulting from the rupture of pseudoaneurysm of the renal artery branch.

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Case report

A 61-year-old patient presented with sudden abdominal pain, palpable right flank mass and weakness. During the transport to an emergency diagnostic center for suspected rupture of abdominal aorta aneurysm, the patient developed hypotension and developed shock.

CT of the aorta demonstrated normal findings. Incidentally, CT revealed a change in morphology of both kidneys. Multiple lesions of fat density with dilated blood vessels were found in the left kidney. The right retroperitoneum was obliterated with giant heterogeneous mass originating from the right kidney with massive hemorrhage, active extravasations, compression of the inferior vena cava and intraperitoneal collections (Figure 1).

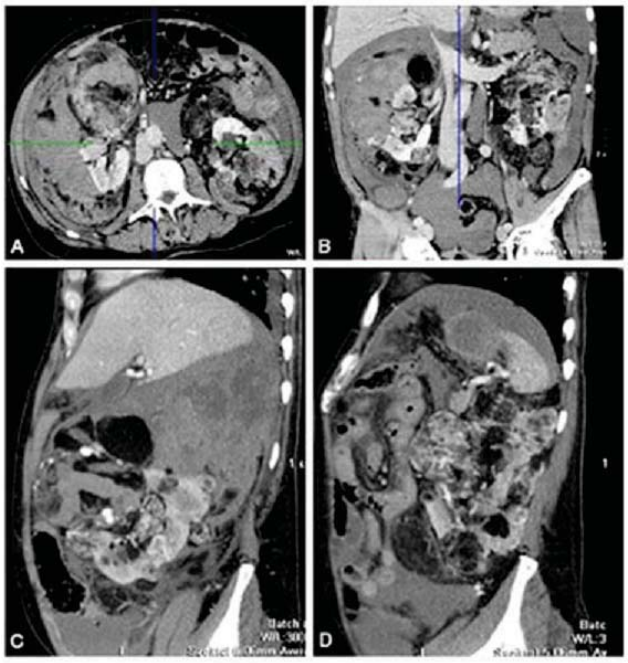

Fig. 1 – Postcontrast multiple detector computed tomography (MDCT): A) Sagittal and B) Coronal plane, show bilateral angiomiolipomas of the kidney with right sided retroperitoneal haemorrhage; C) Right sided kidney angiomiolipoma with haemorrhage; D) Left sided kidney angiomiolipoma.

Radical right nephrectomy was performed on the same day and samples were sent for pathological verification.

Grossly, the lesion was predominantly pale yellow, 7 cm in size with massive areas of hemorrhage (Figure 2).

Microscopically, the lesion had the relative proportions of fat, smooth muscles, and blood vessels. The adipose tissue was composed of uniform fat cells with large cytoplasmic vacuoles and small peripheral nucleus. The smooth muscle cells were typically spindle shaped but occasionally they were epithelioid and had abundant eosinophilic cytoplasm. The vascular components consisted of large thick walled tortuous blood vessels.

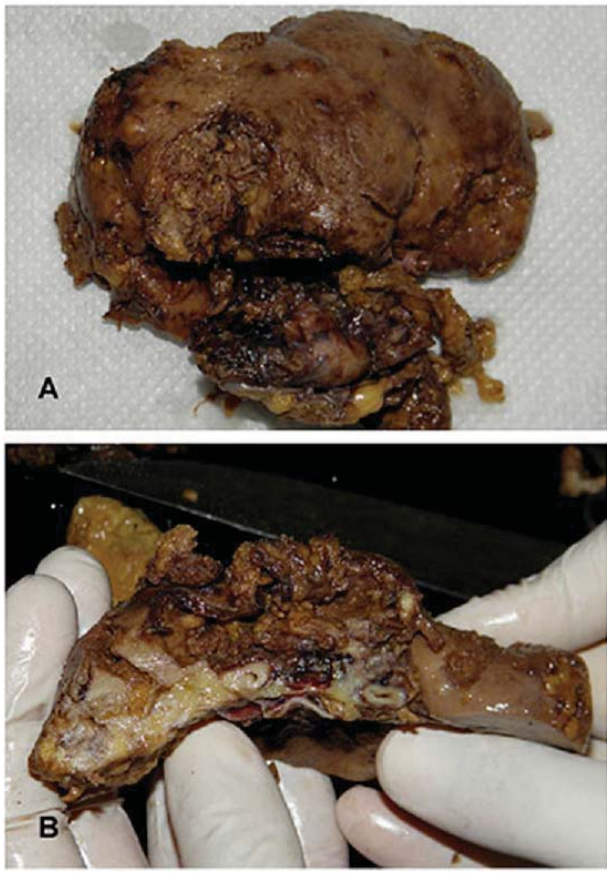

Fig. 2 – A) The tumor measuring 7 cm in its maximum diameter, protruded from the renal capsule in the upper-pole; B) The cut surface exhibited yellowish solid and hemorrhagic degeneration zones.

According to immunohistochemistry, tumor cells were positive for melan-A, HMB-45, CD117, CD68. Moreover, tumor cells were negative for S-100 protein, as well as for epithelial markers such as cytokeratin and epithelial membrane antigen (Figure 3).

Incidentally, we found a small renal adenoma. The cells had round to oval nuclei with chromatin that ranges from stippled to clumped, as well as inconspicuous nucleoli (Figure 4).

Because of the increase of nitrogen products in blood, the patient underwent dialysis. However, after dialysis creatinine values w ere still increasing.

After achieving diuresis of 1,000 mL, on the postoperative day 4, control CT examination was performed.

CT of the abdomen presented completely distorted morphology of the left, remaining kidney, caused by multifocal angiomyolipoma, with patches of preserved renal parenchyma.

