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Review paper
Revijski rad

GENETIC POLYMORPHISM OF MATRIX METALLOPROTEINASE 9 AND SUSCEPTIBILITY TO CHRONIC OBSTRUCTIVE PULMONARY DISEASE: A META-ANALYSIS

GENETSKI POLIMORFIZAM MATRIKS METALOPROTEINAZE 9 I OSETLJIVOST NA OPSTRUKTIVNU BOLEST PLUĆA: META ANALIZA

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Summary

Background: To systematically analyze the influence of genetic polymorphisms of matrix metalloproteinase 9 (MMP9) on susceptibility to chronic obstructive pulmonary disease (COPD).
Methods: Relevant literatures reporting MMP9 and susceptibility to COPD in PubMed, Web of Science, VIP, Wanfang and CNKI databases were searched using the key words »matrix metalloproteinases 9/MMP9, COPD/chronic obstructive pulmonary disease«. Data of eligible literatures were extracted and analyzed for the odds ratio (OR) and corresponding 95% CI.
Results: A total of 16 independent studies reporting MMP9-1562C/T and COPD patients were enrolled and analyzed. None of the genetic models revealed the relationship between MMP9-1562C/T and susceptibility to COPD. Subgroup analyses identified lower risk of COPD in Chinese population carrying the TT genotype for theMMP-9 rs3918242 relative to those carrying CT and CC genotypes (P=0.03, OR=0.67, 95% CI=0.46–0.97).
Conclusions: Chinese population carrying the TT genotype for the MMP-9 rs3918242 present lower susceptibility to COPD relative to those carrying CT and CC genotypes.

Keywords: MMP9, polymorphism, COPD, meta-analysis

Kratak sadržaj

Uvod: Sistematska analiza uticaja genetskih polimorfizama matriks metaloproteinaze 9 (MMP9) na osetljivost hronične opstruktivne bolesti pluća (HOBP).
Metode: Relevantna literatura koja izveštava o MMP9 i podložnosti HOBP u bazama podataka PubMed, Web of Scince, VIP, Wanfang i CNKI pretraživana je korišćenjem ključnih reči »matriks metaloproteinaze 9/MMP9, COPD/hronična opstruktivna bolest pluća«. Podaci iz kvalifikovane literature su ekstrahovani i analizirani za odnos šanse (OR) i odgovarajući 95% CI.
Rezultati: Ukupno je uključeno i analizirano 16 nezavisnih studija koje su izveštavale o pacijentima sa MMP9-1562C/T i HOBP. Nijedan od genetskih modela nije otkrio vezu između MMP9-1562C/T i osetljivosti na HOBP. Analize podgrupa identifikovale su niži rizik od HOBP kod kineske populacije koja nosi TT genotip za MMP-9 rs3918242 u odnosu na one koji nose CT i CC genotipove (P=0,03, OR=0,67, 95% CI=0,46–0,97).
Zaključak: Kineska populacija koja nosi TT genotip za MMP-9 rs3918242 predstavlja manju osetljivost na HOBP u odnosu na one sa CT i CC genotipovima.

Ključne reči: MMP9, polimorfizam, HOBP, meta-analiza

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# Xiaoping Yang and Yuanyuan Yu contributed equally to this work

Introduction

Chronic obstructive pulmonary disease (COPD) is a worldwide disease affecting approximately 3 million people. It is estimated that COPD will be the third leading cause of death by 2020 (1). As a chronic airway inflammatory disease, COPD is characterized by incomplete reversible airflow limitation, inflammatory cell infiltration, excessive mucus secretion, and airway remodeling (2). The precise molecular mechanism underlying the pathogenesis of COPD remains unclear. At present, it is generally believed that several risk factors are directly related to the pathogenesis of COPD, including host and environmental factors (3). Among environmental factors, smoking, exposure to chemicals, indoor and outdoor air pollution are risk factors for COPD (4). Host factors of COPD include antitrypsin-1, excessive deposition of extracellular matrix (ECM), corticosteroids, inflammatory stimuli, and metabolic imbalances (5, 6).

Matrix metalloproteinases (MMPs) are members of the metformin group and they are capable of degrading ECMs and regulating extracellular signaling networks (7). MMPs are important in COPD. They degrade matrix proteins (elastin, collagen) during the disease progression (8). In the past decade, abundant researches have been conducted to analyze the relationship between single nucleotide polymorphisms (SNPs) of MMPs and COPD risk in some populations (9–12). However, the conclusions were controversial. Some reports demonstrated the certain influence of MMPs on the occurrence of COPD (13–18), while others did not (9, 12, 19, 20). These conflicting findings may be explained by limited sample size, false positive results, and publication bias. In this paper, we performed a comprehensive meta-analysis to assess the influence of MMP polymorphisms on COPD.

Materials and Methods

Search strategy of literatures

Relevant literatures reporting the relationship between polymorphisms of MMP9-1562C/T and susceptibility to COPD in PubMed, Web of Science, VIP, Wanfang and CNKI databases were searched using the key words »matrix metalloproteinases 9/MMP9, COPD/chronic obstructive pulmonary disease«. There were no limitations on published languages. Citations in each literature were manually reviewed.

Inclusive and exclusive criteria

Inclusive criteria were as follows:1) Case-control studies conducted in humans; 2) Literatures published complete data or raw data that could calculate the genotype distribution; 3) COPD patients underwent diagnosis of pulmonary function index; 4) Literatures were conducted on the influence of polymorphisms of MMP9-1562C/T on susceptibility to COPD.

Exclusive criteria were as follows: 1) Repeated literatures; 2) Literatures lacked valid raw data; 3) Reviews, comments, animal experiments, researches on mechanism and case reports;4) The latest studies or those with a larger sample size were selected if data overlapping; 5) Unpublished data.

Flow diagram of literature searching was depicted in *Figure 1*.

Data extraction

Data were independently extracted and analyzed by two researchers, and the third one was responsible for solving any disagreement. Extracted data included: 1) Baseline data of literatures, including publication origin, first author, year or publication, and etc.; 2) Basic characteristics of subjects, including sample size, research country, genotype number and distribution, HWE in control group and etc.

Statistical analysis

Heterogeneity test was conducted by calculating odds ratio (OR) and the corresponding 95% CI with the I^2 test and the Q test. The pooled OR in studies lacking the heterogeneity was calculated by the fix-effects model. Otherwise, a random-effects model was used. Sensitivity analysis was performed by removing one study each time and analyzing the remaining in a combination way. The HWE of control genotype distribution was evaluated using the χ^2 test and $P<0.05$ considered as inequivalent. Publication bias was evaluated by depicting funnel plots and quantified by Egger's test. Data analyses were carried out using RevMan 5.3 and STATA12.0.

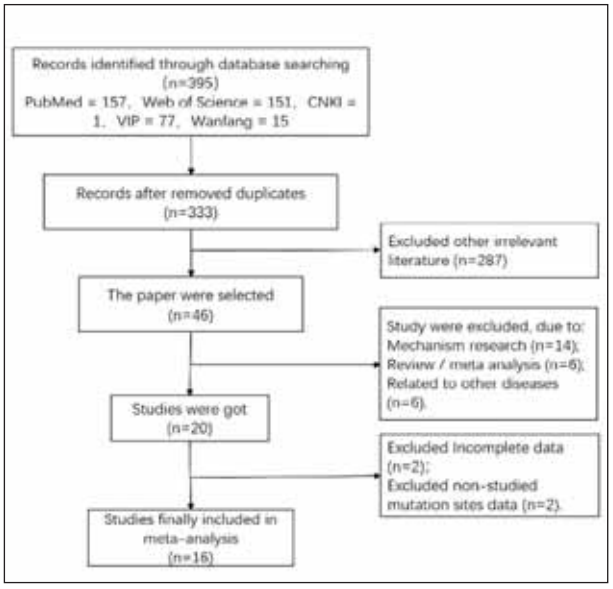

Figure 1 Flow diagram of the publication selection process.

Results

Baseline characteristics of eligible literatures

Initially, 157 literatures in PubMed, 151 in Web of Science, 1 in CNKI, 77 in VIP and 15 in Wanfang database were searched out, with a total of 395 literatures. A total of 62 replicates and 287 irrelevant literatures were excluded after the first-round screening. Subsequently, 14 literatures on mechanisms, 6 reviews, 6 literatures reporting other diseases, 2 literatures without complete data and 2 reporting other mutant sites were excluded. Finally, 16 literatures were included in this study (*Figure 1*).

Table I Main characteristics of studies included in the meta-analysis.

Author	Year	Country	Journal name/ publication origin	Genotyping methods	SNP loci (P_{HWE})	Sample size	Control	Sample
Zhou	2004	China	Chinese Medical Journal	PCR-sequence	rs3918242 (p_{HWE}=0.92)	100 (male=98, female=)	100 (male=99, female=1)	Whole blood
Isao Ito	2005	Japan	Am J Respir Crit Care Med	PCR-RFLP	rs3918242 (p_{HWE}=0.41)	84 (male=81, female=3)	85 (male=69, female=16)	
Zhang Rongbao	2005	China	Chin J Epidemiol	PCR-RFLP	rs3918242 (p_{HWE}=0.09)	147 (male=135, female=12)	120 (male=110, female=10)	Whole blood
Han	2006	Asian	Chin J Tuberc Respir Dis	PCR-RFLP	rs3918242 (p_{HWE}=0.48)	60	52	Whole blood
Testaigzi	2006	Caucasian	Int J Chron Obstruct Pulmon Dis	PCR-RFLP	rs3918242 (p_{HWE}=0.39)	123	262	Whole blood
Korytina	2008	Russia	Russian Journal of Genetics	PCR-RFLP	rs3918242 (p_{HWE}=0.53)	318	319	Whole blood
Shih-Lung Cheng	2009	Taiwan (China)	Biochem Genet	PCR-RFLP	rs3918242 (p_{HWE}=0.23)	184 (male=152, female=32)	212 (male=182, female=30)	Whole blood
H. Schirmer	2009	Brazil	Genetics and Molecular Research	PCR	rs3918242 (p_{HWE}=0.60)	89	97	Whole blood
Shih-Yup Lee	2010	Korean	Basic Science Investigations	PCR-sequence	rs3918242 (p_{HWE}=0.376)	301	333	Whole blood
Hua	2010	China	Int J Respi	PCR-RFLP	rs3918242 (p_{HWE}=0.04)	180 (male=142, female=38)	180 (male=130, female=50)	Whole blood
Korytina	2012	Russia	Molecular Biology	PCR-RFLP	rs3918242 (p_{HWE}=0.67)	391	514	Whole blood
Sarra Bchir	2015	Tunisia	Mol Diagn Ther	PCR-RFLP	rs3918242 (p_{HWE}=0.02)	138 (male=122, female=16)	216 (male=155, female=61)	Whole blood
Marja Stankovic	2016	Serbia	Environmental and Molecular Mutagenesis. PCR-RFLP	rs3918242 (pHWE=0.28)	86	100		Whole blood
Marja Stankovic	2017	Serbia	JOURNAL OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE	PCR-RFLP	rs3918242 (p_{HWE}=0.28)	122	100	Whole blood
Tan Jie	2017	China	Journal Of Inner Mongolia Medical Universit	PCR-RFLP	rs3918242 (p_{HWE}<0.001)	186 (male=92, female=294)	219 (male=105, female=112)	Whole blood
Lwona Gilowska	2018	Poland	BioMed Research International	PCR-RFLP	rs3918242 (p_{HWE}=0.33)	335 (male=87, female=248)	309 (male=229, female=80)	Whole blood

SNP=Single nucleotide polymorphism; HWE = Hardy-Weinberg equilibrium; pHWE=p-value of Hardy-Weinberg Equilibrium test in controls for each locus; PCR = polymerase chain reaction

Baseline characteristics of eligible literatures were listed in *Table I*. Briefly, 16 case-control studies were published from 2004–2018, including 13 studies published in English-language scientific journals and 3 in Chinese-language scientific journals. Genotyping methods were conducted using polymerase chain reaction (PCR), PCR-RFLP and PCR-sequence. Identification of single nucleotide polymorphisms (SNPs) was conducted by extracting blood samples of subjects.

In the 16 eligible literatures, 5 analyzed Chinese population, 1 analyzed Japanese population, 2 analyzed Russian population, 1 analyzed Brazilian population, 1 analyzed Korean population, 1 analyzed Tunisian population, 2 analyzed Serbian population, 1 analyzed Poland population, 1 analyzed Asian population and 1 analyzed Caucasian population. Sample size of each literature was 60-391.

Meta-analysis

A total of 2011 COPD patients and 2249 healthy controls were enrolled. The influence of MMP9 (-1562) C/T on susceptibility to COPD was assessed using different genetic models. No relationship was found between the CC *vs.*TT genotype of MMP9 rs391842 and susceptibility to COPD in the allele model (P=0.41, OR=1.12, 95% CI=0.86-1.47) (*Figure 2 A-C*). The other three genetic models obtained the same conclusion, including the dominant model (CC *vs.* CT+TT, P=0.13, OR=0.82, 95% CI=0.63–1.06), recessive model (TT *vs.* CC+CT, P=0.87, OR=0.97, 95% CI=0.65–1.43) and over-dominant model (CT *vs.* CC+TT, P=0.51, OR=1.13, 95% CI=0.79–1.61).

Subgroup analyses were performed based on the ethnic populations, involving Asian population (8 literatures), European population (3 literatures), Caucasian population (3 literatures) and African population (2 literatures). The random-effects model was utilized owing to the different degrees of hetero-

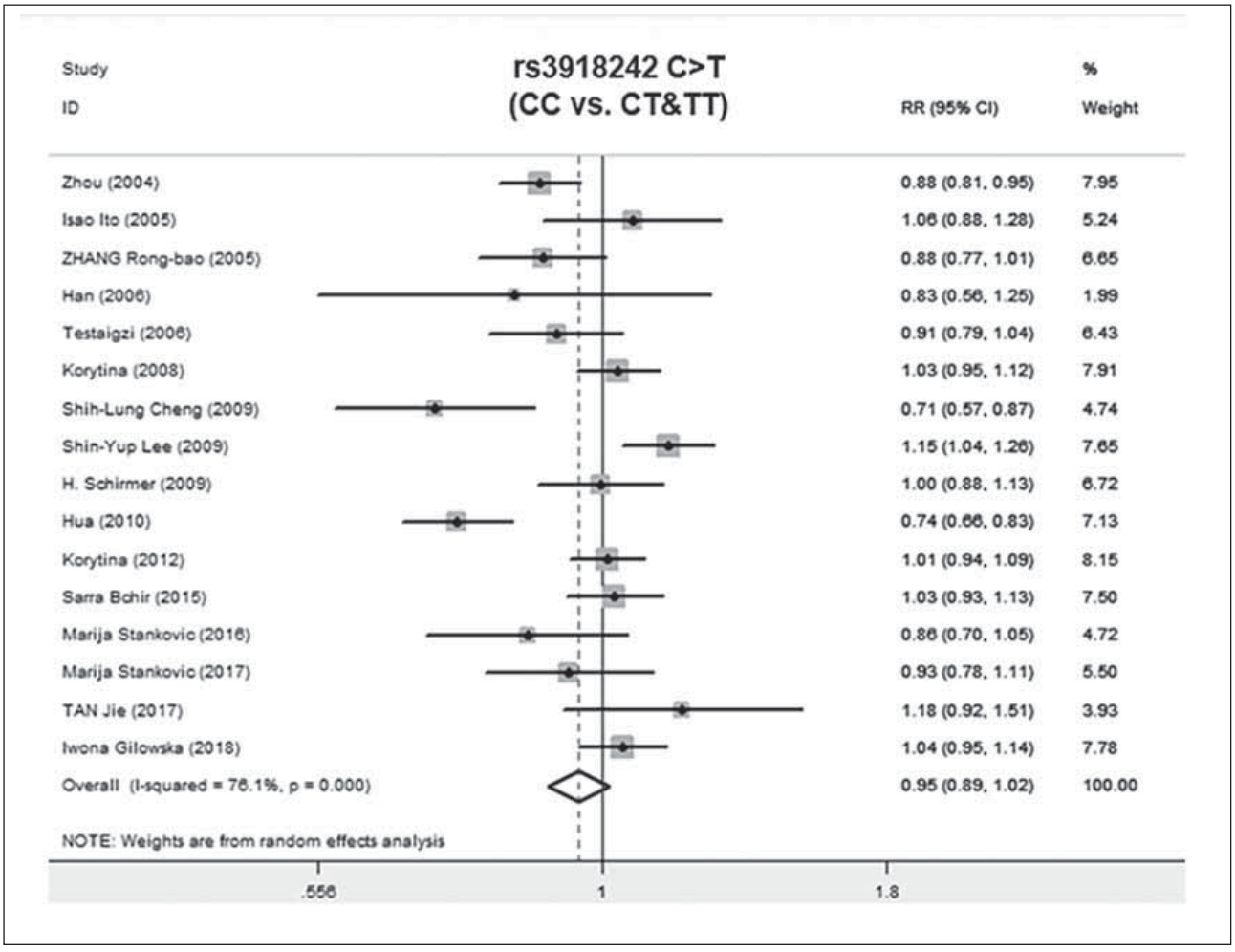

Figure 2A Forest map of the relationship between the SNP of MMP-9 rs3918242 and susceptibility to COPD.

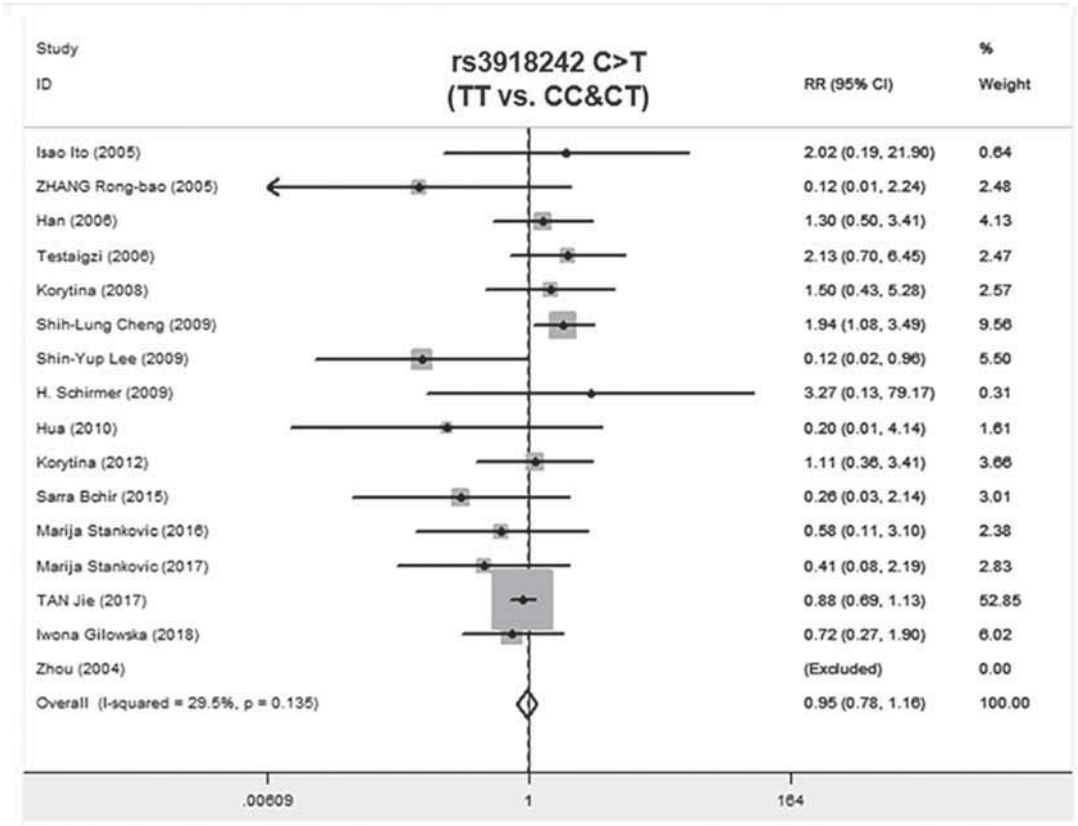

Figure 2B Forest map of the relationship between the SNP of MMP-9 rs3918242 and susceptibility to COPD.

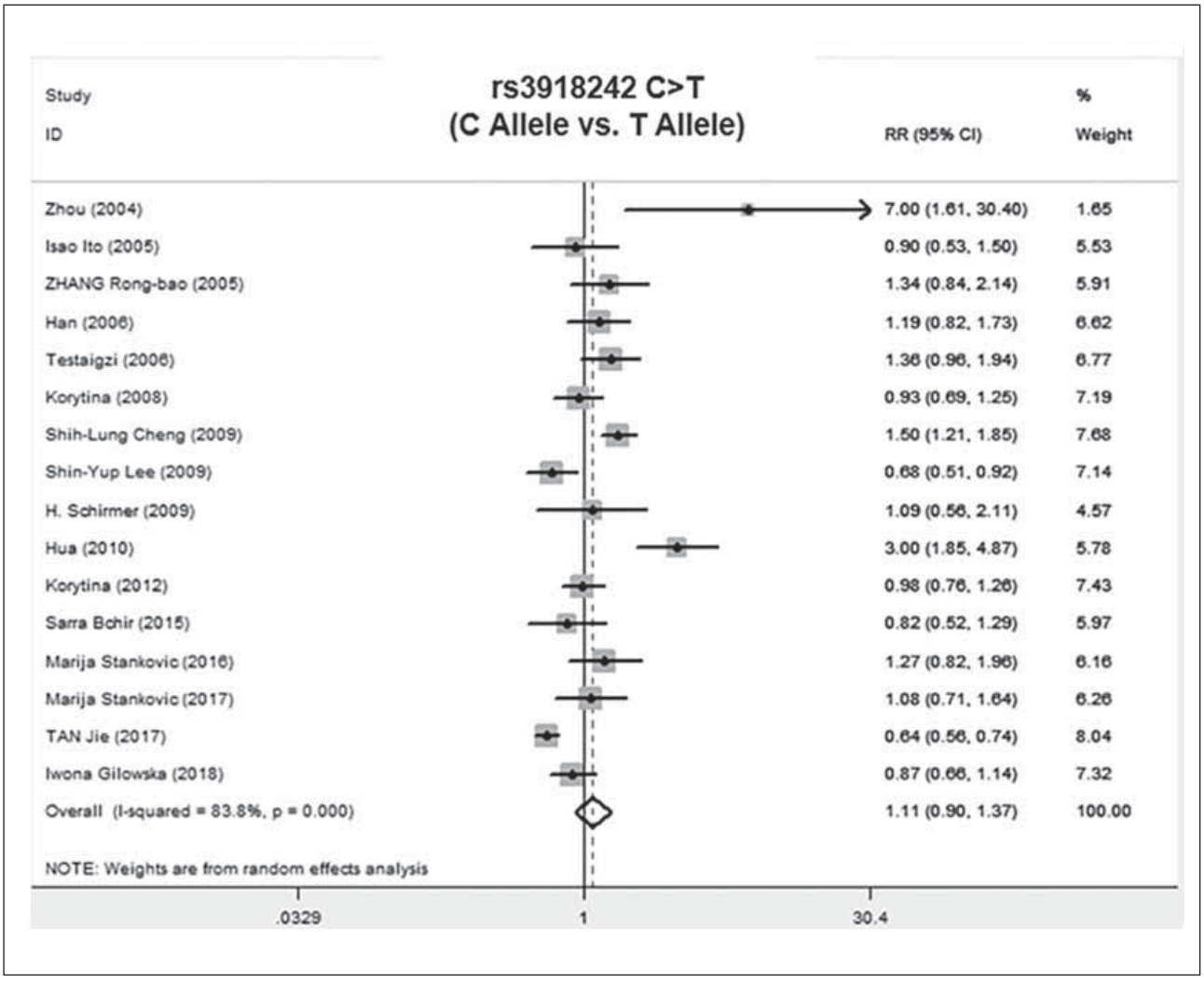

Figure 2C Forest map of the relationship between the SNP of MMP-9 rs3918242 and susceptibility to COPD.

geneity (I^2>50%, P<0.05). The data showed no relationship between MMP9 polymorphisms and COPD risk under the different genetic models (P>0.05) (*Figure 3 C-D*).

Subsequently, we individually analyzed the relationship between MMP9 polymorphisms and COPD in Chinese population, involving 5 literatures (15, 18, 21–23). Except for the recessive model (TT *vs*. CC&CT) analyzed by the fix-effects model (P=0.13, I^2=46%), the remaining were assessed using the random-effects model (I^2>50%, P<0.05) (*Figure 4*). Our data showed that Chinese population carrying the TT genotype for the MMP-9 rs3918242 was closely related to susceptibility to COPD relative to those carrying CT and CC genotypes (P=0.03, OR=0.67, 95% CI=0.46–0.97). Such a difference was not observed in the dominant model (CC *vs*. CT&TT), over-dominant model (CT *vs*. CC&TT) and allele model (C Allele *vs*. T Allele) (P>0.05) (*Figure 4*).

Heterogeneity and sensitivity analysis

Significant heterogeneity was identified in the dominant model, over-dominant model and allele model analyzing the relationship between MMP9 (-1562) C/T and susceptibility to COPD (all P<0.001). No remarkable changes in I^2 and P values were observed after removing a single study. In addition, sensitivity analysis was not altered by removing any study each time (data not shown).

In the subgroup analyses based on different ethnic populations, all genetic models showed the results of I^2>50% and P<0.05. We did not find any changes in I^2 and P values after removing a single study. Sensitivity analysis was not influenced by removing a single study (data not shown).

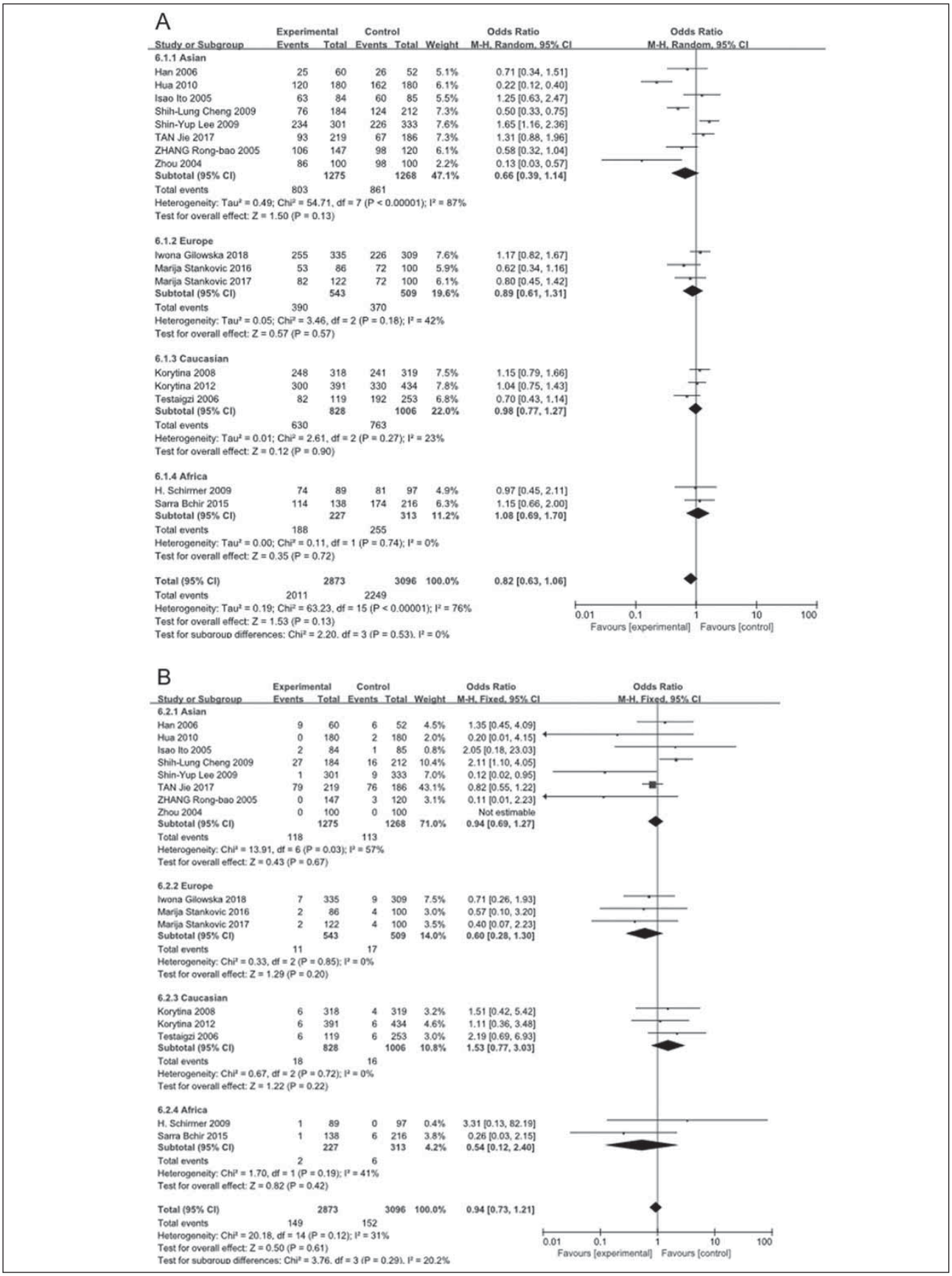

Figure 3A, B Subgroup analyses of the relationship between the SNP of MMP-9 rs3918242 and susceptibility to COPD in different regions and different pairs of comparisons.

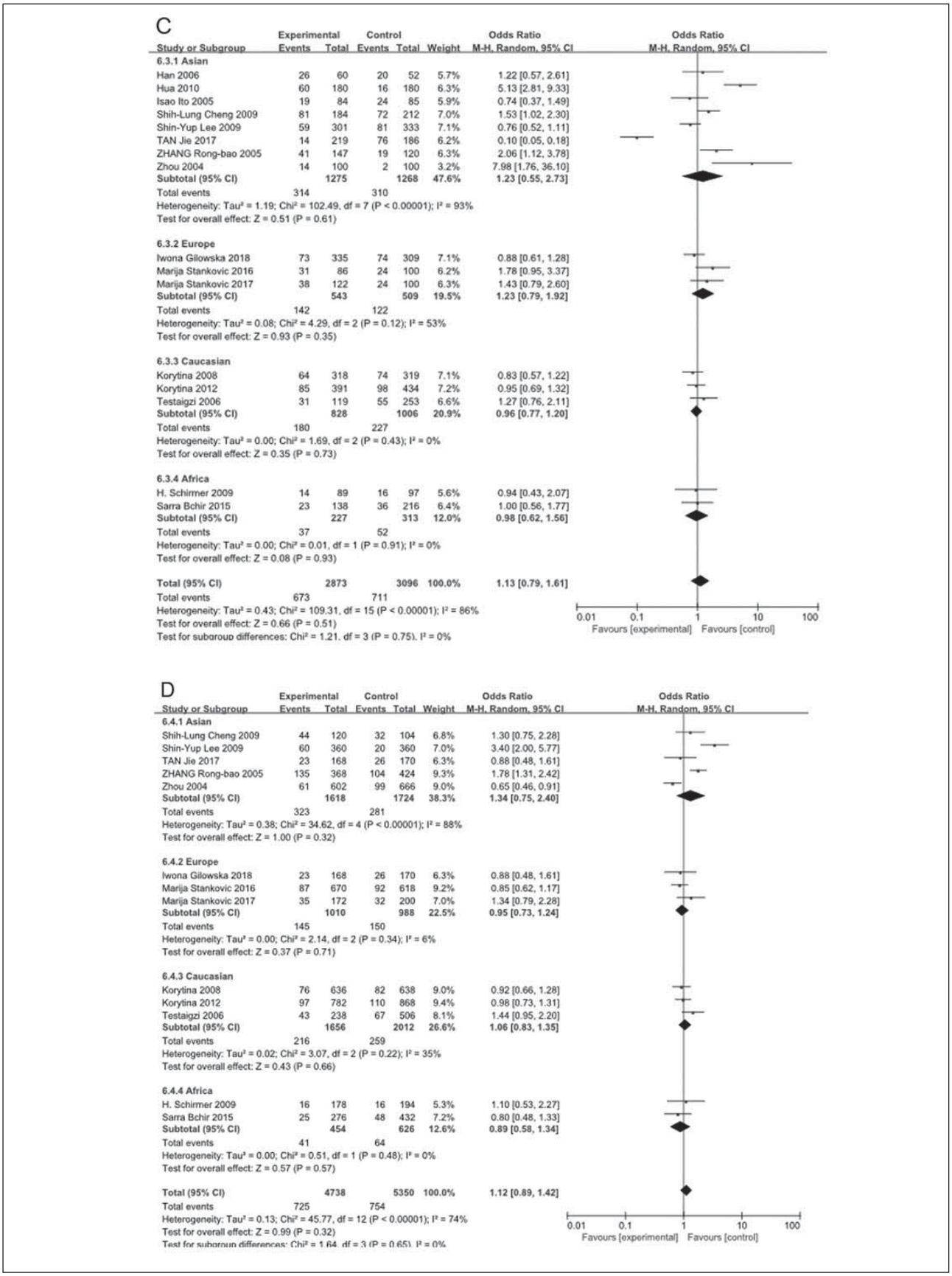

Figure 3C, D Subgroup analyses of the relationship between the SNP of MMP-9 rs3918242 and susceptibility to COPD in different regions and different pairs of comparisons.

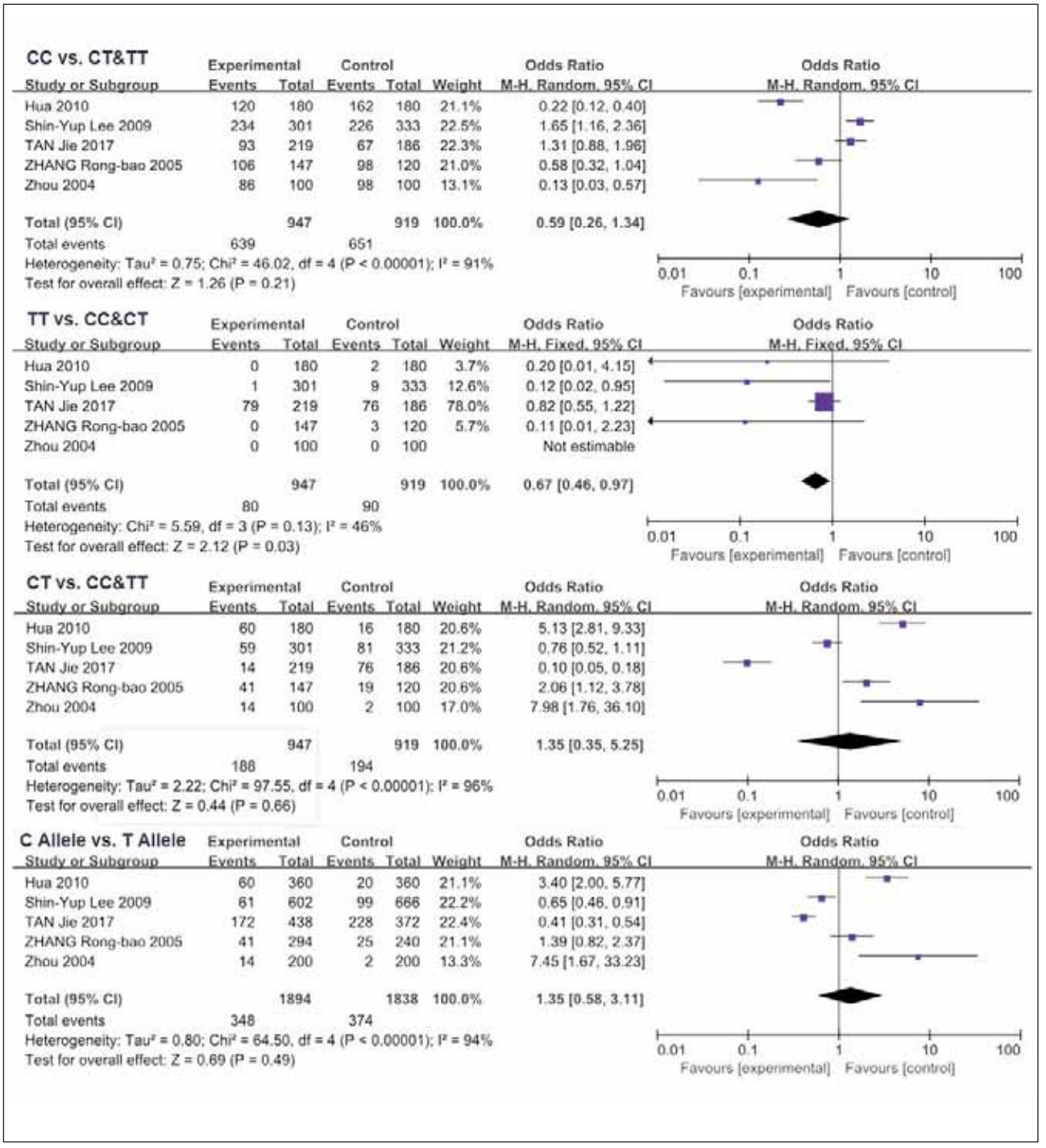

Figure 4 Subgroup analyses of the relationship between the SNP of MMP-9 rs3918242 and susceptibility to COPD in Chinese population and different pairs of comparisons.

