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VITAMIN D AND THIOL-DISULFIDE HOMEOSTASIS LEVELS IN POST-MENOPAUSAL WOMEN WITH OVERACTIVE BLADDER SYNDROME

VITAMIN D I NIVOI TIOL-DISULFIDNE HOMEOSTAZE KOD ŽENA U POSTMENOPAUZI SA SINDROMOM PREAKTIVNE BEŠIKE

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Summary

Background: This study aimed to find a relationship between vitamin D concentration and thiol-disulfide homeostasis in the pathophysiology of overactive bladder (OAB) syndrome in postmenopausal women.
Methods: A total of 76 postmenopausal women, referred for routine controls, were recruited between January and March 2018 to participate in this study. Participants with an overactive bladder questionnaire (OAB-q) score of >11 (n = 34) were included in the OAB syndrome group, while those with a score of <5 (n = 42) were included in the control group. Serum total antioxidant capacity, ischemia-modified albumin, C-reactive protein, 25-hydroxy vitamin D levels, and thiol-disulfide homeostasis were measured.
Results: Patients with OAB syndrome had waist circumferences of 106 ± 11 cm, and their body mass indexes (BMIs) were 30.8 ± 4.8 kg/m^2. The control groups' waist circumferences were 102 ± 11 cm and their BMIs were 28.9 ± 4.3 kg/m^2 (p = 0.069 and p = 0.098, respectively). The level of vitamin D in the control group was 33.7 (IQR: 30.7) nmol/L and 27.0 (IQR: 27.5) nmol/L (p = 0.081) in the OAB syndrome group.

Kratak sadržaj

Uvod: Cilj ove studije je bio da se nađe veza između koncentracije vitamina D i tiol-disulfidne homeostaze u patofiziologiji sindroma preaktivne bešike (OAB) kod žena u postmenopauzi.
Metode: Između januara i marta 2018. godine ukupno je odabrano 76 žena u postmenopauzi da bi učestvovale u ovoj studiji, koje su potom upućene na rutinske kontrole. Učesnice studije koje su imale skor od > 11 (n = 34) na upitniku za preaktivnu bešiku su uključene u grupu sa OAB sindromom, dok su one sa skorom od < 5 (n = 42) uključene u kontrolnu grupu. Izmereni su ukupni antioksidativni kapacitet seruma, albumin modifikovan ishemijom, C-reaktivni protein, 25-hidroksi nivoi vitamina D i tiol-disulfidna homeostaza.
Rezultati: Pacijentkinje sa OAB sindromom su imale obim struka od 106 ± 11 cm, a njihovi indeksi telesne mase (BMI) bili su 30,8 ± 4,8 kg/m^2. Obim struka kontrolne grupe bio je 102 ± 11 cm, a njihovi BMI bili su 28,9 ± 4,3 kg/m^2 (p = 0,069 i p = 0,098, respektivno). Nivo vitamina D u kontrolnoj grupi bio je 33,7 (IQR: 30,7) nmol/L, u grupi sa OAB sindromom 27,0 (IQR: 27,5) nmol/L (p = 0,081).

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Conclusions: We were not able to demonstrate with certainty any significant relationships between serum 25-hydroxy vitamin D levels and thiol-disulfide homeostasis parameters and OAB syndrome.

Keywords: C-reactive protein, disulfides, postmenopause, urinary bladder, overactive, vitamin D

Zaključak: Nismo bili u mogućnosti da sa sigurnošću dokažemo bilo kakve značajne veze između nivoa 25-hidroksi vitamina D u serumu i parametara tiol-disulfidne homeostaze i OAB sindroma.

Ključne reči: C-reaktivni protein, disulfidi, postmenopauza, mokra na be ika, preaktivna, vitamin D

Introduction

Overactive bladder (OAB) syndrome is a common clinical condition that affects millions of people worldwide. It is defined by urgency, urinary incontinence, and frequency (>8/24 hour) in the absence of metabolic (e.g. diabetes) or local pathological factors (1). Advanced age (>40 years), menopause, parity >2, constipation, and high body mass index (BMI) are risk factors for OAB syndrome (1). Detrusor overactivity and involuntary contractions during the filling phase of the bladder result in decreased functional bladder capacity and associated symptoms (1).

Vitamin D, a fat-soluble prohormone, is biologically inert when derived from diet or elicited in the skin from sunlight, and requires two consecutive hydroxylations in the human body for activation. Vitamin D plays an important role in the human body and its deficiency, related to many health problems, is a global issue (2).

Vitamin D receptors are found in nearly 30 different tissues, including the human bladder (3). The pelvic floor musculature, which provides a constrictor mechanism for the urethra, expresses a vitamin D receptor that plays a significant role in attaining urinary continence (4). It has been suggested that vitamin D deficiency might lead to bladder dysfunction through its effect on the smooth muscles of the detrusor and pelvic musculature (3–5). Higher dietary vitamin D intake has also been shown to lower the risk of OAB syndrome onset (6).

The loss of balance between reactive oxygen species (ROS) and antioxidant defence mechanisms against them is defined as oxidative stress. Vitamin D has been shown to have in vitro anti-oxidant and anti-inflammatory effects, which might link vitamin D deficiency to an increased probability of developing diseases (7, 8). In a recent study, Dokumacioglu et al. (9) showed that the levels of the oxidative stress markers, urinary malondialdehyde and 8-hydroxy-2'-deoxyguanosine, increased in women with OAB syndrome compared with a healthy control group.

Oxidative stress has been shown to contribute to the etiopathogenesis of some diseases, and it can be measured by a new marker, dynamic thiol-disulfide homeostasis (10, 11). This technique is simple and fast, and it can be used in routine laboratory practice to assess and monitor oxidative stress. Alvarez et al. (8) demonstrated that serum 25(OH)D concentrations were independently associated with major plasma thiol/disulfide redox systems, suggesting that vitamin D status may be involved in redox-mediated pathophysiology.

In this study, we aimed to determine the relationship between vitamin D concentration and thiol-disulfide homeostasis, as an oxidative stress marker, in the pathophysiology of OAB syndrome in postmenopausal women.

Materials and Methods

The study was conducted in accordance with the Declaration of Helsinki and approved by the research ethics committee. All the subjects gave their written, informed consent, and all the authors followed the ICMJE's requirements for privacy.

Postmenopausal women who had been referred for routine controls (aged 50 or older), recruited between January and March 2018, were invited to participate in the study, and an OAB examination was performed in the urology clinic.

Women with urinary tract infections, urinary stones, infections, or other urinary system pathologies; a history of malignancy; current active malignant neoplasm; cardiovascular disease; chronic neurological, hematologic, infectious, musculoskeletal, psychiatric, or endocrine disease; stress urinary incontinence; those who smoked; take antioxidant drugs or vitamins; or receiving treatment for OAB syndrome were excluded from the study. The participants who were accepted into the study answered an overactive bladder questionnaire (OAB-q) (12). The OAB-q included eight questions about the severity of a patient's complaints that were answered using a 6-point scale, ranging between no (0), very few (1), a little (2), quite a few (3), a lot (4), and too many (5). The total score ranged between 0 and 40.

In our study, those with an OAB-q score of >11 were evaluated as having OAB syndrome. Those with an OAB-q score <5 were included in the control group.

The patients' height, weight, and waist circumference were recorded, and a BMI was calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2). Serum calcium, phosphorus, triglyceride, high-density lipoprotein (HDL), low-

Table I Characteristics of the study population.

Characteristics	Women without OAB	Women with OAB	p
Number of subjects (n)	34	42	
Age (years)	54.0 ± 3.4	54.6 ± 4.5	0.600
OAB-q	1 (2.0)	18 (12.0)	< 0.001
Waist circumference (cm)	102 ± 11	106 ± 11	0.069
BMI (kg/m^2)	28.9 ± 4.3	30.8 ± 4.8	0.098
Waist circumference/height	0.64 ± 0.07	0.67 ± 0.07	0.098
Triglyceride (mmol/L)	1.86 ± 1.12	1.84 ± 1.08	0.927
Total cholesterol (mmol/L)	6.05 ± 1.13	5.58 ±1.08	0.071
HDL (mmol/L)	1.42 ± 0.38	1.42 ± 0.43	0.835
LDL (mmol/L)	3.7 ± 0.98	3.36 ± 1.00	0.089
AIP	0.046 ± 0.26	0.048 ± 0.31	0.982
CRP (nmol/L)	29.5 (0.9)	29.5 (0.9)	0.994
Ca (mmol/L)	2.39(0.07)	2.37(0.12)	0.724
Hba1c (%)	5.9(0.7)	5.8(0.5)	0.363
Vit D (nmol/L)	33.7 (30.7)	27.0(27.5)	0.081
PTH (pmol/L)	6.15 (3.9)	6.68 (4.0)	0.715
FRAP (µmol/L)	1120 ± 264	1135 ± 283	0.842
Alb (g/L)	55 ± 17	46 ± 10	0.151
IMA (AU)	0.569 ± 0.219	0.629 ± 0.257	0.335
Native thiol (µmol/L)	356 ± 73	331 ± 64	0.156
Total thiol (µmol/L)	394 ± 70	365 ± 65	0.095
Disulfide (µmol/L)	19.0 ± 6.2	17.0 ± 4.2	0.118
Disulfide/native thiol	5.1 (1.8)	5.3 (2.8)	0.710
Disulfide/total thiol	4.8 (1.4)	4.8 (2.2)	0.720
Native thiol/total thiol	90.2 (2.9)	90.2 (4.5)	0.725

Data are expressed as mean ± standard deviation when normally distributed, otherwise as median (interquartile range). OAB-q: overactive bladder questionnaire; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; Ca: calcium; Vit D: vitamin D, AIP: atherogenic index of plasma; CRP: C reactive protein; OAB: overactive bladder; IMA: ischemia-modified albumin; FRAP: ferric reducing power of plasma, AU: absorbance unit. *$p < 0.05$ was considered significant for statistical analyses. Mann-Whitney U or student t used for statistical analysis.

density lipoprotein, and total cholesterol were measured using commercially available assay kits with an auto-analyzer (Olympus AU 2700; Beckman Coulter, Germany). HbA1c levels were measured with a HbA1c analyzer (G8; Tosoh Corporation, Tokyo, Japan); parathormone (PTH) and 25-hydroxy vitamin D levels were measured using an immunoassay system (Advia Centaur XP; Siemens Healthcare Diagnostics, USA), and C reactive protein (CRP) was measured with a nephelometer (BN II System; Siemens Healthcare Diagnostics, USA). The atherogenic index of plasma (AIP) was calculated as log (triglyceride/HDL-c). Total antioxidant capacity was measured using the ferric reducing ability of plasma method (FRAP) (13). Reduced cobalt to albumin-binding capacity levels (IMA) were determined according to the method defined by Bar-Or et al. (14). Thiol-disulfide homeostasis was measured by the method developed by Erel (15).

Statistics

Statistical analyses were performed using the SPSS program, Version 15 (SPSS Inc., Chicago, IL, USA). The normality of the continuous variables was analyzed using the Kolmogorov–Smirnov test. The results were expressed as the mean ± the standard deviation (SD) or the median (interquartile range). Normally distributed continuous variables were compared using the independent sample t-test, but the Mann-Whitney U test was used if the distribution was skewed. The relationships among the variables were examined using Spearman's correlation coefficient.

Results

A total of 76 menopausal women were included in the study. The patients, diagnosed with OAB syn-

drome in the urology clinic, were divided into two groups according to their OAB-q scores: those with a score of <5 (n = 34) were included in the healthy control group, and those with a score of >11 (n = 42) were classified as OAB syndrome. Fourteen patients with scores between 5 and 10 were not evaluated.

The age of the patients and the healthy participants were similar at the time of the examination (p = 0.600). Patients with OAB syndrome had waist circumferences of 106 ± 11 cm and BMIs of 30.8 ± 4.8 kg/m^2. The control group had waist circumferences of 102 ± 11 cm and BMIs of 28.9 ± 4.3 kg/m^2 (p = 0.069 and p = 0.098, respectively) (*Table I*).

The level of vitamin D in the control group was 33.7 (IQR: 30.7) nmol/L and 27.0 (IQR: 27.5) nmol/L in the OAB syndrome group (p = 0.081) (*Table I*). However, the correlation between OAB-q and vitamin D using Spearman's correlation was statistically insignificant (r = -0.095, p = 0.418).

The AIP was 0.048 ± 0.31 in women with OAB syndrome and 0.046 ± 0.28 in the control group (p = 0.982).

We found native thiol 356 ± 83 μmol/L vs 331 ± 64 μmol/L (p = 0.444), total thiol 394 ± 70 μmol/L vs 365 ± 67 μmol/L (p = 0.095), and disulfide 19.0 ± 6.2 μmol/L vs 17.0 ± 4.2 μmol/L (p = 0.118) in the control vs the OAB syndrome patients, respectively (*Table I*).

Vitamin D levels showed a weak negative correlation with waist circumference (r = −0.339, p = 0.004), waist circumference to height ratio (r = −0.362, p = 0.002), and BMI (r = −0.68, p = 0.021). AIP correlated with waist circumference (r = 0.823, p < 0.001), waist circumference to height ratio (r = 0.349, p = 0.003), BMI (r = 0.384, p = 0.001), and FRAP (r = 0.345, p = 0.007).

CRP had a weak positive correlation with waist circumference (r = 0.399, p = 0.001), waist circumference to height ratio (r = 0.420, p <0.001), and BMI (r = 0.369, p = 0.01). There was no correlation between the OAB-q score and any of the examined parameters. The thiol-disulfide homeostasis parameters did not correlate with any parameters investigated in the patient group.

Discussion

We found that the postmenopausal patients with OAB syndrome levels of vitamin D were non-significantly low compared to the control group. Supporting our study, low 25-hydroxy vitamin D blood test levels have been suggested as contributing to pelvic floor muscle weakness, which is involved in urinary incontinence and OAB syndrome (16). High-dose vitamin D therapy has been proven to reduce the severity of urinary incontinence in postmenopausal women (17, 18), and in a survey of 5,816 women aged over 40 years, high dietary vitamin D intake was found to reduce the risk of developing OAB syndrome (19). However, some studies did not find an association between lower urinary tract symptoms and vitamin D deficiency (20). Similarly, in a Korean-patient group, low serum vitamin D was not significantly related to female urinary incontinence matched for risk factors such as menopause, number of pregnancies, hypertension, diabetes, and BMI (21).

The vitamin D levels in our study negatively correlated with waist circumference and BMI. Several studies have demonstrated evidence of an association between low plasma concentrations of 25-hydroxy vitamin D and obesity (22). BMI and waist circumference were higher in the OAB syndrome patients. This was consistent with a recent meta-analysis by Zhu et al. (23) that showed that an increase in BMI is a risk factor for OAB syndrome. The exact mechanisms, explaining the link between obesity and OAB syndrome, are not well-known. In the current study, the inflammatory marker CRP was positively correlated with waist circumference, waist circumference to height ratio and BMI. These findings are in agreement with previously published reports (24).

ROS are normal products of aerobic metabolism. However, excess production of ROS is a common feature of various pathophysiological bladder conditions, although its possible role in the physiopathology of bladder dysfunction has still not been clarified (25, 26).

Masuda et al. (27) suggested that oxidative stress might play a role in the development of bladder dysfunction by increasing detrusor muscle contractility and stimulating bladder afferent fibres. With ageing, there seems to be a decrease in antioxidant mechanisms, and ageing increases the sensitivity of detrusor contraction to oxidative damage (28). Some studies have demonstrated that ROS mediate detrusor muscle activity, which provides insight into possible mechanisms (27, 29, 30).

We speculated that the interplay between vitamin D and oxidative stress might affect the severity of OAB syndrome. However, in our study, total antioxidant capacity, measured as FRAP, and thiol-disulfide homeostasis in the OAB syndrome group were similar to those of the control group, and no correlation was found between the severity of the syndrome. No previous study has investigated thiol-disulfide homeostasis in patients with OAB syndrome.

There was no relationship between vitamin D and CRP in our study. Accordingly, Jorde et al. (31) could not find a correlation between 25-hydroxy vitamin D blood test levels and a number of cytokines and inflammation markers. Yiu et al. (32) also demonstrated that vitamin D did not have a signifi-

cant effect on the serum biomarkers of inflammation and oxidative stress. In addition, we could not find an association between FRAP and vitamin D or vitamin D and CRP. This might be explained by the in vivo studies that have demonstrated that active vitamin D in very high concentrations has an immunoregulatory effect (33).

Alvarez et al. (8) showed that in a large cohort of ambulatory adults, the serum 25-hydroxy vitamin D concentration is related to the plasma circulating major thiol-disulfide redox systems; namely plasma glutathione (GSH), cysteine (Cys), and their associated disulfides. However, we found no correlation between vitamin D and thiol-disulfide homeostasis.

Our study involves certain limitation. For instance, the number of participants in both groups was low. Another drawback of the study is that this cross-sectional study was conducted in only one centre and, thus generalizability may be limited.

In conclusion, we were not able to demonstrate with certainty any significant relationships between serum 25-hydroxy vitamin D levels and thiol-disulfide homeostasis parameters and OAB syndrome.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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CAN NEUTROPHIL/LYMPHOCYTE RATIO BE USED AS AN INDICATOR OF INFLAMMATION IN PATIENTS WITH HYPERTHYROIDISM?

DA LI SE ODNOS NEUTROFILA/LIMFOCITA MOŽE KORISTITI KAO INDIKATOR UPALE KOD PACIJENATA SA HIPERTIREOIDIZMOM?

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Summary

Background: In our study, we aimed to evaluate changes in the neutrophil and lymphocyte series and investigate whether the neutrophil/lymphocyte ratio (NLR) is indicative of inflammations in patients with hyperthyroidism.
Methods: A total of 161 patients were enrolled, 121 of which had hyperthyroidism (71 Graves' Disease (GD) and 50 non-Graves hyperthyroidism (NGH) patients) and 40 of which were control group members. Retrospectively, patients' neutrophil and lymphocyte counts were taken, and the NLR was calculated.
Results: While the number of neutrophils was significantly lower in the GD group ($p = 0.003$), there was no significant difference between the NGH and the control group. In the GD group, NLR values were significantly lower than the other two groups (median 1.39 for GD, median 1.84 for NGH and median 1.83 for the control group, $p < 0.001$). Only three patients in the GD group had neutropenia. There was also a significant negative correlation between free T3 and neutrophil count and NLR in hyperthyroid patients ($r = –0.28$, $p = 0.001$ and $r = –0.34$, $p < 0.001$, respectively).
Conclusions: In our study, we found that NLR did not increase in hyperthyroid patients and that this ratio decreased due to the decrease in neutrophil levels in GD. We thus concluded that NLR is not a suitable indicator of hyperthyroidism.

Keywords: hyperthyroidism, lymphocyte, neutrophil, ratio

Kratak sadržaj

Uvod: Cilj naše studije je bio da procenimo promene u seriji neutrofila i limfocita i istražimo da li je odnos neutrofila/limfocita (NLR) indikativan za upale kod pacijenata sa hipertireoidizmom.
Metode: Ukupno je bio uključen 161 bolesnik, od kojih je 121 imao hipertireoidizam (71 sa Grejvsovom bolešću (GD) i 50 njih sa hipertireoidizmom koji nije u vezi sa Grejvsovom bolešću (NGH)), dok su 40 njih bili članovi kontrolne grupe. Retrospektivno su uzeti broj neutrofila i limfocita pacijenata i izračunat je NLR.
Rezultati: Dok je broj neutrofila bio značajno niži u GD grupi ($p = 0,003$), nije bilo značajne razlike između NGH i kontrolne grupe. U GD grupi su vrednosti NLR bile značajno niže od ostalih dveju grupa (medijan 1,39 za GD, medijan 1,84 za NGH i medijan 1,83 za kontrolnu grupu, $p < 0,001$). Samo tri pacijenta u GD grupi su imala neutropeniju. Takođe, postojala je značajna negativna korelacija između slobodnog T3 i broja neutrofila i NLR kod hipertireoidnih pacijenata ($r = -0,28$, $p = 0,001$ i $r = -0,34$, $p < 0,001$, redom).
Zaključak: U našoj studiji smo otkrili da se NLR nije povećao kod hipertireoidnih pacijenata i da se taj odnos smanjio zbog smanjenja nivoa neutrofila u GD. Tako smo zaključili da NLR nije pogodan pokazatelj hipertireoidizma.

Ključne reči: hipertiroidizam, limfocit, neutrofil, odnos

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Introduction

Thyrotoxicosis refers to a clinical condition resulting from inappropriate high thyroid hormone effects in tissues. Hyperthyroidism is a type of thyrotoxicosis due to increased hormone synthesis and secretion in the thyroid gland. The two most common causes of hyperthyroidism are Graves' disease (GD) and toxic nodular goitre (1). The prevalence of hyperthyroidism is 0.8% in Europe and 1.3% in the USA (2, 3).

Hyperthyroidism is a form of inflammation caused by the systemic effects of increased thyroid hormones (4). This inflammation is much more evident in autoimmunity-related GD. Since antibodies against thyroid stimulating hormone (TSH) receptors primarily affect the thyroid, they also affect many cells such as adipocytes, fibroblasts, and bone cells (5, 6).

Neutrophil/lymphocyte ratio (NLR) has gained increasing importance in recent years and is an easily identifiable marker of inflammation (7). There are studies that NLR may be a marker of inflammation for a host of diseases such as familial Mediterranean fever, ankylosing spondylitis, rheumatic valve diseases, ulcerative colitis, psoriasis, coronary artery disease, malignancies, diabetes mellitus, hypertension, and chronic autoimmune thyroiditis (8–11). There are also opinions that NLR can be used for predicting progression and mortality, and that it can be used as a disease activity indicator for certain diseases (12). Considering these findings, it is conceivable that NLR may be an indicator of inflammation in patients with hyperthyroidism, which is an inflammatory disease. Although it is known that hyperthyroidism may cause changes in a number of hematological parameters, the relationship between hyperthyroidism with NLR and inflammation is not yet known (13). Therefore, we have aimed to evaluate the changes in the neutrophil and lymphocyte series in hyperthyroid patients, as well as to investigate whether NLR could be an indicator of inflammation in this study.

Materials and Methods

First of all, approval was asked for from the local ethical commission before beginning the study. The work was conducted according to the principles of the Helsinki Declaration, and a written consent concerning the study was obtained from each of the participants.

The study included 150 hyperthyroid patients who had applied to the Endocrinology Outpatient Clinic between January 1, 2014, and January 1, 2016, as well as 40 healthy volunteers who had also applied to the same outpatient clinics within the same date range. Twenty-nine patients with active infection or malignancy, alongside those who had previously received anti-thyroid drugs for any reason, were not included in the study. 71 of 121 hyperthyroid patients had GD. Participants were assessed according to three groups: GD, non-GD hyperthyroidism (NGH) and control group. Data on age, gender, TSH, free T3, free T4, anti-thyroglobulin antibody (anti-Tg), anti-thyroid peroxidase antibody (anti-TPO), thyroid receptor antibody (TRAb), and whole blood counts were retrospectively obtained. Neutrophil and lymphocyte counts were determined, whereupon NLR values were calculated for each group.

Statistical analysis of the data was done using SPSS 22.0 software. For statistical significance, $p < 0.05$ was considered significant.

Descriptive statistics of patients and control groups were performed. Categorical values were reported in terms of number and percentage. Kolmogorov-Smirnov test and histogram graphs of the data were used to assess whether or not the data corresponded to normal distribution. Normal distributed data were expressed in terms of mean and standard deviation. Data that did not correspond to normal distribution were expressed in terms of median and minimum-maximum values. The Student's *t*-test was used in order to assess countable data that met the normal distribution, and the Mann-Whitney U test was used to assess any countable data that did not fit the normal distribution. The Chi-Square test was used to compare the categorical variables. Pearson and Spearman correlation tests were applied during the analysis of correlations.

Results

69% of the GD group, 70% of the NGH group and 75% of the control group were female. There was no statistically significant difference between the groups regarding gender. There was no significant difference in age between the GD and NGH groups. The control group consisted of patients who were younger than those of the other two groups (median age of 45 for the GD, 45.5 for the NGH, and 33.5 for the control groups, $p < 0.001$). In the GD group, the number of neutrophils was significantly lower than the other two groups ($p = 0.003$). There was no significant difference in the number of neutrophils between the NGH and control groups. The NLR values in the GD group were significantly lower than the other two groups (median of 1.39 for the GD, 1.84 for the NGH, and 1.83 for the control groups, $p < 0.001$). There was no significant difference between NGH and control group in terms of NLR. Neutropenia was found in 3 patients in the GD group. However there was no neutropenia in the other two groups.

When the thyroid hormone and antibody parameters were examined, free T3 (fT3) values were significantly higher in the GD group than in the NGH group (9.11 versus 13.75; $p = 0.001$). There was no

Table I Demographic characteristics of groups, full blood count and NLR values.

	GD n=71	NGH n=50	Control n=40	p
Female (%)	49 (69%)	35 (70%)	30 (75%)	–
Age (years)	45 (19–69)	45.5 (20–83)	33.5 (21–67)	<0.001
Leukocyte count (cells/μL)	6700 (3200–12300)	7400 (3900–13000)	7200 (4800–13000)	–
Neutrophil count (cells/μL)	3400 (1000–7500)	4000 (1800–9300)	4300 (2100–8100)	0.003
Lymphocyte count (cells/μL)	2400 (600–5300)	2250 (1100–4900)	2200 (1200–3600)	–
NLR	1.39 (0.41–5.33)	1.84 (0.97–4.92)	1.83 (1–4.26)	<0.001
fT3 (pg/mL)	13.75 (3.01–30)	9.11 (4.47–30)	–	0.001
fT4 (ng/mL)	3.86 (1.01–10)	3.36 (1.14–9.20)	0.97 (0.68-1.49)	<0.001
TSH (μU/mL)	0.004 (0.001–0.068)	0.005 (0.004–0.21)	2.08 (0.53–4.45)	<0.001
TRAb (U/L)	4.81 (0.72–405)	3.47 (1.07–59)	–	0,03
Anti-TPO (U/mL)	154.65 (2.65–2000)	9.5 (0.7–1521)	–	<0.001
Anti-Tg (U/mL)	46.07 (5–5000)	12.1 (5–1329)	–	0.03

Contractions; GD: Graves' Disease, NGH: Non Graves' Disease Hyperthyroidism, NLR: Neutrophil Lymphocyte Ratio, fT3: free T3, fT4: free T4, TSH: Thyroid Stimulating Hormone, TRAb: Thyroid Receptor Antibody, Anti-TPO: Anti-thyroid peroxidase antibody, Anti-Tg: anti-thyroglobulin antibody

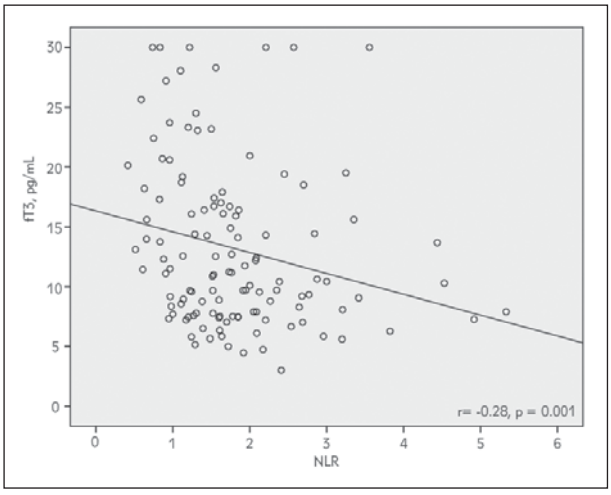

Figure 1 Correlation between fT3 and NLR.

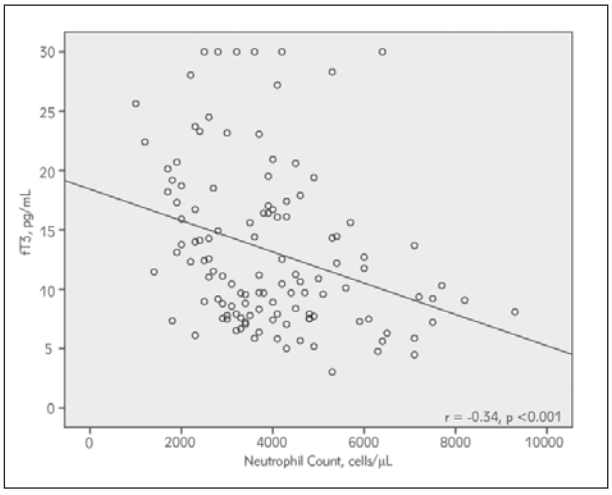

Figure 2 Correlation between fT3 and neutrophil count.

significant difference in free T4 (fT4) and TSH values between the two groups. TRAb, anti-TPO, and anti-Tg were significantly higher in the GD group ($p = 0.03$, $p < 0.001$ and $p = 0.03$, respectively). When the control, GD, and NGH groups were compared in terms of fT4 and TSH, it was found that the TSH values were significantly higher and the fT4 values were significantly lower in the control group ($p < 0.001$ and $p < 0.001$, respectively). The relevant data are provided in *Table I*.

Significant differences were found only in fT3 levels between TRAb positive patients and TRAb negative patients (13.7 vs 9.8, $p = 0.03$). However, no significant difference was found in terms of other parameters.

When the hyperthyroid patients (GD and NGH) were evaluated together, there was a significant negative correlation between the fT3 and NLR, as well as between fT3 and neutrophil counts ($r = –0.28$, $p =$

0.001 and r = –0.34, p < 0.001, respectively) (*Figures 1* and *2*). Beyond this, no other significant correlation was found.

Discussion

In our study, we found that the NLR did not increase in hyperthyroid patients and that this ratio decreased due to the reduction in neutrophil levels in Graves' patients.

Neutropenia is generally defined as having an absolute neutrophil count below 1500 cells/microL. However, there are opinions based on different figures (14). The effects of anti-thyroid drugs on the granulocytic series (agranulocytosis and neutropenia) are well known; however, the data on the direct effect of thyroid diseases on granulocytes are limited. In addition, data on lymphocyte subpopulation distributions in thyroid patients with or without neutropenia are also inadequate (15, 16). Indeed, the relationship between thyroidopathy and neutropenia dates back over 100 years (16). Subsequently, studies have been carried out in hyperthyroid patients with hematological disorders (such as leukopenia) (13, 17). In various studies, the prevalence of neutropenia in hyperthyroid patients was found to be between 14.1% and 30% (18–20). Recently, Aggerwal et al. (18) found that 29 of the 209 Graves' patients were found to have neutropenia at the time of diagnosis. In this study and several previous small scale studies, neutropenia was also shown to improve after patients become euthyroid with treatment (17, 19, 21). The rate of neutropenia in our study is rather low compared to previous studies. 71 of 3 Graves' patients (4.2%) had neutropenia (neutrophil < 1500). Ethnic differences within the patient populations as well as differences in the hyperthyroid levels of the patients (especially fT3 levels) in the studies may have caused this. The difference in neutropenic reference values (e.g. < 1800 and < 2000) may have led to this situation. In our study, the patients' post-treatment status could not be assessed.

Three possible mechanisms in the thyroid-associated neutropenia are thought to play a role. These are humoral and cellular mechanisms as well as toxicity associated with direct thyroid hormone (16). Panossi et al. (22) have shown that a reduction in abnormal granulopoiesis and bone marrow granulocyte reserve in GD plays a role in the mechanism of neutropenia development. Shaw and Mehta (23) have also suspected that thyroid hormones might have an effect that directly inhibits the maturation and differentiation of pluripotent stem cells in the early stages of hematopoiesis. Moreover, experimental data has shown that normally high or low thyroid hormone exposure induces apoptosis of $CD34^+$ progenitor cells (24). Furthermore, pernicious anemia, which may accompany autoimmune thyroid diseases, is thought to contribute to the development of neutropenia (16).

In our study, we found a negative correlation between fT3 and neutrophil levels in GD patients. This was a finding supporting the direct toxic effect of thyroid hormone levels on neutrophils. As a matter of fact, in a number of previous studies, a negative correlation between fT3 level and the number of neutrophils was found (16, 18). In our study, only the detection of neutropenia in autoimmune thyroid patients suggests that immune reactions besides the hormone level may also be indicative of an important role in the development of neutropenia. However, no significant relationship was found between TRAb level and neutrophil count. Nevertheless, in some previous studies, a relationship between autoimmune thyroid disease and anti-neutrophil antibody (anti-PMN) was detected (16, 25). In 1985, Weitzman et al. (25) found that 50–55% of GD patients had a positive anti-PMN level, however, only 2% of them had neutropenia. Anti-PMN antibodies were detected in 37.2% of thyroid patients in the study of Kyritsi et al. (16), whereby the majority of which were autoimmune in origin. Considering previous studies in the literature, it can be considered that certain antibodies in addition to thyroid-related antibodies play a role in the development of some immuno-mediated hematologic disorders in autoimmune thyroid diseases. In our study, PMN antibody levels were not measured.

There was no significant difference in lymphocyte levels between the groups in our study. There are some studies in the literature reporting changes in peripheral lymphocyte subgroups in thyroid diseases (26, 27). However, these studies have very insufficient and contradictory results.

There are reports that NLR can be used as a systemic inflammation marker in many diseases with inflammation. In the studies of Keskin et al. (11), NLR was found to be significantly higher in euthyroid Hashimoto patients, and moreover, this ratio also showed a positive correlation with the autoantibody level. In the study of Kocer et al. (28) NLR was significantly higher in patients with papillary thyroid cancer. However, there are studies with different results and that show that this ratio is not increased in papillary thyroid cancer (29). This data suggests that NLR may be used as an inflammatory marker in thyroid diseases as well as in other diseases. However, in our study, changes in the granulocyte series in Graves' patients alongside no significant difference in NGH compared to the control group showed that NLR was not a suitable parameter to be used in these patients.

There are some restrictive factors in our study. First of all, our study was retrospective. Other inflammation markers such as sedimentation rate and C-reactive protein were not evaluated. Furthermore, the anti-PMN antibody level thought to play a role in the development of neutropenia was not examined at all.

There was also a significant age difference between the hyperthyroid patients and the control group. However, there was no significant age difference between the GD and NGH groups. It is thought that age is not an important factor in the results.

In conclusion, we believe that it would be inappropriate to use NLR as an inflammation marker in patients with hyperthyroidism. Nevertheless, we feel that there is a need for more extensive work in this regard.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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MEASURING PLASMA FERRITIN LEVELS WITH TWO DIFFERENT METHODS: A COMPARISON OF ROCHE COBAS E601 VERSUS ROCHE COBAS C501 (INTEGRATED MODULAR SYSTEM ROCHE COBAS 6000)

MERENJE NIVOA PLAZMA FERITINA SA DVE RAZLIČITE METODE: POREĐENJE ROCHE COBAS E601 SA ROCHE COBAS C501 (INTEGRISANI MODULARNI SISTEM ROCHE COBAS 6000)

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Summary

Background: The aim of our study is to compare plasma ferritin levels found to be high or low in terms of reference range by means of electrochemiluminescence (ECLIA) and immunoturbidimetric method and to examine whether they can be used interchangeably.
Methods: 84 patients with high plasma ferritin level and 153 patients with low ferritin level according to the reference range were included in the study. Plasma samples measured in Cobas e601 device with ECLIA were also measured as immunoturbidimetric Cobas c501 device. For method comparison, CLSI EP9-A3 Guideline was used. While the consistency between the methods were specified with Passing-Bablok regression analysis and Spearman correlation analysis, bias error between the methods (bias%) was determined through Bland-Altman analysis.
Results: Both high and low plasma ferritin levels measured with Cobas e601 module and determined high in terms of reference range were compared with the results found with cobas c501 module. The difference was found to be statistically significant ($p<0.001$). According to regression and correlation (for low plasma ferritin levels; r: 0.993, $p<0.001$, for high plasma ferritin levels; r: 0.966, $p<0.001$) results, the methods were in consistency with each other. Additionally, while the bias% value was found to be 10.4% for low plasma ferritin levels, it was found to be 12.6% for high ferritin levels.
Conclusions: Accordingly, we believe that, comparison with more samples especially in terms of different clinical decision levels is required in order to examine interchangeable use of immunoturbidimetric method in integrated devices and ECLIA.

Keywords: plasma ferritin, electrochemiluminescence, immunoturbidimetric

Kratak sadržaj

Uvod: Svrha ovog izučavanja je bila da se uporede nivoi plazma feritina bilo visoki ili niski u odnosu na referentne vrednosti određeni elektrohemiluminescencijom (ECLIA) i imunoturbidimetrijskom metodom i da se proceni da li mogu da se koriste prema potrebi.
Metode: U proučavanja je uključeno 84 pacijenta sa visokom vrednošću feritina i 153 pacijenta sa niskom vrednošću u odnosu na referentne vrednosti. Uzorci plazme mereni su na Cobas e601 sa ECLIA metodom kao i imunotuirbidimetrijski na Cobas e501. Za poređenje je korišćen protokol CLSI EP9-A3. Takođe je primenjivana Passing-Bablok regresiona analiza i Spearman korelaciona analiza, dok je greška odstupanja između metoda (bias%) određivana Bland-Altmanovom analizom.
Rezultati: Visoke i niske vrednosti feritina u plazmi izmerene na Cobas e601 modulu su poređene u odnosu na one dobijene na Cobas c501 modulu. Nađena je statistički značajna razlika ($p < 0,001$). Prema korelacionoj i regresionoj analizi (za niske nivoe plazma feritina; $r = 0,993$, $p < 0,001$, za visoke nivoe plazma feritina; $r = 0,996$, $p < 0,001$) metode su bile u saglasnosti jedna sa drugom. Dodadatno, vrednost bias% bila 10,4% za nizak nivo feritina u plazmi, a za visoke nivoe feritina u plazmi 12,6%.
Zaključak: Shodno ovim istraživanja zaključujemo da su potrebna mnogo obimnija ispitivanja u prvom redu radi donošenja ispravnih kliničkih odluka a u vezi primene imunoturdidimetrijske metode integrisane sa ECLIA sistemom.

Ključne reči: plazma feritin, elektrohemiluminescencija, imunoturbidimetrija

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Introduction

Plasma ferritin levels are sensitive parameters used for evaluating condition of iron in the body non-invasively and for diagnostic reagents of iron deficiency anemia (1–3). Additionally, high plasma ferritin levels reflect systemic inflammation as acute phase reactants (4, 5). Although there are different methods for the measurement of plasma ferritin levels such as immunoturbidimetric method, electrochemiluminescence (ECLIA), Enzyme-Linked Immunosorbent Assay (ELISA), Radioimmunoassay (RIA), Chemiluminescence immunoassay (CLIA), the use of two methods in clinical laboratories stands out more. These methods are immunoturbidimetric method and electrochemiluminescence (ECLIA) method (6). As these measurement methods used in clinical laboratories have different test principles, their reference ranges and measurement limits are also different from each other. While anti-ferritin antibodies (rabbit) are used in immunoturbidimetric method, antibodies against human liver, spleen and heart ferritins are used in immunoassay method. The one with the highest analytic specificity among these antibodies is liver ferritin (7, 8).

Hormone and biochemistry module-integrated-systems (Roche Cobas 6000) were used in our laboratories for measurement (or determination) of plasma ferritin. While plasma ferritin levels result in 9 minutes in biochemistry module (Cobas c501) with immunoturbidimetric method, they result in 18 minutes in hormone module (Cobas e601) with ECLIA method due to long incubation period. The fact that tests examined in hormone module and analyzed in biochemistry module provides advantages for the laboratory in terms of saving time and obtaining practical results. The aim of our study is to examine whether there is a difference between plasma ferritin levels identified as high and low in terms of laboratory reference range with different methods in our hormone and biochemistry module-integrated-systems (Roche Cobas 6000), and whether they can be used interchangeably.

Materials and Methods

The ferritin levels of 153 (86 female, 68 male) patients whose sample acceptance was performed by Amasya Central Public Health Laboratory were found to be low in terms of reference range and 84 (41 female, 43 male) patients found to have high plasma ferritin levels in terms of reference range. Both values were studied in Cobas c501 biochemistry module and Cobas e601 hormone module in order to make a comparison. No additional samples were taken from the patients. The written and signed consent forms regarding their voluntary participation were received from the participants.

Regarding the method comparison, Clinical Laboratory Institute (CLSI) EP9-A3 Guidelines was used (9). Blood samples were selected among daily received blood samples between 01/08/2018 – 30/09/2018 from the patients who were suitable for the inclusion criteria of the study. Since it could affect the measurement method, patients with immunological diseases, biotin use history (at least 8 hours should pass after use), having iron treatment, with malignancy were excluded from the study. Moreover, as indicated in kit insert, patients who had RF levels higher than 2500 U/mL were excluded from the study by measuring rheumatoid factor (RF) levels of the patients. Furthermore, hemolysis, lipaemia and icterus indexes of the patients were evaluated and the samples having the level of hemolysis, lipaemia and icterus for interference were excluded from the study. Patient samples were taken to 5 mL tubes with mechanic separators and Barricor (BD, Becton Dickinson) in order to prevent artefact-based problems in gel separator tubes. After being centrifuged at 4000 RCF (g) for 3 minutes, plasma samples were measured in Cobas c501 (Roche Diagnostics, Mannheim, Germany) biochemistry module within 20 minutes following pipetting procedure in Cobas e601 (Roche Diagnostics, Mannheim, Germany) hormone module. Systematic error (bias%) comparison was carried out on the basis of data obtained from different sources (Royal College of Pathologist Australasia (RCPA): 3 μg/L (<40 μg/L for results), 7.5% (>40 μg/L for ferritin results), Wisconsin State Laboratory of Hygiene (WLSH):15%; College of American Pathologists (CAP):8%; Canadian Fixed Limits (CFX): 9.7%; American Association of Bioanalysts (AAB): 15%. Biological Variation (BV): 8.7%) (11).

Cobas c501 biochemistry module measurement method: Human-driven ferritin shows agglutination with latex particles covered with anti-ferritin antibodies in expanded particle surface immunoturbidimetric test. Precipitation was to be turbidimetric at 570/800 nm.

Cobas e601 hormone module measurement method: First incubation: sample with monoclonal antibody with biotin specific to ferritin and monoclonal antibody specific to ferritin marked with ruthenium complex creates a sandwich complex. Second incubation: After streptavidin-covered micro-particles are added, by way of interaction between biotin and streptavidin, complex is linked to a solid phase. Reaction mixture is aspired into measurement cell where micro-particles are magnetically caught by electrode surface. Voltage application on electrode causes chemiluminescence emission, and this is measured by a photon counter (photomultiplier).

Method characteristics for ferritin measurement in Cobas c501 and Cobas e601 devices are shown in *Table I*.

Table I Characteristic of methods for immunoturbidimetric assay and ECLIA

	Immunoturbidimetric assay (Cobas c501)	ECLIA (Cobas e601)
Testing time	9 minutes	18 minutes
Test principle	Expanded particle surface immunoturbidimetric test	sandwich
Calibration traceability	This method has been standardized against a selected manufacturer's measurement procedure (immunological method) *	The Elecsys Ferritin assay (REF 04491785) has been standardized against the Elecsys Ferritin assay (REF 11820982). The Elecsys Ferritin assay (REF 11820982) has been standardized against the Enzymun-Test Ferritin method. This in turn has been standardized against the 1st International Standard (IS) NIBSC (National Institute for Biological Standards and Control) »Reagent for Ferritin (human liver)« 80/602.
Limitations-interference	– Icterus: No significant interference up to an I index of 60 (approximate conjugated and unconjugated bilirubin concentration: 1026 μmol/L). – Hemolysis: No significant interference up to an H index of 500 (approximate hemoglobin concentration: 310 μmol/L). – Lipemia (Intralipid): No significant interference up to an L index of 1000. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration. – Rheumatoid factors up to 1200 U/mL do not interfere.	– The assay is unaffected by icterus (bilirubin < 1112 μmol/L) hemolysis (Hb < 0.31 mmol/L), lipemia (Intralipid < 3.3 g/L), and biotin < 205 nmol/L. – In patients receiving therapy with high biotin doses (i.e. > 5 mg/day), no sample should be taken until at least 8 hours after the last biotin administration. – No interference was observed from rheumatoid factors up to a concentration of 2500 U/mL. – There is no high-dose hook effect at ferritin concentrations of up to 100000 μg/L.
Reference range	30–400 ug/L for males and 15–150 μg/L for females.	30–400 μg/L for males and 15–150 μg/L for females.
Measuring range	5–1000 ng/mL (5–1000 μg/L)	0.5–2000 ng/mL (0.5–2000 μg/L).

* Data on file at Roche Diagnostics (kit insert).

Table II Evaluation of intra-day and intra-day method accuracy

	Cobas c501 biochemistry module				Cobas e601 hormone module			
	Intra-day mean (μg/L) (CV%)		Inter-day mean (μg/L) (CV%)		Intra-day mean (μg/L) (CV%)		Inter-day mean (μg/L) (CV%)	
	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
Laboratory	99.8 (0.73)	193.2 (1.3)	98.7 (1.78)	196.2 (2.8)	142.5 (1.09)	911.7 (2.25)	149.7 (4.59)	951.7 (4.1)
Manufacturer	223 (0.7)	568 (0.9)	226 (1.2)	558 (1.7)	19.4 (3)	234 (3.1)	14.7 (4)	361 (4.4)
IQC of Laboratory	99.3 (1.08)	197.9 (1.57)	98.7 (0.98)	194.1 (1.98)	161.3 (2.9)	965.3 (1.26)	158.1 (3.84)	949.5 (4.04)
IQC of Manufacturer	125 (0.8)	306 (0.6)	119 (1.1)	310 (1.3)	22.2 (2.1)	221 (1.9)	23.8 (4.3)	247 (4.9)

IQC: Internal Quality Control, CV: Coefficient of variation

Intra-day and inter-day repeatability were performed with the plasma pool obtained from patient samples. For inter-day repeatability, plasma pool was aliquoted as 21 pieces and stored under -20 degrees. The repeatability was conducted by using a single piece every day. On the other hand, intra-day repeatability was performed by using the prepared human plasma pool consecutively 21 times under the same conditions. The results were found as coefficient of variation (CV). Certainty study was performed according to EP15-3A protocol of CLSI. (10) Furthermore, different-level-controls for these two methods submitted by the manufacturer firm were repeated 5 times in 3 different days. Results were calculated as coefficient of variation (%CV) and compared to the values given by the manufacturer firm (*Table II*).