Considering high rate of comorbidity with tuberous sclerosis, especially in bilateral angiomyolipomas, the patient underwent CT of the brain, which demonstrated normal findings.

Ten months after the first intervention the patient underwent radical left nephrectomy. As suspected pathological diagnosis was also angimyolipoma.

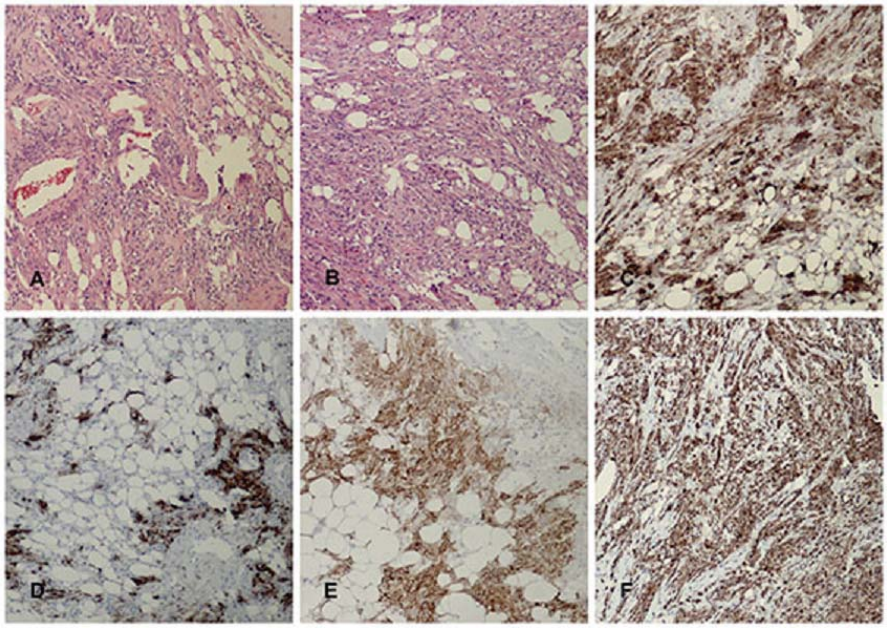

Fig. 3 – A) and B) Angiomyolipoma composed of thick-walled blood vessels, smooth muscle, and fat (hematoxylin-eosin, original magnification ×10); C) Cytoplasmic granular diffuse immunopositivity for Melan-A in the tumor cells (original magnification ×10); D) Cytoplasmic granular focal immunopositivity for HMB-45 in the tumor cells (original magnification ×10); E) Cytoplasmic granular immunopositivity for CD-117 in the tumor cells (original magnification ×10); F) Cytoplasmic granular immunopositivity for CD-68 in the tumor cells (original magnification ×10).

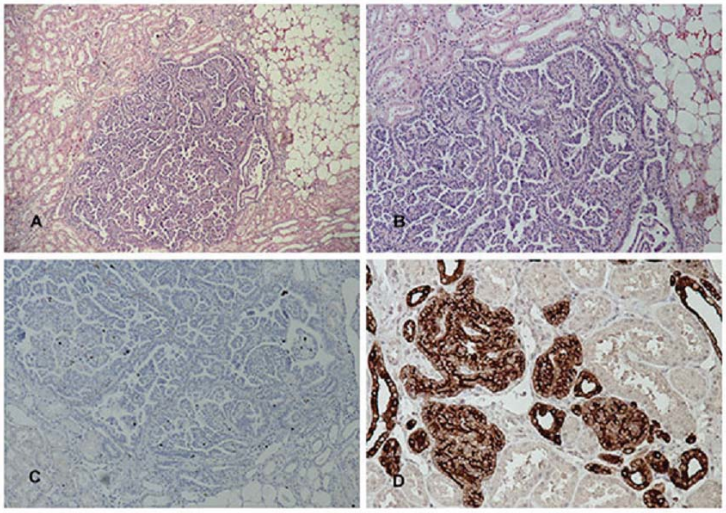

Fig. 4 – A) Cortical adenoma (hematoxylin-eosin, original magnification ×4); B) Cortical adenoma (hematoxylin-eosin, original magnification ×10); C) Neoplastic cells are Ki-67 negative (original magnification ×10); D) Neoplastic cells are strong EMA positive (original magnification ×20).

Discussion

Renal angiomyolipoma is the second most common benign tumor of the kidney, and accounts for 3.7% of all renal masses [12].

It is composed of variable amounts of mature adipose tissue, smooth muscle, and thick-walled blood vessels derived from perivascular epithelioid cells [6, 11].

AML occurs with overall female predominance of approximately 4 : 1 to 8 : 1, suggesting the role of female hormones in tumor growth [4]. There are two clinical forms: as isolated phenomenon or a part of the syndrome associated with tuberous sclerosis. Isolated AML occurs sporadically, accounts for 80% of cases and is usually solitary, while those associated with tuberous sclerosis are typically larger, have multifocal or bilateral disease, tend to occur in younger patients and bleed more often than sporadic AMLs [4, 13].

At presentation, most patients are asymptomatic, with AML presenting as an incidental finding on imaging done for other reasons [14]. Although most AML are benign and asymptomatic, symptoms develop in 68–80% of patients when tumor size reaches 4 cm or more [2]. Symptomatic patients classically suffer from flank pain (53%), a palpable tender mass (47%) and gross hematuria (23%); this is known as “Lenk's triad” [15]. Clinical manifestations less frequently include nausea or vomiting, fever, anemia and blood pressure changes [2].

More than 51% of symptomatic cases are presented with haemorrhage [2].