Publication bias

A wide range of search strategies was carried out to minimize potential publication biases. After quantification using Egger's test, the data showed no publication biases between MMP9 (-1562) C/T and susceptibility to COPD in the three genetic models except for the allele model (CC *vs.* CT+TT, P=0.325; TT *vs.* CC+CT, P=0.541; CT *vs.* CC&TT, P=0.553; C allele *vs.* T allele, P=0.017) (*Figure 5*).

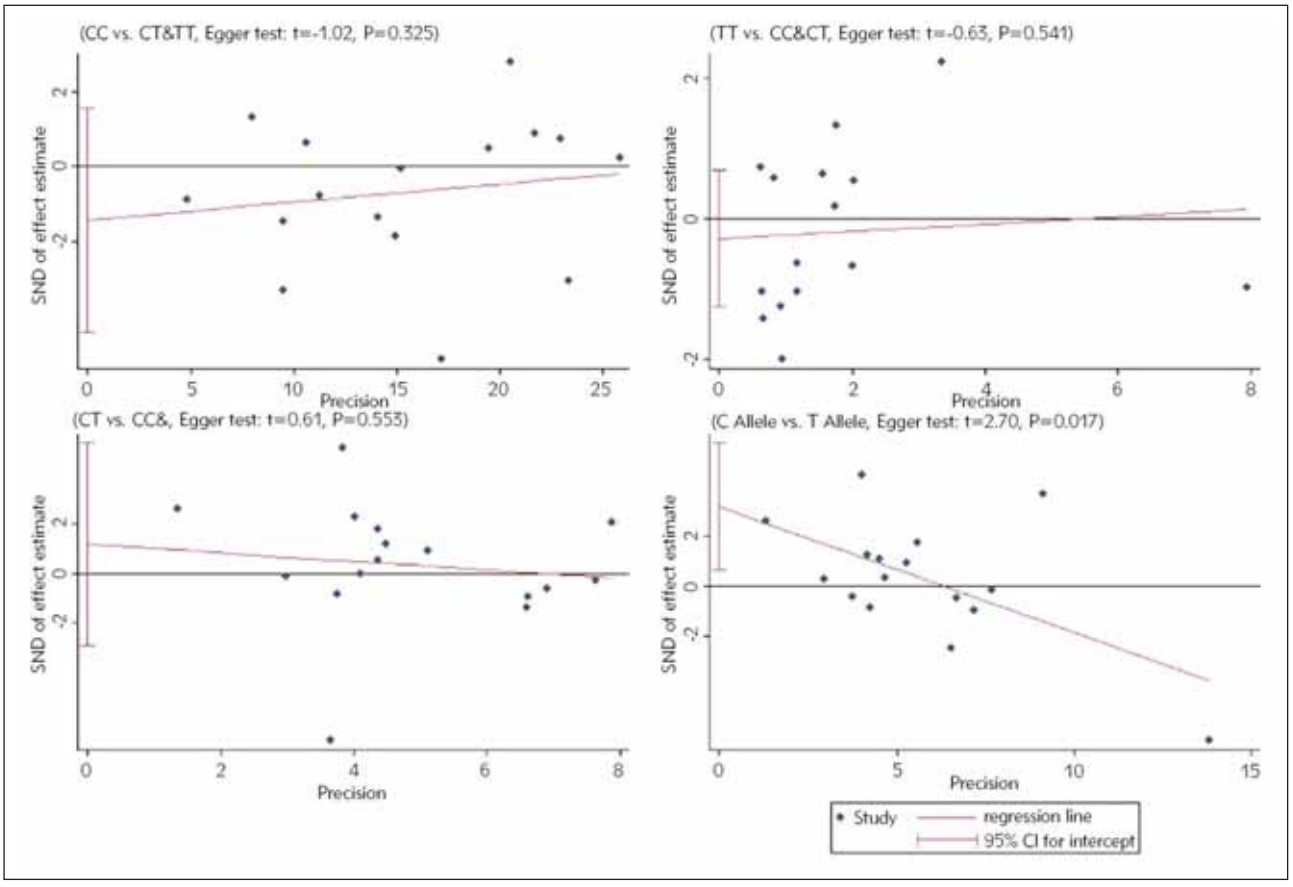

Figure 5 Subgroup analyses of the relationship between the SNP of MMP-9 rs3918242 and susceptibility to COPD in Chinese population and different pairs of comparisons.

Discussion

MMPs are a class of zinc-dependent endopeptidases that degrade major protein components of the ECM. They participate in development- and inflammation-related tissue remodeling and repair (7). MMP-9 (gelatinase B) can degrade ECM proteins, such as type IV collagen and gelatin (24). In addition, it exerts a vital role in airway inflammation and remodeling (25, 26). MMP-9 protects ventilator-induced lung injury by reducing infiltration of alveolar neutrophils (27).

COPD is a common respiratory disease characterized by airflow limitation. The pathogenesis of COPD is complex, involving inflammatory response, oxidant-antioxidant imbalance, and MMPs-induced proteolysis of the alveolar wall. MMP9, one of the most widely studied MMPs, decomposes most of the components of ECM by degrading structural proteins, such as collagen and elastin (28). Many studies have reported the involvement of MMP9 in the development of lung diseases (29). MMP9 polymorphism is identified to increase the susceptibility to respiratory diseases (30–33). Multiple SNPs of MMP9 have been discovered. Among them, C/T mutation on MMP9 (-1562) rs3918242 results in the increased promoter activity owing to the deletion of the transcriptional repressor binding site (34).

So far, studies focusing on the correlation between MMP9 -1562 C/T polymorphism and COPD are relatively rare and uncertain. Studies with a small sample size lack the statistical power and often lead to contradictory conclusions. Meta-analysis provides convincing evidences by calculating data extracted from multiple studies. In this paper, we obtained the conclusion that MMP9 -1562 C/T polymorphism was not associated with susceptibility toped in different putative genetic models. Subgroup analyses showed that Chinese population carrying the TT genotype for the MMP-9 rs3918242 are risky of COPD relative to those carrying CT and CC genotypes.

Inconsistent with our results, some studies have demonstrated that the MMP9 -1562 C>T polymorphism indeed influences COPD risk. Zhou et al. (35) illustrated that the TT genotype of MMP9 -1562 C/T polymorphism is a genetic risk factor for severe COPD. Korytina et al. (36) have indicated the correlation between the TT genotype of MMP9 -1562 C/T polymorphism and COPD severity. Similarly, a study conducted in Russia showed a significant difference in the frequency distribution of MMP9 -1562 C>T

among COPD patients with different severity levels (37).

Some shortcomings in this study should be pointed out. First of all, many complex factors were not adjusted, such as gender, age, and smoking history. Secondly, some studies (16, 20, 23) had small sample sizes and did not have enough capacity to detect the risk of COPD. Thirdly, the lack of raw data limited the further analysis of the potential interactions between genetic risks and environmental factors in COPD. Studies with large sample sizes in a multicenter hospital are required for further validation.

Conclusions

Chinese population carrying the TT genotype for the MMP-9 rs3918242 present lower susceptibility to COPD relative to those carrying CT and CC genotypes.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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EVALUATION OF OXIDATIVE STRESS, 3-NITROTYROSINE, AND HMGB-1 LEVELS IN PATIENTS WITH WET TYPE AGE-RELATED MACULAR DEGENERATION

PROCENA OKSIDATIVNOG STRESA, NIVOA 3-NITROTIROZINA I HMBG-1 KOD PACIJENATA SA VLAŽNOM VRSTOM MAKULARNE DEGENERACIJE

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Summary

Background: This study aims to compare serum HMGB-1, 3-nitrotyrosine (3-NT), TAS, TOS, and OSI levels in Wet-type Age-Related Macular Degeneration (wAMD) patients and healthy controls to determine the correlation of these parameters with each other.
Methods: Thirty patients with Wet-type Age-Related Macular Degeneration (wAMD) and 27 healthy adults, as controls were enrolled in the study. We determined the TAS and TOS levels in serum samples of both groups using commercial kits on a microplate reader. Serum HMGB-1 and 3-NT levels were measured with the enzyme-linked immunosorbent assay method.
Results: HMGB-1 levels were significantly higher in the patient group (137.51 pg/mL, p=0.001), while there was no difference between the two groups in serum 3-NT levels (p = 0.428). A statistically significant difference found in the levels of TOS and OSI (p = 0.001 and p = 0.045, respectively) between the patients and controls, however, no significant difference was observed between the groups in terms of TAS levels (p = 0.228).
Conclusions: Oxidative stress and HMGB-1 levels were increased in wAMD patients and enhanced oxidative stress

Kratak sadržaj

Uvod: Ova studija ima za cilj da uporedi serumske nivoe HMBG-1, 3-nitrotirozina (3-NT), TAS, TOS i OSI kod pacijenata sa vlažnom starosnom makularnom degeneracijom (vAMD) i zdravih kontrola kako bi se utvrdila korelacija ovih parametri međusobno.
Metode: Trideset pacijenata sa vlažnom starosnom makularnom degeneracijom (wAMD) i 27 zdravih odraslih osoba su uključene u studiju. Određivali smo nivoe TAS i TOS u uzorcima seruma obe grupe pomoću komercijalnih kompleta na čitaču mikroploča. Serumski nivoi HMBG-1 i 3-NT mereni su pomoću enzimsko-imunolo ke analize.
Rezultati: Nivoi HMBG-1 bili su značajno veći u grupi pacijenata (137,51 pg/mL, p = 0,001), dok nije bilo razlike između dve grupe u serumskim nivoima 3-NT (p = 0,428). Statistički značajna razlika pronađena u nivoima TOS i OSI (p = 0,001 i p = 0,045, respektivno) između pacijenata i kontrola, međutim, nije primećena značajna razlika između grupa u pogledu nivoa TAS (p = 0,228).
Zaključak: Oksidativni stres i nivo HMBG-1 su povećani kod pacijenata sa wAMD-om, a pojačani oksidativni stres može biti povezan sa povećanom nekrozom tkiva i upalom. Stoga

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may be associated with increased tissue necrosis and inflammation. Thus administration of antioxidant treatment in addition to routine therapy should be considered in wAMD.

Keywords: HMGB-1, 3-Nitrotyrosine, TAS, TOS, OSI, wet type AMD

bi primenu antioksidativnog tretmana pored rutinske terapije trebalo razmotriti wAMD-u.

Ključne reči: HMBG-1, 3-nitrotirozin, TAS, TOS, OSI, mokri tip AMD

Introduction

Age-related macular degeneration (AMD) is one of the most common causes of central vision loss in the elderly (1). As a result of abnormalities in the Bruch's membrane, retinal pigment epithelium (RPE), photoreceptor, and choroid complex, AMD can often result in regional atrophy and/or neovascularization. For this reason, the disease is basically divided into two subgroups: wet and dry type (2).

AMD, is associated with various pathological factors such as chronic oxidative stress, decrease in autophagy, and chronic inflammation (3). Although several local and systemic inflammatory molecules are recommended as markers for AMD, a specific and reliable marker has not been established.

High Mobility Group Box-1 (HMGB-1), a non-histone protein, is found in large amounts in the nucleus and plays a role in DNA transcription, replication, and repair (4). HMGB-1 is a newly described inflammatory molecule that is a mediator of endotoxin shock and is elevated in the blood of septic patients (5). The expression of HMGB-1 increases significantly in dry-type AMD and also increases the aging rate of RPE cells (6, 7). In addition, the application of recombinant HMGB-1 initiates a pro-inflammatory response in human endothelial cells (5). Considering the importance of these molecules and inflammatory status in AMD pathogenesis, it is understood that HMGB-1 is important in the prognosis and pathogenesis of this disease. However, apart from a few limited studies, which are all based on cell culture, there are no studies investigating the role and level of serum HMGB-1 in macular diseases in humans.

Recent research has provided a lot of evidence on the relationship between inflammation drusen and oxidative stress in the development of wet type AMD (wAMD). During inflammation, nitric oxide NO plays an important role and it may be released in high amounts with stimulation. Higher levels of NO in plasma have been reported in AMD (8). NO is an important physiological regulator but it can form other reactive intermediates such as nitrite (NO_2), peroxynitrite (ONOO), which can change the structure of tissues. Peroxynitrite changes the structure of molecules by adding a nitro group to the phenolic ring of tyrosine in proteins or in free form. Thus, 3-nitrotyrosine (3-NT) is a specific marker for oxidative damage to proteins and NO production (9). In this case, 3-NT levels in AMD patients can provide information on pathogenesis and damage.

The formation and removal of free radicals in the living system occur in perfect balance, and the organism is not affected by these reactive molecules if this sensitive condition stabilizes the oxidative balance. If one of the steps to achieve this balance is interrupted, it causes oxidative stress that can lead to cell damage. Therefore, while oxidative stress is so important in AMD pathogenesis, plasma oxidant and antioxidant levels also gain importance. The total effects of all antioxidants in serum and other body fluids are measured according to the total antioxidant status (TAS), and the total effects of oxidants are determined by the total oxidant status (TOS). In addition, a global oxidative stress index (OSI) reflecting both oxidative and antioxidant responses is informative (10).

The relationship of AMD, whose inflammatory base has been demonstrated in many studies, with HMGB-1, an inflammation-related molecule, has not been previously examined and we aimed to contribute to the literature on this matter. We measured 3-NT levels which is a marker of nitrosative stress and hasn't studied in patients with AMD; and TOS, TAS, and OSI levels which is an indicator of oxidative stress that has been shown to be associated with AMD before, in serum of wAMD patients.

Material and Methods

Study population

The study was approved by the Noninvasive Ethics Committee of Niğde Ömer Halisdemir University (The Decision Number: 2020/52) and written consent was obtained from each patient before taking the first blood samples. The study was conducted in accordance with the principles of the Helsinki Declaration. 30 patients with wAMD and 27 healthy controls constituted the study group of our research. All individuals included in the study groups were over 50 years old. Patients with other eye pathologies, such as glaucoma, uveitis, who had undergone intraocular surgery, smoking, and systemic disease were not included in the study. The diagnosis of wAMD was carried out by examination, fundus photographs, optical coherence tomography, and fundus fluorescein angiography. The control group of our study consisted of individuals without the

systemic disease who had a complete healthy eye examination.

Blood sample collection

Five mL of venous blood samples were taken into sterile biochemistry tubes with gel from the patient and control groups. Then serum samples were obtained by centrifuging at 1600xg for ten min. Sera samples were kept at -80 °C until analysis day.

Determination of Serum HMGB-1 and 3-Nitrotyrosine Levels

HMGB-1 and 3-Nitrotyrosine levels were measured with ELISA kits (Cusabio; CSB-E08223h and Yehua; YHB0033Hu-96, respectively) according to the instructions of the manufacturers.

Measurement of TAS and TOS levels in the serum samples

TOS and TAS levels were measured using commercially available kits (Rel Assay, Mega Tıp, Gaziantep, Turkey) by Erel's colorimetric method. The results were expressed mmol Trolox equivalents/L for TAS; μmol H_2O_2 equiv/L for TOS (11, 12).

Determination of oxidative stress index

OSI was calculated through the following formula:

OSI =[TOS (mmol Trolox equivalent/L)/TAS (μmol H_2O_2 equivalent /L)] X100

Statistical analysis

Statistical analysis was made with IBM SPSS Statistics version 23 software. The normal distribution of the variables was assessed with the Shapiro–Wilks test. Statistical data were stated as mean ± standard deviation ($\bar{x}$ ± SD), or median and inter-quartile range for normally and non-normally distributed variables, respectively. Comparisons between groups for continuous variables were performed using the Student t-test (normal distribution) or the Mann-Whitney U test (non-normal distribution). Data were considered statistically significant at a value of $p<0.05$.

Results

As can be seen in *Table I*, there were 13 females and 17 males in the patient group while 10 females and 17 males in the control group. The mean age was 71.13 ± 9.37 years in the patient group, and 66.51 ± 8.09 years in the controls. There was no significant difference between the groups in terms of gender ($p = 0.138$) and age ($p=0.053$).

Table I Demographic data of patients and the control group.

	Patient Group (N=30)	Control Group (N=27)	*p*
Mean Age (year±SD)	71.13±9.37	66.51±8.09	0.053
Sex (Female/Male)	13/17	10/17	0.138

SD, standard deviation; * $p < 0.05$

Table II HMGB-1, 3-NT, TAS, TOS, and OSI levels in patients with wAMD and the control group.

	Contor Group (N=27)	Patient Group (N=30)	*p*
HMGB-1 (pg/mL)	95.93 (92.90–114.63)	137.51 (126.47–166.17)	0.001*
3-NT (nmol/L)	900.33±197.47	951.53±236.62	0.428
TAS (mmol Trolox Equiv. /L)	1.73 (0.73–2.96)	2.00 (1.06–2.89)	0.228
TOS (μmol H_2O_2 Equiv. /L)	7.93 (6.99–9.18)	17.03 (7.11–30.79)	0.001*
OSI (%)	0.56 (0.25–1.13)	0.79 (0.56–1.42)	0.045*

HMGB-1, TAS, TOS, and OSI levels were expressed as the median (min-max), whereas 3-NT levels as the mean ± standard deviation. HMGB-1, High Mobility Group Box-1; 3-NT, 3-nitrotyrosine; TAS, Total antioxidant status; TOS, Total oxidant status; OSI, Oxidative stress index. * $p < 0.05$.

Table II summarizes the statistical comparison of HMGB-1, 3-NT, TAS, TOS, and OSI levels determined in the sera samples of the study groups. Median levels of HMGB-1 were 137.51 pg/mL in wAMD patients and 95.93 pg/mL in controls. The median 3-NT levels were 951.53 nmol/L in the patient group and 900.33 nmol/L in the control group. The levels of HMGB-1 were meaningfully higher in the patient group than in the control group while there was no difference between the two groups in terms of 3-NT levels. Furtheremore, while there was no significant difference between TAS values, TOS and OSI levels were significantly higher in the patients then the control (*Table II*). Lastly, we found a significant correlation coefficient between the OSI and HMGB-1 levels measured in the patient group ($r^2 =0.6$; $p=0.0001$).

Discussion

The retina is one of the tissues with the highest oxygen consumption in humans (13). Indeed, Hanus et al. (14) reported that RPE cells undergo necrosis due to increased oxidative stress. As it is known, ROS attacks the cells, especially the lipids in the

membrane, DNA, RNA, and proteins by disrupting their functions. Such a situation may increase the necrosis of RPE cells in wAMD patients. It has been suggested that RPE cell injury and death caused by oxidative stress promote the release of damage-associated molecular pattern molecules (DAMP) due to intracellular and extracellular damage. DAMP can stimulate immune and inflammatory responses and lead to AMD progression (15). Consistent with these findings, we found that levels of TOS and OSI were higher in wAMD patients in our study ($p < 0.05$).

HMGB-1 is a large DAMP molecule that releases from the nucleus during necrosis and is secreted into the extracellular matrix and induces inflammation (16). It has been reported that HMGB-1 is secreted from also necrotic RPE cells and induces inflammation (17). HMGB-1 is a non-histone DNA binding protein that was first described by Goodwin and Johns in 1973 (18). It plays an important role in DNA transcription, replication, and repair also contributes to the collection of nuclear proteins. In the cytoplasm, it functions as a signal regulator and takes a role in the inflammatory cascade, and acts as a pro-inflammatory cytokine in the extracellular environment (19). HMGB-1 serum levels, which have an important role in mediating inflammation, have been shown to be high in various diseases (20). Furthermore, HMGB1plays an important regulatory role in angiogenesis and it affects many angiogenesis-related conditions such as proliferative diabetic retinopathy, cancer, and wound healing via p53. Thus, HMGB1 is a promising therapeutic target in many malignancies such as prostate and colon cancer, and epidermal tumors (21–24).

HMGB-1, which is intertwined with inflammation and oxidative stress, also attracts attention in the field of ophthalmology. Sakamoto et al. (25) showed that HMGB-1 induced cell death of retinal ganglion cells with increased oxidative stress in the rat retina. Chang et al. (26) reported that the release of HMGB-1 peptides caused by hypoxia-regulated the production of angiofibrogenic factors in RPE cells, thereby contributing to the pathogenesis of hypoxia-associated diabetic retinopathy. Watanabe et al. (27) reported that the extracellular HMGB-1 contributes to ocular inflammation in autoimmune uveoretinitis. Murakami et al. (28) investigated the vitreous of retinitis pigmentosa (RP) patients and found a significant release of extracellular HMGB-1 associated with necrotic cell death and they reported that HMGB-1 could be a new therapeutic target in RP. In a study on pluripotent stem cells from healthy individuals, Sun et al. (7) showed that HMGB-1 caused RPE cell aging. They also reported that HMGB-1 upregulated Caveolin-1, which is also associated with RPE cell aging. With these results, they demonstrated that HMGB-1 may be associated with RPE cell aging and may play a role in AMD pathogenesis, thus suggesting that it is a potential therapeutic target to prevent the progression of RPE cell aging. We found significantly higher HMGB-1 levels in wAMD patient sera (137.51 pg/mL) compared to the control group (95.93 pg/mL) in our study and to our knowledge this was the first study in the literature to examine the HMGB-1 level in the serum of wAMD patients ($p=0.001$).

Nitrotyrosine is an indicator of the production of reactive nitrogen species and this end product causes damage to cellular components. Therefore, tyrosine nitration has been recently investigated in the pathogenesis of diseases (29). In a study by Thomson (30), 3-nitrotyrosine levels were found to be associated with higher cardiovascular risk. Bandookwala et al. (31) reported that the change in the nitrotyrosine profile occurred in the presymptomatic stage of the neurodegenerative diseases and this could be used for early diagnosis. Qian et al. (32) found that nitrotyrosine level was associated with mortality in patients with acute kidney injury. Khan et al. (33) reported that serum 3-NT has increased in SLE patients and preventing nitrosative damage could be new treatment methods for some diseases. Nitrosative stress also attracts attention in the field of ophthalmology. It was shown that the formation of nitrotyrosine significantly increases in the diabetic retina and is an inflammatory element in the development of diabetic retinopathy (29). Wang et al. (34) reported that 3-nitrotyrosine levels in blood serum were higher in type 2 diabetic patients than in healthy individuals. In RP, nitrosative stress has been reported to be effective in cone photoreceptor deaths (35). In another study, it has been reported that retinal nitrosative stress, plays an important role in retinal ganglion cell loss in glaucoma (36). In a study in the tissue culture, it was reported that the non-enzymatic nitration of the RPE basement membrane may have significant harmful effects on the RPE function and they found that the accumulation of 3-nitrotyrosine increased with the age of the patient on the human Bruch membrane (8). Wang et al. (37) reported that nitrite-induced changes in the normal basement membrane can mimic the harmful effects of the aging Bruch membrane on RPE function. In line with these findings, in our study, in which we planned to evaluate the importance of 3-nitrotyrosine in AMD pathogenesis, we found that the level of serum 3-nitrotyrosine did not show a statistically significant difference between the two groups ($p=0.428$). Although our findings indicate that nitrosative stress is not significantly effective in patients with AMD, different results can be obtained in large-scale studies conducted with study groups consisting of more individuals, thus we consider that additional studies are needed.

Free radicals are reactive molecules and these molecules damage cell components, and oxidative stress occurs (38). The formation and removal of free radicals in the body occur in perfect balance,

imbalance between these two processes causes oxidative stress that can cause cell damage in the living systems. Oxidative stress plays an important role in the pathogenesis of many diseases (39). Icel et al. (40) reported that TOS level was found to be significantly higher while TAS was found to be low in the study of diabetic rats. Oruç et al. (41) found that the TOS value was high while the TAS value was found low in humorous aqueous of pseudoexfoliation patients, and they reported that normal these levels may slow the progression of pseudoexfoliation syndrome. In a study conducted by Altını ık et al. (42) in patients with retinal vein occlusion, it was found that the oxidative stress index in humor aqueous was high, while TAS values were low. Recently, the relationship between AMD and oxidative stress has drawn attention in a study (13, 14). Elbay et al. (43) investigated wAMD patients and reported that serum TOS levels increased and TAS values decreased in wAMD patients. In the literature, there is no other study examining TOS and TAS values in wAMD patients. In our study, it was found that TOS levels increased and there is no difference between TAS values. We believe that our study is the second study on TOS and TAS values in AMD patients. As a result of high TOS values, OSI was found significantly higher in the patient group.

Conclusion

We have found significantly higher OSI in wAMD patients and thus oxidative stress may have important roles in AMD pathogenesis. The high TOS and normal TAS values that we have found indicate increased oxidative stress and a sufficient antioxidant system in wAMD patients. Furthermore, increased HMGB-1 levels in patients with wAMD and determined the significant correlation between OSI and this parameter may suggest that increased tissue necrosis and inflammation in these patients are associated with OSI. However, further studies are needed to examine the effects of OSI and inflammation on the pathogenesis of wAMD and their relationship in this disease.

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Declarations

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Research involving Human Participants and/or Animals

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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DOWNREGULATION OF MAPK/MAK/MRK OVERLAPPING KINASE 1 IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF PEDIATRIC PATIENTS WITH TYPE 1 DIABETES MELLITUS

NISHODNA REGULACIJA MAPK/MAK/MRK PREKLAPAJUĆE KINAZE U MONONUKLEARNIM ĆELIJAMA PERIFERNE KRVI PEDIJATRIJSKIH PACIJENATA SA TIP1 DIIABETES MELLITUS-OM

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Summary

Background: Type 1 diabetes mellitus (T1DM) is one of the most common endocrine diseases in children. T-cell autoreactivity toward β-cells is controlled by significant changes in metabolism of T cells. Mammalian target of rapamycin (mTOR) is an important intracellular regulator of metabolism and cell growth. MAPK/MAK/MRK overlapping kinase 1 (MOK1) is one of the less known regulators of mTOR. We sought to investigate if MOK1 and mTOR mRNA levels in peripheral blood mononuclear cells (PBMCs) of T1DM pediatric patients are different compared to healthy subjects.
Methods: This study included 172 adolescents with T1DM and 36 healthy adolescent volunteers designated for control group (CG). MOK1 and mTOR mRNA levels were determined in PBMCs by qPCR.
Results: T1DM patients have significant downregulation of MOK1 mRNA levels in PBMCs compared CG (P=0.018), while there was no significant difference in mTOR mRNA levels (P=0.891). Furthermore, in T1DM patients, MOK1 significantly correlated with age, triglycerides and mTOR, while mTOR correlated significantly with BMI and systolic blood pressure. Overweight T1DM subjects had significantly lower MOK1 (P=0.034) and mTOR (P=0.017) mRNA levels, together with significantly higher levels of systolic blood pressure (P<0.001), total cholesterol (P=0.001), LDL-cholesterol (P=0.001) and CRP (P<0.001). Multivariate analysis showed that MOK1 was independently negatively associated with T1DM when adjusted for sex, age,

Kratak sadržaj

Uvod: Dijabetes melitus tip 1 (T1DM) jedno je od najčešćih endokrinih oboljenja kod dece. Autoreaktivnost T-ćelija prema β-ćelijama kontroliše se značajnim promenama u metabolizmu T ćelija. Cilj delovanja rapamicina kod sisara je važan unutarćelijski regulator metabolizma i rasta ćelija. MAPK/MAK/MRK preklapajuće kinaze koja se preklapa (MOK1) jedan je od manje poznatih regulatora mTOR-a. Cilj istraživanja je bio da se ispita da li su nivoi iRNK MOK1 i mTOR u mononuklearnim ćelijama periferne krvi različiti kod pedijatrijskih pacijenata sa T1DM u odnosu na zdrave ispitanike.
Metode: Ovo istraživanje je obuhvatilo 172 adolescenta sa T1DM i 36 zdravih adolescenata dobrovoljaca koji su činili kontrolnu grupu (CG). Nivoi MOK1 i mTOR mRNA određeni su u PBMC-ima pomoću qPCR-a.
Rezultati: Pacijenti sa T1DM imali su značajno niže nivoe iRNK MOK1 u PBMC u odnosu na CG, dok razlike u nivoima iRNK mTOR nisu bile značajne (P = 0,891). Štaviše, kod pacijenata sa T1DM, MOK1 je značajno korelirao sa godinama, trigliceridima i mTOR, dok je mTOR značajno korelirao sa BMI i sistolnim krvnim pritiskom. Ispitanici sa prekomernom težinom T1DM imali su značajno niže nivoe iRNK MOK1 (P = 0,034) i mTOR (P = 0,017), zajedno sa značajno većim nivoima sistolnog krvnog pritiska (P <0,001), ukupnog holesterola (P = 0,001), LDL-holesterola (P = 0,001) i CRP (P <0,001). Multivarijantna analiza je pokazala da je MOK1 nezavisno negativno povezan sa

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List of abbreviations: MOK1, MAPK/MAK/MRK overlapping kinase1; mTOR, Mammalian target of rapamycin; mTORC1, Mammalian target of rapamycin complex 1; NLRP3, NLR family pyrin domain containing 3; PBMC, Peripheral blood mononuclear cell; T1DM, Type 1 diabetes mellitus; T2DM, Type 2 diabetes mellitus.

HDL-C and CRP (OR=0.417 (95%CI: 0.175–0.997), p=0.049).

Conclusions: Our study demonstrated for the first time that T1DM is associated with MOK1 downregulation. In addition, downregulation of both mTOR and MOK1 gene expressions was associated with cardiovascular risk factors in overweight T1DM patients.

Keywords: type 1 diabetes mellitus, Mammalian target of rapamycin, MAPK/MAK/MRK overlapping kinase 1

T1DM kada je prilagođen polu, starosti, HDL-C i CRP (OR = 0,417 (95%CI: 0,175–0,997), p = 0,049).

Zaključak: Naša studija je prva koja je pokazala da je T1DM udružen sa nishodnom regulacijom MOK1. Pored toga, snižena regulacija ekspresije gena mTOR i MOK1 bila je povezana sa kardiovaskularnim faktorima rizika kod pacije nata sa T1DM sa prekomernom težinom.

Ključne reči: dijabetes melitus tip 1, meta rapamicina kod sisara, MAPK/MAK/MRK kinaza 1 koja se preklapa

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic disease characterized by dysfunction of pancreatic islet β-cells and concomitant insulin deficiency and hyperglycemia. It is considered as one of the most common endocrine and metabolic diseases in children, and over 500,000 children are currently living with this condition worldwide (1, 2). Although observed loss of pancreatic islet β-cell's function is currently poorly understood, it is evident that autoreactive T lymphocytes play a critical pathological role in this process (1, 3). Early stages of T1DM are associated with B cells mediated activation of $CD4^+$ and $CD8^+$ T cells that lead to selective loss of β-cells (1, 3). The analysis of Langerhans islets in *post mortem* samples obtained close to T1DM diagnosis showed rare cellular infiltrates dominated by $CD4^+$ and $CD8^+$ T lymphocytes (4). It seems that T cell autoreactivity in β-cells autoimmunity is controlled by significant changes in metabolic pathways (5). Previous studies have shown that these metabolic shifts are highly related to T cells activation, proliferation and differentiation into different subsets, the generation of memory T cells, and the capacity to respond to recall antigen in the long term (5). Metabolic changes in T cells are even considered as targets for therapy in preclinical models of autoimmunity and transplantation (5). Mammalian target of rapamycin (mTOR) is considered to be an important intracellular regulator of metabolism and cell growth (5, 6). This serine/threonine kinase, which is a part of phosphoinositide-3-kinase (PI3K)-AKT pathway, is related to functional regulation of T cells, B cells, neutrophils, macrophages, dendritic cells, mast cells, and natural killer cells (4). In addition, mTOR seems to be an important player in cytotoxic T cell proteome shaping (7). mTOR signaling dysfunction has been associated with type 2 diabetes, cancer, neurodegeneration and ageing (6). One of the less known regulators of mTOR is MAPK/MAK/MRK overlapping kinase1 (also known as renal tumour antigen-1, and serine/threonine kinase 30) (MOK1). It was suggested that MOK1 negatively regulates cilium length of renal epithelial cells by inhibition of mTOR complex 1 (mTORC1) signaling (8). MOK1 was firstly identified in renal carcinoma cells as an antigen recognized by autologous cytolytic T cells (9). So far, relatively little is known about MOK1 functions or its upstream and downstream regulators. MOK1 overexpression has been mostly associated with different types of cancer, and this overexpression might be caused by hypomethylation of its promoter (10). In a recent transcriptome-wide twin study, Huang et al. (11) showed that upregulation of MOK1 in peripheral blood mononuclear cells (PBMC) is associated with hypertension. Principal component analysis of proteins has also revealed MOK1 as relevant to type 2 diabetes mellitus (12).

Considering the fact that 70–90% of PBMCs are T lymphocytes (12), as well as their important role in pathogenesis of T1DM, the main aim of this study was to analyze for the first time whether gene expression levels of MOK1 and mTOR in PBMCs are changed in pediatric patients with T1DM. In order to get a deeper insight into association of these two kinases with other factors tied with T1DM, we sought to investigate if their mRNA levels are related to different demographic, clinical and laboratory characteristics of T1DM patients.

Materials and Methods

This study included 172 adolescents with T1DM (84 males and 88 females) and 36 healthy adolescent volunteers (6 males and 30 females) without family history of T1DM designated for control group (CG). Recruitment of subjects was done in The Mother and Child Health Care Institute of Serbia »Dr Vukan Čupić«, Belgrade, Serbia, during regular follow-up in the outpatient clinic. Criteria from Serbian national guidelines of good clinical practice for diagnosis and treatment of diabetes mellitus were used for diagnosis of diabetes (13, 14). Tanner scale was used for assessment of puberty stages. The groups were matched by pubertal stage and body mass index (BMI).

All T1DM patients were on insulin therapy. 162 patients were on the intensive insulin therapy regime that included multiple daily insulin injections, and 10 patients were treated with continues subcutaneous insulin infusion through insulin pump. The patients were not on any antihypertensive or lipid-lowering therapy, and there was no clinical nor laboratory evidence of any diabetic complications.

This study was conducted according to guidelines laid down in the Declaration of Helsinki and

approved by the Ethics Committees of University of Belgrade-Faculty of Pharmacy and Mother and Child Health Care Institute of Serbia »Dr Vukan Čupić«. All the subjects and their parents were thoroughly informed about all aspects of the study, and written inform consent was obtained.

Whole blood was obtained from all subjects after 12 hours fasting period. Immediately after plasma separation, PBMCs were isolated using density gradient (Ficoll-Paque® PLUS gradient-gel), added to Trizol™ (Invitrogen Life Technologies, Foster City, USA) and stored at -80 °C.

Glucose, total cholesterol, HDL-cholesterol, and triglycerides levels were measured in serum using routine laboratory methods. Friedewald formula was used to calculate LDL-cholesterol. HbA1c level was determined by competitive turbidimetric inhibition immunoassay. C-reactive protein (CRP) was measured using immunoturbidimetric method. All the analyses were performed on Roche/Hitachi c501 automated analyzer (Roche, Mannheim, Germany).

Protocols for RNA isolation, reverse transcription and real-time PCR were described elsewhere (15). In brief, total RNA was isolated using TRIZOL™-chloroform extraction with modified protocol (16). RNA concentration and contamination for proteins and organic solvents was assessed using UV analysis at 260 nm, 280 nm and 230 nm respectively. Integrity of isolated RNA was checked using electrophoresis on 1% agarose gel.

Reverse transcription was performed on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

MOK1 gene expression levels were measured by quantitative PCR on 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan® 5'-nuclease gene expression assays (Applied Biosystems, Foster City, CA, USA) for MOK1 (Hs00179504_m1) and HOT FIREPol® DNA Polymerase (Solis BioDyne, Tartu, Estonia). MOK1 mRNA levels were normalized to beta-actin (Hs01060665_g1) as a housekeeping gene.

MTOR gene expression levels were measured by quantitative PCR on Lightcycler II (Roche diagnostics) using specific primers (F: 5 -AGGCCGCATTGTCTCTATCAA-3 ; R: 5 -GCAGTAAATGCAGGTAGTCATCCA-3) and 5x HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia). MTOR mRNA levels were normalized to GAPDH (F: 5 -TGCACCACCAACTGCTTAGC-3 ; R: 5 -GGCATGGACTGTGGTCATGAG-3) as housekeeping gene.

Statistical Analysis

Statistical analysis was performed using a statistical program IBM® SPSS® Statistics version 22 (SPSS Inc., Chicago, USA). Normality of data distribution was tested with Shapiro-Wilk test. Data did not follow normal distribution and were presented as median with interquartile range. Comparisons between the tested groups were done by Mann-Whitney test. Categorical data were given as absolute frequencies and compared by Chi-square test for contingency tables. Associations between clinical data were tested by Spearman's bivariate correlation analysis.