Statistical Analysis: For the statistical analysis, Medcalc (Mariakerke, Belgium) 18.9.1 version was used. Descriptive statistics were presented for categorical variables as number and percentage, average for numerical variable, median, standard deviation and interquartile range (IQR). Normal distribution skewness was determined by the examination of kurtosis values, Kolmogorov-Smirnov (Lilliefors Significance Correction), Shapiro-Wilk tests and distribution of histogram graphs. As the numerical variables had not met the normal distribution condition, two connected group comparisons were made with Wilcoxon Runk Sum test. Inter-methods relation was found with Spearman correlation and Passing Bablok regression analysis used for non-parametric test condition. The measurement difference between the methods were found with Bland-Altman analysis.

Results

When the results of patients grouped in terms of low (n=153) and high (n=84) plasma ferritin levels were evaluated with different devices by means of different methods, both high and low levels of plasma ferritin measured in hormone module (Cobas e601) were statistically much higher than results measured in biochemistry module (Cobas c501) ($p<0.001$). In addition, both low plasma ferritin results (r:0.993, $p<0.001$) and high ferritin results (r:0.966, $p<0.001$) of the two methods revealed a strong correlation positively. In Passing Bablok regression analysis, while y= 1.285 + 0.767x (intersection confidence interval: 0.7657 – 1.6695, slope confidence interval: 0.7292 – 0.8088) equation was found for low level plasma ferritin method comparison, y = 5.719 + 0.859x (intersection confidence interval: -3.8540 – 16.7387, slope confidence interval: 0.8195 to 0.9048) equation was found for high level plasma ferritin method comparison (*Figure 1* and *Figure 2*). In Bland-Altman graph, when differences between the two methods were compared, low plasma ferritin levels measured with ECLIA method were found to be 10.4% (1.44 µg/L) (bias%) higher

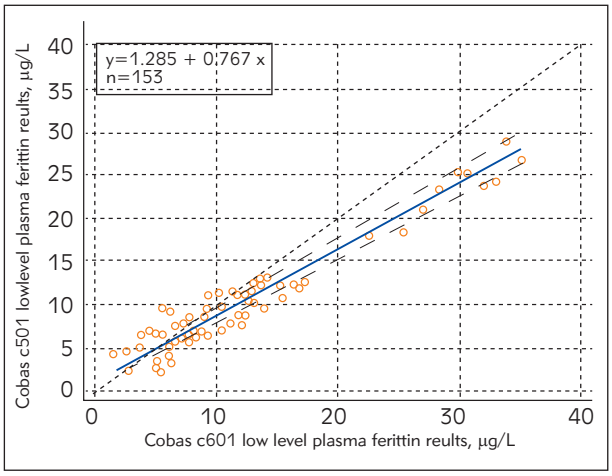

Figure 1 Comparison of Cobas c501 and Cobas e601 methods for low plasma ferritin values by Passing Bablok regression analysis.

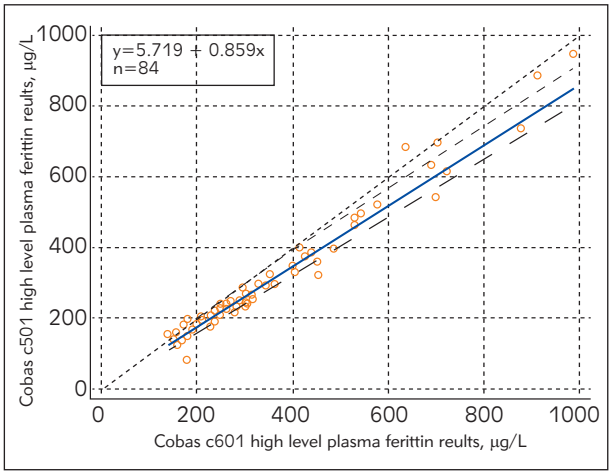

Figure 2 Comparison of Cobas c501 and Cobas e601 methods for high plasma ferritin values by Passing Bablok regression analysis.

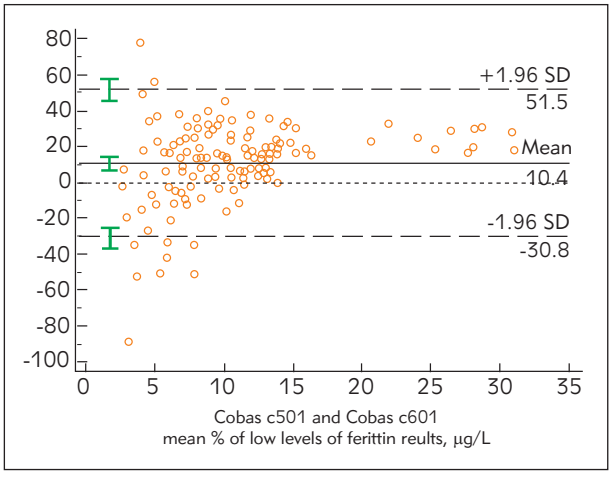

Figure 3 Differences between Cobas c501 and Cobas e601 methods for low plasma ferritin values by Bland Altman analysis (bias%= 10.4%).

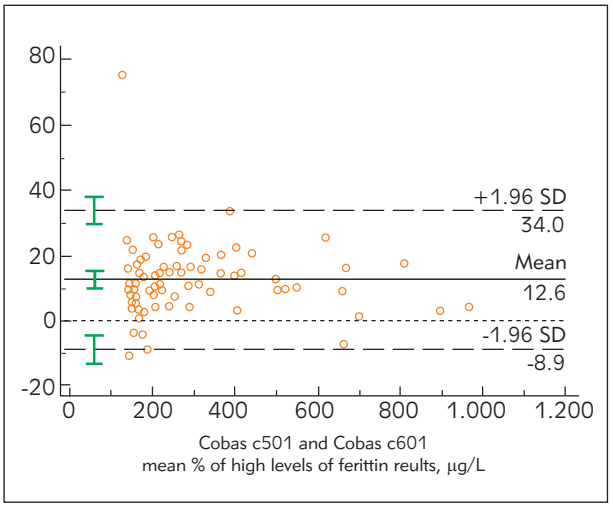

Figure 4 Differences between Cobas c501 and Cobas e601 methods for high plasma ferritin values by Bland Altman analysis (bias%= 12.6%).

compared to plasma ferritin levels measured with immunoturbidimetric method. Furthermore, high plasma ferritin levels measured with ECLIA were about 12.6% (bias%) higher than plasma ferritin levels measured with immunoturbidimetric method. While inter-methods bias value (1.44 µg/L) of low plasma ferritin level was lower than the acceptable bias value (3 µg/L) declared by RCPA for results lower than 40 µg/L, bias% value (10.4%) was found to be lower than bias% values suggested by WLSH (bias%: 15%) and AAB (bias%: 15%). However, it was found to be higher than bias% values suggested by CAP, BV and CFX (8%, 8.7%, 9.7% respectively). Inter-methods bias% value of high plasma ferritin levels (12.6%) was found lower than bias% values suggested by WLSH (bias%: 15%) and AAB (bias%: 15%). Yet, it was found to be higher than bias% values suggested by RCPA, CAP, BV and CFX (7.5%, 8%, 8.7%, 9.7% respectively) (*Figure 3* and *Figure 4*).

Discussion

In this study, plasma ferritin levels identified as high and low were measured with both methods in Cobas 6000 modular system in our laboratory according to reference range of Cobas c501 biochemistry analyzer and Cobas e601 hormone analyzer. Whether there is a difference between different levels of plasma ferritin between two methods and whether these methods could be used interchangeably have also been evaluated.

The first result of the study regarding whether there is a difference among plasma ferritin levels according to the methods have revealed that the difference was statistically significant. Secondly, the results of the two methods were in consistency according to Passing-Bablok regression analysis and Spearman correlation analysis. When the difference between methods were evaluated with Bland-Altman analysis, bias% value for low plasma ferritin levels was found to be 10.4% (1.44 µg/L) and bias% value for high plasma ferritin levels was found to be 12.6%. Acceptable bias% values for plasma/serum ferritin levels obtained from various sources (RCPA: 3 µg/L (<40 µg/L for results), 7.5% (>40 µg/L for ferritin results), WLSH: 15%; CAP: 8%; CFX: 9.7%; AAB: 15%. BV: 8.7%) and bias% value obtained for low plasma ferritin results were found to be lower than bias value recommended for low ferritin levels by RCPA and bias% values recommended by WLSH and AAB. bias% value found in the study regarding high plasma ferritin results was only lower than bias% values recommended by WLSH and AAB. These results show that deviation in high plasma ferritin levels between the two methods is higher than deviation in low plasma ferritin levels.

Previously, serum/plasma ferritin levels were evaluated with different methods and the results were shown below. In the study where nce et.al evaluated randomly selected patients' serum ferritin levels with unintegrated AU5800 biochemistry analyzer and Cobas e601 hormone analyzer, it was noted that there was a positive correlation between serum ferritin results evaluated with different measurement methods and indicated that the two methods can be used interchangeably as the difference between them was within clinically acceptable limits (11). In another study, Dupuy et al. (12) compared chemiluminescence and immunoturbidimetric method with radioimmunoassay (RIA) method. As a result of their Bland Altman analysis, it was highlighted that the methods of which serum ferritin levels were compared were in consistency with each other and indicated that these methods could be used instead of RIA method (12). Karakochuk et al. (13) examined serum ferritin levels in non-pregnant women having iron deficiency with four different immunoassay methods and they observed that serum ferritin results were in different concentrations in systems using different calibrator, ferritin isoforms and antibodies. Despite those different results, they stated that it correctly reflected iron deficiency prevalence (13). Zhang et al. (14) compared patient samples in different concentrations in Architect i2000 (Abbott Laboratories) and Cobas e601 (Roche Diagnostics) systems with two different methods and revealed that the average of serum ferritin levels made in Cobas e601 around 60.6 ng/mL (µg/L) were found to be higher than the average of Architect 2000 auto-analyzer. As a result, it has been concluded that both methods show correlation; however, they cannot be used interchangeably, and patients' serum ferritin results should always be observed with the same method (14, 15).

Conclusion

According to our results, the difference between high and low results depending on plasma ferritin reference range was found to be statistically significant. On the other hand, there was a coherence found between the different levels of both methods based on correlation and regression analysis. However, while bias% results were lower than WLSH and AAB % bias results, they were higher than CAP, CFX and BV's bias% results. As a result, it has been believed that, these two methods should be compared with more samples especially in different clinical decision levels in order to examine interchangeable use of immunoturbidimetric method and ECLIA in integrated devices.

Limitations

Despite the fact that the study has fulfilled its purpose, there were some limitations. Firstly, plasma ferritin levels of patients only determined as low or high depending on reference range were examined. Patient samples which are at the clinical decision limits can be compared with these two methods both for serum and plasma samples and the difference between the methods can be analyzed. Secondly, a comparison between the two methods can be performed by including normal plasma ferritin levels within the reference range for future studies.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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THROMBIN GENERATION IN DIFFERENT COMMERCIAL SODIUM CITRATE BLOOD TUBES

STVARANJE TROMBINA U RAZLIČITIM KOMERCIJALNIM EPRUVETAMA ZA KRV SA NATRIJUM CITRATOM

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Summary

Background: This study aimed to verify whether blood drawn into six different commercial coagulation tubes generated comparable results of thrombin generation.
Methods: Blood was sequentially collected from 20 healthy subjects into different brand and draw volume 3.2% sodium citrate tubes (4.3 mL Sarstedt, 3.0 mL Greiner, 2.7 mL Becton Dickinson, 2.0 mL Kima, 1.8 mL Sarstedt and 1.0 mL Greiner). Thrombin generation was measured in plasma with the fully-automated ST Genesia analyzer using the weakest trigger (STG-BleedScreen).
Results: Different values of lag time (LT), time to reach thrombin peak (TP), thrombin peak height (PH) and endogenous thrombin potential (ETP) were commonly found in different tubes. Thrombin generation was the lowest in 4.3 mL Sarstedt tubes and the highest in 1.0 mL Greiner tubes. Other tubes displayed intermediate values. In multiple comparisons, LT was significantly different in 6/15 cases (40%), whilst PH, TP and ETP were significantly different in 14/15 (93%), 13/15 (87%) and 13/15 (87%) cases. The mean percent bias of LT, PH, TP and ETP ranged between -6% and +1%, -27% and +116%, -22% and +8%, and between -18% and +65%. The intra-assay imprecision of LT, PH, TP and ETP was exceeded in 0/15 (0%), 13/15 (87%), 6/15 (40%) and 13/15 (87%) comparisons. The correlation of LT, PH, TP and ETP values in different tubes ranged between 0.718–0.971, 0.570–0.966, 0.725–0.977 and 0.101–0.904.

Kratak sadržaj

Uvod: Ovo izučavanje je imalo za cilj da potvrdi da li krv uzeta u šest različitih komercaijalnih epruveta za koagulaciju proizvodi uporedive rezultate stvaranja trombina.
Metode: Krv je uzastopno uizimana od 20 zdravih osoba u različite zapremine epruveta sa 3,2% natrijum citrata (4,3 mL Sarstedt, 3,0 mL Greiner, 2,7 mL Becton Dickinsion, 2,0 mL Kima, 1,8 mL Sarstedt i 1,0 mL Greiner). Stvaranje trombina mereno je u plazmi na potpuno automatizovanom ST Genesia analizatoru primenom slabog okidača (STG-BleedScreen).
Rezultati: Različite vrednosti lag vremena (LT), vremena dostizanja trombinskog pika (TP), visine trombinskog pika (ETP) su nađeni u različitim epruvetama. Stvaranja trombina bilo je najniže u 4,3 mL Sarstedt epruvetama a najviše u 1,0 mL Greiner epruvetama. U drugim epruvetama nađene su srednje vrednosti. U multiplom poređenju, LT se značajno razlikovao u 6/15 slučajeva (40%), dok su se PH, TP i ETP značajno razlikovali u 15/15 (93%), 13/15 (87%) i 13/15 (87%) slučajeva. Srednji procenat odstupanja LT, PH, TP i ETP kretao se između -6% i +1%, -27%, i +116%, -22% i +8% i između -18%, i +65%. Unutrašnja nepreciznost za LT, PH, TP i ETP bila je povećana u 0/15 (0%), 13/15 (87%), 6/15 (40%) i 13/15 (87%) slučajeva. Korelacija LT, PH, TP i ETP vrednosti u različitim epruvetama kretala se između 0,718–0,971, 0,570–0,966, 0,725–0,977 i 0,101–0,904.
Zaključak: Sakupljanje krvi zahteva lokalnu standardizaciju primenom identičnih epruveta za vrstu i zapreminu i

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Conclusions: Blood collection for thrombin generation assays requires local standardization using identical tubes for brand and draw volume, and reference ranges calculated according to type of tubes.

Keywords: preanalytical variability; blood tubes; blood collection; thrombin generation

referentne vrednosti izračunate prema tipu epruveta koje se koriste za uzimanje krvi.

Ključne reči: preanalitička varijabilnost, epruvete za krv, sakupljanje krvi, stvaranje trombina

Introduction

Laboratory hemostasis represents an essential part of diagnostic reasoning and clinical decision making in patients with hemostasis disorders, either hemorrhagic or thrombotic (1). Among the various hemostasis tests, thrombin generation is now emerging as a valuable investigative tool, since it helps in obtaining essential clinical information for monitoring the global hemostasis potential of patients with inherited (i.e., hemophiliacs) or acquired (i.e., severely injured or heavily traumatized) bleeding disorders, for assessing the risk of development or recurrence of venous thromboembolism, as well as for monitoring both anti-hemorrhagic and anti-thrombotic treatments (2, 3). Although thrombin generation assays have been empirically used since early 1950s, it was only recently that a standardized calibrated automated thrombin generation (CAT) assay has been developed by Hemker et al. (4). This method allows assessing thrombin generation in both platelet-poor and platelet-rich plasma, whereby the latter sample matrix would provide results also dependent on platelet function (4). The main aspects evaluated with the standardized CAT assay include the lag time (LT; reflecting the time necessary for initial thrombin generation after adding the trigger), the time to reach the thrombin peak (TP; mirroring the speed of thrombin generation), the thrombin peak height (PH; reflecting the highest value of thrombin generated) and the endogenous thrombin potential (ETP; underscoring the total amount of thrombin generated). The combination of these different parameters contributes to accurately define the hemostatic potential in the test plasma, as reflected by the speed and amount of thrombin that can be actually generated (5).

As with any other laboratory and hemostasis tests (6–8), thrombin generation assays are highly vulnerable to the impact of many preanalytical variables, which may ultimately influence the final quality of test results. The Scientific and Standardization Committee (SSC) of the International Society of Thrombosis and Hemostasis (ISTH) has recently published an official document aimed at highlighting the many preanalytical variables directly impacting the reliability of this test (9), which encompass the procedures used for drawing blood, for transportation of samples and for their preparation and storage before testing. Notably, the SSC of the ISTH has also highlighted that some important differences may be noted using different brands of blood tubes, thus hampering within- and between-laboratory standardization of this important measurement. Therefore, this study was essentially aimed at verifying whether or not blood drawn into different commercial coagulation blood tubes would generate comparable results of thrombin generation on a newly commercialized fully automated thrombin generation system, ST Genesia, using the weakest trigger available with it, as most differences are reported at low trigger activity (9, 11).

Materials and Methods

The study population consisted on 20 ostensibly healthy subjects recruited among the laboratory staff (mean age: 40±10 years; 10 women and 10 men). Blood was sequentially collected by the same expert phlebotomist, early in the morning, by straight needle venipuncture into different brand and draw volume 3.2% sodium citrate blood tubes, as follows: 13×75 mm, 4.3 mL non-evacuated S-Monovette (Reference number: 04.1922.001; Lot number: 8034811; Sarstedt, Nümbrecht, Germany); 13×75 mm, 3.0 mL evacuated Vacuette (Reference number: 454334; Lot number: A180848F; Greiner Bio-One, Kremsmünster, Austria); 13×75 mm, 2.7 mL Vacutainer (Reference number: 363048; Lot number: 8242840; Becton Dickinson [BD], Plymouth, United Kingdom); 13×75 mm, 2.0 mL evacuated Vacutest (Reference number: 14074; Lot number: A2908; Kima, Padova, Italy); 1.8 mL non-evacuated S-Monovette (Reference number: 04.1955.001; Lot number: 9030111; Sarstedt, Nümbrecht, Germany); 13×75 mm, 1.0 mL evacuated Vacuette (Reference number: 454320; Lot number: A181039T; Greiner Bio-One, Kremsmünster, Austria). The sequence of the tubes was varied among subjects, by escalating one tube for each next subject in the sequence. Blood was drawn in both Sarstedt tubes, by manual rather than by vacuum aspiration (i.e., blood was aspirated by slowly withdrawing the plunger until complete filling). Plasma was immediately separated after blood collection (i.e., within 15 min), by centrifugation at 1500×g, for 15 min, at room temperature.

Thrombin generation parameters (i.e., LT, TP, PH and ETP) were measured, immediately after plasma separation, by using the novel fully-automated

analyzer ST Genesia with STG-BleedScreen (Diagnostica Stago, Asnières sur Seine, France), which is essentially based on the reference CAT assay developed by Hemker et al. (4). This method encompasses the assessment of thrombin generation based on measurement of fluorophore aminomethylcoumarin (AMC) generation, after adding a standard amount of human recombinant tissue factor and synthetic phospholipids to the test plasma. AMC generation is monitored every 15–20 seconds at 450 nm, and mirrors the quantity of thrombin generated throughout the measuring range. All measurements were carried out in duplicate, during a single analytical session, and final results were reported as mean of the two duplicate measures. According to manufacturer's specifications, the intra-assay imprecision of thrombin generation with ST Genesia and STG-BleedScreen is 7.0% for both LT and TP, 7.5% for PH and 6.2% for ETP.

Normality of value distributions were verified with Shapiro-Wilk Test. The statistical analysis was hence performed using parametric tests and results were finally reported as mean and 95% confidence interval (95% CI). More specifically, the difference and correlation of values among all tubes were evaluated with Student's T and Pearson's tests, respectively. Due to the lack of biological variation studies for thrombin generation, results obtained using different tubes were considered significant when the percent bias of values between two tubes was larger than the intra-assay imprecision of each thrombin generation parameter. The statistical significance was set at $p<0.05$. The statistical analysis was performed with Analyse-it (Analyse-it Software Ltd, Leeds, UK). All subjects provided a written informed consent for participating to this study, which was cleared by the local Ethics Committee (970CESC; July 20, 2016).

Results

The results of this study are shown in *Tables I* and *II*. The lowest thrombin generation in plasma was observed in 4.3 mL Sarstedt blood tubes, as mirrored by the longest values of LT and TP as well as by the lowest values of PH and ETP. The highest thrombin generation in plasma was instead found in 1.0 mL Greiner blood tubes, as reflected by the shortest values of LT and TP as well as by the highest values of PH and ETP. All other blood tubes exhibited intermediate values of all thrombin generation parameters. Notably, in multiple comparisons among the different blood tubes (*Table II*), LT was found to be significantly different in 6/15 cases (40%), whilst PH, TP and ETP were found to be significantly different in 14/15 (93%), 13/15 (87%) and 13/15 (87%) cases, respectively. The mean percentage bias of LT, PH, TP and ETP among the different blood tubes was comprised between -6% and +1%, -27% and +116%, -22% and +8%, and between -18% and +65%, respectively (*Table II*). Notably, although in no case did the bias among the different blood tubes exceed the intra-assay imprecision of LT (i.e., ±7.0), the intra-assay imprecision of PH (±7.5%), TP (±7.0%) and ETP (±6.2%) was exceeded in 13/15 (87%), 6/15 (40%) and 13/15 (87%) comparisons, respectively. The correlation (r) of LT, PH, TP and ETP values among the different sodium citrate blood tubes was comprised between 0.718–0.971, 0.570–0.966, 0.725–0.977 and between 0.101–0.904, respectively. The multiple correlations among blood tubes were all good or acceptable and statistically significant (i.e., $p<0.05$), except for ETP measured in 1.0 mL Greiner blood tubes compared to any other blood tube (i.e., p values comprised between 0.074 and 0.672).

In multiple linear regression analysis, where brand and draw volume of blood tubes were entered as dependent variables and the different thrombin

Table I Mean and 95% confidence interval of thrombin generation parameters in plasma collected with different blood tubes.

Parameters	Sarstedt 4.3 mL	Greiner 3.0 mL	BD 2.7 mL	Kima 2.0 mL	Sarstedt 1.8 mL	Greiner 1.0 mL
Tube name	S-Monovette	Vacuette	Vacutainer	Vacutest	S-Monovette	Vacuette
Type of tube	Non-evacuated	Evacuated	Evacuated	Evacuated	Non-evacuated	Evacuated
Lag time (min)	3.06 (2.79–3.33)	3.11 (2.86–3.35)	3.09 (2.81–3.36)	3.05 (2.80–3.29)	2.96 (2.71–3.20)	2.93 (2.73–3.12)
Peak Height (nM)	137 (111–162)	194 (170–217)	142 (118–167)	167 (144–190)	157 (130–184)	295 (274–316)
Time to peak (min)	6.68 (6.18–7.18)	6.29 (5.88–6.70)	6.82 (6.32–7.31)	6.41 (6.01–6.80)	6.55 (6.04–7.05)	5.35 (5.00–5.69)
ETP (nM*min)	994 (871–1117)	1251 (1144–1358)	1029 (910–1148)	1145 (1043–1247)	1147 (1026–1269)	1644 (1531–1757)

Table II Mean percent variation, statistical significance and correlation of thrombin generation parameters in plasma collected with different blood tubes.

Blood tubes	Greiner 3.0 mL	BD 2.7 mL	Kima 2.0 mL	Sarstedt 1.8 mL	Greiner 1.0 mL
Sarstedt 4.3 mL	LT: +1%; p=0.152; r=0.959§ PH: +42%; p<0.001; r=0.902§ TP: -6%; p=0.001; r=0.918§ ETP: +26%; p<0.001; r=0.852§	LT: +1%; p=0.343; r=0.906§ PH: +4%; p=0.143; r=0.922§ TP: +2%; p=0.017; r=0.972§ ETP: +4%; p=0.122; r=0.891§	LT: +1%; p=0.425; r=0.790§ PH: +22%; p<0.001; r=0.957§ TP: +4%; p=0.010; r=0.914§ ETP: +15%; p<0.001; r=0.898§	LT: -3%; p=0.003; r=0.971§ PH: +15%; p<0.001; r=0.966§ TP: -2%; p=0.012; r=0.977§ ETP: +15%; p<0.001; r=0.880§	LT: +4%; p=0.033; r=0.876§ PH: +116% p<0.001; r=0.595§ TP: -20%; p<0.001; r=0.725§ ETP: +65%; p<0.001; r=0.185*
Greiner 3.0 mL	–	LT: -1%; p=0.377; r=0.922§ PH: -27%; p<0.001; r=0.894§ TP: +8%; p<0.001; r=0.930§ ETP: -18%; p<0.001; r=0.832§	LT: -2%; p=0.211; r=0.834§ PH: -14%; p<0.001; r=0.925§ TP: +2%; p=0.113; r=906§ ETP: -8%; p<0.001; r=0.904§	LT: -5%; p=0.001; r=0.949§ PH: -19%; p<0.001; r=0.901§ TP: +4%; p=0.014; r=0.918§ ETP: -8%; p=0.003; r=0.842	LT: -6%; p=0.001; r=0.914§ PH: +52%; p<0.001; r=0.771§ TP: -15%; p<0.001; r=0.924§ ETP: +31%; p<0.001; r=0.316*
BD 2.7 mL	–	–	LT: -1%; p=0.227; r=0.924§ PH: +17%; p<0.001; r=0.875§ TP: +6%; p=0.001; r=0.913§ ETP: +11%; p=0.003; r=0.793§	LT: -4% p=0.045; r=0.860§ PH: +10%; p=0.004; r=0.930§ TP: -4%; p<0.001; r=0.971§ ETP: +12%; p<0.001; r=0.856§	LT: +5%; p=0.032; r=0.824§ PH: +107%; p<0.001; r=0.570§ TP: -22%; p<0.001; r=0.763§ ETP: +60%; p<0.001; r=0.101*
Kima 2.0 mL	–	–	–	LT: -3%; p=0.173; r=0.739§ PH: -6%; p=0.029; r=0.935§ TP: +2%; p=0.139; r=0.878§ ETP: 0%; p=0.472; r=0.837§	LT: -4%; p=0.095; r=0.718§ PH: +77%; p<0.001; r=0.670§ TP: -17%; p<0.001; r=0.745§ ETP: +44%; p<0.001; r=0.409*
Sarstedt 1.8 mL	–	–	–	–	LT: -1%; p=0.309; r=0.860§ PH: +88%; p<0.001; r=0.648§ TP: -18%; p<0.001; r=0.734§ ETP: +43%; p<0.001; r=0.368*

§, p value of correlation <0.05; *, p value of correlation >0.05
LT, lag time; PH, thrombin peak height; TP, time to reach the thrombin peak; ETP; endogenous thrombin potential.

generation parameters were entered as independent variables, ETP was found to be significantly associated with both brand (p=0.042) and draw volume (p<0.001), PH with draw volume (p<0.001) but not with brand (p=0.087), TP with draw volume (p=0.001) but not with brand (p=0.296), whilst LT was not associated with either draw volume (p=0.267) or brand (p=0.493).

Discussion

Although the SSC of the ISTH has recently stated that blood drawn into different brands of coagulation tubes may be a source of bias in thrombin generation studies, limited evidence for this presumption has been published in the scientific literature to the best of our knowledge. Earlier information was published by Dargaud and Negrier (10), who showed that the overall thrombin generation (i.e., ETP) measured in platelet-rich plasma was considerably higher when blood was drawn by forced aspiration into evacuated BD Vacutainer tubes than with slow manual aspiration in Sarstdet Monovette tubes. In a subsequent study, Loeffen et al. (11), assayed thrombin generation in seven different sodium citrate tubes (12), and also found that ETP was consistently lower in plasma collected into 4.3 mL Sarstedt Monovette tubes (i.e., 270 nM*min) than in standard evacuated blood tubes such as 2.7 mL BD Vacutainer (i.e., 465 nM*min) and 9.0 mL Greiner Vacuette (i.e., 490 nM*min), whilst the other parameters remained virtually similar.

According to our data, the direct comparison of thrombin generation measured in plasma collected into 1.8 mL Sarstedt tubes and 2.0 mL Kima tubes does not support the hypothesis that slow (manual)

aspiration of blood would results in lower pre-activation of blood coagulation, since all thrombin generation parameters were found to be non-statistically different between these two tubes, the mean difference was always comprised within the intra-assay imprecision, and the Spearman's correlation of the various parameters was also satisfactory (i.e., comprised between 0.739 and 0.935) (*Table II*). On the other hand, data obtained in plasma collected into the 4.3 mL Sarstedt tube were comparable to those obtained in the 2.7 mL BD tube, since in no case the percent variation calculated on these two tubes was larger than the intra-assay imprecision. This would lead us to conclude that both draw volume and tube composition may produce a larger impact on thrombin generation than the mode of blood aspiration (i.e., manual or forced by the vacuum).

Therefore, the first conclusion that can be made according to our data, is that the accurate assessment of thrombin generation would need standardized blood collection by always using identical tubes for brand and draw volume. This procedure seems actually unavoidable since specific tube-dependent reference ranges should be defined for all the different parameters, and is even more important for enabling a reliable longitudinal monitoring of patient's data. Irrespective of the highly anomalous values obtained using plasma collected into 1.0 mL Greiner tubes, the difference observed comparing the values of the other five tubes were also generally noteworthy, since the intra-assay imprecision was exceeded in 8/10 (80%) of cases for both PH and ETP (*Table II*). Another important aspect that emerged from this investigation is that the use of very low-draw citrate collection tubes (i.e., 1.0 mL) may be unsuitable for measuring thrombin generation, since their use may substantially increase the speed and total amount of thrombin generation, so producing values that are not at all comparable with those of other tubes, as clearly shown by the correlation coefficients reported in *Table II*. It is indeed conceivable that very low volume blood draw was the main source of this variation, since the comparison of the two Greiner blood tubes (which share the same composition and additive) yielded mean differences of PH, TP and ETP as high as 116%, -20% and +65%, in all circumstances largely exceeding the intra-assay variability of the method. This also means that any studies assessing thrombin generation which may potentially use low volume versus standard volume tubes (e.g., in neonates and adults, respectively) would lead to substantial bias in the results of such study.

Conclusions

In conclusion, the results of our study further emphasize the importance of standardizing the preanalytical phase for obtaining reliable and consistent results of thrombin generation to be used in clinical practice. In particular, we suggest that blood collection needs to be locally standardized by always using identical tubes for brand and draw volume, and that any reference ranges for the different thrombin generation parameters must be locally calculated according to the type of tubes used for blood collection.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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THE RELATIONSHIP BETWEEN SEX STEROIDS, INSULIN RESISTANCE AND BODY COMPOSITIONS IN OBESE WOMEN: A CASE-CONTROL STUDY

ODNOS IZMEĐU POLNIH STEROIDA, INSULINSKE REZISTENCIJE I TELESNE KOMPOZICIJE KOD GOJAZNIH ŽENA: KONTROLISANA STUDIJA SLUČAJA

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Summary

Background: Obesity causes many health problems and affects the quality and duration of life negatively. We aimed to investigate the relationship between sex steroids, insulin resistance and body compositions in obese women.
Methods: This study was carried out on a sample of 150 premenopausal women who were referred to the Outpatient Clinic of Family Medicine between 2014–2015. A survey about their socio-demographic characteristics was carried out, and anthropometric parameters were measured. LDL-C, HDL-C, total cholesterol, triglyceride, glucose, insulin, sex hormone binding globulin (SHBG), estradiol, dehydroepiandrosterone sulfate (DHEA-S), total/free testosterone levels were measured in the blood. Body compositions were assessed with a bioelectrical impedance device. For insulin resistance, Homeostasis Model Assessment (HOMA-IR) was calculated.
Results: In our study, a significant association was found between high glucose, total cholesterol, LDL-C, TG, insulin, insulin resistance and low HDL-C, SHBG, DHEA-S levels with obesity ($p<0.05$). There was no statistically significant relationship between estradiol, total/free testosterone and obesity ($p>0.05$).
Conclusions: In our study, high glucose, total cholesterol, LDL-c, TG, insulin, insulin resistance and low HDL-C, SHBG, DHEA-S levels were associated with obesity. This relationship leads to many diseases, especially diabetes mellitus and cardiovascular disease. Therefore, obesity is a disease that needs to be monitored closely, frequently and treated properly.

Keywords: obesity, sex steroids, insulin resistance, female

Kratak sadržaj

Uvod: Gojaznost izaziva mnoge zdravstvene probleme i negativno utiče na kvalitet života i životni vek. Cilj nam je bio da istražimo vezu između polnih steroida, insulinske rezistencije i telesne kompozicije kod gojaznih žena.
Metode: Ova studija je sprovedena na uzorku od 150 žena u perimenopauzi koje su posetile ambulantu porodične medicine u periodu 2014–2015. Primenjeno je istraživanje o njihovim socio-demografskim karakteristikama i izmereni su antropometrijski parametri. Iz krvi su izmereni LDL-C, HDL-C, ukupni holesterol, trigliceridi, glukoza, insulin, globulin koji vezuje polni hormon (SHBG), estradiol, dehidroepiandrosteron sulfat (DHEA-S), i nivoi ukupnog/slobodnog testosterona. Telesne kompozicije su procenjene pomoću uređaja sa bioelektričnom impedansom. Za rezistenciju na insulin, izračunat je Homa model (HOMA-IR, Homeostasis Model Assessment).
Rezultati: U našoj studiji je pronađena značajna povezanost između visoke glukoze, ukupnog holesterola, LDL-C, TG, insulina, rezistencije na insulin i niskog nivoa HDL-C, SHBG, DHEA-S i gojaznosti ($p<0,05$). Nije bilo statistički značajne veze između estradiola, ukupnog/slobodnog testosterona i gojaznosti ($p>0,05$).
Zaključak: Naša studija je ukazala da su sledeći faktori bili povezani sa gojaznošću: visoka glukoza, ukupni holesterol, LDL-C, TG, insulin, insulinska rezistencija i niski nivoi HDL-C, SHBG, DHEA-S. Ovaj odnos dovodi do mnogih bolesti, posebno dijabetes melitusa i kardiovaskularnih bolesti. Dakle, gojaznost je bolest koju treba pažljivo pratiti i redovno tretirati na odgovarajući način.

Ključne reči: gojaznost, polni steroidi, rezistencija na insulin, žena

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Introduction

Obesity is a complicated and multifactorial disease which occurs as a result of the interaction of genetic, metabolic, social, behavioural and cultural factors. Obesity provides a basis for many diseases, reduces the quality of life and life's duration and it causes deaths. For this reason, it has become an important public health concern on a global scale (1).

Obesity causes gender-specific issues as well as similar comorbidities in men and women. Gender-specific issues start with early puberty, amenorrhoea and dysovulation, and they go forward with polycystic ovary syndrome (PCOS), infertility, obstetric problems and endometrial and breast cancer incidence which increase after menopause (2).

In premenopausal women, the increase in body fat ratio can cause an imbalance in sex steroids. Estrogen aromatization of androgens increases the amount of estrogen in stromal vascular cells in fatty tissue. While the estrogen/androgen ratio increases in obese individuals, there is a decrease in sex hormone-binding globulin (SHBG). Androgen transition accelerates depending on this and androgen synthesis increases to correspond to this. As a result, free estrogen and testosterone levels increase (3).

In 65% of patients with type 2 diabetes, obesity is responsible for etiology. Type 2 diabetes risk rises with the degree and duration of obesity and the existence of abdominal obesity. Free fatty acids which are extremely secreted by visceral adipocytes cause insulin resistance both in skeletal muscle and in the liver. It is known that the disruption of hormone regulation which is secreted from adipose tissues also affects insulin resistance (4).

In this study, we aimed to investigate the relationship between sex steroids, insulin resistance and body composition in obese and non-obese women who came to our polyclinic.

Materials and Methods

Study design, setting and population

This analytical case-control study was carried out on a sample of women aged 18 and older who came to Family Practise Polyclinic for any reasons between October 1, 2014, and March 1, 2015.

The individuals were classified as normal weight people (BMI<25 kg/m^2), overweight people (BMI 25–29.9 kg/m^2) and obese people (BMI 30 kg/m^2) in terms of their body mass index (BMI). The groups were similar in terms of ages and residences.

In our study, the number of subjects included in the study was calculated using $n = t^2 \cdot p \cdot q / d^2$ formula because the number of individuals in the universe was not known. According to this calculation, 150 women were included in our study.

Exclusion criteria

Those with diabetes mellitus, disorder of thyroid, liver and kidney failure and infertility, and those who were pregnant, lactating women, in the period of menopause, ones who were receiving oral contraceptive and hormone replacement therapy, using medications containing cortisol, ones who had hysterectomy or oophorectomy operations, who had a drug history story to affect SHBG levels and those who did not agree to participate in the study were not included in the research.

Ethical Authorisation of the study

Before the study started, an ethics committee approval was received from Necmettin Erbakan University, Meram Medical Faculty (Number: 2013/31 Date: October 23, 2013).

During the application phase, patients were informed about the objectives of the study, and they gave their oral and written consent.

Collection of data

The survey that questioned sociodemographic characteristics of the participants was carried out through face to face interview technique.

Tall statures, body weights, hip and waist circumferences (WC) of the participants were measured with a standard bascule, and a length meter and BMI was calculated. According to WHO obesity criteria, waist circumference cut-off value was accepted to be 88 cm in women (5). Blood pressure in lower and upper extremities was measured by a sphygmomanometer while patients were lying on their backs.

Bioelectrical impedance device was used to measure and evaluate the body compositions of the individuals. Body fat ratio, water ratio, bone mass, muscle mass, basal metabolism and visceral fat ratio were measured too.

LDL-C, HDL-C, total cholesterol, triglyceride, fasting blood glucose (FBG), fasting insulin, SHGB, total testosterone, free testosterone, estradiol and DHEA-S levels were measured from blood samples of patients after 12-hour fasting. Fasting blood glucose (by the hexokinase method), total cholesterol (by cholesterol esterase method), LDL-C (by Friedewald formula), triglyceride and HDL-C (by enzymatic colourimetric method) were measured using the Abbott Architect c16000 Systems autoanalyser. SHBG, estradiol and insulin (by chemiluminescent microparticle immunoassay method) were measured using Abbott Architect i4000 SR autoanalyser. DHEAS (by competitive chemiluminescent enzyme immunoassay method) was measured using Siemens IMMULITE 2000 XPi autoanalyser. Total testosterone

and free testosterone (by ELISA method) were measured using the Siemens Advia Centaur Immunoassay System.

For insulin resistance, HOMA-IR was calculated with the formula below by using fasting plasma glucose and insulin levels. A cut-off value of HOMA-IR was taken as 2.5 (6).

HOMA-IR=Serum glucose (mmol/L)×plasma insulin (μU/mL)/405.

Statistical analysis

SPSS 20.0 packet program was used to statistically evaluate the results obtained in the study. Descriptive statistics for continuous variables were given in terms of average and standard deviation, and descriptive statistics for categorical data were given in terms of frequency and percentage. To compare quantitative data in doublet groups, the student t-test was used if they corresponded to normal distribution hypothesis and Mann-Whitney U test was used if they did not correspond to normal distribution hypothesis and if they showed a skew distribution. To compare quantitative data in the triad, one-way ANOVA test was used in the parameters which showed normal distribution. Triads which did not show normal distribution were compared by using the Kruskal-Wallis test. Double comparisons were made by Mann-Whitney U test. Chi-square test was used to compare categorical data. Results were evaluated at a 95% confidence interval, and significance was evaluated in $p<0.05$ level.

Results

The mean age of 150 women participating in the study was 31.27±8.39. When the women were classified into 3 groups according to BMI, there was not a difference between the groups regarding the average age ($p=0.151$). There was a significant association between obesity and being low educated ($p<0.001$). The prevalence of obesity was higher in housewives ($p<0.001$). The prevalence of being overweight and obesity was higher in married women ($p<0.001$). The prevalence of obesity in the low-income group was significantly higher ($p<0.001$) (*Table I*).

Table I Comparison of sociodemographic characteristics between body mass index groups.

Sociodemographic characteristics	Normal weight		Overweight		Obese		X^2	p
	n	%	n	%	n	%		
Marital status								
Married	25	24.0	34	32.7	45	43.3	18.875	<0.001
Single	25	54.3	16	34.8	5	10.9		
Working status								
Working	21	38.9	25	46.3	8	14.8	13.715	<0.001
Non-working	29	30.2	25	26.0	42	43.8		
Education level								
≤ Secondary education	7	12.3	14	24.6	36	63.2	38.879	<0.001
≥ High school education	43	46.2	36	38.7	14	15.1		
Income status								
Less than TL 1000	13	28.3	8	17.4	25	54.3	26.832	<0.001
TL 1000-2000	12	21.4	24	42.9	20	35.7		
More than TL 2000	25	52.1	18	37.5	5	10.4		
Residence								
Rural area	4	33.3	3	25.0	5	41.7	0.549	0.760
Urban area	46	33.3	47	34.1	45	32.6		
Smoking								
Yes	6	35.3	6	35.3	5	29.4	0.133	0.936
No	44	33.1	44	33.1	45	33.8		
Doing exercises								
Yes	6	27.3	11	50.0	5	22.7	3.303	0.192
No	44	34.4	39	30.5	45	35.2		
Daily activities								
High	13	35.1	11	29.7	13	35.1	2.105	0.716
Middle	25	30.9	31	38.3	25	30.9		
Low	12	37.5	8	25.0	12	37.5		

Table II Comparison of laboratory parameters between body mass index groups.

	Normal weight (a)	Overweight (b)	Obese (c)		
	Mean±SD	Mean±SD	Mean±SD	F	p
FBG (mmol/L)	4.94±0.47	5.33±0.59	5.39±0.41	12.044	$<0.001^{ab}$ <0.001ac
Total-C (mmol/L)	4.56±0.89	4.68±0.91	5.12±1.03	4.859	0.010^{ac}
LDL-C (mmol/L)	2.73±0.74	2.89±0.75	3.32±0.83	7.731	0.001^{ac} 0.017^{bc}
HDL-C (mmol/L)	1.36±0.25	1.22±0.23	1.18±0.25	7.110	0.018^{ab} 0.001^{ac}
TG (mmol/L)	0.97±0.54	1.20±0.68	1.45±0.79	6.439	0.001^{ac}
	Median (min-max)	Median (min-max)	Median (min-max)	χ^2	p
Insulin (pmol/L)	40.41 (12.29–378.22)	49.30 (13.40–396.14)	54.24 (24.23–201.40)	14.403	0.026^{ab} $<0.001^{ac}$
*SHBG (nmol/L)	59.8 (15.0–246.0)	40.9 (15.6–321.9)	35.8 (8.1–122.4)	19.000	0.001^{ab} $<0.001^{ac}$
Estradiol (pmol/L)	381.7 (73.4–1266.4)	436.8 (110.1–3061.6)	295.5 (73.4–1457.3)	1.629	0.443
**DHEA-S (µmol/L)	5.30 (0.14–14.16)	5.00 (0.04–13.20)	3.79 (0.85–11.14)	6.149	0.021^{ac}
T. Testosterone (nmol/L)	1.45 (0.13–2.63)	1.14 (0.34–3.08)	1.04 (0.34–5.69)	1.811	0.404
F. Testosterone (pmol/L)	9.75 (0.52–52.88)	9.61 (2.01–51.46)	7.98 (1.04–49.72)	1.852	0.396

*SHBG= sex hormone-binding globulin, **DHEA-S= dehydroepiandrosterone sulfate, F= One-way ANOVA, χ^2= Kruskal-Wallis
a: Normal weight group, b: Overweight group, c: Obese group

Obesity prevalence in married women was 6.254 times higher than in single women [OR=6.254, %95 CI; (2.287–17.107)], obesity prevalence in non-working women was 4.472 higher than in working women [OR=4.472, %95 CI; (1.907–10.487)], obesity prevalence in the middle school and under-educated women was 9.673 times higher than in high school and highly educated women [OR=9.673, %95 CI; (4.422–21.160)], and these differences were highly statistically significant ($p<0.001$).

Systolic blood pressures in the obese group were significantly higher than the normal weight ($p<0.001$) and overweight ($p=0.023$) group. Diastolic blood pressures in the obese group were significantly higher than in the normal weight group ($p=0.014$).

FBG, total cholesterol, LDL-C, triglyceride values in the normal weight group were significantly lower than in the overweight ($p<0.001$) and the obese group ($p<0.001$); HDL-C values were higher. Whereas insulin values in normal weight group were significantly lower than in overweight ($p=0.026$) and obese group ($p<0.001$), their SHBG and DHEA-S values were significantly higher ($p<0.001$). When BMI and estradiol, total and free testosterone values were compared, no statistically significant relation was found (*Table II*).

In our study, insulin values in the group with WC>88 cm was significantly higher than those with WC 88 cm ($p=0.001$). Insulin resistance in the group where WC was >88 cm was significantly higher than those with WC 88 cm ($\chi^2=5.697$, $p=0.017$). When waist circumferences were compared to SHGB, DHEA-S and total testosterone levels, SHBG ($p<0.001$), DHEA-S ($p=0.042$) and total testosterone ($p=0.037$) values were significantly lower in the group with WC>88 cm than those with WC 88 cm. However, estradiol and free testosterone values did not show a significant difference between the groups.

When BMI and body compositions were compared in our study, it was discovered that fat ratio, visceral fat, basal metabolism, metabolic age, muscle and bone ratios in the obese group were significantly higher than the normal weight and overweight groups but the water ratio was significantly lower ($p<0.001$) (*Table III*).

Table III Comparison of body composition between body mass index groups.

Parameters	Normal weight (a)	Overweight (b)	Obese (c)		
	Mean±SD	Mean±SD	Mean±SD	F	p
Fat ratio (%)	28.31±5.51	36.24±3.49	43.72±4.42	143.252	$<0.001^{ab.bc.ac}$
Basal met. (kcal)	1329±121	1477±98	1758±678	14.678	$<0.001^{ac}$ 0.002^{bc}
Basal met. (kJ)	5563±508	6183±412	6863±1118	37.730	$<0.001^{ab.bc.ac}$
Metabolic age (year)	27.1±10.0	43.16±8.9	49.1±8.8	74.362	$<0.001^{ab.ac}$ 0.005^{bc}
Water ratio (%)	52.9±4.1	47.4±2.6	42.2±3.0	129.443	$<0.001^{ab.bc.ac}$
Visceral fat (kg)	2.62±1.3	5.56±1.6	10.4±3.1	163.550	$<0.001^{ab.bc.ac}$
Bone ratio (%)	2.19±0.20	2.42±0.15	2.66±0.28	55.761	$<0.001^{ab.bc.ac}$
Muscle ratio (%)	40.9±3.84	45.3±3.0	50.0±5.5	56.805	$<0.001^{ab.bc.ac}$

a: Normal weight group, b: Overweight group, c: Obese group

Discussion

Obesity is a health problem with gradually increasing prevalence all over the world, particularly in developed countries. When the relationship between obesity and sociodemographic characteristics was investigated in our study, obesity was significantly higher in the middle school and under-educated women, whereas normal weight was higher in the high school and highly educated women. Obesity prevalence was 9.6 times higher in middle school and under-educated women. Various studies have shown that obesity prevalence decreases as the educational status of women increases (7, 8). Knowledge and thinking ability obtained from education seems to be important to prevent the increase in body weight in adulthood.