AML is the most common cause of spontaneous renal hemorrhage which, presented with the classic triad of symptoms – acute abdominal pain, palpable mass and hypovolemic shock, is referred to as Wunderlich syndrome. Wunderlich syndrome appears in up to 10% of patients with AML, thus considered the most severe complication of these lesions [16–18].

The histological appearance of AML may vary. Nuclear pleomorphism may be pronounced and mitotic figures may be present. But these findings have no adverse prognostic significance in most cases. In some cases, angiomyolipomatous tissue has been found in regional lymph nodes and spleen. This finding should not be misinterpreted as metastatic sarcoma. Occasionally, angiomyolipoma invades the renal vein or vena cava; all these patients are cured surgically, so this does not indicate malignancy.

AMLs are typically positive for melan-A and HMB-45 antibody raised against melanosome-related antigen. They are also known to be positive for other melanocytic markers such as HMB-50, tyrosinase, and microphthalmia-associated transcription factor. Other markers for AML are CD117 and CD68 [19, 20]. AMLs exhibit variable immunopositivity for myoid markers such as smooth muscle actin, musclespecific actin, desmin, and calponin. About 25% of AMLs express estrogen and progesterone receptors. Angiomyolipomas are typically negative for S100 protein and epithelial markers such as cytokeratin and epithelial membrane antigen [21, 22].

Because of the benign nature of renal AML, the principles of management are resolution of symptoms and preservation of renal function. The choice between current management approaches (observation with monitoring of tumor size, selective arterial embolization, renal-conserving surgery and total nephrectomy) is made based on the following: size of the tumor; the presence of significant symptoms such as pain, severe hemorrhage and risk of rupture; and suspicion of a malignant tumor [23]. Nephrectomy, partial or radical, is indicated if there are persistent hemorrhage, suspicion of malignancy, or failed embolisation [24].

Incidental finding of bilateral AML, not associated with tuberous sclerosis, in an elderly male patient, developing symptoms only after spontaneous rupture and hemorrhage, is, in our opinion, considered extremely rare. On the other hand, considering positive correlation between the size of the lesion and risk of bleeding [2, 4], giant AML of the right kidney in our patient was extremely prone to rupture. At the moment of presentation, AML was already complicated by rupture followed by Wunderlich syndrome, which is one of the most feared complications of renal AML and required aggressive management.

Surprisingly, beside histopathological verification of AML of the right kidney, adenoma was also found in tissue of the operatively removed kidney. To our knowledge, there are only two reported cases of concurred occurrence of adenoma with AML, both of them adrenal adenoma. One of them is in the homolateral adrenal gland, and the other one intrarenal, ectopic, adrenal adenoma [25, 26].

Conclusion

The true nature of AMLs is unclear, but they are usually classified as hamartomas. Angiomyolipomas are generally benign lesions, although the epithelioid angiomyolipoma, a subtype that occurs in about 3% of cases, can behavior aggressively.

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Invalidity and deformity in the art of Weimar Republic

Invaliditeti i deformiteti u umetnosti Vajmarske Republike

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Key words:
world war I; germany; art; disabled persons.

Ključne reči:
prvi svetski rat; nemačka; umetnost; invalidi.

Introduction

World War One (WWI) brought enormous losses to entire Europe. Eight million soldiers lost their lives and 7 million suffered untreatable injuries and were forced to spend the rest of their lives living with disability. Germany lost 15.1% of their active male population. It can be said that after the Great War, Europe was unrecognizable. It is hard to say whether people could even comprehend the tremendous losses they have suffered and to mourn their dead before they were forced to deal with the great crises and the rise of Nazi party.

Needless to say that the Great War has brought great loss to Europe and Germany especially and the consequences that Germany had to face were enormous. A society destroyed by war was incapable to retrieve its faith in life. The faith in life and the faith in the world were beginning to die in the hearts of men. Remarque's words best describe these feelings: *We were eighteen and had begun to love life and the world; and we had to shoot it to pieces. The first bomb, the first explosion, burst in our hearts* [1].

The Great War had wounded the minds of millions of people which later on produced a lot of opposing ideological movements. The war had caused political maturing of the arts, but also a great protest against the society from which a great cataclysm was born.

A war is a destruction force that destroys everything in its path, but out of all the ruins, art never fails to rise up and heal the souls of those who had survived the devastating claws of war. We must remember the importance of art and the role it plays in our lives, especially in times of crises.

Art after the war

What was Germany left with after WWI? Crises, poverty, decadence. However, in the art field, this period was very productive. Many artists, confronted with the horrors of war, expressed themselves through their art. The artists who were working in Germany in the postwar period were surrounded with disturbing images which they presented in their work portraying a society of a war racked country.

George Grosz, having felt the devastating forces of war on his own skin, used his art as a weapon for criticizing the society. He had become an activist of the pacifist left wing. He published his satiric drawings in many periodicals and participated in different protests and social riots. In his 1946 Autobiography, Grosz remembers what had affected him the most after he was discharged from the army and had returned to Berlin: 'The Berlin to which I returned was cold and grey… The same soldiers who were seen in cafes and wine cellars singing, dancing and clinging drunkenly to the arms of prostitutes, were to be seen later dirty and unkempt, dragging their weary from station to station… My drawings expressed my despair, hate and disillusionment. I had utter contempt for mankind in general… I drew soldiers without noses; war cripples with crustacean-like steel arms; two medical soldiers putting a violent infantryman into a straitjacket made of a horse blanket; a one-armed soldier saluting a lady decorated with medals who was putting a cookie on his bed; a colonel, his fly open, embracing a nurse, a medical orderly emptying into a pit a pail filled with various parts of the human body [2]. The drawings and the paintings from this period represent a severe criticism of what was seen by Grosz as one society's decay.