Multivariate binary regression analysis was conducted to determine possible independent association of MOK1 mRNAs and T1DM using data significantly different between tested groups and data which correlate significantly with MOK1 mRNA levels as covariates (sex, age, HDL-C and CRP). Statistically significant p-value was less than 0.05.

Results

Demographic, clinical and laboratory characteristics of T1DM and healthy controls are presented in the *Table I*. The T1DM group had significantly higher percentage of males ($P<0.001$) and was significantly younger compared to CG ($P=0.003$). Glucose, HbA1c, CRP and HDL-C levels were significantly higher in T1DM patients ($P<0.01$, $P<0.001$ and $P=0.009$, $P=0.001$ respectively). In addition, there was no significant difference in systolic blood pressure, total cholesterol, LDL-C and triglycerides levels between observed groups.

Normalized MOK1 mRNA levels were significantly downregulated in T1DM patients compared to CG ($P=0.018$) (*Figure 1A*), while there were no significant differences in normalized mTOR mRNA levels between observed groups ($P=0.891$) (*Figure 1B*). MOK1 gene expression levels were not significantly different between males in females in CG ($P=0.865$), nor in T1DM group ($P=0.709$). Correlation analysis in T1DM group revealed significant negative correlation of normalized MOK1 mRNA levels with age, and triglycerides and positive association with normalized mTOR mRNA levels, as well as negative association of normalized mTOR mRNA levels with BMI percentiles and systolic blood pressure. (*Table II*). In addition, we have observed negative trend between BMI percentiles and MOK1 mRNA levels ($P=0.091$)

In order to further explore association between MOK1 and mTOR gene expression with BMI, we have divided T1DM patients according to 85th percentile BMI. Namely, subjects with BMI larger then 85th percentile according to age and gender were considered as overweight. Interestingly, we have observed significant downregulation of MOK1 ($P=0.034$) and mTOR ($P=0.017$) in overweight subjects, together with significantly higher levels of systolic blood pressure ($P<0.001$), total cholesterol ($P=0.001$), LDL-cholesterol ($P=0.001$) and CRP ($P<0.001$). Overweight subjects also had higher

Table I Demographic, clinical and laboratory characteristics of CG and T1DM patients.

Parameter	CG	T1DM	P
Sex (male/female)[a]	6/30	84/88	<0.001
Age (years)[b]	17 (13–18)	(12–16)	0.003
Tanner stage			0.088
Tanner I	3	28	
Tanner II	1	13	
Tanner III	3	27	
Tanner IV	3	26	
Tanner V	26	81	
Diabetes duration (years)[b]	/	7 (5-8)	
BMI percentiles[b]	54.4 (33.6–78.4)	56.4 (33.4–78.1)	0.831
Systolic blood pressure (mm Hg) b	110 (94–120)	110 (100–115)	0.678
Glucose (mmol/L)[b]	4.79 (4.66–5.13)	10.20 (7.72–13.85)	<0.001
HbA1c (%)	5.2 (4.8–5.0)	7.6 (7.0–8.7)	<0.001
Total cholesterol (mmol/L)[b]	4.01 (3.56–4.46)	3.98 (3.54–4.62)	0.600
HDL-cholesterol (mmol/L)[b]	1.49 (1.26–1.64)	1.63 (1.42–1.88)	0.001
LDL-cholesterol (mmol/L)[b]	2.21 (1.87–2.51)	2.02 (1.68–2.50)	0.246
Triglycerides (mmol/L)[b]	0.81 (0.62–1.03)	0.71 (0.58–0.93)	0.224
CRP (mg/L)[b]	0.30 (0.20–0.50)	0.70 (0.30–1.70)	0.009

a – categorical variables (compared with Chi-square test); b – data are presented as median and interquartile range (compared with Mann-Whitney test)

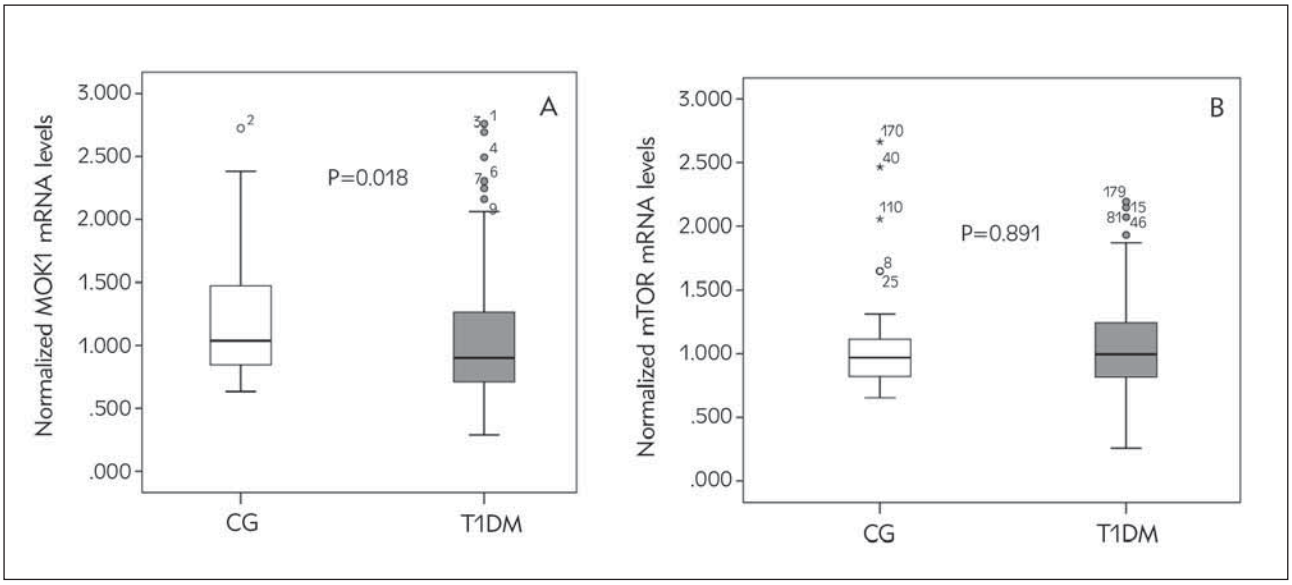

Figure 1 Normalized mRNA levels of MOK1 (A) and mTOR (B) in CG and T1DM patients.

Tablel II Spearman correlation analysis of MOK1 and mTOR mRNA levels with demographic, clinical and laboratory parameters in T1DM patients.

Parameter	Normalized MOK1 mRNA ρ / P	Normalized mTOR mRNA ρ / P
Age (years)	-0.168 / 0.027	-0.086 / 0.259
BMI percentiles	-0.117 / 0.091	-0.190 / 0.006
Systolic blood pressure (mm Hg)	-0.134 / 0.068	-0.173 / 0.019
Glucose (mmol/L)	-0.080 / 0.302	-0.039 / 0.613
Total cholesterol (mmol/L)	0.005 / 0.945	0.091 / 0.233
HDL-cholesterol (mmol/L)	0.101 / 0.192	0.024 / 0.758
LDL-cholesterol (mmol/L)	-0.016 / 0.841	0.093 / 0.229
Triglycerides (mmol/L)	-0.159 / 0.037	0.010 / 0.892
CRP (mg/L)	-0.066 / 0.397	-0.145 / 0.062
HbA1c (%)	0.051 / 0.502	0.122 /0.111
Normalized MOK1 mRNA	/	0.350 /<0.001
Normalized mTOR mRNA	0.350 /<0.001	/

Table III Demographic, clinical and laboratory characteristics of CG and T1DM patients.

Parameter	BMI < 85 percentile	BMI 85 percentile	P
Sex (male/female)[a]	69/71	13/14	0.914
Age (years)[b]	14 (12–17)	(13–16)	0.641
Systolic blood pressure (mm Hg)[b]	106 (100–110)	120 (110–120)	<0.001
Glucose (mmol/L)[b]	10.19 (7.74–14.16)	11.59 (7.33–14.04)	0.838
HbA1c (%)	7.6 (6.9–8.6)	7.8 (7.4–9.0)	0.231
Total cholesterol (mmol/L)[b]	3.91 (3.48-4.56)	4.53 (4.14–5.15)	0.001
HDL-cholesterol (mmol/L)[b]	1.64 (1.42–1.89)	1.63 (1.46–1.93)	0.793
LDL-cholesterol (mmol/L)[b]	1.91 (1.62–2.36)	2.48 (2.04–3.08)	0.001
Triglycerides (mmol/L)[b]	0.69 (0.57–0.93)	0.79 (0.67–1.16)	0.052
CRP (mg/L) [b]	0.60 (0.30–1.20)	1.70 (0.90–3.10)	<0.001
Normalized MOK1 mRNA[b]	0.962 (0.709–1.319)	0.805 (0.654–1.019)	0.034
Normalized mTOR mRNA[b]	1.023 (0.842–1.245)	0.840 (0.769–1.039)	0.017

a – categorical variables (compared with Chi-square test); b - data are presented as median and interquartile range (compared with Mann-Whitney test)

triglycerides levels, but this was of borderline significance (P=0.052) (*Table III*).

Binary logistic regression analysis was used to test associations of MOK1 with the presence of T1DM. Univariate analysis revealed significant negative association between MOK1 mRNA and T1DM (OR=0.449 (95%CI: 0.224–0.897), p=0.023). In multivariate analysis, when MOK1 was adjusted for sex, age, HDL-C and CRP, it demonstrated significant independent negative association with T1DM (OR=0.417 (95%CI: 0.175–0.997), p=0.049).

Discussion

This study, for the first time, demonstrated that MOK1 gene expression levels were downregulated in PBMCs of patients with T1DM compared to healthy controls, whereas there was no significant difference in gene expression level of mTOR between groups. In addition, MOK1 mRNA showed positive correlation with mTOR mRNA, and negative correlation with age, and TG, while mTOR correlated negatively with systolic blood pressure and BMI percentiles.

Although MOK1 was identified 20 years ago as an antigen in renal carcinoma cells that can bind to autologous cytolytic T cells, not much is known about its role in cell signaling (9). Since its discovery, MOK1 overexpression was observed in numerous types of cancer cells (10). Quite surprisingly, changes in MOK1 expression levels in PBMCs along with 12 other genes were associated with hypertension in transcriptome-wide twin study (11). Our study is the first to report that downregulation of MOK1 in PBMCs is associated with T1DM. This association was independent of sex, age, HDL-C and CRP levels. The effects of observed MOK1 downregulation on functions of PBMCs in T1DM are currently not known. One study conducted in cell models for amyotrophic lateral sclerosis suggested that MOK1 inhibition might lead to inflammatory response. Namely, this study showed that aggregates of pathological protein TDP-43 bind to MOK1, disrupting its phosphorylation status supposedly leading to NLRP3/caspase-1 inflammasome activation and secretion of IL-1b and IL-18 (17). NLRP3/caspase-1 inflammasome activation stimulates secretion of IL-1b and IL-18 (17, 18) and increases systemic inflammation, while IL-1 alone is a major regulator of T-cell proliferation and function leading to polarization of T cells towards proinflammatory immunity (18). Therefore we can presume that the observed downregulation of MOK1 in PBMCs could lead to increased proinflammatory activity of T cells and contribute to the overall proinflammatory scenery seen in patients with long standing T1DM.

Obesity is considered as a major risk factor for atherosclerosis development and progression and subsequent cardiovascular complications in general population, especially in T1DM patients. It is characterized by proatherogenic lipid profile and increased systemic inflammation (19). In our study, T1DM patients above the threshold for overweight children (85th percentile BMI according to age and gender), along with higher levels of systolic blood pressure, total cholesterol, LDL-cholesterol, triglycerides and CRP, had significantly lower levels of MOK1 and mTOR gene expressions. Namely, the hormonal changes of normal puberty cause a transient physiologic state of insulin resistance. This insulin resistance is markedly exaggerated in adolescents T1DM, especially in obese ones, leading to defects in both the plasma glucose and lipid-lowering effects of insulin (20). In addition, peripheral insulin resistance, both in well controlled and poorly controlled T1DM, is reflected by impaired insulin suppression of fatty tissue lipolysis and lowering of plasma free fatty acids and glycerol levels (20, 21), followed by increased triglycerides levels, which contribute to cardiovascular risk (19, 20). Therefore, it is not surprising that overweight T1DM patients in our study showed higher levels of proatherogenic lipids (total cholesterol, LDL-cholesterol, triglycerides) compared to non-overweight ones. Along with that, MOK1 was negatively correlated with serum triglycerides, and downregulated in overweight T1DM group, suggesting potential link to higher risk of cardiovascular disease development in T1DM patients.

Furthermore, mTORC1 represents a regulator of cellular nutrient and energy status through stimulation of protein, lipid and nucleotide synthesis (22, 23). It has been found that attenuation of mTOR signaling in the form of protein complex mTORC1 is associated with increased lipolysis in adipose tissue, as well as with enhanced autophagy in adipocytes of obese patients with T2DM (24). In addition, it was suggested that the relation between mTORC1 activity and insulin resistance follows a U-shaped curve, where too little or too much mTORC1 activity has a negative impact on systemic metabolism (25). In that way, downregulation of mTOR seen in overweight T1DM could further aggravate processes leading to peripheral insulin resistance. Taken all together, our findings imply that downregulation of both MOK1 and mTOR genes, together with proatherogenic lipid profile and increased SBP, could contribute to increased risk of atherosclerosis development and cardiovascular complications seen in these patients. However, our conclusions are drawn from different studies that explored function of MOK1 and mTOR in various cell types. Our findings are limited to PBMCs, and require further functional studies to back up our premises.

Limitations

Our study has several limitations. Firstly, this study was performed on relatively small number of

healthy participants. Considering that the participants were pediatric population, we had to take into account the underlying ethical rationale. Next, the percentage of females was significantly higher in CG compared to T1DM group. However, mRNA levels of MOK1 and mTOR were not significantly different between males in females in CG, nor in T1DM group, suggesting that uneven sex distribution between groups did not significantly influence our conclusions. Thirdly, CG was significantly older in comparison to T1DM group. Even though MOK1 exhibited a significant negative correlation with age, we find that this correlation has no significant effect on our conclusions. Namely, our study demonstrated negative association of MOK1 levels and age, and since our CG is older we can presume that this age difference didn't affect the observed difference in MOK1 mRNA levels. Moreover, multivariate binary logistic regression showed that MOK1 is associated with T1DM independently of sex, age, HDL-C and CRP levels.

Conclusion

Our study demonstrated for the first time that MOK1 downregulation is associated with T1DM, which could lead to increased proinflammatory state in T1DM. In addition, downregulation of both mTOR and MOK1 gene expressions was associated with cardiovascular risk factors in overweight T1DM patients.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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PREOPERATIVE PREALBUMIN-TO-FIBRINOGEN RATIO TO PREDICT SURVIVAL OUTCOMES IN HEPATOCELLULAR CARCINOMA PATIENTS AFTER HEPATIC RESECTION

PREOPERATIVNI ODNOS PREALBUMIN-FIBRINOGEN ZA PREDVIĐANJE PREŽIVLJAVANJA KOD PACIJENATA SA HEPATOCELULARNIM KARCINOMOM NAKON HEPATEKTOMIJE

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Summary

Background: This study aimed to evaluate the clinical application of the preoperative prealbumin-to-fibrinogen ratio (PFR) in the clinical diagnosis of hepatocellular carcinoma (HCC) patients and its prognostic value.
Methods: The clinical and laboratory data of 269 HCC patients undergoing surgical treatment from January 2012 to January 2017 in Taizhou Hospital were retrospectively analysed. The Cox regression model was used to analyse the correlation between the PFR and other clinicopathological factors in overall survival (OS) and disease-free survival (DFS).
Results: Cox regression analysis showed that the PFR (hazard ratio (HR)=2.123; 95% confidence interval (95% CI), 1.271–3.547; *P*=0.004) was an independent risk factor affecting the OS of HCC patients. Furthermore, a nomogram was built based on these risk factors. The C-index for the OS nomogram was 0.715.
Conclusions: Nomograms based on the PFR can be recommended as the correct and actual model to evaluate the prognosis of patients with HCC.

Keywords: hepatocellular carcinoma, prealbumin-to-fibrinogen ratio, survival analysis, nomogram

Kratak sadržaj

Uvod: Cilj ove studije je bio da proceni kliničku primenu preoperativnog odnosa prealbumin-fibrinogena (PFR) u kliničkoj dijagnozi pacijenata sa hepatocelularnim karcinomom (HCC) i njegovu prognostičku vrednost.
Metode: Sprovedena je retrospektivna analiza kliničkih i laboratorijskih podataka 269 pacijenata sa HCC koji su bili na hirurškom lečenju od januara 2012. do januara 2017. u Taizhou bolnici. Za analizu korelacije između PFR-a i drugih kliničko-patoloških faktora u ukupnom preživljavanju (OS) i preživljavanju bez bolesti (DFS) korišćen je Koksov regresioni model.
Rezultati: Koksova regresiona analiza je pokazala da je PFR (indeks rizika (HR) = 2,123; 95% interval pouzdanosti (95% CI), 1,271–3,547; P = 0,004) bio nezavisni faktor rizika koji je uticao na ukupno preživljavanje pacijenata sa HCC. Dodatno, na osnovu ovih faktora rizika je urađen nomogram. C-indeks za nomogram ukupnog preživljavanja je bio 0,715.
Zaključak: Nomogrami zasnovani na PFR-u se mogu preporučiti kao ispravan i stvarni model za procenu prognoze kod pacijenata sa HCC.

Ključne reči: hepatocelularni karcinom, odnos prealbumina i fibrinogena, analiza preživljavanja, nomogram

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Introduction

Hepatocellular carcinoma (HCC) is a globally prevalent malignant tumour with a 5-year survival rate (<5%) (1). HCC is mainly diagnosed by imaging examinations, serological markers (alpha-fetoprotein, α-L-fucosidase, abnormal prothrombin, and so on), and liver histopathological diagnoses. Moreover, China has the highest incidence of liver cancer. According to informal statistics, the number of Chinese people who die of liver cancer each year is approximately 110,000, accounting for 45% of the world's liver cancer deaths (2).

Fibrinogen is an important acute-phase protein. Recent studies have found that fibrinogenaemia is strongly related to various tumours' occurrence, development, and prognosis. These tumours mainly include renal cell carcinoma, lung cancer, ovarian cancer, and hepatocellular carcinoma (3–6). Furthermore, prealbumin levels can often be used to assess a patient's nutritional status. Consequently, prealbumin levels can reflect postoperative effects such as cervical cancer, metastatic renal cell carcinoma, and non-small-cell lung cancer (7–9). Furthermore, a new prognostic indicator has been reported, which is a combination of prealbumin and fibrinogen. Several articles have shown that prealbumin and fibrinogen (PFR) ratio has good prognostic significance in acute pancreatitis (10).

Therefore, the trend of early detection and early intervention is crucial for HCC treatment and mortality reduction. Finding new HCC biomarkers to predict the prognosis of HCC patients after surgery is urgent. Thus, this study aimed to use nomograms to analyse the relationship of the preoperative PFR in the prognosis of HCC patients to determine its prognostic value in HCC.

Materials and Methods

Patients

This study recruited 367 HCC patients admitted to the Department of Hepatobiliary Surgery of Taizhou Hospital of Zhejiang Province, Taizhou, China, from January 2012 to January 2017. The inclusion criteria were as follows: (a) complete clinical and laboratory data (current medical history, previous medical history, family history, personal history, physical examination, and complete pathological data); (b) abdominal computed tomography or ultrasound to exclude liver abscess, liver-occupying lesions, and other diseases; (c) the use of required pathological data to exclude secondary liver cancer; (d) postoperative TNM clinical-pathological stages I, II, and III; and (e) exhaustive whole blood cell analysis, biochemical analysis, and examinations. The preoperative clinical diagnosis was clear, and the tumour location, size, number, pathological stage, and differentiation degree were obtained through pathological examination. The intraoperative lymph nodes were examined to determine the lymph node metastasis in each study group. Preoperative blood examination data was exhaustive. Furthermore, the exclusion criteria were (a) patients with other benign liver tumours (e.g., haemangiomas and hepatic adenomas) and (b) patients with infections or other inflammatory diseases. Finally, 269 patients were included.

Treatment and follow-up

All patients underwent a 1-year regular follow-up visit. The follow-up deadline was April 22, 2017. The recurrence and survival times were all recorded in months, from surgery to recurrence or death. According to the follow-up data criteria, tumour recurrence or metastasis was the DFS endpoint. Furthermore, the overall survival (OS) endpoint was death.

Statistical analysis

The Statistical Package for the Social Sciences, version 22.0, and X-tile, version 3.6.1, were used for analysis. The optimal PFR cut-off value was calculated using the X-tile plot. The *X″* test was used to analyse the differences between the categorical variables. Moreover, the Cox proportional hazards model was used for univariate and multivariate survival analyses to determine the risk factors for prognosis. Finally, the nomogram for prediction value was established using R software. Consequently, model accuracy was evaluated by Harrell's concordance index (c-index), calibration plots, decision curves, and clinical impact curves. *P* values ≤ 0.05 were considered statistically significant.

Results

Analysis and calculation of the PFR optimal cut-off value

This study group enrolled 269 patients. Taking postoperative mortality of the patients as the endpoint, 0.8 was the optimal PFR cut-off value calculated by the X-tile plot (*Figure 1*).

Patient characteristics

The patients' baseline and clinicopathological characteristics stratified by the PFR are described in *Table I*. Furthermore, the table shows that the patients were divided into two groups for further analysis (PFR<0.8 and PFR 0.8). Moreover, the PFR was associated with the Barcelona Clinic Liver Cancer (BCLC) stage, Child-Pugh score, complications (hepatic rupture, portal hypertension, and intraoperative

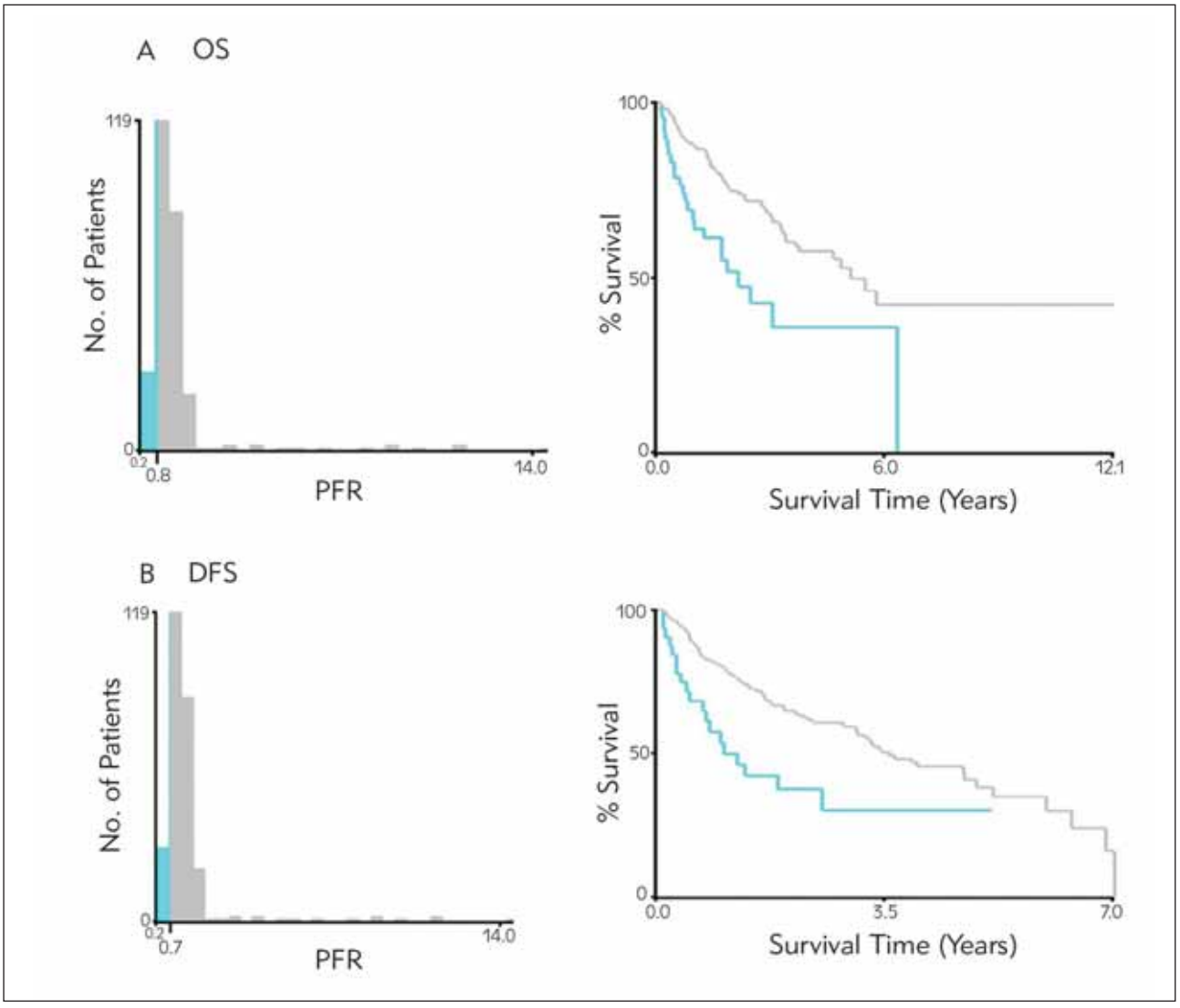

Figure 1 X-tile OS analyses were performed using patient data to determine the optimal PFR cut-off values. The optimal cut-off values are shown in the histograms of the entire cohort (left panels), and Kaplan–Meier plots are displayed (right panels). The P values were determined by using the cut-off values defined in the training sets and applying them to the validation sets. A. For OS, the optimal cut-off value of the PFR was 0.8. B. For DFS, the optimal cut-off value of the PFR was 0.7.

ascites), survival, tumour length, and red cell distribution width. The average age of the 269 patients was 57 years, with 219 (81.4%) males and 50 (18.6%) females. Moreover, patients with stages A and B–C, using the BCLC staging system, accounted for 55.8% and 44.2% of the total patients, respectively. However, patients with grades A and B accounted for 89.6% and 10.4% of the total patients' Child-Pugh scores, respectively. Patients with complications of liver rupture bleeding, portal hypertension, and hepatic encephalopathy accounted for 7.8%, 6.3%, and 0.7% of the total patients, respectively. Consequently, 173 patients survived (64.3%). Of the total patients, 167 (62.1%) and 102 (37.9%) were diagnosed with tumours <5 and 5 cm, respectively.

Prognostic value of the PFR

Univariate analysis was carried out using the Cox regression model. Consequently, the clinicopathological parameters predicting OS and DFS were further studied. In the univariate analysis, the BCLC stage, nerve invasion, vascular invasion, T stage, M stage, tumour size, number of tumours and PFR were significantly related to OS ($p<0.05$). Furthermore, the BCLC stage, portal hypertension, vascular invasion, T stage, M stage, tumour size, number of tumours and PFR were associated with DFS ($p<0.05$). For the multivariate Cox regression OS model, the PFR (hazard ratio (HR)=2.123; 95% confidence interval (95% CI), 1.271–3.547; $P=0.004$), vascular invasion (HR=2.272; 95% CI, 1.032–5.003; $P=0.041$), M stage (HR =8.095; 95% CI, 3.518–18.627;

Table I Comparison of baseline clinicopathological characteristics based on PFR.

		Cases	PFR		*P*
		No. (%)	<0.8	≥0.8	
Age (years)	≤60	164 (61%)	25	139	0.312
	>60	105 (39%)	21	84	
Gender	Male	219 (81.4%)	35	184	0.308
	Female	50 (18.6%)	11	39	
Smoking	No	140 (52%)	25	115	0.731
	Yes	129 (48%)	21	108	
Drinking	No	196 (72.9%)	37	159	0.205
	Yes	73 (27.1%)	9	64	
BCLC stage	A	150 (55.8%)	18	132	0.013
	B, C	119 (44.2%)	28	91	
Child-Pugh score	A	241(89.6%)	31	210	0.000
	B	28 (10.4%)	15	13	
Liver rupture bleeding	No	248 (92.2%)	33	215	0.000
	Yes	21 (7.8%)	13	8	
Portal hypertension	No	252 (93.7%)	37	215	0.000
	Yes	17 (6.3%)	9	8	
Hepatic encephalopathy	No	267 (99.3%)	45	222	0.215
	Yes	2 (0.7%)	1	1	
Recrudescence	No	146 (54.3%)	21	125	0.197
	Yes	123 (45.7%)	25	98	
Survival situation	Survival	173 (64.3%)	23	150	0.026
	Mortality	96 (35.7%)	23	73	
Cirrhosis	No	69 (25.4%)	7	62	0.075
	Yes	200 (74.3%)	39	161	
Liver capsule invasion	No	220 (81.8%)	37	183	0.795
	Yes	49 (18.2%)	9	40	
Liver margin	No	247 (91.8%)	39	208	0.056
	Yes	22 (8.2%)	7	15	
Nerve invasion	No	259 (96.3%)	45	214	0.543
	Yes	10 (3.7%)	1	9	
Vascular invasion	No	201 (74.7%)	30	171	0.103
	Yes	68 (25.3%)	16	52	
T stage	T0–T1	157 (58.4%)	24	133	0.350
	T2–T4	112 (41.6%)	22	90	
N stage	N0	264 (98.1%)	45	219	0.862
	N1	5 (1.9%)	1	4	
M stage	M0	263 (97.8%)	45	218	0.977
	M1	6 (2.2%)	1	5	
Tumour size (cm)	<5 cm	167 (62.1%)	20	147	0.004
	5 cm	102 (37.9%)	26	76	
Number of tumours	1	209 (77.7%)	38	171	0.379
	2	60 (22.3%)	8	52	
Hepatitis B infection	No	47 (17.5%)	43	4	0.097
	Yes	222 (82.5%)	181	41	
PLT	300×10^9/L	259 (96.3%)	45	214	0.543
	$>300 \times 10^9$/L	10 (3.7%)	1	9	
AFP	20 μg/L	116 (43.1%)	15	101	0.114
	>20 μg/L	153 (56.9)	31	122	
CEA	≤5 ng/mL	232 (86.2%)	41	191	0.533
	>5 ng/mL	37 (13.8%)	5	32	

Table II Univariate and multivariate survival analyses of OS and DFS in HCC patients.

	OS				DFS			
	Univariate analysis HR (95% CI)	*P*	Multivariate analysis HR (95% CI)	*P*	Univariate analysis HR (95% CI)	*P*	Multivariate HR (95% CI)analysis	*P*
Age (years)		0.489		0.389		0.937		0.525
≤60	1.000		1.000		1.000		1.000	
>60	1.157 (0.766–1.749)		1.010		0.985 (0.680–1.427)		1.133 (0.772–1.663)	
Gender		0.948		0.796		0.985		0.991
Male	1.000		1.000		1.000		1.000	
Female	0.982 (0.574–1.682)		0.927 (0.520–1.651)		0.995 (0.621–1.595)		0.997 (0.614–1.621)	
Smoking		0.545				0.424		
No	1.000				1.000			
Yes	1.132 (0.758–1.690)				1.157 (0.809–1.653)			
Drinking		0.536				0.386		
No	1.000				1.000			
Yes	1.146 (0.744–1.766)				1.189 (0.804–1.757)			
BCLC stage		0.000		0.421		0.001		0.852
A	1.000		1.000		1.000		1.000	
B, C	2.171 (1.445–3.263)		0.766 (0.401–1.466)		1.800 (1.257–2.579)		0.945 (0.523–1.709)	
Child's score		0.055				0.123		
A	1.000				1.000			
B	1.742 (0.988–3.073)				1.532 (0.891–2.635)			
Liver rupture bleeding		0.083				0.238		
No	1.000				1.000			
Yes	1.746 (0.930–3.278)				1.433 (0.789–2.604)			
Portal hypertension		0.068				0.002		0.062
No	1.000				1.000		1.000	
Yes	1.842 (0.955–3.551)				2.462 (1.378–4.397)		1.870 (0.970–3.604)	
Hepatic encephalopa-		0.547				0.727		
No	1.000				1.000			
Yes	1.833 (0.255–13.188)				1.421 (0.198–10.193)			
Cirrhosis		0.699				0.403		
No	1.000				1.000			
Yes	1.100 (0.678–1.787)				1.203 (0.780–1.858)			
Liver capsule invasion		0.328				0.881		
No	1.000				1.000			
Yes	1.270 (0.787–2.048)				1.035 (0.661–1.621)			
Liver margin		0.085				0.234		
No	1.000				1.000			
Yes	1.782 (0.923–3.439)				1.459 (0.784–2.717)			
Nerve invasion		0.034		0.330		0.168		
No	1.000		1.000		1.000			
Yes	2.456 (1.071–5.633)		1.596 (0.623–4.086)		1.787 (0.783–4.078)			
Vascular invasion		0.000		0.041		0.001		0.151
No	1.000		1.000		1.000		1.000	
Yes	2.472 (1.619–3.774)		2.272 (1.032–5.003)		1.938 (1.315–2.856)		1.709 (0.823–3.550)	
T stage		0.000		0.938		0.001		0.778
T1	1.000		1.000		1.000		1.000	
T2, T3, T4	2.111 (1.412–3.157)		0.966 (0.408–2.286)		1.823 (1.276–2.603)		0.894 (0.411–1.947)	
N stage		0.876				0.623		
N0	1.000				1.000			
N1	1.119 (0.272–4.598)				0.698 (0.167–2.925)			
M stage		0.000		0.000		0.000		0.000
M0	1.000		1.000		1.000		1.000	
M1	13.114 (5.515–31.184)		8.095 (3.518–18.627)		8.556 (3.679–19.897)		5.015 (2.253–11.163)	
Tumour length(cm)		0.000		0.007		0.001		0.073
<5 cm	1.000		1.000		1.000		1.000	
≥5 cm	2.320 (1.551–3.471)		2.188 (1.240–3.859)		1.868 (1.306–2.671)		1.611 (0.957–2.711)	
Number of tumours		0.025		0.224		0.004		0.103
1	1.000		1.000		1.000		1.000	
≥2	1.658 (1.067–2.577)		1.532 (0.770–3.048)		1.770 (1.197–2.617)		1.714 (0.896–3.278)	
Hepatitis B infection		0.630				0.674		
No	1.000				1.000			
Yes	0.816 (0.357–1.867)				0.849 (0.395–1.822)			
PFR		0.001		0.004		0.007		0.092
≥0.8	1.000		1.000		1.000		1.000	
<0.8	2.261 (1.411–3.624)		2.123 (1.271–3.547)		1.829 (1.175–2.848)		1.525 (0.934–2.491)	

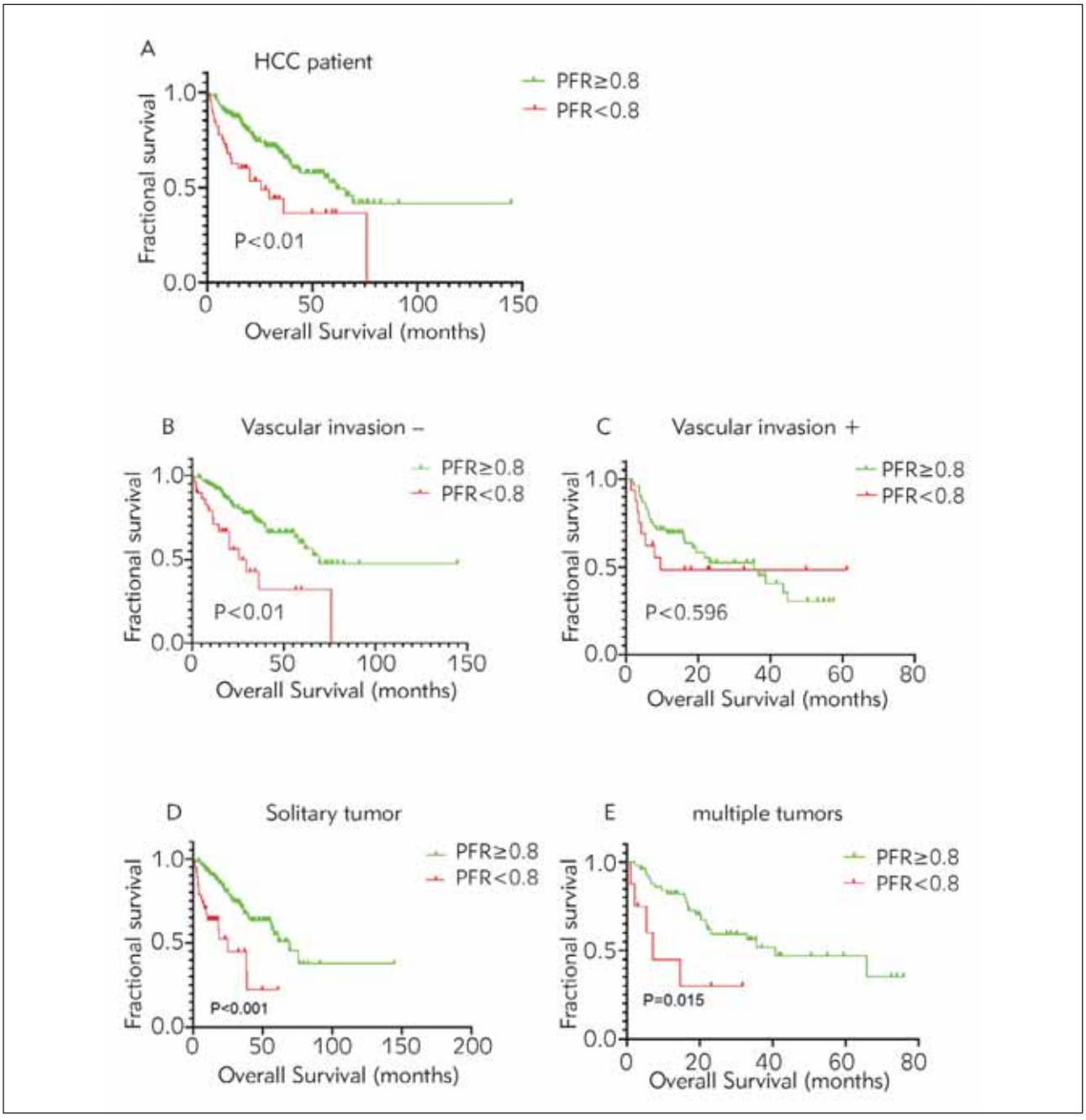

Figure 2 Kaplan–Meier curves for OS according to the PFR in each subgroup and the total HCC patients. A. total HCC patients, B. vascular invasion-negative subgroup, C. vascular invasion-positive subgroup, D. solitary tumour subgroup, and E. multiple tumours subgroup.