In our study, there was a significant relationship between marital status and BMI. Obesity was 6.2 times higher in married women than single ones. In long-time monitoring in the USA, it was seen that women used to gain weight after they got married even when they were standardized in terms of their educational status and family income (9). Low prevalence of obesity in single women may result from the fact that women in this group have never been pregnant, their age average is low, and this group may have been more sensitive to weight control.

In our study, obesity was significantly lower in working women. Obesity prevalence in non-working women showed a 4.4-fold increase compared to working women. In TURDEP I study performed in our country, it was found out that obesity prevalence was the highest in the group of housewives (10). Employed women are more conscious about protecting their body composition and are more successful than housewives in this respect.

In our study, there was a significant relationship between blood pressures and BMI. Blood pressure averages in the obese group were significantly higher. Epidemiological studies showed that there was a continual and strict correlation between body weight and blood pressure (11, 12). It is thought that hypertension is associated with liquid retention in obesity.

It is known that insulin resistance and hyperinsulinemia in people with type 2 diabetes increase VLDL and LDL-C formation in the liver and this causes hypertriglyceridaemia and low HDL-C levels (13). In studies, dyslipidaemia in obesity, total cholesterol, triglyceride and high LDL-C were defined as low HDL-C (14). In our study, as BMI increased in women, FBG, total cholesterol, LDL-C and TG levels also increased significantly, but the HDL-C level decreased.

It was found out that there was a significant relationship between obesity and HOMA-IR, and HOMA-IR value increased with weight gain as in insulin (15). Obese people need more insulin to keep blood glucose at normal limits than normal individuals. For this reason, a high level of insulin is secreted all the time. However, body fat distribution is a more important risk factor for insulin resistance (16). In our study, a significant relation was found between HOMA-IR and waist circumference which indicates abdominal obesity. Sex hormone-binding globulin (SHBG) is responsible for regulating the biological activities of sex hormones and the main carrier protein for estradiol and testosterone. In our study, while we were investigating the relationship between SHBG level and BMI and waist circumference, we found out that SHBG level

decreased significantly as BMI and waist circumference increased in women. In many studies, the results are found similar to our study (17–19). When the relation between insulin resistance and SHBG was investigated in our study, SHBG levels were significantly lower in the group with insulin resistance. In obesity, the increased insulin levels in the circulation suppress SHBG syntheses in the liver, and this is thought to be the basic mechanism of increased body weight to reduce SHBG levels (20).

The only estrogen source resulting from the stopping of estrogen production in the postmenopausal ovary is a transition of androgen in adipose tissue. Accordingly, it is known that plasma estrogen levels are associated with overweight in postmenopausal women. In the literature, there are different data in the studies performed on premenopausal women (17). In our study, we could not find a relationship between BMI, waist circumference and estradiol level.

In our study, we found out that the level of DHEA-S decreased significantly as BMI and waist circumference increased in women. In many studies, the results are found similar to our study (21–23). Hernandez-Morante et al. (24) presented that DHEA-S treatment increased adiponectin gene expression and that this mechanism was effective in preventing obesity. The same researchers found out that DHEA-S treatment increased lipolysis in subcutaneous fat tissue of women and visceral fat tissue of men.

When we investigated the relationship between total/free testosterone levels and BMI/ waist circumference in our study, the total testosterone level was significantly low in the group with large waist circumference. There was not a significant relationship between the other parameters. In obese people, androgen transition depending on the decrease of sex hormone binding globulin accelerates, and androgen syntheses increase to correspond to this. As a result, the free testosterone level increases (25).

Conclusion

In our study, it was found out that high FBG, total cholesterol, LDL-C, TG, insulin, insulin resistance and low HDL-C, SHBG, DHEA-S levels were associated with obesity. This relationship causes many diseases, particularly diabetes mellitus and cardiovascular diseases. Besides, it is seen that obesity changes overall body metabolism and hormone metabolism in women. Infertility, polycystic ovary syndrome and endometrial cancer prevalence are high in obese women. For this reason, obesity is a disease which needs frequent follow-up and treatment.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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GLUCOSE METERS: WHAT'S THE LABORATORY REFERENCE GLUCOSE?

GLUKOMETRI: ŠTA JE LABORATORIJSKI REFERENTNO ZA GLUKOZU?

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Summary

Background: The accuracy of glucose meters is evaluated by comparing their results with those from a reference laboratory glucose analyser. The main scientific societies recommend the use of a prompt glycolysis inhibitor such as citrate for an accurate glucose determination. In the present preliminary study, we discuss the bias between capillary and plasma glucose measured concentrations, determined in two Italian clinical laboratories, using tubes containing an NaF and citrate mixture in liquid and granular form.
Methods: 139 volunteers in whom 75 g OGTT was requested were recruited. Basal capillary glucose was determined using Abbott FreeStyle Precision Neo in Brescia (n=63), while clinical laboratory reference P-glucose was determined using tubes containing NaF/K_3EDTA and liquid NaF/Na_2EDTA/citrate. Basal capillary glucose was determined using a Roche Cobas Accu-Chek Inform II in Vicenza (n=76), while P-glucose was determined using tubes containing NaF/K_2Ox and NaF/Na_2EDTA/citrate in granulated form. Reference P-glucose was determined with a hexokinase method on Dimension Vista systems. Differences between capillary and reference P-glucose were evaluated according to ADA/ISO 15197:2013 specifications.
Results: 96.82% and 97.37% of capillary determinations were within specifications when liquid and granular citrate

Kratak sadržaj

Uvod: Tačnost glukometra se procenjuje poređenjem njihovih rezultata sa rezultatima dobijenim referentnim analizatorom za glukozu. Stručna zajednica preporučuje upotrebu brzog inhibitora glikolize kao što je citrat za precizno određivanje glukoze. U ovoj preliminarnoj studiji razmatrana su odstupanja između izmerenih koncentracija kapilarne glukoze i glukoze u plazmi, koje su obavljene u dve italijanske referentne laboratorije, primenom epruveta sa mešavinom NaF i citrata u tečnom i granularnom obliku.
Metode: Odabrano je 139 dobrovoljaca i od njih zatraženo da urade OGTT test sa 75 g glukoze. Bazalna kapilarna glukoza je određena korišćenjem Abbott FreeStyle Precision Neo u Breši (n=63), dok je laboratorijska referentna P-glukoza određivana korišćenjem NaF/K_3EDTA i tečnih NaF/Na_2EDTA/citrata koji sadrže epruvete. Bazalna kapilarna glukoza je određena korišćenjem Roche Cobas Accu-Chek Inform II u Vićenci (n=76), dok je P-glukoza određena pomoću NaF/K_2Ok i NaF/Na_2EDTA/citrata u granularnoj formi koja sadrži epruvete. Referentna P-glukoza je određena metodom heksokinaze na Dimension Vista sistemima. Razlike između kapilarne i referentne P-glukoze su procenjene prema ADA/ISO 15197:2013 specifikacijama.
Rezultati: 96,82% i 97,37% kapilarnih određivanja bilo je u okviru specifikacija kada su korišćene epruvete sa tečnom i

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List of abbreviations: ADA, American Diabetes Association; BGM, blood glucose meter; ISO, International Organization for Standardization; CLSI, Clinical and Laboratory Standard Institute; EDTA, ethylenediaminetetraacteic acid; FDA, US Food and Drug Administration; K_2Ox, Potassium Oxalate; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; GMECC, How should Glucose Meters be Evaluted in Critical Care; NACB, National Academy of Clinical Biochemistry; NaF, Sodium fluoride; OGTT, Oral Glucose Tolerance Test; POC, Point of Care; QC, Quality Control; SIBioC, Italian Society of Clinical Biochemistry; SID, Italian Society of Diabetology; SIPMeL, Italian Society of Clinical Pathology and Laboratory Medicine; WG, Working Group.

mixture tubes were used, respectively. Conversely, only 73.02% and 80.26% of determinations were within criteria using NaF.

Conclusions: It's important to know what is the laboratory reference glucose in evaluating glucose meters' accuracy. The evaluation of glucometers' accuracy with respect to a reference laboratory may be wrong if tubes containing only NaF are used due to in vitro glycolysis. Only tubes containing citrate mixture permit the correct evaluation of glucose meters' accuracy.

Keywords: blood glucose, blood glucose meters, citrate buffer, sodium fluoride

granularnom mešavinom citrata. Nasuprot tome, samo 73,02% i 80,26% određivanja je bilo u okviru kriterijuma pomocu NaF.

Zaključak: Važno je znati šta je laboratorijska referentna glukoza u proceni tačnosti glukometra. Ispitivanje preciznosti glukometra u odnosu na referentu laboratoriju može biti pogrešno ako se koriste epruvete koje sadrže samo NaF zbog in vitro glikolize. Samo epruvete koje sadrže citratnu mešavinu omogucavaju ispravnu procenu tačnosti glukometra.

Ključne reči: glukoza u krvi, glukometri, citratni pufer, natrijum fluorid

Introduction

The measurement of glucose represents a cornerstone in the diagnosis of carbohydrate metabolism disorders and in the care of all people with diabetes (1). Point of Care (POC) glucose meters (from this point onwards, referred to only as glucose meters) are commonly used to quickly obtain glucose determinations at hospital and home. The American Diabetes Association (ADA) recommends their use for self-monitoring at home or in clinical settings (2). Glucose meters are easy to use and cost-effective and have also become the method of choice in many laboratories in the context of the evaluation of basal glucose determination in the Oral Glucose Tolerance Test (OGTT). There is some debate over what constitutes good technical accuracy when comparing glucose meters with a laboratory method. Multiple performance goals have been proposed for portable glucose meters. These targets vary widely. The ADA specifies the criteria for total error of glucose meters: At least 95% of a glucose meter's results have to be within $<\pm 0.8$ mmol/L at glucose concentrations <5.6 mmol/L and within $\pm 15\%$ at glucose concentrations ≥ 5.6 mmol/L. The international standard ISO 15197:2013 specifies the same criteria for accuracy of glucose meters but introduces a second trueness criterion relating to the consensus error grid that assigns pairs of glucose meters' results and a corresponding reference value to a clinical risk zone: at least 99% of results have to be within the consensus error zones A and B (4). The more stringent CLSI POCT12-A3 recommendations are that, for 95% of the samples, the difference between glucose meter and laboratory measurements of glucose have to be (*a*) $<12.5\%$ when the laboratory glucose value is >5.6 mmol/L and (*b*) <0.7 mmol/L of the laboratory glucose value when the glucose concentration is ≤ 5.6 mmol/L (5). The Food and Drug Administration (FDA) 2016 criteria state that 95% of glucose values must be within 15% of reference and 99% of glucose values must be within 20% of reference (6).

In any case, glucose meters' accuracy is based on a comparison of POC and laboratory reference glucose results. Moreover, for quality assurance, the ADA recommends a periodic comparison of the results from glucose meters with those from referenced laboratories (3). In-vitro instability of whole blood glucose can introduce an important bias in the accuracy assessment of glucose meters. For this reason, all steps in the analytical and pre-analytical processes require careful attention (7, 8). In vitro glycolysis, which results in the breakdown of glucose, is the principal source of uncertainty in glucose determinations. An average reduction of glucose concentration of 5–7%/hour can occur, especially when high leukocyte blood counts and high temperatures are present (9). For accurate glucose determinations, tubes containing anti-glycolytic agents are used (10). The most commonly used anti-glycolytic agent, sodium fluoride (NaF), acts inhibiting enolase activity (11). NaF is a long-term glucose stabilizer, but it does not prevent a drop during the first hours after blood drawing (12). In most Italian clinical laboratories, glucose is usually determined in tubes containing sodium fluoride (NaF) (13). However, to prevent in vitro glycolysis, the Italian working group on diabetes of the Italian Society of Clinical Biochemistry (SIBioC) and the Italian Society of Clinical Pathology and Laboratory Medicine (SIPMeL), together with representatives of the Italian Society of Diabetology (SID), the American Diabetes Association (ADA) and the National Academy of Clinical Biochemistry (NACB), recommend the collection of blood into tubes containing a rapid glycolysis inhibitor, i.e., a citrate buffer, in a granular or liquid form that acts immediately inhibiting hexokinase, the first enzyme of the glycolytic pathway, instead of using NaF alone (3, 14).

Since the instability of glucose in blood can introduce important bias in the assessment of glucose meters, we have planned a preliminary study to quantify the bias of glucose measured by two different glucose meters (i.e., Abbott FreeStyle Precision Neo and Roche Cobas Accu-Chek Inform II) based on ADA and ISO 15197:2013 requirements with respect to reference glucose measured in laboratories using NaF and the two universally commercially available recommended citrate-containing tubes (GlucoEXACT from Sarstedt and FC-Mix from Greiner Bio-One) (10, 12, 16).

Materials and Methods

The study was conducted at Spedali Civili Central Clinical Chemistry Laboratory (Brescia, Italy) and at the Laboratory of St. Bortolo Hospital (Vicenza, Italy) in summer 2018. One hundred thirty-nine volunteers (13 men, 126 women), median age 35 years (interquartile range (IQR): 31–48), in whom a 75 g OGTT was requested, were recruited.

Basal capillary whole blood glucose was determined using Abbott FreeStyle Precision Neo in Brescia and a Roche Cobas Accu-Chek Inform II in Vicenza. All capillary whole blood glucose measurements were carried out by the same trained nurses, and all venepunctures were performed in each study by a single experienced phlebotomist to minimize venepuncture bias.

To evaluate the glucose meters' performance, internal quality control (QC) materials (high and low levels) were tested daily according to the manufacturers' instructions.

Reference plasma glucose was determined in the tube containing NaF-K_3EDTA, 2.7 mL draw (Ref. 04.1918 from Sarstedt Verona, Italy), and the tube containing Na_2EDTA, NaF, citric acid, and Na-citrate in a liquid form, GlucoEXACT, 3.1 mL draw (Ref. 04.1945.001 from Sarstedt Verona, Italy), in Brescia.

Reference plasma glucose was determined in the tube containing NaF-K_2 oxalate (NaF/K_2Ox), 2 mL draw (Ref. 454514 from Vacutest Kima, Arzegrande, Italy), and the tube containing Na_2EDTA, NaF, citric acid, and Na-citrate in a granular form, FC-MIX, 2 mL draw (Ref. 454511 from Greiner Bio-One, Cassina de Pecchi, Italy), in Vicenza.

Plasma glucose concentration measurements were performed by Dimension Vista 1500 analyzers (Siemens Healtheers, Milan, Italy) using a hexokinase method (within-laboratory CV=2.1% at Brescia and 2.8% at Vicenza laboratory) upon arrival at the clinical laboratory, between 3 and 4 hours after blood drawing. QC was performed according to the laboratory specifications.

Glucose concentrations measured in GlucoEXACT tubes containing a liquid mixture of NaF, Na_2EDTA, and citrate were multiplied by 1.16 as recommended by the manufacturer in order to overcome the sample's dilution by liquid addictive.

Ethics

The study was conducted according to the principles of the revised Helsinki Declaration, adopted in 2013 (Fortaleza, Brazil), and written informed consent was obtained from all participants.

Statistics

Normal distributions for all the datasets were evaluated by using the Kolmogorov-Smirnov test. Since normal distribution was not confirmed, the Wilcoxon test for paired samples was used to compare reference laboratory glucose in tubes containing NaF and citrate to those measured by glucose meters. The biases between capillary glucose and venous reference glucose in NaF and citrate mixture tubes were calculated as $B = ((Glu_{Glucose\ meter}/Glu_{Reference}) \times 100)-100$. The acceptance criteria were defined according to ADA and ISO 15197:2013 (total error for 95% of samples to 15% at glucose concentrations ≥5.6 mmol/L and to <0.8 mmol/L at glucose concentrations <5.6 mmol/L and difference plots created. The statistical analysis was performed with MedCalc software, version 18.10.2 (Ostend, Belgium). Values of $p<0.05$ were considered statistically significant.

Results

The median glucose concentration in the whole blood capillary sample using the Abbott FreeStyle Precision Neo glucose meter (n=63) was 5.11 (IQR:4.68–5.50–99.0) mmol/L, and reference venous plasma glucose using tubes containing NaF/K_3EDTA was 4.46 (IQR:4.13–4.95) mmol/L ($p<0.0001$), while that obtained using a liquid form of NaF/Na_2EDTA/citrate (i.e., GlucoEXACT) was 5.00 (IQR: 4.60–5.48–98.6) mmol/L ($p=0.0059$).

The median glucose concentration in the whole blood capillary sample using the Roche Cobas Accu-Chek Inform II glucose meter (n=76) was 5.22 (IQR: 4.78–5.83) mmol/L, and reference venous plasma glucose using tubes containing NaF/K_2Ox was 4.64 (IQR: 4.11–5.17) mmol/L ($p<0.0001$), while that obtained using a granular form of NaF/Na_2EDTA/citrate (i.e., FC-Mix) was 4.92 (IQR: 4.39–5.64) mmol/L ($p<0.0001$).

The difference plots illustrating the comparisons of glucose results from the whole blood capillary sample measured with the Abbott FreeStyle Precision Neo and the Roche Cobas Accu-Chek Inform II and the reference plasma glucose measured in tubes containing NaF and NaF/Na2EDTA/citrate in liquid and granular form are presented in *Figures 1* and *2*. Only 73.02% and 80.26% of capillary glucose determinations were within ADA – ISO 15197:2013 criteria when NaF/K_3EDTA and NaF/K_2Ox were used. Conversely, 96.82% and 97.37% of capillary glucose determinations were within ADA–ISO 15197:2013 criteria when liquid and granular citrate mixture tubes were used, respectively.

The correlation between capillary whole blood glucose using both the FreeStyle Precision Neo and the Roche Cobas Accu-Chek Inform II with respect to

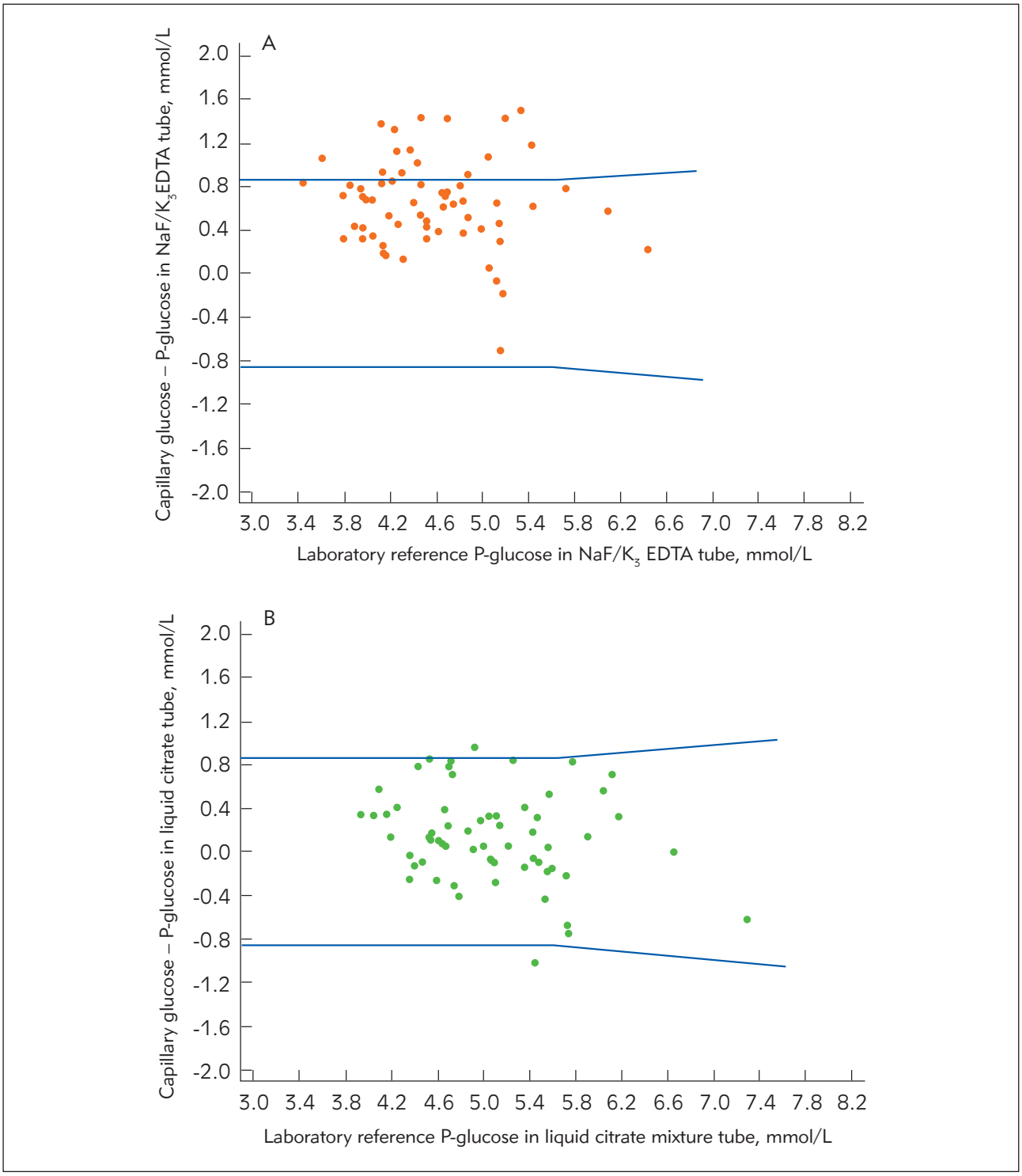

Figure 1 Difference plots of capillary glucose and laboratory venous plasma reference glucose in (A) NaF/K_3EDTA (NaF) tubes and (B) NaF/Na_2EDTA/citrate tubes in a liquid form (GlucoEXACT). Blue lines indicate ADA and ISO 15197:2013 accuracy limits.

the plasma glucose concentration using hexokinase methods on Dimension Vista 1500 were r=0.80 when NaF and citrate liquid mixture (*Table I*) were used, respectively.

The correlation between capillary whole blood glucose on the Roche Cobas Accu-Chek Inform II and plasma glucose concentration were r=0.94 and r=0.92 when NaF and citrate granular mixture (*Table I*) were used, respectively.

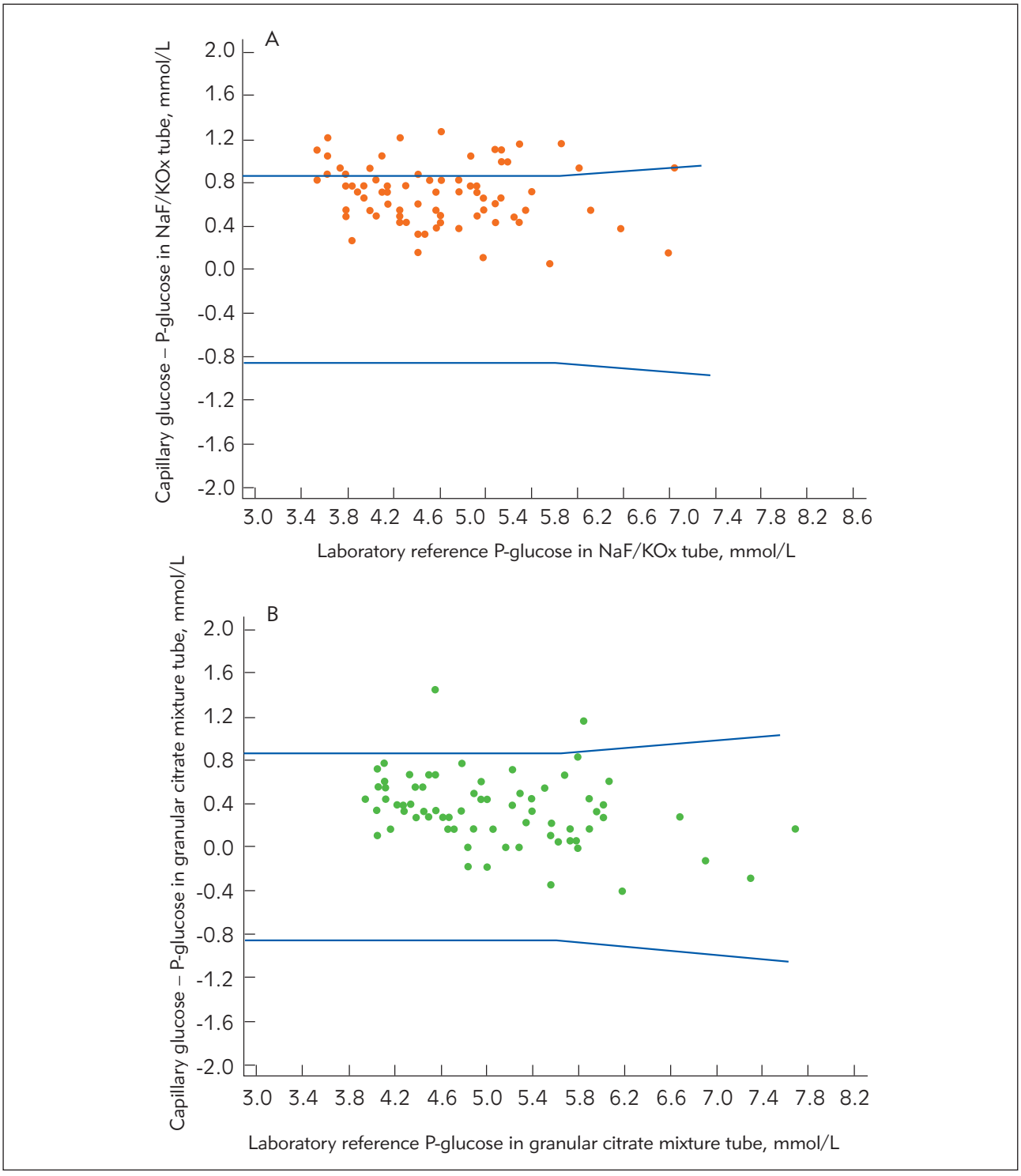

Figure 2 Difference plots of capillary glucose and venous plasma laboratory reference glucose in (A) NaF/K_2Ox tubes and (B) NaF/Na_2EDTA/citrate tubes in a granular form (FC-Mix).Blue lines indicate ADA and ISO 15198:2013 accuracy limits.

Discussion

Glucose meters are often used for a fast evaluation of glucose concentrations in the hospital and at home. Many studies and reviews have evaluated glucose meters' technical trueness (17). Trueness is defined as the closeness of agreement between a test result and the true value of that analyte. An important but often neglected aspect that can affect POC system trueness results is the measurement procedure used (5).

Table I Least square regression equations comparing capillary glucose in two different glucometers and plasma glucose in the tubes evaluated.

x	y	N	Regression equation	Slope 95% CI	Intercept 95% CI	r	$S_{y/x}$
P-glucoseNaF/K_3EDTA	Capillary glucose (Abbott FreeStyle Neo)	63	y=0.9204 x+1.0164	0.7411–1.0997	0.1924–1.8404	0.796	0.4148
P-glucose Citrate mixture (liquid – GlucoEXACT))			y=0.8349 x+0.9853	0.6738–0.9960	0.1631–1.8076	0.796	0.4123
P-glucoseNaF/K_2Ox	Capillary glucose (Roche Cobas Accu-chek Inform II)	76	y=0.9429 x + 0.9681	0.8580–1.0278	0.5647–1.3715	0.932	0.2754
P-glucose Citrate mixture (granular-Fc-MIX)			y=0.8721 x + 0.9825	0.7872–0.9569	0.5481–1.4170	0.922	0.2944

In fact, during transport to the laboratory to compare results with laboratory methods, glucose concentrations have a tendency to decrease due to in-vitro glycolysis. Delay in transportation can lead to biases between glucose meters and reference laboratory methods (18). A study from Salacinski and colleagues (19) suggested that the glucose meter provided poor-validity and-reliability results compared to the results provided by the reference laboratory analyser using lithium-heparin plasma samples. They indicated that portable glucose meters should be used for patient management but not for diagnosis, treatment, or research purposes (19). Fluoride-containing tubes are often used to reduce glycolysis, especially when accurate results are required, but it is known that the inhibitory effect of fluoride upon glycolysis can take to 2–3 h, with a consequent lowering of the glucose concentration in the first hours (20).

In a recent study, Blaurock and colleagues (21) evaluated the impact of glucose measuring systems on diagnosis rates of diabetes mellitus using POCT and core laboratory glucose methods. In the study limitations section, they reported that the effect of glycolysis could not be completely excluded because NaF was used (21). Already in 1996, the FDA suggested that fluoride should not be used as a preservative for venous specimens when using glucose meters (22). Hung et al. (22), using stored samples to evaluate glucose meters' accuracy with respect to their laboratory method, called this recommendation into question. However, in their letter, they did not describe how much time had passed between the sampling and the centrifugation of the NaF sample (22). Recently, IFCC Working Group WG-GMECC wrote a document on the clinical practice of using glucose meters and the requirements they must fulfil in order to be used in critical care, which emphasizes that anticoagulant preservatives such as NaF-oxalate are not completely effective in inhibiting glycolysis and that the addition of citrate is the most effective (23). Our study is a preliminary one on the evaluation of the bias of two different glucose meters (i.e., Abbott FreeStyle Precision Neo and Roche Cobas Accu-Chek Inform II) in referencing venous plasma glucose using the new recommended citrate-containing tubes. In our study, capillary glucose concentrations obtained from glucose meters and venous plasma reference glucose determined by laboratory instruments using NaF and citrate tubes were evaluated as in typical scenarios experienced in patient care. The novelty of our study is that is the first to compare capillary whole blood glucose to reference plasma glucose using the two universally available tubes containing citrate in a liquid and granular form together with the old NaF-containing tubes (10, 16). Significant biases in glucose concentrations were observed between glucose meters and reference plasma glucose when different plasma tubes were used. Our data suggest that for an efficient comparison of glucose measured by glucose meter systems with the reference laboratory measurements, only plasma samples containing an acidified citrate mixture should be used in reference laboratories. NaF plasma tubes need to be abandoned because they exert biases exceeding analytical goals for bias according to ADA and ISO 15197:2013. Maintaining the use of NaF as a comparison system can cause incorrect evaluations of the quality of the glucose meters used. Previously, only van den Berg and colleagues' (24) study had considered citrate-containing tubes in POC evaluation. That study was about the screening of gestational diabetes mellitus. The glucose concentration determined by routine laboratory analysis using lithium-heparin tubes was well correlated with POC analysis but was lower at both T0 and T120 (24). The bias was lower if lithium-heparin tubes were centrifuged immediately. If citrate-containing tubes were used, the glucose concentrations determined within 60 minutes strongly correlated at both 0 and 120 minutes of OGTT.

Because the FDA suggests that fluoride should not be used as a preservative for venous specimens when using glucose meters, we could hypothesize that samples with an acidified mixture in liquid and granular form could be used as an efficient alternative.

In this real-life study, there are some limitations. A limited number of participants were recruited, and plasma and capillary glucose measurements were made as single determinations and not in duplicate, as stated in the CLSI EP09c document (25). Because capillary glucose determinations are part of the basal evaluation of OGTT, only low and medium glucose concentrations were available, so an assessment of higher glucose concentrations is still required. Additional glucose meters need to be investigated since only two devices were considered in the present study.

Eventually, tight control of the pre-analytical procedures is needed, even using POC devices, as stated by the Working Group for the Preanalytical Phase of the European Federation for Clinical Chemistry and Laboratory Medicine (26).

Conclusions

We have found that comparison of POC glucose analysis versus reference laboratory analysis may be wrong if tubes containing only NaF are used due to in vitro glycolysis. Furthermore, we have shown that the use of the new tubes with the acidified mixture, in either granular or liquid form, can be adequate for screening subjects who undergo an OGTT in order to correctly compare results to the reference laboratory plasma glucose, as well as for an accurate evaluation of glucose meters according to ADA and ISO 15197:2013 standards.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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EFFECTS OF AN EIGHT-WEEK EXERCISE PROGRAM ON PARAMETERS OF THE LIPID PROFILE OF FEMALE STUDENTS

EFEKTI OSMONEDELJNOG PROGRAMA VEŽBANJA NA LIPIDNI PROFIL STUDENTKINJA

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Summary

Background: Increased lipid levels are one of the major risk factors for the development of cardiovascular diseases. The aim of the current study was to evaluate the effect of short-term (8 weeks) aerobic exercise of moderate to vigorous intensity on lipid profile in young healthy females.
Methods: 27 female students (mean age 20.5 ± 1 year) completed 8 weeks of aerobic training that included two exercise sessions of continuous aerobic activity of moderate intensity (running 35–60 min) and one exercise session with interval training of vigorous intensity. Intervention aerobic capacity and lipid profile were examined before and after the exercise.
Results: Exercise intervention has caused a decrease of low density lipoprotein levels per 9.8% (from 2.52 ± 0.47 to 2.27 ± 0.53 mmol/L; $p < 0.001$) and significant improvement of high density lipoprotein (HDL) levels per 22.7% (from 1.29 ± 0.24 to 1.59 ± 0.24 mmol/L; $p < 0.001$), total cholesterol/HDL ratio per 17.2 % and aerobic capacity ($VO_{2\ max}$) per 3.8 % (43.9 ± 3.7 to 45.56 ± 3.63 $mLO_2/kg/min$).
Conclusions: The results support the hypothesis that a short-term aerobic exercise intervention of moderate to vigorous intensity may have significant effects on blood lipid profile in young healthy females.

Keywords: lipid profile, female, aerobic training

Kratak sadržaj

Uvod: Povećani nivo lipida je jedan od značajnih faktora rizika za razvoj kardiovaskularnih bolesti. Cilj ovog istraživanja je da se procene efekti kratkog (8 nedelja) aerobnog vežbanja, umerenog do visokog intenziteta, na lipidni profil.
Metode: 27 studentkinja (prosečne starosti 20,5 ± 1 godina) je izloženo osmonedeljnom aerobnom vežbanju koje se sastojalo od treninga kontinuirane aerobne aktivnosti umerenog intenziteta (trčanje 35–60 min) dva puta nedeljno i intervalnog treninga visokog intenziteta jednom nedeljno. Lipidni profil je određen pre i posle programa vežbanja.
Rezultati: Program vežbanja je doveo do smanjenja nivoa »lošeg« holesterola za 9,8% (od 2,52 ± 0,47 na 2,27 ± 0,53 mmol/L; $p < 0,001$), značajnog povećanja »dobrog« holesterola (HDL) za 22,7% (od 1,29 ± 0,24 na 1,59 ± 0,24 mmol/L; $p < 0,001$), odnosa ukupnog holesterola/HDL za 17,2% i poboljšanja aerobnog kapaciteta ($VO_{2\ max}$) za 3,8% (od 43,9 ± 3,7 na 45,56 ± 3,63 $mLO_2/kg/min$).
Zaključak: Rezultati istraživanja podržavaju hipotezu da kratkotrajno aerobno vežbanje od umerenog do visokog intenziteta ima značajan uticaj na lipidni profil mladih, zdravih, ženskih osoba.

Ključne reči: lipidni profil, žene, aerobno vežbanje

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List of abbreviations: TC, Total Cholesterol; HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein; TG, Triglycerides; $VO_{2\ max}$, maximal oxygen uptake.

Introduction

Hypercholesterolemia is an important risk factor for the development of atherosclerosis, ischemic heart disease and stroke (1, 2). According to the American Heart Association, 54% of adult Europeans aged > 25 suffer from increased cholesterol levels higher than 5.17 mmol/L (3). Decreasing of Low-Density Lipoprotein (LDL) levels over a lifetime for 1 mmol/L is associated with a 55 % lower risk for cardiovascular diseases (4).

Among different forms of physical activity, the authors have highlighted the importance of aerobic exercise. Special training programs can have a significant impact on improving the quality of life of individuals or their skills. Walking and running are the cyclical activities most used to develop aerobic fitness, which is important physical ability for health. Developing interest in a healthy population for this form of physical activity is important in order to include it in their everyday life as an integral component of a healthy lifestyle.

Regular physical activity has a number of positive effects on blood lipids profile (5–7). Cross-sectional studies have clearly shown that well-trained endurance athletes (runners, cyclists, and triathletes) have lower triglycerides (TG) (19–50%) and LDL (7–21%) levels and higher High-Density Lipoprotein (HDL) levels (5–59%) in comparison with untrained individuals (8). Current exercise guidelines recommend 150 minutes/week of moderate intensity exercise or 75 minutes/week of vigorous intensity exercise on most or at least five days per week for the improvement of health parameters (6, 9–12).

Studies have shown that the exercise threshold for the effects on lipid profile is between 1200–2200 kcal/week and that the volume and intensity of exercise are positively correlated with the beneficial effects on lipid profile (8, 13), as HDL will increase 0.008 mmol/L per each kilometre of the running distance (14). The response of the specific lipid profile components to exercise is different. HDL and TG are much more sensitive to the exercise-related changes than are LDL and total cholesterol (TC), as only 25% of the studies have shown that exercise will induce some changes in the LDL and TC levels (8, 13).

Although there is a general agreement that exercise is beneficial for the improvement of lipid profile, it is still not known in terms of exercise intensity what is the most beneficial exercise regimen, volume, and duration of the exercise intervention. In the past few years, high-intensity interval training (HIIT) has become very popular as important for health effects on lipid profile (15, 16).

The main purpose of this study was to evaluate the effects of 8-week moderate to vigorous aerobic exercise on the lipid profile in young healthy females, with a wish to investigate the preventive effects of aerobic exercise.

Materials and Methods

Participants

The research was conducted on a sample of 27 female students (mean age 20.5 ± 1 year; body mass 60.1 ± 5.5 kg, body height 168.1 ± 6.2 cm) in the second year of undergraduate studies at the Faculty of Sport and Physical Education, the University of Belgrade, whose instruction syllabus already contain the 8-week training program of preparation for cross-country running. All the subjects were healthy and had no neurological, orthopaedic, cardiovascular and metabolic disorders, nor were using medications that could influence research results. Participants entered the study after being informed about the purpose, potential benefits, and possible risks of the training program. They were also required to sign an informed consent document.

The study protocol was approved by the Ethics Committee of the Faculty of Sport and Physical Education, University of Belgrade.

Study design

Exercise intervention that was used in the study was created as a training program for students to apply one of the methods for developing aerobic endurance and to prepare students for the 4000 m cross-country race. Participants were also instructed to maintain their normal diet (17) and daily activities throughout the duration of the study. There was no nutritional intervention during the study. Before and after the exercise intervention, all participants underwent the anthropometric and body composition testing and shuttle run test. Blood samples for full lipid profile were also taken before and after the exercise intervention.

Exercise intervention

Based on the results of initial measurements, an individual program was created, which was subsequently implemented by each student. The individual training program was structured in two ways: based on the running velocity realized at the initial shuttle run measurement and based on the value of heart rate (by determining maximum heart rate). The exercise lasting 45–60 minutes was performed 3 times a week, and it included 10 minutes' warm-up followed by a main running session that included three exercise zones. Zone 1 was long a slow distance run lasting 45 minutes at 60–80% of the maximal heart rate (6–7 MET). Zone 2 training was 35–45 minutes run at 81–90% HR_{max} (8–10 MET), and zone 3 included interval runs: 2×1600 m or 2×2400 m race pace at 91–100% HR_{max} (11–15 MET) (18, 19, 20). Each participant wore a Suunto dual belt HR monitor during the process to control the intensity of training.

Measurements

Shuttle run test

In order to assess the aerobic capacity (VO_{2max}), the multistage progressive 20-meter »shuttle run« test was used. This test was performed on a hard synthetic surface located in an indoor sports arena. Subjects ran between two lines 20 m apart in time with a sound signal which was emitted from an audio cassette. The frequency of the sound signals increased every minute. The test was terminated when the subject was no longer able to follow the set pace and did not reach the targeted line on three consecutive occasions. The level attained and the number of shuttles at that level allowed a prediction of VO_{2max}, as suggested by Ramsbottom et al. (21).

Blood samples

To monitor the exercise effects on the lipid profile, the following variables were selected: TC (mmol/L), HDL (mmol/L), TC/HDL ratio, LDL (mmol/L) and plasma TG concentration (mmol/L). Blood samples were taken between 7 and 8 a.m. to avoid variations in circadian rhythm. A complete blood count was determined on the haematology cell counter Coulter LH750 (Beckman Coulter, Inc., Fullerton, CA). Total cholesterol, HDL, LDL and TG were routinely determined by automatic analyzer BT 2000 (Biotehnica, Milan, Italy) using automated methods and bioMérieux (Marcy l'Etoile, France) reagents.

Statistical analysis

Mean and standard deviation were used to report basic descriptive parameters. The differences between the initial and final tests were analyzed using the Student's t-test for paired samples. The impact of training on the calculated variables was determined using ETA squared test. The normal distribution of the results was examined by the Shapiro-Wilk test. The extreme points were established by a boxplot. It was found that there were no extreme points in the used variables. All data were analyzed using SPSS software (20.0, SPSS, Chicago, IL). A significance level of .05 was used for all tests.

Results

The effect of exercise intervention on aerobic capacity

Mean values of shuttle run tests, velocity and duration, and $VO_{2\ max}$ in the initial and final measurements are shown in *Table I*.

Table I Mean values of velocity and duration of shuttle run and VO_2 max in the initial and final measurements.

Variables		M	SD	p	ETA
Shuttle run – velocity (m/s)	In	11.68	0.61	0.007	0.24
	Fin	11.96	0.62		
Shuttle run – duration (min)	In	6:49	1:12	0.001	0.36
	Fin	7:22	1:12		
$VO_{2\ max}$ (mLO_2/kg/min)	In	43.89	3.66	0.001	0.36
	Fin	45.56	3.63		

$VO_{2\ max}$ – maximal oxygen uptake; In – Initial measurement; Fin – Final measurement
ETA – Eta-squared; 0.01 low impact; 0.06 medium impact; 0.14 high impact

Values of ETA squared test higher than 0.14 examined dependent variables confirm that the impact of training programs on health parameters of the test is significant.

The initial measurement of VO_{2max} was 43.9 ± 3.7 mL O_2/kg/min and the final measurement 45.56 ± 3.63 mL O_2/kg/min, which represents a significant increase ($p < 0.001$) between the initial and final measurements in the amount of 1.67 mL O_2/kg/min (3.8%). Overall, there was an 8% increment in the shuttle run performance and running velocity ($p < 0.01$). These values are expected as the training program was based predominantly on the type of endurance training (22).

The effects of exercise intervention on blood lipid profile

Mean values of the TC, HDL, LDL, and TG in the initial and final measurements are shown in *Table II*.

Table II Parameters of lipid status at the initial and final phase of measurements.

		Mean	SD	p	ETA
TC (mmol/L)	In	4.12	.62	0.278	0.04
	Fin	4.19	.59		
HDL (mmol/L)	In	1.29	.24	0.000	0.83
	Fin	1.59	.24		
TC/HDL (%)	In	3.25	0.52	0.000	0.80
	Fin	2.68	0.46		
LDL (mmol/L)	In	2.52	.47	0.000	0.38
	Fin	2.27	.53		
TG (mmol/L)	In	.619	.219	0.009	0.23
	Fin	.717	.223		

TC = Total cholesterol, HDL = High Density Lipoprotein, LDL = Low Density Lipoprotein, TG = triglycerides
$VO_{2\ max}$ – maximal oxygen uptake; In – Initial measurement; Fin – Final measurement

The average TC did not change after the exercise intervention ($p < 0.05$). However, survey results show that components of the lipid profile were improved. HDL increased from 1.29 to 1.59 mmol/L, ($p < 0.001$), and LDL decreased from 2.52 to 2.27 mmol/L, ($p < 0.001$). There was also a significant improvement of the TC/HDL ratio ($p < 0.001$), while the TG level has increased ($p < 0.01$).

Discussion

There were various exercising programs presented in scientific studies to examine the effect of physical exercises on lipid status in healthy population through exercising programs of different duration ranging from 20 days to 24 months (8, 13). In our research, the selected program of aerobic exercises was established as a model running program for the preparation of physically active adults which lasted long enough to improve aerobic endurance.

The main finding of the present study is that 8 weeks of mainly aerobic activity of moderate to vigorous intensity caused significant improvements in blood lipid profile and aerobic capacity in young healthy females. The most important significant changes were (1) improvement of VO_{2max} and the increase in endurance measured through shuttle run test (2), the increase in HDL levels, with concomitant improvement of TH/HDL levels, and (3) the decrease of LDL levels. We have also registered a significant increase in TG levels.

The energy consumption of an exercise intervention was estimated to be between 800–1040 kcal/week and was of sufficient intensity to improve aerobic capacity and running endurance. In the current investigation, certain improvement of the running endurance was expected in the study subjects, while that was otherwise physically active population and the study protocol was their first experience with the structured aerobic endurance exercise program. There are probably two possible explanations for this outcome. First of all, it is likely that the improvement of the running endurance was related to the functional and biochemical adaptations that could be best noticed through the significant improvements of VO_{2max} levels. Secondly, we believe that there were also some neuromuscular adaptations related to the running economy and better functioning of the stretch-shortening cycle that is important in the running. The 3.8% improvement of VO_{2max} was relatively large and comparable only to the results reported in well-trained female athletes (23). In such trained athletes we could expect progress at the intensities higher than used in our study (e.g., 95% HR_{max}) (24). The results from the studies investigating the effects of aerobic training on untrained females show much greater improvements in the range of 10 to 20% (25). However, our participants belonged to the regularly physically active population (although not trained athletes) and their VO_{2max} levels were relatively high even at the beginning of the study which might explain fewer improvements than expected. It is also possible that a short period of exercise intervention (8 weeks instead of the usual 12 weeks) could have been the additional explanation of such an outcome.