In the portrait of a society, the people in Grosz's work don't represent specific individuals. They are allegoric figures that represent different classes and different troubles that have rained on the German society. The use of allegory has enabled Grosz to criticize the society without moving to far away from the ideals of portraying a modern vision of reality. In the painting Grey Day (Figure 1) we can see in the foreground a well-dressed man, behind him a partially build

*** At the time the study was conducted the author was empoloyed at this institution**

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Fig. 1 – George Grosz: Grey Day, 1921, Oil on canvas, 115 × 80 cm (Staatsliche Museen zu Berlin, Nationalgalerie, Berlin, Germany).

wall and a worker. Far away in the background a war veteran can be seen. He is still in his uniform, a cane in one hand and without the other. He is walking down the street, his face expressionless. The main subject of the painting Republican Automatons (Figure 2) is the transformation of man into ma-

Fig. 2 – George Grosz: Republican Automatons, 1920; Watercolor on paper, 60 × 47.3 cm (The Museum of Modern Arts, New York, NY, USA).

chine, but the image of a war veteran is also present and painfully obvious. The two figures are obviously war veterans, invalids without their arms and legs. These two faceless automatons with prosthetics and cylindrical, machine-like limbs stand in front of a background of rectangular buildings and empty streets. The automaton on the right is a war cripple, his arms amputated, but he is still a determined military man which is expressed by his Iron cross and the slogan '1, 2, 3, Hurra' which is coming out of his empty head. The clothes of the figure on the left label him as middle class, and his wooden leg and functional arm prosthetic reveal him to be a war cripple. He is holding a black, red and golden flag of the Republic with his metal claw. The flag is on shaky grounds if these crippled, mechanical men are viewed as its base.

George Grosz didn't include the war cripples in his work by accident. As a portraitist of a society who tries to present the cruel reality of the world in the most satirical way, Grosz incorporates in his work elements from reality which surrounds him. War cripples were the reality of the German cities. They could be seen on the streets of the cities all over the country. After the war, Germany fell into a crisis, and the government couldn't take care of the people who had fought for it and became invalids in the process. Unfit to work, powerless and helpless, they were rejected by their own country and were forced to live on the street as bums and beggars filled with the feeling of nonsense that was surrounding them as they wondered the streets aimlessly.

Otto Dix also portrays war cripples who were forced to live on the street and to make their living by begging. The war veterans in his works are crippled by war, ignored and pitied by passers-by. They are drowning in poverty. In the painting The Match Seller (Figure 3) we can see a war vet-

Fig. 3 – Otto Dix: The Match-box Seller, 1920; Oil and collage on canvas (Staatsgalerie, Stuttgart, Germany).

eran, recognizable by his old hat that was once a part of a uniform, sitting on the curve. Black glasses indicate that he is blind. He has also lost both his arms and both his legs which are replaced by two wooden ones. His amputated legs are made much more conspicuous because of the contrast they form with the long legs of the passers-by who are trying to bypass him. The only living thing that acknowledges the man's existence is a dog urinating on the stumps of his legs. This painting evokes pity in the viewer because of the isolation and the poverty of the portrayed man who is not pre-

sented as an officer decorated with medals, but as a homeless person trying to make a living by selling matches on the street. His wooden legs only emphasize his helplessness and his vulnerability.

Another street scene is presented in Dix's Prague Street (Figure 4) where two cripples are presented. In the background of this popular shopping street in Dresden are two shop windows: one is that of a cosmetics shop (representing women's sphere) and the other is a window of a shop selling prosthetics (representing men's sphere). This juxtaposition indicates a feminized and passive position of crippled veterans. One of the men is sitting on the ground in front of the shops. He has absurd prosthetics, his legs look like two sticks and his left arm is a strange mechanical composition. His right arm is outstretched, awaiting charity. The other veteran who is wearing a war medal is missing the entire lower part of his body. He is set up on a platform with wheels which he moves with two sticks. While the first veteran has a lonesome gaze, the pretentious military posture of the other half-man expresses a grotesque effect of the painting [3].

Fig. 4 – Otto Dix: Prague Street, 1920; Oil on canvas (Staatsgalerie, Stuttgart, Germany).

Dix was familiar with postwar mass demonstrations organized by war victims and these images served as inspiration for his painting War Cripples (Figure 5). Four grotesque war invalids are presented in the painting. They are wearing their medals and parading down the street in front of a shoemaker's shop. They all have primitive or fantastic prosthetics with the exception of the third man who is a torso in a wheelchair pushed by the fourth man. The wavy lines and blurred image of the second man mark him as a 'shiverer'. This is a rare opportunity where a psychically traumatized person can be seen, especially a World War I veteran, but these people could be seen on the streets and Dix found a way to show his suffering as well. In the background of the painting a hand points to Dix's own profile with crosshairs over it, perhaps indicating that it was only by chance that he had escaped a similar fate during his military service. The subtitle of the painting, Four of These Don't Add up to a Whole Man, makes explicit Dix's passionate critique of the inhuman uses of technology. At the same time, the pomposity of these wrecked figures and their absurd attempt to keep their military dignity and to keep marching makes them appear as worthless remains of the Prussian army. The war machinery has produced these human wrecks but Dix's portrait of their grotesque prosthetics shows that the peace machinery was not able to put these men together [3]. After the Nazis came into power, the painting was confiscated and exhibited at the Entartete Kunst [4] exhibition in Munich in 1937 under the title Slander against the German Heroes of the World War. After the exhibition the painting went missing and it is presumed to have been destroyed together with many other works of 'degenerate art'.

Fig. 5 – Otto Dix: War Cripples, 1920, Oil on canvas (location unknown).