P=0.000), and tumour size (HR=2.188; 95% CI, 1.240–3.859; *P*=0.007) were verified to be independent prognostic factors in patients with HCC (*Table II*).

Survival PFR analysis

This study used Kaplan-Meier analysis to determine the prognostic value of the PFR. Low PFR levels (<0.8) were associated with short OS (*Figure 2A*). Based on vascular invasion and the number of tumours, a separate subgroup analysis was also conducted to investigate the significance of the PFR for the prognosis of HCC patients. Moreover, short OS was found in patients with low PFR in the solitary and multiple tumour subgroups ($P<0.001$ and $p<0.001$, respectively) and the vascular invasion-negative subgroup ($P<0.001$) but not in the vascular invasion-positive subgroup ($p=0.596$; *Figure 2B*, *2C*, *2D*, *2E*).

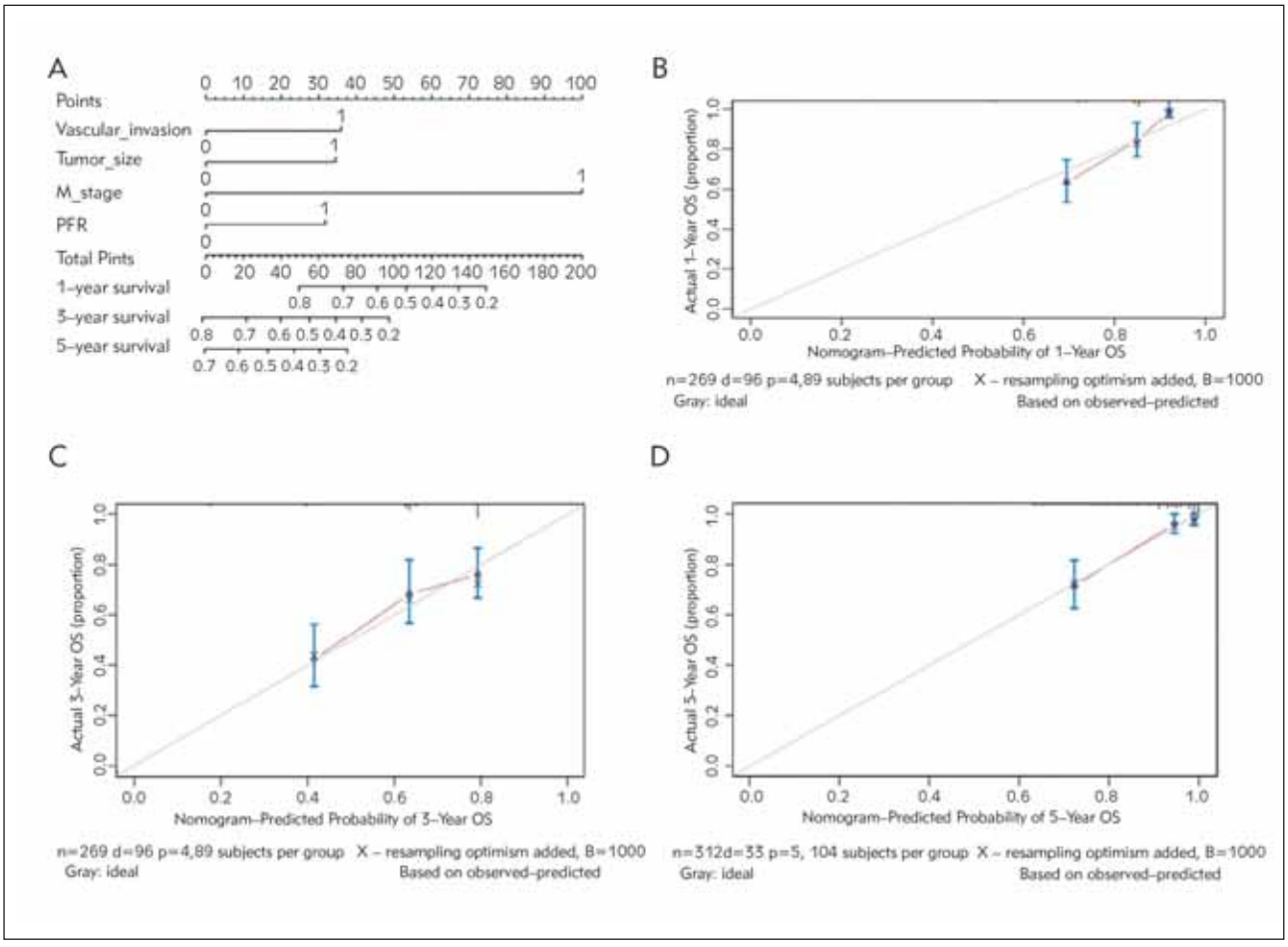

Figure 3 The PFR was an independent predictive factor for disease progression in HCC patients. A. Nomogram predicting the OS of HCC patients. B. 1-year OS calibration plot. C. 3-year OS calibration plot. D.5-year OS calibration plot.

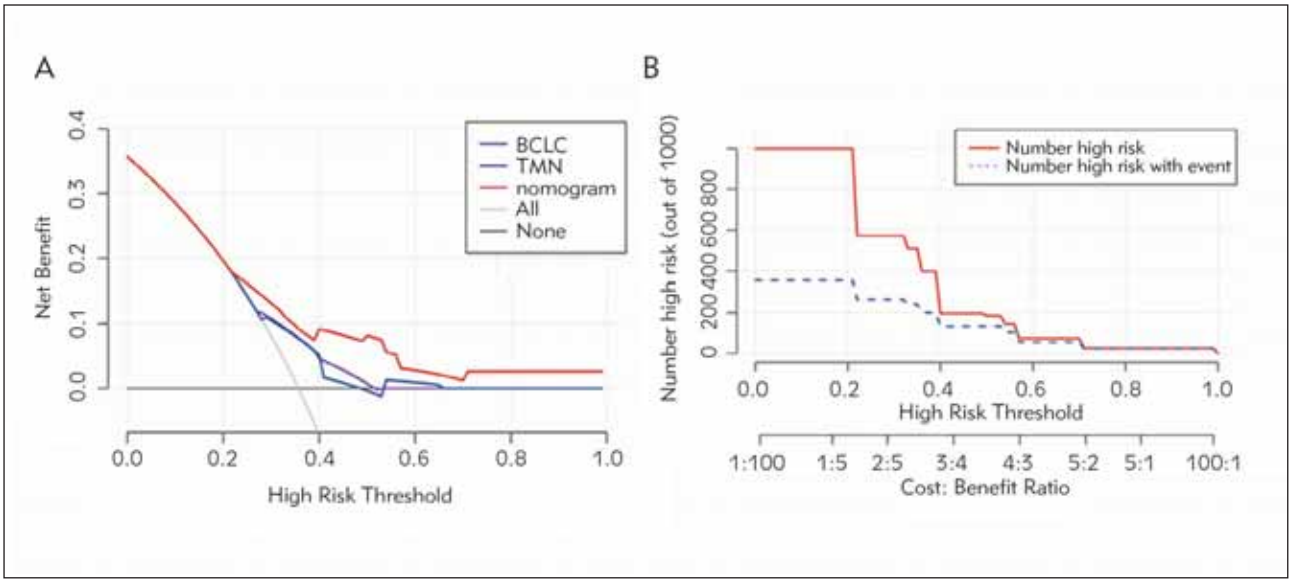

Figure 4 Decision curve for the disease progression of HCC patients. A. Nomogram, *red line;* BCLC stage, *blue line*. TMN stage, *purple line*. The abscissa of this graph is the threshold probability, and the ordinates are the net benefit. B. Clinical Impact Curve.

Development and validation of nomograms for predicting OS in HCC patients

The nomograms can be explained by adding up the number of points assigned to each variable at the top of the scale. At the bottom of the scale, the total score translates into predicting the patient's 5-year probability of mortality. A nomogram, based on independent risk factors (the PFR, vascular invasion, M stage, tumour size), was established to predict OS in HCC patients (*Figure 3A*). The C-index for the OS nomogram was 0.715 (95% CI, 0.662–0.768). Moreover, the calibration curves by internal validation demonstrated good agreement between the predicted and actual probability of 1-, 3- and 5-year OS (*Figure 3B, 3C, 3D*). The decision curve analysis found that the nomogram model that included the PFR had better net benefits than the model that included the BCLC and TNM stages to identify OS for HCC patients (*Figure 4*).

Discussion

Malnutrition and fibrinogen abnormalities are common in cancer patients and have major effects on their quality of life, treatment outcomes, and prognosis (11, 12). This study indicates that a low preoperative PFR (<0.8) is an independent risk factor for OS in HCC patients.

Chronic infections, including HCC, cause >15% of malignancies worldwide (13). Studies have shown that systemic inflammatory responses boost angiogenesis and tumour invasion by upregulating cytokines (14–16). This shows that the inflammatory response plays a critical role in tumorigenesis and tumour development. Furthermore, the two functions of prealbumin may be related to the occurrence and prognosis of tumours. The first is that inflammation is associated with decreased prealbumin levels in several studies (17, 18). Moreover, prealbumin levels may be affected in other ways during inflammation because cytokines (e.g., IL-6, IL-1, and TNF-α) can downregulate synthesis (19) and increase vascular permeability (20). The second is that prealbumin can respond to the nutritional status of the reaction body. Serum prealbumin has a shorter half-life than albumin and is synthesised by hepatocytes. However, synthesis rapidly declines when hepatocytes are damaged. Furthermore, tumours cause protein malabsorption in the body and can also cause prealbumin levels to decline. Studies have recently shown that hypoproteinaemia is a poor prognostic indicator of oesophageal and colorectal cancers (21, 22).

Fibrinogen is the most acutely reactive plasma protein (1). It plays an important role in activating the coagulation cascade (23). Moreover, fibrinogen is also the key factor in regulating the inflammatory cascade through the interaction of ligand-receptor mechanisms involving immune cells (e.g., monocytes and microvasculature) (2, 24, 25). Some studies have indicated that fibrinogen can be endogenously synthesised by cancer cells (26, 27). Meanwhile, highly concentrated fibrinogen induces epithelial-mesenchymal transition, which increases cancer cell invasion and metastasis using a cell line model by increasing vimentin expression and decreasing E-cadherin expression (28).

Therefore, in theory, prealbumin and fibrinogen are two valuable markers for monitoring HCC progression. Furthermore, this study showed that the PFR, an inflammatory marker, is a potential prognostic factor for HCC patients, combining the two factors of HCC patients' nutritional status and inflammatory response status and having the advantages of low cost and convenience, rapidity, and easy detection.

This study tried to explain the prognostic value of the PFR for HCC patients. X-tile was used to calculate the optimal PFR cut-off value of 0.8. Moreover, many studies previously used the receiver operating characteristic (ROC) curve to select the cut-off value. However, most of the ROC curves included only the event outcomes and experimental indicators but did not include important factors for cancer prognosis. The X-tile software precisely includes the time worth choosing as the cut-off value. Thus, the cut-off value of this article may be more accurate. Furthermore, this study suggests that the PFR is associated with OS and DFS in HCC patients in univariate analysis, and the PFR is related to the patient's OS (HR=2.123; 95% CI, 1.271–3.547; P=0.004) and has nothing to do with DFS in multivariate analysis. The risk of mortality of HCC patients with a low PFR is significantly increased. Moreover, the nomogram was used to establish the prognostic model of liver cancer, and the accuracy of the nomogram was proven by calibration, decision, and clinical influence curves. The nomogram shows that the PFR has an important predictive value.

Although the PFR can predict OS in HCC patients, there are some limitations in this study. First, this single-centre retrospective study may have selection bias as it only included HCC patients undergoing surgical resection. Moreover, this study does not represent HCC patients who refuse surgery for different reasons. Second, verification queues are lacking to verify whether the findings of this study are commonly used. Therefore, the results of this study need to be further verified in forward-looking and large-scale cooperative research.

Conclusion

In conclusion, the results of this study suggest that the PFR (<0.8) is a prognostic indicator of OS in HCC patients. Thus, a PFR-containing nomogram can be used as a more practical model for evaluating OS in HCC patients.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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SPECIFIC IMPACT OF CARDIOVASCULAR RISK FACTORS ON CORONARY MICROCIRCULATION IN PATIENTS WITH SUBCLINICAL HYPOTHYROIDISM

SPECIFIČAN UTICAJ KARDIOVASKULARNIH FAKTORA RIZIKA NA KORONARNU MIKROCIRKULACIJU U PACIJENATA SA SUBKLINIČKOM HIPOTIREOZOM

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Summary

Background: Although thyroid hormones have significant effect on cardiovascular system, the impact of subtle thyroid dysfunction such as subclinical hypothyroidism (SCH) remains to be determined. We investigated coronary flow reserve (CFR) in patients with subclinical hypothyroidism.
Methods: Thirty two subjects with SCH and eighteen control subjects with normal serum thyroid hormones and thyroid-stimulating hormone (TSH) levels were included in the study. TSH, free thyroxine, free triiodothyronine, glucose, insulin, HbA1c, cholesterol, triglyceride and plasma levels of C-reactive protein were measured. Coronary diastolic peak flow velocities in left anterior descending coronary artery were measured at baseline and after adenosine infusion. CFR was calculated as the ratio of hyperemic to baseline diastolic peak velocity.
Results: CFR values were not significantly different between the two groups (SCH 2.76±0.35 vs controls 2.76±0.42). There was a significant correlation of CFR with waist to hip ratio, hypertension, smoking habits, markers of glucose status (glucose level, HbA1c, insulin level, HOMA IR), cholesterol, LDL-cholesterol and triglyceride levels in SCH group, whereas only cholesterol level showed significant correlation with CFR in controls. There was no correlation between CFR and thyroid hormones.

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Kratak sadržaj

Uvod: Poznato je da tiroidni hormoni imaju značajan efekat na kardiovaskularni sistem, ali i dalje ostaje da se utvrdi uticaj suptilnih promena na nivou tiroidne osovine kao što je subklinička hipotireoza (SHT). Ispitivali smo koronarnu rezervu protoka (KRP) kod pacijenata sa subkliničkim hipotireoidizmom.
Metode: Trideset dva ispitanika sa subkliničkom hipotireozom i osamnaest kontrolnih ispitanika sa urednim tiroidnim hormonskim statusom su bili uključeni u studiju. Mereni su TSH, fT4, fT3, glukoza, insulin, HbA1c, holesterol, triglyceridi i CRP. Koronarne dijastolne brzine protoka u levoj prednjoj silaznoj koronarnoj arteriji merene su na početku i nakon infuzije adenozina. Koronarna rezerva protoka je izračunata kao odnos hiperemijske i osnovne dijastolne brzine protoka.
Rezultati: Vrednosti koronarne rezerve protoka se nisu značajno razlikovale između dve grupe (SHT 2,76 ± 0,35 u odnosu na kontrole 2,76 ± 0,42). Postojala je značajna korelacija KRP sa odnosom struka i kukova, hipertenzijom, navikama pušenja, markerima glikoregulacije (nivo glukoze, HbA1c, nivo insulina, HOMA IR), holesterolom, LDL-holesterolom i nivoom triglicerida u SHT grupi, dok je samo nivo holesterola pokazao značajnu korelaciju sa KRP u kontrolnoj grupi. Nije bilo korelacije između KRP i hormona štitaste žlezde.

List of abbreviations: SCH, subclinical hypothyroidism; CFR, coronary flow reserve; LAD, left anterior descending coronary artery; DPFV, diastolic peak flow velocity

Conclusions: We concluded that there is a different impact of cardiovascular risk factors on CFR in SCH patients compared to healthy control and that these two groups behave differently in the same circumstances under the same risk factors. The basis for this difference could be that the altered thyroid axis »set point« changes the sensitivity of the microvasculature in patients with SCH to known risk factors.

Keywords: cardiovascular risk factors, coronary flow reserve, subclinical hypothyroidism, thyroid

Zaključak: Zaključili smo da postoji drugačiji uticaj kardiovaskularnih faktora rizika na koronarnu rezervu protoka kod pacijenata sa subkliničkom hipotireozom u poređenju sa zdravom kontrolom i da se ove dve grupe ponašaju različito u istim okolnostima, pod istim faktorima rizika. Osnova za ovu razliku mogla bi biti da promenjen »set point« tiroidne osovine menja osetljivost mikrovaskulature kod pacijenata sa SHT na poznate faktore rizika.

Ključne reči: kardiovaskularni faktori rizika, koronarna rezerva protoka, subklinička hipotireoza, štitasta žlezda

Introduction

Subclinical hypothyroidism (SCH) is defined as mild elevation of thyroid-stimulating hormone (TSH) in the presence of normal free thyroxine (fT4) and free triiodothyronine (fT3) levels (1). It is well known that overt hypothyroidism has negative impact on cardiovascular function (2, 3). The clinical importance of subclinical hypothyroidism in cardiovascular disease and mortality is still controversial, because of the inconsistent results (4–6), on the impact of SCH on cardiovascular function. Several studies including meta-analyses have suggested that there is an association between SCH and cardiovascular diseases (2, 3, 7–10), and that SCH is an independent risk factor for atherosclerosis and myocardial infarction in elderly women (11). Razvi et al. (12) in their meta-analysis postulated that SCH is associated with increased cardiovascular morbidity and mortality only in younger subjects. On the other hand, in the last few years there were several studies that did not show relation between SCH and cardiovascular disease or cardiovascular and all-cause mortality (5, 13, 14). Likewise, treatment of subclinical hypothyroidism in older persons did not show any clinical benefit (15).

Coronary flow velocity reserve (CFR) is defined as the ratio of hyperemic coronary blood flow velocity to baseline and reflects functional integrity of coronary microcirculation. It has been shown that reduced CFR is an early manifestation of atherosclerosis and coronary artery disease (16). CFR measured by transthoracic Doppler echocardiography (TTDE) has an excellent correlation with CFR measured by positron emission tomography, which has been validated as a gold standard for noninvasive CFR measurement (17).

The present study was designed to investigate the impact on persistent SCH on the value of CFR, as assessed by transthoracic Doppler echocardiography, and consequently microcirculatory function.

Materials and Methods

The study group consisted of 32 patients with newly diagnosed persistent SCH (31 female, one male; mean age 52.6±14.8 years), and 18 healthy controls (17 female, one male; mean age 50.1±15.4 years). SCH was diagnosed on the basis of persistent TSH increase with free thyroid hormones level within the referent range. Patients were included in the study only if they had stable SCH which was demonstrated by repeated thyroid hormone profile after minimum four weeks. The institutional ethics committee approved the study protocol, and all participants signed informed consent to the study.

The exclusion criteria for SCH group as well as for the control group were history of coronary artery disease, valvular or congenital heart disease, cardiac rhythm abnormalities, diabetes mellitus, systemic, hepatic or renal diseases. Controls had a normal thyroid hormonal status.

All blood samples were collected between 08.00 and 09.00 h in the morning after overnight fast. Serum lipid levels (total cholesterol, high-density lipoprotein cholesterol, triglyceride), HbA1c and fasting glucose levels were measured using spectrophotometry commercial kits on an automatic analyzer c501 (Roche Diagnostics, GmbH, Mannheim, Germany). C-reactive protein values were analyzed by Immunoturbidimetric assay for the in vitro quantitative determination on a Cobas c501 analyzer (Roche Diagnostics, GmbH, Mannheim, Germany), using the latex-enhanced immunoturbidimetric assay. Low density lipoprotein cholesterol was calculated by Friedewald's formula. The serum TSH, fT4, fT3, TPOAb and insulin levels were measured using a electrochemiluminescence immunoassay (ECLIA) on the Roche Cobas e601 automated analyzer (Roche Diagnostics, Mannheim, Germany) and using a chemiluminescent microparticle immunoassay (CMIA) on an Alinity instrument (Abbott Diagnostics, Wiesbaden, Germany). Normal range for TSH was 0.27–4.2 mIU/L, for fT3 was 3.1–6.8 pmol/L, for fT4 12–22 pmol/L and for TPOAb 0–34 IU/mL). Body mass index (BMI), waist-to-hip ratio (WHR) and HOMA IR were also calculated, using standard formulas. Systolic and diastolic blood pressures (BP) were measured on the right arm of subjects in an upright sitting position after at least 5 min of rest using a sphygmomanometer.

CFR was performed using the Acuson Sequoia C 256 (Siemens Medical Solutions, Mountain View, CA, USA) with 4-MHz transducer. With the patient

positioned in the left lateral decubitus, coronary flow was searched for in the mid/distal portion of the left anterior descending (LAD) coronary artery with the transducer placed at the cardiac apex or one intercostal space higher in order to obtain modified, three-chamber view. Color Doppler imaging was performed by decreasing the Nyquist limit to 1624 cm/s. With a sample volume 3–5 mm wide and positioned on the LAD color flow signal in diastole, pulsed Doppler tracings of peak flow velocities were recorded. After acquiring Doppler tracings in baseline conditions, under continuous echocardiographic monitoring, adenosine 140 mg/kg/min was administrated over 2 min and peak diastolic coronary flow velocities were obtained during maximal hyperemia. Three optimal flow profiles at rest and during hyperemia were obtained and results were averaged. CFR was calculated as the ratio of hyperemic to baseline diastolic flow velocities. Preserved CFR was defined as 2.0. All patients abstained from caffeine-containing drinks for at least 12 hours before the tests.

Statistical analysis

Results were presented as mean ± standard deviation, frequency (percent) and median (range) in case of not normal distribution of data. Chi-square test was used to test differences between nominal data (frequencies). For parametric data independent samples t-test was used to test differences between groups. For numeric data with non-normal distribution and ordinal data Mann-Whitney U test was used. Chi-square test or Fisher's exact test were used to test differences between nominal data (frequencies). Correlation between the CFR for LAD as dependent variable and potential predictors was analyzed by linear regression. All p-values less than 0.05 were considered significant.

Results

Age, gender, BMI, glucose, insulin levels, HOMA IR, cholesterol, HDL, LDL, levels, systolic and diastolic blood pressure, smoking habits were similar in SCH group and in controls. Triglyceride levels were higher in SCH group, whereas CRP also showed borderline higher values in SCH group. The fT3/fT4 ratio was

Table I Patients characteristic.

	SCH group (n=32)	Control group(n=18)	P
Age	52.6±14.8	50.1±15,4	0.509
Male/female	1/31	1/17	0.595
BMI	26.6±5.1	24.2±3.0	0.069
WHR	0.84±0.07	0.82±0,06	0.620
Hypertension (%)	40.6%	38.9%	0.904
Systolic BP (mmHg)	120.3±11.6	119.4±10.1	0.792
Diastolic BP (mmHg)	75.8±7.8	73.3±7.3	0.283
Smokers (%)	21.9%	22.2%	0.923
Glycose (mmol/L)	5.5±0.7	5.4±0.6	0.708
Insulin (mIU/L)	8.5 (1.4–23.5)	6.8 (1.4–16.1)	0.983
HbA1c (%)	5.7±0.4	5.5±0.3	0.212
HOMA IR	1.8 (0.3–6.7)	1.6 (0.3-3.6)	0.861
Cholesterol (mmol/L)	5.60±1.02	5.63±1.08	0.914
HDL (mmol/L)	1.48±0.32	1.67±0.53	0.113
LDL (mmol/L)	3.47±0.85	3.46±0.70	0.986
Triglyceride (mmol/L)	1.30 (0.48–3.66)	0.97 (0.52–2.19)	0.044
CRP (nmol/L)	1.3(0.3–9.6)	0.7(0.1–2.1)	0.051
fT4 (mgl/L)	11.8±1.6	12.6±1.7	0.082
fT3 (pmol/L)	4.03±0.42	4.01±0.42	0.881
fT3/fT4	0.35±0.05	0.31±0.04	0.015
TSH (mIU/L)	7.70 (4.60–15.35)	2.08 (0.51–4.14)	<0.001
TPOAb (IU/mL)	248.5 (4–7413.5)	14.4(0.3–793.3)	<0.001

BMI – body mass index; WHR – waist to hip ratio

Table II Coronary flow velocity values in SCH and controls.

	SCH group (n =32)	Control group (n=18)	P
Baseline DPVF of LAD (cm/s)	0.27±0.04	0.26±0.06	0.402
Hyperemic DPVF of LAD (cm/s)	0.75±0.18	0.70±0.17	0.379
CFR	2.76±0.35	2.76±0.42	0.999

DPVF – diastolic peak flow velocity; CFR – coronary flow reserve; LAD – left arterior descending coronary artery

Table III Univariate linear regression with CFR for LAD as dependent variable.

Variable	SCH group		Control group	
	B	p	B	p
Age	-0.012	0.003	-0.019	0.002
BMI	-0.013	0.282	-0.018	0.610
WHR	-2.090	0.013	-1.897	0.307
Hypertension	0.249	0.045	0.362	0.075
Systolic tension	-0.010	0.066	-0.013	0.192
Dyastolic tension	-0.010	0.231	-0.013	0.362
Smokers	-0.256	0.037	0.033	0.877
Glucose	-0.249	0.006	-0.311	0.063
HbA1c	-0.357	0.021	-0.547	0.168
Insulin	-0.023	0.024	-0.013	0.629
HOMA. IR	-0.102	0.009	-0.116	0.326
Cholesterol	-0.165	0.005	-0.179	0.056
HDL	-0.077	0.701	-0.334	0.082
LDL	-0.160	0.028	-0.166	0.153
Triglyceride	-0.195	0.020	0.146	0.496
CRP	-0.008	0.798	-0.043	0.815
fT4	0.038	0.337	-0.016	0.810
fT3	0.057	0.693	0.340	0.216
fT3/fT4	-0.790	0.496	4.873	0.150
TSH	0.009	0.698	0.121	0.169
TPOAb	<0.001	0.802	0.001	0.124

significantly higher in SCH group, as well as titer of thyroid peroxidase (TPOAb) autoantibodies (*Table I*).

Baseline diastolic peak flow velocity (DPFV) of LAD was similar between the groups, as well as hyperemic DPFV. Accordingly, there was no statistically significant difference in CFR for LAD between the SCH group and the control group, and all the values in both groups were above the preserved limit of CFR (2.0), but with a wide range of scatterplot data (*Table II*).

By univariate linear regression analysis with CFR for LAD as dependent variable, CFR was inversely associated with the age and total cholesterol values in controls, whereas in SCH, CFR was related to the age, hypertension, smoking, total and LDL cholesterol, triglycerides, and glucose metabolism deterioration, and waist-hip ratio, implicating specific contributory effect of cardiovascular risk factors on CFR in patients with SCH (*Table III*). There was no association between TSH level and CFR nor between fT4 level and CFR in both control and SCH group.

Discussion

We have shown that in patients with SCH, microcirculatory function as assessed by 2D Doppler echocardiography derived CRF is generally preserved with wide scatter of data and without significant differences to patients with normal thyroid function. However, it seems that in patients with SCH, in comparison to normal thyroid function, the value of CFR is more dependent on traditional cardiovascular risk factors including hypertension, smoking, high cholesterol, and glucose metabolism deterioration. CFR by Doppler echocardiography, over last 10 years has been shown to be highly reproducible, efficacious and feasible noninvasive to assess microcirculatory dysfunction in different clinical scenarios affecting coronary microcirculation (18, 19).

Based on a large number of studies and meta analyses conducted in the last twenty years, it is clear that SCH leads to a somewhat increased risk for cardiovascular disease, cardiovascular mortality and overall mortality (2, 3, 6, 9, 11, 12, 20, 21), but the pathophysiologic mechanisms involved in this phenomenon are still to be defined.

Since subclinical hypothyroidism is a laboratory finding, the diagnosis of SCH should be made with caution. Different physiological conditions as well as other diseases can change the pituitary-thyroid axis, i.e. lead to a transient increase in TSH. There is also an increase in TSH with age, and this increase does not lead to increased cardiovascular mortality CVD (22). One way to overcome such doubts is to prove persistently elevated TSH over a period of time, and as Hashimoto's thyroiditis is the most common cause of both overt and subclinical hypothyroidism (1), finding of elevated TPOAb could reinforce the diagnosis of SCH (23). In our study the SCH group showed a significantly higher titer of TPOAb compared to the control group, thus confirming the existence of an autoimmune process in the thyroid gland in SCH group. We also confirmed persistently higher TSH values which, after initial elevated values, were confirmed by TSH re-determination. There was a significant increase in the fT3/fT4 ratio in the SCH group

in our study, which we know to represent the adaptive mechanism of the thyroid axis due to increased activity of deiodinase 2 (D2), which mediates T4 to T3 conversion, as well as due to higher TSH-induced increase of T3 synthesis and secretion from the thyroid gland (24).

Of all anthropometric and biochemical parameters, only C-reactive protein (CRP) and triglyceride level were significantly higher in the SCH group than in the controls. This agrees with studies that have shown similar results (25, 26). CRP is known to be a strong independent risk factor for cardiovascular events (27), not only among those with stable and unstable angina (28) but also among individuals with no current evidence of cardiovascular disease (29). Triglyceride level was also higher in SCH group in two large observational studies (30, 31), and it is well known that elevated plasma triglyceride level is an independent risk factor for cardiovascular disease (32). The values of cholesterol, its fractions and glycose related parameters (basal glucose level, insulin, HOMA IR) did not differ significantly between groups, which was also shown in some of the published papers (30, 33), but there are also papers that show a significant difference between groups in relation to these parameters (34, 35).

Only four studies have previously evaluated CFR in middle-aged patients with SCH (36–39). The two of them used dipyridamole (36) and adenosine (37) as a stressor, and both adenosine and dipyridamole induce a hyperaemic stimulus that relaxes vascular smooth muscle cells in mostly endothel-independent way. In the third study, conducted by the Oflaz et al. (38) CFR for LAD was evaluated before and after the introduction of levothyroxine replacement therapy. The fourth study evaluated endothelial-mediated CRF in SCH subjects using cold pressor test to induce endothelium-dependent vasodilation (39). Importantly, in comparison to previous studies our study did not find significant deterioration of CFR in SCH patients (36, 37, 39).

In particular, Baycan et al. (36) in 50 SCH patients and 30 controls (hyperaemia was induced by dipyridamole), showed no significant difference in anthropometric and biochemical parameters between the groups (BMI, lipids, CRP), but a significant deterioration of CFR due to blunted hyperaemic response in SCH group (2.38 ± 0.44 vs. 2.98 ± 0.47, $p<0.0001$) (36).

Oflaz et al. (37) with a smaller group of subjects (18 SCH, 24 controls) and adenosine as a stimulus of hyperaemia-endothelium independent vasodilation obtained similar results for CFR (SCH 1.97 ± 0.09 vs. controls 2.58 ± 0.08.The same authors evaluated CFR for LAD before and after the introduction of levothyroxine replacement therapy and showed that there was a significant increase in CFR for LAD in SCH group after six month levothyroxine substitution (2.03 ± 0.13 vs 2.54 ± 0.18) but the study was conducted on only ten patients with SCH (38).

Biondi et al. (39) also showed a significant difference in CFR between the SCH and control group (SCH 20, control 15), but they induced endothelium-dependent vasodilation and hyperaemia using a cold pressor test as an inducer (SCH 1.4 ± 0.2 vs. controls 1.9 ± 0.3 $p< 0.0001$) (39).

It is challenging to explain about the significant differences between our and previous results in CFR values, but few points should be emphasized regarding our study population, methodology and results. Our study population was older (SCH 52.6 ± 14, 8; controls 50.1 ± 15, 4) than study populations in Oflaz et al. (37) (SCH 45 ± 2; controls 48 ± 2 years), Baycan et al. (36) (41.4 ± 9.5; controls 41.3 ± 9.4 years) and Biondi et al. (39) (SCH38.4 ± 12.1; controls 41.4 ± 14.5 years), and since CRF is significantly negatively correlated with age, it is possible that the subtle vascular changes that might be detected in SCH are outweighed by changes due to aging. Further, in the study by Biondi et al. (39) CFR was measured after induction of endothelium-dependent vasodilatation, while in the remaining tree studies, including our study, endothelium-independent vasodilatation was induced. And third, all of these studies were performed on a relatively small number of subjects.

If we look at the dependence of CFR for LAD in our study, it is expected that in both groups there is a significant dependence of CFR on the age of the subjects. However, apart from age, there is only a significant dependence of CFR for LAD on total cholesterol in the control group. It is interesting, however, that in the SCH group, the dependence of CFR for LAD on several anthropometric and metabolic parameters (WHR, HTA, smoking, glycemia, HbA1c, basal insulin, HOMA IR, cholesterol, LDL, triglycerides) was obtained. These results suggest that individuals with subclinical hypothyroidism are more sensitive to certain metabolic, proatherogenic parameters, and this finding could be one of the explanations for the increased morbidity and mortality from cardiovascular disease in patients with SCH, which has been shown in several studies and meta-analyzes (2, 3, 9, 10). Since CRP is shown to be higher in patients with SCH compared to healthy controls, low but prolonged chronic inflammation could be the basis for greater sensitivity of the microvasculature in patients with SCH to other known cardiovascular risk factors and mechanism linking SCH and CVD, i.e. that SCH facilitate the effect of traditional risk factors on microvascular function.

Study limitations

Our study reflects a single-center experience with a relatively small number of participants. Second,

the cross-sectional design of our study limits its ability to establish causality between SCH and CFR, and long-term effects of SCH on microcirculatory and cardiovascular function.

Conclusion

Our study has shown that people with subclinical hypothyroidism have a higher risk of chronic inflammation, which plays an important role in the development of atherogenesis and thus an increased risk of developing CHD. We also showed that in patients with SCH several known risk factors for atherogenesis have a significant impact on CFR for LAD which is not the case in the control group. Although we did not find a significant difference between groups in relation to CFR for LAD, the different impact of cardiovascular risk factors on CFR for LAD suggests that these two groups behave differently in the same circumstances under the same risk factors. The basis for this difference could be that the altered »set point« of the thyroid axis changes the sensitivity of the microvasculature in patients with SCH to known risk factors, making them more susceptible for low prolonged chronic inflammation. Further investigations on a larger number of participants are needed to address in depth the relation between SCH, CFR, chronic inflammation and cardiovascular risk factors.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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PERFORMANCE EVALUATION BETWEEN TWO AUTOMATED BIOCHEMICAL ANALYZER SYSTEMS: ROCHE COBAS 8000 AND MINDRAY BS2000M

PROCENA PERFORMANSI DVA AUTOMATIZOVANA SISTEMA BIOHEMIJSKIH ANALIZATORA: ROCHE COBAS 8000 I MINDRAY BS2000M

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Summary

Background: The values of biomarkers play a central role in routine clinical decision-making. Whereas the performance of different automated chemical analyzers remains unclear. To determine the performance of different platforms, we compared the consistency and accuracy between Roche Cobas 8000 and Mindray BS2000M.
Methods: A total of 1869 remaining serum samples were collected. CK, LDH-1, RBP, Cys-C, IgA, IgM, and IgG were assessed using paired t-test, Passing-Bablok regression analysis, and Bland-Altman analysis according to CLSI EP5-A3.
Results: There were significant differences in the average bias of all items between the two machines ($P<0.001$). Because the 95% confidence interval of intercept A included 0, CK, LDH-1, Cys-C and IgG did not show systematic error in Passing-Bablok regression analysis. The confidence interval of 95% of the slope B in IgM contained 1, and there was no difference in the two measurements in IgM. Except for IgA, the *r* values and correlation coefficient of all items were higher than 0.91, which showed that the correlation and consistency were good. The Bland-Altman analysis showed that two instruments had more than 95% of the points apart from CK, LDH-1, and IgA.