The results have also shown a specific response of lipid profile to aerobic exercise intervention. The increase of HDL levels was above the mean values of 4.6% as reported in the meta-analysis of the effects of aerobic exercise on cholesterol and lipid profile (13). The findings are consistent with the known fact that aerobic activity of moderate intensity (like as a long slow distance running 45–60 min at about 70% HR max in our study) targets HDL levels. The observed reduction in LDL in our study could be explained through the HDL accelerated removal of LDL and by the direct impact of vigorous and high vigorous exercise (high-intensity interval runs) on lowering LDL also. It was reported before that the lowering of LDL, as well as TG levels, requires greater exercise intensity and longer duration of exercise intervention (more than 8 weeks) (13). Furthermore, even the rise of TG levels could in part support the fact that exercise intensity is related to the specific changes of lipid profile. This was also noticed in the study that investigated the effect of aerobic exercise at different intensity, on TG levels where TG levels have raised for 12% in a group with moderate intensity exercise (26). In contrast to that, the same study has reported that in a group with high-intensity interval training TG levels have decreased by 50%. To sum up, our study supports the previous findings that exercise intensity is related to the specificity of lipid profile changes in response to exercise. Moderate intensity exercise will increase HDL levels, while high-intensity exercise will additionally decrease LDL and TG levels. This could have an important implication for the creation of exercise programs in recreational sport and individuals with overweight, obesity and dyslipidemia problems. These persons usually poorly tolerate high-intensity exercises and the exercise protocol used in our study could be a good starting alternative for them to gain aerobic capacity and prepare themselves for high-intensity exercise later on.

The increase of HDL levels is related to the volume of aerobic exercise. Cross-sectional studies have shown that the threshold for this effect (the increase of HDL per 0.05–0.08 mmol/L) is around 1200 and 2200 kcal/week which is in line with current guidelines (10–12) about the health-enhancing levels of physical activities for healthy adults. However, estimated energy consumption in our study was only 800–1200 kcal/week or 18 km of running/week (3×300–400 kcal/exercise session) and it was still enough to cause significant improvements of HDL levels that are in line with other studies (27, 28, 15) that reported the even short-term vigorous-intensity exercise could have beneficial health effects on lipid profile.

Limitations

Initial physical activity readiness bias may be one of the study limitations. As such, the results of the current study should be evaluated in larger clinical trials.

Conclusions

The current study reveals that even short-term (8 weeks) moderate to vigorous intensity aerobic exercise at low energy consumption (around 1000 kcal/week) has a beneficial effect on the lipid profile and aerobic capacity at young adult healthy females.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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CHANGES IN THE BLOOD FATTY-ACID PROFILE ASSOCIATED WITH OXIDATIVE-ANTIOXIDANT DISTURBANCES IN CORONARY ATHEROSCLEROSIS

PROMENE U PROFILU MASNIH KISELINA U KRVI POVEZANO SA OKSIDATIVNO-ANTIOKSIDATIVNIM POREMEĆAJIMA KOD KORONARNE ATEROSKLEROZE

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Summary

Background: The objective of this work was to study the profile of fatty acids and to search for associations of fatty acids with oxidative-antioxidant parameters and an oxidative-inflammatory biomarker (lipoprotein-associated phospholipase A2) in men with coronary atherosclerosis and coronary heart disease.
Methods: Analysis of 20 fatty acids was performed in 60 men with angiographically confirmed coronary atherosclerosis and coronary heart disease and in a control group of men without coronary heart disease. Serum fatty-acid content was evaluated by high-performance gas-liquid chromatography. The blood levels of oxidative stress, total antioxidative defence, and lipoprotein-associated phospholipase 2 were analyzed.
Results: In the group of men with coronary atherosclerosis the levels of myristic and palmitic fatty acids were higher by 59% and 22%, respectively. An increase in the weight percentage of monounsaturated fatty acids was noted, such as palmitoleic, oleic, and octadecenic. Significantly lower levels of polyunsaturated fatty acids, such as linolic, eicosadienoic, eicosatrienoic, arachidonic, eicosapentaenoic, γ-linolenic, docosapentaenoic, and docosahexaenoic were

Kratak sadržaj

Uvod: Cilj ovog rada bio je da se ispita profil masnih kiselina i da se istraže veze masnih kiselina sa oksidativno-antioksidativnim parametrima i oksidativno-inflamatornim biomarkerom (lipoprotein – udružena fosfolipaza A2) kod muškaraca sa koronarnom aterosklerozom i koronarnom bolešću srca.
Metode: Sprovedena je analiza 20 masnih kiselina kod 60 muškaraca sa angiografski potvrđenom koronarnom aterosklerozom i koronarnom bolešću srca i kod kontrolne grupe muškaraca bez koronarne bolesti srca. Sadržaj masnih kiselina u serumu je procenjen gasnom-tečnom hromatografijom visokih performansi. Analizirani su nivoi oksidativnog stresa u krvi, ukupne antioksidativne odbrane i lipoprotein-asocirane fosfolipaze A2.
Rezultati: U grupi muškaraca sa koronarnom aterosklerozom, nivo mirističkih i palmitinskih masnih kiselina bio je veći za 59% i 22%, respektivno. Uočen je porast u težinskom procentu mononezasićenih masnih kiselina, kao što su palmitoleinska, oleinska i oktadeceinska. U grupi sa koronarnom arterosklerozom otkriveni su znatno niži nivoi polinezasićenih masnih kiselina, kao što su linolna, eikosadienoična, eikosatrienoična, arahidonska, eikos-

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List of abbreviations: AT, Agilent Technologies; CA, Coronary atherosclerosis; CBS, Coronary bypass surgery; CHD, Coronary heart disease; FA, Fatty acid; FORD, Free Oxygen Radicals Defense; FORT, Free Oxygen Radicals Testing; HDL, High-density lipoproteins; LDL, Low-density lipoprotein; Lp-PLA2, Lipoprotein-associated phospholipase A2; Me, Median; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid; SFA, Saturated fatty acids; VSMCs, Vascular smooth muscle cells.

detected in the group with coronary atherosclerosis. The lipoprotein-associated phospholipase A2 level was higher by 48%. Oxidative stress was higher by 17%, and the total antioxidant defence in serum was lower by 45%. We found correlations between fatty acids and oxidative-antioxidative alterations. The relative risk of vulnerable atherosclerotic plaques correlated with increased levels of palmitic, stearic, oleic, and linolic fatty acids.

Conclusions: Significant alterations in the profile of fatty acids are associated with oxidative-antioxidative alterations and are accompanied by an increase in free-radical formation, which can probably serve as a risk factor of atherosclerosis.

Keywords: atherosclerosis, atherosclerotic plaque, fatty acid, lipid, lipoprotein-associated phospholipase 2, oxidative stress

apentaenska, γ-linolenska, dokosapentaenoična i dokosaheksaenoična. Nivo lipoprotein-asocirane fosfolipaze A2 bio je viši za 48%. Oksidativni stres je bio veći za 17%, a ukupna antioksidativna odbrana u serumu bila je niža za 45%. Pronašli smo korelacije između masnih kiselina i oksidativno-antioksidativnih promena. Relativni rizik od nestabilnih aterosklerotskih plakova korelira sa povećanim nivoima palmitinske, stearinske, oleinske i linolne masne kiseline.

Zaključak: Značajne promene u profilu masnih kiselina povezane su sa oksidativno-antioksidativnim promenama i praćene su porastom formiranja slobodnih radikala, što verovatno može da posluži kao faktor rizika za aterosklerozu.

Ključne reči: ateroskleroza, aterosklerotski plak, masne kiseline, lipid, lipoprotein-asocirane fosfolipaze A2, oksidativni stres

Introduction

There is significant interest in the problem of lipid accumulation in the arterial wall in atherosclerosis. However, some aspects concerning both the mechanisms of atherosclerotic lesions of arterial vessels and the causes of dyslipidemia remain unclear. Studies that are intended to reveal the role of fatty acids (FAs) in the pathogenesis of atherosclerosis have been growing in number, and priority is given to the separate contribution of every FA instead of the whole set of FAs (1–7). FAs are believed to play a dual role in atherogenesis. Saturated FAs (SFAs) increase the risk of cardiovascular disease, whereas unsaturated FAs suppress atherosclerosis development, and the latter effect is associated not only with a reduction in the total cholesterol level but also with a shift of some cholesterol into high-density lipoproteins (HDLs) and accumulation of polyunsaturated FAs (PUFAs) inside phospholipids in cells (1, 5, 8). Thus, a modification of the FAs composition of the blood can play an important role in the pathogenesis of atherosclerosis. Lipoprotein-associated phospholipase A2 (Lp-PLA2), an enzyme whose increased level in the circulation is linked with a high risk of atherosclerotic-plaque destabilization, is being actively studied. Besides, this enzyme has been suggested to be a highly specific marker of intravascular inflammation (9).

The present study was aimed at investigating the balance of FAs, the search for associations of FAs with oxidative-antioxidant parameters and oxidative-inflammatory biomarker LP-FLA2 in men with coronary atherosclerosis (CA) and coronary heart disease (CHD).

Materials and Methods

This study was conducted within the framework of R&D topics of Government contracts No. 0324-2018-0002 and 0324-2017-0048 and with the financial support of RFBR grant No. 17-04-02120.

The study protocol was approved by the local Ethics Committee of the Institute of Internal and Preventive Medicine (a branch of the Institute of Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia). Each patient gave written informed consent to be examined and to participate in the study.

The study was carried out in the two groups comparable by sex, age (56.38±8.5), and without metabolic syndrome. Study exclusion criteria were acute infectious inflammatory diseases, and exacerbation of chronic conditions, renal failure, active hepatic disorders, and cancer, taking steroid and non-steroidal anti-inflammatory drugs. In addition, the experimental group exclusion criteria included myocardial infarction that happened less than 6 months ago. The experimental group consisted of 60 patients with CHD, angiographically confirmed CA, with stable effort angina, and without the acute coronary syndrome, who were admitted to the hospital for coronary bypass surgery (CBS) in Meshalkin National Medical Research Center of the Ministry of Health of the Russian Federation. The patients underwent endarterectomy in coronary arteries in the course of CBS by intraoperative indications. All the endarterectomy samples were sent for histological analysis. The control group consisted of 20 peers without CHD. The presence of CHD was excluded in the control group based on clinical examination and exercise testing.

Collection of blood samples from the basilic vein was performed in both the experimental (before CBS) and control group after overnight fasting for a minimum of 12 hours and after overnight rest, restraining from physical exercises, smoking, and alcohol for at least 12h before laboratory measurements. These were determined in the blood serum: 20 FAs, triglycerides, FORD, FORT, Lp-PLA2. The FAs profile of all the samples was determined after extraction and methanolysis by high-performance capillary gas-liquid chromatography on an Agilent Technologies (AT)

6890N chromatograph with a plasma ionization detector and by chromatography with mass spectrometry on an AT 6890N chromatograph with mass-selective detector AT 5975N. The study included SFAs, such as myristic (C 14:0), pentadecanoic (C 15:0), palmitic (C 16:0), steatic (C 18:0), arachic (C 20:0), and docosanoic (C 22:0), and unsaturated FAs, such as palmitoleic (C 16:1), oleic (C 18:1), octadecenic (C 18:1), eicosenoic (C 20:1), docosanoic (C 22:1), linoleic (C 18:2, ω-6), α-linolenic (C 18:3, ω-3), γ-linolenic (C 18:3, ω-6), eicosadienoic (C 20:2, ω-6), eicosatrienoic (C 20:3, ω-6), eicosapentaenoic (C 20:5, ω-3), docosapentaenoic (C 22:5, ω-3), docosahexaenoic (C 22:6, ω-3), and arachidonic (C 20:4, ω-6). The quantitative assessment of FAs was made in relation to their total amount.

Triglyceride content of blood was evaluated by an enzyme method using Thermo Fisher Scientific kits on a KonelabPrime 30i biochemical analyzer (Thermo Fisher Scientific, Finland). Oxidative stress (test FORT – Free Oxygen Radicals Testing) and total antioxidative defence in the blood (test FORD – Free Oxygen Radicals Defense) were evaluated using a FORM Plus CR3000 analyzer (Callegary, Italy). The level of oxidative stress (test FORT) was evaluated as a release of active oxygen forms by colourimetric registration. This method is based on the ability of metal ions to catalyze the formation of free radicals in the presence of hydroperoxides. Test FORD is based on the assessment of decreased absorption of previously formed active radicals, which is proportional to the concentration of the antioxidants in the blood. The concentration of Lp-PLA2 in serum was evaluated by an enzyme immunoassay with an ELISA kit (Cloud-Clone Corp., USA).

Histological analysis of the pieces of intima-media coronary arteries was performed under a binocular microscope Axiostar Plus after a macroscopic sample characterization (possible expansion of an atherosclerotic plaque, the degree of artery luminal occlusion, areas of calcification, and thrombi), standard hematoxylin-eosin staining, and Van Gieson's staining. Vulnerable atherosclerotic plaques were identified via the following criteria: a damaged plaque with a fibrous cap less than 65 μm, infiltrated by macrophages and T lymphocytes (more than 25 cells per field of view 0.3 mm), with a large lipidic nucleus (>40%) (10). According to the results of the histological analysis of intima-media pieces, 53.2% of the patients had vulnerable atherosclerotic plaques in coronary arteries (subgroup A), whereas 46.8% patients were found to have only stable atherosclerotic plaques (subgroup B).

Statistical analysis of the results was performed in the SPSS software (version 20.0). Median (Me) and interquartile ranges (25%; 75%) of various parameters are presented in tables. The Kolmogorov-Smirnov test was conducted for the assessment of the data distribution. The nonparametric criterion was used to compare two groups by the Mann-Whitney test in case of a non-normal distribution. In addition, multivariate logistic regression analysis was performed. Spearman's test assessed correlations. Differences were considered statistically significant at $p < 0.05$.

Results

In total, 20 FAs were identified in the serum samples, including six SFAs and 14 unsaturated FAs (*Table I*). Comparative analysis showed significant elevation of SFA levels in the group of patients with CHD and CA in comparison with the patients without CHD, mainly owing to an increase of the myristic acid level by 59% ($p<0.01$) and the palmitic acid level by 22% ($p<0.01$). The weight proportion of stearic acid had only a slight tendency to be elevated in the group with CHD and CA. When we studied the content of MUFAs, we found a significant increase in the mass proportion of palmitoleic acid (by 52%, $p<0.01$), oleic acid (by 38%, $p<0.01$), and octadecenoic acid (by 25%, $p<0.05$) in the group of patients with atherosclerosis.

The percentages of omega-3 FAs (α-linolenic, eicosapentaenoic, docosapentaenoic and docosahexaenoic) were significantly lower (*Table I)* compared with the control group. The level of eicosapentaenoic acid decreased by 22%, the level of docosapentaenoic acid by 28%, and that of docosahexaenoic acid by 33% ($p<0.05$). Although the level of α-linolenic acid decreased 3.3-fold, it did not show any statistically significant difference from the control group which was probably due to the small sample size of patients.

We also revealed a statistically significant decrease in the proportion of omega-6 PUFAs in the experimental group. The weight proportion of linolic acid was lower by 25%, of eicosadienoic acid by 21%, and arachidonic acid by 44% ($p<0.01$). It should be mentioned that the eicosatrienoic acid level decreased by 15%, while the γ-linolenic acid level decreased by 32% in men with atherosclerosis.

In our study, patients with CA and CHD, as expected, had significantly higher triglyceride levels (by 68%, $p<0.05$) as compared with the control group (*Table II*). As expected, in the analysis of inflammation and oxidative-stress biomarkers, it was found that the indicator of oxidative stress (test FORT) was higher by 17% in the experimental group ($p<0.05$), while the overall degree of antioxidant protection (test FORD) was lower by 45% ($p<0.01$) compared with the control group (*Table II*).

In addition, the concentration of the principal enzyme of phospholipid hydrolysis, Lp-PLA2, was

Table I Weight percentages of FAs in the serum of men with CA and CHD, and in their peers without CHD.

Fatty acids	Patients with CHD and coronary atherosclerosis Me (25%; 75%), %	Patients without CHD Me (25%; 75%), %	Value
Saturated fatty acids			
Myristic (C 14:0)	0.94 (0.75; 1.28)	0.59 (0.48; 0.92)	<0.01
Pentadecanoic (C 15:0)	0.25 (0.17; 0.29)	0.32 (0.18; 0.39)	–
Palmitic (C 16:0)	26.84 (25.43; 29.27)	22.08 (19.12; 24.48)	<0.01
Stearic (C 18:0)	7.89 (7.19; 8.71)	7.72 (7.21; 9.53)	–
Arachic (C 20:0)	0.37 (0.15; 0.49)	0.29 (0.17; 0.52)	–
Docosanoic (C 22:0)	0.09 (0.08; 0.11)	0.12 (0.09; 0.18)	–
Monounsaturated fatty acids			
Palmitoleic (C 16:1)	2.48 (0.19; 2.69)	1.63 (1.01; 2.12)	<0.01
Oleic (C 18:1)	23.02 (21.45; 24.60)	16.73 (15.31; 20.49)	<0.01
Octadecenic (C 18:1)	3.79 (3.21; 4.39)	3.03 (2.54; 3.76)	<0.05
Eicosenoic (C 20:1)	0.27 (0.20; 0.33)	0.25 (0.16; 0.57)	–
Docosanoic (C 22:1)	0.14 (0.10; 0.19)	0.14 (0.11; 0.37)	–
Polyunsaturated fatty acids			
Linolic (C 18:2, ω-6)	24.07 (22.34; 27.64)	32.32 (27.63; 34.24)	<0.01
γ-linolenic (C 18:3, ω-6)	0.29 (0.22; 0.37)	0.43 (0.26; 0.68)	<0.05
α-linolenic (C 18:3, ω-3)	0.90 (0.84; 1.07)	2.95 (0.61; 10.75)	–
Eicosadienoic (C 20:2, ω-6)	0.33 (0.28; 0.39)	0.42 (0.34; 0.55)	<0.01
Eicosatrienoic (C 20:3, ω-6)	1.22 (1.02; 1.47)	1.43 (1.24; 1.68)	<0.05
Arachidonic (C 20:4, ω-6)	3.29 (2.38; 4.84)	5.88 (4.35; 7.29)	<0.01
Eicosapentaenoic (C 20:5, ω-3)	0.45 (0.31; 0.65)	0.58 (0.49; 0.81)	<0.05
Docosapentaenoic (C 22:5, ω-3)	0.31 (0.19; 0.41)	0.43 (0.35; 0.56)	<0.01
Docosahexaenoic (C 22:6, ω-3)	1.22 (0.87; 1.69)	1.81 (1.50; 2.13)	<0.01

Table II Triglyceride levels, characteristics of oxidative stress and of antioxidant defense, and Lp-PLA2 levels in the serum of patients with CA and CHD, and in their peers without CHD.

Characteristics	Patients with CHD and coronary atherosclerosis Me (25%; 75%), %	Patients without CHD Me (25%; 75%), %	value
Triglycerides (mmol/L)	1.66 (1.27; 2.18)	0.99 (0.82; 1.46)	<0.05
Lp-PLA2 (ng/mL)	109.91 (66.58; 188.27)	74.04 (44.42; 98.89)	<0.05
FORT (mmol/L)	2.27 (1.64; 2.76)	1.94 (1.47; 2.39)	<0.05
FORD (mmol/L)	0.59 (0.25; 0.80)	1.08 (0.82; 1.28)	<0.01

Table III Logistic regression analysis of a relative risk of the presence of vulnerable atherosclerotic plaques in coronary arteries in relation to FAs.

Fatty acids	Exp(B)	95.0% C.I. for Exp(B)		p
		Lower	Upper	
Myristic (C 14:0)	1.179	0.039	35.930	0.925
Pentadecanoic (C 15:0)	0.540	0.015	19.069	0.734
Palmitic (C 16:0)	16.591	2.011	136.866	0.009
Stearic (C 18:0)	6.308	1.112	35.766	0.037
Palmitoleic (C 16:1)	0.267	0.010	7.322	0.435
Oleic (C 18:1)	6.751	1.358	33.568	0.020
Eicosenic (C 20:1)	1462.15	0.000	49E9	0.410
Linolic (C 18:2, ω -6)	7.522	1.497	37.804	0.014
γ-linolenic (C 18:3, ω -6)	51.836	0.003	8E5	0.422
Eicosadienoic (C 20:2, ω -6)	18E5	0.001	23E14	0.177
Arachidonic (C 20:4, ω -6)	2.928	0.567	15.111	0.200
Eicosatrienoic (C20:3, ω -6)	62.413	0.499	7806.245	0.093
Eicosapentaenoic (C 20:5, ω -3)	1.830	0.001	4850.686	0.881
Docosapentaenoic (C 22:5, ω -3)	23132.16	0.001	61E10	0.249
Docosahexaenoic (C22:6, ω -3)	38.256	0.654	2237.713	0.079
Patient age	0.939	0.779	1.132	0.511

higher by 48% ($p<0.05$) in the circulation in men with CA and CHD in comparison with the control group. This enzyme is directly involved in atherogenesis by oxidizing free FAs as well as by inducing oxidation of free FAs, lipid modification, and initiation of intravascular inflammation (9).

Correlation analysis showed direct correlations of the triglyceride level with myristic and palmitic FAs ($r=0.422$, $r=0.248$, $p<0.05$, respectively) and with palmitoleic and oleic FAs ($r=0.455$, $r=0.561$, $p<0.01$, respectively). We revealed negative correlations between triglycerides and PUFAs, such as linolic ($r = –0.454$, $p<0.01$) and arachidonic ($r = 0.258$, $p<0.05$).

When conducting a correlation analysis between the weight proportion of individual FAs with parameters FORT, FORD, and Lp-PLA2, we identified a negative correlation between γ-linolenic acid and Lp-PLA2 ($r= –0.224$, $p<0.05$); between FORT and γ-linolenic, arachidonic, docosapentaenoic, and docosahexaenoic acids ($r= –0.288$, $–0.283$, $–0.230$, and $–0.259$, $p<0.05$, respectively); and between FORD and myristic, palmitic, palmitoleic, and oleic acids ($r= –0.224$, $p<0.05$; $r= –0.427$, $–0.465$, and $–0.398$, $p<0.01$, respectively). We also uncovered positive associations between octadecenoic acid and Lp-PLA2 ($r=0.233$, $p<0.05$); between FORT and oleic, octadecenoic, and eicosenic acids ($r=0.237$, 0.359, and 0.378, respectively, $p<0.05$); and between linolic acid and FORD ($r=0.468$, $p<0.01$).

The data on patients with CA and CHD were processed using multivariate logistic regression analysis. The presence of vulnerable atherosclerotic plaques in coronary arteries was considered a dependent variable. All of the studied FAs and the age of the patients served as independent variables (*Table III*). The results indicated that the relative risk of the presence of vulnerable atherosclerotic plaques in coronary arteries was associated with increased levels of palmitic, stearic, oleic, and linolic acids.

For an increase in the palmitic acid weight percentage by 1%, the risk of formation of vulnerable plaques increased 16-fold ($p<0.01$). For an increase in the weight percentage of stearic or oleic acid by 1%, the risk increased more than sixfold ($p<0.05$). For an increase in the linolic acid level by 1%, the risk of formation of vulnerable atherosclerotic plaques increased 7.5-fold ($p<0.01$).

Discussion

SFAs with a long and medium carbon chain are constituents of lipoproteins and circulate in the blood. These SFAs are used for the synthesis of other lipoid compounds in the body, e.g., cholesterol and LDL, and therefore SFAs are associated with an increased risk of cardiovascular disease (1). Nonetheless, there are studies showing that SFAs have no significant association with the risk of CHD (11, 13).

Our data on the changes in the levels of SFAs are consistent with the previously obtained results of Chen X. et al. (3), who revealed that the level of palmitic acid (from 16:0) was eightfold higher, and the level of stearic acid (from 18:0) was threefold higher in patients with atherosclerosis compared to the levels of these acids in the control group. These FAs have been identified as potential biomarkers for the clinical diagnosis of atherosclerosis. A number of researchers have suggested that high levels of palmitic acid may initiate the processes of inflammation and apoptosis (14, 15).

Unsaturated FAs are subdivided according to the degree of unsaturation into monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs).

We detected a significant increase in the concentration of MUFAs, owing to oleic and palmitoleic acid, in patients with atherosclerosis. This change could switch vascular smooth muscle cells (VSMCs) from the contractile to synthetic type and stimulate VSMC proliferation and migration in the subendothelium, which contributes to the formation of an organized atherosclerotic plaque (16, 17).

During a comparison of the effects PUFAs on atherosclerosis and CHD risk, it is important to distinguish between omega-3 and omega-6 PUFAs. Omega-3 PUFAs are traditionally considered protective factors, which are associated with a decreased risk of cardiovascular disease (4, 5). Despite the lack of a direct association between a high level of omega-3 FAs and a lower risk of CHD in several studies, there is an association with a decrease in total mortality (2). Moreover, there are studies showing that treatment with omega-3 FAs significantly decreases triglyceride levels in blood among men with hyperlipidemia (3, 6, 7, 12, 13). According to our data, the triglyceride content in the control group is lower, and PUFA levels are much higher.

Our results on the relation of low levels of omega-6 PUFAs with coronary artery disease and atherosclerosis are consistent with the findings of L. Wang et al. (18), who have also shown the relation between low levels of omega-6 PUFAs and the development of CHD.

The changes of the decrease in the content of individual PUFAs in the blood serum in patients with atherosclerosis (owing to increased lipid peroxidation processes, which most strongly affect PUFAs) underlines the importance of oxidative stress in the pathogenesis of CHD (14, 15).

To date, only a few researchers have studied the association of omega-6 and omega-3 PUFAs with Lp-PLA2 concentration and activity. In our study, we detected a negative association of omega-6 PUFAs (γ-linolenic) with Lp-PLA2; these data are partially consistent with the results of Steffen et al. (19). Unlike us, Schmidt et al. (20), observed the opposite relation: a correlation of omega-3 PUFA levels (EPA and DHA; rather than omega-6) with Lp-PLA2 concentration.

We determined that high levels of SFAs and MUFAs can contribute to blockage of the absorption of linoleic and linolenic LDL by the cell and a deficiency of PUFAs in the cell. These problems may promote the atherosclerotic process.

Considering the identified correlations, it is possible that during atherosclerosis in the human body, triglycerides contain mainly palmitic, oleic, myristic, and palmitoleic FAs.

The uncovered moderate negative correlations of PUFAs with biomarkers of inflammation and oxidative stress suggest that a decrease in the level of these FAs is accompanied by an increase in free-radical formation. The latter can probably increase the risk of atherosclerosis.

Conclusion

Thus, individual contributions of 20 FAs to atherogenesis were analyzed in patients with atherosclerosis. Increased levels of SFAs and MUFAs, such as myristic, palmitic, palmitoleic, oleic and octadecenic, were detected in blood serum. Furthermore, significant downregulation of all the studied omega-3 and omega-6 PUFAs was uncovered. These alterations are prominent and associated with signs of oxidative-antioxidative imbalance in the blood and an increased concentration of an oxidative-inflammation biomarker, Lp-PLA2. The relative risk of the presence of vulnerable atherosclerotic plaques in coronary arteries is associated with higher levels of palmitic, stearic, oleic, and linolic acids.

These findings probably indicate that alterations in the profile of FAs are accompanied by an increase in free-radical formation, which can probably serve as a risk factor of atherosclerosis.

Ethics approval and consent to participate

The study protocol was approved by the local Ethics Committee of the Institute of Internal and Preventive Medicine (a branch of the Institute of

Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia). Written informed consent to be examined and to participate in the study was obtained from each patient.

Availability of data and material

The datasets before and after analysis in this study are available from the corresponding author on reasonable request.

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Consent for publication

All the authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work as a whole, and have given final approval to the version to be published.

Competing interests

The authors declare that they have no conflicts of interest associated with publication of this article. Authors' contributions Yuliya I. Ragino: study conception and design and critical revision of the manuscript Viktoriya S. Shramko: analysis and interpretation of the biochemical data, literature review, drafting of the manuscript, and participation in discussion Ekaterina M. Stakhneva: collection of the clinical data, analysis and interpretation of the data on oxidative stress (test FORT) and total antioxidative defense in blood (test FORD), and literature review Elena V. Chernyak: analysis and interpretation of the data of chromatographic analysis Sergey V. Morozov: analysis and interpretation of the data of chromatographic analysis and participation in discussion Elena V. Shakhtshneider: revision of the manuscript and participation in discussion Yana V. Polonskaya: a collection of the clinical data, analysis and interpretation of the enzyme immunoassay data, and drafting of the manuscript Liliia V. Shcherbakova: statistical analysis Alexander M. Chernyavskyi: a collection of the clinical data, analysis and interpretation of the data on all endarterectomy samples from patients with angiographically confirmed CA, and participation in discussion.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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PLATELET GLUTAMATE DEHYDROGENASE ACTIVITY AND EFFICACY OF ANTIPSYCHOTIC THERAPY IN PATIENTS WITH SCHIZOPHRENIA

AKTIVNOST TROMBOCITNE GLUTAMAT DEHIDROGENAZE I EFIKASNOST ANTIPSIHOTIČNE TERAPIJE U PACIJENATA SA SHIZOFRENIJOM

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Summary

Background: Evaluation of possible relationship between platelet glutamate dehydrogenase (GDH) activity and mental state of schizophrenia patients after antipsychotic pharmacotherapy.
Methods: Patients (n = 50) with chronic paranoid schizophrenia (F20.0) initially in acute psychotic state were examined before and after a treatment course with antipsychotics. When assessing the patients' states using PANSS, the »responder« category was attributed to those patients who had not less than 30% reduction in the score for the corresponding PANSS »subscale«. The control group (n = 48) was age- and gender-matched with the patient group. Platelet glutamate dehydrogenase (GDH) activity was measured in patients twice, before and after the treatment course, and once in controls.
Results: Significantly reduced GDH activity was found in patients compared with controls. The patient group was divided into two subgroups according to median GDH activity at baseline: above and below the median GDH, subgroup 1 and subgroup 2, respectively. GDH activity significantly increased from its level at baseline after antipsychotic treatment in subgroup 2. Distribution of non-

Kratak sadržaj

Uvod: Proučavan je mogući odnos između aktivnosti trombocitne glutamat dehodrogenaze (GDH) i mentalnog stanja pacijenata sa shizofrenijom nakon antipsihotične terapije.
Metode: Ispitivani su pacijenti (n = 50) sa hroničnom paranoidnom shizofrenijom (F20.0) inicijalno u akutnom psihotičnom stanju pre i nakon tretmana sa antipsihoticima. Kada je procenjivano stanje pacijenata primenom PANSS, »resoponder« kategorija je odgovarala onima koji su imali manje od 30% smanjenja u odnosu na odgovarajuću PANSS »subskalu«. Kontrolnu grupu činilo je (n= 48) osoba sličnog pola i starosti. Aktivnost trombocitne glutamat dehidrogenaze (GDH) merena je kod pacijenata dva puta, pre i posle tretmana i jedanput kod kontrola.
Rezultati: Značajno umanjena aktivnost GDH nađena kod pacijenata upoređena je sa vrednošću kod kontrole grupe. Grupa pacijenata podeljena je u dve podgrupe prema srednjoj vrednosti GDH, kod niske aktivnosti kao i iznad i ispod srednje MDH vrednosti kod podgrupe 1 i podrupe 2. Aktivnost GDH je bila značajno ispod nivoa osnovne vrednosti nakon primene antipsihotične terapije kod podgrupe 2. Distribucija nonrispondera/rispondera nakon antiopsihotične terapije (prema PANSS skorivima) značajno se

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List of abbreviations: GDH, glutamate dehydrogenase; PANSS, Positive and Negative Syndrome Scale; NMDA, N-methyl-D-aspartate

responders / responders to antipsychotic treatment (by PANSS scores) was significantly uneven among subgroups 1 and 2. In subgroup 1, GDH activity levels significantly correlated with PANSS scores after the treatment course.
Conclusions: Baseline platelet GDH activity might serve as a predictor of antipsychotic therapy efficacy in schizophrenia patients.

Keywords: glutamate dehydrogenase, platelets, schizophrenia

razlikovala između podgrupa 1 i 2. U podgrupi 1 aktivnost GDH značajno je bila u korelaciji sa PANSS skorovima nakon primene terapije.
Zaključak: Osnovna aktivnost trombocitne GDH može da posluži kao prediktor efikasnosti antipsihotične terapije kod pacijenata sa shizofrenijom.

Ključne reči: glutamat dehidrogenaza, trombociti, shizofrenija

Introduction

Impairment of glutamatergic system regulation contributes to the development of psychosis in schizophrenia (1–3). Glutamate concentration and the activity and concentration of glutamate receptors and transporters are altered in the brain of patients with schizophrenia in comparison with mentally healthy persons; besides, strong antagonists of NMDA glutamate receptors induce psychotomimetic effects. These facts serve a basis for the »glutamate hypothesis« of schizophrenia pathogenesis that is prominent today (4). Glutamate neurotransmitter signal transduction depends on amounts and activity of glutamate receptors and transporters, as well as on activity of glutamate converting enzymes. Glutamate concentration in tripartite synapses is determined by equilibrium between its transport, binding by specific receptors, and metabolism by neuronal and glial enzymes, and the concentration can change due to impairment of glutamate metabolism leading to neurological or mental pathologies, including psychoses (5–7). As a result of investigations of human autopsy brain, characteristic features of glutamate metabolism in schizophrenia have been revealed in comparison with mentally healthy individuals. These features consist in changing both the concentration and intracellular localization of key glutamate metabolizing enzymes such as glutamine synthetase, glutamine synthetase-like protein, glutamate dehydrogenase isoenzymes (GDH), and regulatory links that control the ratio of these enzymes (8). Neurochemical studies of the brain are important for understanding the pathogenesis of schizophrenia, but the search for biochemical changes in the blood of schizophrenic patients is relevant and important in revealing biomarkers helping to choose the right antipsychotic therapy and predict its efficacy (9). There is a need for an inexpensive and reliable pathogenesis-related biomarker for prediction of antipsychotic therapy efficacy in patients with schizophrenia. From this point of view, blood platelets deserve special attention because they possess glutamate system components such as glutamate receptors, transporters (glutamate-dependent ion channels) (10), and some glutamate metabolizing enzymes (11, 12).

The key glutamate metabolizing enzyme, glutamate dehydrogenase (GDH, EC 1.4.1.3), catalyzes the reaction:

$$\alpha\text{-Ketoglutarate} + NH_4^+ + NAD(P)H \Leftrightarrow \text{L-Glu} + NAD(P) + H_2O$$

Three isoforms of GDH (GDHI, GDHII, and GDHIII) have been described in the human brain, differing in their strength of association with the cell membrane (12–15). Using antibodies to the human brain GDH isoforms, two isoenzymes of GDH, similar to GDHI and GDHII, are found in extracts of human platelets, whereas the amount of membrane-associated GDHIII isoform is below the threshold of immunoblotting detection (11). The presence of GDH in platelets is confirmed not only by immunoblotting, but also by the presence of its enzymatic activity (16, 17).

In this study, we evaluated the relationship between the activity of platelet GDH and the efficacy of antipsychotic pharmacotherapy in a group of patients with chronic acute schizophrenia.

Materials and Methods

Participants

A group of patients with paranoid schizophrenia (F20.0 according to ICD-10) with chronic course of the disease, all inpatients due to their illness (n = 50: 47 men and 3 women aged 25–56 years, median 34 years), was examined in initially acute psychotic state (hallucinatory paranoid or paranoid syndrome), before and after a treatment course with typical and atypical antipsychotics. A total of 50 patients entered the study, and for 45 patients the study was completed. Exclusion criteria were organic central nervous system diseases and acute and chronic somatic diseases.

Informed consent was obtained from all the participants of the study, the study was performed in conformance with the Declaration of Helsinki ethical guidelines, and ethical approval for the study was obtained from the Ethics Committee of the Mental Health Research Centre (Protocol N343, 14.04.2017). The patients were assessed twice by the attending psychiatrist, before and after the treatment course, using the Positive and Negative Syndrome Scale (PANSS) (18). In accordance with the peculiarities of the mental state of each patient, psychopharma-

Table I Patient Demography and Clinical Data.

Gender	47 m / 3f	
Education: secondary/ secondary special/ incomplete higher/higher (number of patients)	14 / 17 / 9 / 10	
Hereditary burden (+/–)	11 / 39	
Predominant psychopathology: delusional/ hallucinatory/disorganization (number of patients)	21 / 21 / 8	
Hospitalizations: 1/2/3 (number of patients)	5 / 8 / 37	
Antipsychotic therapy: atypical/typical/mixed (number of patients)	19 / 19 / 12	
	Median; minimum – maximum	25-, 75% – quartiles
Age (years)	34; 20–56	28; 42
Disease duration (months)	96; 2–420	48; 240
Age of manifestation (years)	25; 17–42	19; 28
PANSS positive at baseline	23; 10–35	21; 28
PANSS negative at baseline	26; 12–39	21; 29
PANSS psychotic at baseline	47; 25–71	43; 53
PANSS total at baseline	96; 58–141	89; 106

The control group (n = 48) consisted of 45 men and 3 women aged 21–59 years (median 38 years). Exclusion criteria were the same as for the patient group.

cotherapy individually selected by clinicians was carried out using typical or atypical antipsychotics. The end of the treatment course was determined as improvement in the patient's condition sufficient for discharge from the hospital (about two months after the initiation of the therapy).

When assessing patients' state using PANSS, »responder« category for each PANSS subscale (PANSSpositive subscale, PANSSnegative subscale, and PANSS subscale of general psychopathology) was assigned to those patients who had not less than 30% reduction of the corresponding PANSS subscale score. *Table I* contains data on the patients.

Blood sampling

Blood samples were collected after overnight fasting into evacuated tubes containing anticoagulant agent (3.2% Na-citrate buffer).

Each blood sample was treated individually within 2 hours after blood collection. Initially, the whole blood was centrifuged for 15 min at 200 *g* and 20 °C. The supernatant (platelet-rich plasma) was used for the subsequent isolation of platelets.

The platelet-rich plasma was centrifuged for 20 min at 2000 *g* at 4 °C. The precipitate was resuspended in 0.105 mol/L Na-citrate buffer with 0.105 mol/L glucose (pH 5.7) and centrifuged again for 20 min at 2000 *g* at 4 °C. The platelet precipitate was resuspended in 0.05 mol/L Tris-HCl buffer, pH 7.0, with 50% glycerol, and stored at –20 °C.

Immediately before determining the activity of GDH, 50 mmol/L K-phosphate buffer pH 7.4 containing dodecyl-β-D-maltoside was added to the platelet sample to final concentration of 1%. Lysis was carried out for 10 min at 25 °C, then the samples were centrifuged for 10 min at 4000 g at 4 °C. The resulting supernatant was diluted 5-fold in 50 mmol/L K-phosphate buffer, pH 7.4 (16).

GDH activity measurement

The activity of GDH was determined by a spectrophotometric method (19) with modifications (17). The sample volume added to 400 μL of the reaction mixture was 125 μL. The reaction mixture composition was 0.1 mmol/L NADH, 0.1 mol/L NH_4Cl, 10 mmol/L α-ketoglutarate, 1 mmol/L ADP, and 2.6 mmol/L EDTA in 0.01 mol/L Tris-HCl pH 8.0 buffer. The measurement of the rate of reaction (1), in this case directed to the right, is based on the loss of absorption of NADH recorded by spectrophotometry at 340 nm for 3 min. To calculate the enzymatic activity, the value of the molar extinction coefficient of NADH, 6.22×10^{-3} $l\times mol^{-1}\times cm^{-1}$ was taken, and the specific activity of GDH per 1 mg of protein is given in *Figures 1* and *2*.

Protein concentration was determined by the Lowry spectrophotometric method using a Bio-Rad DC Protein Assay Kit (USA) in accordance with the protocol and using bovine serum albumin (Sigma-Aldrich) as a protein standard for calibration.

Statistical analysis

The significance of differences between experimental groups was determined using »nonparametric analysis«. The significance of differences, changes in the parameters, and connections between them were assessed using the Mann–Whitney U-test, Chi-square test with Yates correction, Spearman correlation coefficients determination, and the Wilcoxon matched pair test. Differences and correlations were considered significant at $p<0.05$.

Results

The schizophrenia-patient and control groups were matched in age and gender, allowing between-group comparison of GDH activity. Significantly reduced GDH activity was found in the patient group both before and after the treatment course compared with the control group (Mann–Whitney U-test, $p<0.01$ and $p<0.04$, respectively), thus we reproduced our previous data (17).

The results of GDH activity determination for the schizophrenia patients before (baseline) and after the treatment course are shown in *Figure 1*.

When regarding the individual values of GDH activity in the patient group before the treatment course, substantial variation in values was noted. Therefore, this group of patients was divided into two subgroups by the median of the baseline (initial) GDH activity for further statistical analysis. The Wilcoxon matched-pair test showed that the GDH activity significantly increased after the treatment ($p = 0.002$) in the subgroup with the activity of baseline GDH below the median. *Figure 2* shows obvious dynamics of GDH activity during the treatment in this subgroup: the majority (18 patients) demonstrated an increase, and the other 5 showed no change or a decrease in GDH activity, whereas no significant changes in GDH activity were observed after the treatment in the subgroup with the baseline GDH activity above the median.

After the treatment course, significant correlations of GDH activity levels with PANSS negative and PANSS total score were revealed in the subgroup with the baseline GDH activity above the median (Spearman correlation coefficients $R = 0.56$, $p = 0.007$ and $R = 0.45$, $p = 0.04$, respectively). Besides, there was a significantly higher number of responders in this subgroup (for instance, by PANSS positive subscale, Chi-square with Yates correction = 9.1, $p = 0.001$) (*Table II*).

Table II Distribution of Responders and Non-responders (in Percentages of Total Group) by PANSS between the Subgroups of Patients with Baseline GDH Activity Below and Above the Median.

PANSS subscale	Below median GDH		Above median GDH	
	Responders	Non-responders	Responders	Non-responders
Positive	29	20	44	7
Negative	0	49	9	42
General psycho-pathology	7	42	18	33
Total	11	38	22	29

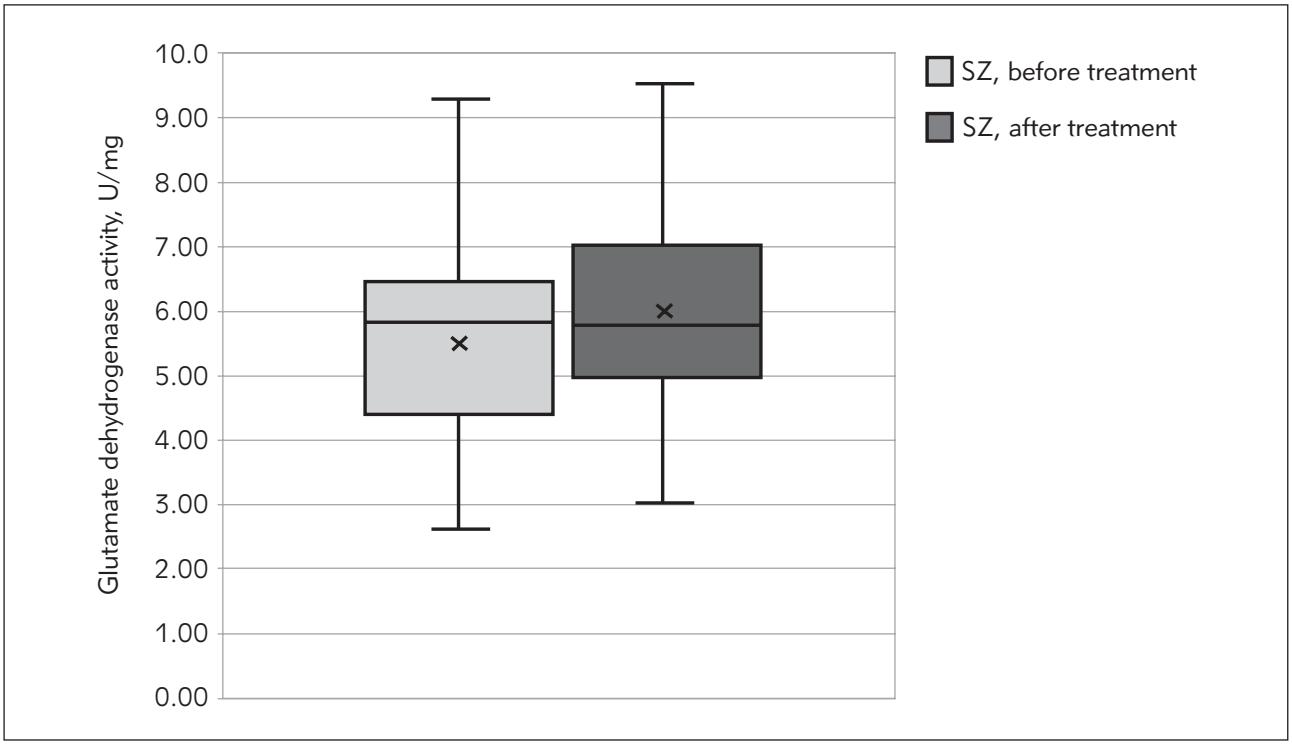

Figure 1 GDH activity in patients with schizophrenia (SZ) before (baseline) and after the treatment course.

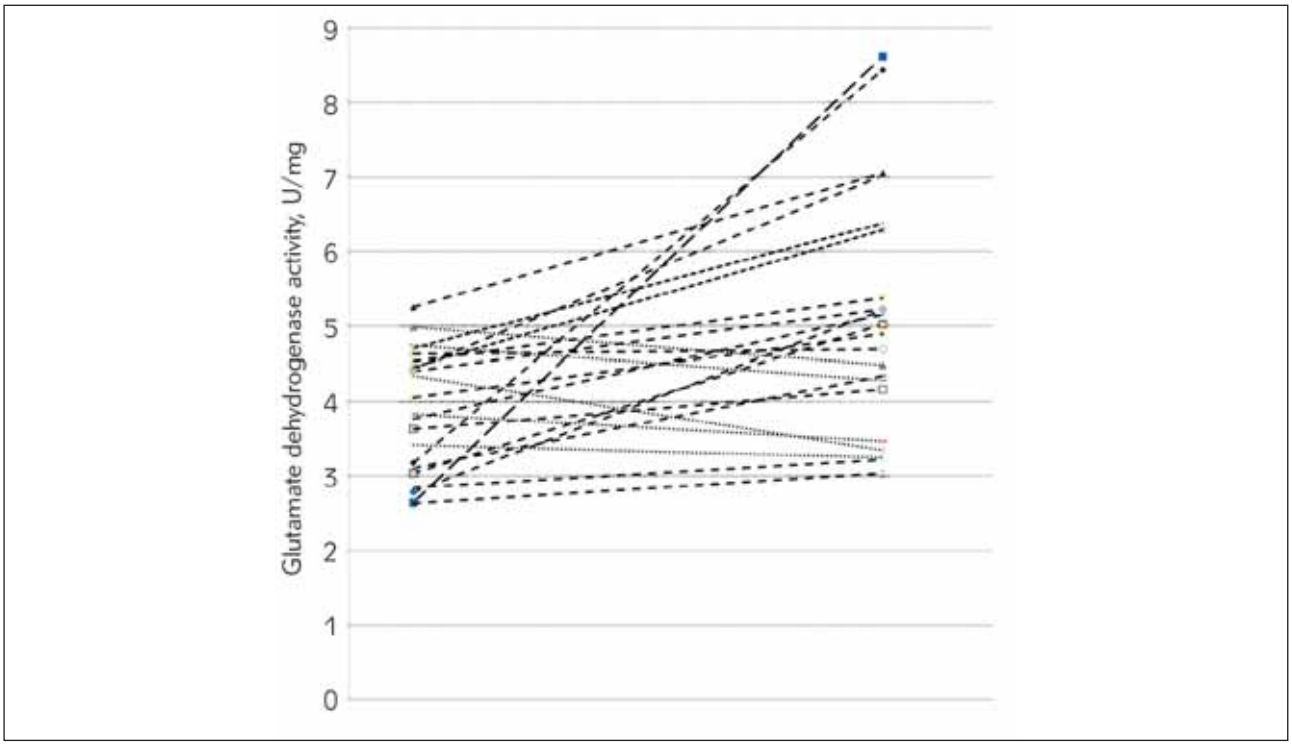

Figure 2 Dynamics of GDH activity for patients with baseline GDH activity below the median.