The painting The Skat Players (Figure 6) portrays three German officers mutilated by war who are playing cards. Two of them have lost their legs, while the one portrayed on the left

Fig. 6 – Otto Dix: Skat Players, 1920; Oil on canvas with photomontage and collage, 110 × 87 cm (Nationalgalerie Staatliche Museen zu Berlin, Berlin, Germany).

is using his leg to hold the cards, since he has lost both his arms. Their prosthetic limbs are intertwined with the table legs in the bottom of the painting. Two of the portrayed men have artificial jaws and one is missing an ear and instead of it has a long, snakelike ear trumpet. All the figures are very badly mutilated. The images of these people were very common in Weimar Republic during the 1920s, as well as in the rest of Europe. They were unfit, unwanted people who were a sore reminder of war and failure. Being reminded about death, loss and weakness were things everyone tried to avoid. The whole society was in a state of denial, turning their heads from the reality that was too horrible to bear. Otto Dix was one of the people who didn't turn his head away. His artistic eye noticed all the people's troubles and pain that overflew German cities. He felt all the horrors that were surrounding him and he felt the need to express them.

Berlin of the 1920s was a city of contrasts. On the one hand, it was the city of leisure, fun and entertainment, and on the other it was a city full of pain, misery, poverty, homelessness and prostitution. Metropolis (Figure 7) is a representation of exactly this Berlin. In the middle part of the triptych a typical cabaret scene is presented. Loud music and ladies dressed in silk and gentlemen in flawless suits with bowties are dancing Charleston. This is only an illusion of the big city. The reality is depicted in the right wing of the triptych where a street scene in a rich neighbored is presented. Here, we can see well-dressed ladies with rich makeup on their faces, ugly and stupid, with arrogant expressions, obviously of less than reputable nature. Under the feet of these morally gray passers-by we can see a war cripple sitting. He is dressed in rags. He is without a nose and without legs. He is the central figure of the scene. Pushed into a corner, hidden it the shadows he is a reminder of the social injustice that is the foundation of the big city. The left part of the triptych finally reveals the ugly truth: it depicts the pour neighbored. In an ugly street, between modest houses, daughters of beggars are selling their love. A war cripple who is still wearing his ragged uniform waddles on his crutches, another war veteran is lying on the street drunk or dead while a small dog barks at passers-by. Metropolis shows the capitalist Weimar. On one hand we can see the illusion of the easy life, on the other we can see the reality based on social differences

Fig. 7 – Otto Dix: Metropolis, 1927–28; Wood, distemper, 181 × 404 cm (Kunstmuseum Stuttgart, Stuttgart, Germany).

and injustices and also the horrifying poverty that runs deep in the core of the society. When presenting poverty, Dix does not hide anything. On the contrary, he presents everything as it is, he emphasizes the ugliest aspects of life trying to reveal the truth hidden behind the cabarets' lampions and that is the misery of the residents of the rich neighbored [5].

Max Beckman portrays invalids in his ten lithograph series named Hell. The first plate, The Way Home (Figure 8), presents the mutilated veteran, the pimp, the prostitute, the harsh lights and the symbolic snarling dog [6]. In the foreground we can see two figures facing each other beneath a street lamp. One is a veteran whose face has been largely blown away. He is without a nose and almost eyeless. The

Fig. 8 – Max Beckmann: The Way Home (Hell), 1919; Lithograph, 87.00 × 61.00 cm (The Metropolitan Museum of Art, New York, USA).

stump of his arm protrudes from his sleeve, which the other figure, Beckmann himself, grips with one hand while pointing 'the way home' with the other. In the background, two crippled veterans hobble along on crutches behind a prostitute. It is not clear whether the wounded veteran can see where Beckmann is pointing. The second plate, The Street (Figure 9), includes a disabled veteran using a clumsy wheelchair along with a blind beggar in the chaotic clinch of bodies on the street during the November Revolution. Disability becomes a formal organizing principle in this fragmented, compressed jumble of limbs. The viewer must look closely to discern where one body ends and another begins; where body and inanimate object merge.

Fig. 9 – Max Beckmann: The Street (Hell), 1919; Lithograph, 87.00 × 61.00 cm (The Metropolitan Museum of Art, New York, USA).

The disabled and the post-war society

The question is: where was the place of the disabled veterans in postwar Germany and its society? Did they have the same rights as the rest of the citizens and did they even have the right to exist? The 'cult of health and beauty' associated with the life reform movement since the late 19th century still flourished after the war, serving in many ways to create a hostile atmosphere toward those viewed as ill, disabled or ugly. Similarly, the discourses of degeneracy and eugenics had also begun in the late 19th century. The perception that the war had killed or disabled many of the healthiest young German men, however, gave a strong impetus both to postwar advocates of eugenics who opposed squandering the nation's resources on the 'unfit' and thus wanted to limit their reproduction and to proponents of outright 'euthanasia' such as Karl Binding and Alfred Hoche. The disabled were unwanted by society whether their disability was a product of war or not. People preferred not to see them. War invalids were mostly destined to live on the street and off of charity. Some lived hidden from the eye of the public, locked away by their families, while about 70,000 war invalids died of hunger in psychiatric facilities. Some appeared in 'freak shows' at fairs such as in Christian Schad's painting Agosta, the Winged Man and Rasha the Black Dove (Figure 10). In the picture, both Agosta and his

Fig. 10 – Christian Schad: Agosta, the Pigeon-Chested Man, and Rasha, the Black Dove, 1929; Oil on canvas, 1200 × 800 (Tate Modern, London, United Kingdom).

companion are looking out at the spectator with the serene expression of those used to public scrutiny. Our attention is immediately drawn to Agosta's deep pectus excavatum with outward deformities of the lower halves of the anterior rib cage. A long and thin thorax and a relatively long left arm can be observed. In addition, there is an obvious kyphosis or kyphoscoliosis affecting his shoulder girdle. The second and third fingers of his right hand are awkwardly positioned with hyperextension of proximal and distal interphalangeal joints suggesting joint laxity. His face is straight with slant-down eyes. His extraordinarily large arm span, chest and back deformities together with the possibility of joint and tissue hypermobility as well as his facial appearance suggest the diagnosis of Marfan's syndrome [7].