Kratak sadržaj

Uvod: Vrednosti biomarkera igraju centralnu ulogu u rutinskom kliničkom donošenju odluka. S druge strane, performanse različitih automatizovanih hemijskih analizatora ostaju nejasne. Kako bismo utvrdili performanse različitih platformi, izvršeno je upoređivanje konzistentnosti i tačnosti između analizatora Roche Cobas 8000 i Mindray BS2000M.
Metode: Prikupljeno je ukupno 1869 preostalih uzoraka seruma. Vrednosti CK, LDH-1, RBP, Cys-C, IgA, IgM i IgG su određene korišćenjem uparenog t-testa, Passing-Bablok regresije i Bland-Altmanove analize prema CLSI EP5-A3.
Rezultati: Pokazale su se značajne razlike u prosečnoj pristrasnosti svih stavki između dve mašine ($P < 0,001$). Pošto je interval poverenja od 95% preseka A uključivao 0, CK, LDH-1, Cys-C i IgG nisu pokazali sistematsku grešku u Passing-Bablok regresionoj analizi. Kod intervala poverenja od 95% nagiba B u IgM koji je sadržao 1, nije bilo razlike u dva merenja u IgM. Osim za IgA, *r* vrednosti i koeficijent korelacije kod svih stavki su bili veći od 0,91, što je pokazalo dobru korelaciju i konzistentnost. Bland-Altmanova analiza je pokazala da su dva instrumenta imala više od 95 procentna poena osim za CK, LDH-1 i IgA.

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List of abbreviations: CLSI, Clinical and Laboratory Standards Institute; CK, creatine kinase; AMI, acute myocardial infarction; LDH-1, lactate dehydrogenase-1; RBP, retinol-binding protein; Cys-C, Cystatin-C; IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G; CSF, cerebrospinal fluid; NABL, the National Laboratory Accreditation Board; CI, confidence interval; CV, coefficient of variation; LOA, the limit of agreement; Min, minimum; Max, maximum; CC, correlation coefficient.

†These authors contributed equally to this work and should be considered as co-first authors.

Conclusions: It can be considered that the two instruments have good correlation and consistency in CK, LDH-1, RBP, Cys-C, IgM, and IgG, and the two instruments are interchangeable and can replace each other.

Keywords: Analytical techniques and equipment, validation, Roche Cobas 8000, Mindray BS2000M, comparison

Zaključak: Može se reći da dva instrumenta imaju dobru korelaciju i konzistentnost u pogledu CK, LDH-1, RBP, Cys-C, IgM i IgG, te se mogu koristiti bez razlike.

Ključne reči: analitičke tehnike i oprema, validacija, Roche Cobas 8000, Mindray BS2000M, poređenje

Introduction

The biomarkers in clinical laboratories have played a key role in medical decisions for patients' diagnosis, treatment, and prognosis (1–4). Therefore, the results tested by diagnostic machines must be more precise, accurate, sensitive, and specific. Technological advances have greatly improved the development of laboratory medicine and met the growing demands in routine biochemistry analysis, such as high throughput analysis of multi-parameters, leading to the increasing use of new analyzers. Implementing an automation analyzer in laboratory diagnostics provides advantages and convenience for requiring a high degree of value with precision and accuracy (5). However, the detected results of the same samples by different machines are sometimes inconsistent. For example, some common clinical chemistry analytes have shown that comparable problems still exhibited unacceptable or suboptimal bias compared to the true value (6). Imprecise or incorrect results might lead to immeasurably serious consequences for patients, clinicians, and even the entire health care system. Hence, emphasis should be placed on examining the standardized protocol (7).

However, it remains controversial whether the values of the different equipment in an identical medical laboratory from the same specimen may be inconsistent and not the same according to the standardized operating procedures. It is high time that the emphasis is placed on creating different reference intervals for different machines. Recently, most previous studies have compared automated hematology analyzers about Beckman Coulter, Sysmex, and Mindray (8–11), automated hemostasis analyzer between Sysmex and Atellica (12), automated bacterial identification, and drug sensitivity analyzer between GENECUBE and Vitek (13). However, some research has studied other different automated chemistry analyzers, such as Abbott, Roche, Beckman Coulter, and Hitachi (6, 14, 15).

Nikolac Gabej et al. (14) recently focused on three parameters about hemoglobin and bilirubin intralipid in Abbott, Roche, and Beckman Coulter. Having included 21 items, Lim et al. (6) emphasized liver and kidney function and blood lipid. Though Leitner-Ferenc et al. (1) study underlined the reference intervals on the Roche Cobas 8000 platform based on the Clinical and Laboratory Standards Institute (CLSI), their study only focused on gender difference. As we all know, automated chemistry analyzers can detect organ functions and all kinds of metabolites. These published studies have indicated excellent performance in the precision and accuracy of automated biochemical analyzers. Mindray BS2000M, a new generation automated chemistry analyzer, is a high test, less reaction volume, and multi-wavelengths system. None of the studies have focused on the performance and evaluation of Mindray BS2000M. Moreover, none of these studies have defined the performance at low, normal, and high concentration. Therefore, we designed the present study and paid attention to the differences in myocardial enzyme, kidney function, and immunoglobulin in Roche and Mindray. As far as we know, we are first to compare the two machines' performance. For myocardial enzymes, such as creatine kinase (CK), which appears very early after the attack of acute myocardial infarction (AMI), its sensitivity reaches 98% in the diagnosis after the onset of the disease of AMI. Moreover, a previous study revealed that patients with high CK had a worse prognosis (16). Another enzyme, lactate dehydrogenase-1 (LDH-1), owing to being increased in blood 5~10 hours after AMI, was also treated as an early biomarker of AMI (16). As mentioned, many studies have shown the discrepancies of common kidney biomarkers, for instance, creatinine, blood urea nitrogen, and uric acid, between different measurements. We focused on other markers that were more sensitive and specific. Retinol binding protein (RBP), which can remain stable in acid urine and quickly appear after an early renal proximal injury, is considered to be a reliable and sensitive parameter for kidney injury (17). Cystatin-C (Cys-C) improves the risk classification of patients with chronic kidney disease, death, cardiovascular disease (3), and end-stage renal disease (18). As the effector molecules of the adaptive humoral immune system, high or low levels of immunoglobulins cause an allergic reaction or immunodeficiency diseases. Since immunoglobulin A (IgA) can limit antigen access to host tissues, it was referred to as the mucosal barrier in immune exclusion and shed light on the importance of regulating food allergen sensitization (19). At the same time, the patients with Crohn's disease or ulcerative colitis also showed that serum IgA in blood was elevated (20). While for common variable immunodeficiency and primary immunodeficiency diseases, the level of IgA of patients may be deficient (21). Immunoglobulin M (IgM), involved in both immune protection and immunoregulatory functions, is treated as the first line of humoral defense

against pathogens (22). Reducing IgM might increase the risk of infection, exacerbate autoimmunity as well as atherosclerosis (23). High immunoglobulin G (IgG) helps to diagnose autoimmune hepatitis (24) and IgG in cerebrospinal fluid (CSF), which is useful for the diagnosis of multiple sclerosis (15). In addition to the mean bias in the two instruments, test characteristics related to consistency and correlation in two measurements were investigated.

Measurement of laboratory analytical errors falls into two main categories, systematic error and random error. Systematic errors are predictable problems that influence observations consistently in one direction, while random errors are more unpredictable. Sources that contribute to uncertainty may include samples, calibrators, reference materials, input quantities, equipment, and environmental conditions.

Materials and Methods

Samples

A total of 1869 remaining serum samples were collected from outpatients and inpatients at the Second Affiliated Hospital of Guangxi Medical University from July 2019 to October 2019 for diagnostic accuracy. All samples were tested within 2 hours after centrifugation of 4000 g for 5 minutes. Specimens that could not be tested immediately were refrigerated at 4 after centrifugation, and tests were completed within 24 hours. Samples must be thawed to room temperature and mixed thoroughly after refrigeration. After being tested on Cobas 8000 c702 (Roche, Basel, Switzerland), those serum samples were immediately tested on Mindray BS2000M (Mindray Bio-Medical Electronics Co., Ltd, Shenzhen, China) to guarantee the consistency of time and the accuracy of results. Those samples were categorized as being of abnormally high, abnormally low, or normal value. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Guangxi Medical University.

Reagents

All of the procedures were carried out according to the manufacturer's protocols. In brief, CK tested by Cobas 8000, and Mindray BS2000M used colorimetry and phosphocreatine substrate method, respectively. LDH-1tested by Cobas and Mindray used the rate method and lactic acid substrate method, respectively. Latex immunoturbidimetry by using Cobas-reagents was used for RBP, Cys-C analysis, while they were examined by latex enhanced immunoturbidimetry in Mindray. For IgA, IgM, and IgG, all were detected by immunoturbidimetry in two automatic analyzers. All methods in seven parameters are summarized in *Table I*.

Table I Characteristics of the compared methods between Cobas 8000 and Mindray BS2000M.

Parameter	Cobas 8000 method	Mindray BS2000M method
CK	Colorimetry	Creatine phosphate substrate method
LDH-1	Rate method	Lactic acid substrate method
RBP	Latex immunoturbidimetry	Latex enhanced immunoturbidimetry
Cys-C	Latex immunoturbidimetry	Latex enhanced immunoturbidimetry
IgA	Immunoturbidimetry	Immunoturbidimetry
IgM	Immunoturbidimetry	Immunoturbidimetry
IgG	Immunoturbidimetry	Immunoturbidimetry

Quality control

All reagents, quality control products, and calibration products were original reagents that matched with the machine. The instrument was calibrated according to the manufacturer's guidelines using calibration samples provided by the manufacturer. High, normal, and low control samples were run every day to monitor the system's performance according to the National Laboratory Accreditation Board (NABL) guideline and CLSI EP5-A3 (25). To evaluate the quality of our results from two machines, two levels of control in seven parameters were detected every time, including Lot 32419602 and 32419602 in CK and LDH-1, Lot 1293uN, and 983uE in RBP and Cys-C, and Lot 48902 and 48903 in IgA, IgM, and IgG. The coefficient of variation of quality control in all parameters was less than 10% which means that the results of quality control were in control. There was nothing unusual in control, which demonstrates that the quality of controls was acceptable. Then the serum samples were tested according to the manufacturer's instruction and strictly followed standard operating procedure.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and MedCalc v18.2.1 (Ostend, Belgium). The paired t-test was used to compare the mean bias of results in two instruments. Bland-Altman plot (26, 27) was used to evaluate the consistency of the two machines. Passing-Bablok regression analysis (28) was used to evaluate the regression equation and the correlation of the two instruments. If the 95% confidence interval (CI) of intercept A does not contain 0, there are systematic errors in the two instruments. The slope B was used to measure the difference in the ratio between the two instruments. The 95% CI for slope B

did not include 1, which means that there are a few differences between the two methods. The Cusum test for linearity was used to test the applicability of the Passing-Bablok regression. If $P<0.05$, it indicates that there is no linear relationship between the two apparatuses, so this method is not applicable. When the correlation coefficient r is lower than 0.4, the correlation degree is low. If r is more than 0.4 but lower than 0.7, the correlation degree is moderate. If r is higher than 0.7, the correlation degree is high. All comparison with P-value <0.05 was considered statistically significant.

Results

Descriptive analysis of different methods

As shown in *Table II*, the IgG in Cobas 8000 had a minimum CV value of 2.64%, while CK in Cobas 8000 reached 7.10%. However, all CVs of the parameter in the two instruments were lower than 10%. The paired t-test was performed, and the results revealed a statistically significant difference in all items (both $P<0.001$). All methods of different items between the two platforms were summarized in *Table I*.

Comparison methods

Based on the clinical significance of these parameters level, serum samples were divided into two levels (low and high level) and three levels (low, normal, and high level). Three of seven items (LDH-1, RBP, and Cys-C) and four of seven items (CK, IgA, IgM, and IgG) were divided into either two or three levels according to the clinical reference range. All subgroups of these parameters are shown in *Table III*.

The comparison between seven items of two instruments was carried out using Passing and Bablok regression analysis and Bland-Altman plots. The results of this statistical analysis are shown in *Table III* and *Table IV*. A high correlation was obtained for analysis compared with two instruments for most parameters in all results but not subgroups in six items (r ranging from 0.904 to 0.995) except for IgA ($r=0.857$) by Spearman rank correlation analysis. However, the high level of IgA (>4.53 g/L) between the two instruments showed little correlation ($r=0.089$). Moreover, there was a high correlation between 7 parameters in the two machines according to correlation coefficient (CC) results. All CC of items were more than 0.7, whether the items had low, moderate, and high values, except when IgA was more than 4.53 g/L (CC: 0.605, 95%CI 0.426–0.738) (*Table III*). All correlations were statistically significant (P<0.001).

On the Bland-Altman plot, the average bias in Cys-C, IgA, and IgM was close to zero (0.520, 0.189, and 0.046, respectively), while the average bias of CK and LDH-1 in the two machines were -11.938 and 12.180, respectively (*Table III*). In particular, the comparison of Cobas 8000 and Mindray data showed a significant negative bias for CK while the bias was positive for LDH-1 and RBP (*Figure 1*). In addition, three-sevenths of two instruments had more than 95% of the points within the 95% consistency limit (RBP 96.4%, IgM 95.6%, and IgG 95.0%) in Bland-Altman analysis, meeting the consistency requirements. The remaining four items were also more than 90% (data not shown). The absolute value of the difference between the two machines was less than 10% which demonstrates that the difference is clinically acceptable.

Table II Evaluation of the imprecision between the two measurements.

Parameter (units)	Cases	Instrument	Min	Max	Mean	CV (%)	P
CK (U/L)	241	Cobas 8000	11	1026	147.5	7.10	<0.001
		Mindray BS2000M	25	1000	159.44	6.99	
LDH -1 (U/L)	261	Cobas 8000	25	572	97.48	4.79	<0.001
		Mindray BS2000M	10	500	85.3	4.83	
RBP (mg/L)	275	Cobas 8000	7.9	157.5	44.19	3.47	<0.001
		Mindray BS2000M	10	150	38.63	4.14	
Cys-C (mg/L)	272	Cobas 8000	0	10	1.49	5.17	<0.001
		Mindray BS2000M	0.4	6.34	0.971	5.84	
IgA (g/L)	311	Cobas 8000	0.06	8.61	2.8654	3.72	<0.001
		Mindray BS2000M	0.4	6.06	2.6767	3.40	
IgM (g/L)	271	Cobas 8000	0.056	4.02	1.2744	4.71	0.001
		Mindray BS2000M	0.35	3.6	1.3206	4.43	
IgG (g/L)	238	Cobas 8000	1.99	36	12.8547	2.64	<0.001
		Mindray BS2000M	1.03	27.6	10.501	2.81	

Min: minimum, Max: maximum, CV: coefficient of variation

Table III Spearman rank correlation, Bland-Altman plot analysis and correlation coefficient in the two systems.

Parameter	Group	N	r	Average bias (95%CI)	LOA	CC (95%CI)	P
CK	All	241	0.995	-11.938 (-13.968 to -9.907)	(-43.303 to 19.428)	0.997 (0.997 to 0.998)	<0.001
	7–40 (U/L)	65	0.807	-3.846 (-4.636 to -3.056)	(-10.095 to 2.402)	0.955 (0.927 to 0.972)	<0.001
	40–300 (U/L)	127	0.994	-7.937 (-9.175 to -6.699)	(-21.756 to 5.882)	0.997 (0.995 to 0.998)	<0.001
	>300 (U/L)	49	0.975	-33.041 (-39.782 to -26.300)	(-79.040 to 12.958)	0.933 (0.884 to 0.962)	<0.001
LDH-1	All	261	0.956	12.180 (10.053 to 14.307)	(-22.020 to 46.380)	0.977 (0.971 to 0.982)	<0.001
	<90 (U/L)	125	0.910	7.288 (6.190 to 8.386)	(-4.873 to 19.449)	0.933 (0.905 to 0.952)	<0.001
	≥90 (U/L)	136	0.934	16.677 (12.852 to 20.501)	(-27.522 to 60.874)	0.898 (0.860 to0.927)	<0.001
RBP	All	275	0.910	5.579 (4.635 to 6.524)	(-10.019 to 21.178)	0.980 (0.974 to 0.984)	<0.001
	<70 (U/L)	218	0.913	5.845 (5.299 to 6.391)	(-2.167 to 13.856)	0.962 (0.950 to 0.970)	<0.001
	≥70 (U/L)	57	0.459	4.563 (0.4200 to 8.706)	(-26.042 to 35.169)	0.886 (0.814 to 0.932)	<0.001
Cys-C	All	272	0.981	0.520 (0.475 to 0.564)	(-0.208 to 1.246)	0.954 (0.942 to 0.964)	<0.001
	<1.03 (mg/L)	119	0.618	0.313 (0.300 to 0.327)	(0.168 to 0.459)	0.770 (0.686 to 0.835)	<0.001
	≥1.03 (mg/L)	153	0.981	0.679 (0.611 to 0.748)	(-0.157 to 1.516)	0.963 (0.950 to 0.973)	<0.001
IgA	All	311	0.851	0.189 (0.107 to 0.271)	(-1.252 to 1.630)	0.935 (0.919 to 0.948)	<0.001
	<0.82 (g/L)	57	0.145	-0.220 (-0.344 to -0.095)	(-1.139 to 0.700)	0.839 (0.740 to 0.902)	<0.001
	0.82–4.53	188	0.957	0.070 (0.041 to 0.099)	(-0.328 to 0.468)	0.983 (0.977 to 0.987)	<0.001
	>4.53 (g/L)	67	0.089	0.867 (0.560 to 1.174)	(-1.601 to 3.334)	0.605 (0.426 to 0.738)	<0.001
IgM	All	271	0.950	0.046 (-0.073 to -0.020)	(-0.481 to 0.389)	0.981 (0.976 to 0.985)	<0.001
	<0.46 (g/L)	68	0.436	-0.058 (-0.082 to -0.034)	(-0.255 to 0.139)	0.829 (0.736 to 0.891)	<0.001
	0.46–3.04	174	0.912	-0.071 (-0.097 to -0.044)	(-0.421 to 0.279)	0.962 (0.950 to 0.972)	<0.001
	>3.04 (g/L)	29	0.251	0.129 (-0.049 to 0.307)	(-0.787 to 1.045)	0.695 (0.441 to 0.846)	<0.001
IgG	All	238	0.904	2.354 (2.139 to 2.569)	(-0.945 to 5.652)	0.947 (0.932 to 0.958)	<0.001
	<7.51 (g/L)	46	0.737	0.793 (0.569 to 1.018)	(-0.688 to 2.274)	0.861 (0.761 to 0.921)	<0.001
	7.51–15.60	127	0.751	2.343 (2.139 to 2.546)	(0.0712 to 4.615)	0.860 (0.807 to 0.900)	<0.001
	>15.60 (g/L)	65	0.739	3.479 (2.960 to 3.999)	(-0.633 to 7.592)	0.820 (0.720 to 0.886)	<0.001

LOA: limit of agreement, CC: correlation coefficient, CI: confidence intervals

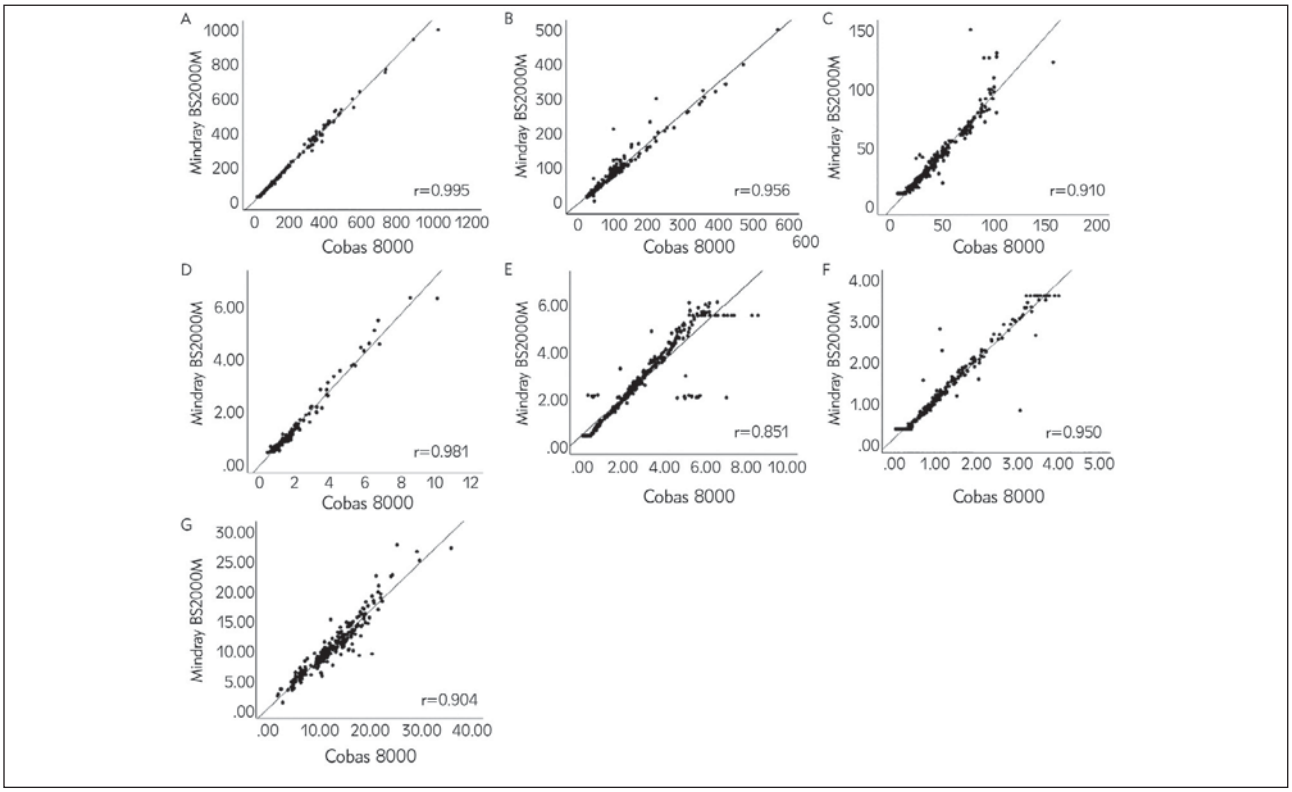

Figure 1 Spearman rank correlation of evaluated parameter between the two machines (A) CK, (B) LDH-1 (C) RBP, (D) Cys-C, (E) IgA, (F) IgM, (G) IgG.

Table IV A Passing-Bablok regression analysis for the two analyzers.

Parameter	Regression equation	Intercept A (95%CI)	Slope B (95%CI)
CK	y = 0.769 + 1.077 x	0.769 (-0.027 to 1.333)	1.077 (1.067 to 1.087)
LDH-1	y = -0.206 + 0.8504 x	-0.206 (-1.579 to 0.775)	0.851 (0.838 to 0.868)
RBP	y = -4.351 + 0.947 x	-4.351 (-4.960 to -3.705)	0.947 (0.928 to 0.966)
Cys-C	y = -0.023 + 0.653 x	-0.023 (-0.050 to 0.003)	0.653 (0.628 to 0.678)
IgA	y = 0.092 + 0.925 x	0.092 (0.066 to 0.118)	0.925 (0.914 to 0.936)
IgM	y = 0.044 + 0.995 x	0.044 (0.029 to 0.056)	0.995 (0.981 to 1.011)
IgG	y = -0.238 + 0.840 x	-0.238 (-0.600 to 0.115)	0.840 (0.808 to 0.871)

CI: confidence intervals

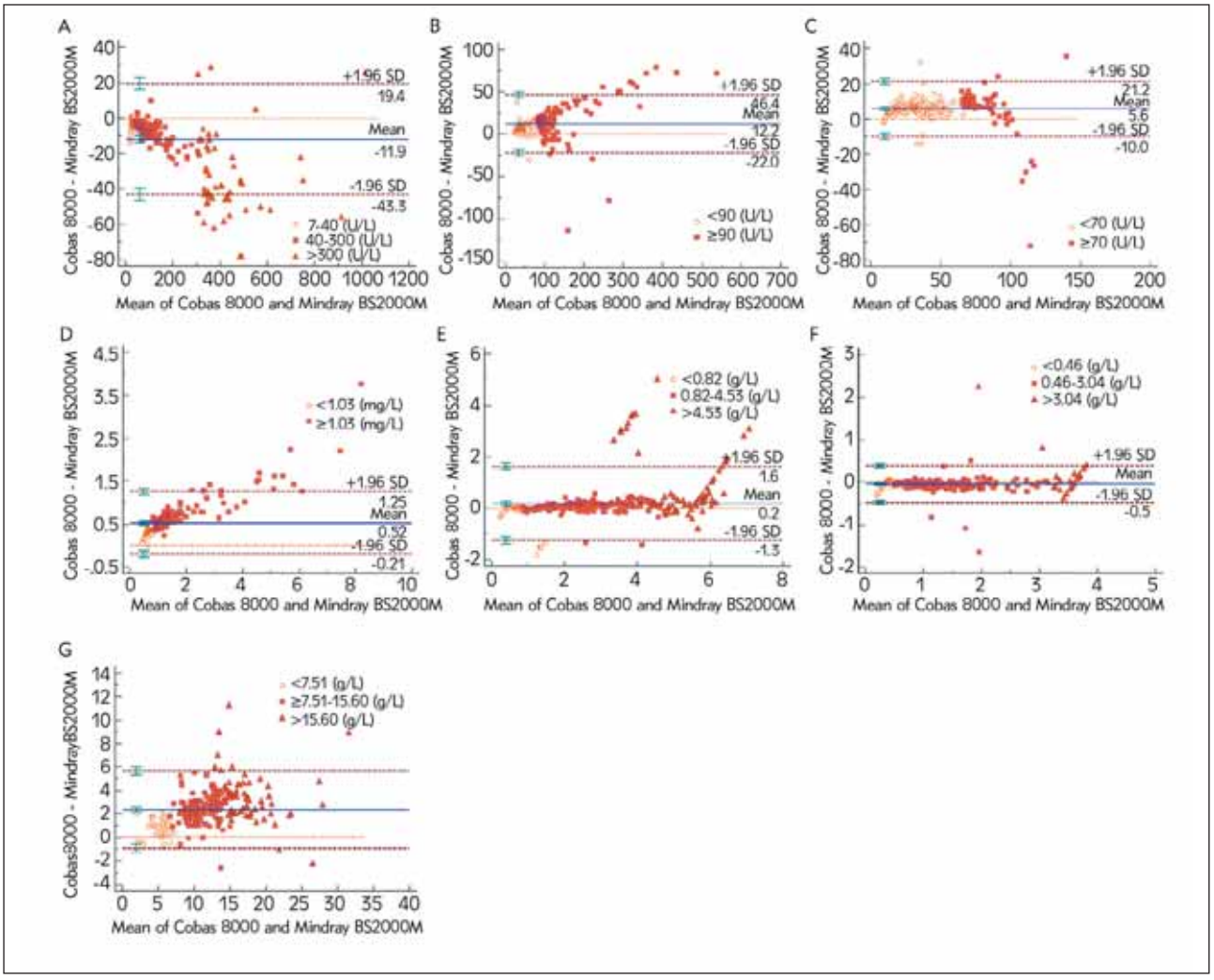

Figure 2 Bland-Altman plots of the difference of the evaluated parameter between the two machines (A) CK, (B) LDH-1 (C) RBP, (D) Cys-C, (E) IgA, (F) IgM, (G) IgG.

According to Passing and Bablok regression analysis, 95% CI for the intercept A of the regression equation for CK, LDH-1, Cys-C, and IgG includes 0, and there is no systematic error between the two instruments. For IgA and IgM, the 95% CI for intercept A was very close to zero. Only a relatively high intercept A can be found in RBP (intercept A: -4.351, 95% CI -4.960 to -3.705). Except for IgM, the 95% CI of the slope B contains 1 (0.9806–1.0105), another the slope B of CK, RBP, and IgA were almost equal

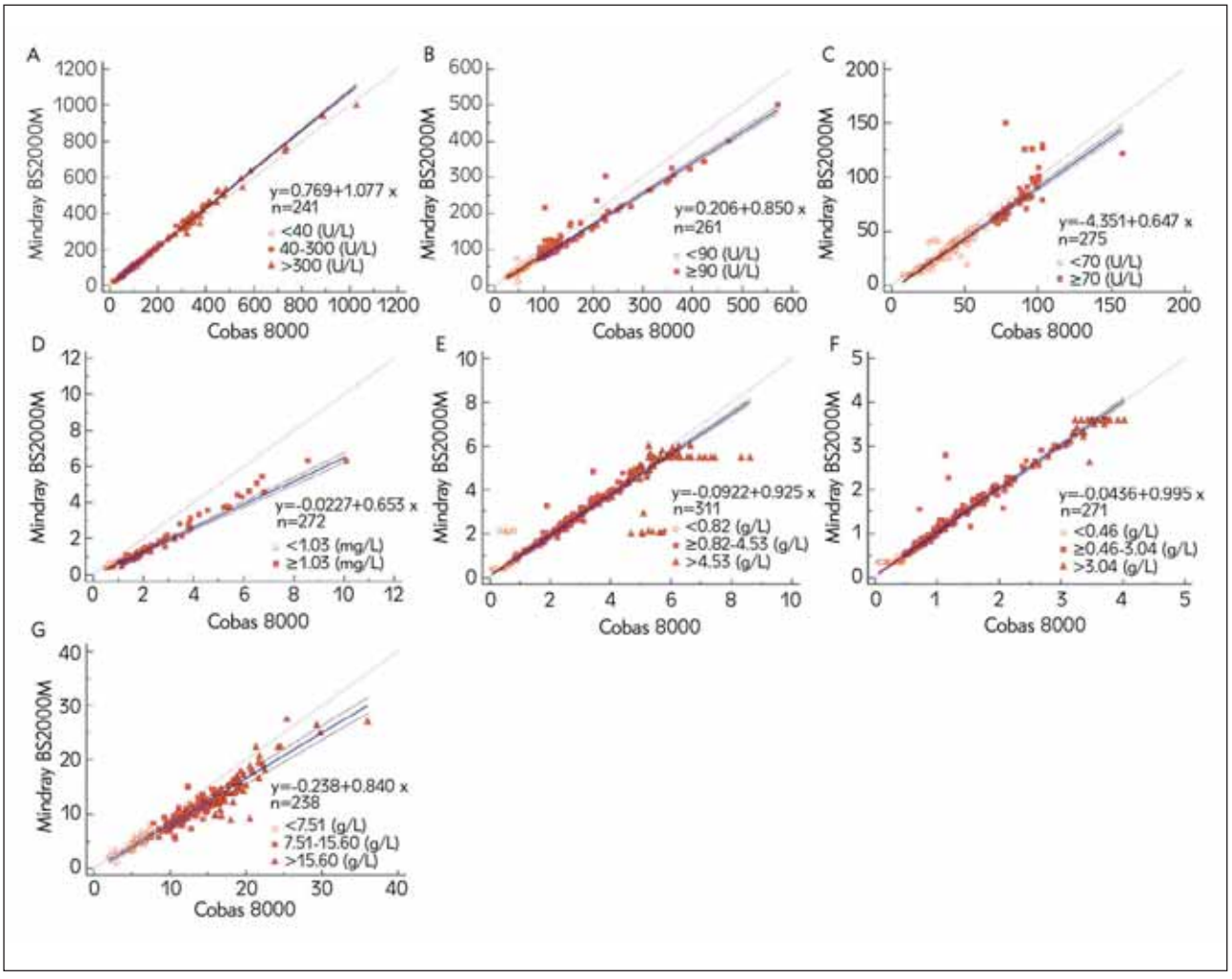

Figure 3 Passing-Bablok regression of the difference of the evaluated parameter between the two machines (A) CK, (B) LDH-1 (C) RBP, (D) Cys-C, (E) IgA, (F) IgM, (G) IgG.

to 1 (1.077, 0.947, and 0.925, respectively). For LDH-1, Cys-C, and IgG, slope B did not contain 1 (0.851, 0.653, and 0.840, respectively), which shows some proportional differences between the two instruments (*Table IV*). Therefore, it can be considered that the results of the two pieces of equipment are consistent, and the two devices are interchangeable.

Discussion

The availability of rapid and automated methods regarded as a major breakthrough in the laboratory can decrease the labor force and increase consistency and repeatability. Indeed, in addition to improving the clinical effectiveness, the new generation of automated analyzers increases laboratory efficiency by reducing working time and costs associated with the optical validation of the results. At present, the most regularly used chemistry platforms in the laboratory are Abbott, Beckman Coulter, Roche Cobas, and Mindray. Different detection systems using different methods will produce different results for different samples on different detected platforms, and this difference may affect routine clinical decision-making. Hence, when utilizing different analyzers to disclose the same items, the instrument needs to be contrasted with guaranteeing the consistency and conformity of the detected results. Numerable studies have focused on comparing biomarkers in Abbott, Hitachi, and Roche (6, 14). As previously described in the literature, these clinical chemistry assays are accurate and reliable and are readily applicable on various platforms. Some newly launched and advanced chemical and immune analyzers remain uncertain. This study aimed to compare basic biochemistry parameters between Roche Cobas 8000 and Mindray BS2000M.

To our knowledge, this is the first large study using two automated chemistry platforms Roche Cobas 8000 and Mindray BS2000M, to assess the

equivalence of common organ function parameters. A total of 1869 samples were screened in our study. The ultimate objective was to evaluate whether the detected values in different analyzers were identical and therefore interchangeable when informing clinicians' decisions in diagnosis, treatment, and prognosis. All items in the two platforms were appraised according to CLSI protocols (7, 25).

In the assessment of linearity, the *r* for all analytes at all levels was more than 0.9 except for IgA ($r=0.851$). All items within the clinical reference range showed excellent linearity. However, the linearity of RBP, Cys-C, IgA, and IgM at a low or high level was verified outside the range as claimed by the manufacturer. Regarding the correlation of parameters in two systems, we found that the correlation of all analytes at all levels was highly relevant (CC> 0.95, $P<0.001$). However, the CC of IgA and IgM at a high level showed a low correlation (0.605 and 0695, respectively). According to the regression equation between Roche Cobas 8000 and Mindray BS2000M, CK, IgA, and IgM performance were excellent in our study, which did not show a statistically significant proportional error or constant error. On the contrary, RBP in the two instruments displayed a significant constant error (intercept A=-4.351), and Cys-C showed obviously proportional error (slope B=0.653). There remained a small proportional error in LDH-1 (slope B=0.851) and IgG (slope B=0.840).

In Bland-Altman's plot, Cys-C, IgA, IgM, RBP, and IgG showed a low average bias (0.520, 0.189, 0.046, 5.579, and 2.354, respectively), and their mean bias in the former three almost closed to 0. While for CK and LDH-1, the mean differences were higher (-11.938 and 12.180, respectively) and the same as the limit of agreement (LOA), proportionally increasing with the growing levels (CK: -43.303 to 19.428, LDH-1: -22.020 to 46.380). For instance, their average bias showed significant differences in CK (-3.846 to -33.041) and LDH-1 (7.288 to 16.677), compared with the Cys-C, IgA, and IgM. We suggest three possible explanations for why the average bias of CK and LDH-1 was so wide. One possible reason is that the two platforms use the different detection methods for CK (colorimetry vs. creatine phosphate substrate method) and LDH-1 (rate method vs. lactic acid substrate method). The study of He et al. demonstrated that the coefficient of variation of Cys-C showed a significant difference (P=0.016), very low pass rates, and widespread distributions (from 3.63% to 6.74%) in internal quality control of laboratories using different systems from 2014 to 2017 in China (29). Meanwhile, Han et al. (30) study also showed that LDH-1 should be improved their precision and accuracy at the same time after being evaluated sigma index, further supporting our investigation. Another factor caused by the significant mean difference was that the detection limits of different platforms are different. If the true value of parameters exceeds upper detection limits, one of the common solutions in regular work of laboratories to solve high-level samples is for an operator to dilute the sample by adding low level serum or matrix (31). A previous study also demonstrated that substrate depletion plays a key role in causing negative results. The enzyme linearity extension function in BS-2000M2 can effectively solve the risk of false-negative results for high-level samples (32). Hence, to avoid unnecessary misleading and misconceptions, the sample from one patient should not be detected separately on different methods of different systems in the same laboratory. One should not use sample internal quality control rule if it is necessary to use a different sample to verify or review the values of parameter. Moreover, it is wise and advisable for different laboratories to establish reference ranges and dilute high levels samples beyond upper limitation.