On the other hand, there was a significantly higher number of non-responders in the subgroup with baseline GDH activity lower than the median. Among these were non-responders by PANSS negative subscale score (Chi-square with Yates correction = 7.5, p = 0.006), by PANSS psychotic (Chi-square with Yates correction = 5.7, p = 0.02), and by PANSS total score (Chi-square with Yates correction = 3.8, p = 0.04).

Discussion

As we indicated in the Introduction, we previously found two GDH forms (similar to GDHI and GDHII) in human platelet extracts (9, 11) when the proteins were extracted using detergent (sodium dodecylsulfate, SDS) and the samples boiled with SDS according with the ECL-immunoblotting protocol developed for evaluation of GDHI and GDHII in human brain extracts using the same immunoprobes (i.e. antibodies recognizing human brain GDHI and GDHII) (8). When estimating GDH amount by immunoblotting, the sum of GDHI and GDHII forms was taken into consideration, and no significant difference was found between the group of patients with paranoid schizophrenia and controls (11).

In the present work, a soft extraction method was employed for GDH enzymatic activity measurement using n-dodecyl β-D-maltoside, which preserves the enzymatic activity. The GDH activity was found to be significantly higher in the control group than in the patient group. Obviously, SDS and n-dodecyl β-D-maltoside may differently extract the GDH forms from platelets, causing different dissociation of the GDH forms particularly from platelet mitochondria.

We would like to emphasize that the method of GDH enzymatic activity determination is more easily standardized and is not so expensive and time consuming in comparison with ECL-Western immunoblotting, and thus the activity determination is preferable for routine clinical analyses.

Generally, only in the subgroup of patients with baseline GDH activity below the median was the GDH activity significantly increased after the treatment, and a significantly higher number of non-responders to antipsychotic treatment was found in this subgroup.

Correspondingly, a significantly higher number of responders was found in the other subgroup with baseline GDH activity above the median, and significant correlations of GDH activity levels with PANSS negative scores and PANSS total scores were revealed after the treatment in this subgroup.

Conclusion

Significantly different uneven distribution of responders and non-responders between two subgroups of patients with paranoid schizophrenia (with baseline GDH activity above and below the GDH

median) indicates that measurement of platelet GDH activity in patients with paranoid schizophrenia before the course of antipsychotic treatment might be useful for individual prediction of the efficacy of antipsychotic pharmacotherapy.

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Conflict of interest statement

The authors state that they have no conflicts of interest regarding the publication of this article.

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PERIPHERAL NEURAL RESPONSE AND SEX HORMONES IN TYPE 1 GAUCHER DISEASE

PERIFERNI NEURALNI ODGOVOR I POLNI HORMONI KOD GOŠEOVE BOLESTI TIPA 1

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Summary

Background: In a rare Gaucher disease, reduced activity of lysosomal β-glucocerebrosidase incompletely blocks glucosphingolipid catabolism. Accumulation of the unhydrolyzed substrate glucosylceramide within lysosomes results in progressive, multisystem Gaucher disease, classified into three types. Both parkinsonism and peripheral neuropathy are observed in cases of putative non-neuronopathic type 1 disease. In the current study we investigated whether the peripheral neural response in type 1 Gaucher disease patients, with no neural manifestations is conditioned by the influence of sex hormones.
Methods: The catalytic activity of β-glucocerebrosidase in peripheral blood leukocytes was determined spectrofluorometrically. Direct sequencing of the *GBA1* gene was performed. Somatosensory evoked potentials were recorded after electrical stimulation of the median nerve of both arms. Stimuli of 0.2 ms duration at a frequency of 5 Hz were used. Sex hormones were determined by radioimmunoassay using a gamma scintillation counter.
Results: Analysis of the somatosensory evoked potentials revealed significant differences in peak latencies on periphery between men and women in both control and type 1 Gaucher disease groups. Analysis by gender showed significant associations between latencies and sex hormones

Kratak sadržaj

Uvod: Kod retke Gošeove bolesti, snižena aktivnost lizozomske β-glikocerebrozidaze uzrokuje nepotpuni blok katabolizma glikosfingolipida. Nagomilavanje nehidrolizovanog supstrata glukozilceramida u lizozomima dovodi do progresivne, multisistemske Gošeove bolesti klasifikovane u tri tipa. Ispostavilo se da se kod obavezno ne-neuronopatskog tipa 1 ipak javlja neuralna patologija – parkinsonizam i periferna neuropatija. U aktuelnoj studiji, istraživali smo da li je kod pacijenata sa Gošeovom bolešću tipa 1 koji su bez ikakvih neuroloških manifestacija periferni neuralni odgovor uslovljen uticajem polnih hormona.
Metode: Katalitička aktivnost β-glikocerebrozidaze u leukocitima periferne krvi utvrđena je spektrofluorometrijskim metodom. Analiza gena *GBA1* izvedena je direktnim sekvenciranjem. Snimljeni su somatosenzorni potencijali evocirani električnom stimulacijom nervusa medijanusa obe ruke. Nadražajima trajanja 0,2 ms delovalo se frekvecijom od 5 Hz. Polni hormoni su mereni radioimunološkim metodom pomoću gama scintilacionog brojača.
Rezultati: Metodom somatosenzornih evociranih potencijala otkrili smo značajnu razliku u latencama kod muškaraca i žena na periferiji, kako u kontrolnoj grupi tako i u grupi Gošeove bolesti tipa 1. Analiza po polu pokazuje značajne korelacije između latenci i polnih hormona samo kod

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only in female patients: negative correlation between oestradiol concentration and N9 peak latency, and a strong negative correlation of testosterone levels with all peak latencies on the periphery (N9-N13).

Conclusions: A relationship between testosterone concentrations and the latencies of potentials evoked on peripheral nerves exists only in females with type 1 Gaucher disease. We point out sexual dimorphism in the development of this entity.

Keywords: Type 1 Gaucher disease, somatosensory evoked potentials, latencies, oestradiol, testosterone

ženskih pacijenata: značajno negativnu korelaciju između koncentracije estradiola i latence N9, i jaku negativnu korelaciju nivoa testosterona sa svim latencama na periferiji (N9-N13).

Zaključak: Dokazom da povezanost koncentracija testosterona i latenci potencijala izazvanih na perifernim živcima postoji samo kod žena sa tipom 1 Gošeove bolesti ukazujemo na seksualni dimorfizam u razvoju toga entiteta.

Ključne reči: Gošeova bolest tip 1, somatosenzorni evocirani potencijali, latencije, estradiol, testosteron

Introduction

Gaucher disease (GD) is a rare lysosomal storage sphingolipidosis caused by compound heterozygous or homozygous mutations in the *GBA1* gene (Online Mendelian Inheritance in Man (OMIM) #606463) (1). Consecutive unnatural configuration and insufficient activity of lysosomal β-glucocerebrosidase leads to incomplete block in glucosphingolipid catabolism (2, 3). Since sphingolipids and their metabolites, as structural elements of membranes and signaling molecules, play a vital role in cell physiology, accumulation of the unhydrolyzed substrate glucosylceramide within lysosomes results in progressive, multisystem GD with a wide phenotypic spectrum (4). GD is classified into mandatory non-neuronopathic type 1 (OMIM #230800), acute neuronopathic type 2 (OMIM #230900) and chronic neuronopathic type 3 (OMIM #231000) disease. However, the occurrence of parkinsonism and peripheral neuropathy different from the specific characteristics defined by type 2 and 3 has been observed in a number of patients with type 1 GD (5, 6).

The aim of this study was to determine whether the peripheral neural response in type 1 GD is under the influence of sex hormones.

Materials and Methods

Patients

In this cross-sectional study, 20 type 1 GD patients with no clinical neurological manifestations (10 women and 10 men) aged 19 to 61 years were compared with healthy controls matched for gender and age. Among the patients, three were treatment-naïve, fourteen had received enzyme replacement therapy and three substrate-reductive therapy for more than 5 years.

Compliance with ethical standards

Approval for the study was obtained from the Ethics Committee of the Medical Faculty, University of Belgrade (decision number 29/III-12 from 27.03. 2015.).

Data aquisition methods

The algorithm for setting the GD diagnosis included clinical findings, assessment of biomarker activity followed by determination of specific enzyme activity and was confirmed by identification of *GBA1* gene mutations.

Biochemistry

The activity of the biomarker chitotriosidase in serum was measured spectrofluorometrically using the fluorogenic substrate 4-methyl-umbelliferyl-β-D-N-N'-N"-triacetylchitotrioside (Sigma Chemical Co, USA) on an SPF-500™C spectrofluorometer (SLM Instruments Inc, USA). The fluorogenic substrate 4-methylumbelliferyl-β-D-glucoside (Sigma Chemical Co, USA) was used for determining the catalytic activity of β-glucocerebrosidase in peripheral blood leukocytes spectrofluorometrically using the same spectrofluorometer.

The following ranges of control values (as 2.5 and 97.5 percentiles) for a healthy population were established at the Clinical Center of Serbia: for chitotriosidase activity 1.80–146.56 nmol/mL/h; for β-glucocerebrosidase activity 6.65–15.90 nmol/mg/h.

Molecular genetics

The *GBA1* gene was analyzed by direct sequencing (Applied Biosystems 3500, Hitachi, USA).

Neurophysiology

Somatosensory evoked potentials (SSEP) were recorded after electrical stimulation of the right and left median nerve at the wrist (MedelecSynergy, Viasys Healthcare, UK). Stimuli of 0.2 ms duration were given at a frequency of 5 Hz.

Hormones

Oestradiol (E2) (ESTR-US- CT, Cisbio Bioassays, France, coefficient of variance,CV, 2.8%) and testos-

Table I Anthropological data and sex hormones levels.

	Control (N = 20)			Gaucher (N = 20)			
	Women	Men	*p*	Women	Men	p	*p* (Con vs. GD)
Age (years)	36.5 ± 4.4	44.4 ± 3.9	0.19	38.2 ± 4.1	43.8 ± 4.2	0.52	0.93
Height (cm)	171.5 ± 1.3	180.2 ± 1.8	0.01	164 ± 2.7	180.0 ± 2.6	0.01	0.24
Testosterone (nmol/L)	1.07 ± 0.12	21 ± 2	0.01	0.89 ± 0.17	19.4 ± 1.3	0.01	0.63
Oestradiol (pmol/L)	252 ± 60	129 ± 10	0.06	274 ± 76	115 ± 13	0.07	0.88

Data are presented as mean values ± standard error.

terone (TESTO- CT2, Cisbio Bioassays, France, CV 3.1%) were measured by radioimmunoassay using a gamma scintillation counter (CliniGamma 1272, LKB-Wallac, USA).

Statistics

Normal distribution of the data was examined by the Shapiro-Wilk test. We used two way ANOVA (group, gender) to assess differences between the control and type 1 GD groups. Pearson's correlation coefficient was employed to estimate linear relationships between the variables. Data are presented as means ± standard error. Differences were considered statistically significant at $p < 0.05$. We used SPSS 19 (IBM) software for statistical analysis.

Results

Biochemistry

Pretreatment chitotriosidase activity levels ranged from 2404 to 26930 nmol/mL/h. Residual levels of β-glucocerebrosidase activity were between 0.50 to 3.14 nmol/mg/h.

Molecular genetics

Two patients were homozygous for the *GBA1*mutation c.1226A>G, while all the others had compound heterozygous mutations. In each control participant, the wild type *GBA1* gene was confirmed in both alleles.

Anthropology

There was no age difference between the groups (*Table I*). In terms of height, significant differences existed in each group: women were shorter than men in both groups; women with type 1 GD were shorter than the control women ($p = 0.03$) (*Table I*).

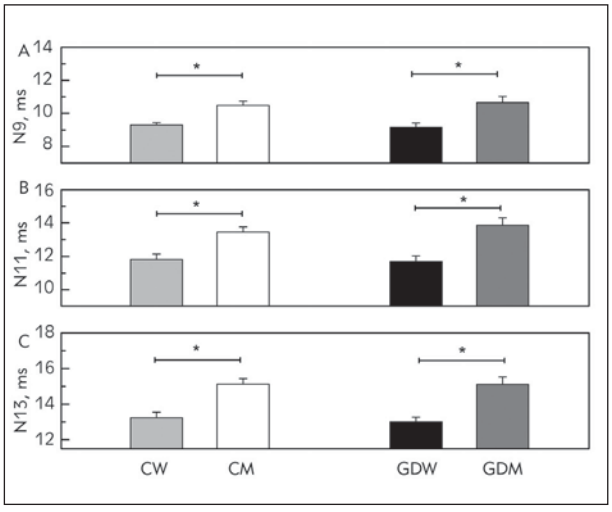

Figure 1 Peak latencies plus standard error in control women (CW), control men (CM), type 1 GD female patients (GDW), type 1 GD male patients (GDM), * $p < 0.01$.

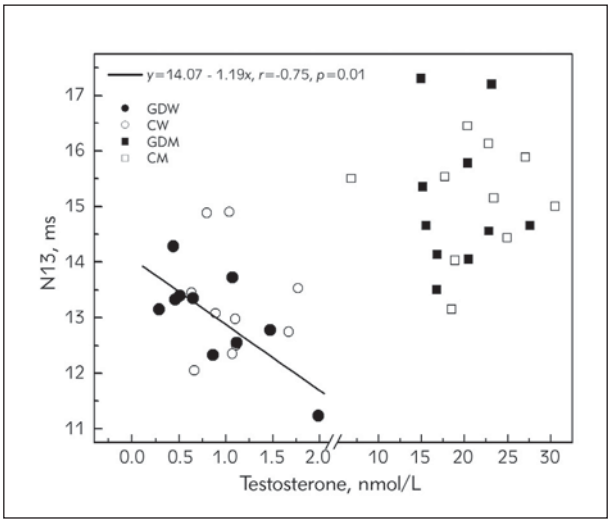

Figure 2 Relationship between peak latency N13 and plasma testosterone levels in GDW (solid circle), CW (open circle), GDM (solid square) and CM (open square).

SSEP

Regarding the average latency values there were no difference for all parameters between the left and right median nerves (latency of the N9, N11 and N13 waves), no when the control and GD groups were compared in total and for each gender separately. However, within each groups, a significant differences in peak latencies between men and women were identified from N9 to N13 ($p \leq 0.01$) (*Figure 1*).

Sex hormones

As expected both groups showed statistically significant differences in testosterone concentrations between women and men (*Table I*). There was a tendency for the difference between the sexes for oestradiol concentration to approach statistical significance both groups (*Table I*).

Correlations

Statistically significant latency correlations with the height of subjects were present only in women with type 1 GD: N9 ($r = 0.78$, $p < 0.01$), N11 ($r = 0.58$, $p = 0.08$, near), N13 ($r = 0.70$, $p = 0.03$).

Considering men and women together, there were significant positive correlations between all latencies and testosterone concentrations in both the control and type 1 GD groups: N9 ($r = 0.57$ vs. $r = 0.58$, $p < 0.01$, respectively), N11 ($r = 0.60$ vs. $r = 0.62$, $p < 0.01$, respectively), and N13 ($r = 0.65$ vs. $r = 0.67$, $p < 0.01$, respectively) (*Figure 2*).

However, when the analysis was performed for each gender, significant latency correlations with sex hormones were identified only in female patients with type 1 GD: negative correlation between estradiol concentration and N9 peak latency ($r = -0.63$, $p = 0.05$) and negative correlations of testosterone levels with all peak latencies on the periphery N9 ($r = -0.58$, $p = 0.08$, tendency), N11 ($r = -0.76$, $p = 0.01$), N13 ($r = -0.75$, $p = 0.01$).

Discussion

A crucial large-scale prospective observational cohort study, employing strictly defined criteria, provided evidence for polyneuropathy as part of the natural course of type 1 GD (6). In 103 patients enrolled at 18 to 75 years old, 13.6% were untreated and 86.4% received enzyme replacement therapy. Among them, 10.7% were diagnosed with sensory motor axonal polyneuropathy at baseline using standardized electrophysiological assessment. Six new cases of polyneuropathy were revealed during two-years monitoring (2.9 per 100 person-years). The same diagnostic procedure was used for the 25 healthy subjects. Since prevalence and incidence of polyneuropathy in the general population were estimated to be between 0.09 and 1.3% and 0.0046 and 0.015 per 100 person-years, respectively, it was concluded that both prevalence and incidence of polyneuropathy in type 1 GD patients are greater than for the general population. Therefore, for this study we enrolled only patients with no peripheral neurological manifestations.

Until now, only one multimodal neurophysiological investigation (including SSEP) has been performed in adult subjects with type 1GD (7). It involved eight female and four male adult patients aged 17 to 48 years. Findings were obtained from the right median nerve. In all subjects normal recordings from the periphery (N9, N11, N13) were read out. We have confirmed such normal findings but have gone further. Namely the present cross-sectional SSEP study on the median nerve periphery revealed gender differences – shorter latency peaks in women from both groups (healthy, diseased) which has not been noticed, so far.

Neuroactive steroids include those produced by the nervous system and hormones originating from the gonads and adrenal glands. The peripheral nervous system (PNS) not only synthesizes and metabolizes neuroactive steroids, but peripheral nerves also express receptors for neuroactive steroids and, therefore, are a target for their activity (8, 9). Steroids acting in the nervous system realize their effects via classical intracellular androgen, progesterone, oestrogen, glucocorticoid and mineralocorticoid receptors, as well as via non-classical steroid receptors expressed by different cellular components of the PNS (10, 11). Therefore, regulating PNS physiology over various signaling pathways, neuroactive steroids, including testosterone, can influence different peripheral nerves functions, among which Schwann cells proliferation and myelination have been studied in particular (8, 11).

Striking sexual dimorphism of white matter growth in adolescent brains has already been observed, but not explained (12, 13). In order to assess the role of the androgen receptor (AR) in mediating the effect of testosterone on white matter growth, 204 male and 204 female adolescents were studied (14). Functional polymorphism in the AR gene (number of CAG repeats in exon 1) was genotyped, togather with measurement of plasma testosterone concentration, computational analysis of magnetic resonance images and calculation of the magnetization transfer ratio (MTR) for white matter throughout the brain as an indirect index of myelination. Evidence emerged that a genetic variation in the AR gene moderates the effect of testosterone on white matter volume during male adolescence. Namely, the testosterone-related increase of white matter volume was stronger in male adolescents with lower versus higher numbers of CAG repeats in exon 1 of the AR gene. The MTR results indicated age-related growth in volume, but this could not be

explained by an increase in myelination. It was assumed that testosterone effected axonal diameter, rather than myelin sheath thickness. The direct consequence of increased axonal diameter under the influence of testosterone is a decrease in the number of fibers per unit volume, which is manifested as a lower index of myelination and lower MTR values (14). Likewise, the latter might explain the peripheral nerve response data obtained in the current study. Within the total testosterone concentration range (males plus females), the positive correlation between plasma testosterone levels and all peak latencies in all participants in each group is very significant. Since the number of nerve fibers is a key factors determining the efficiency of peripheral nerve transmission (15), the smaller number of myelinated fibers in the median nerve, due to their large testosterone-induced caliber in men, could be the reason for longer latencies.

When females were analyzed *per se*, using the lower range of testosterone values in women (up to 1.9 nmol/L), only female patients with type 1 GD showed a statistically significant negative correlations of plasma testosterone levels with latencies from N9 to N13. This association indicates that a normal SSEP finding alone is not sufficient to draw conclusions about undisturbed morphological and functional nerve integrity on the periphery in type 1 GD females. Why small changes in testosterone concentrations in women with type 1 GD affect the duration of impulse propagation should be clarified. Experimental models have indicated that pathology affects the concentration of neuroactive steroids present in peripheral nerves in a sex-dimorphic way (16, 17).

We have entered a serious area that has yet to be examined in detail due to insufficient available data on this matter.

Conflict of interest statement

The authors state that they have no conflicts of interest regarding the publication of this article.

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LEUKOCYTOSIS INTERFERENCE IN CLINICAL CHEMISTRY: SHALL WE STILL INTERPRET TEST RESULTS WITHOUT HEMATOLOGICAL DATA?

INTERFERENCIJA LEUKOCITOZE U KLINIČKOJ HEMIJI: DA LI ĆEMO I DALJE INTERPRETIRATI REZULTATE TESTA BEZ HEMATOLOŠKIH PODATAKA?

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Summary

Background: Extreme leukocytosis is known to induce remarkable variations of some clinical chemistry tests, thus leading to possible clinical misinterpretation. This study aimed to define whether also moderate leukocytosis may influence the stability of glucose and blood gases.
Methods: Blood samples are sent to the local laboratory through a pneumatic tube system. Clinical chemistry testing is routinely performed using lithium-heparin tubes (for glucose) and heparin blood gases syringes (for blood gas analysis). Stability of glucose (in uncentrifuged blood tubes) and blood gases (in syringes) was hence evaluated in samples maintained at room temperature. Results were also analyzed in 2 subgroups of samples with different leukocyte counts, i.e., those with leukocytes $<15 \times 10^9$/L and those with leukocytes $>15 \times 10^9$/L.
Results: An accelerated decrease of pH was observed in blood gases syringes with leukocytosis (i.e., $>15 \times 10^9$/L), while no difference was noted for other blood gases parameters (PCO_2, PO_2). Spurious and time-dependent hypoglycemia was noted in uncentrifuged blood tubes of patients with leukocytosis.
Conclusions: The results of our study suggest that even modest leukocytosis (i.e., around 15×10^9/L), which is

Kratak sadržaj

Uvod: Poznato je da ekstremna leukocitoza izaziva značajne varijacije nekih kliničkih hemijskih testova, što dovodi do moguće pogrešne kliničke interpretacije. Cilj ovog rada bio je da se utvrdi da li i umerena leukocitoza može uticati na stabilnost glukoze i gasova u krvi.
Metode: Uzorci krvi su poslati u lokalnu laboratoriju preko pneumatskog sistema cevi. Klinička hemijska ispitivanja se rutinski izvode koristeći litijum-heparinske epruvete (za glukozu) i heparinske gasove (za analizu gasa u krvi). Stabilnost glukoze (u necentrifugiranim epruvetama krvi) i gasova u krvi (u špricevima) je zbog toga procenjena na uzorcima koji su održavani na sobnoj temperaturi. Rezultati su takođe analizirani u 2 podgrupe uzoraka sa različitim brojem leukocita, to jest, sa leukocitima $< 15 \times 10^9$/L i sa leukocitima $> 15 \times 10^9$/L.
Rezultati: U špricu sa gasovima u krvi sa leukocitozom (tj. $> 15 \times 10^9$/L) primećeno je ubrzano smanjenje pH vrednosti, pri čemu nije zabeležena razlika za druge parametre gasova krvi (PCO_2, PO_2). Lažna i vremenski zavisna hipoglikemija zabeležena je u necentrifugiranim epruvetama pacijenata sa leukocitozom.
Zaključak: Rezultati našeg istraživanja ukazuju da čak i skromna leukocitoza (tj. oko 15×10^9/L), koja se često

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frequently encountered in clinical and laboratory practice, may be associated with significant variations of both glucose and pH. This would lead us to conclude that results of these parameters shall be accompanied by those of hematologic testing to prevent clinical misinterpretation, namely with leukocyte counts.

Keywords: glucose, blood gases, preanalytical variability

sreće u kliničkoj i laboratorijskoj praksi, može biti povezana sa značajnim varijacijama i glukoze i pH. To nas je navelo na zaključak da rezultati ovih parametara moraju biti propraćeni rezultatima hematološkog testiranja kako bi se sprečila pogrešna klinička interpretacija, a sve zbog broja leukocita.

Ključne reči: glukoza, gasovi u krvi, preanalitička varijabilnost

Introduction

It has been convincingly shown that some clinical chemistry parameters may display spurious changes in samples with extreme leukocytosis, thus potentially disturbing both clinical decision making and patient management.

Monitoring blood glucose is essential, since hyperglycemia may be associated with many symptoms such as infections or late-onset nerve damage, while hypoglycemia may also cause serious health consequences. The basic symptoms of hypoglycemia are poorly specific and include fatigue, mood changes, difficulty in concentrating and completing mental tasks, blurred vision, extreme hunger, nervousness, headache, excessive perspiration and tachycardia. Extremely low glucose levels can trigger convulsions and coma (1). An accurate diagnosis of hypoglycemia is also essential for diagnosing metabolic diseases (2).

Glucose is usually measured in clinical laboratories using plasma, serum or whole blood samples. The term »artifactual hypoglycemia« was proposed when results of laboratory measurement do not reflect the actual blood glucose concentration, regardless of the presence of symptoms of both hypo- or hyperglycemia (3). Artifactual hypoglycemia has also been related to *in vitro* glucose consumption by blood cells after blood sample has been drawn (4, 5).

Sodium fluoride is largely used as glycolysis inhibitor in fluoride tubes since it assures longer glucose stability in uncentrifuged or non-separated blood tubes. Nevertheless, lithium-heparin blood tubes without glycolysis inhibitors are very frequently used to measure glucose in clinical and laboratory practice, since it helps to reduce the number of samples drawn and minimizing blood wasting, especially in critically ill patients with severe anemia (6, 7).

A recent study showed that normal blood cell counts and volumes might both have an impact on glucose concentration in uncentrifuged serum and lithium-heparin blood tubes (8), while no data are available on the impact of non-leukemic leukocytosis on glucose stability in blood samples to the best of our knowledge. We suspect that the impact on glucose may be even greater with leukocytosis than with normal leukocyte counts.

Blood gases are conventionally requested as emergency tests. By measurement of dissolved gases in blood and pH, blood gas analysis provides essential clinical information for monitoring alveolar ventilation and acid-base balance (9). The stability of these parameters in blood samples is strongly influenced by storage temperature and by the possible presence of hemolysis (10, 11). Previous studies showed that blood gases are stable for up to 30 minutes after collection (12), while the analysis of blood gases in samples (typically syringes) after this period may yield unreliable results. Since blood gas analysis is an essential test for patients with acute conditions, and recollection of a new blood sample is not always feasible in short-stay units, blood gas analysis is occasionally carried out after the recommended 30-min stability threshold. Nevertheless, blood cells are known to gradually modify the acid-base balance over time, by consuming both oxygen and glucose (13–15), and thus potentially leading to unreliable test results. A case of pseudohypoxemia in a patient with chronic lymphocytic leukemia was also identified, showing that blood gases stability is inversely correlated with the leukocyte count (16).

Therefore, the aim of this study was to define whether moderate leukocytosis may also influence the stability of some clinical chemistry parameters, namely glucose and blood gases.

Materials and Methods

Patient selection and data collection

The study population consisted of patients undergoing routine laboratory testing at Lille University Hospital. Patients received detailed information that their clinical data and/or residual blood samples could be used for research purposes after routine testing had been completed. All data were retrieved from a human biological database authorized by the French Ministry of Research (No. DC-2008-642). Therefore, no written informed consent was collected from this population.

The stability of glucose was assessed in lithium-heparin tubes (4 mL BD Vacutainer™; Becton Dickinson, Franklin Lakes, NJ, United States) and blood gases in heparin syringes (3 mL Smiths Medical; Minneapolis, MN, United States).

To perform our study, we selected samples containing enough blood to allow repeated analyses (full draw). Samples were selected upon arrival from

patients who had had a recent leukocyte count available. Samples were divided into 2 groups according to the leukocyte count: i.e. $<15 \times 10^9$/L or $>15 \times 10^9$/L.

Laboratory measurements

Glucose concentrations were measured using Roche Cobas 8000® (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Blood gas parameters were measured on Radiometer ABL800 FLEX (Radiometer, Copenhagen, Denmark). White blood cell count (WBC), hematocrit and platelets count were assayed in BD Vacutainer™ ethylenediaminetetraacetic acid tubes (Becton Dickinson, Franklin Lakes, NJ, United States), using Sysmex XN (Sysmex Corporation, Kobe, Japan).

All blood tubes and syringes were sent to the laboratory by a pneumatic tube system (PTS) (Swisslog, Switzerland) within 30 min of collection.

For glucose stability experiments, one blood sample (i.e., lithium-heparin blood tube) was collected from each patient and was immediately centrifuged after arrival at 4000×g for 5 min. Lithium-heparin tubes were maintained on a roller mixer at room temperature throughout the study to prevent sedimentation of blood cells in between measurements. Blood cells would this way continue *in vitro* glucose consumption and better reflect the impact of a delay in sample handling. Before each plasma glucose measurement, samples were centrifuged at 4000×g for 5 min.

Plasma glucose was successively measured at 0, 2, 4, 6 and 8 hours after the reception in the laboratory, and its variation was compared in 2 different groups of patients, displaying WBC count $<15 \times 10^9$/L or $>15 \times 10^9$/L.

For blood gases stability experiments, one whole blood sample (i.e., heparin blood gas syringe) was collected from each patient. Blood gas analysis was then carried out at 0, 60, 120 and 180 min after the reception in the laboratory, on the same syringe maintained at room temperature. Results were compared in 2 groups of patients, i.e., those with leukocyte counts $<15 \times 10^9$/L or $>15 \times 10^9$/L. Before each measurement, the syringe was gently mixed to avoid sedimentation and kept sealed afterwards.

The local reference ranges were 65 to 100 mg/dL (3.58–5.50 mmol/L) for glucose and 4–10 $\times 10^9$/L for WBC count.

Statistical analyses

Data analyses were performed with paired Wilcoxon ranked test and Pearson correlation using R software (www.R-project.com) and GraphPad Prism software v6.0. The statistical methods used included t-tests, ANOVA and regression analysis. Statistical significance was set at $p < 0.05$.

Results

Glucose

A total number of 64 lithium-heparin samples were included in our analysis, which were sorted according to WBC count, platelet count and hematocrit values, as shown in *Table I*. The baseline glucose

Table I Grouping of patients (n=64) according to leukocytosis levels (threshold: 15×10^9/L) and the measure of baseline glucose levels, White Blood Cells counts, Platelets counts and Hematocrit levels (Mean +/– SEM) measured in blood. Regression and correlation between glucose and time for each group, p-value obtained by Pearson t-test.

		Glucose t0 (mmol/L)		White Blood Cells ($x10^9$/L)		Platelets ($x10^9$/L)		Hematocrit (%)		Glucose-Time Regression		
	N	Mean (SE)	Min-Max	Mean (SE)	Min-Max	Mean (SE)	Min-Max	Mean (SE)	Min-Max	Slope	r	p-value
Low Leukocytes ($<15 \times 10^9$/L)	54	6.32 (1.39)	3.77–12.9	7.77 (2.75)	0.3–14.51	249 (110)	24–785	31 (4.8)	19.9–44.2	-0.34	-0.42	<0.001
High Leukocytes ($>15 \times 10^9$/L)	10	6.32 (1.49)	3.55–9.55	109 (88)	15–346	214 (206)	13–682	27 (4.3)	19.4–36.6	-0.63	-0.67	<0.001
Low Platelets ($<150 \times 10^9$/L)	18	6.93 (1.83)	3.55–12.9	57 (71)	0.3–346	61 (30)	13–148	26.9 (4)	19.4–42.5	-0.27	-0.22	0.028
High Platelets ($>150 \times 10^9$/L)	46	6.05 (1.22)	3.77–10.7	10 (4)	2.7–41.26	316 (109)	157–785	32.5 (4.2)	23.5–44.2	-0.39	-0.26	<0.001
Low Hematocrit (<30%)	32	6.49 (1.49)	3.55–14.1	37 (47)	0.3–346	118 (116)	13–413	26 (2.3)	19.4–29.5	-0.31	-0.36	<0.001
High Hematocrit (>30%)	32	6.16 (1.27)	3.77–10.7	9.5 (3.5)	3.8–40.5	300 (142)	24–785	36 (3.1)	30.2–44.2	-0.41	-0.56	<0.001

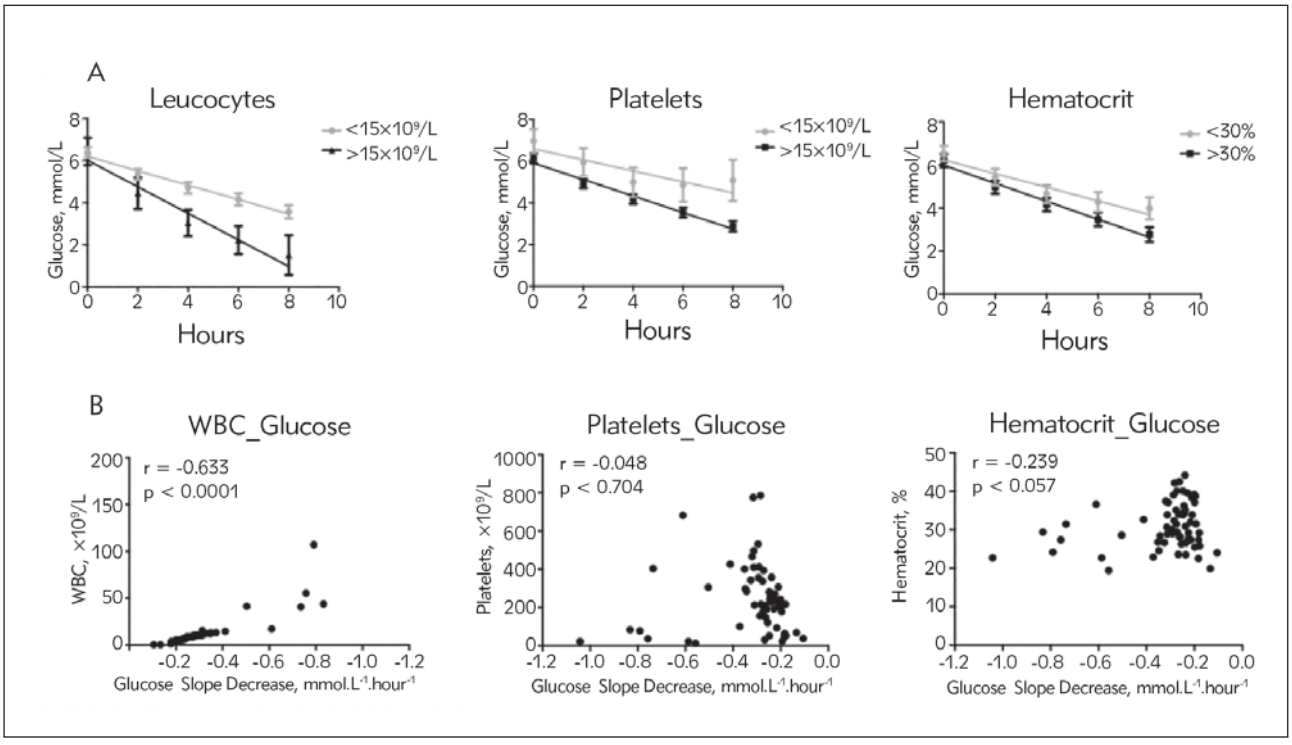

Figure 1 Glucose values (Mean +/– SEM) measured at 2 hours intervals in blood from patients with white blood cell (WBC) values < 15 × 10^9/L (gray lines) or > 15 × 109/L (black lines), with platelets <15 × 10^9/L (gray lines) or >15 × 10^9/L (black lines), and hematocrit < 30% (grey lines) and > 30% (black lines). B. Correlation between variation of glucose (mmol.L-1.hour-1) and WBC, platelets or hematocrit.

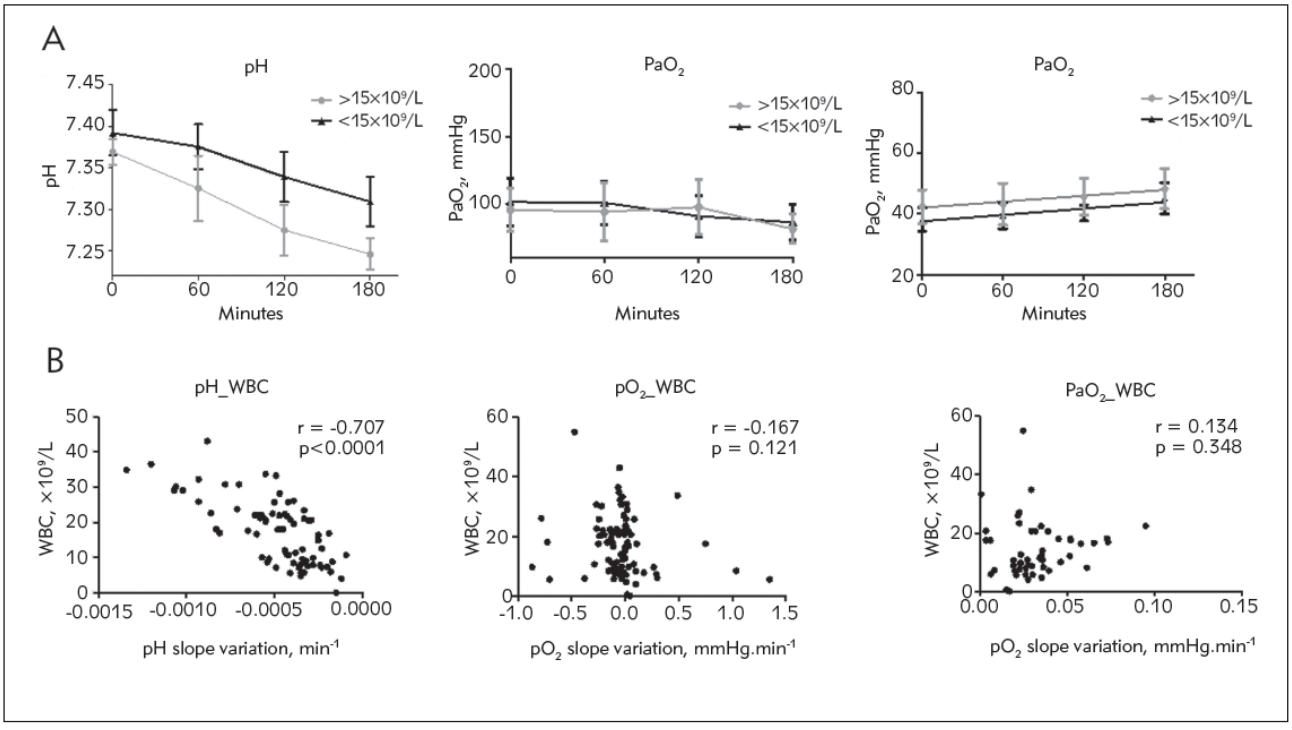

Figure 2 pH, PaO_2 and PCO_2 values (Mean +/– SEM) measured at 60 minutes intervals in whole blood collected from patients with white blood cell (WBC) values < 15 × 10^9/L (black lines) or >15 × 10^9/L (grey lines). B. Correlation between WBC count and variation of pH, pO_2 and pCO_2 in min^{-1}.

Table II Grouping of patients (n = 77) according to leukocytosis levels (threshold: 15 × 10^9/L) and measure of baseline pH, PaO_2, PCO_2 and White Blood Cells counts (Mean +/– SEM) measured in blood. Regression and correlation between time and each blood gas parameters (pH, pO_2 and pCO_2), p-value obtained by Pearson t-test.

		Low Leukocytes (<15×10^9/L) n = 32		High Leukocytes (>15×10^9/L) n = 45	
		Mean (SE)	Min-Max	Mean (SE)	Min-Max
pH t0		7.39 (0.12)	6.96–7.57	7.36 (0.08)	7.14–7.60
pO_2 t0 (mmHg)		101 (32)	33–333	96 (40)	33–302
pCO_2 t0 (mmHg)		36 (6)	22–78	46 (14)	18–94
White Blood Cells (×10^9/L)		8.4 (2.1)	0.07–14.92	24.3 (5)	16.4–43
Regression (pH)	Slope	-0.0004718		-0.0006996	
	r	-0.204		-0.430	
	p-value	0.028		<0.0001	
Regression (PaO_2)	Slope	-0.0907		-0.0658	
	r	-0.130		-0.110	
	p-value	0.125		0.243	
Regression (PCO_2)	Slope	0.035		0.032	
	r	0.243		0.172	
	p-value	0.018		0.115	

concentration did not significantly differ in each subgroup. No difference was noted when the analysis was carried out in subgroups of patients with different values of platelets and hematocrit (*Figure 1A*).

Interestingly, a strong association was found between the value of WBC and decrease of glucose values (r = –0.633, p < 0.0001), but not between glucose and platelet count (r = 0.048; p = 0.704) or hematocrit (r = 0.239; p = 0.057) (*Figure 1B*). Glucose variations over time (from baseline to 2, 4, 6 and 8 hours after blood draw) was significantly accelerated in samples with WBC count > 15x10^9/L (n = 10) as opposed to in those with WBC count < 15 × 10^9/L (n = 54).

Blood gases parameters

Overall, 77 blood gases samples were included in our analysis. The results, sorted according to WBC counts, are shown in *Table II*. No significant difference was found for baseline values of pH, pO_2 and pCO_2 between the two groups.

A larger decrease of pH values over time was observed in samples with WBC count > 15 × 10^9/L (n = 45) compared to those with WBC count < 15 × 10^9/L (n = 32) (*Table II* and *Figure 2A*). However, no difference in pO_2 and pCO_2 values were noted between these two groups (*Figure 2A*).

The variations of blood gases values (pH, pO_2 and pCO_2) over time (i.e., at baseline and 60, 120 and 180 min afterwards) were analyzed with Pearson's correlation, in which a significant association was found between time and pH in the two groups (r = –0.204; p = 0.0284 and r = –0.430; p < 0.0001), and pCO2 in the low leukocyte count cohort (r = 0.243; p = 0.018). On the contrary no difference was observed for pO_2 in both groups (r = –0.130; p = 0.126 and r = –0.110; p = 0.243), and pCO_2 in the high leukocyte cohort (r = 0.172; p = 0.116). Notably, a highly significant association was noted between WBC count and pH changes over time (r = –0.707; p < 0.0001), but not between WBC count and variation of both pO_2 (r = –0.167; p = 0.121) or pCO_2 (r = 0.134; p = 0.348) values over time (*Figure 2B*).

Discussion

The stability of laboratory parameters in blood samples is an important matter of concern, since spurious variations occurring before testing may have a profound impact on clinical decision making and care management, thus ultimately jeopardizing patient safety. An important aspect that emerged from our study is that plasma glucose undergoes a substantial, time-dependent consumption in plasma samples displayed in even modest leukocytosis (i.e., ~15×10^9/L). The maximum allowable time before sample centrifugation shall hence be reduced in these patients since glucose values would become otherwise unreliable. The use of glycolysis inhibitors might also be mandatory in all patients with leukocytosis. However, the complete effect of fluoride on gly-

colysis inhibition could take up to 4h according to leukocytes values, and concentration of glucose in the blood tube may meanwhile considerably decrease (17). Alternatively, a comparison with glucose results obtained with a glucometer may be advisable in case of suspicious results.

Unlike previous studies (18, 19), we failed to observe an association between WBC count and variation of pO_2 and pCO_2, while we noted a significant association between WBC values and pH, and this may be attributable to the fact that glucose metabolism by leukocytes may contribute to increased lactic acid production, thus generating acidosis.

In conclusion, the results of our study suggest that even modest leukocytosis (i.e., around 15 × 10^9/L), which is frequently encountered in clinical and laboratory practice, may impair the stability of glucose and pH in blood samples. This would lead us to conclude that the results of these parameters should be perhaps accompanied by those of hematologic testing, namely the leukocyte count, to prevent clinical misinterpretation. In case of suspicious biochemistry results associated with high leucocyte levels, verification could be performed short-circuiting pre-analytical transport using glucometer or point of care device.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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Original paper
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APPLICATION OF TARGETED NEXT GENERATION SEQUENCING FOR THE MUTATIONAL PROFILING OF PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

PRIMENA CILJANOG SEKVENCIRANJA NOVE GENERACIJE U ANALIZI MUTACIONOG PROFILA PACIJENATA SA AKUTNOM LIMFOBLASTNOM LEUKEMIJOM

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Summary

Background: Acute lymphoblastic leukemia (ALL) is the most common cancer in children, whereas it is less common in adults. Identification of cytogenetic aberrations and a small number of molecular abnormalities are still the most important risk and therapy stratification methods in clinical practice today. Next generation sequencing (NGS) technology provides a large amount of data contributing to elucidation of mutational landscape of childhood (cALL) and adult ALL (aALL).
Methods: We analyzed DNA samples from 34 cALL and aALL patients, using NGS targeted sequencing TruSeq Amplicon – Cancer Panel (TSACP) which targets mutational hotspots in 48 cancer related genes.
Results: We identified a total of 330 variants in the coding regions, out of which only 95 were potentially protein-changing. Observed in individual patients, detected mutations predominantly disrupted Ras/RTK pathway (*STK11*, *KIT*, *MET*, *NRAS*, *KRAS*, *PTEN*). Additionally, we identified

Kratak sadržaj

Uvod: Akutna limfoblastna leukemija (ALL) je najčešće maligno oboljenje kod dece, dok je kod odraslih njena učestalost mnogo niža. U današnjoj kliničkoj praksi kao najvažnije metode stratifikacije pacijenata u određene grupe rizika koriste se metode identifikacije citogenetičkih aberacija i malog broja molekulanih markera. Tehnologija sekvenciranja nove generacije (SNG) obezbeđuje veliku količinu podataka koji doprinose razjašnjavanju mutacionog profila dečje (dALL) i adultne ALL (aALL).
Metode: Uzorci DNK iz 34 dALL i aALL pacijenata analizirani su primenom SNG ciljanog sekvenciranja (»TruSeq Amplicon Cancer Panel – TSACP«) kojim se sekvenciraju »hotspot« mutacije u 48 gena povezanih sa kancerom.
Rezultati: Identifikovano je ukupno 330 varijanti u kodirajućim regionima, od kojih je samo 95 njih za posledicu imalo potencijalnu promenu u proteinu. Posmatrano kod pojedinačnih pacijenata, detektovane mutacije su pretežno remetile Ras/RTK signalni put (*STK11*, *KIT*, *MET*, *NRAS*,

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List of abbreviations: cALL, childhood acute lymphoblastic leukemia; aALL, adult acute lymphoblastic leukemia; NGS, next generation sequencing; NFM mutations, nonsense, frameshift and missense mutations.