People with disabilities, whether they were the consequence of the war or not, were marginalized, both consciously and unconsciously and there was a tendency towards excluding these people from the social sphere. The term 'degeneracy' was coined in the late 19th century by Max Noradu in his book Entartung (Degeneracy) and it provided the theoretical basis for further marginalization of the disabled. Under the influence of Social Darwinism, Nordau was advocating the persecution of the 'degenerate' by the healthy. The degenerate were not only inferior, but were a threat for the upstanding society. Paul Schultze-Naumburg's Kunst und Rasse (Art and Race) criticizes modern art, including Expressionism and other art movements which he defamed as degenerate by comparing them to photographs of disabled people. Photographs of people suffering from Mongoloid idiocy, paralysis of eye muscles, microcephaly, idiocy, elephantiasis, rickets, anencephaly, acromegaly of hands and lower face, severe harelip, chondrodystrophy, obesity, cretinism, nervous disorder of late-stage syphilis and encephalitis were compared with the works of Picasso, Kokoschka, Modigliani, Hofer, Nolde, Schmidt-Rottluff and others in order to prove their degeneracy.

When the Nazis came into power in 1933, one of the first actions they took was the attack on contemporary authors, burning of books and the attack on modern art of the previous period. The Nazis discarded and censured everything that was present on the art scene of 20th century prior to 1933. Abstract and figural art, landscapes and portraits of August Macke, the expressionist works of the art group Bridge, Kirchner, Nolde, Schmidt-Rottluff, Beckmann were proclaimed as degenerate. The unwanted were George Grosz, Otto Dix, Kathe Kollwitz, because of their non-German way of representing the German people. The Nazis couldn't allow the presentation of weaknesses of the German, Arian society. Therefore, the first attack was pointed towards the art that represented crippled war veterans, prostitution and other unwanted aspects of the life in Germany from the period between the two wars [8].

The artists whose work was labeled as degenerate were forced to stop practicing their art, so they turned to alternative ways for making a living. Many had left the country, among them Georg Grosz who moved to the USA, while those who stayed were sentenced to isolation, constant pressure, threats and a life in fear and poverty. Otto Dix in his letter written to a friend in the USA describes the horrible conditions in which the artists lived in Nazi Germany: 'We live in very difficult conditions. I worry constantly how I am going to buy bread and heat… In Germany the painters do not exhibit, unless they are members of the *Reichskulturkammer*. I work and don't look around. I paint landscapes and self-portraits with children' [5].

The portrait of German reality after WWI and before the Nazi rule was labeled as degenerate and doomed. Not much time will pass before similar pictures will once again fill the streets of Germany after the monstrous act against humanity, WWII.

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LETTER TO THE EDITOR / PISMO UREDNIKU UDC: 929:61-05(044.4)

Chekhov speaks to us

Čehov nam govori

To the Editor:

Anton Pavlovich Chekhov (1860–1904) was a medical doctor and writer. Celebrations commemorating Chekhov's centenial were organized in 2004 from Russia to Germany, France, and England, all the way to the United States and Canada and other countries. Colby College in Waterwille, Maine was one of the sites in the USA that hosted a Chekhov festival. As a physician and author, I was invited to make a presentation at the Chekhov Centenary Conference at the Colby College. In addition, there was a symposium "Chekhov the Immigrant: Translating a Cultural Icon", and performances of film versions of Chekhov's works, as well as his plays (The Seagull, Three Sisters). The festival lasted for three weeks. That people in the USA promoted this rich program comes as no surprise, as Chekhov's plays are frequently performed here. (Only Shakespeare is performed in the USA more often than Chekhov).

Chekhov graduated from medical school in 1884 and started working as a physician in a suburban Moskow hospital. Eight years later, he purchased the rundown Melikhovo estate south of Moscow. He lived there until his health declined due to lung tuberculosis and forced him to move to the milder climate of Yalta.

In Melikhovo, where Chekhov lived with his parents and sister, he saw hundreds of patients, made more than a thousand house calls, and daily fought cholera and illiteracy among his patients. He was not a wealthy man, but he did not charge his patients; he even bought medicines to give to the poorest ones. At night, he wrote literary gems: his stories and two magnificent plays, "The Seagull" and "Uncle Vanya". His publisher, who was also his friend, advised him to give up medicine and concentrate on writing. Chekhov's response was:

You advise me not to chase after two hares at once and to forget about practicing medicine. Well, I don't see what's so impossible about chasing two hares at once ... Medicine is my lawful wife, literature my mistress. When I tire of the one, I spend the night with the other... This is somewhat disorganized, but then again it's not boring, and anyway, neither looses anything by my duplicity.

Through his writing, Chekhov showed how the symbiosis of the muse and Æsculapius can work. The doctor-writer situation may seem to some to be an apparent contradiction, yet it also describes Chekhov, the man. His artist role differed-but did not contradict-his role as a physician. As a doctor, he was a man of action. He founded schools and clinics for the peasants in the Melikhovo region, donated books to libraries and pursued humanitarian causes. In contrast, many of the protagonists in his stories and plays are passive. Chekhov realized that scientific rationalism cannot provide answers to important questions, such as what is the meaning of life; hence, he often presents man as a victim in an absurd world. Regarding the ideological shortcomings of his characters, Chekhov often treated them as a joke. In 1888, he wrote to a friend:

I still lack a political, religious and philosophical view. I change it every month, and so I'll have to limit myself to descriptions of how my heroes love, marry, give birth, die, and how they speak.