This study has mentioned limitations. One of them was that the performance of our study only compared with two analyzers (Roche Cobas 8000 and Mindray BS2000M) and did not include more clinical chemistry platforms, such as Abbott and Hitachi. Due to the small volume of samples, there was no possibility of repeating the analysis with every analyzer once more. Another disadvantage was that the samples included in our study contained all kinds of patients and healthy people. Further study on the performance of biochemical or immune items by various analyzers in a more significant number of cases and multicenter should be performed to validate the findings of this study. Based on the data in our study, we can conclude that the analytical performances of RBP, Cys-C, IgA, IgM, and IgG are excellent, while CK and LDH-1 need to be improved to decrease or remove the systematic error as much as possible.

Taken together and to the best of our knowledge, this is the first study to describe the performance characteristics of the Roche Cobas 8000 and Mindray BS2000M systems. The two platforms have good correlation and bias for detecting CK, LDH-1, RBP, Cys-C, IgM, and IgG analytes. They have a high method agreement in CK, LDH-1, IgA, IgM, and IgG. In summary, Cobas and Mindray clinical chemistry assays are reliable and precise, and applicable to different analytic platforms.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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MAGNESIUM SUPPLEMENTATION AND IRON STATUS AMONG FEMALE STUDENTS: THE INTERVENTION STUDY

SUPLEMENTACIJA MAGNEZIJUMA I STATUS GVOŽĐA KOD STUDENTKINJA: STUDIJA INTERVENCIJE

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Summary

Background: Literature data indicate the benefit of magnesium (Mg) supplementation. The aim of this study was to examine the effect of short-term Mg supplementation on iron status in healthy female participants.
Methods: One hundred healthy female students of the University of Belgrade - Faculty of Pharmacy participated the study during eleven intervention days. Students ingested Mg preparations with the same dose of the active substance. The analysis included the measurement of serum iron, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), total Mg (tMg), ionized Mg (iMg), complete blood count, met-, carboxy- and oxy-haemoglobin (metHgb, COHgb, O_2Hgb). Transferrin concentrations and percentage of transferrin saturation (SAT) were calculated manually. The association among the analyzed biochemical parameters was examined using polynomial regression. A principal component analysis (PCA) was used for the evaluation of interdependence between the analyzed parameters.
Results: A statistically significant trend for change in O_2Hgb (%) by tertiles of iMg concentrations was found ($P = 0.029$). Serum tMg reached significant positive correlation with the SAT at concentration levels greater than 0.9 mmol/L, after 11 days of intervention (R^2=0.116). Ionized Mg in a concentration higher than 0.6 mmol/L is positively correlated with SAT and serum Fe (R^2=0.214; 0.199, respectively).

Kratak sadržaj

Uvod: Literaturni podaci ukazuju na benefit suplementacije magnezijumom (Mg). Cilj ove studije bio je da se ispita uticaj kratkotrajne suplementacije Mg na status gvožđa kod zdravih žena.
Metode: Sto zdravih studentkinja Univerzitet u Beogradu – Farmaceutskog fakulteta je učestvovalo u istraživanju tokom jedanaest dana intervencije. Studenti su uzimali preparate Mg sa istom dozom aktivne supstance. U serumu je određivano gvožđe, nezasićen kapacitet vezivanja gvožđa (UIBC), ukupan kapacitet vezivanja gvožđa (TIBC), ukupan Mg (tMg), jonizovni Mg (iMg), kompletna krvna slika, met-, karboksi- ioksi- hemoglobin (metHgb, COHgb, O_2Hgb). Transferin i saturacija transferina (SAT) su izračunati ručno. Povezanost analiziranih biohemijskih parametara je ispitana pomoću polinomalne regresije. Za procenu međuzavisnosti između analiziranih parametara korišćena je analiza glavnih komponenti (PCA).
Rezultati: Utvrđen je statistički značajan trend promene O_2Hgb (%) po tertilima koncentracija iMg ($P = 0,029$). Ukupan Mg je dostigao značajnu pozitivnu korelaciju sa SAT pri koncentracijama većim od 0,9 mmol/L, nakon 11 dana intervencije ($R^2 = 0,116$). Jonizovani Mg u koncentraciji većoj od 0,6 mmol/L pozitivno korelira sa SAT i gvožđem ($R^2 = 0,214$; 0,199, redom). PCA analizom je pokazana varijabilnost od 64,7% za dve ose nakon 11 dana.

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PCA revealed variability of 64.7% for two axes after 11 days. **Conclusions:** Mg supplementation leads to an improvement in the certain iron status parameters even in individuals with optimal levels of these indices. However, caution should be exercised when supplementing Mg, and laboratory monitoring of the interaction is required.

Keywords: magnesium, supplementation, iron status, female, students

Zaključak: Suplementacija Mg dovodi do poboljšanja određenih parametara statusa gvožđa čak i kod pojedinaca sa optimalnim nivoima ovih parametara. Međutim treba biti oprezan pri suplementaciji Mg, a dodatno je neophodno i laboratorijsko praćenje ovih interakcija.

Ključne reči: magnezijum, suplementacija, status gvožđa, žene, studenti

Introduction

Magnesium (Mg) plays an important role in many physiological functions. As a cofactor for over 600 enzymes and an activator of an additional 200 enzymes, Mg is involved in almost all major biochemical and metabolic processes in the body. Magnesium has an important role in macronutrient and energy metabolism, neuromuscular function, bone development, cell proliferation and signalling pathways (1, 2). Magnesium is an essential micronutrient, and therefore it must be supplied regularly via food sources to reach the recommended intake and prevent deficiency. Insufficient dietary intake of Mg is one of the most important causes of hypomagnesaemia (3). Recommendations for Mg intake for adults in the United States (Recommended Daily Allowances, RDA) are 320 mg/day for women and 420 mg/day for men and in Europe 300 mg/day for women and 350 mg/day for men (Dietary Reference Values, DRVs) (4, 5). Although Mg is widely distributed in plant and animal foods as well as in beverages, literature data indicate that the dietary intake of Mg is below the recommended levels in a large percentage of European and US populations where there is a higher prevalence of the Western dietary pattern (6–9). There is also evidence that many young women in various European countries and in the US fail to achieve these recommended intakes (2, 10). Furthermore, epidemiological studies have shown that people who follow a Western-style diet, characterized by a high intake of processed foods, have an inadequate intake of several micronutrients, with dietary Mg intake of less than 30–50% of RDA (11, 12).

In recent decades, insufficient Mg intake and consequent hypomagnesaemia have been associated with several cardiovascular conditions, including hypertension, an increased risk of glucose intolerance, metabolic syndrome, and type 2 diabetes (1–3, 13). Moreover, a meta-analysis of eight prospective cohort studies has reported a significant inverse association between Mg intake and risk of type 2 diabetes in a dose-response manner (14). In addition, Mg deficiency is closely related to the development of anaemia (15, 16).

There is accumulating research regarding the importance and modalities for achieving adequate Mg intake. Magnesium supplementation may be a good strategy for preventing deficiency and preventing associated diseases, when the recommended daily intake cannot be provided solely by the diet (1–3, 13). According to the recently conducted survey, Mg supplements were among ten most popular dietary supplements used in adult population (17). Magnesium supplementation led to improved anemia in thalassemic mice and improved erythrocyte membrane transport abnormalities in patients with sickle cell disease (18, 19), while in athletes, Mg supplementation increased erythrocyte count and haemoglobin levels (20). Moreover, according to the Shi et al. (16) Mg supplementation may be a safer alternative than iron (Fe) supplementation in the prevention of anemia.

Although previously published data suggest the beneficial effects of increased Mg intake on Fe status among anaemic individuals, research on this issue is lacking for the non-anaemic population (15, 16). It is generally accepted that the female population is more prone to Fe deficiency anemia and more vulnerable on Fe status than men (21, 22). Therefore, it is important to ensure stable Fe status, particularly in the reproductive period of life. The aim of this study was to examine the effect of short-term Mg supplementation in doses of 375 mg corresponding to the 100% of Mg Nutritive Referent Value NRV on Fe status in healthy female participants.

Materials and Methods

Ethics statement

This study was conducted following the guidelines laid down in the Declaration of Helsinki and the study protocols were approved by the Ethics Commission of the University of Belgrade - Faculty of Pharmacy, Belgrade, Serbia (approval number: 188/2, 2020). All subjects went through verbal and written consent processes.

Study design and subjects

One hundred healthy female students of the University of Belgrade – Faculty of Pharmacy agreed to participate in this study. Eligible students were approached in the Faculty setting. Recruitment brochure contained detailed information regarding the purpose of the study, procedures involved as well

as rights and expectations of the potential participants. The main inclusion criteria were: age between 18 and 30 years, body mass index (BMI) between 18.5 and 29.9 kg/m^2 and willingness to maintain regular dietary habits throughout the study. The study did not include students who had altered Fe status (primarily based on complete blood count analysis), who had taken Mg supplements over the previous three months, or students who had confirmed chronic kidney and/or gastrointestinal tract disease. Finally, after applying the exclusion criteria, 46 respondents remained in the analytical sample.

Anthropometric parameters were measured at the beginning of the study. Height was measured to the nearest 0.1 cm (Perspective Enterprises, Kalamazoo, MI, USA). Body weight and body fat percentage were determined using the bioimpedance method (BC-418MA, Tanita, USA). Body Mass Index (BMI) was calculated as weight (kg) / height^2 (m^2).

During eleven intervention days, students ingested Mg preparations (citrate, oxide and carbonate) with the same dose of the active substance (375 mg/day). The subjects were randomly assigned to three groups according to the form of the Mg preparation.

Dietary intake assessment

Dietary intake data was collected via participants' subjective retrospective reports in two study points. Participants were administered 24h dietary recalls on two consecutive days before the initiation of the supplementation (t0) and after 11 days of using the provided Mg supplements, within the follow-up assessment (t2). Dietary information and relevant contextual data were obtained over the course of face-to-face structured interviews led by a trained researcher. In order to improve the accuracy of the report, enhance participants' memory, and assure the provision of detailed descriptions of consumed items multiple-pass methodological approach was applied. To assist respondents in portion size quantification interviewers used validated, 135-item Food Atlas featuring coloured photographs of increasing portion sizes for a selection of Balkan region-specific simple foods and composite dishes (23). Additionally, subjects reported quantities of foods consumed based on standardized household measures, natural units and labelling information for packaged products. Diet Assess & Plan – original software-based platform for comprehensive nutritional assessment was applied in questionnaire processing (24). Subsequent conversion of food consumption information into energy and nutrient intake estimates was performed according to data compiled in National Serbian Food Composition Database (25). Age and gender-adjusted nutritional recommendations proposed by EFSA i.e., Dietary Reference Values (DRVs) were applied for micronutrient adequacy evaluation (26).

Biochemical assessment

Serum biochemical parameters were analyzed before the initiation of the intervention, at baseline (t0), on the fifth day (t1) and the eleventh day (t2) of the intervention period. Blood samples were collected by professional phlebotomists via venipuncture between 7:00 am and 9:00 am. All studied participants were donated blood samples after the 12h of overnight fast. Standard operating procedures for blood collection and sample preparation were followed (27). A closed venipuncture system, Beckton Dickinson (BD) 22 Standard Wire Gauge (SWG), a reusable adapter and vacutainers were used. Vacutainers with clot activator (BD Vacutainer® SST™ Tubes) were used to obtain serum samples. Serum samples were used for measurement of serum Fe, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC) and total Mg (tMg). Transferrin concentrations and percentage of transferrin saturation (SAT) were calculated according to the formula proposed by Dacie et al. (28). Complete blood count was determined using the whole blood samples collected in a tube with liquid anticoagulant (ethylenediaminetetraacetic acid, EDTA) (BD Vacutainer® EDTA Tubes). For the analyses of met-, carboxy- and oxy-haemoglobin (metHgb, COHgb, O_2Hgb) and ionized Mg (iMg), lithium-heparin spray-coated tube (BD Vacutainer® Heparin Tubes) were used. Serum Fe, UIBC, TIBC and tMg concentrations were measured using the spectrophotometric method with commercial reagents, on Olympus AU400 biochemical analyzer (Beckman Coulter, Inc., California, USA). Complete blood count was measured in whole blood samples using electrical impedance on Coulter Ac•T diff Hematology Analyzer (Beckman Coulter, Inc., California, USA). Total haemoglobin (tHgb) concentrations were measured by spectrophotometry on Coulter Ac•T diff Hematology Analyzer. MetHgb, COHgb, O_2Hgb and iMg were determined approximately up to 60 min after collection using Stat Profile Prime Plus Critical Care blood gas analyzer (Nova Biomedical, USA). The precision and accuracy of the methods were verified using commercial control samples for all the listed parameters.

Statistical analysis

The normality of the data distribution was analysed using the One-sample Kolmogorov-Smirnov test. Normally distributed data were presented as mean and standard deviation (SD). Non-normally distributed data were presented as a median and interquartile range (IQR), by subtracting the first from the third quartile of the distribution. For data that were not normally distributed, values were log-trans-

formed before analysis. Linear regression analysis was performed to evaluate the relationship between investigated parameters. The association among serum Fe and Mg (as tMg, iMg and iMg/tMg) with certain (some) biochemical parameters was presented as polynomial regression. A principal component analysis (PCA) was used for the evaluation of interdependence between serum Fe and Mg (as tMg, iMg and iMg/tMg) and biochemical parameters (UIBC, TIBC, Transferrin, tHgb, hematocrit, SAT and Fe). We have considered p-value < 0.05 as statistically significant. All statistical analyses were performed using IBM SPSS version 24 (SPSS Inc., USA).

Results

Baseline participants' characteristics are presented in *Table I*. Participants were on average 23 years old, with a BMI of 21.8 kg/m^2. Erythrocyte indices and biochemical parameters indicated a favourable Fe status. All presented parameters were within the reference values routinely used in the laboratory, and recommended by the test manufacturer. Given that the aim of this study was to examine the effect of the applied dose of magnesium of 375 mg, in which magnesium is most often found in dietary supplements on the Serbian market (dose that meets 100% nutritional reference value for Mg), data was further analysed without partition of subjects into sub cohorts in relation to different types of supplemented magnesium (citrate, oxide and carbonate).

Estimated daily energy and nutrient intakes are presented in *Table II*. Based on the average of dietary recalls for two consecutive days at baseline evaluation (t0) and after 11 days (t2) of using provided dietary supplements, the results obtained indicate that food intake did not change over time.

Estimated intake levels of eight food groups and their corresponding contributions to daily iron intake based on repeated 24 h dietary recalls are presented in *Table III*. Dominant Fe dietary sources were grains and cereal products (28.6%), meat and meat products (22.3%) and vegetables and vegetable products (15.7%).

A statistically significant trend for change in O_2Hgb (%) by tertiles of whole blood iMg concentrations was found. With the increase of iMg, the percentage of O_2Hgb decreases. Additionally, we found a significant increase in changes of SAT (%) by quartile until the third quartile of tMg values. Interestingly, in the fourth quartile of serum tMg values, a significant decrease in SAT (%) was observed.

Based on polynomial regression analyses, serum tMg reached significant positive correlation with the SAT at concentration levels greater than 0.9 mmol/L, after 11 days of supplementary intervention (R^2=0.116; *Figure 1B*). Before the intervention, there was no statistically significant correlation between serum tMg and SAT (*Figure 1A*). Ionized Mg in a concentration higher than 0.6 mmol/L is positively correlated with SAT and serum Fe (R^2=0.214; 0.199, respectively; *Figure 2B*) after supplementation, which was not the case before the supplementation, because at the mentioned concentration the serum Fe decreased slightly (*Figure 2A*).

Table I Baseline participant characteristics (N=46).

Parameters	Mean±SD[a] Median (IQR)[b]
Age, years	23 (2)
BMI, kg/m^2	21.8 (2.8)
Total body fat, %	25.49±4.85
Systolic pressure (mmHg)	113.8±10.6
Diastolic pressure (mmHg)	80.3±8.6
WBC, 10^9/L	7.29±1.13
Lymphocytes, %	33.5±5.7
Monocytes, %	6.0±1.6
Granulocytes, %	60.4±5.9
Lymphocytes, #	2.4±0.5
Monocytes, #	0.4 (0.1)
Granulocytes, #	4.4±0.9
RBC, 10^{12}/L	4.51±0.28
tHgb, g/L	140 (4)
Hct, L/L	0.439±0.029
MCV, fL	89.6±4.2
MCH, pg	29.9±1.7
MCHC, g/L	334±7
RDW, %	14.5±1.7
iMg, mmol/L	0.59±0.032
tMg, mmol/L	0.89±0.054
Fe, μmol/L	14.89±5.65
UIBC, μmol/L	51.8±13.8
TIBC, μmol/L	66.7±12.5
SAT, %	23.9±9.8
Transferrin, g/L	2.6 (0.7)
MetHgb, %	0.4 (0.15)
COHgb, %	3.66±2.02
O_2Hgb, %	40.6±19.7

[a]Mean±SD, the standard deviation for normal distribution
[b]Median (IQR); IQR, interquartile range (quartile3-quartile1) for not normally distribution
WBC, white blood cells (Leucocytes); RBC, red blood cells; tHgb, haemoglobin; Hct, hematocrit; MCV, mean cell volume; MCH, mean haemoglobin concentration; MCHC, amount of haemoglobin per unit volume in a single red blood cell; Fe, iron; UIBC, unsaturated iron-binding capacity; TIBC, total iron-binding capacity; SAT, total transferrin saturation

Table II Daily energy and nutrient intakes among study participants assessed by the average of dietary recalls for two consecutive days at baseline evaluation (t0) and after 11 days (t2) of using provided dietary supplements.

Intervention Group (N=46)			
Energy/Nutrients	t0	t2	P
Energy (kcal)	1733.6±550.6	1804.9±485.6	0.501
Carbohydrates (TEI%)	29.9±9.8	28.6±7.8	0.516
Proteins (%TEI)	10.5±3.9	11.7±3.3	0.066
Fats (%TEI)	29.0±13.1	31.9±13.1	0.283
Fe (mg)	7.8±3.3	8.6±3.0	0.177
Mg (mg)	236.2±85.1	230.8±74.1	0.741
Zn (mg)	7.3±3.3	8.4±3.2	0.052
Folic acid (μg)	189.2±96.6	210.9±78.2	0.269
Vitamin B12 (μg)	2.5±1.3	2.6±1.2	0.053
Vitamin C (mg)	61.5±7.3	68.9±6.0	0.631

%TEI, percentage of total energy intake; $p < 0.05$ – statistically significant difference between t0 and t2 within the same intervention group.
[a]Mean±SD, the standard deviation for normal distribution

Table III Daily intake levels presented as the median levels and 5th and 95th percentiles of eight food groups and their corresponding contributions to daily iron intake based on repeated 24 h dietary recalls among study participants.

Food groups	Intake of the food group (mg/day)			The average contribution to the total iron intake (10.52 mg/day)	
	Median	5th percentile	95th percentile	%	Iron intake (mg/day)
Milk and dairy products	0.18	0.01	0.44	2.25	0.24
Eggs and egg products	0.63	0.15	3.34	8.01	0.84
Meat and meat products	1.64	0.38	5.92	22.28	2.34
Grains and cereal products	2.28	0.71	5.60	28.57	3.00
Nuts and seeds	0.42	0.03	2.24	6.94	0.73
Vegetables and vegetable products	1.29	0.14	4.03	15.67	1.65
Fruit and fruit products	0.51	0.04	1.54	4.84	0.51
Sugar and confectionary products	0.21	0.01	1.95	4.37	0.46

Table IV Associations between estimated O_2Hgb and tertiles of whole blood ionized Mg at the eleventh day.

Biochemical parameter	Whole blood ionized Mg on the eleventh day			
	T1 (N=17) <0.59	T2 (N=16) 0.60–0.63	T3 (N=13) 0.64–0.68	P for trend
O_2Hgb (%)	49.9 (38.7–61.1)	41.3 (29.4–53.2)	27.3 (20.9–33.7)	0.029

$p < 0.05$ – a statistically significant difference for trend
T, tertil; O_2Hgb, oxy-haemoglobin; N, number of participants

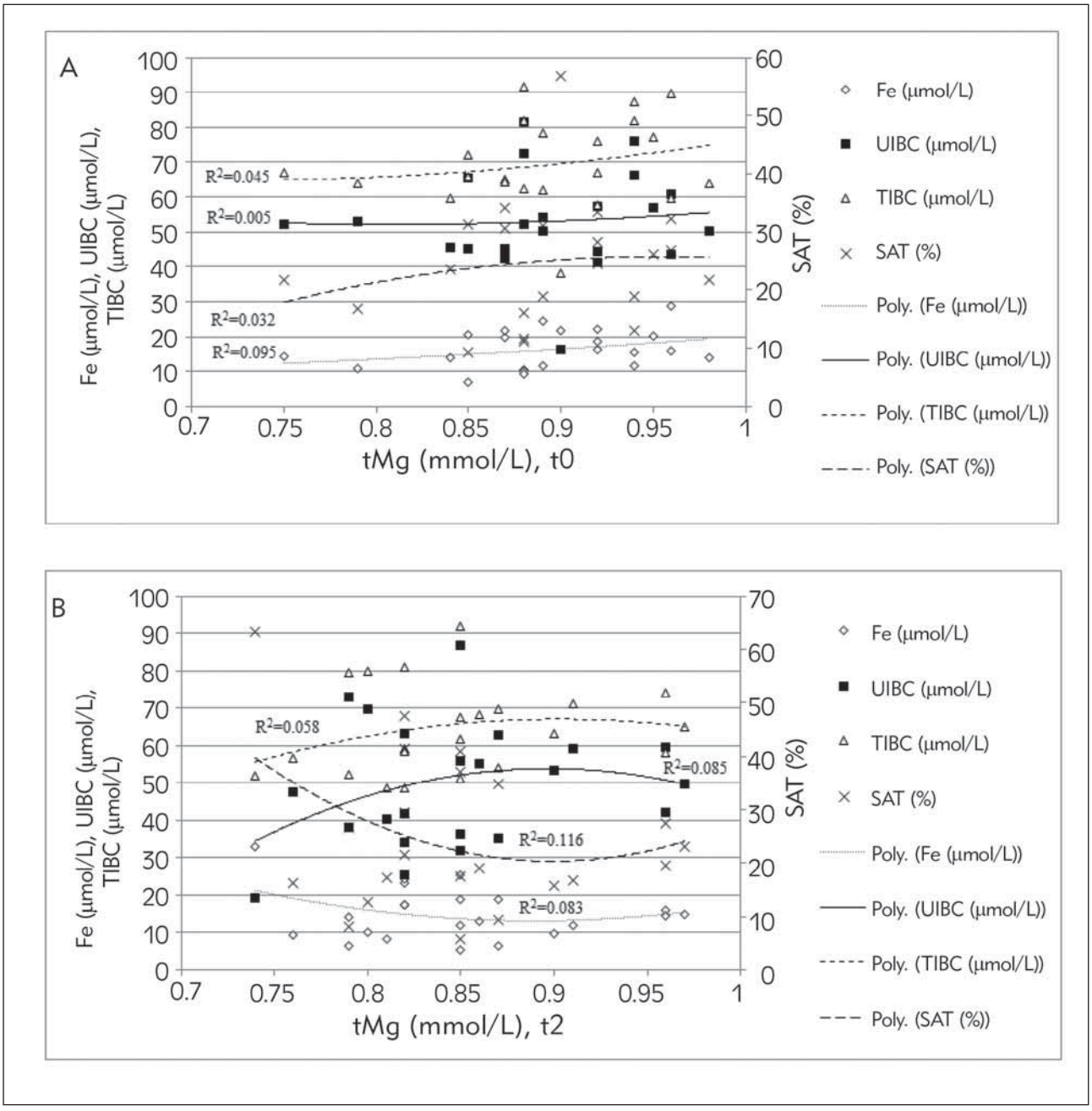

Figure 1 Interdependence between serum total magnesium (tMg) with serum iron (Fe),unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC) and transferrin saturation (SAT) at the beginning (A) and after 11 days (B) of supplementary intervention.

PCA was applied to integrate results of biochemical parameters, to discover the possible correlations among measured parameters, and to classify the parameters in a factor plane. PCA is a factor model in which the factors are based on summarizing the total variance. The first two factors should correspond to a high % of the variance to ensure that the maps based on the first two factors are a good quality projection of the initial multi-dimensional table. At the beginning of the experiment, PCA revealed that two axes participated in total variability with 55.8% (F1: 34.6% and F2: 21.2%), and after 11 days, the axes participated in total variability with 64.7% (F1: 46.7% and F2: 18.0%). According to the PCA, Transferrin, TIBC, UIBC, SAT, iMg/tMg and iMg correlated mainly with the first axis - factor (0.952; 0.952; 0.940; -0.607; -0.579 and -0.520, respectively), while serum Fe, tHgb, SAT, Mg; hematocrit; iMg/tMg, and MetHgb were mainly connected to the second axis - factor (0.758; 0.721; 0.588; 0.442;

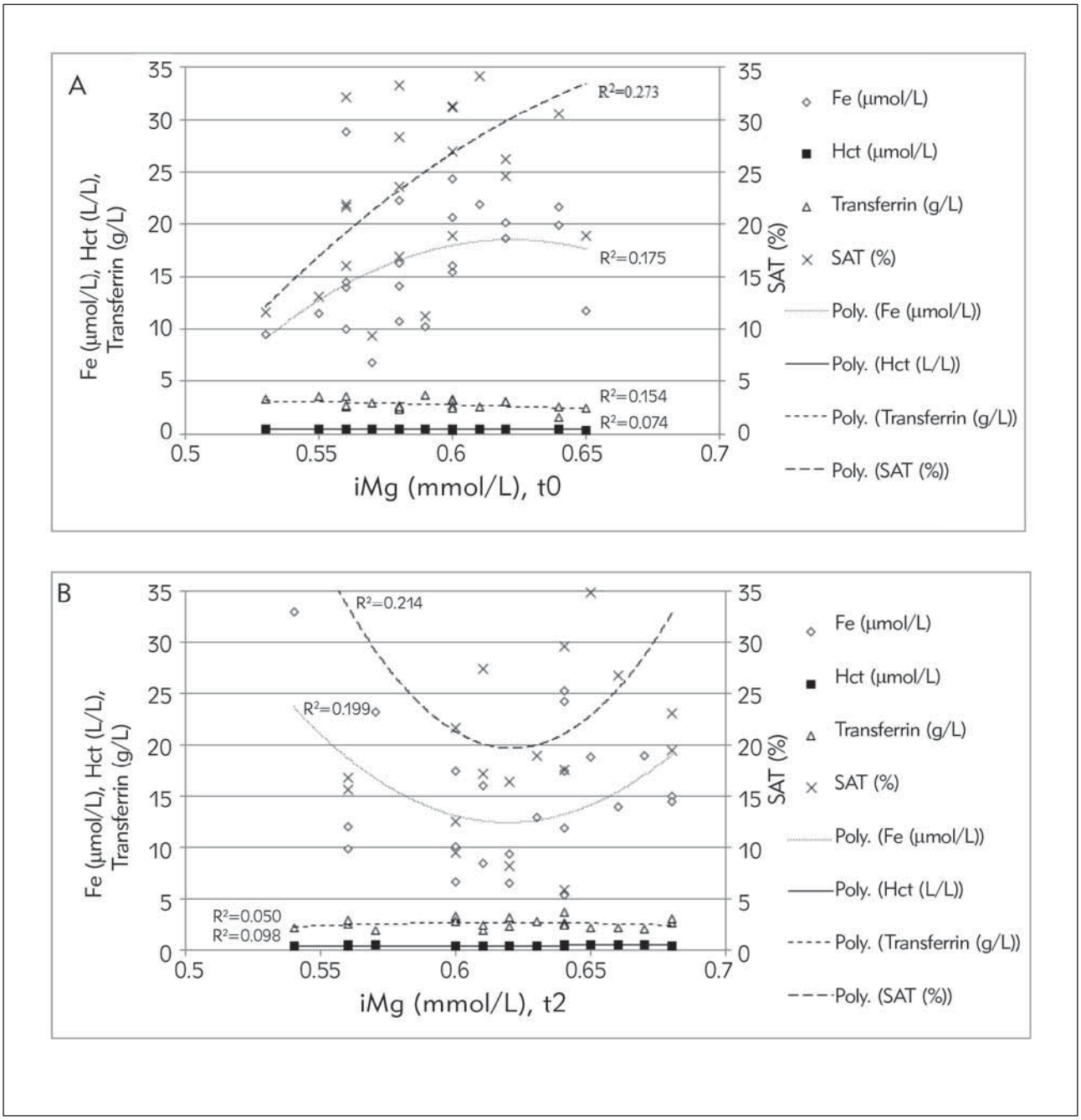

Figure 2 Interdependence between serum ionized magnesium (iMg) with serum iron (Fe), hematocrit (Hct), transferrin and transferrin saturation (SAT) at the beginning (A) and after 11 days (B) of supplementary intervention.

0.317; -0.554; -0.510; respectively), *Figure 4A*. In the first factor (F1) there is a strong positive correlation between TIBC, UIBC and Transferrin, while SAT, iMg/tMg and iMg are negatively correlated. The second factor (F2) is positively correlated with serum Fe, tHgb, SAT, serum Mg; hematocrit, and negatively with iMg/tMg, and MetHgb. After 11 days of the study, F1 was determined with UIBC, TIBC, Transferrin; SAT; serum Fe; tHgb and hematocrit (-0.982, -0.890, -0.890, 0.890, 0.822, 0.777, and 0.698, respectively), whereas F2 was determined with iMg/tMg; iMg and serum Mg (0.981; 0.720 and -0.616; respectively), *Figure 4B*. The first factor (F1) is positively correlated with SAT; serum Fe; tHgb and hematocrit, and negatively with UIBC, TIBC and Transferrin, while second factor (F2) is positively correlated with iMg/tMg and iMg, and negatively with serum tMg.

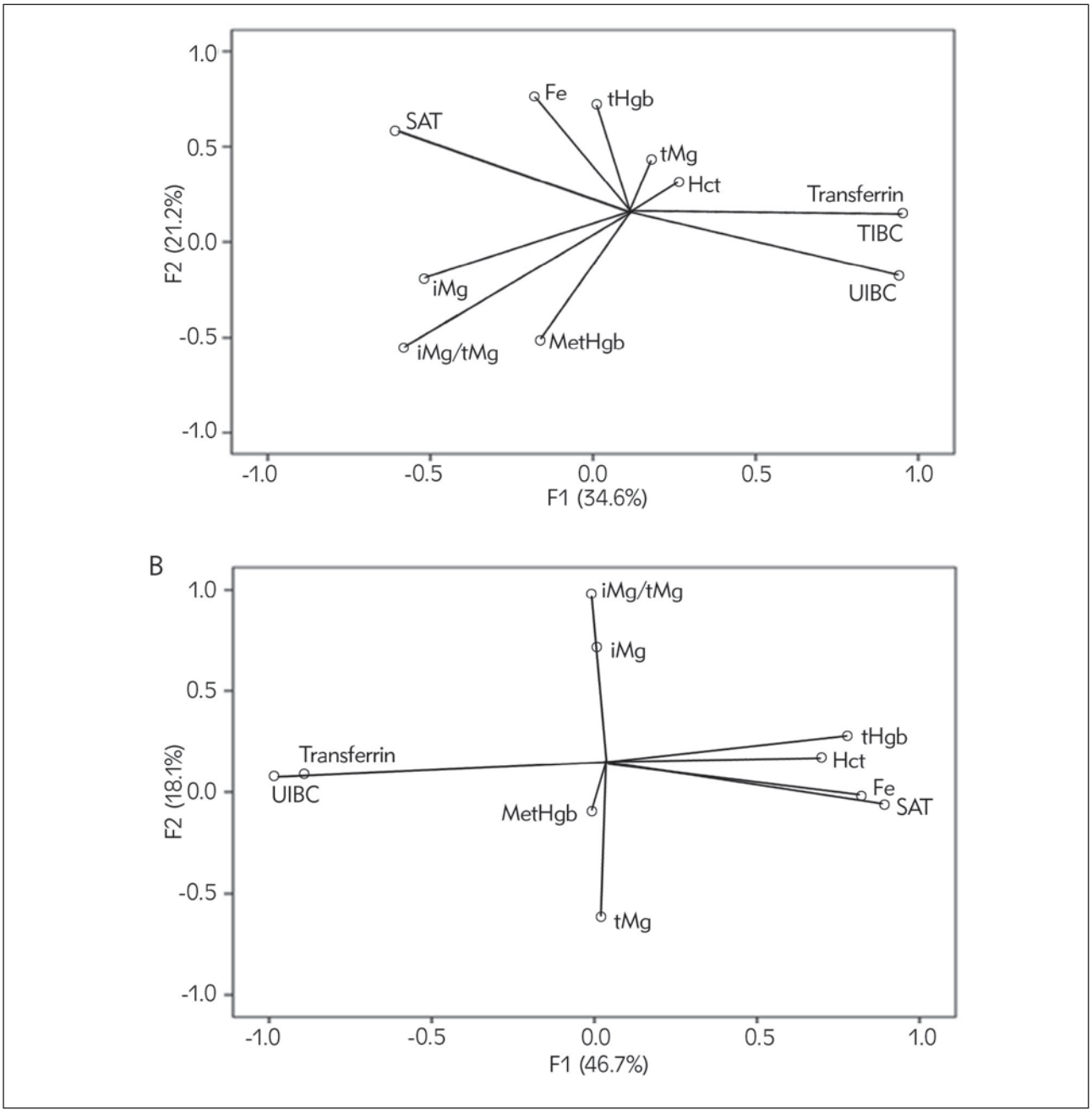

Figure 3 Principal Component Analysis for serum iron (Fe) and serum magnesum (Mg) (as total (tMg), ionized (iMg) and iMg/Mg) and biochemical parameters (unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), transferrin, transferrin saturation (SAT), total haemoglobin (tHgb), MetHgb and hematocrit (Hct)) content at the beginning (A, t0) and after 11 days (B, t2) of supplementary intervention.

Discussion

To the best of our knowledge this is the first study examining the short-term effects of Mg supplementation in daily doses corresponding to 100% NRV on Fe status in young healthy women. Intervention study was conducted among young female subjects with an aim to explore the effects of short-term Mg supplementation on indices of iron status. Namely, the literature data indicate that the human body adapts to the additional intake of magnesium, calcium and phosphorus in the period from 7 to 10 days and that the mineral balance is achieved in the period of a few days after supplementation intake (29). After 11 days of supplementation in a dose corresponding to Mg DRV (i.e. 375 mg), direct association was found between the serum tMg concentration and SAT. Furthermore, whole blood iMg correlated positively with SAT and serum Fe. These observations

were also confirmed by polynomial regression. These finding suggest that Mg supplementation and increased Mg levels, both serum tMg and blood iMg, might exert beneficial impact on obtaining favorable Fe status among young female population.

In this sample, the average iron intake was significantelly bellow the DRI and DRV for women, i.e., 18 mg/day and 16 mg/day, respectively (4, 5). These findings are consistent with the results of other studies which reported dietary iron intake in women of reproductive age in Europe (30, 31). Regardless the suboptimal dietary intake of Fe, biomarkers of Fe status were within the normal range. This can be explained by the following facts: iron metabolism was primarily regulated at the level of absorption, and the examined population did not have gastrointestinal disorders. Also, the amount of iron that enters the body from food is regulated by the body's need for iron. Other studies also indicate that a significant proportion of the UK population has Fe intake below recommendations and a low prevalence of poor Fe status (32–34). This might be because there are important uncertainties in the DRVs for Fe intake which may be too high, particularly for girls and women of reproductive age. It is recommended that the DRVs for iron should be reviewed when more data becomes available. Good quality dose-response data are required to enable a reassessment of the DRVs for iron. Knowledge of the systemic regulation and mediation of iron homeostasis should be applied to characterize better the responses to increased and reduced systemic needs for Fe and development, or better validation, of existing markers used to assess the adequacy of Fe status in populations and individuals. The main food sources of Fe among participants in our study were grains and cereal products with contribution of more than a quarter of total dietary intake of this nutrient followed by meat and meat products and vegetables. These three food groups together contributed to 65% of estimated iron intake in the participants. These observations are in accordance with previously published data for European population (35, 36). Furthermore, dietary intake assessment revealed that Mg intake among female student population were also below the recommended level although mean baseline tMg concentration was adequate.

There is a lack of literature data regarding the effect of increased Mg intake on Fe status in young women in the reproductive period. This issue needs to be addressed taking into account that Mg supplements are one of the most popular dietary supplements used in adult population (17) and the fact that anemia is most prevalent among females of childbearing age (36).

In this study, we tried to explore the connection between serum Fe and serum Mg (as tMg, iMg and iMg/Mg) and other biochemical indices of Fe status using the PCA approach, before and after Mg supplementation. Given that the diet did not change over intervention period intake of Mg and Fe were not considered in PCA. At the beginning of the study, before the initiation of the supplementation, there was a strong positive correlation between UIBC, TIBC and transferrin, but after 11 days of supplementation we found a strong negative correlation among the same analyzed parameters. Analyses revealed that even in a short period of intervention there is a noticeable effect of Mg supplementation on Fe status parameters (serum Fe, tHgb, hematocrit). PCA analysis revealed a positive correlation between serum Fe and SAT after 11 days of supplementation. Reddy et al. have explained similiar associations in Fe status parameters but in patients with functional anemia in chronic kidney disease (37). Anemia could be present as a latent condition, mostly in young women who are in the reproductive period. In order to optimize Fe status it is important to monitor biochemical parameters and routinely examine relevant markers (38).