5 patients with the same mutation in *HNF1A* gene, disrupting both Wnt and Notch signaling pathway. In two patients we detected variants in *NOTCH1* gene. *HNF1A* and *NOTCH1* variants were mutually exclusive, while genes involved in Ras/RTK pathway exhibit a tendency of mutation accumulation.

Conclusions: Our results showed that ALL contains low number of mutations, without significant differences between cALL and aALL (median per patient 2 and 3, respectively). Detected mutations affect few key signaling pathways, primarily Ras/RTK cascade. This study contributes to knowledge of ALL mutational landscape, leading to better understanding of molecular basis of this disease.

Keywords: acute lymphoblastic leukemia, next generation sequencing, somatic mutations

KRAS, *PTEN*). Pored toga, identifikovano je 5 pacijenata sa istom mutacijom u *HNF1A* genu, koja je uzrokovala poremećaje u Wnt i Notch signalnom putu. Kod dva pacijenta otkrivene su varijante u *NOTCH1* genu. Nije detektovano istovremeno prisustvo varijanti u *HNF1A* i *NOTCH1* genu, dok su geni uključeni u Ras/RTK signalni put pokazali tendenciju ka akumuliranju mutacija.

Zaključak: Naši rezultati pokazuju da ALL sadrži mali broj mutacija, bez značajnih razlika između dALL i aALL (medijana po pacijentu 2 odnosno 3). Detektovane mutacije izazivaju poremećaje u nekoliko ključnih signalnih puteva, prvenstveno Ras/RTK kaskade. Ova studija doprinosi ukupnom znanju o mutacionom profilu ALL, što vodi ka boljem razumijevanju molekularne osnove ovog oboljenja.

Ključne reči: akutna limfoblastna leukemija, sekvenciranje nove generacije, somatske mutacije

Introduction

Acute lymphoblastic leukemia (ALL) is a heterogenous malignant disorder resulting from the accumulation of aberrantly transformed B or T lymphoid progenitors at different developmental stages (1). ALL is the most common cancer in children, representing about 80% of acute leukemia, whereas it is less common in adults (20%). The heterogenity of this disease originates from various clinical, morphological and immunological phenotypes, but also from the fact that ALL is a genetically complex entity (2).

Contemporary approach to ALL treatment implies precise stratification into different risk groups that is primarily based on specific clinical and genetic characteristics. Significant advances have been made in the treatment of ALL with the cure rates for childhood ALL approaching 90%. Still, survival in the adult ALL population is only about 40% and decreases with age (3).

It has been noted that the frequency of recurring cytogenetic abnormalities present in both childhood and adult ALL, differ between these groups of patients. This difference may be the basis for the reported discrepancy in the survival rates in these age groups. Moreover, the frequency of high risk T-ALL, is much higher in adults than among children. Childhood ALL is in 85% of cases B-cell type, characterized by the presence of high hyperploid (>50 chromosomes) and t (12; 21) (p13; q22) i.e. *ETV6/RUNX1* rearrangement, both associated with favorable prognosis. Conversely, in adult leukemia, aberrations with poor prognostic significance, like the presence of hypodiploidy (30–39 chromosomes), translocations t (4; 11) (q21; q23) and t (9; 22) (q34; q11), i.e. *MLL/AFF1* and *BCR/ABL1* rearrangements, are much more frequent (4, 5).

Above-mentioned chromosomal rearrangements are common in ALL and are critical events in leukemogenesis. These, so called primary genetic events, usually affect lymphoid differentiation and proliferation processes, but for the induction of full-blown leukemia, multiple mutations are required. New technologies like next generation sequencing (NGS) offers great potential for variants identification and genomic profiling of ALL. Utilization of NGS has enabled detection of additional submicroscopic alterations in the genes involved in tumor suppression, apoptosis, and cell-cycle regulation, contributing to more comprehensive insight into leukemogenesis. And not only that, these new markers have been used in diagnosis, risk-stratification and targeted therapy application, leading to improvement of current protocols and patient management (6, 7).

In this study, we applied targeted next generation sequencing on MiSeq System for analyzing somatic mutations in groups of adult (aALL) and childhood (cALL) ALL patients, in order to facilitate recognition and better understanding of the genetic profile of the disease.

Materials and Methods

Subjects

Bone marrow samples from the 17 adult and 17 childhood ALL patients at diagnosis were collected. Adult ALL patients came from the Clinic of Hematology, Clinical Center of Serbia, and childhood patients came from the Department of Hematology, University Children Hospital in Belgrade. The study was approved by the Ethics Committee of the Clinical Center of Serbia. Research was conducted in accordance with the ethical standards of the World Medical Association's Declaration of Helsinki. Informed consent was obtained from each patient or patient's parent or guardian.

Mononuclear cells were separated by Ficoll density gradient centrifugation and cryopreserved until mutational analyses. Some clinical characteristics of the patients are listed in *Tables I* and *II*.

Table I Clinical characteristic of cALL patients.

Patient No.	Sex	Age	immunophenotype	karyotype	RT-PCR analysis
1	F	34	B-ALL (common)	NA	TEL/AML1
2	F	30	B-ALL (common)	46,XX [20]	neg
3	M	46	B-ALL (common)	NA	neg
4	F	18	B-ALL (common)	hyperdiploidy (51–55, XX)	neg
5	F	76	B-ALL (common)	hyperdiploidy (49–52, XX)	neg
6	M	97	B-ALL (common)	NA	TEL/AML1
7	F	29	B-ALL (common)	NA	neg
8	M	23	pre-B-ALL	46,XY [20]	neg
9	F	30	pre-B-ALL	46,XX [20]	BCR/ABL
10	M	61	B-ALL (common)	hyperdiploidy (55–60, XY)	neg
11	F	30	B-ALL (common)	46,XX [20]	TEL/AML1
12	F	125	B-ALL (common)	46,XX [20]	neg
13	F	77	B-ALL (common)	hyperdiploidy (47, XX)	TEL/AML1
14	M	63	B-ALL (common)	NA	neg
15	M	213	pre-B-ALL	NA	neg
16	M	145	pre-B-ALL	NA	TEL/AML1
17	M	126	B-ALL (common)	NA	NA

M – male, F – female; NA – not available

Table II Clinical characteristic of aALL patients.

Patient	Sex	Age (years)	immunophenotype	Karyotype	RT-PCR analysis
1	M	29	pro B-ALL	46,XY [20]	ND
2	F	26	B-ALL (common)	46,XX [6]	MLL/AFF1
3	M	37	B-ALL (common)	46,XY, t(4,11)(q21;q23))[2]/ 62–82,XY,t(4,11)(q21;q23) [18]	ND
4	M	44	B-ALL (common)	46,XY [20]	neg
5	M	19	B-ALL (common)	46XY [20]	neg
6	F	64	B-ALL (common)	46,XX [20]	neg
7	M	40	T-ALL	46,XY [13]	BCR/ABL
8	F	24	B-ALL (common)	46,XX,del(cq)[5]/46,XX,t(9;22)(q34;q11) [2]/46,XX [7]	BCR/ABL
9	F	45	B-ALL (common)	NA	BCR/ABL
10	M	33	B-ALL (common)	46,XY, t(8,22)(q24:q11) [20]	ND
11	F	19	B-ALL (common)	46 XX/46XX, -B, -C, +M1, +M2	ND
12	M	41	B-ALL (common)	46XX [20]	ND
13	M	28	B-ALL (common)	46XY[20]	ND
14	F	61	B-ALL (common)	46,XX [20]	BCR/ABL
15	M	43	B-ALL (common)	NA	ND
16	M	28	T-ALL	46, XY [20]	ND
17	F	35	B-ALL (common)	46, XX [20]	ND

M – male, F – female; NA – not available; ND – not done

TruSeq Amplicon – Cancer Panel library preparation and sequencing

TruSeq Amplicon – Cancer Panel, TSACP (Illumina Inc., San Diego, CA, USA) targets mutational hotspots in 48 cancer-related genes. TSACP consists of 212 amplicons captured by pairs of oligonucleotides designed to hybridize flanking targeted regions of interest. Genomic DNA from mononuclear cells was extracted using Qiagen Blood Mini kit (Heidelberg, Germany). The library preparation was performed using 250 ng of genomic DNA, according to the manufacturer's protocol. The equal volumes of normalized libraries were pooled and prepared for subsequent cluster generation and sequencing on the MiSeq system (Illumina Inc., San Diego, CA, USA). Paired-end sequencing was performed using the MiSeq Reagent Kit v3 (600-cycle) and the sequencing quality was demonstrated by the percentage of bases having the Q30 score (1 error in 1000 bases) of 97.2%.

Bioinformatics Analysis

After library sequencing, FASTQ files were processed in multiple stages starting with quality control and trimming, alignment and pre-processing, followed by additional quality control, variant calling and filtration. The bioinformatics pipeline was designed by Seven Bridges Genomics (SBG), containing open source bioinformatics tools as well as in-house developed tools. The basic quality control was performed with FastQC (8) and after that, low quality bases were trimmed of from read ends using FastqMcf (9). Then the production of BAM file(s) was done with BWA-MEM (10–12). This step involved the alignment of sequences with the reference genome GRCh37. The insertions/deletions (indel) realignment over the reads overlapping target regions was performed using RealignerTargetCreator and IndelRealigner (13, 14). SBG developed custom scripts that was used for additional quality control which consisted of counting reads of each amplicon and identifying amplicons presenting low read-coverage across all samples. The variant calling and filtration was done by UnifiedGenotyper and VariantFiltration tools (13, 14). Comparison between resulting sequence and the reference genome GRCh37 sequence was done using UnifiedGenotyper which applies a Bayesian approach and produces a VCF file containing single nucleotide variants (SNVs) and insertions/deletions (indel) variants. To filter out low quality variants from VCF file, Variant Filtration tool was used, and for variant annotation we used Ensembl Variant Effect Predictor (VEP) (15). At the final step, a report has been generated for each sample, containing all amplicones with the reading depth for each one, with present mutation (if called) both on DNA and protein level and dbSNP identifier. The Integrated Genomics Viewer was used for visual evaluation of the data (16).

Results

We have analyzed approximately 11.9×10^8 bp sequence from 34 ALL patients (17 cALL and 17 aALL) by targeted NGS using TSACP. The average coverage of high-quality sequences was 2609× per amplicon. Ten genes were discarded due to insufficient coverage, therefore a total of 183 amplicons from 38 genes was used for subsequent analysis. Variants were identified in relation to the GRCh37 reference genome by applying a Bayesian approach and compared to public genetic variation databases.

We identified 72 different variants across the samples in both coding and non-coding targeted regions, out of which 37 (21 in cALL, 22 in aALL) variants were in the coding regions and 35 (28 in cALL, 30 in aALL) outside of the targeted amplicons. Among the 37 different variants in the coding region, we found 3 different types of insertions/deletions (indels) in 3 cALL patients and in 2 aALL patients, while in the non-coding regions we found 9 different types of indels (in 9 cALL and in 8 aALL patients). We also identified 34 different single nucleotide variants-SNVs (in 18 cALL and in 20 aALL samples) in the coding and 26 different SNVs (in 19 cALL and in 22 aALL patients) in the non-coding regions.

Additionally, in all patients' samples, we identified a total of 330 (157 in cALL, 173 in aALL) variants (including synonymous variants) in the coding regions, (median per patient: 9, range: 6–12; median per cALL: 9, range: 6–12; median per aALL: 10, range: 7–12) (*Figure 1*). In the non-coding regions, we found 429 (211 cALL, 218 aALL) (median per patient: 13 range: 10–15; median per cALL: 13, range: 10–14; median per aALL: 13, range 10–15) (*Figure 1*).

Only mutations located within the coding regions were considered for further analysis, and from those only protein-changing mutations (nonsense, frameshift and missense (NFM) mutations). The total number NFM mutations was 95 (45 in cALL, 50 in aALL), median per patient 2, range: 1–7 (median per cALL: 2, range: 1–6; median per aALL: 3, range: 1–5). The majority of patients had no more than 3 NFM mutations, whereas only one cALL patient (#4) had 6 (*Figure 2*).

Our analysis revealed that 21 different genes had at least one NFM mutation in the coding regions (17 in cALL, 15 in aALL). Out of these, we identified variants in 6 cALL-specific genes (*CDKN2A*, *GNAQ*, *HRAS*, *PTPN11*, *AKT1*, and *ERBB2*) and 4 genes containing NFM mutations only in aALL patients (*NRAS*, *CSF1R*, *RET*, and *FLT3*). Mutations identified in the coding regions of following targeted genes: *KIT* (5 cases), *HNF1A*, *STK11* and *KRAS* were present in at least 4 cases (more than 10%), whereas substitution variants *KDR* Q472H and *TP53* P72R were detected in at least 18 cases (more than 50%). The

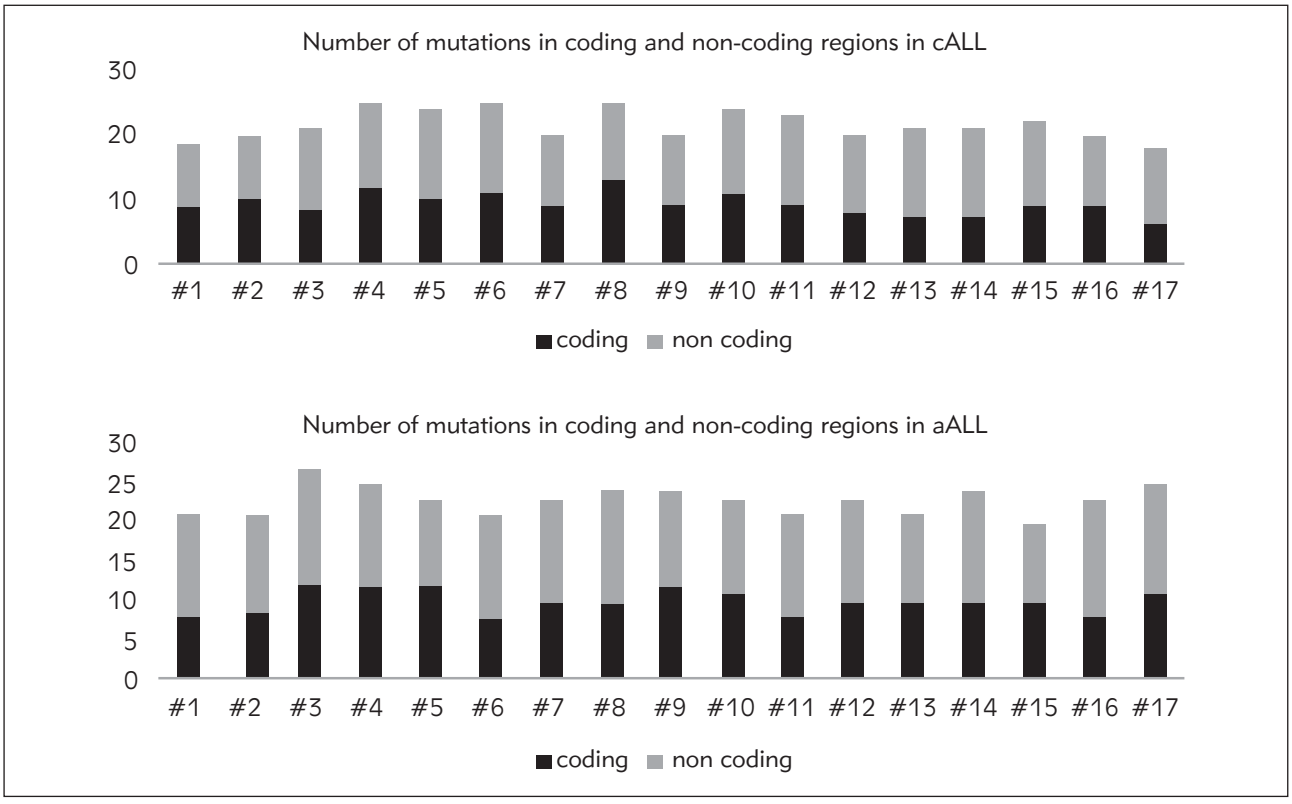

Figure 1 Total number of mutations in coding and non-coding regions identified by targeted NGS in cALL and aALL patients.

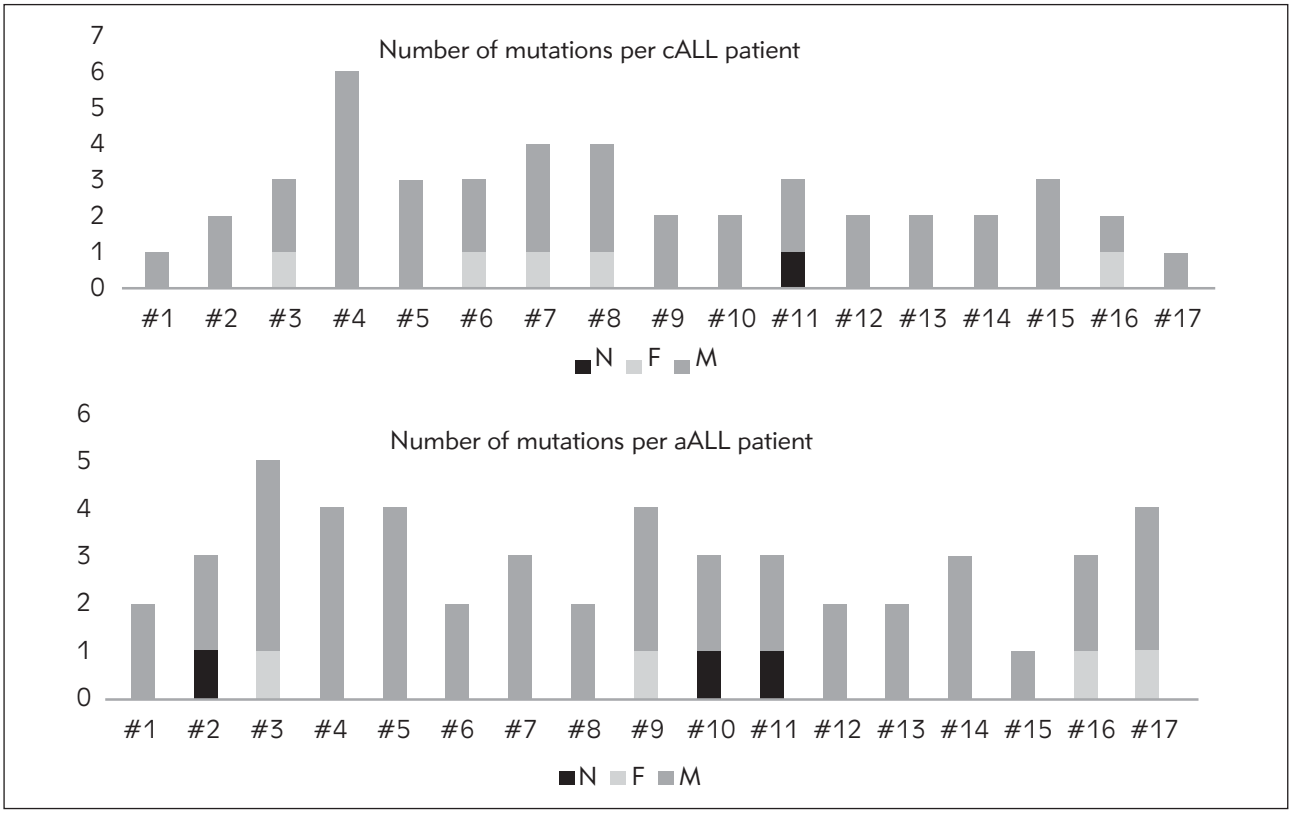

Figure 2 Distribution of nonsense (N), frameshift (F), and missense (M), mutations in the coding regions of targeted genes per cALL and aALL patient.

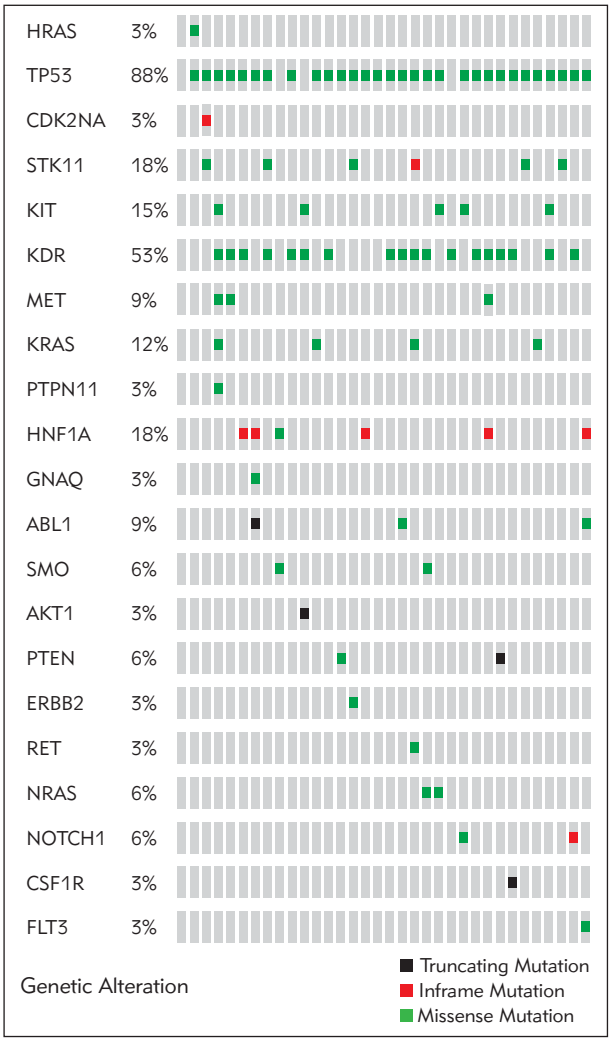

Figure 3 OncoPrint showing the distribution of genetic alterations in 38 targeted tumor suppressor and oncogenes in 34 ALL patients. The type of mutations are labeled in the color legend, particular genes in rows and tumor samples in columns.

list of the genes and their mutational types per patients are represented in OncoPrint (*Figure 3*).

In seven cALL patients and in eight aALL patients we detected 16 unreported NFM mutations in 10 genes (*Table III* and *Table IV*). The largest number of new mutations were detected in the *STK11* gene with four, followed by *ABL1* gene with three and *NOTCH1* gene with two mutations. These mutations were prevalently substitutions – missense type, but we also detected three nonsense and three frameshift truncating mutations.

Discussion

Acute lymphoblastic leukemia represents hematopoietic malignancy whose main feature is its clinical heterogeneity reflecting the heterogeneity that exists on the genetic level. As the development of full-blown leukemia implies a multistep process of gradual accumulation of genetic and epigenetic alterations, ALL represents a mixture of the sub clones, characterized by a special combination of the mutations (17). Each mutation, characterized as »driver« or «passenger« mutation, in its own way contributes to complete leukemic phenotype and clinical characteristics. In order to study such a complex nature of the disease next generation sequencing (NGS) methodology was used, enabling the detection of new somatic mutations that are contributing to the pathogenesis of ALL.

In this study, the application of TSACP cancer panel to analyze the mutational pattern of childhood and adult ALL samples we have analyzed the role of genes previously described primarily in solid tumors. Moreover, by applying targeted re-sequencing method we have achieved a high accuracy in variant detection, with an average coverage of 2609× per amplicon. High coverage is required for detection of somatic mutations in the samples with large number of sub clones, characteristic for hematological malignancies.

We have detected 95 potentially protein-changing variants, (45 in cALL and 50 in aALL patients). Our finding of low number of mutations in both cALL (median per patient 2, range 1–6) and aALL (median per patient 3, range 1–5) is in accordance with previous studies with reported frequency of 0–7 mutations per patient (18, 19). Moreover, in comparison to other types of both adult and childhood cancers, acute leukemias were described as low mutation rate cancers (20, 21). In particular, in many of our patients we were not able to detect any of the mutations, excluding common germline polymorphism in *TP53*, and in *KDR* gene (*Table III* and *Table IV*). Polymorphism P72R in *TP53* gene, characteristic for 70% of European population, was found in 32 patients, while eighteen out of 34 contained Q472H variant in *KDR* gene (22).

In this study, we have noticed that commonly mutated genes belong to Ras/RTK signaling pathway, which is in accordance with previously published data (18, 23). Deregulation of Ras signaling pathway is very common feature among all cancers, because activated RAS proteins affect multiple downstream pathways (Raf/MEK/ERK and PI3K/Akt), and thus deregulate many important cellular processes (24).

One of the main mechanism of Ras deregulation is through acquisition of oncogenic mutations in 3 *RAS* genes: *NRAS*, *KRAS*, and *HRAS* (25). In our study, we identified hotspot mutations in *HRAS* gene affecting Glycine at G48, in *KRAS* gene affecting Glycine at positions G12 and G13, and in *NRAS* gene the same amino-acid at position G12 and Alanine at A59. Identified mutations were mutually exclusive. This observation is in accordance with the traditional concept according to which only one

Table III Mutations identified in childhood ALL patients using NGS.

Sample No.	Mutation detected by MySeq	Mutation Status	dbSNP	COSMIC
1	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
2	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
	HRAS, c.142G>A, p.G48R	Heterozygous		COSM55555612
3	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
	STK11, c.1023G>T, p.L341F	Heterozygous	unreported	unreported
	CDKN2A, c.175_176insG, p.V59fs*61	Heterozygous		COSM13715
4	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
	KIT, c.1621A>C, p.M541L	Heterozygous	rs3822214	COSM28026
	MET, c.3029C>T, p.T1010I	Heterozygous	rs56391007	COSM707
	KRAS, c.35G>A, p.G12D	Heterozygous	rs121913529	COSM521
	PTPN11, c.205G>A, p.E69K	Heterozygous	rs397507511	COSM13013
5	MET, c.3029C>T, p.T1010I	Heterozygous	rs56391007	COSM707
	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
6	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
	HNF1A, c.864_865insC p.P292fs*25	Heterozygous		COSM4611384
7	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
	HNF1A, c.864_865insC p.P292fs*25	Heterozygous		COSM4611384
	GNAQ, c.842A>G, p.E281G	Heterozygous	unreported	unreported
	ABL1, c.754C>A, p.Q252*	Heterozygous	unreported	unreported
8	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
	STK11, c.769delG, pG257fs*28	Heterozygous	unreported	unreported
	STK11, c.802G>A, p.G268R	Heterozygous		COSM4559384
9	HNF1A, c.862G>T,p.G288W	Heterozygous	rs539507291	
	SMO, c.1916T>C, p.V639A	Heterozygous	unreported	unreported
10	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
11	KIT, c.1621A>C, p.M541L	Heterozygous	rs3822214	COSM28026
	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	AKT1, c.66G>A, p.22*stop	Heterozygous	unreported	unreported
12	KRAS, c.38G>A, p.G13D	Heterozygous	rs112445441	COSM532
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
13	KDR, c.1416A>T, p.Q472H	Homozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
14	PTEN, c.64G>A, p.D22N	Heterozygous	unreported	unreported
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
15	ERBB2, c.2341C>T,p.R811W	Heterozygous	unreported	unreported
	STK11, c.1087A>G, p.T363A	Heterozygous	unreported	unreported
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
16	HNF1A, c.864_865insC p.P292fs*25	Heterozygous		COSM4611384
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
17	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061

Table IV Mutations identified in adult ALL patients using NGS.

Sample No.	Mutation detected by MySeq	Mutation Status	dbSNP	COSMIC
1	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
2	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
	ABL1, c.782G>A, p.W261*stop	Heterozygous	unreported	unreported
3	KRAS, c.38G>A, p.G13D	Heterozygous	rs121913488	COSM532
	RET, c.2383A>T, p.S795C	Heterozygous	unreported	unreported
	STK11, c.769delG, p.G257fs*28	Heterozygous	unreported	unreported
	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
4	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
	SMO, c.1916T>C, p.V639A	Heterozygous	unreported	unreported
	NRAS, c.176C>A, p.A59D	Heterozygous		COSM253327
5	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
	NRAS, c.35G>A, p.G12D	Heterozygous	rs121913237	COSM564
	KIT, c.1621A>C, p.M541L	Heterozygous	rs3822214	COSM28026
	TP53, c.614A>T, p.Y205F	Heterozygous		COSM11351
6	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
7	KIT, c.1621A>C, p.M541L	Heterozygous	rs3822214	COSM28026
	NOTCH1, c.4690C>T, p.H1564Y	Heterozygous	unreported	unreported
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
8	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
9	MET, c.3029C>T, p.T1010I	Heterozygous	rs56391007	COSM707
	HNF1A, c.864_865insC	Heterozygous		COSM4611384
	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
10	PTEN, c.19G>T, p.E7*stop	Heterozygous		COSM5298
	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
11	CSF1R, c.2862C>A, p.C954*stop	Heterozygous	unreported	unreported
	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
12	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
	STK11,c.1046A>G, p.E349G	Heterozygous	unreported	unreported
13	KRAS, c.35G>T, p.G12V	Heterozygous	rs121913529	COSM520
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
14	KIT, c.1621A>C, p.M541L	Heterozygous	rs3822214	COSM28026
	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	TP53, c.215C>G, p.P72R	Homozygous	rs1042522	COSM250061
15	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
16	KDR, c.1416A>T, p.Q472H	Heterozygous	rs1870377	COSM149673
	NOTCH1,c.4729_4734delGTGGTG	Heterozygous	unreported	unreported
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
17	FLT3, c.2503G>T, p.D835Y	Heterozygous	rs121913488	COSM783
	HNF1A,c.864_865insC	Heterozygous		COSM4611384
	TP53, c.215C>G, p.P72R	Heterozygous	rs1042522	COSM250061
	ABL1, c.880A>G, p.K294E	Heterozygous	unreported	unreported

mutation in each pathway is sufficient for disease development. In one model of ALL genesis, it was suggested that G12D variant in *KRAS* is a first genetic event responsible for malignant transformation of hematopoietic stem cells (26). Still, the majority of studies have focused on *KRAS* and *NRAS* mutant forms and suggest that oncogenic RAS alone is insufficient to drive leukemogenesis and cooperating genetic events are necessary for full-blown leukemia. It was found that mutations affecting *RAS* gene family and entire Ras pathway as well, were associated with other aberrations (27). In our cohort of patients, it was the case in MLL1/AF4–driven leukemogenesis.

Deregulation of Ras/RTK signaling pathway can also occur due to constitutive activation of protein tyrosine kinase located upstream of RAS (25). Many studies described receptor tyrosine kinases as a key regulator of the process of hematopoiesis, as well as leukemogenesis (28). In our cohort of ALL patients, we have found mutations of missense type in receptor tyrosine kinase genes *FLT3* and *ERBB2* (at positions D835Y and R781W, respectively). Additionally, we identified variants in non-receptor tyrosine kinase *ABL1* gene including two stop gain mutations at position Q252* and W261*, as well as one substitution mutation K294E in one patient. All of these mutations are unreported, although mutations in *ABL1* gene have been previously associated with ALL occurrence or therapy resistance (7). We have also detected one mutation in *PTPN11* gene coding for non-receptor tyrosine phosphatase SHP2. SHP2 is a putative positive regulator of the Ras signaling pathway and mutations in *PTPN11* gene have been described as a «driver« mutations in B-ALL development (19).

Mutations in *HNF1A* gene, encoding transcriptional factor affect both Ras/RTK and Notch1 pathways. We have detected six patients with mutations in this gene; five frameshift mutations (P292fs*25), and 1 missense variant (G288W). In our study, these frameshift mutations were associated with the presence of *TEL/AML1* rearrangement in cALL, probably as a «second hit« mutation, as an additional genetic event required for development of full-blown leukemia (30, 31). Changes in the *HNF1A* gene are associated with liver/pancreatic tumors, not with hematological malignancies (32, 33).

Another gene whose mutations are not usually associated with hematological malignancies is *STK11* (*LKB1*) gene. *STK11* gene is encoding serine/threonine kinase protein, which has been involved in the cell cycle and apoptotic processes. It is assumed that this gene has the role of a classical tumor suppressor gene, because its loss-of-function somatic mutations lead to deactivation of the PI3K/Akt signaling pathway (34). Significant frequency of somatic mutations in *STK11* gene were reported only in lung and cervical tumors , while in other types of human cancers, the occurrence of these mutations is a sporadic event (35, 36). In our study, it was one of the most mutated one with one frameshift (G257fs*28) and four missense mutations (G268R, L341F, E349G, and T363A) found in 6 patients. All of the detected *STK11* mutations were previously unreported, and mutations in this gene are not specific for leukemias.

In *NOTCH1* gene, we identified one missense and one frameshift mutation in 2 T-ALL patients. Activating mutations in Notch1 signaling pathway have been described as a crucial factor in T-ALL development and their identification could lead to prognostic marker discovery and therapy improvement (37, 38). In five patients, 2 cALL and 3 aALL, we identified M541L mutation in *KIT* gene that encodes proto-oncogene receptor tyrosine kinase, while in another receptor tyrosine kinase coding proto-oncogene *MET*, we detected T1010I mutation that was present exclusively among cALL patients. *KIT* gene belongs to receptor tyrosine kinase gene family involved in hematopoietic stem cells (HSCs) self-renewal and differentiation, suggesting that any activating mutation in the receptor could alter hematopoietic development. Mammalian cells transformed with *KIT* gene that contained activating mutations exhibited increased growth both *in vitro* and *in vivo*, suggesting important role of this mutation in leukemogenesis (39). Pathogenic mutations in *KIT* tyrosine kinase gene have been reported in various diseases. One of the recently published results emphasizes the role of M541L variant in the therapy response in chronic eosinophilic leukemia patients (40).

In conclusion, by using targeted NGS method in studying the mutational landscape of our cohort of ALL patients, we have found that low number of mutations are implicated in the pathogenesis of this disease. The impact of the detected mutations is focused on few key signaling pathways, primarily on Ras/RTK cascade. Our findings provide additional information for mutational portrait of ALL and the results could be used as a supplement to classical therapy stratification methods. In that way, we would be able to apply therapeutics that target specific signaling pathways in each individual patient. It is possible that better outcome among ALL patients of all ages could finally be accomplished through such personalized treatment approach.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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URINARY NEPHRIN IS EARLIER, MORE SENSITIVE AND SPECIFIC MARKER OF DIABETIC NEPHROPATHY THAN MICROALBUMINURIA

URINARNI NEFRIN JE RANIJI, OSETLJIVIJI I SPECIFIČNIJI MARKER DIJABETESNE NEFROPATIJE NEGO MIKROALBUMINURIJA

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Summary

Background: Diabetic nephropathy (DN) is a leading cause of end-stage renal disease. Progressive damage and decline in the number of podocytes often occur in the early stages of DN. Thus, nephrin as a podocyte-specific protein may be regarded as a potential biomarker of early detection of DN. The aim of this study is to determine whether urinary nephrin is an earlier marker in DN than microalbuminuria and to test the significance of urinary nephrin as a marker for early detection of DN.
Methods: Our cross-sectional study included 90 patients with type 2 diabetes mellitus (T2DM), 30 patients with diagnosed DN and 60 patients without diagnosed DN. As a control group, we used 30 healthy subjects. All patients with T2DM were classified into three subgroups according to urinary microalbumin/creatinine ratio (UMCR): normoalbuminuric, microalbuminuric and macroalbuminuric patients. Nephrin in urine was measured by immunoenzyme assay, microalbumin with turbidimetric and creatinine with the photometric method. In blood sera, we measured a few standard biochemical parameters.
Results: Nephrinuria was found to be present in 100% of patients with T2DM and macroalbuminuria, in 88% with microalbuminuria, as well as 82% of patients with T2DM and normoalbuminuria. A concentration of urinary nephrin was significantly increased in all groups of subjects with T2DM compared to the control group ($p<0.05$).

Kratak sadržaj

Uvod: Dijabetesna nefropatija (DN) je vodeći uzrok završnog stadijuma bubrežne bolesti. Progresivno oštećenje i opadanje broja podocita često se javlja u ranim fazama DN, tako da se nefrin kao specifični protein podocita može smatrati potencijalnim biomarkerom ranog otkrivanja DN. Cilj ove studije je da utvrdi da li je urinarni nefrin raniji marker DN u odnosu na mikroalbuminuriju i da testira značaj urinarnog nefrina kao markera za rano otkrivanje DN.
Metode: Naša studija preseka je obuhvatila 90 pacijenata sa dijabetesom tipa 2 (T2DM), 30 bolesnika sa dijagnozom DN i 60 pacijenata bez dijagnoze DN. Za kontrolnu grupu smo koristili 30 zdravih ispitanika. Svi bolesnici sa T2DM klasifikovani su u tri podgrupe prema odnosu urinarnog mikroalbumina/kreatinina (UMCR): normoalbuminurni, mikroalbuminurni i makroalbuminurni pacijenti. Nefrin u urinu je meren imunoenzimskim testom, mikroalbumin uz korišćenje turbidimetrijske i kreatinin koristeći fotometrijsku metodu. U krvnim serumima, merili smo nekoliko standardnih biohemijskih parametara.
Rezultati: Ustanovljeno je da je nefrinurija prisutna u 100% pacijenata sa T2DM i makroalbuminurijom, u 88% sa mikroalbuminurijom, kao i 82% pacijenata sa T2DM i normoalbuminurijom. Koncentracija urinarnog nefrina značajno je povećana u svim grupama ispitanika sa T2DM u poređenju sa kontrolnom grupom ($p < 0,05$). Nefrinurija je korelirala statistički negativno sa eGFR ($r = -0,54$). ROC

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Nephrinuria correlated statistically negative with eGFR (r=-0.54). ROC analysis showed that nephrin has a total predicted probability of 96% in patients with DN.
Conclusions: Urinary nephrin is earlier, more specific and sensitive marker than microalbumin in early detection of DN.

Keywords: nephrin, microalbumin, diabetic nephropathy

analiza je pokazala da je kod pacijenata sa DN ukupna predviđena verovatnoća nefrina 96%.
Zaključak: Urinarni nefrin je raniji, specifičniji i osjetljiviji marker od mikroalbumina u ranom otkrivanju DN.

Ključne reči: nefrin, mikroalbumin, dijabetesna nefropatija

Introduction

Diabetic nephropathy (DN) is a clinical syndrome characterized by the following: persistent albuminuria (>300 mg/d) that is confirmed on at least 2 occasions 3–6 months apart, progressive decline in GFR and elevated arterial blood pressure. It is the most common complication in patients with type 2 diabetes mellitus (T2DM). Almost 40% of diabetic patients develop DN, but the incidence is much larger in new onset cases of T2DM. DN is a leading cause of the end-stage renal disease (ESRD), which require renal replacement therapies, dialysis or transplantation. These therapies have a great economic impact – an economic loss to people with diabetes and their families, national economies and health systems through direct medical costs and loss of work and wages (1–4). Thus, early detection of renal involvement in patients with T2DM is important for timely treatment and to slow the disease progression to ESRD. Microalbuminuria was previously considered s a gold standard in early detection of DN despite being a non-specific marker, concomitantly present in other pathological conditions such as urinary tract infections, cardiovascular disease, in non-diabetic patients, etc. In addition, the body of evidence indicates a high percentage of patients with T2DM, who have renal involvement despite the absence of microalbuminuria (5, 6). Studies have shown a progressive decline in the number of podocytes and disappearance of foot processes which occur in the early stages of DN due to apoptosis or shedding of podocytes. Therefore, urinary podocytes and their specific proteins may be regarded as potential biomarkers of podocyte injury. These facts motivated us to investigate novel biomarkers for early detection of DN. Currently, new and more specific markers for early detection and prediction of DN that appear in the urine before microalbuminuria are being evaluated, and the studies are generally focused on the podocyte-specific protein products because it is difficult to detect urinary podocytes directly (7–10). Recently particular attention has been paid to the role of specific podocyte proteins such as nephrin, podocalyxin, synaptopodin, podocin, mindin, etc., in early detection of DN. Among these new urinary biomarkers, the podocyte protein – nephrin has been evaluated as the most promising one in early detection of DN (11).

Podocytes, visceral epithelial cells, are key structural elements of the glomerular filtration barrier (12). Nephrin is a transmembrane glycoprotein which plays an essential role in the structure of the filtration diaphragm and provides the ultimate physical barrier for plasma proteins. Decreased number of podocytes at a glomerular level and presence of podocytes in urine – podocyturia were reported in DN (13–15). The loss of podocytes leads to glomerulosclerosis and together with the damage of the podocyte's cytoskeleton and filtration diaphragm lead to proteinuria (16–18). Damage of podocyte cells and their shedding result into the presence of podocyte-specific proteins in urine, such as nephrin, rendering it as an attractive marker for non-invasive early diagnosis or prediction of DN.

Our study aimed to: 1) determine whether urinary nephrin is an earlier marker in DN than microalbuminuria by classification of all diabetic patients into three subgroups according to UMCR and comparison of concentration of urinary nephrin between subgroups of patients and healthy subjects and 2) test the diagnostic significance of nephrinuria in early detection of DN by comparison of concentration of urinary nephrin among selected groups and among classified subgroups of patients and healthy subjects, by correlation among urinary nephrin concentration and eGFR and testing the predicted probability of urinary nephrin in patients with DN.

Materials and Methods

Subjects

This cross-sectional study included 90 patients with T2DM. We selected two groups of patients with T2DM: patients with T2DM with diagnosed DN (n=30) and patients with T2DM without diagnosed DN (n=60). Healthy subjects (n=30) were used as a control group. Patients with diagnosed DN were recruited from the University Department of Nephrology at the Medical Faculty in Skopje. Inclusion criteria for patients with nephropathy were the presence of T2DM with clinically diagnosed nephropathy – defined by macroalbuminuria or macroalbuminuria and abnormal renal function, represented by an abnormality in serum creatinine and glomerular filtration rate (GFR). We used this group of patients to determine whether urinary nephrin is elevated in all patients with diagnosed DN and to test the diagnostic performance of nephrin in patients with DN. Diabetic patients without DN were recruited from the Primary Health Care Offices. Inclusion criteria for patients with T2DM with-

out nephropathy were: duration of diabetic disease for no more than 5 years (generally new-onset cases), absence of pregnancy in women and absence of any type of renal disease.

All patients with T2DM were diagnosed in the Centre of Diabetes at the University Department of Endocrinology and Metabolic Diseases.

All subjects with T2DM (n=90) were classified into three subgroups depending on UMCR: patients with normoalbuminuria – UMCR <30 mg/g (n=56), microalbuminuria – UMCR 30–300 mg/g (n=25) and patients with macroalbuminuria – UMCR >300 mg/g (n=9). Classification was done according to KDIGO – Kidney Disease: Improving Global Outcomes – the giudelines from 2012 (19).

Samples and methods

We used the first midstream morning urine and blood sera. Urine (10 mL) was collected in clean plastic containers, without any preservative. After centrifugation of blood at 3.000 rpm for 10 min, serum was collected in 1.5 mL Eppendorf tubes. Firstly, a chemical analysis of the fresh urine was done by using urinary dipsticks. Then, microalbumin concentration in urine was measured by a turbidimetric method and creatinine by using the Jaffe reaction on biochemical analyser ChemWell (2910 Awareness Technology, Inc.). The rest of the urine samples after centrifugation were stored at -80 °C until quantification of the nephrin.

Microalbumin/creatinine ratio was determined by using the mathematical formula from the assessed concentration of microalbumin and creatinine in urine. The glomerular filtration rate (GFR) was calculated by Cocroft and Gault formula (20).

The concentration of nephrin in urine was determined using commercially available kits (Exocell Inc., Philadelphia, PA). The method is an indirect competitive ELISA with polyclonal antibodies used against nephrin. Antigens – nephrin from a urine sample and immobilized nephrin antigens (at the bottom of polystyrene plates) compete for anti-nephrin rabbit antibodies. Antirabbit HRP (HRP-horseradish peroxidase) conjugate is used for detection of bound antibodies. After rinsing, the remaining concentration of antibody conjugate bound to immobilized nephrin antigens is measured photometrically at 450 nm wavelength. The colour intensity is inversely proportional to the concentration of nephrin in the urine sample. The concentration of nephrin was read from a standard curve constructed on commercial standards. The results are presented in ng/mL.

In blood sera, we measured the concentrations of the following parameters: urea, creatinine, glucose, total protein, and albumin. All parameters were measured by photometric method on biochemical analyser ChemWell. Data on age, sex, height, weight, duration of the disease, blood pressure and glycaemic control were collected from the patients' records and completed questionnaires.

All analyses were performed at the Institute of Medical and Experimental Biochemistry, Medical Faculty in Skopje. Informed written consents were obtained from all participants. The study was approved by the Ethical Committee of the Medical Faculty in Skopje, Macedonia.

Statistical analysis

Statistical analyses were performed by using the Statistical package SPSS and R. We perform statistical analysis by using: Kolmogorov-Smirnov test, Mann-Whitney U test, ONE-way analysis of variance (ANOVA), Kruskal-Wallis test, Spearmanžs rank-order correlation and binary logistic regression with Receiver Operating Characteristic (ROC) curve. Statistical significance was defined as $p<0.05$.

Results

Clinical data of subjects

Clinical characteristics of the subgroups of patients with T2DM and healthy subjects are shown in *Table I* and *II*.

The concentration of urinary nephrin in all patients with T2DM and healthy subjects

We performed a comparison of concentration of urinary nephrin between all patients with T2DM and healthy subjects by Mann-Whitney test. The results are shown in *Figure 1*.

Reference values of urinary nephrin

Reference values of urinary nephrin were calculated by using the distribution of frequencies of urinary nephrin in healthy subjects. Considering the nonparametric distribution of urinary nephrin in healthy subjects, reference values were calculated by using the interquartile range (IQR) formula: 50 percentiles + (75 percentiles - 25 percentiles 1.5), respectively we calculate 136+119=255 ng/mL (255 ng/mL). Hence, pathological values for urinary nephrin are 255 ng/mL. Data for urinary nephrin in healthy subjects are shown in *Table III*.

We determined the percentile of subjects with elevated urinary nephrin (255 ng/mL) in subgroups of subjects according to UMCR, and we found that nephrin was elevated in 100% of subjects with macroalbuminuria, 88% of subjects with microalbuminuria and it is particularly high (82%) in subjects with normoalbuminuria. Only 10% of healthy subjects have had elevated urinary nephrin. The results are shown in *Figure 2*.