Despite his self-depreciation, Chekhov remains a singularly talented author. Thanks to his literary talent and approach to life, he elevated both the modern story and the modern play. It is difficult to decide if he accomplished more as a dramatist or as a story teller. Through his association with his so-called mistress, Chekhov created hundreds of characters who often show weakness and ineffectivness. His people were not the heroes or monumental personalities, like those described by Dostoevsky or Tolstoy. They lived in capital cities and provinces, villages and new industrial zones. They came from Europen Russia, Siberia and the Far East. They included officials, nobleman, priests, doctors, children, students, prisoners, hunters in the field, ladies, peasant women, old men, the sane, and the mad. With these diverse characters, Chekhov created a new kind of story. He was an author-observer who avoided ideological excess and moral judgments. His characters lived their lives fully. Like us, they are citizens of a world that does not always make sense. Tolstoy was struck by Chekhov's originality.

Chekhov is an artist of life...[he] has created new forms of writing, compeltly new, in my opinion, to the whole world, the like of which I have not encountered anywhere...Chekhov has his own special form, like impressionists.

Unlike the endless and eternal sky above the vast steppes that so deeply impressed Chekhov, we have but diminutive role in a life that is too short. Recognition of this

fact frequently leads us to isolation and disappointment. Unfulfilled desires prompt us to ask the big questions: How do we manage life? Are we but pawns in an absurd world?

Although Chekhov's life span was a short 44 years, he was in good company; Pushkin, Byron, Lermontov, Njegoš, Mayakovsky, Blok, Lorca, Vallejo, Orwell, and many other prominent writers also had short lives. Like these masters, Chekhov's innovative and prolific literary output secured him a place among the greatest men of letters. His works still strongly appeal to readers and theater lovers all over the world, and they speak deeply to our moral and philosophical selves.

Chekhov's numerous characters also include more than 30 medical doctors. For the greater part, these characters are people burdened with obligations to their patients, who are hindered by various life problems and poor working conditions. Some, like Dr. Ragin in the story, "Ward No 6", succumb to mental illness. Others are blinded by one-sided medical observation and neglect the patient, like poor Anyuta, who deserves at least a little human attention. Dr. Ragin is in charge of a district provincial hospital for twenty years. Initially, he was energetic physician, but time erodes his enthusisam, and he concludes that his efforts made no difference. The hospital is very poorly equipped, and the socioeconomic state of his patients is beyond his control. Despite his efforts, mortality does not decrease in his town. Dr. Ragin meets the brilliant but paranoid, Gromov, who is confined for proclaiming that truth and justice must triumph one day. Ragin becomes obsessed with Gromov and becomes even more disfunctional. His superiors trap him in his own ward, where he dies after a beating by a nurse. When the novelist Nikolay Leskov read this story, he said "Ward No 6 is Russia."

Chekhov was faithful to his family and his friends. [He was less faithful to the women who fell in love with him. One exception was a young actress in the Moscow Art Theater, Olga Knipper, who he married in 1901.] His devotion to his friends was commendable. For example, when in 1902 Maxim Gorky was elected as a member of the Russian Akademy, Czar Nicolas II annulated this election. In protest, Chekhov resigned from the Academy. Only one other member of the Academy, Vladimir Korolenko, joined in his protest. [According to Stefan Zweig, both Russia and whole Europe were fascinated by Gorky's unique literary voice that appeared from the bottom strata of society. Gorky was an advocate of Russian social and cultural changes, a man who publically opposed the czarist regime.]

In 1890, Chekhov traveled across Siberia to Sakhalin Island, off the far eastern coast of Russia to study conditions in the site where Russian convicts were kept and to take a census of the population. The hardships of that journey (the railway was not yet built) and his 3-month sojourn on the island were frightful. His report to the medical society and general public in the documntary book Sakhalin Island (1893), made a huge impression on the Russian public. He described in details the brutal beatings of the prisoners that he witnessed first hand. Thanks to the influence of the medical doctors that followed Chekhov's report, the Czar abolished corporal punishment for women in 1897 and for men in 1904, and he also introduced reforms for prison administrations.

Even today, torture and brutal treatment of prisoners occurs frequently in many prisons. Torture has been reported in the Guantanamo Bay detainment camp, secret prisons in several European countries, as well as in regular prisons all over the world. We do not need Chekhov to tell us that prisoners in these places are often mistreated. Media reports clearly show the brutality. For serious crimes, death or life imprisonment may be necessary, but ongoing cruelty is not. Many of us remain ignorant of the inhumane behavior of prison guards. And, shockingly, some of the brutality is accompished with the help of staff physicians.

Unfortunately, many of our top current physicians, scientsts, artists, and other influential people rarely rise their voices against such atrocities. They seem loath to provoke a public outcry sufficient to enlist governments and politicians, as Chekhov did when his report forced the Czar's decree more than a century ago. Anton Pavlovich Chekhov has told us to be bold and to speak loudly when we recognize problems. The solution will come afterwards. Thus, from time to time, we hear bold voices that help us create more humane societies. We must make sure that they are heard.

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a) Naslov treba da bude kratak, jasan i informativan i da odgovara sadržaju rada. Podnaslove treba izbegavati.

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c) Navode se puni nazivi ustanove i organizacijske jedinice u kojima je rad obavljen i mesta u kojima se ustanove nalaze, sa jasnim obeležavanjem odakle je autor, koristeći standardne znake za fus-note.