Previously published data suggest an interaction between the resorption of divalent cations such as Mg and Fe. Namely, the deficiency of one divalent cation in the intestine can lead to increased resorption of other divalent cations (39). In an animal model, it has been shown that Fe deficiency can lead to increase in intestinal absorption of Mg, calcium and phosphorus since the same receptor may be involved in the resorption of these chemically similar cations (40). Low dietary intake of Mg in rats has been shown to increase Fe resorption (41). Furthermore, *in vitro* studies have demonstrated that Mg salts can negatively affect absorption of Fe by raising the pH value as the availability of Fe salts in the intestinal tract is pH dependent (42). Moreover, certain Mg salts can absorb Fe and thus interact with its absorption (42).

On the contrary, there are studies linking Mg intake and the risk of developing anemia (15, 16, 42–44). In our study, we have demonstrated that short term supplementation with Mg and increased level of serum tMg and iMg could have benefical effects on % SAT and serum Fe. Magnesium is a cofactor of a large number of enzymes, with an important role in the synthesis of hemoglobin. Accordingly, Mg deficiency can interrupt hemoglobin synthesis and erythrocyte energy metabolism and result in anemia. In addition, Mg deficiency has been reported to be associated with an inflammatory process, which could lead to anemia (45). Therefore, the question arises as to whether Mg supplementation is required in persons who are not deficient in this trace element, and leaves space to the future studies to examine longitudinal effects of Mg supplementation on Fe status indices.

The key limitation of the present study is missing data regarding the leukocyte, thrombocyte, and erythrocyte counts as well as ferritin level, after the inter-

vention period although changes in these parametres couldn't be expected during short intervention period. Additionally, acknowledge that calculated transferrin concentrations provide limited information. Furthermore, the duration of the Mg supplementation may have been short to explore the dynamical changes of the supplements' effects on biomarkers of Fe status. Similar studies are of interest in the male population, as well.

Conclusion

This study results indicate that Mg supplementation leads to an improvement in the certain iron status parameters even in individuals with optimal levels of these indices. Additionally, the analyzed parameters were significantly correlated, and after the intervention period, a significant positive association among the analyzed parameters was achieved. However, caution should be exercised when supplementing Mg, and laboratory monitoring of the interaction is required. Further research is warranted regarding the possible impact of the forms of Mg preparations that exist on the market, and whether they equally affect biochemical changes of iron status in healthy young people as well as in specific target groups of patients.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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IMPACT OF GENETIC POLYMORPHISMS AT THE PROMOTER AREA OF IL-10 GENE ON TACROLIMUS LEVEL IN JORDANIAN RENAL TRANSPLANTATION RECIPIENTS

UTICAJ GENETSKIH POLIMORFIZAMA U OBLASTI PROMOTERA GENA IL-10 NA NIVO TAKROLIMUSA U JORDANSKIH PACIJENATA SA TRANSPLANTACIJOM BUBREGA

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Summary

Background: Tacrolimus is a widely used immunosuppressant that prevents solid organ transplant rejection. The pharmacokinetics of Tacrolimus show considerable variability. Interleukin-10 (IL-10), in the host's immune response after transplantation, contributes to the variable CYP3A-dependent drug disposition of Tacrolimus. In the current study, we aim to evaluate the impact of single nucleotide polymorphisms (SNP) in the promoter region of IL-10 on Tacrolimus dose requirements and the Dose Adjusted Concentration (DAC) of Tacrolimus among kidney transplantation recipients.
Methods: Blood levels of Tacrolimus were measured using Microparticle Enzyme Immunoassay (MEIA) for six months post-transplantation. Genotyping analysis was utilized using specific Polymerase Chain Reaction (PCR) followed by sequencing methods for 98 Jordanian kidney transplant recipients.
Results: Genotyping frequencies of IL-10 (-592) were (CC/CA/AA: 38, 46.7, 15.2%); IL-10 (-819) were

Kratak sadržaj

Uvod: Takrolimus je široko korišćen imunosupresiv koji sprečava odbacivanje presađenog čvrstog organa. Farmakokinetika takrolimusa pokazuje značajnu varijabilnost interleukin-10 (IL-10), u imunološkom odgovoru domaćina nakon transplantacije, doprinosi promenljivoj dispoziciji leka zavisne od CIP3A kod takrolimusa. U ovoj studiji, cilj nam je da procenimo uticaj polimorfizama pojedinačnih nukleotida (SNP) u promotorskom regionu IL-10 na zahteve za dozom takrolimusa i koncentraciju prilagođenu dozi (DAC) takrolimusa među primaocima transplantacije bubrega.
Metode: Nivoi takrolimusa u krvi su mereni korišćenjem imunoeseja mikročestica enzima (MEIA) tokom šest meseci nakon transplantacije. Analiza genotipizacije je izvedena korišćenjem specifične lančane reakcije polimeraze (PCR) praćene metodama sekvenciranja za 98 jordanskih primalaca transplantiranih bubrega.
Rezultati: Učestalosti genotipizacije IL-10 (-592) su (CC/CA/AA: 38, 46,7, 15,2%); IL-10 (-819) su (CC/CT/TT: 40,4, 44,1, 15,1%); i IL-10 (-1082) su (AA/AG/GG: 42,6,

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List of abbreviations: ESRD, End-stage renal disease; SNPs, Single-nucleotide polymorphisms; SD, Standard deviation; BSA, Body surface area; DAC, Dose-adjusted concentration

(CC/CT/TT: 40.4, 44.1, 15.1%); and IL-10 (-1082) were (AA/AG/GG: 42.6, 44.7, 12.8%). The impact of IL-10 (-1082) on Tacrolimus DAC was gender dependent. Men carrying at least one A allele had significantly lower DAC than men carrying GG genotyping only in the first month post-transplantation [88.2±32.1 vs. 117.5±22.5 ng/mL per mg/kg/day, p=0.04].

Conclusions: Our current study showed that the interaction between gender and IL-10 -1082 affects Tacrolimus DAC in Jordanian kidney transplant recipients during the first-month post-transplantation.

Keywords: IL-10 genetic polymorphism, kidney transplantation, pharmacokinetics, tacrolimus

44,7, 12,8%). Uticaj IL-10 (-1082) na takrolimus DAC zavisio je od pola. Muškarci koji su nosili najmanje jedan alel A imali su značajno niži DAC od muškaraca koji su nosili genotipizaciju GG samo u prvom mesecu nakon transplantacije [88,2±32,1 naspram 117,5±22,5 ng/mL po mg/kg/dan, p=0,04].

Zaključak: Naša trenutna studija je pokazala da interakcija između pola i IL-10-1082 utiče na takrolimus DAC kod jordanskih primalaca transplantacije bubrega tokom prvog meseca nakon transplantacije.

Ključne reči: IL-10 genetski polimorfizam, transplantacija bubrega, farmakokinetika, takrolimus

Introduction

End-stage renal disease (ESRD) is the final stage of kidney failure, characterized by a decreased glomerular filtration rate and increased urinary albumin excretion (1). Kidney transplantation is considered the most effective treatment of End-stage renal disease (ESRD) when compared to dialysis (2, 3). In 1954, Murray, and Merrill (4) performed the first successful kidney transplant operation; it was made possible because the donor and recipient were monozygotic identical twins. The first kidney transplantation in the Arab world was performed in Jordan in 1972, the kidney was obtained from a deceased donor (5).

Transplantation patients during their post-operative phase run a great risk of developing major life-threatening complications which include: (1) cardiovascular diseases most likely caused by calcification of vessels and left ventricular hypertrophy associated originally with ESRD; (2) delayed graft function which is defined as the use of dialysis due to poor kidney function in the first week of graft life; (3) infection due to the high level of immunosuppressants given to the patient, but the improved use of antimicrobials and antimicrobial regimens has decreased infection severity; (4) graft rejection (6–9).

To solve these complications, immunosuppressants are essential for successful organ transplantation as they suppress rejection and inhibit the autoimmune process, however, they also lead to undesired consequences such as immunodeficiency, infection or malignancy, and non-immune toxicity (10). Tacrolimus is a fermentation product of *Streptomyces* and belongs to the family of calcineurin inhibitors. It is a widely used immunosuppressive drug for preventing solid-organ transplant rejection (11). But its usage is complicated due to its narrow therapeutic index and considerable inter-and intra-individual pharmacokinetic variability (12).

Many single-nucleotide polymorphisms (SNPs) have been studied concerning the pharmacokinetics of Tacrolimus, especially CYP3A4, CYP3A5, and ABCB-1; as their alleles have been involved in the metabolism of calcineurin inhibitors showing promising prospects in Tacrolimus dose individualization (13).

The IL-10 promoter area is highly polymorphic; many studies have been conducted showing variations in IL-10 expression linked to promoter area polymorphisms. Five SNPs tagging the promoter area of IL-10 have been widely studied and they are (-3575), (- 2763), (-1082), (-819), and (-592) (14).

IL-10 production level showed that the GG genotype -1082 is higher versus (AA and AG) genotypes, independently of the polymorphisms at positions -819 and -592, and also associated with higher serum concentration (15–17). Furthermore, *in vivo* study showed that higher IL-10 decreases CYP3A activity, which is involved in the metabolism of tacrolimus among renal transplantation recipients (18, 19).

Our current study aimed to investigate the association between the dose required to reach the target level of Tacrolimus and genetic variations in renal transplantation recipients through the study of IL-10 (-592, rs1800872, A/C); IL-10 (–819, rs1800871, T/C) and IL-10 (–1082, rs1800896, A/G). The SNPs were selected due to the reported relationship with IL-10 production and level (15, 20).

Materials and Methods

Patients and ethical approval

Ninety-eight adult renal transplant recipients, who had received a renal graft between 2009 and 2011 from Jordanian Royal Medical Services, were included in the study. The inclusion criteria were patients with a newly transplanted kidney and who were on a Tacrolimus-based immunosuppressive maintenance therapy starting immediately following transplantation. Tacrolimus was given in two equally divided doses. All patients treated with Tacrolimus used the capsule formulation Prograf® (Fujisawa, Munich, Germany). Patients who received medications known to interact with Tacrolimus were excluded from the study.

Table I Primers, PCR conditions of genotyping analysis for *IL-10 -1082A/G*, *IL-10 -592A/C* and *IL-10 -819T/C*.

Allele	Positiona	Primers	PCR conditions
IL-10 -1082A/G	rs1800896	Forward primer 5′ GGCTTCCTACAGTACAGGCG 3′ Reverse Primer 5′ GGTAGAGCAACACTCCTCGC 3′	Denaturing 95 °C for 1 min Annealing 60 °C for 1 min Extension 72 °C for 1 min 35 cycles Size of PCR product 447 bp and 783 bp
IL-10 –592A/CIL-10 –819T/C	rs1800872 rs1800871	Forward primer 5′GATGAATACCCAAGACTTCTCCT 3′ Reverse Primer 5′ CCTTCCCCAGGTAGAGCAACAC 3′	

This study was approved by local Research Ethics Committees of Jordanian Royal Medical Services (IRB: TF1/3/ethics obtained on June 27th, 2016); and has been performed following the ethical standards laid down in the 2000 Declaration of Helsinki as well as the Declaration of Istanbul 2008. Written informed consent was obtained from all participants. Details that might disclose the identity of the subjects in the study were omitted.

Tacrolimus blood level measurements

Blood samples were collected before the administration of the morning dose of Tacrolimus for the determination of trough blood concentrations of the drug. The trough concentration was measured in whole blood by IMx Tacrolimus II assay which utilizes MEIA in the Abbott IMx system (Tacrolimus II; Abbott Laboratories, IL. USA). This measurement was performed in the laboratory of Jordanian Royal Medical Services, and the dose-adjusted concentration was calculated by dividing the pre-dose concentration by the corresponding 24-hour dose in milligram Tacrolimus per kilogram body weight.

Genomic DNA isolation and genotype analysis

Genomic DNA was isolated from 300 μL EDTA-treated whole blood using a Commercial kit (Wizard genomic DNA purification kit, Promega,WI, USA). The procedure was carried according to the kit manufacturer's recommendation.

Genotyping analysis for detection of 3 SNPs of IL-10s was performed for all patients by using specific PCR primers. *Table I* describes primers used and PCR conditions. PCR was performed in a total volume of 25 μL using 100 ng of genomic DNA with 1.5 μL of 10 μmol/L of each primer and 12.5 μL of 2X KAPA2G Fast ReadyMix PCR Kit (Kappa Biosystems, USA). PCR amplifications were performed in PTC-100 Peltier Thermal Cycler (MJ Research, MA, USA).

PCR reaction products were sequenced using Big Dye Terminator version 3.1 kit (Applied Biosystems, Waltham, MA, USA). Samples were run on an ABI Prism Genetic Analyzer system 3130xl (Applied Biosystems, Waltham, MA, USA).

Statistical analysis

Data were coded and entered into Statistical Packages for Social Sciences (SPSS version 20.0, 2012). Data were summarized as counts and percentages for categorical data and as means and standard deviation (SD) for continuous data. A data set was tested for normality of distribution using the Shapiro-Wilk test. Homogeneity of variance was assessed by Levene's test. Comparison between categorical data was conducted using the Fisher exact test or Chi-square test, when appropriate. Comparison between continuous data was performed utilizing independent t-test; ANOVA, Mann-Whitney or Kruskal Wallis, based on which was most appropriate. A *p*-value of 0.05 or less was considered statistically significant.

Results

Ninety-eight kidney transplant recipients met our inclusion criteria. The age, weight and gender of donors and recipients are comparable. Demographic data of recipients and corresponding donors are summarized in *Table II*. The most common cause of chronic kidney disease among our patients was hypertensive nephropathy (49%). Other identifiable causes of chronic kidney disease included glomerulonephritis (8.2%), chronic pyelonephritis (6.1%), diabetic

Table II Demographic data of Jordanian kidney transplant recipients and corresponding donors.

Parameter		Donors	Recipients	p
Gender N (%)	Male	53 (54.1%)	60 (61.2%)	0.38
	Female	45 (45.9%)	38 (38.8%)	
Age, years, mean (±SD)		34.1±8.9	35.6±9.6	0.26
Weight, kg, mean (±SD)		70.9±16.4	72.1±17.4	0.62

Chi-Squared with one degree of freedom

Table III Medical history data for Jordanian kidney transplant recipients.

Parameter		N (98)
Causes of chronic kidney disease, N (%)	Glomerulonephritis	8 (8.2%)
	Chronic pyelonephritis	6 (6.1%)
	Diabetic nephropathy	4 (4.1%)
	Hypertensive nephropathy	48 (49.0%)
	Polycystic kidney disease	4 (4.1%)
	Undetermined	8 (8.2%)
	Others	20 (20.4%)
Transplantation events N (%)	First	95 (96.9%)
	Second	3 (3.1%)
Type of donors N (%)	Relative, living	91 (92.9%)
	Non relative, living	7 (7.1%)
Immunosuppressant use		
Prednisolone N (%)		95 (96.9%)
	Total daily dose (mean±SD), mg	11.8±6
Azathioprine N (%)		6 (6.1%)
	Total daily dose (mean±SD), mg	75 ± 27.4
Mycophenolate N (%)		89 (90.0%)
	Total daily dose (mean±SD), mg	1393.3±491.2

N: number of recipients. SD: standard deviation.

nephropathy (4.1%), and polycystic kidney disease (4.1%). Ninety-seven percent of patients underwent the transplantation operation for the first time, with the majority of them receivingthe graft from a living relative (93%). The medical history of kidney transplant recipients is summarized in *Table III*.

Genotypes and alleles frequencies

Among the 98 kidney transplant recipients, some cases were not genotyped due to unsuccessful PCR results. The allele frequencies of the 3 SNPs in all patients were in accordance with the Hardy-Weinberg Equilibrium equation (P>0.05). Genotype frequencies of patients are summarized in *Table IV*.

Effect of recipients genotypes on Tacrolimus pharmacokinetics parameters

Daily dose (mg/day), concentration level (ng/mL), weight-adjusted daily dose (mg/kg/day), body surface area (BSA) adjusted dose (mg/m^2) and dose-adjusted concentration (DAC) (ng/mL per mg/kg/day) of Tacrolimus were compared among recipients with different allelic statuses of three SNPs

Table IV Genotype frequencies of Jordanian kidney transplant recipients.

Genotype	N (%)		Allele frequency %		X^2	P
IL-10 (-592, rs1800872, C/A)	CC	35 (38)	Minor	61	0.01	0.99
	CA	43 (46.7)	Major	36		
	AA	14 (15.2)				
IL-10 (–819, rs1800871, C/T)	CC	38 (40.9)	Minor	37	0.1	0.96
	CT	41 (44.1)	Major	63		
	TT	14 (15.1)				
IL-10 (–1082, rs1800896, A/G)	AA	40 (42.6)	Minor	34	0.06	0.97
	AG	42 (44.7)	Major	66		
	GG	12 (12.8)				

N: number of recipients. χ^2: Chi-Squared with one degree of freedom.

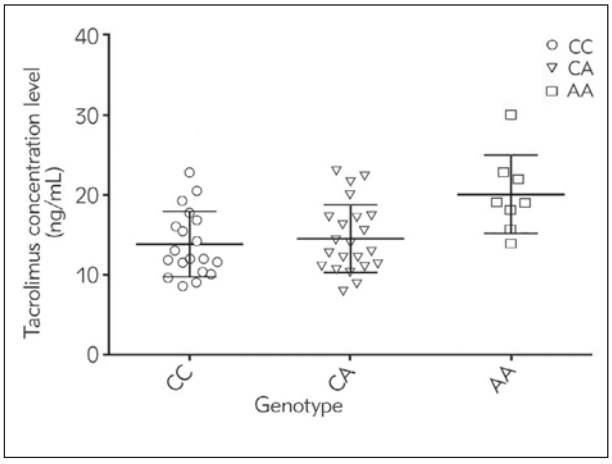

Figure 1 Effect of gender-genotype at IL-10 (-592, rs1800872, C/A) on Tacrolimus concentration level (ng/mL) of Jordanian kidney transplant recipients in the first month post transplantation.

Y-axis is Tacrolimus concentration level (ng/mL), X-axis is the genotypes at IL-10 -592. CC: Homozygous ancestral genotype, AA: Homozygous variant genotype and CA: Heterozygote variant genotype.

of *IL-10*. All mentioned parameters did not differ significantly among *IL-10* (–819, rs1800871, T/C) and *IL-10* (–1082, rs1800896, A/G) during the first six months post-transplantation as shown in Supplementary Tables (*Table I* and *Table III*). However, we found that patients carrying AA at *IL-10* (-592, rs1800872, A/C) had significantly a higher tacrolimus concentration level than those patients carrying AC or CC genotypes in the first month, post-transplantation (AA: 20.1±4.95 ng/mL; AC: 14.58±4.4 ng/mL; and CC: 13.86±4.1 ng/mL, p = 0.01). This difference in Tacrolimus concentration level disap-

peared after the first month as shown in *Figure 1* and *Supplementary Tables (Table II*).

Effect of gender-genotypes interaction of recipients on Tacrolimus pharmacokinetics parameters

Recipients were grouped according to their gender (male vs female) then categorized into two subgroups according to the presence of at least one ancestral allele versus the homozygous variant genotype (-592; CC and CA vs AA), (-819; CC and CT vs TT) and (-1082; AA and AG vs GG). All mentioned parameters did not differ significantly among*IL-10* (-592, rs1800872, A/C); *IL-10* (–819, rs1800871, T/C) during the first six months post-transplantation (as shown in *Supplementary Tables*, *Table V, VI*).

However, we found that patients carrying GG genotype at*IL-10* (–1082, rs1800896, A/G) versus patients carrying at least one A allele (AA or AG) show differences. Men carrying at least one A allele had significantly lower Tacrolimus adjusted concentration than men carrying GG genotype in the first-month post-transplantation. This reduction in DAC, however, disappeared after the first month [88.2±32.1 vs. 117.5±22.5 ng/mL per mg/kg/day, p=0.04]. On the other hand, in women, non of mentioned parameters differed significantly between different *IL-10* -1082 A>G genotype groups during the first six months post-transplantation as shown in *Figure 2* and *Supplementary Tables* (*Table IV*).

Table V Haplotype Distribution of IL-10 –592, –819 and –1082 among renal transplantation recipients one month post transplantation.

Haplotype	Total (%)	<= median N=27 (%)	>median N=23 (%)	P
ATA	31.6	7(27.3)	8(36.7)	0.99
CCG	29.9	6(24.8)	8(35.9)	0.99
CCA	27.6	11(41.1)	3(11.7)	0.04
ACA	3.6	1(1.8)	1(5.7)	0.94
ACG	3.0	0(1.0)	1(5.4)	
CTA	2.1	1(2.0)	1(2.3)	0.94
CTG	2.1	1(2.0)	1(2.3)	0.94

N: number of recipients. C (first): the ancestral allele of IL-10 –592. C (middle): the ancestral allele of IL-10 –819. A (last) the ancestral allele of IL-10–1082. A (first): the variant allele of IL-10 –592. T (middle): the variant allele of IL-10 –819. G (last): the variant allele of IL-10–1082.

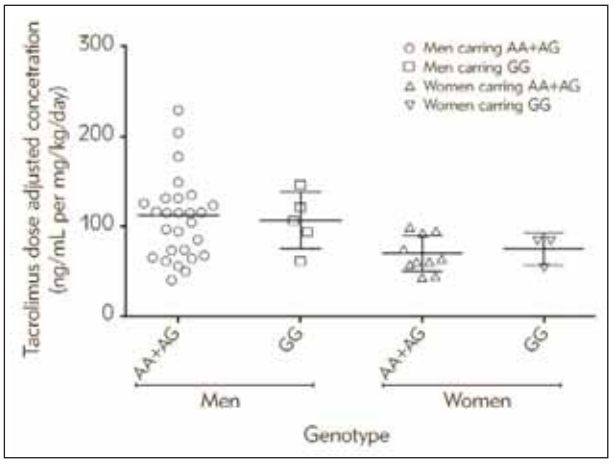

Figure 2 Effect of gender-genotype interaction at *IL-10* (–1082, rs1800896, A/G) on Tacrolimus dose adjusted concentration (ng/mL per mg/kg/day) of Jordanian kidney transplant recipients in the first month post transplantation.
Y-axis is dose adjusted concentration (ng/mL per mg/kg/day), X-axis is the genotypes at *IL-10 –1082*. AA: Homozygote ancestral genotype, GG: Homozygous variant genotype and AG: Homozygous variant genotype.

Discussion

This study examined the contribution of IL-10 (-592, rs1800872, A/C); IL-10 (–819, rs1800871, T/C) and IL-10 (–1082, rs1800896, A/G)polymorphisms in Jordanian renal transplant recipients to Tacrolimuspharmacokinetics parameters within the first six months post-transplantation. The clinical use of Tacrolimus is complicated by its narrow therapeutic range and highly variable pharmacokinetics among individuals. Some patients do not reach target concentrations using the recommended initial doses of Tacrolimus, and therefore, have an increased risk of inadequate immunosuppression and subsequent acute rejection during the early period following organ transplantation (21). The association of the IL-10 gene SNPs with Tacrolimus dose requirements has been recognized as a genetic base for the observed inter-individual differences in pharmacokinetics (22).

Our current study aimed to determine whether the genotype of IL-10 could explain variability in pharmacokinetic parameters of Tacrolimus in kidney recipients during the proposed period. We hypothesized that the recipient's polymorphisms of IL-10 are associated with changes in Tacrolimuspharmacokinetics parameters during the early period post-transplantation.

In our current study, IL-10 alleles frequencies were found to be as follows; the A allele: 65% and the G allele: 35%. this is consistent with data from previously published research on such frequencies among Caucasians (A allele: 58.5–65.6%, G allele: 34–41.5%) (23, 24).

Turner and Williams (15) found that following stimulation, IL-10 production was measured by ELISA showed that the GG genotype -1082 is significantly higher compared to the AA and AG genotypes. This correlation was independent of the polymorphisms at positions -819 and -592. Later, studies found that the G allele at position -1082 is the most important genetic factor in the regulation of constitutive IL-10 mRNA level, and is associatedwith a greater serum concentration (16, 17). Furthermore, an important relationship was noted between IL-10 and cytochrome P450 activity through an *in vivo* study that showed IL-10 to significantly decrease CYP3A activity ($P \leq 0.02$) (18). Interestingly, a previous study conducted among Jordanian kidney transplant recipients revealed a correlation between genetic variations in both CYP3A4 and CYP3A5 enzymes and tacrolimus blood levels among renal transplant recipients (19).

In a previous study of liver transplant recipients, significantly higher Tacrolimus dose-adjusted concentrations were measured in patients carrying -1082 AA versus those carrying GG and GA during an intermediate value within the first three weeks after transplantation (22). On the other hand, a Chinese study demonstrated the impact of IL-10 gene polymorphism on Tacrolimus dosage requirement in 53 liver transplant recipients and found no statistically significant differences in Tacrolimus dose-adjusted concentration among recipients. The same study revealed a significantly higher Tacrolimus dose-adjusted concentration in recipients with donors with the -1082 AA genotype than those whose donors with IL-10 -1082 GA genotype (25).

In a later study including 240 renal transplant recipients, IL-10 (-1082) variants did not show a significant relationship between Tacrolimus metabolism and -1082 genotypes within the first four weeks following transplantation (26). The current study did not find a significant relationship between studied IL-10 SNPs among kidney transplant recipients and Tacrolimus pharmacokinetics parameters. Remarkably, gender analysis revealed that males carrying at least one A allele at IL-10 (-1082) had significantly lower Tacrolimus dose-adjusted concentration than males carrying GG genotype in the first-month post-transplantation. We divided the patients according to their gender due to the differences in liver and renal function between males and females (27).

Our results can be explained by hypothesizing that the -1082 GG allele is associated with increased IL-10 production (15–17), which leads to decreased CYP3A catalytic activity (18). Hence, a lower tacrolimus dose is required to reach a significantly higher Tacrolimus dose-adjusted concentration. This is evident during the early phase after transplantation.

Multiple studies have demonstrated linkage disequilibrium between the polymorphism at position -1082 in the IL-10 promoter area and other SNPs in the same area including SNPs at positions -819 and -592, suggesting that the functional effects may be haplotype-dependent (28–30).

The current study shows that Tacrolimus adjusted concentration is sex-genotype-dependent in Jordanian kidney transplant recipients during the first-month post-transplantation at IL-10 -1082 A>G. This effect was observed in the first-month post-transplantation in male patients carrying at least one A allele who showed significantly lower DAC than male patients carrying the GG genotype. This reduction in DAC disappeared after the first month. On the other hand, non of the mentioned parameters differed significantly between different IL-10 -1082 A>G genotype groups during the first six months post-transplant in the female patients.

Limitations

The number of studied patients was small due to the long follow-up period of 6 months per patient. As well there were cases where data was missing due to the difficulty in interviewing patients, or loss of contact with patients. Because of the small sample size, we couldn't detect rare mutations and their frequency impact on tacrolimus pharmacokinetic parameters. However, it should be noted that our sample size is similar to other previously published studies that were close to (240) or even smaller (53) than the present study (22, 25, 26).

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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ALKALINE PHOSPHATASE INTERFERENCE IN IMMUNO-ENZYMATIC ASSAYS

INTERFERENCIJA ALKALNE FOSFATAZE U IMUNO-ENZIMSKIM ANALIZAMA

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Summary

Background: Alkaline phosphatase (ALP) enzymes are widely used as signal amplifiers in immunoenzymatic methods. Conditions that cause ALP elevations, such as bone or liver diseases, can cause interference in immunoenzymatic methods. We aimed to examine ALP's effect on immunoenzymatic assay by adding isolated pure ALP to the prepared serum pool.
Methods: We prepared a serum pool and divided it into 4 groups. By adding isolated pure ALP at different concentrations to each group, we obtained sample groups containing ALP enzyme at concentrations of 85 U/L, 340 U/L, 870 U/L, and 1570 U/L. 20-repetition of βhCG, ferritin, FT4, TSH, troponin I, and Vit B12 tests were performed in each group. The coefficient of variation, bias, and total error was calculated. All groups were compared by using the Friedman test for paired samples.
Results: After ALP addition, the calculated total error values of FT4, βhCG and troponin I tests were above the acceptable error limits. There were statistically significant differences in βhCG, FT4, troponin I, and Vit B12 tests compared to the baseline ALP level ($P<0.0125$).
Conclusions: Isolated ALP elevations can be a source of interference for immunoenzymatic methods.

Keywords: Alkaline phosphatase, ALP, bias, immunoenzymatic, total error

Kratak sadržaj

Uvod: Enzimi alkalne fosfataze (ALP) se široko koriste kao pojačivači signala u imunoenzimskim metodama. Stanja koja izazivaju povišenje ALP, kao što su bolesti kostiju ili jetre, mogu izazvati smetnje u imunoenzimskim metodama. Naš cilj je bio da ispitamo efekat ALP-a na imunoenzimski test dodavanjem izolovanog čistog ALP-a u pripremljenu grupu uzoraka seruma.
Metode: Pripremili smo veći broj uzoraka seruma i podelili ih u 4 grupe. Dodavanjem izolovanog čistog ALP u različitim koncentracijama svakoj grupi, dobili smo grupe uzoraka koje sadrže ALP enzim u koncentracijama od 85 U/L, 340 U/L, 870 U/L, i 1570 U/L. Urađeno je 20 ponavljanja u svakoj grupi testova za βhCG, feritin, FT4, TSH, troponin I, i Vit B12. Izračunati su koeficijent varijacije, pristrasnost i ukupna greška. Sve grupe su upoređene korišćenjem Fridmanovog testa za uparene uzorke.
Rezultati: Nakon dodavanja ALP, izračunate ukupne vrednosti greške testova FT4, βhCG i troponina I su bile iznad prihvatljivih granica greške. Postojale su statistički značajne razlike u testovima za βhCG, FT4, troponin I i Vit B12 u poređenju sa osnovnim nivoom ALP ($P<0,0125$).
Zaključak: Izolovana povišenja ALP mogu biti razlog smetnji za sprovođenje imunoenzimskih metoda

Ključne reči: alkalna fosfataza, ALP, pristrasnost, imunoenzimski, ukupna greška

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List of abbreviations: ALP, Alkaline phosphatase; βhCG, βeta human chorionic gonadotropin; FT4, Free thyroxine; TSH, thyroid-stimulating hormone; Vit B12, Cobalamin; TE, Total Error; CV, coefficient of variations; MEIA, microparticle immunoassay; FIA, fluorometric immunoassay.

Introduction

Alkaline phosphatases (ALP; orthophosphate mono-ester phosphohydrolase (alkaline optimum) EC 3.1.3.1) are homodimeric and glycoprotein enzymes in the hydrolase group with a molecular weight of 86 kilodaltons. These groups of enzymes are commonly found in nature in both eukaryotes and most prokaryotes. Each monomer is encoded by multiple genes to contain five cysteine residues, two zinc atoms and one magnesium atom, which are vitally important for its catalytic function.

ALP enables the detachment of phosphate groups from a variety of molecules, including nucleotides, proteins and alkaloids, in alkaline pH environments (1).

ALP mainly functions as bound by hydrophobic glucosyl-phosphatidylinositol to the cell membrane (2). It is mostly found in the canalicular membrane of hepatocytes and bile duct epithelium lumen, bone osteoblasts, brush border membrane of the intestinal mucosa, placenta, proximal kidney tubules, and breast tissue during lactation. A healthy human serum contains four different ALP isoenzymes under normal conditions. These are Intestinal ALP, Placental ALP, Germ cell ALP and tissue nonspecific alkaline phosphatase. The difference between the isoenzymes stem from the sialic acid found in them at varying rates, and also, the protein amount of the placental isoenzyme is different (2, 3).

Serum ALP measurement plays a vital role in the diagnosis of hepatobiliary and bone diseases associated with an increased osteoblastic activity. Intrahepatic and extrahepatic cholestasis, space-occupying lesions in the liver, metastasis and infiltrative liver diseases cause an increase in serum ALP levels (4). Apart from the liver diseases, serum ALP levels elevate as a result of Paget's disease (associated with increased osteoblastic activity), osteomalacia and rickets associated with vitamin D deficiency, primary and secondary hyperparathyroidism, and during the healing process of bone fractures. Likewise, placenta-induced ALP enzyme elevation is seen in the third trimester of pregnancy and placental or germ cell malign diseases (4).

ALP is used to provide signal amplification by conjugating it with antibodies in immunoenzymatic methods (4–6). ALP is frequently used in immunochemical methods along with Horseradish Peroxidase due to its substrate diversity, cheapness and accessible possibility. Once conjugated with antibodies, antigens and streptavidin, these enzymes increase the test sensitivity owing to their low background effect, linear reaction rate, and extended incubation time. Using ALP as a signal amplifier provides an enhanced and longer-lasting lumination obtained at the end of the reaction. Thus, the target analyte can be assayed in lesser concentrations and much broader linearity. It was reported that while the endogenous ALP had an interfering effect on immunochemical methods in the previous automated systems, this effect is prevented through the increased washing processes in the renewed automated systems (4, 7).

In our laboratory, we use UniCelDxl 800 and Access II (Beckman Coulter, Brea, CA) auto analyzers that measure by immunoenzymatic method and use ALP conjugates as signal amplifiers. Our purpose is to evaluate the interference caused by the ALP elevation on these systems through βeta human chorionic gonadotropin (βhCG), ferritin, free thyroxine (FT4), thyroid-stimulating hormone (TSH), troponin I and Cobalamin (Vit B12) tests.

Materials and Methods

We created a serum pool with 20 patients' sera who have previously consulted our laboratory in January 2020 and whose ALP, βhCG, ferritin, FT4, TSH, troponin I, and Vit B12 tests were found to be within the reference range.

The prepared serum pool was divided into four groups. Commercially prepared isolated ALP (Toyoba Enzymes, Osaka, Japan), with an activity of 30,000,000 U/L, was added at different doses to the groups. Commercial ALP enzyme is in transparent liquid form, has grade 2 activity (30,000 U/mL or more) and contains Adenosine deaminase and Phosphodiesterase. First of all, due to the enormous enzyme activity, isolated ALP was diluted to a working stock by adding 2 μL of commercial ALP to 998 μL of distilled water. We prepared a stock solution immediately before the assay. We added 5 μL, 10 μL and 20 μL of this stock solution, respectively, to the serum pools of 8 mL each. We obtained four groups, one being our reference group without ALP addition and the others containing ALP at concentrations of 340 U/L, 870 U/L and 1590 U/L after adding isolated ALP.

Biochemical analysis

The ALP was measured spectrophotometrically with a Beckman Coulter AU 5800 (Beckman Coulter, Brea, CA) auto-analyzer. Ferritin, FT4, TSH and Vit B12 tests were measured immunoenzymatically with an UniCelDxl 800 (Beckman Coulter, Brea, CA) auto-analyzer and βhCG and troponin I test with an Access II (Beckman Coulter, Brea, CA) auto-analyzer. All assays are two-site sandwich immunoassay using enzyme-conjugated antibodies with direct chemiluminometric technology. In all groups, each test was performed with 20 repetitions. All measurements were completed on the same day and within-run.

Statistical analysis

In the evaluation of the effect of the ALP enzyme on the immunoenzymatic tests, Total error (TE), Bias and coefficient of variations (CV) were calculated by 20 repetitions of each group.

TE was calculated by the following formula:

$$TE = |Bias\ \%| + 2CV$$

TE = total error

CV = coefficient of variation.

Bias was calculated as follows (8):

$$Bias\ \% = \left(\frac{C_2 - C_1}{C_1}\right) x100$$

C_1 = mean value of reference group and C_2 = mean value of groups added with ALP

We applied the Friedman test to show the difference between groups using Med Calc (MedCalc Software Mariakerke, Belgium) software. We used Bonferroni correction to determine the adjusted significance level as p<0.0125.

Results

CV, Bias, and TE values of each analyte are reported as separate groups in *Table I*. ALP interference was assessed by calculating CV, bias, and TE for each analyte and comparing it to allowable total error (9). Results for each sample pool are summarized in T*able I*. *Figure 1* shows the TE values of βhCG, Ferritin, FT4, troponin I, TSH and Vit B12 between the groups, while *Figure 2* shows the calculated CV's.