Table I Clinical characteristics of subgroups of patients with T2DM classified according to UMCR and healthy subjects.

	Macroalbuminuria	Microalbuminuria	Normoalbuminuria	Healthy subjects	Kruskal – Wallisp-value
Age (years)	59.1±10.6	57.2±8.2	57.4±6.9	47.8±9.3	0.005
Duration of disease (years)	13±6.7	8.5±6.5	4.8±4.2	/	<0.05
BMI (kg/m^2)	29.5±5.6	28.8±3.6	28.7±4.0	25.6±3.8	<0.05
Glucose (mmol/L)	9.9±3.9	8.1±2.9	6.8±2.4	4.2±1.1	<10^{-3}
UMCR (mg/g)	437.8±170.8	89.6±57.9	12.1±6.9	15.1±15.4	<10^{-3}
HbA1c (%)	7.9±0.9	7.4±1.6	6.9±1.1	4.7±0.4	0.025
Total proteins (g/L)	72.1±8.1	64.1±11.4	62.4±10.5	73.7±5.8	<10^{-3}
Albumin (g/L)	39±4.3	36.2±9.2	37.5±6.5	46.4±2.9	<10^{-3}
Urea (mmol/L)	7.9±2.1	8.4±5.4	6.7±1.9	4.3±1.1	<10^{-3}
Creatinine (mmol/L)	109.5±7.5	81.5±15.1	75.9±13	75.1±14.6	<10^{-3}
eGFR (ml min^{-1} 1.73 m^{-2})	48.2±12.5	65.±19.6	67.5±12.5	91.3±5.9	<10^{-3}
Nephrin in urine (ng/mL)	1086.5±550	983.3±1182.5	444.6±237.6	160.5±58	<10^{-3}

Results are shown as mean ± SD. BMI – body mass index, HbA1c – glycated haemoglobin A1c, eGFR – (estimated Glomerular Filtration Rate).

Table II Clinical characteristics of patients with T2DM with and without diagnosed DN and healthy subjects.

	Diabetic patients with diagnosed nephropathy	Diabetic patients without diagnosed nephropathy	Healthy subjects	Kruskal -Wallis p-value
Age (years)	55.8±10.1	57.9±6.7	47.8±9.3	<10^{-3}
Duration of disease (years)	10.7±6.9	4.9±4.5	/	<10^{-3}
BMI (kg/m^2)	28.8±4.2	28.7±4.1	25.6±3.8	0.002
Glucose (mmol/L)	9.3±3.6	6.8±2.3	4.2±1.1	<10^{-3}
UMCR (mg/g)	209.5±206.1	16.9±18.9	15.1±15.4	<10^{-3}
HbA1c (%)	7.7±1.6	6.9±1.1	4.7±0.5	0.020
Total proteins (g/L)	69.5±9.9	61.3±10.8	73.7±5.8	<10^{-3}
Albumin (g/L)	38.3±9.3	36.6±6.3	46.4±2.8	<10^{-3}
Urea (mmol/L)	8.3±5.3	6.7±2.1	4.3±1.1	<10^{-3}
Creatinine (mmol/L)	94.1±18.1	74.9±12.1	75.1±14.6	<10^{-3}
eGFR (ml min^{-1} 1.73 m^{-2})	61.1±21.8	67.1±12.5	91.3±5.9	<10^{-3}
Nephrin in urine (ng/mL)	1043.3±889.5	418.7±233.1	160.5±58	<10^{-3}

Results are shown as mean ± SD. BMI – body mass index, HbA1c – glycated hemoglobin A1c, eGFR – (estimated Glomerular Filtration Rate).

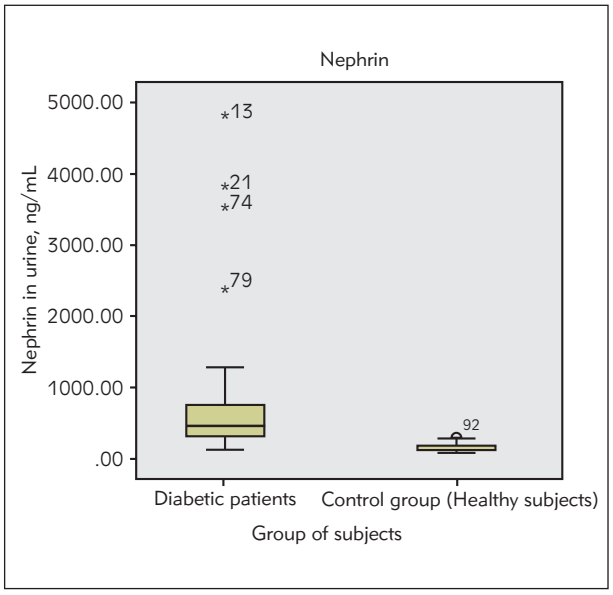

Figure 1 The concentration of urinary nephrin in all patients with T2DM and healthy subjects.

Table III The concentration of urinary nephrin in healthy subjects.

Nephrin (ng/mL)	n	$\bar{x}$	SD	Min.	Max.	Median	Percentile		
							25	50	75
Healthy subjects	30	160	58	94	315	136	110	136	189

The concentration of urinary nephrin in selected groups and classified subgroups of subjects with T2DM

By using Kruskal-Wallis test, we compared the levels of urinary nephrin between the subgroups of subjects classified according to UMCR and healthy subjects. The results show that the elevation of the level of urinary nephrin is statistically significant in all subgroups of subjects with T2DM compared to healthy subjects ($p<0.05$). Results are shown in *Figure 3*. We compared the levels of urinary nephrin between the selected groups of subjects with and

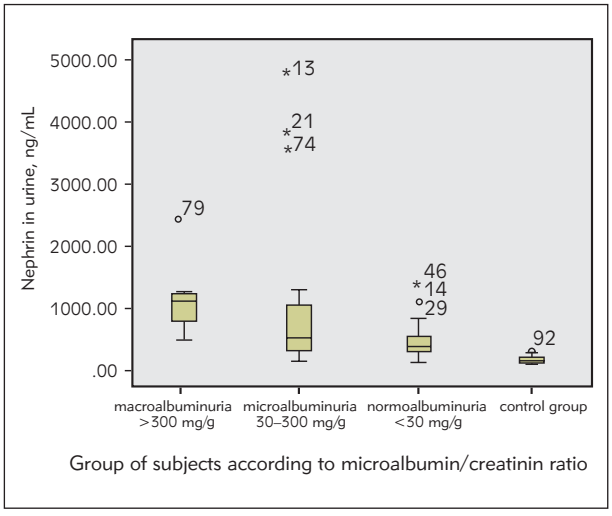

Figure 3 The concentration of urinary nephrin in subgroups of subjects classified according to UMCR.

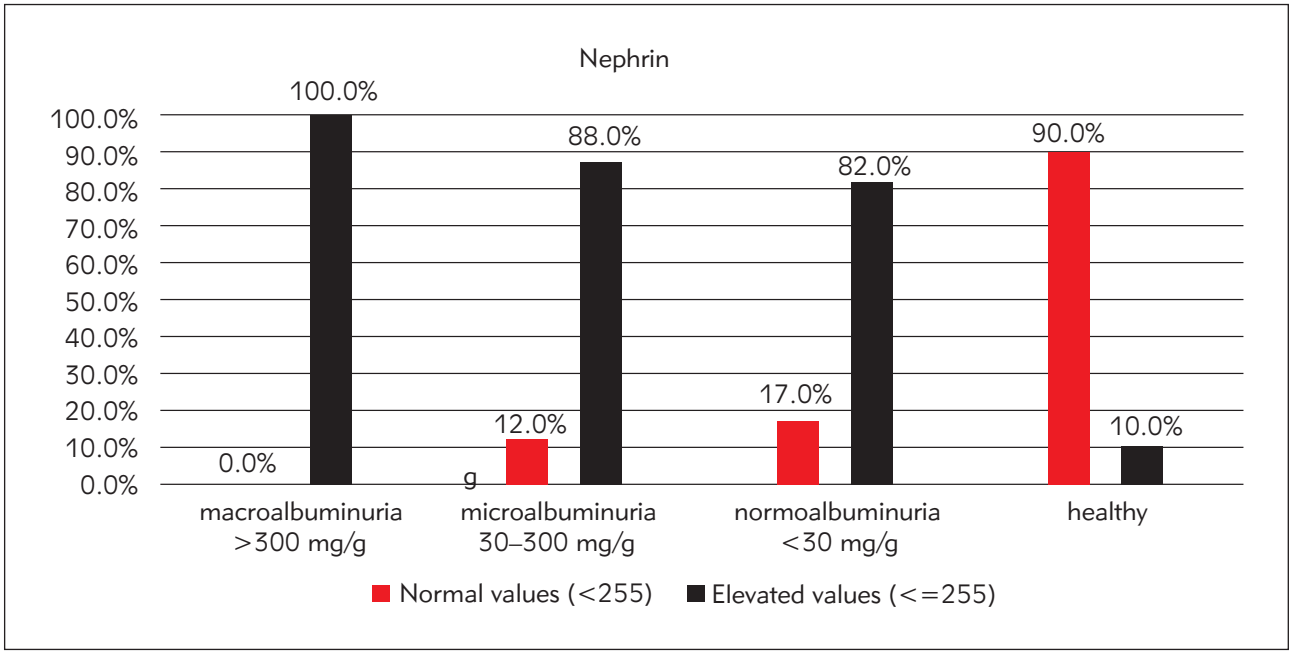

Figure 2 The percentage of subjects with elevated urinary nephrin in subgroups of patients classified according to UMCR.

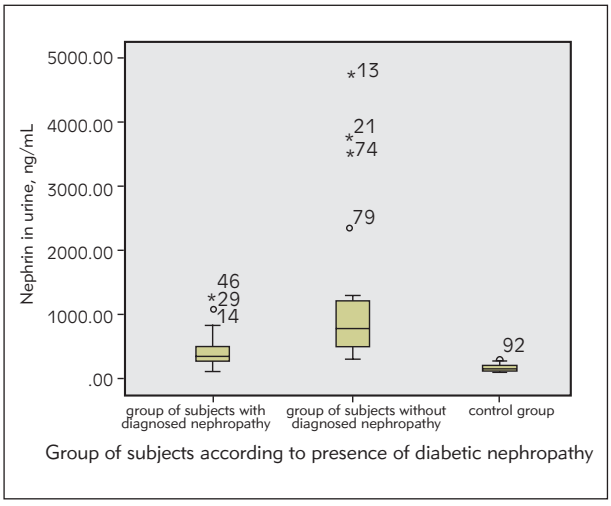

Figure 4 The concentration of urinary nephrin in selected groups of patients – with and without diagnosed DN.

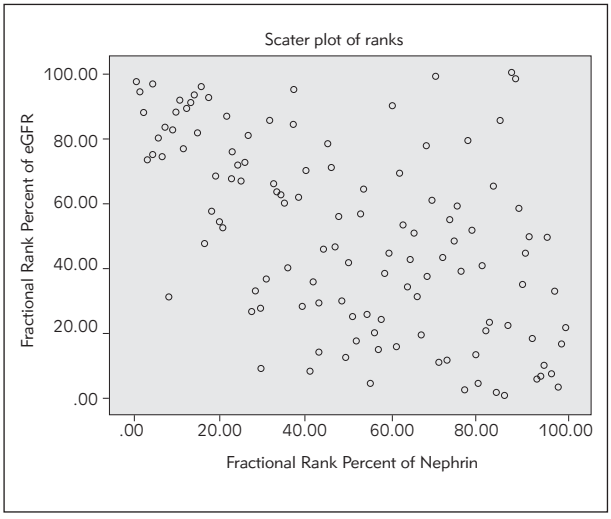

Figure 5 Spearman's correlation rank between urinary nephrin and eGFR.

without diagnosed diabetic nephropathy and healthy subjects. The results show that the level of urinary nephrin in both selected groups of patients with T2DM is statistically significantly higher compared to healthy subjects ($p<0.05$). The results are shown in *Figure 4*.

Correlation between urinary nephrin and eGFR

We ordered correlation rank between the concentration of urinary nephrin and estimated glomerular filtration rate in all subjects with T2DM by Spearman's method. Correlation is negative and statistically significant (Spearman $r=-0.54$, $p<10^{-3}$).

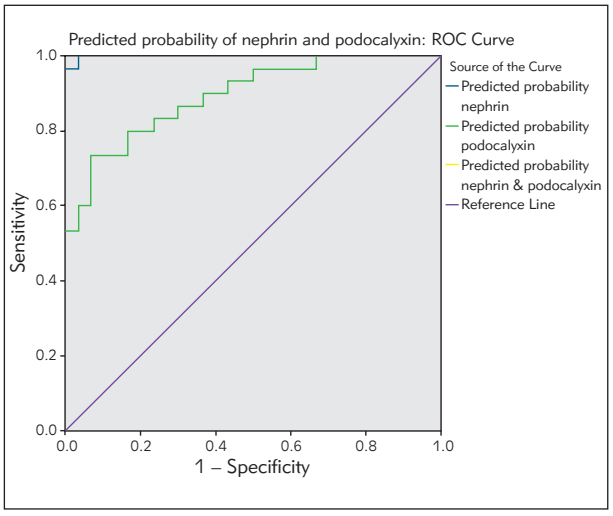

Figure 6 ROC analysis of urinary nephrin.

Non-parametric ROC analysis

We used ROC analysis to assess the diagnostic efficiency of urinary nephrin in discrimination between patients with DN and healthy subjects. We performed binary logistic regression, and we found that nephrin has an accuracy of 96% as a discriminator between patients with DN and healthy subjects.

Discussion

Microalbuminuria was previously considered as a gold standard for early detection of DN. Body of evidence also indicates that microalbumin in urine is a nonspecific and nonsensitive marker for early detection of DN, it is elevated in other pathological conditions such as urinary tract infections, acute illness, cardiovascular diseases (21, 23). Presence of microalbumin in urine suggests damage of all three components of the glomerular filtration barrier (endothelium, glomerular basement membrane, and podocytes), and its diagnostic accuracy is limited by the fact that structural damage might precede microalbumin excretion (24). The presence of nephrin and other podocyte-specific proteins in urine indicate only damage of podocytes, independently of the other two components of the glomerular filtration barrier (16). Thus, it is thought that podocyte damage is present before the appearance of microalbuminuria and proteinuria, hence, podocyte proteins such as nephrin are considered as earlier and more specific markers for diagnosis of DN compared to microalbuminuria.

In our study, we used the ELISA method for the quantification of urinary nephrin. We detected statistically significantly elevated concentrations of urinary nephrin in patients with T2DM compared to healthy subjects. It is particularly important that we found a statistically significant difference in the concentration of urinary nephrin in normoalbuminuric subjects with

T2DM compared to healthy subjects. These results indicate that damage of podocytes is present in subjects with T2DM before the appearance of microalbuminuria. It is important to highlight that we found an elevated concentration of urinary nephrin in 82% of normoalbuminuric subjects. The percent of subjects with elevated urinary nephrin in the subgroup with microalbuminuria was 88%, while in the subgroup with macroalbuminuria was 100 %. Our findings are similar to those published by Jim et al. (18) who detected that 54% of normoalbuminuric patients had increased nephrin and all (100%) patients with macroalbuminuria and microalbuminuria had elevated nephrin.

The level of urinary nephrin correlated negatively with GFR, indicating that nephrinuria is a marker of disordered renal function. In the study of Ng DPK, the same correlation was found, although a different method for measurement of nephrin and GFR was used (25). A significant negative correlation between urinary nephrin and GFR was also found in the study of Jim et al. (18).

ROC analysis showed that nephrin has a total predicted probability of 96% in subjects with DN. This means high discriminatory power between healthy subjects and patients with DN; also this result means high sensitivity and specificity of nephrin as a urinary biomarker in early detection of DN. In the literature, we do not find data on the predicted probability of nephrin.

The limitations of the study are the small sample size and cross-sectional nature of the study, and we do not know if nephrinuria is part of a causal mechanism, or early nephrinuria will consistently predict subsequent DN. To answer these questions, we need to measure urinary nephrin prospectively in diabetic patients with normoalbuminuria. A large, prospective study is recommended to further assess the value of this marker in early detection of DN. If further research confirms that nephrinuria is a marker of preclinical DN, microalbumin as a previous gold standard will be replaced by urinary nephrin in routine laboratory practice as a marker for early detection of DN.

In conclusion, the results of this study indicate that urinary nephrin could be very important in early detection of DN, due to: high percent of normoalbuminuric subjects with elevated levels of urinary nephrin, statistically significant difference in the levels of urinary nephrin among subgroups of patients and healthy subjects and among selected groups of patients and healthy subjects, negative correlation between urinary nephrin concentration and GFR and high diagnostic sensitivity and specificity of nephrin in patients with DN. Furthermore, these results indicate that nephrin has greater diagnostic value in early detection of DN compared to microalbumin.

It could be concluded that urinary nephrin is an earlier marker than microalbumin and at the same time more specific and sensitive marker for detection of DN in an early stage.

Acknowledgments. None declared.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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NLRP3 AND *CARD8* POLYMORPHISMS INFLUENCE RISK FOR ASBESTOS-RELATED DISEASES

UTICAJ *NLRP3* I *CARD8* POLIMORFIZAMA NA BOLESTI U VEZI SA AZBESTOM

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Summary

Background: This study aimed to investigate the association between *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms and the risk of developing pleural plaques, asbestosis, and malignant mesothelioma (MM), and to study the influence of the interactions between polymorphisms and asbestos exposure on the risk of developing these diseases.
Methods: The case-control study included 416 subjects with pleural plaques, 160 patients with asbestosis, 154 subjects with MM and 149 subjects with no asbestos disease. The *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms were determined using real-time PCR-based methods. In the statistical analysis, standard descriptive statistics was followed by univariate and multivariate logistic regression modelling.
Results: Asbestos exposure (medium and high vs low) was associated with the risk for each studied asbestos-related disease. An increased risk of pleural plaques was found for *CARD8* rs2043211 AT + TT genotypes (OR = 1.48, 95% CI 1.01–2.16, p = 0.042). When the analysis was performed for MM patients as cases, and pleural plaques patients as controls, a decreased MM risk was observed for carriers of *CARD8* rs2043211 TT genotype (OR = 0.52, 95% CI 0.27–1.00, p = 0.049). The interactions between *NLRP3* rs35829419 and *CARD8* rs2043211 genotypes did not influence the risk of any asbestos-related disease. However, when testing interactions with asbestos exposure,

Kratak sadržaj

Uvod: Cilj ovog istraživanja bio je da se ispita povezanost između polimorfizama *NLRP3* rs35829419 i *CARD8* rs2043211 i rizika od razvoja pleuralnih plakova, azbestoze i malignog mezotelioma (MM) i da se prouči uticaj interakcija između polimorfizama i izloženosti azbestu na rizik od razvoja ovih bolesti.
Metode: Ova studija slučaja je uključivala 416 ispitanika sa pleuralnim plakovima, 160 pacijenata sa azbestozom, 154 ispitanika sa MM i 149 ispitanika bez azbestne bolesti. Polimorfizmi *NLRP3* rs35829419 i *CARD8* rs2043211 su određivani pomoću metoda zasnovanih na PCR u realnom vremenu. U statističkoj analizi, standardnu deskriptivnu statistiku pratilo je univarijantno i multivarijantno logističko regresiono modeliranje.
Rezultati: Izloženost azbestu (srednja i visoka u odnosu na nisku) bila je povezana sa rizikom za svaku proučavanu bolest povezanu sa azbestom. Povećan rizik od pleuralnih plakova je ustanovljen za *CARD8* rs2043211 AT + TT genotipove (OR = 1,48, 95% CI 1,01–2,16, p = 0,042). Kada je obavljena analiza za pacijente sa MM, kao i za pacijente sa pleuralnim plakovima kao kontrolne slučajeve, primećen je smanjeni MM rizik za nosioce *CARD8* rs2043211 TT genotipa (OR = 0,52, 95% CI 0,27–1,00, p = 0,049). Interakcije između genotipova *NLRP3* rs35829419 i *CARD8* rs2043211 nisu uticale na rizik od bilo koje bolesti povezane sa azbestom. Međutim, kada su testirane interakcije sa izloženošću azbestu, ustanovljen je smanjen rizik

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List of abbreviations: CI, confidence interval; MM, malignant mesothelioma; OR, odds ratio; ROS, reactive oxygen species; RNS, reactive nitric species; SNP, single nucleotide polymorphism.

a decreased risk of asbestosis was found for *NLRP3* CA+AA genotypes (OR = 0.09, 95% CI 0.01–0.60, p = 0.014).

Conclusions: The results of our study suggest that *NLRP3* and *CARD8* polymorphisms could affect the risk of asbestos-related diseases.

Keywords: inflammasome, polymorphism, asbestosis, pleural plaques, malignant mesothelioma

od azbestoze za *NLRP3* CA + AA genotipove (OR = 0,09, 95% CI 0,01–0,60, p = 0,014).

Zaključak: Rezultati našeg istraživanja ukazuju na to da polimorfizmi *NLRP3* i *CARD8* mogu uticati na rizik od bolesti povezanih sa azbestom.

Ključne reči: inflamazom, polimorfizam, azbestoza, pleuralni plakovi, maligni mezoteliom

Introduction

The asbestos-related diseases, including pleural plaques, diffuse pleural thickening and pleural effusion, asbestosis, and several types of cancers, such as lung cancer, malignant mesothelioma (MM) of the pleura and peritoneum, cancer of the larynx, cancer of the ovary, as well as the cancers of the buccal mucosa, the pharynx, the gastrointestinal tract, and the kidney, are still a major public health problem (1–4).

Pleural plaques and diffuse pleural thickening, which can be accompanied by pleural calcification, are among the most common non-malignant effects of asbestos and may occur even after relatively low asbestos exposure (5–11). Asbestosis, one of the most frequent diseases caused by asbestos, is an interstitial pulmonary process which, after a long latency period, slowly develops into diffuse pulmonary fibrosis. The disease continues to progress even after the cessation of exposure and the process is irreversible (10, 12, 13). Among cancers, MM is considered to be a highly aggressive and invasive malignoma that arises from the mesothelium, most commonly from pleura and less frequently from peritoneum or other serosal surfaces (14). As the onset of symptoms is often non-specific and insidious, this malignoma is very difficult to diagnose. However, an early diagnosis is extremely important for timely and more effective treatment (15). Therefore, potential new biomarkers for an earlier diagnosis of MM have been intensively investigated (16, 17).

The pathogenesis of asbestos-induced pleural diseases has been extensively investigated. Asbestos fibres are thought to provoke pleural inflammation from direct toxicity to mesothelial cell. Pleural injury can be elicited by inhaled asbestos fibres indirectly via the release of inflammatory cytokines and growth factors from within the lung (18). The mechanism of cell injury caused by asbestos and affecting the cells of the pleura and lung remains unclear. It has been suggested that inflammation may have an important role in the pathogenesis of asbestos-related diseases (19–21).

Inflammation has been proposed to be involved in the modification and/or calcification of pleural thickenings as well as in the development of asbestosis (22, 23). Chronic inflammation is also known to play an essential role in tumorigenesis and is a major contributing factor in the development of many types of cancer, including MM (22, 24). Evidence suggests that inflammation can also be associated with unfavourable clinical prognosis in cancer patients in general (24–27) as well as in MM (28).

The reactive oxygen species (ROS) and reactive nitric species (RNS) are considered to play an important role in modulating the immune response to inflammatory stimuli (29). Recent studies have led to a better understanding of molecular mechanisms underlying the pathogenesis of asbestos-related diseases, including MM. Asbestos fibres can cause genotoxic damage either directly or indirectly, via generation of reactive oxygen and nitric species (ROS and RNS). ROS may also mediate the activation of the *NLRP3* inflammasome (30).

The *NLRP3* inflammasome is a multiprotein cytoplasmic complex comprised of NOD-like receptors, a family of intracellular sensors that have become known as crucial components of the innate immune responses and inflammation (31, 32). The *NLRP3* inflammasome complex is composed of several components, such as *NLRP3, CARD8* and ASC (23, 33, 34). It is activated by numerous physical and chemical stimuli (33), including asbestos. In response to a variety of pathogens and/or danger-associated molecular patterns, the *NLRP3* inflammasome activates caspase-1, which results in IL-1 secretion and consequently inflammatory response (33, 35). It has been shown that the secretion of IL-1 from alveolar macrophages induced by asbestos is mediated through the *NLRP3* inflammasome (21, 23).

It has been proposed that genetic variants of inflammasome components may influence the function of the complex and that functional polymorphisms in the *NLRP3* and *CARD8* genes may be associated with IL-1 production and severe inflammation (23, 34).

Among the most commonly investigated inflammasome-related single nucleotide polymorphisms (SNPs) is nonsynonymous gain-of-function polymorphism *NLRP3* rs35829419 (p.Gln705Lys; C > A) that leads to an overactive *NLRP3* inflammasome and increased production of IL-1 (36). Another important inflammasome-related SNP is *CARD8* rs2043211 (p.Cys10Ter, A>T) that results in non-functional protein and leads to loss of CARD-8 inhibition of cas-

pase-1 (23, 37). Consequently, both polymorphisms have been proposed to be associated with increased IL-1 production and proinflammatory phenotype (21, 23, 36).

Although asbestos-related diseases are among the most frequently investigated occupational diseases and the association between asbestos exposure and asbestos-related diseases has been well proved, relatively little has been known about the genetic factors that might modify the individual susceptibility to the development of these diseases (4). This study aimed to investigate the association between *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms and the risk of developing pleural plaques, asbestosis, and MM, as well as to study the influence of the interactions between *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms and asbestos exposure on the risk of developing these diseases.

Materials and Methods

The case-control study included 416 subjects with pleural plaques, 160 patients with asbestosis, 154 subjects with MM and 149 subjects with no asbestos disease.

Subjects with pleural plaques, asbestosis and no asbestos disease were occupationally exposed to asbestos and presented at the State Board for the Recognition of Occupational Asbestos Diseases in the period from 1 January 1998 to 31 December 2007. The diagnosis of pleural plaques, asbestosis or »no asbestos-related disease« was confirmed by two groups of experts from the State Board for the Recognition of Occupational Asbestos Diseases, each group consisting of an occupational physician, a pulmonologist, and a radiologist.

Patients with MM were treated at the Institute of Oncology Ljubljana in the period between 1 January 2004 and 31 December 2012. In all patients with MM, thoracoscopy or laparoscopy/laparotomy was performed. The diagnosis of MM was histologically proved by a pathologist skilled in diagnosing this cancer.

Data on smoking were obtained for all subjects during an interview using a standardized questionnaire. The number of pack-years of smoking was calculated from the duration of smoking, and the number of cigarettes smoked per day (38, 39).

To determine the exposure to asbestos, a semi-quantitative method was used. For the subjects with pleural plaques, asbestosis, »no asbestos-related disease« and for 33 patients with MM, data on cumulative asbestos exposure in fibres/cm^3-years were available from the previous study (39). Based on these data, the subjects were divided into three groups: low (< 11 fibres/cm^3-years), medium (11–20 fibres/cm^3-years) and high (> 20 fibres/cm^3-years) asbestos exposure. For patients with MM with no cumulative asbestos exposure data, accurate work history was obtained, and their asbestos exposures were compared with exposures of the group of subjects with known cumulative asbestos exposure and were accordingly divided into three groups with presumed low, medium and high asbestos exposure, as previously described (40). Reliable semi-quantitative data on asbestos exposure could be obtained only for 81 MM patients.

For the genetic analysis, genomic DNA was isolated from peripheral blood leukocytes or FTA Mini Cards (Whatman Bioscience). The *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms were determined by using a fluorescent-based competitive allele-specific polymerase chain reaction (KASPar) assay (LGC Genomics, UK) or real-time PCR-based Taqman assay (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

In the statistical analysis, standard descriptive statistics was first performed, followed by univariate and multivariate logistic regression modelling. The possible interactions between genotypes as well as between genotypes and asbestos exposure were tested by logistic regression models using dummy variables. The dominant and additive genetic models were used in the analysis. All statistical analyses were performed using IBM SPSS Statistics version 21.0 (IBM Corporation, Armonk, NY, USA).

The study was approved by the Republic of Slovenia National Medical Ethics Committee and was carried out according to the Helsinki Declaration.

Results

The study included 879 subjects altogether; among them, 645 (73.4%) were male and 234 female (26.6%). The median age of subjects was 56.70 (inter-quartile 50.00–64.90) years. Regarding smoking, 431 (49.9%) were ever smokers, and 432 (50.1%) of them never smoked. Among smokers, the median number of pack-years was 19 (inter-quartile 7.3–30). Asbestos exposure was low in 538 (67.60%) subjects, medium in 104 (13.10%) and high in 154 (19.30%) subjects.

The baseline characteristics of each subject group (subjects without the asbestos-related disease, patients with pleural plaques, asbestosis, MM) are presented in *Table I*. No statistically significant difference was found among the groups regarding gender and smoking (ever vs never smokers). On the other hand, there was a statistically significant difference considering age and asbestos exposure, as well as the pack-years among smokers. However, in all groups, the highest percentage of subjects had low asbestos exposure (*Table I*).

Table I The baseline characteristics of each subject group (subjects without the disease, patients with pleural plaques, asbestosis or malignant mesothelioma).

Characteristic		No disease (N=149) N (%)	Pleural plaques (N=416) N (%)	Asbestosis (N=160) N (%)	Malignant mesothelioma (N=154) N (%)	P
Gender	Male Female	107 (71.8) 42 (28.2)	298 (71.6) 118 (28.4)	121 (75.6) 39 (24.4)	119 (77.3) 35 (22.7)	0.486 Chi-square=2.444,
Age (years)	Mean±SD Median (25%–75%) Min-max	55.3±9.3 53.5 (47.6–62.6) 35.4–77.8	55.5±9.354.6 (48.8–62.2) 34.5–85.8	58.8±9.2 59.1 (51.3–65.2) 37.2–81.8	63.5±10.4 65 (57–70) 19–84	<0.001 Test-statistic=87.357
Smoking	No Yes	77 (51.7) 72 (48.3)	206 (50.9) [11] 199 (49.1)	79 (49.7) [1] 80 (50.3)	70 (46.7) [4] 80 (53.3)	0.811 Chi-square=0.961,
Pack-years (smokers only)	Mean±SD Median (25%–75%) Min-max	20.7±15.0 [2] 20 (9.4–29.4) 0.1–65.3	17.9±14.7 [1] 15 (5–27.8) 0.05–67.6	24.7±16.7 24 (11.8–32.2) 0.15–64.5	26.0±20.4 [4] 20 (8–40) 1–79.5	0.002 Test-statistic=14.518
Asbestos exposure	Low Middle High	122 (81.9) 11 (7.4) 16 (10.7)	295 (72.3) [8] 41 (10.0) 72 (17.6)	83 (52.5) [2] 29 (18.1) 46 (28.8)	38 (46.9) [73] 23 (28.4) 20 (24.7)	<0.001 Chi-square=333.371, df=3

Missing data are presented in square brackets [].
P-values were calculated using the chi-square test for categorical and Kruskal-Wallis test for continuous variables.

Table II The association between baseline characteristics and asbestos-related diseases (univariate analysis).

Characteristic	Pleural plaques vs. no disease		Asbestosis vs. no disease		MM vs. no disease		MM vs. pleural plaques	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Gender	1.01 (0.67–1.53)	0.967	0.82 (0.49–1.36)	0.447	0.75 (0.45–1.26)	0.276	0.74 (0.48–1.15)	0.178
Age (for 10 years difference)	1.02 (0.84–1.26)	0.812	1.51 (1.18–1.94)	0.001	2.32 (1.79–3.01)	<0.001	2.37 (1.91–2.93)	<0.001
Smoking	1.03 (0.71–1.50)	0.812	1.08 (0.69–1.69)	0.727	1.22 (0.78–1.92)	0.386	1.18 (0.81–1.72)	0.380
Pack-years (smokers only, for 10 pack-year difference)	0.88 (0.74–1.06)	0.172	1.18 (0.96–1.45)	0.127	1.18 (0.98–1.42)	0.081	1.32 (1.13–1.54)	0.001
Asbestos exposure (medium and high vs. low)	1.73 (1.08–2.77)	0.022	4.08 (2.43–6.87)	<0.001	5.11 (2.80–9.3)	<0.001	2.95 (1.81–4.81)	<0.001

In univariate logistic regression analysis, a positive association was observed between age and asbestosis (OR = 1.51, 95% CI 1.18–1.94, p = 0.001), and between age and MM (OR = 2.32, 95% CI 1.79–3.01, p < 0.001). When considering patients with MM as cases and subjects with pleural plaques as controls, an increased risk of MM was found for pack-years of smoking (OR = 1.32, 95% CI 1.13–1.54, p = 0.001) (*Table II*). Asbestos exposure (medium and high vs low) was associated with the risk of each studied asbestos-related disease (*Table II*).

The genotype frequencies for *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms are shown in *Table III*.

The results of univariate analysis of the association between different asbestos-related diseases and *NLRP3* rs35829419 and *CARD8* rs2043211 geno-

Table III Genotype frequencies of investigated polymorphisms in asbestos-exposed subjects.

Polymorphism	Genotype	All subjects (N=879) N (%)	No disease (N=149) N (%)	Pleural plaques (N=416) N (%)	Asbestosis (N=160) N (%)	Malignant mesothelioma (N=154)
CARD8 rs2043211	AA	389 (44.5) [4]	74 (50.0)	167 (40.3) [2]	74 (46.5) [1]	74 (48.1)
p.Cys10Ter	AT	382 (43.7)	57 (38.5)	190 (45.9)	68 (42.8)	67 (43.5)
	TT	104 (11.9)	17 (11.5)	57 (13.8)	17 (10.7)	13 (8.4)
NLRP3 rs35829419	CC	785 (89.7) [4]	133 (89.9) [1]	375 (90.4) [1]	145 (90.6)	132 (86.8) [2]
p.Gln705Lys	CA	88 (10.1)	15 (10.1)	39 (9.4)	14 (8.8)	20 (13.2)
	AA	2 (0.2)	0	1 (0.2)	1 (0.6)	0

0Missing data are presented in square brackets [].

Table IV The association between different asbestos-related diseases and genotypes in univariate analysis.

Polymorphism	Genotype	Pleural plaques vs no disease		Asbestosis vs no disease		MM vs no disease		MM vs pleural plaques	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
CARD8 rs2043211	AA	reference		reference		reference		reference	
p.Cys10Ter	AT	1.48 (0.99–2.21)	0.058	1.19 (0.74–1.92)	0.469	1.18 (0.73–1.90)	0.508	0.80 (0.54–1.18)	0.252
	TT	1.49 (0.81–2.73)	0.201	1.00 (0.48–2.11)	1.000	0.77 (0.35–1.69)	0.506	0.52 (0.27–1.00)	0.049
	AT+TT	1.48 (1.01–2.16)	0.042	1.15 (0.73–1.80)	0.545	1.08 (0.69–1.70)	0.735	0.73 (0.50–1.06)	0.099
NLRP3 rs35829419	CC	reference		reference		reference		reference	
p.Gln705Lys	CA+AA	0.95 (0.51–1.77)	0.861	0.92 (0.43–1.95)	0.822	1.34 (0.66–2.74)	0.416	1.42 (0.80–2.52)	0.229

Table V Multiplicative interaction between *CARD8* rs2043211 and *NLRP3* rs35829419 genotypes and between genotypes and asbestos exposure.

	Pleural plaques vs no disease		Asbestosis vs no disease		MM vs. no disease		MM vs pleural plaques	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
CARD8 rs2043211 and *NLRP3* rs35829419 (both dominant models)	0.74 (0.21–2.66)	0.647	0.41 (0.09–1.91)	0.254	0.37 (0.09–1.58)	0.179	0.50 (0.16–1.61)	0.245
CARD8 rs2043211 and asbestos exposure	0.58 (0.22–1.49)	0.256	0.63 (0.22–1.79)	0.382	0.75 (0.22–2.52)	0.638	1.30 (0.49–3.46)	0.601
NLRP3 rs35829419 and asbestos exposure	0.37 (0.09–1.62)	0.188	0.09 (0.01–0.60)	0.014	0.62 (0.11–3.62)	0.591	1.66 (0.37–7.51)	0.513

Table VI The association between different asbestos-related diseases and genotypes in multivariate analyses.

Polymorphism	Genotype	Pleural plaques vs no disease		Asbestosis vs no disease		MM vs no disease		MM vs plaques	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
CARD8 rs2043211	AA	reference		reference		reference		reference	
p.Cys10Ter	AT	1.47 (0.98–2.20)	0.064	1.19 (0.71–1.99)	0.517	0.91 (0.47–1.77)	0.776	0.67 (0.39–1.14)	0.137
	TT	1.42 (0.77–2.62)	0.260	1.14 (0.51–2.57)	0.752	0.61 (0.19–1.94)	0.403	0.37 (0.14–0.96)	0.040
	AT+TT	1.46 (1.00–2.13)	0.052	1.18 (0.72–1.92)	0.513	0.84 (0.45–1.58)	0.595	0.59 (0.36–0.99)	0.044
NLRP3 rs35829419	CC	reference		reference		reference		reference	
p.Gln705Lys	CA+AA	0.96 (0.51–1.79)	0.887	1.10 (0.49–2.51)	0.813	0.92 (0.35–2.46)	0.874	1.50 (0.70–3.21)	0.301

Pleural plaques vs no disease: adjusted for asbestos exposure
Asbestosis vs no disease, MM vs no disease: adjusted for asbestos exposure, age, gender
MM vs plaques: adjusted for asbestos exposure, age

Table VII Multiplicative interaction between *NLRP3* rs35829419 and asbestos exposure – asbestosis vs no disease.

	Asbestos exposure								Asbestos exposure within *NLRP3*	
	Low				Medium and high					
NLRP3 rs35829419	No disease (N)	Asbestosis (N)	OR (95% CI)	P	No disease (N)	Asbestosis (N)	OR (95% CI)	P	OR (95% CI)	P
CC	110	70	1	reference	23	73	4.99 (0.86–8.70)	<0.001	4.99 (0.86–8.70)	<0.001
CA+AA	11	13	1.86 (0.79–4.38)	0.157	4	2	0.79 (0.14–4.40)	0.274	0.42 (0.07–2.77)	0.369
NLRP3 within asbestos exposure			1.86 (0.79–4.38)	0.157			0.16 (0.03–0.92)	0.040		

types are shown in *Table IV*. An increased risk of pleural plaques was found for the rs2043211 AT + TT genotypes (OR = 1.48, 95% CI 1.01–2.16, p = 0.042). On the other hand, when the analysis was done for MM patients as cases, and pleural plaques patients as controls, a decreased risk of MM was found for carriers of TT genotype (OR = 0.52, 95% CI 0.27-1.00, p = 0.049). No association was found either between other asbestos-related diseases and *CARD8* rs2043211 genotypes or between *NLRP3* rs35829419 and different asbestos-related diseases (*Table IV*). In the subsequent forward conditional logistic regression analysis, the results did not change considerably after adjustment for asbestos exposure, age in and gender (*Table VI*).

The interactions between *NLRP3* rs35829419 and *CARD8* rs2043211 genotypes did not influence the risk of any asbestos-related disease. However, when testing interactions between SNPs and asbestos exposure, a multiplicative interaction was observed with *NLRP3* rs35829419 (OR = 0.09, CI 0.01–0.60, p = 0.014) (*Table V*). A decreased risk of asbestosis was found for *NLRP3* CA + AA genotypes only in subjects with medium or high asbestos exposure (OR = 0.16, CI 0.03–0.92, p = 0.040) (*Table VII*).

Discussion

It has been suggested that in addition to asbestos exposure, genetic factors may also affect the development of asbestos-related diseases (40–42). This study investigated the influence of *NLRP3* rs35829419 and *CARD8* rs2043211 polymorphisms on the risk of different asbestos-related diseases.

As expected, asbestos exposure (medium and high vs low) was associated with an increased risk of developing all asbestos-related diseases studied, which is in agreement with the results of previous studies (18, 43–46).

An important finding of this study is that subjects with *CARD8* rs2043211 AT + TT genotypes had an increased risk of pleural plaques compared to those with rs2043211 AA genotype. This could be explained by observing that A to T transversion in *CARD8* gene can introduce a stop codon at codon 10 (Cys10Stop) and produce a truncated CARD protein, which is unable to suppress NF-kB activity. This results in an increased production of pro-IL-1 and increased inflammation (21, 23, 36, 47, 48). Considering that asbestos fibres may provoke pleural inflammation, this result could be biologically plausible (18). According to our knowledge and available literature, the association between *CARD8* rs2043211 polymorphism and pleural plaques has not been studied yet.

Another interesting result of the current study shows a decreased risk of developing MM compared to those patients with pleural plaques who had *CARD8* rs2043211 TT genotype, which indicates a protective effect. As described above, the activity of truncated *CARD8* protein is decreased, thus facilitating the inflammation response (23, 37) and consequently the increased risk of developing MM, which seems to be contrary to our findings. However, our results are in agreement with studies investigating the association between *CARD8* rs2043211 genotypes and other diseases (48, 49). Moreover, the investigated SNP represents only a fraction of the polymorphic content of the *CARD8* gene (48). Therefore, other SNPs that may be responsible for the altered *CARD8* function need to be further investigated. As *NLRP3* inflammasome is involved in apoptosis (49), a decreased activity of *CARD8* protein may decrease apoptosis. Considering that apoptosis is a key part of the innate tumour-suppression mechanism (50), this could explain the decreased risk of MM for *CARD8* rs2043211 TT genotype.

This study also revealed that *NLRP3* polymorphism could modify the association between asbestos exposure and asbestosis. A decreased risk of asbestosis was found for subjects with *NLRP3* CA and AA genotype. Considering that inflammation has been proposed to be involved in the development of asbestosis and the role of *NLRP3* in the inflammation process, this interaction could be logical and biologically plausible. The results of our study are in agreement also with the findings of Kukkonen et al., who reported that the *NLRP3* inflammasome is important in the development of fibrotic lung disease by associating the *NLRP3* rs35829419 variant allele with increased risk of asbestos-related interstitial lung fibrosis (23).

To our knowledge and available literature, the influence of *NLRP3* and *CARD8* polymorphisms on the risk of asbestos-related diseases has been not studied yet. The results of the study suggest that *NLRP3* and *CARD8* polymorphisms could affect the risk of these diseases. However, further studies including more subjects and also other *NLRP3* and *CARD8* polymorphisms are necessary to elucidate these associations.

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Conflict of interest statement

The authors state that they have no conflicts of interest regarding the publication of this article.

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Short Communication
Kratko saopštenje

DIAGNOSTIC BENEFITS OF MINDIN AS A PROSTATE CANCER BIOMARKER

DIJAGNOSTIČKE PREDNOSTI MINDINA KAO BIOMARKERA RAKA PROSTATE

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Summary

Background: It has been shown that decreased expression and activity of extracellular matrix protein mindin correlate with various types of cancers including breast, colon and lung cancers. The aim of the presented study was to investigate the serum mindin levels in prostate cancer.
Methods: Mindin concentrations in serum were measured in 56 patients with prostate cancer (mean age 68 years) and in control group of 29 healthy men (mean age 64 years) using commercially available enzymatic immunoassay (Cusabio, WuHan, China). The patients were divided with respect to the severity of the disease into two groups according to the EAU guidelines (stage 1, 2 – less severe tumours, stage 3, 4 – severe tumours).
Results: Serum mindin concentrations were significantly elevated in the group of healthy individuals unlike in the patients with prostate cancer (2.12 ng/mL vs 0.78 ng/mL, with P=0.0007, AUC=0.705). Patients with less severe tumours (stage 1, 2) and severe tumours (stage 3, 4) had significantly decreased levels of S-mindin as well (P=0.0037), although the difference in serum mindin concentrations between the patients with less severe and severe tumours was not significant.
Conclusions: Concentrations of mindin were decreased in patients with prostate cancer and reduced in patients with less severe prostate cancer as well. Mindin appears to be a promising diagnostic marker useful in the diagnosis of prostate cancer.

Keywords: mindin, prostate cancer, biomarker

Kratak sadržaj

Uvod: Pokazalo se da je smanjena ekspresija i aktivnost ekstracelularnog proteinskog matriksa mindina korelira sa različitim tipovima raka, uključujući rak dojke, debelog creva i pluća. Cilj prikazane studije je bio da se ispita serumski nivo mindina kod karcinoma prostate.
Metode: Koncentracije mindina u serumu su merene kod 56 bolesnika sa karcinomom prostate (srednja starost 68 godina) i u kontrolnoj grupi od 29 zdravih muškaraca (srednja starost 64 godine) korišćenjem komercijalno dostupnog enzimskog testa (Cusabio, VuHan, Kina). Pacijenti su prema težini bolesti podeljeni u dve grupe prema EAU smernicama (faza 1, 2 – manje teški tumori, faza 3, 4 – teški tumori).
Rezultati: Koncentracije serumskog mindina bile su značajno povišene u grupi zdravih pojedinaca, za razliku od pacijenata sa rakom prostate (2,12 ng/mL u odnosu na 0,78 ng/mL, sa P=0,0007, AUC=0,705). Pacijenti sa manje ozbiljnim tumorima (faza 1, 2) i teškim tumorima (faza 3, 4) imali su i značajno snižene nivoe S-mindina (P=0,0037), iako razlika u koncentracijama mindina u serumu izme u pacijenata sa manje teškim i ozbiljnim tumori nije bila značajna.
Zaključak: Koncentracije mindina su smanjene kod pacijenata sa rakom prostate, a smanjene su i kod pacijenata sa manje teškim oblikom raka prostate. Izgleda da je mindin obećavajući dijagnostički marker i da je koristan u dijagnostici raka prostate.

Ključne reči: mindin, rak prostate, biomarker

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List of abbreviations: AUC, Area under the curve; CV, Coefficient of variation; EAU, European Association of Urology; ELISA, Enzyme-linked immunosorbent assay; IQR, interquartile range; Pca, prostate carcinoma; PHI, prostate health index; PSA, Prostate-specific antigen; PSADT, Prostate-specific antigen doubling time; ROC, Receiver operating characteristics.

Introduction

Prostate cancer (PCa) is one the most frequently diagnosed tumours of men and the third most common cause of cancer death of Czech men (1). Currently, the only biomarker in wide clinical use for the diagnosis and prognosis of prostate cancer is the prostate-specific antigen (PSA). Other markers useful for the diagnostics of prostate cancer are free PSA and Free/Total PSA ratio, complexed PSA, pro PSA - 2proPSA and Prostate Health Index (PHI). Total PSA, % fPSA, f/tPSA, and other PSA derivatives, PSAD, PSA velocity, PSADT, age-specific PSA do not decrease the number of unnecessary biopsies performed for diagnostic purposes. Despite all of these markers, a 100% conclusively precise diagnostic test for PCa has not been introduced yet (2).