2. ***Apstrakt i ključne reči***

Na drugoj stranici nalazi se strukturisani apstrakt sa naslovom rada. Kratkim rečenicama na srpskom i engleskom jeziku iznosi se **uvod i cilj** rada, osnovne procedure - **metode** (izbor ispitanika ili laboratorijskih životinja; metode posmatranja i analize), glavni nalazi - **rezultati** (konkretni podaci i njihova statistička značajnost) i glavni **zaključak**. Naglasiti nove i značajne aspekte studije ili zapažanja. Strukturisani apstrakt (**250** reči) ima podnaslove: *uvod/cilj, metode, rezultati* i *zaključak*. Za apstrakte na engleskom dozvoljeno je i do **450** reči. Strukturisani apstrakt je obavezan za metaanalize (istog obima kao i za originalne članke) i kazuistiku (do 150 reči, sa podnaslovima *uvod, prikaz slučaja i zaključak*). Ispod apstrakta, pod podnaslovom „Ključne reči“ predložiti 3–10 ključnih reči ili kratkih izraza koji oslikavaju sadržinu članka.

3. ***Tekst članka***

Tekst sadrži sledeća poglavlja: **uvod**, **metode**, **rezultate** i **diskusiju**. **Zaključak** može da bude posebno poglavlje ili se iznosi u poslednjem pasusu diskusije. U **uvodu** ponovo napisati naslov rada, bez navođenja autora. Navesti hipotezu (ukoliko je ima) i ciljeve rada. Ukratko izneti razloge za studiju ili posmatranje. Navesti samo strogo relevantne podatke iz literature i ne iznositi opširna razmatranja o predmetu rada, kao ni podatke ili zaključke iz rada o kome se izveštava.

Metode. Jasno opisati izbor metoda posmatranja ili eksperimentnih metoda (ispitanici ili eksperimentne životinje, uključujući kontrolne). Identifikovati metode, aparaturu (ime i adresa proizvođača u zagradi) i proceduru, dovoljno detaljno da se drugim autorima omogući reprodukcija rezultata. Navesti podatke iz literature za uhodane metode, uključujući i statističke. Tačno identifikovati sve primenjene lekove i hemikalije, uključujući generičko ime, doze i načine davanja. Za ispitivanja na ljudima i životinjama navesti saglasnost etičkog komiteta.

Rezultate prikazati logičkim redosledom u tekstu, tabelama i ilustracijama. U tekstu naglasiti ili sumirati samo značajna zapažanja.

U **diskusiji** naglasiti nove i značajne aspekte studije i izvedene zaključke. Posmatranja dovesti u vezu sa drugim relevantnim studijama, u načelu iz poslednje tri godine, a samo izuzetno i starijim. Povezati zaključke sa ciljevima rada, ali izbegavati nesumnjive tvrdnje i one zaključke koje podaci iz rada ne podržavaju u potpunosti.

Literatura

Literatura se u radu citira kao superskript, a popisuje rednim brojevima pod kojima se citat pojavljuje u tekstu. Navode se svi autori, ali ako broj prelazi šest, navodi se prvih šest i dodaje et al. Svi podaci o citiranoj literaturi moraju biti tačni. Literatura se u celini citira na engleskom jeziku, a iza naslova se navodi jezik članka u zagradi. Ne prihvata se citiranje apstrakata, sekundarnih publikacija, usmenih saopštenja, neobjavljenih radova, službenih i poverljivih dokumenata. Radovi koji su prihvaćeni za štampu, ali još nisu objavljeni, navode se uz dodatak „u štampi“. Rukopisi koji su predati, ali još nisu prihvaćeni za štampu, u tekstu se citiraju kao „neobjavljeni podaci“ (u zagradi). Podaci sa *Interneta* citiraju se uz navođenje datuma.

Primeri referenci:

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Balint B. From the haemotherapy to the haemomodulation. Beograd: Zavod za udžbenike i nastavna sredstva; 2001. (Serbian)

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Tabele

Sve tabele pripremaju se sa proredom 1,5 na posebnom listu. Obeležavaju se arapskim brojevima, redosledom pojavljivanja, u desnom uglu (**Tabela 1**), a svakoj se daje kratak naslov. Objašnjenja se daju u fusnoti, ne u zaglavlju. Za fus-notu koristiti sledeće simbole ovim redosledom: ***, †, ‡, §,** ||**, ¶, **, ††, ...** Svaka tabela mora da se pomene u tekstu. Ako se koriste tuđi podaci, obavezno ih navesti kao i svaki drugi podatak iz literature.

Ilustracije

Slikama se zovu svi oblici grafičkih priloga i predaju se kao dopunske datoteke u sistemu **aseestant**. Slova, brojevi i simboli treba da su jasni i ujednačeni, a dovoljne veličine da prilikom umanjivanja budu čitljivi. Slike treba da budu jasne i obeležene brojevima, onim redom kojim se navode u tekstu (**Sl. 1; Sl. 2** itd.). Ukoliko je slika već negde objavljena, obavezno citirati izvor.

Legende za ilustracije pisati na posebnom listu, koristeći arapske brojeve. Ukoliko se koriste simboli, strelice, brojevi ili slova za objašnjavanje pojedinog dela ilustracije, svaki pojedinačno treba objasniti u legendi. Za fotomikrografije navesti metod bojenja i podatak o uvećanju.

Skraćenice i simboli

Koristiti samo standardne skraćenice, izuzev u naslovu i apstraktu. Pun naziv sa skraćenicom u zagradi treba dati kod prvog pominjanja u tekstu.

Detaljno uputstvo može se dobiti u redakciji ili na sajtu:

www.vma.mod.gov.rs/vsp/download/uputstvo_za_autore.pdf.

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