PSA can be detected in the serum of a blood sample and is considered to be currently the most useful tumour marker (3). PSA can be used for prostate cancer screening and monitoring of the response to the treatment. PSA seems to be useful for the detection of prostate cancer of the men whose total PSA concentration in the 4–10 μg/L range (4). Investigation of the serum PSA levels does not have a direct correlation with increasing grade and stage of prostate cancer (5). 20 % of patients have PSA levels less than 4 μg/L, 25 % of the patients have PSA levels in the interval of 4–10 μg/L. Moreover, some aggressive forms of prostate cancer can be PSA negative (6). Due to the limitations of PSA as a biomarker, there is still a need for new biomarkers that can be used as prognostic indicators of prostate cancer for effective differentiation between indolent and aggressive disease (7).

Mindin, also called spondin 2, is an extracellular matrix protein which is encoded by the SPO2 gene located in the chromosome 4p16.3. Spondin 2 belongs to the F-spondin family of secreted extracellular matrix proteins. The members of F-spondin family have three domains: FS1 (for F-spondin), FS, and thrombospondin type 1 repeats. Mindin exerts a broad spectrum of effects on the innate immune system and its role in cancer is currently investigated (8). Recently, mindin is mentioned as a candidate biomarker for prostate cancer diagnosis (9).

The study aimed to investigate serum levels of mindin as a potential diagnostic biomarker in patients with prostate cancer.

Materials and Methods

Serum samples of patients with prostate cancer were obtained in the morning before prostatectomy. The cancer diagnosis was performed by histological examination of tumour specimens obtained by prostate resection. The samples of patients and healthy individuals were frozen immediately, aliquoted and kept at -70 °C until mindin was analyzed. Serum concentrations of mindin were measured in 56 patients (mean age 68 years, range 45–82 years) with prostate cancer, and in the control group consisting of 29 healthy men (mean age 64 years, range 55–78 years). Informed consent was obtained for all of the individuals included in the study. The characteristics of the patients and healthy subjects are summarized in *Table I*.

Serum mindin levels were assayed using commercially available immunoassay technique ELISA kit (Cusabio, WuHan, China). The analytic characteristics of the diagnostic kit were as follows: detection limit 0.78 ng/mL and working range 3.12–200 ng/mL, with a mean coefficient of variation (CV)=11%. The patients were previously clinically investigated and classified according to the European Association of Urology (EAU) guidelines (10). For further investigation, the patients were divided into two groups according to the severity of the disease. The first group consisted of patients with less severe tumours (stages 1 and 2), the second group consisted of patients with severe tumours (stages 3 and 4).

Results

Differences between subgroups were tested for statistical significance by the nonparametric Mann-Whitney test. The value of $P<0.005$ was considered statistically significant. Receiving operation analysis (ROC) was used to investigate the diagnostic efficiency. The analysis of variance was used to evaluate the relationship of mindin levels with the age and PSA levels. Statistical software MedCalc version: 18.02.01 (Ostende, Belgium) was used for statistical analysis.

Serum levels of mindin in patients with prostate cancer were significantly decreased in the control group expressed as medians (2.12 ng/mL vs 0.78 ng/ml, P=0.0007, Mann-Whitney test, *Figure 1*). Diagnostic efficiency of serum mindin expressed as AUC calculated from the ROC analysis was 0.705 (specificity=73 % and sensitivity=64 %) (*Figure 2*). Serum levels of mindin in patients with less severe tumours (stages 1 and 2) and severe tumours (stages 3 and 4) were significantly decreased compared with the control group as well. (P=0.0037, One-way analysis of variance, *Figure 3*).

Discussion

The results of the pilot study confirm the relevant role of mindin in the prostate cancer diagnosis. We proved that the serum levels of mindin were significantly decreased in patients with prostate cancer compared with healthy individuals. These results differ from the results of other studies showing elevated mindin levels in patients with prostate cancer

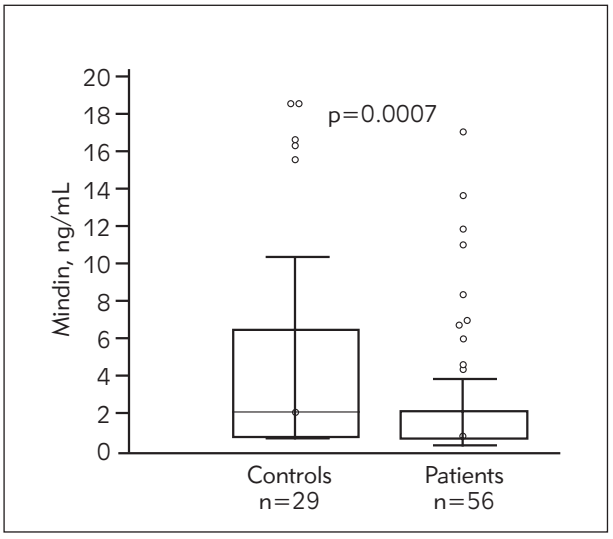

Figure 1 Serum mindin levels in healthy individuals and patients with prostate cancer.
Results are expressed as Box-and-whisker plots with medians (IQR 25. – 75. percentile).

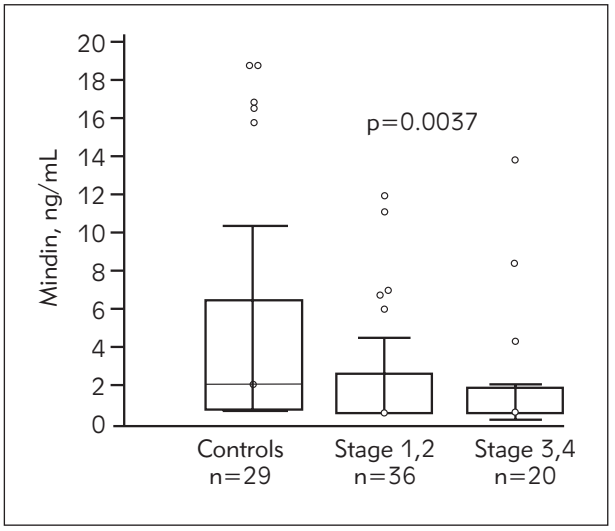

Figure 3 Serum mindin levels in patients with less severe and severe prostate cancer tumours.
Results are expressed as Box-and-whisker plots with medians (IQR 25. – 75. percentile)
Stage 1, 2 – patients with less severe tumours
Stage 3, 4 – patients with severe tumours

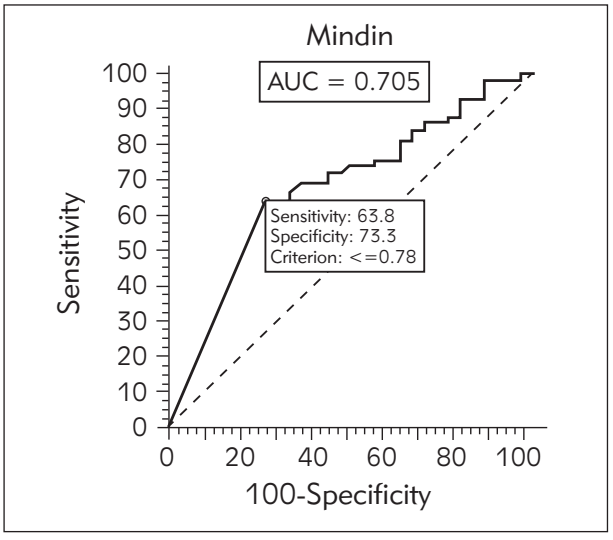

Figure 2 Diagnostic efficiency of mindin.

(11, 12, 14). Presented results are in concordance with the study of Wang et al. (8) showing significantly decreased serum mindin in tumour lesions of patients compared with adjacent control tissues by colon, lung, gastric, oesophageal, and breast cancer. Wang et al. (8) mentioned an important role of EGR-1, which directly regulates mindin expression at the transcriptional level, and this regulates both mindin mRNA and protein expression in vitro to further define EGR-1 mediated regulation of mindin expression. The relationship between EGR-1 expression and prostate cancer was previously mentioned. Gregg et al. (12) showed that EGR-1 is downregulated in patients with prostate carcinoma according to their clinical considerations (18 of 20 patients in pT2 and pT3 clinical stages showed normal or downregulated EGR-1 expression). Contrary to the previously mentioned reports of Wang et al. (8) and Gregg et al. (12), studies of other authors showed that in patients with prostate cancer EGR-1 stimulates tumour cell growth and its expression level increases with the degree of malignancy (11, 13). This seems to be specific to the prostate tumour cells because, in mammary and lung tumours as well as most normal tissues, EGR-1 expression is low. This contradictory findings regarding EGR-1 expression confirmed the bivalent role of EGR-1 either as a tumour suppressor or oncogene with respect to EGR-1 regulation and the degree of the malignancy.

Our results show good diagnostic sensitivity of mindin with AUC of 0.705. We prove that serum mindin levels are not dependent on the PSA levels, and the age (P=0.42, analysis of variance). The serum levels of mindin differ in patients with prostate cancer. We found significant differences between serum mindin concentrations related to the staging of cancer. The patients with less severe tumours belonging to stages 1 and 2 had lower serum mindin level than patients with severe tumours stages 3 and 4 as shown in *Figure 3*. These results correlate with the results found in other cancer types presented by Wang et al., (8) who presented decreased levels in patients with less severe tumours belonging to stages 1 and 2. In our study, the difference between patients in stages 1 and 2 and 3 and 4 is not significant.

The presented study is a pilot study, and thus the results need to be confirmed on a large number of samples. Various enzymatic immunoassays used for

mindin evaluation show discrepant results, as indicated in the presented study and the study of Wang et al. (8) with similar results, which are different from the study of Luccarelli et al. (14). This finding seems to indicate that the concentrations of mindin in patients with prostate cancer are assay dependent.

The concentration of mindin is decreased in patients with prostate cancer. Mindin concentration is not related to the age and PSA levels. Mindin appears to be a promising diagnostic marker useful in the diagnosis of prostate cancer.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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Original paper
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ASSESSMENT OF ABBOTT ARCHITECT 25-OH VITAMIN D ASSAY IN DIFFERENT LEVELS OF VITAMIN D

PROCENA ODREĐIVANJA 25-OH VITAMINA D NA ABBOTT ARCHITECT ANALIZATORU PRI RAZLIČITIM NIVOIMA DEFICITA VITAMINA 25-OH D

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Summary

Background: There is increasing requests of Vitamin D test in many clinical settings in recent years. However, immunoassay performance is still a controversial topic. Several diagnostic manufacturers have launched automated 25-hydroxyvitamin D (25-OH D) immunoassays in the past decade. We compared the performance of Abbott Architect 25-OH D Vitamin immunoassay with liquid chromatography-tandem mass spectrometry systems (LC-MS/MS) to evaluate immunoassay performance, especially in deficient groups.
Methods: Eighty human serum samples were analyzed with Architect 25-OH D vitamin kit (Abbott Diagnostics, Lake Forest, IL, USA) and LC-MS/MS systems (Zivak Technology, Istanbul, Turkey). The results of the immunoassay method were compared with the LC-MS/MS using Passing-Bablok regression analysis, Bland-Altman plots and correlation coefficient analysis. We also evaluated results in four levels of D vitamin as a severe deficiency, deficiency, insufficiency, and sufficiency.
Results: Architect showed 9.59% bias from LC-MS/MS with smaller mean. Passing-Bablok regression analysis demonstrated the value of 0.95 slope and had a constant bias with an intercept value of -4.25. Concordance correlation coefficient showed moderate agreement with the value of 0.918 (95% CI 0.878–0.945). Two methods revealed

Kratak sadržaj

Uvod: Poslednjih godina povećan je zahtev za određivanjem vitamina D u mnogim kliničkim slučajevima. Međutm, imunoodređivanje je i dalje kontraverzno. Nekoliko dijagnostičkih proizvođača razvilo je poslednjih godina automatsko imunoodređivanje 25-hidroksivitamina D (25-OH D). Mi smo poredili imunohemijsko određivanje 25-OH vitamina D pomoću Abbott Architect analizatora sa tandem sistemom tečna hromatografija-masena spektrometrija (LS-MS/MS) kako bi procenili imunohemijsko određivanje naročito u deficijentnim grupama.
Metode: Analizirano je 80 humanih uzoraka seruma primenom Architect 25-OH D vitamin reagensom (Abbott Diagnostics, Lake Forest, IL, USA) i LC-MS/MS (Zivak Technology, Istanbul, Turkey). Dobijeni rezultati primenom imunoodređivanja bili su u korelaciji sa rezultatima dobijenim sistemom tečna hromatografija-masena spektrometrija (LS-MS/MS) korišćenjem Passing-Bablok regresionom analizom, Bland-Altman metodom i analizom korelacionog koeficijenta. Takođe smo procenjivali rezultate četiri nivoa vitamina D kao tešku deficijenciju, deficijenciju, insuficijenciju i suficit.
Rezultati: Arhitect je prikazao 9,59% odstupanje od LC-MS/MS sa neznatnim značajem. Passing-Bablok regresiona analiza ukazala je na vrednost nagiba od 0,95 i konstantom Korelacioni koeficijent se umereno slagao sa vrednostima 0,918 (95% CI 0,878–0,945). Dve metode su imale dobro

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good interrater agreement (kappa = 0.738). While the smallest bias determined in deficiency (9.95%) group, the biggest was in insufficiency (15.15%).

Conclusions: Architect 25-OH D vitamin immunoassay can be used in routine measurements but had potential misclassification of vitamin D status in insufficient and deficient groups. Although there are recent standardization attempts in 25-OH D measurements, clinical laboratories must be aware of this method.

Keywords: vitamin D, method comparison, deficiency, insufficiency, 25-Hydroxyvitamin D

slaganje (kappa = 0,738). Najmanje odstupanje je određeno u grupi sa deficijencijom (0,95%), a najveće u grupi sa insuficijencijom (15,15%).

Zaključak: Imunodređivanje sa Arhitect 25-OH D vitaminom može da se koristi kao rutinsko merenje ma da ima potencijalni nedostatak pri klasifikaciji statusa vitamina D u grupama sa insuficijencijom i deficijencijom. Ma da postoje skorašnji pokušaji standardizacije merenja 25-OH D kliničke laboratorije moraju ovo da imaju na umu.

Ključne reči: vitamin, poređenje metoda, deficijencija, insuficijencija, 25-hiodroksivitamin D

Introduction

Vitamin D deficiency is a worldwide health problem caused mainly by insufficient exposure to sunlight and dietary consumption (1). It is affecting more than one billion people, especially prevalent among elderly with the several clinical findings such as muscle weakness, orthostatic hypotension, eczema etc. (2). Some studies suggested that Vitamin D deficiency were related to rheumatologic and autoimmune diseases, cancer and the other clinical conditions (3, 4).

Chemiluminescence immunoassays (CLIA), radioimmunoassay (RIA), high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are the most common techniques used for measuring of vitamin D (5–6).

National Health and Nutritional Examination Survey (NHANES) recommended LC-MS/MS as the best method for quantifying vitamin D metabolites due to improved sensitivity, accuracy, and reproducibility (7). Although LC-MS/MS is not simple to use, inherit some limitations; those are high costly requirement specifications, complex assay procedures, requiring of an experienced analyst. LC-MS/MS is still the advanced method in determining D vitamin status, hence the new methods have more technique restrictions (8–9).

Serum 25-hydroxyvitamin D (25-OH D) levels have been determined by using **C**hemi**l**uminescent **I**mmuno**a**ssay (CLIA) in the recent years. It is simple, cost and time-effective method when compared to LC-MS/MS systems (6). The main limitation of immunochemical methods is the cross reactivity of the antibodies used in the assay. This method has an inability to discriminate the D2 and D3 forms of the 25-OH D metabolite (9).

Vitamin D Standardization Program (VDSP) (10) is an international effort collaborated with many constitutions etc. Central Disease Center (CDC), National Institute of Standards and Technology (NIST), NHANES, Belgian Laboratory for Analytical Chemistry, Faculty of Pharmaceutical Sciences, Ghent University (11). One of the objectives of VSDP is standardize 25-OH D assays with NIST traceable measurement procedures (12). Standard reference materials (SRM) 2972 and 972 has been recommended for improving traceability and harmonization of 25-OH vitamin D measurement (10, 12).

Recently, Abbott diagnostic claimed, their 25-OH Vitamin D kit could determine 25(OH) Vitamin D metabolites, with excellent accuracy and sensitivity. This kit also calibrated against NIST SRM 2972. Some studies in literature, investigate the verification of Architect 25-OH Vitamin D assay (Catalog no: 3L52-25, 510k Abbott) (13–15). In the present study, we aim to compare NIST traceable Architect 25-OH Vitamin D assay with LC-MS/MS systems. To the best of our knowledge in the literature there is not any study evaluated these methods in different levels of Vitamin D. For further examination, immunoassay performance evaluated in severe deficiency/deficiency/insufficiency/sufficiency groups.

Material and Methods

Serum specimens

We randomly chosen 80 serum specimens from the patients' samples pool during four days period. The sample consisted of 40 male and 40 female, with a mean age of 50 ± 16.4 years, ranging from 18 to 83 years old without any chronic diseases. Serum specimen aliquot into three parts. First part was measured with Architect 25-OH D immunoassay. Within 24 h period, second and third parts were analyzed with LC-MS/MS methods in another laboratory. All samples stored at -80 °C until the transfer period. While transferring period, sun exposure and heating avoided. There was not any calcium/phosphor metabolism defect. One patients' sample was excluded who received vitamin D supplement. The Non-Interventional Medical Ethical Committee of Pamukkale University of Faculty of Medicine approved the study protocol.

Architect 25-OH D immunoassay

Immunochemical assays analyzed on Abbott Architect i-2000 (Abbott Park, IL, USA). The original Architect (Abbott Diagnostics, Lake Forest, IL, USA) 25-OH Vitamin D assay determine 25(OH) D2 and D3 in human serum and plasma. It is a delayed one-step immunoassay including a sample pre-treatment for the quantitative determination of vitamin D in competitive chemiluminescent microparticle immunoassay (CMIA) technology with flexible assay protocols. This chemiflex method was used microparticles coated with anti-vitamin D IgG antibody, and biotinylated Vitamin D antibiotin IgG acridinium-labelled conjugated complex. Architect 25-OH Vitamin D 5p02 calibrators (16) were standardized against NIST SRM 2972 and traceable to the LC-MS/MS Vitamin D reference measurement procedure (University of Ghent). In Architect kit insert, limit of blank (LOB), limit of detection (LOD), limit of quantitation (LOQ) and reportable range of the assay were given as 4, 5.5, 6, 8.5–389.75 nmol/L respectively (16).

Zivak LC-MS/MS Multitasker LC-MS/MS system

LC-MS/MS analysis were performed in Zivak Multitasker Fully Automated Sample Preparation and Injection System coupled to a MS detector. This spectrometer equipped with a Macherey–Nagel Nucleoder C18 Gravity column (125×2 mmi.d., 5 µm particle size). This system can report patient data within 6 minutes including the solid phase extraction (SPE) and sample preparation and mass spectrometry analysis time. Gören et al. (17) has been verified this method, and determined uncertainties in 2015. After verification and standardization of this method started to use in routine laboratory analysis. Certified standard reference material SRM 972a was used to monitor the LC-MS/MS traceability. Sample Chromatograms and validation results for Zivak LC-MS/MS Multitasker LC-MS/MS system were given at *Figure 1* and *Table I*.

Table I Diagnostic performance characteristics of Zivak 25-OH Vitamin D2/D3 LC-MS/MS Analysis Kit.

	25-OH Vitamin D3	25-OH Vitamin D2
LOD (nmol/L)	3.7	2.65
LOQ (nmol/L)	12.25	8.75
Accuracy (%)	94.7	96.4
Intra-assay precision %CV)	2.16	4.6
Inter-assay precision (%CV)	2.74	4.74
Linearity (R2)	0–200 (0.993)	0–200 (0.992)

Measurement comparison

Method comparison was designed according to CLSI EP09-A3 guideline. Inter-assay imprecision of the Architect 25-OH D assay was estimated according to CLSI EP15-A3 guidelines by measuring three levels commercial quality control samples during 5 days with five replicates per day (n = 25). Intra- assay imprecision estimation was conducted on measuring five replicates on the same day. We determined trueness via Biorad External Quality System running three sample from different levels. We determined severe deficiency/deficiency/insufficiency/sufficiency as 0–25, 25–50, 50–75, >75, respectively (nmol/L) (4, 17–19).

Statistical Analysis

Concordance correlation analysis, Passing-Bablok regression analysis, Box Whisker graphics and the Bland-Altman method were performed to determine agreement between two methods. Lin's concordance correlation coefficient for continuous data and Kappa interrater agreement for categorical data was

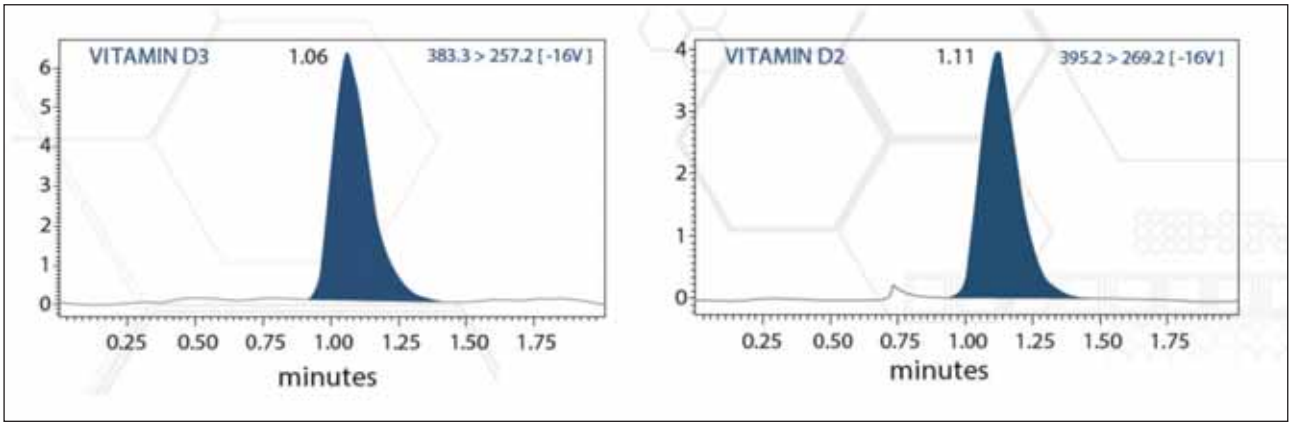

Figure 1 Sample Chromatograms of Zivak 25-OH Vitamin D2/D3 LC-MS/MS Analysis Kit.

used to determine consistency of two methods (κ < 0.4 poor, 0.4–0.70 fair to good, and >0.70 excellent agreement). The level of statistical significance was set at $p \leq 0.05$. Statistical analysis was performed using SPSS v.24.0 for Windows (SPSS Inc., Armonk, NY, USA), R (version 3.4.3, Vienna, Austria) in R Studio (Version 1.1.463 - © 2009-2018 RStudio, Inc.). Packages used for the analysis were mcr and epiR (20, 21).

Results

We studied 80 samples by two methods within 48-hour period. The median value of 25-OH D (2.5–97.5 percentiles; nmol/L) of eighty samples 37.75 nmol/L (9.68–139.7 nmol/L), and 45 nmol/L (12.86–126.16 nmol/L) based on the values obtained from the immunoassay, and LC-MS/MS systems. Box and whisker plots show the distribution of results for the two methods in *Figure 2*. At three concentration of 25-OH D internal quality control inter-assay CV% was 4.9%, 3.0%, and 2.4% and intra-assay CV% was 1.5%, 1.1%, and 1.0%. Deviation percent results from the three external quality reports were -4.2%, 8.6% and -0.9%. Architect 25-0H D Vitamin assay deviated negatively from gold standard (11.5%). Architect 25-OH D vitamin assay demonstrated a good correlation with the results obtained with the LC-MS/MS method (r^2=0.871, $p < 0.001$).

The Passing-Bablok regression analyses for immunoassay against LC-MS/MS system shown in *Figure 3*. Architect 25-OH D assay deviated from the linearity with the value of 0.95 slope and had a constant bias with an intercept value of – 4.25 (*Table II*).

Bland-Altman plots for all results and four groups were given in *Figure 4–5 A-B-C-D*. The first one was for all sample results. The other four graphics for four different Vitamin D level groups. Architect 25-OH showed 9.59% bias when compared to LC-MS/MS system. Agreement between two methods under the four levels of 25-OH D vitamin were given in *Table III*. Classified results under four headings revealed biases as 11.43%, 9.95%, 15.5%, -5.82% for severe deficiency, deficiency, insufficiency and suf-

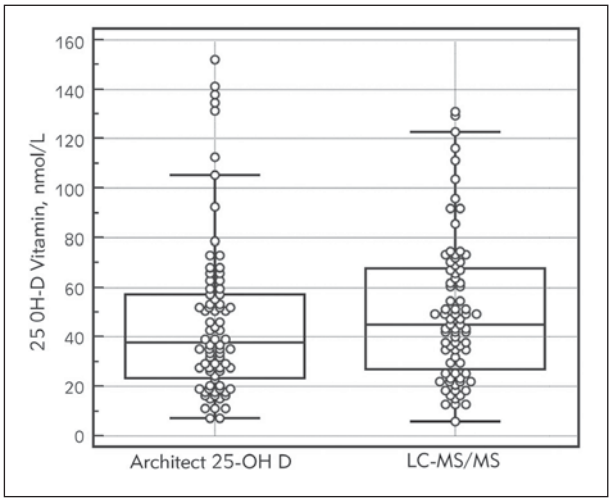

Figure 2 Box and whisker plots of LC-MS/MS and Architect 25-OH D assay for 25-OH D measurements.. Each box shows 25 to 75 percentile range. Horizontal line in each box represents group median.

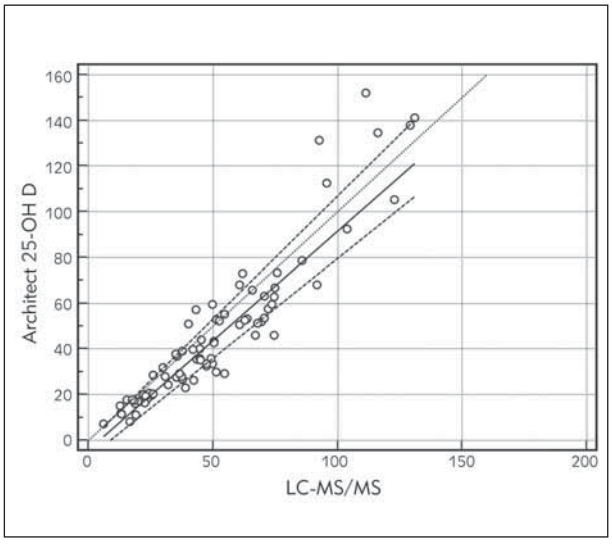

Figure 3 Passing-Bablok regression analysis of Architect 25-OH D immunoassay compared to LC-MS/MS. The regression equation is presented as y = a + bx, y = -4.2512+0.9575 x. Significant deviation from linearity (P<0.05).

Table II Method comparison results of immunoassay for Architect 25-OH D measurement with LC-MS/MS.

Method	Passing-Bablok Regression Analyses		Concordance Correlation Analyses			Bland-Altman Analyses	Interrater agreement
	Slope	Intercept	CCC(CI)	P	Cb	Bias (%)	Kappa (CI)
LC-MS/MS	0.95+(0.87–1.07)	-4.2512 (-7.54–0.55)	0.918 (0.878–0.945)	0.934	0.983	9.59	0.738* (0.604–0.848)

+Significant deviation from linearity (p< 0.05), CCC: Concordance correlation coefficient, +p< 0.05 Cb: bias correction factor (accuracy).

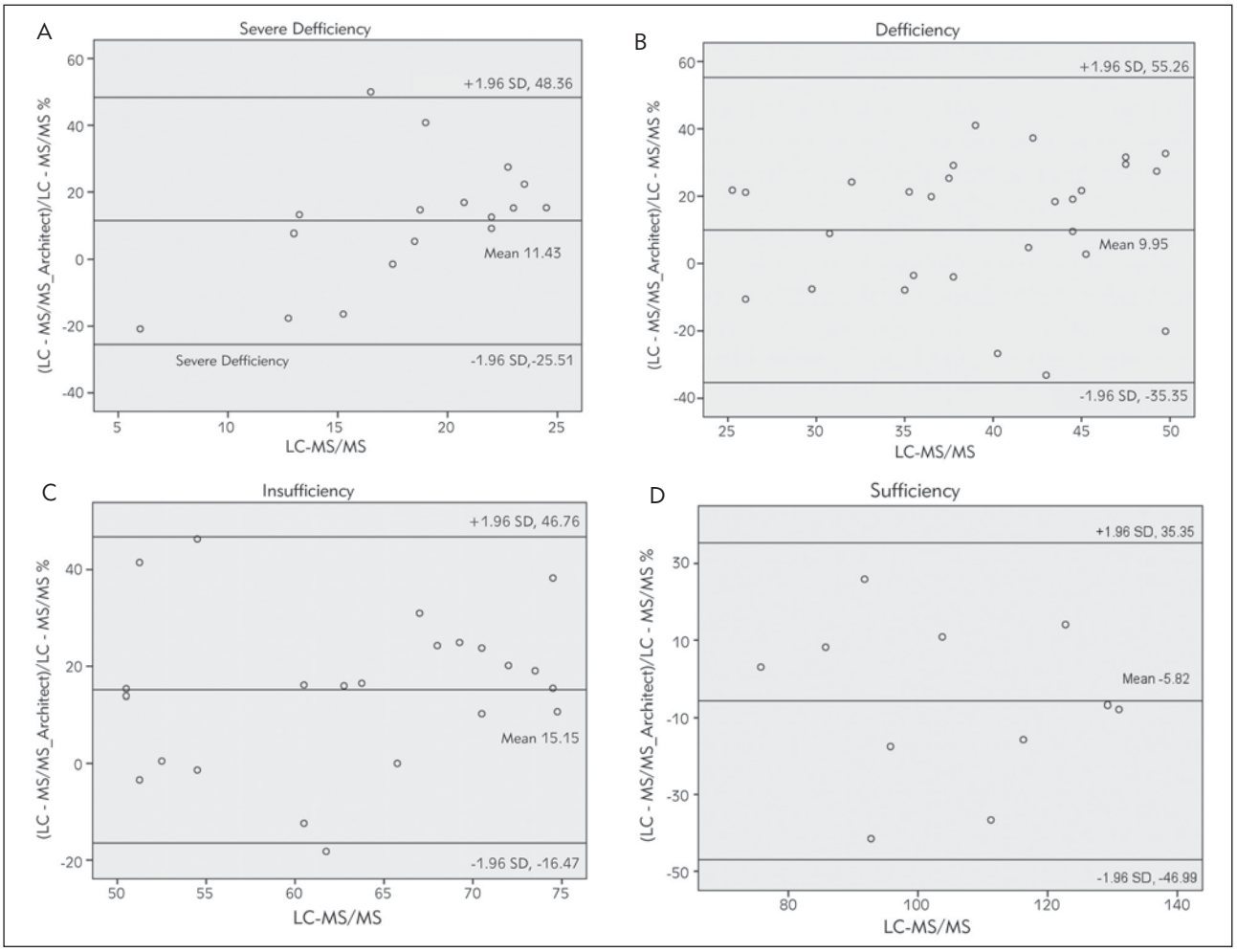

Figure 4 Bland-Altman plots of Architect 25-OH D and LC-MS/MS measurements for four different groups. Mean (thick solid line) – percentage bias (means of paired differences). Dashed lines demonstrate the 95% limits of agreement (bias ± 1.96 standard deviation).

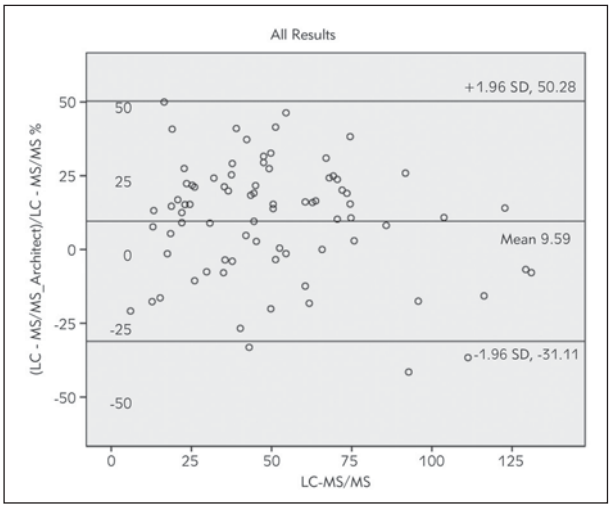

Figure 5 Bland-Altman plots of Architect 25-OH D and LC-MS/MS measurements for all results. Mean (thick solid line) - percentage bias (means of paired differences). Dashed lines demonstrate the 95% limits of agreement (bias ± 1.96 standard deviation).

ficiency groups respectively. However, one of them was < %5 that VSDP recommended. Concordance correlation analyses showed moderate agreement with LC-MS/MS with 0.918 concordance coefficient (95% CI, 0.87 to 0.94). Precision between two methods was 0.93 with the 0.98 corrected bias factor. Kappa coefficients of interrater agreement was found 0.738 (95% CI 0.604–0.848) indicated excellent agreement. Method comparison data were shown in *Table II*.

Discussion

Architect 25-OH D vitamin assay revealed excellent precision with a total coefficient of variance (CV %) <5%. When we compared to LC-MS/MS as the reference method, NIST traceable immunoassay showed a higher r-value (r^2=0.871) and a proportional bias (9.59%). Nonetheless, Architect 25-OH D vitamin assay revealed moderate agreement based on the value CCC (0.918). Two method showed excel-

Table III Agreement between two methods under the four levels of 25-OH D vitamin.

		Architect 25-OH D Vitamin Assay Number of samples (percentage %)				
		Severe deficiency	Deficiency	Insufficiency	Sufficiency	Sum
LC-MS/MS system Number of samples (percentage %)	Severe deficiency	17 (21.3%)	–	–	–	17 (21.3%)
	Deficiency	4 (5.1%)	21 (26.6%)	3 (3.8%)	–	28 (35.5%)
	Insufficiency	–	6 (7.6%)	17 (21.5%)	–	23 (29.1%)
	Sufficiency	–	–	2 (2.5%)	9 (11.4%)	11 (13.9%)
	Sum	21 (26.4%)	27 (34.2%)	22 (27.8%)	9 (11.4%)	79 (100%)

Kappa coefficient: 0.738; Standard error: 0.062 p=0.000

lent agreement; kappa=0.738. In terms of biases immunoassay deviated negative when compared to LC-MS/MS.

Many immunoassays are suitable to determine 25-OH D3 form. Cross reaction between total 25-OH D and 25-OH D2 is the major problem (6). In Abbott kit insert manufacturer claimed 25-OH D vitamin concentrations, at the level of 51.5 nmol/L assay has 80.5% and with the level of 140 nmol/L assay has 82.4% cross reactivity with 25-OH D2 (15). Abbott 25-OH D has 100% cross reactivity with 25-OH D3. However, failure to detect the 25-OH D2 form is not an important issue in our country, hence the supplements mostly used do not contain Vit D2. Anyway, we could not detect measurable amounts of 25-OH D2 in any patients' sera. This issue might be a problem in France or USA, because of treating D vitamin deficiency with D2 supplements (14). Probability of 3-epi-25-OH D3 epimers- presence in the pediatric population (22), we did not include children and patients given D vitamin treatment in this study. On the other hand, both immunoassay and LC-MS/MS cannot separate 3-epi-25(OH) D3 epimer from the D3 form, so negative bias for immunoassay systems cannot be explained on the 3-epimer contribution (14).

In the literature, immunoassay methods showed good agreement when compared reference methods, however revealed negative biases likely our study (11, 14, 22). Annema et al. (15) reported, Architect assay showed a good correlation (r=0.901) in both vitamin D-insufficient and vitamin D-supplemented subjects when compared to the LC- MS/MS method, but had negative biases (17.4% and 8.9%, respectively). Madenci (11) and Ozcan (23) revealed different negative biases for Access immunoassay system (-19.2% and -2.9% respectively), but had good concordance (0.901, 0.952) with LC-MS/MS systems. Topçuoğlu and her colleagues (24) found r and CCC value 0.957 and 0.916 with the bias of 9.5% for Access Total 25(OH) Vitamin D immunoassay on the Beckman Coulter Unicel DXI 800 analyzer.

Different from previous studies, we classified our results under four categories as; severe deficiency (< 25 nmol/L), deficiency (26–50 nmol/L), insufficiency (51–75 nmol/L and sufficiency (>75 nmol/L) (18–19). While the smallest bias between Architect 25-OH D and LC-MS/MS system was in deficiency group (9.95%), the biggest was determined in insufficiency group (15.15%) (*Figure 5*C). Architect 25-OH D vitamin assay determine some sufficient samples in insufficient area. This might lead unnecessary treatment for patients. However, World Health Organization (WHO) defined vitamin D insufficiency as serum 25-OH D is below 50 nmol/L (25). Some studies show a large variance in the plateau level of PTH, ranging from a serum 25-OH D of 45 nmol/L to 75 nmol/L (19). Lack of PTH test as a limitation of our study, sufficiency cut-off was taken as 75 nmol/L (26). Some experts recommended deficiency cut-off should be taken as < 50 nmol/L, based upon evidence related to bone health. Because of the necessity in older adults to minimize the risk of falls and fracture, the others suggested < 75 nmol/L as the deficiency cut-off (27). We divide <75 nmol/L results into three groups, to take the attention capability of immunoassay detection in these groups. Based on the LC-MS/MS data, 8.9% in deficiency, 7.6% of insufficiency, and 2.5% of sufficiency group data were misclassified as having vitamin D deficiency by the Architect 25-OH D vitamin immunoassay (*Table III*).

Annema et al. (15) revealed negative bias (-17.2%) for new restandardized Abbott Architect 25-OH Vitamin D assay, on the Vitamin D deficiency population. Nevertheless, Annema gave proportional small bias in the Vitamin D supplementation group. Evaluation of Abbott 25-OH D assay against LC-MS/MS system, Cavalier et al. (28) has evaluated; immunoassay showed good agreement with the bias of −3.2% (±1.96 SD 8.6 to −15.1). Both two studies dwelled on the misdiagnosis of Architect immunoassay systems in the deficiency groups similar to our study.

In our study, we pointed out NIST traceable Architect 25-OH D assay failed to diagnose deficiency and insufficiency. This issue might be a problem in several clinical conditions such as osteoporosis, osteomalacia etc. Recently, Cavalier et al. (29) reported the analytical and clinical validation of new developed Abbot Architect 25-OH D assay named as »5P02«. They revealed the new standardized 5P02 clearly improved diagnosis especially in osteoporotic patients and in patients from the intensive care unit.

LC-MS/MS is still currently the best method for the precise quantification of 25-OH D3 and 25-OH D2 (9).

However, reported in literature the LC-MS/MS systems have some major problems as matrix related issues, analytical, instrumental, epimeric and isobaric interferences. Insufficiency in separating epimers and isobars resulted in overlapping with Vitamin D metabolites in chromatograms (30). Accuracy of LC-MS/MS systems were performed by DEQAS with the acceptable biases varied from -8.9% to 1.9% in our study. In the DEQAS reports we could see a large discrepancy of CV% for LC-MS/MS systems varied from 2% to 10.6%. Biases for Architect 25-OH D vitamin assay from the EQAS external quality program were -12.2%, -8.6% and -0.9% during three month period.

Based on the present study findings, Architect 25-OH D vitamin assay can be used in routine 25-OH D measurements, still properly making the diagnosing of patients' status. Standardization efforts in improving immunoassay techniques do not seem to contribute too much to clinical diagnosis. Especially, in deficient/insufficient samples, laboratory experts should be aware of the misinterpretation of results. Our study offered an insight into D vitamin deficiency analyzing and further examinations.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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News from the Society of Medical Biochemists of Serbia

SOCIETY OF MEDICAL BIOCHEMISTS OF SERBIA THE TWENTY SECOND ANNUAL PROFESSOR IVAN BERKEŠ SCIENTIFIC CONFERENCE

Prepared by dr Snežana Jovičić
Liaison Member of the IFCC eNewsletter Working Group

Society of Medical Biochemists of Serbia (SMBS) and the Scientific Foundation »Professor Ivan Berkeš« organized for the twenty second time the annual Scientific Conference dedicated to the life and work of the esteemed prof. dr Ivan Berkeš, one of the founders of medical biochemistry in former Yugoslavia. The Conference is the occasion when the best graduate students of the Faculty of Pharmacy, University of Belgrade are awarded by the Scientific Foundation «Professor Ivan Berkeš«.

The 2019 Annual Scientific Conference «Professor Ivan Berkeš« was co-organized and hosted by the Faculty of Pharmacy, University of Belgrade. It gathered over 200 participants – students, older colleagues who were the students of Professor Berkeš, young graduated medical biochemists, and teachers of the Faculty of Pharmacy. Traditional guests were the family members of Professor Berkeš – his son and grandson, with their families. This traditional meeting of students and professors of the Faculty of Pharmacy, honoring the legacy of one of its most distinguished professors. was held on 28 November 2019. Prof. Nada Majkić-Singh, traditionally the organizer, with her opening words greeted the participants and reminded us of the history, the idea of foundation, and the significance of the Conference, as well as of the life and work of the Professor Ivan Berkeš, whom it honors. Following the welcoming address of the Dean of the Faculty of Pharmacy, prof. dr Slađana Šobajić, prof. Majkić-Singh, presented awards of the Foundation. This year's recipients were Tijana Vučković, Master of Pharmacy-Medical Biochemist, and Teodora Bulog, Master of Pharmacy. During the scientific part of the program, chaired by prof. dr Svetlana Ignjatović and prof. dr Vesna Spasojević-Kalimanovska, this year's defended doctoral thesis at the Departments of Medical Biochemistry and Toxicology of the Faculty of Pharmacy, University of Belgrade, were presented. This year, their doctoral theses also presented the colleagues from the Departments of Biochemistry of the Faculty of Medicine, University of Niš and of the Faculty of Medicine, University of Novi Sad. The first speaker was dr Tamara Gojković, with her thesis on the influence of the importance of cholesterol synthesis and abrsorbtion markers determination in healthy subjects and patients with ischemic heart disease. Dr Danijela Ristovski Kornic's thesis was about the determination of myeloperoxidase and lipoprotein subclasses distribution in children and adolescents with chronic kidney disease. Antidotal efficacy of newly synthesized oximes K203 and K027 in rats acutely exposed to dichlorvos was the topic of the lecture of dr Evica Antonijević. The lecture on the effect of melatonin on parameters of oxidative damage, inflammation and neoangiogenesis in the retina of rats with streptozotocin/nicotinamide induced type 2 diabetes mellitus by dr Branka Djordjević followed. The conference closed the doctorate of dr Dragana Milošević on the connection between selected parameters of complete blood count, glycoregulatuon and the presence of degenerative complications in type 2 diabetes mellitus.

Dean of the Faculty of Pharmacy, prof. dr Slađana Šobajić, and prof. Nada Majkić-Singh.

Laureat of the award Tijana Vučković, Master of Pharmacy-Medical Biochemist, with prof. Nada Majkić-Singh.

Laureat of the award Teodora Bulog, Master of Pharmacy, with prof. Nada Majkić-Singh.

Laureats, lecturers and chairs of the Conference, from left to right – Tijana Vučković, Teodora Bulog, prof. Svetlana Ignjatović, prof. Vesna Spasojević-Kalimanovska, dr Danijela Ristovski-Kornic, prof. Nada Majkić-Singh, dr Evica Antonijević, dr Dragana Milošević, dr Branka Djordjević, and dr Tamara Gojković.

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INSTRUCTIONS FOR AUTHORS

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Journal of Medical Biochemistry (J Med Biochem) is the official journal of the Society of Medical Biochemists of Serbia with international peer-review. The Journal publishes original scientific and specialized articles on all aspects of clinical and medical biochemistry, molecular medicine, hema tology, immunology, microbiology, virology, genetic epidemiology, drug measurement, evaluation of diagnostic markers, new reagents and laboratory equipment, reference materials, reference values, laboratory organization, automation and quality control, clinical metrology and all related scientific disciplines where chemistry, biochemistry, molecular biology and immunochemistry are dealing with the study of normal and pathologic processes in human beings. All manuscripts are reviewed and, after final decision, are classified in the following categories: a) personal view, b) review articles, c) original papers, d) professional papers, e) preliminary reports, and f) reviews of scientific meetings. There are also different reports and news, book reviews, reports on the activity of the Society of Medical Biochemists of Serbia and IFCC and other related organizations, letters to the editor, and information about innovations, new reagents and instruments in the field of clinical chemistry.

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The conventional use of statistical significance is $P \leq 0.005$. If a different significance level needs to be used, then the reasons for this must be clearly stated in the statistical method section.

Discussion

Statistical significance should not be equated to importance and P values should not be compared between different statistical tests. Association should not be interpreted as causation without additional evidence.

Problem Areas

Multiple comparisons can produce spurious and misleading significance values. The primary hypothesis should always be clearly stated, and associations detected by retrospective analysis should be interpreted with caution. Whenever possible a single overall statistical test should be applied first e.g. ANOVA. If this is not significant, then multiple comparisons must not be applied. If it is significant then some form of multiple range test can be applied. If a single overall test is not possible, then multiple comparisons must use a Bonferroni type significance level.

With paired data the differences between individual pairs of data and the variability of the differences are more important than the individual values. Graphical representation should also show the difference between individual pairs, e.g. by plotted lines joining the paired data points.

Standard regression analysis requires data points to be independent (repeated measurements are not independent). The independent variable should be measurements without significant error, e.g. age or time, and the points should be evenly distributed over the range and

have no outliers (this can be easily examined with a scatter plot). These requirements are rarely satisfied with biological data.

Method comparison using regression and correlation coefficients is inappropriate and should be performed using Altman and Bland difference plots (4). If a standard scatter plot and regression line are thought to be useful they can be given along with the Altman – Bland plot. Remember, if two methods are supposed to be measuring the same thing, then it is extremely likely they will be correlated so that a statistical tool correlation not tell you anything new.

If you are carrying out complicated statistical analyses, e.g. multivariate analysis, ROC analysis etc., then it is recommended that you seek advice from a statistician.

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2. Moher D, Schultz KF, Altman DG, for the CONSORT Group. The CONSORT statement: revised recommendations for improving the quality of reports of parallel-group randomization trials. Lancet 2001; 357: 1191–4.
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4. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986; 1 (8476): 307–10.

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