After adding ALP, in βhCG, calculated TE values at ALP concentrations of 340 U/L and 1590 U/L were found to be above the acceptable error limits. In the FT4 test, the calculated TE value at ALP concentrations of 340 U/L was found to be above the acceptable error limits. Calculated TE values were found to be above the acceptable error limits in all groups for the troponin I test. We observed that the calculated CV values for the βhCG test increased with

	ALP (85 U/L)	ALP (340 U/L)			ALP (870 U/L)			ALP (1590 U/L)			
Test Name	Mean (CV)	Mean (CV)	Bias %	TE	Mean (CV)	Bias %	TE	Mean (CV)	Bias %	TE	aTE
βHCG, IU/L	0.68 (3.29)	0.77 (3.74)	13.23	20.71	0.72 (4.50)	5.88	14.88	0.84 (8.63)	23.5	40.76	17.00
Ferritin, pmol/L	107.57 (2.55)	105.46 (4.02)	-1.96	10.00	104.42 (2.82)	-2.92	8.56	104.62 (4.06)	-2.73	10.85	13.50
FT4, pmol/L	10.94 (4.88)	11.58 (4.2)	5.88	14.28	11.19 (3.73)	2.35	9.81	10.94 (3.76)	0.05	7.57	13.00
Troponin I, ng/L	19.78 (3.74)	15.19 (6.07)	-23.2	35.34	16.20 (3.5)	-18.02	25.02	15.29 (5.29)	-22.6	33.18	20.00
TSH, mIU/L	2.43 (2.70)	2.46 (1.92)	6.99	10.83	2.44 (1.85)	0.40	4.10	2.40 (2.39)	-1.23	6.01	13.50
vitB12, pmol/L	160.10 (4.60)	169.69 (4.72)	5.99	15.43	166.74 (4.80)	4.14	13.74	166.74 (3.84)	4.14	11.82	25.00

ALP, alkaline phosphatase; aTE, allowable total error; CV, coefficient of variations; FT4, free thyroxine; TE, total error; TSH, thyroid-stimulating hormone; Vit B12, cobalamin; The values marked as bold indicate TE values that exceed the upper limit that recommended by Rili-BAEK (9).

Table II P-values of statistical association analysis by Friedman test with Bonferroni correction for comparison of ALP effects on each test. P<0.0125 is considered statistically significant.

		ALP (340 U/L)	ALP (870 U/L)	ALP (1570 U/L)
ALP (85 U/L)	βHCG, IU/L	P<0.0125	P<0.0125	P<0.0125
	Ferritin, pmol/L	p>0.0125	p>0.0125	p>0.0125
	FT4, pmol/L	P<0.0125	p>0.0125	p>0.0125
	Troponin I, ng/L	P<0.0125	P<0.0125	p>0.0125
	TSH, mIU/L	p>0.0125	p>0.0125	p>0.0125
	vitB12, pmol/L	P<0.0125	P<0.0125	P<0.0125

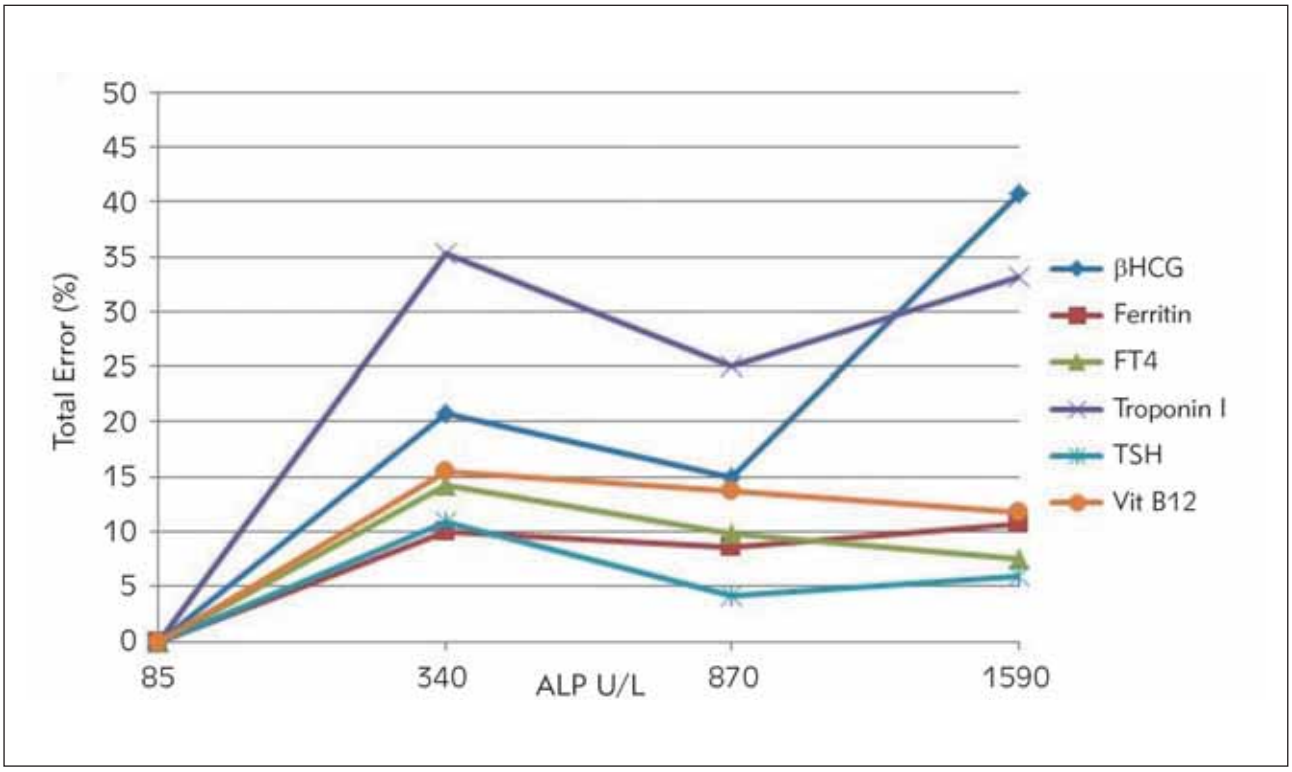

Figure 1 Total error distributions of βhCG, Ferritin, FT4, Troponin I, TSH and Vit B12 tests at different ALP concentrations. Groups containing different ALP concentrations are shown on the x-axis and total error values on the y-axis.

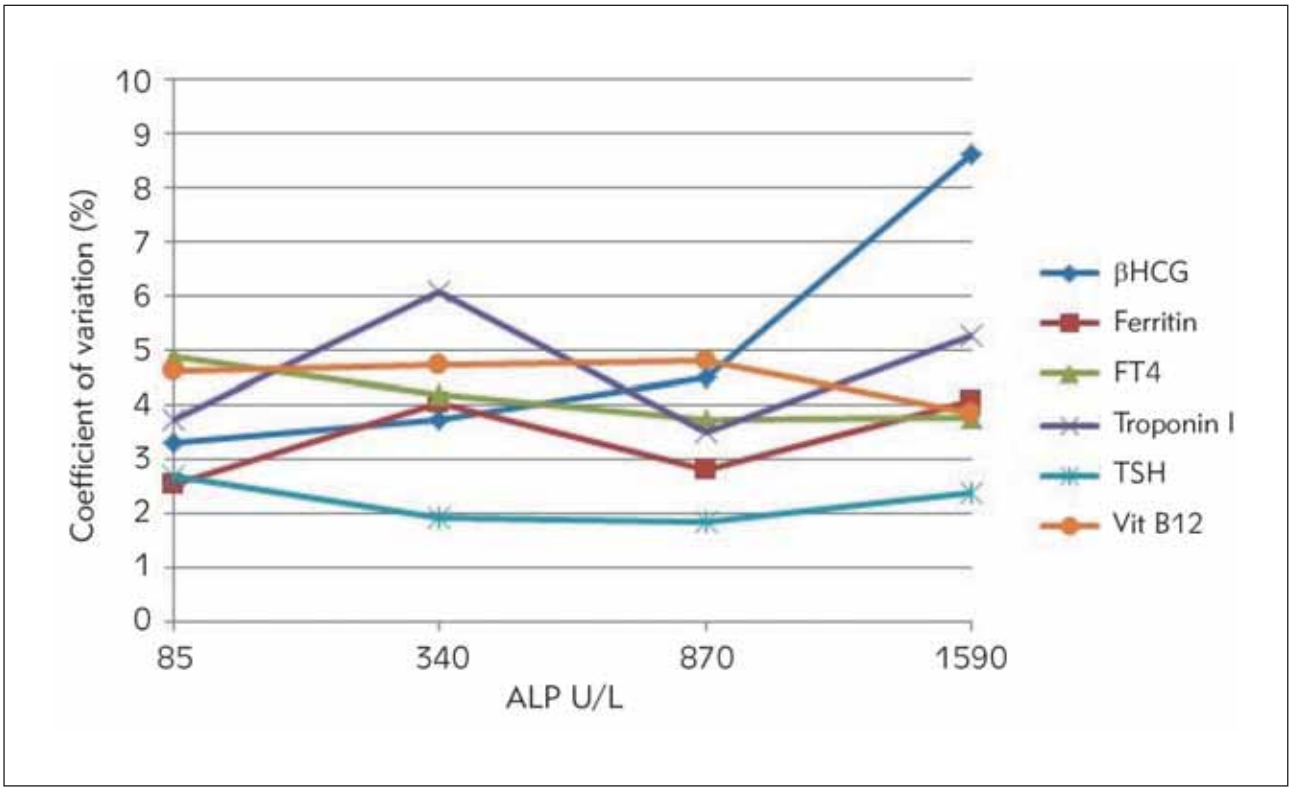

Figure 2 Coefficient of variations of each test at different ALP concentrations. Groups containing different ALP concentrations are shown on the x-axis, and the calculated coefficient of variations on the y-axis.

increasing ALP concentrations. In the troponin I test, calculated CV's at ALP concentrations of 340 U/L and 1590 U/L were found to be higher than the group without ALP added.

Table II shows the p values obtained by comparing the groups with different ALP concentrations with the group containing baseline ALP. We observed that there were statistically significant differences in all groups for βhCG and Vit B12, in the concentration of ALP 340 U/L for FT4, and in concentrations of 340 and 870 U/L for troponin I when compared to the baseline ALP level ($P<0.0125$). There were no statistically significant differences in ferritin and TSH among the groups.

Discussion

In our study, ALP interference was observed in immunoenzymatic assays for βhCG, FT4, troponin I and Vit B12 tests. We observed that TE and CV values increased after ALP addition, especially in troponin I and βhCG tests. Likewise, Sofronescu et al. (10) observed that their patient who used ALP enzyme externally for treatment had low Total testosterone levels than usual. They decided to measure total testosterone by liquid chromatography-mass spectrometry for comparison and found the result in the normal range. They observed negative interference as we observed in the troponin I test. They concluded that ALP could potentially interact and cause interference after binding the antibody (10). There are some similarities between their study and ours. They used the same auto-analyzer and manufacturer's kit as us. But they calculated neither TE nor CV. At the end of their study, they mentioned inadequate washing of the unbound analyte could also lead to false results (10). Similarly, Herman et al. (5) reported that βhCG and troponin I measurements were found to be incorrectly high as a result of improper washing steps of samples containing elevated ALP. Herman et al. (5) reported that this effect was seen especially above ALP> 1000 U/L concentrations.

We observed that ALP addition could cause interference on βhCG, FT4, troponin I and Vit B12 tests. While Herman et al. (5) found an erroneous elevation in both of the tests after the addition of ALP, we found an erroneous elevation in the βhCG test and an erroneously low reading in the troponin I test. We used Access II auto-analyzer for these tests, while Herman et al. (5) used DXI-800 auto-analyzer. Although Access II and DXi-800 use the same kits and calibrators and are manufactured by the same manufacturer, they are systems whose operation performances are different from each other. These systems perform the washing process in three cycles by using special washing solutions and eliminate any unbound molecules from the medium by creating a magnetic field using a magnet. Three critical components of the system are pipetting, washing, and checking the luminometer. If system updates and weekly maintenance are skipped, and the washing performance of the autoanalyzer is not working efficiently, high ALP values may cause interference.

In a way similar to our study, Dasguptaet et al. (11) evaluated ALP interference on troponin I assayed by microparticle immunoassay (MEIA) and fluorometric immunoassay (FIA). They did not observe ALP interference in the MEIA method, while they observed that interference increased with the elevation of ALP enzyme concentrations in the FIA method (11). Similarly, Butch et al. (12) demonstrated the interference of endogenous ALP on troponin I measurement by the FIA method. They evaluated and reported that the reason for this interference may be related to the washing performance of the system they use; they contacted the device manufacturers and reported that the interference was reduced by improving the washing steps (12).

Similar to our study, Marinheiro et al. (13) compared two troponin I methods using ALP as conjugate (Beckman Coulter Access AccuTnI+3®) and acridinium as conjugate (Abbott Architect STAT high sensitive TnI®). They reported that troponin I was falsely higher in the method which uses ALP. Interestingly, like in our case, the ALP level was normal in their case report. They concluded that endogenous ALP may interfere with the assay by interacting with microparticles even if it is in the normal reference range (13).

While preparing the study plan, we had to determine the final ALP concentration that we would reach. The linearity upper limit of the system that we used (Beckman AU5800) was set at 1500 U/L for the ALP test. Nargis et al. (14) analyzed patients with persistent ALP elevations in their study. They have reported that in the population they studied, ALP values were above 3000 U/L in only 3% of the patient group (14). So, we decided to stay within the values that we might encounter clinically in the daily workflow. Although we could elevate the ALP levels to much higher levels by adding ALP externally, we preferred to keep our upper limit within the linearity limits of the assay as we did not want to exceed the general patient population.

The interference effect of the presence of heterophilic antibodies on the test for immunoenzymatic methods is reported as a generally erroneous test result (15). One of the limitations of our study is that we did not examine the samples used in the serum pool for the presence of heterophilic antibodies. If some of the selected samples had contained heterophilic antibody would have possibly affected our entire pool. To avoid this, we could have used heterophilic antibody inhibitors. However, since we did not want to create a different interference source by using a heterophilic antibody inhibiting tube, we ignored the presence of heterophilic antibodies.

Other studies investigating ALP interference are often presented as case reports and are not statistically strong. They tried to understand and show the interference through a patient. Our study design was structured very well, and we compared our results to the reported guideline.

Based on the findings obtained from our study, we determined that elevated ALP caused interference on βhCG, FT4, troponin I and Vit B12 tests, but it did not cause a significant interference on Ferritin and TSH tests. Especially in terms of misdiagnosing myocardial infarction, it should be considered that troponin I could be affected by high ALP levels. It would be beneficial to repeat the βhCG and troponin I tests with DXI-800. There is a need for repeating the study with samples free of any heterophilic antibodies and with samples containing higher rates of ALP.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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CLINICAL EVALUATION OF NON-INVASIVE PRENATAL SCREENING IN 32,394 PREGNANCIES FROM CHANGZHI MATERNAL AND CHILD HEALTH CARE HOSPITAL OF SHANXI CHINA

KLINIČKA PROCENA NE-INVAZIVNOG SKRININGA U 32.394 TRUDNICE U CHANGZHI PORODILIŠTU I DEČIJOJ BOLNICI U SHANXI U KINI

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Summary

Background: Non-invasive prenatal screening (NIPS) is a highly sensitive and specific screening test to detect fetal chromosomal abnormalities. The primary objective of this study was to evaluate the NIPS as an effective method for prenatal detection of aneuploidies in both high-risk and low-risk pregnancies.
Methods: In current study, we performed NIPS in 32,394 pregnancies, out of which results were available in 32,361 (99.9%) of them. Illumina sequencing was performed for NIPS screening. Hypothesis Z test was used to classify fetal autosomal aneuploidy of T21, T18, and T13. Karyotyping was performed to determine the true negative and true positive NIPS results.
Results: Among the 32,361 confirmed samples, 164 cases had positive results and 32197 cases had negative results. Of these positive cases, 116 cases were trisomy 21, 34 cases were trisomy 18 and 14 cases were trisomy 13. No false negative results were found in this cohort. The overall sensitivity and specificity were 100% and 99.91%, respectively. There was no significant difference in test performance between the 7,316 high-risk and 25,045 low-risk pregnancies, (sensitivity, 100% vs 100% (P>0.05); specificity, 99.96% vs 99.95% (P > 0.05)). Factors contributing to false-positive results included fetal copy number variants (CNVs), fetal mosaicism and typically producing Z scores between 3 and 4. Moreover, we analyzed NIPS whole-

Kratak sadržaj

Uvod: Neinvazivni prenatalni skrining (NIPS) je veoma osetljiv i specifičan skrining test za otkrivanje fetalnih hromozomskih abnormalnosti. Primarni cilj ove studije bio je da se proceni NIPS kao efikasan metod za prenatalno otkrivanje aneuploidije u trudnoćama visokog i niskog rizika.
Metode: U trenutnoj studiji, NIPS smo uradili u 32.394 trudnoće, od kojih su rezultati bili dostupni u 32.361 (99,9%) trudnoća. Illumina sekvenciranje je izvršeno za NIPS skrining. Z test hipoteze je korišćen za klasifikaciju fetalne autozomne aneuploidije T21, T18 i T13. Kariotipizacija je urađena da bi se utvrdili pravi negativni i istinski pozitivni rezultati NIPS.
Rezultati: Među 32.361 potvrđenim uzorkom, 164 slučaja je imalo pozitivne rezultate, a 32.197 slučajeva je imalo negativne rezultate. Od ovih pozitivnih slučajeva, 116 slučajeva je bilo trizomija 21,34 slučaja trizomija 18 i 14 slučajeva trizomija 13. Nisu pronađeni lažno negativni rezultati u ovoj kohorti. Ukupna osetljivost i specifičnost bile su 100% i 99,91%, respektivno. Nije bilo značajne razlike u performansama testa između 7.316 visokorizičnih i 25.045 trudnoća sa niskim rizikom, (osetljivost, 100% naspram 100% (P>0,05); specifičnost, 99,96% naspram 99,95% (P > 0,05)). Faktori koji su doprineli lažno pozitivnim rezultatima uključivali su varijante broja kopija fetusa (CNV), fetalni mozaicizam i tipično stvaranje Z rezultata između 3 i 4. Štaviše, analizirali smo sekvenciranje celog genoma NIPS da bismo istražili povezanost polimorfizama jednog nukleotida

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genome sequencing to investigate the Single Nucleotide Polymorphisms (SNPs) associations with drug response or risk of disease. As compare to the 1000g East Asian genome data, the results revealed a significant difference in 7,285,418 SNPs variants of Shanxi pregnant women including 19,293 clinvar recorded variants and 7,266,125 non-clinvar recorded.

Conclusions: Our findings showed that NIPS was an effective assay that may be applied as routine screening for fetal trisomies in the prenatal setting. In addition, this study also provides an accurate assessment of significant differences in 7,285,418 SNPs variants in Shanxi pregnant women that were previously unavailable to clinicians in Shanxi population.

Keywords: NIPS, trisomy, false positive, true positive, Z score, SNPs variants

(SNP) sa odgovorom na lek. ili rizik od bolesti. U poređenju sa podacima o genomu istočne Azije od 1000 g, rezultati su otkrili značajnu razliku u 7.285.418 varijanti SNP-a kod trudnica u Shanki-u, uključujući 19.293 zabeležene varijante klinvara i 7.266.125 zabeleženih ne-clinvara.

Zaključak: Naši nalazi su pokazali da je NIPS bio efikasan test koji se može primeniti kao rutinski skrining za fetalne trisomije u prenatalnom okruženju. Pored toga, ova studija takođe pruža tačnu procenu značajnih razlika u 7.285.418 varijanti SNP-a kod trudnica u Shanki-u koje su ranije bile nedostupne kliničarima u populaciji Shanki-a.

Ključne reči: NIPS, trizomija, lažno pozitivan, istinski pozitivan, Z skor, SNP varijante

Introduction

China has the largest number of birth defects in the world, with about 900,000 new cases of birth defects every year (1), including about 240,000 cases of chromosomal abnormalities, most of which cannot be cured till today (2). In 1997, Lo et al. (3) discovered the presence of fetal cell free DNA in maternal peripheral (also named cfDNA). The cfDNA in maternal plasma grows in concentration with the gestational age, and it also harbors genomic information of the fetus. When a fetus has an abnormal number of chromosomes (aneuploidy), the cfDNA ratio regarding that chromosome will be altered (4). However, the concentration of cfDNA in the maternal plasma circulation may vary widely (5, 6), ranging from 4% to over 30% (7). Thus, advanced technologies such as digital polymerase chain reaction or massively parallel sequencing have been used to study cfDNA in maternal blood, differentiate fetal DNA from maternal DNA, and detect fetal chromosomal abnormalities (8). These discoveries made non-invasive prenatal screening (NIPS) of fetal aneuploidies a clinical reality and led to a new era of non-invasive prenatal screening with high sensitivity and specificity in multiple clinical centers (9, 10). NIPS had been commercialized in China since May 2010, and the clinicians showed strong interest and attempted to adopt the technology for detection of fetal aneuploidies (11). Find Gene NIPS is one of the earliest NIPS assays developed in China. In November 2019, based on retrospective clinical trials conducted in multiple clinical centers, FindGene NIPS got approval from the National Medical Products Administration for screening fetal trisomies (T) 21, 18, and 13 in China. Moreover, sufficiently large NIPS samples also indicate population genetics databases such as SNPs and allele frequency, genomic locations, and functional annotations. Single Nucleotide Polymorphisms (SNPs) are known to contribute to variation in various diseases and drugs responses. The primary objective of this study was to evaluate the NIPS as an effective method for prenatal detection of aneuploidies in both high-risk and low-risk pregnancies. Moreover, we also investigated the SNPs induced risk of disease based on 32,394 samples obtained from the Changzhi Maternal and Child Health Care Hospital, and SNPs induced variation in drug response.

Materials and Methods

Study design

This study was approved by the institutional review board of the Changzhi Maternal and Child Health Care Hospital. This was a prospective, large-scale, blinded cohort study conducted from 11 October 2016 to 26 June 2019. Only the patients with written consent were included in this study. The inclusion criteria for participants were: pregnant women, at least 18 years old, and a gestational age of 11 to 30 weeks. The women who were excluded included those: (i) who had recent stem cell therapy or immune therapy; (ii) who had cancer diagnosed; (iii) who had known chromosome abnormalities or whose partners had known aneuploidy.

NIPS screening

Five milliliter peripheral blood of pregnant women was collected by EDTA anticoagulant tube and delivered to the laboratory within 6 hr. of collection. The plasma was isolated from the peripheral blood by two-step centrifugation including at 1,600 $\times$ g for 10 min and then at 16,000 $\times$g for 10 min at 4 °C. After that the samples were frozen and delivered to Shanghai Findgene clinical laboratories (Shanghai, China) at which plasma was prepared for library construction, quality control and pooling. The plasma DNA was extracted from 600 µL plasma of each sample using Circulating Nucleic Acid Kit from Findgene (Chengdu Findgene Medical Equipment Co., Ltd). The detailed general technical procedure was described previously (12). The prepared library was quantified using real time PCR, 96 barcoded

libraries which were titrated and evenly pooled and sequenced with 36-cycles single-end multiplex sequencing strategy on an Illumina Next Seq. 500 platform (Illumina, USA) (13).

Hypothesis Z test was used to classify fetal autosomal aneuploidy of T21, T18, and T13. An approach integrating dynamic GC reference library (14) and LOESS (locally weighted scatterplot smoothing) regression (15) was applied to correct the GC bias before Z test. Integrating maternal copy number variant (CNV) and fetal fraction correction (2) were applied to correct the Z score after Z test. The sequences of each sample were mapped to the reference genome of human and Z scores were calculated for each chromosome (16, 17). $Z \geq 3$ would represent a chromosomal aneuploidy and $-3.0 < Z < 3.0$ would represent a chromosomal euploid. Fetal fraction of male fetuses was calculated based on the Y chromosome fraction. Re-sequencing, retesting and resampling was applied when samples did not meet quality control (QC) standards. Re-sequencing was performed on samples with insufficient unique reads only. Common reasons for retested sample included insufficient cfDNA content after library preparation quantification and unsuitable GC content to the reference or Z score chaos ($Z \geq 3$ or $Z \leq -3$) for more than five chromosomes. Samples with hemolysis, gestational age of less than 12 weeks, insufficient fetal fraction, and repeating Z score chaos would be resampled. Only one chance of resampling was allowed.

Clinical follow-up

Patients with negative NIPS screening results were advised for regular prenatal care; genetic counseling was provided if routine ultrasound examination showed abnormalities. Karyotyping was performed for all NIPS screening samples. On the basis of karyotyping a negative NIPS result was considered as a true negative if the prenatal or neonatal karyotyping results were normal, or if the neonate looked phenotypically normal. By contrast, a negative NIPS result was defined as a false negative if the prenatal or neonatal showed aneuploidy in karyotyping, or if the newborn was phenotypically abnormal. Pregnant women with positive NIPS results were recommended for confirmatory invasive prenatal diagnosis like amniocentesis. The positive NIPS result was defined as true positive upon karyotyping invasive confirmation or clinical follow-up results. The false positive was defined as high risk trisomy report in a case that was subsequently shown to be without aneuploidy upon invasive diagnostic confirmation or clinical physical examination. Patients without confirmatory diagnostic results were excluded from calculation of test parameters (sensitivity, specificity, positive perspective value (PPV), and negative perspective value (NPV)).

Statistical analysis

Statistical analysis between the different groups was performed using a chi square test or Fisher's exact test, and P values of <0.05 were considered statistically significant.

Results

NIPS failure rate

Between 11 October 2016 and 26 June 2019, a total of 32,394 maternal blood samples were obtained for NIPS screening at Findgene from the Changzhi Maternal and Child Health Care Hospital in Shanxi, China. Out of these samples, 33 cases yielded no NIPS results including 24 cases of haemolysis and 9 cases of cancellation. The rate of NIPS failure was 0.1% (33/32394).

Demographic characteristics of pregnant women undergoing non-invasive prenatal screening (NIPS) for aneuploidies

The demographic characteristics of the remaining 32,361 samples are shown in *Table I*. Data from a total of 32,361 cases were included in this study, which mainly consisted of women from Shanxi province, China. The mean maternal age was 31 years, with

Table I Demographic characteristics of pregnant women undergoing NIPS for aneuploidies between 11 October 2016 and 26 June 2019.

Maternal age	
Mean age (year)	31
Advanced maternal age > = 35	7,316 (22.8%)
Pregnancies with the age < 35	25,045 (77.2%)
18–20 year	89
20–24 year	4,012
25–29 year	12,260
30–34 year	8,684
35–40 (year)	6,272
>40 (year)	1,044
In Vitro Fertilization sample	549
Twin samples	875
Single pregnant sample	31,486
Gestational age at blood sampling	
Median (week)	18.5
Range (week)	11–30
11 to 15 weeks (n, %)	1,060 (3.3%)
16 to 20 weeks (n, %)	25,651 (79.2%)
20 to 25 weeks (n, %)	4,819 (15%)
26-30 weeks (n, %)	831 (2.5%)

Table II Efficiency of NIPS for T21/T18/T13.

Trisomies	TP	FP	FN	Sensitivity	Specificity	PPV	NPV
T21	65	17	0	100%	99.95%	79.27%	100%
T18	17	8	0	100%	99.98%	68%	100%
T13	4	4	0	100%	99.99%	50%	100%
Total	86	29	0	100%	99.91%	74.78%	100%

TP = true positive, FP = false positive, NIPS = noninvasive prenatal screening, PPV = positive predictive value, NPV= Negative predictive value.

22.8% maternal age ≥ 35 years upon delivery. In this cohort, 31,486 cases were single pregnant, 875 cases were twins and 549 cases were *in-vitro* fertilization (IVF). The gestational age (GA) ranges from 11 to 30 weeks, with a mean GA of 18 weeks.

Efficiency of NIPS for T21/T18/T13

Among the 32,361 confirmed cases who obtained NIPS results, 164 were positive and 32,197cases were negative (*Table I*). Pregnancies with the NIPS positive results were recommended for confirmatory invasive prenatal diagnosis using chromosomal microarray analysis. After informed consent, only 114 cases agreed to go through the confirmatory invasive prenatal diagnosis while, 50 cases declined for further diagnosis. *Table II* shows the prenatal diagnosis results. The NIPS sensitive, specificity, positive predictive values and negative predictive values were 100%, 99.91%, 74.78%, 100% respectively.

Efficiency of NIPS in high-risk pregnancies and low-risk pregnancies

In present study, the women whose age ≥ 35 (high-risk pregnancies) years old were 7,316 cases. Among 7,316 advanced maternal age women, 29 cases were the NIPS positive and 12 cases were NIPS false positive results of T21/T18/T13 (*Table II*). Of all 25,045 cases of the women whose age < 35 (low risk pregnancies), there were 56 cases with NIPS positive results and 17 cases with false positive results. False negative cases were not found after at least 10 months of follow-up. There was no significant difference in test performance between the 7,316 high-risk and 25,045 low-risk subjects (sensitivity, 100% vs. 100% (P >0.05); specificity, 99.96% vs. 99.95% (P > 0.05)). These results suggested that the application of NIPS could significantly reduce the cost of invasive prenatal diagnosis, which had a positive yield of only 0.56% (41/7,316) in high-risk group and 0.29% (73/25,045) in low-risk group.

Table III Performance of non-invasive prenatal screening (NIPS) in high-risk pregnancies and low-risk pregnancies.

Category	Verified	TP	FP	Sensitivity	Specificity
T13 (<35yrs)	3	2	1	100%	99.99%
T18 (<35yrs)	13	10	3	100%	99.99%
T21 (<35yrs)	56	44	13	100%	99.96%
T13 (>=35yrs)	5	2	3	100%	99.99%
T18 (>=35yrs)	11	7	4	100%	99.99%
T21 (>=35yrs)	25	21	5	100%	99.98%
Low-risk pregnancies (<35yrs)	73	56	17	100%	99.95%
High-risk pregnancies (>=35yrs)	41	29	12	100%	99.96%

TP = true positive, FP = false positive, NIPS = noninvasive prenatal screening, T13 = trisomy 13, T18 = trisomy 18, T21 = trisomy 21.

Z score and gray zone

Out of 29 false positive cases, 20 cases (69%) had Z score with 3 < Z < 4 and 9 cases (31%) had Z score > 4. In contrast, 85 cases got true positive results of NIPT, 80 (95%) cases got Z > 4, and only 5 cases (5%) cases got Z score ranging from 3 to 4. These results indicated that Z score between 3 and 4 represented a gray zone where most false positive cases resided.

Follow-up investigation of test negative cases

At the time of writing, all pregnant women included in this series had given birth. To date, among 32,197 NIPS negative cases, no abnormalities were reported through our feedback mechanism.

Characteristics of single-nucleotide polymorphisms (SNPs) in the Shanxi pregnant genome

As compared to 1000 g Eastern Asian population there were significant difference alleles frequencies in 7,285,418 SNPs variants (p<0.05) (Supplementary 1). Out of these SNPs variants, 19, 293 SNPs variants were recorded by clinivarhttps://www.ncbi.nlm.nih.gov/clinvar/including 17,167 benign variants, 67 pathogenic variants, 5 affects variants, 82 associated variants, 408 not provided/other variants, 8 protective variants, 85 risk factor variants, 977 uncertain significance variants, 387 conflicting interpretation of pathogenicity, and 107 drug response variants.

Discussion

In the past few years, NIPS has been widely used as a powerful screening tool to detect the chromosomal aneuploidies such as T21, T18, and T13 (18).

Table IV The characteristics of Z score in the false and true positive cases.

	Z score	T21	T18	T13	Total
False positive (n=29)	3<Z<4	14	3/7	3/4	20/29 (69%)
	Z>4	4	4/7	1/4	9/29 (31%)
True positive (n=85)	3<Z<4	4	1	0	5/85 (5%)
	Z>4	60	16	4	80/85 (95%)

T13 = trisomy 13, T18 = trisomy 18, T21 = trisomy 21.

This prospective study was performed to evaluate the efficiency of NIPS in the Changzhi Maternal and Child Health Care Hospital. The results indicated a significant difference in 7,285,418 SNPs variants of Shanxi pregnant women including 19,293 clinvar and 7,266,125 nonclinvar recorded variants. Our data also showed that the sensitivity, specificity, PPV and NPV were 100%, 99.91%, 74.56% and 100% respectively, which were very competitive compared to earlier reports (19–21). When compared to studies in high-risk pregnancies, our results were comparable to those of Qi et al. (22) and Hu et al. (23), showing that the efficacy of NIPS screening was consistent from low-risk to high-risk pregnancies.

In parallel, false positives were analyzed in detail. Some possible reasons of false positives in NIPS had been reported in literature, including low Z scores, fetal pathogenic CNVs and placental mosaicism. Bianchi et al. (24) demonstrated that a Z-score between 2.5 and 4 should be considered as a borderline value (24), and false-positives were likely to occur at borderline Z scores (25). Indeed, this study indicated that 20 NIPS positive cases (80%) with Z score from 3 to 4 were later confirmed to be false positives (*Table IV*). The algorithm of NIPS is Z test with the standard normal distribution. If the true negative is judged to be negative by Z 3 (99.87%), the probability of false positive would be 0.13%. Theoretically, the probability of false positives of 0.13% can be accepted. We also developed an algorithm to exclude the effect of maternal CNV and refined the Z-score that can determine fetal aneuploidy. However, biological factors such as fetal pathogenic CNV played an important role in causing the false positives (26). This study indicated that 2 cases of false positives were related to fetal pathogenic CNV, including a case T13 for 1450KB amplification at 13q12.12 and a case T21 for amplification at 21B, 21I points. Fetal mosaicism had also been reported to cause false results (27). Moreover, our data also showed that 2 cases of false positive were fetal mosaicism by invasive diagnostic confirmation. These factors must therefore be taken into account when interpreting NIPS results, and post-test genetic counseling should be provided to pregnant women following recommendations, such as those of the National Society of Genetic Counselors' statement.

In present study, the percentage of positive NIPS was 0.51%, which was lower than those reported in other studies, ranging from 1.6 to 3.2 % (23, 28). This was because this study included a large number of low-risk pregnancies (25,046 cases (77.3%)) for NIPS with a mean maternal age of 31 years. Out of the 7,316 advanced maternally aged women, there were only 29 patients (0.39%) showing positive results; Among 25,046 low-risk cases (<35 years), there were 55 cases (0.22%) showing positive results. Although NIPS was often recommended for maternal ages above 35, we demonstrated its efficacy in low-risk pregnancies. It could significantly reduce the cost of invasive prenatal diagnosis, many of which would be unnecessary. In fact, in this study, application of NIPS significantly reduced the rate of invasive prenatal diagnosis to 0.56% (41/7316) in high-risk group and 0.29% (73/25046) in low-risk group (*Table III*). Surprisingly, this study found 44/64 cases (69%) of Down syndrome coming from the low-risk pregnancies (*Table III*), this highlighted the value of NIPS in this often-overlooked subgroup.

Conclusion

In conclusion, this study showed that NIPS was an effective method for prenatal detection of aneuploidies (29). It showed comparable results when applied to both high-risk and low-risk pregnancies, and was able to provide valuable information for more cost-effective utilization of invasive diagnostic methods. Although our study provides an authentication of NIPS as effective method to detect the prenatal detection of aneuploidies on the basis of being highly sensitivity and specificity with lower false-positive rates, but still there are some limitations to NIPS and our study. Although NIPS has high sensitivity and specificity, the overall specificity and sensitivity is not uniform for all chromosomes because of the variation in GC content of sequences. Another limitation is that selected patients for NIPS may not reflect a general obstetrical population. Also, our study didn't include the abnormalities determined using ultrasound in comparison to NIPS. Further studies are needed to overcome these issues to narrower the gap between diagnosis and treatment.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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FOCUS-PDCA CAN EFFECTIVELY OPTIMIZE THE CRITICAL VALUE OF TEST ITEMS

FOCUS-PDCA MOŽE EFIKASNO DA OPTIMIZUJE KRITIČNU VREDNOST ISPITIVANJA

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Summary

Background: To optimize the critical value of test items using FOCUS-PDCA (find, organize, clarify, understand, select, plan, do, check and act), and to set the personalized critical value of the test for different departments.
Methods: We searched for literature reporting on the critical value and FOCUS-PDCA published over recent 5 years in order to understand the significance and status quo of critical value and FOCUS-PDCA. We also collected and analyzed the critical value data of hospital tests performed in Sichuan province hospitals in 2019, which were later compared to data from 2020 to determine the FOCUS-PDCA cycle.
Results: The proportion of critical values in the whole hospital decreased from 3.5% before optimization to 2.5% to 3% after optimization. The critical values of ICU, hematology, nephrology, urology, and neonatal departments after optimization significantly decreased compared with those before optimization, while the critical values of cardiac sur-

Kratak sadržaj

Uvod: Svrha rada je optimizacija kritične vrednosti ispitivanja pomoću FOCUS-PDCA (pronaći, organizovati, razjasniti, razumeti, izabrati, planirati, uraditi, proveriti i delovati), i postaviti personalizovanu kritičnu vrednost testa za različita odeljenja.
Metode: Tražili smo literaturu koja izveštava o kritičnoj vrednosti i FOCUS-PDCA objavljenoj u poslednjih 5 godina da bismo razumeli značaj i status kritične vrednosti i FOCUS-PDCA. Takođe smo prikupili i analizirali podatke kritične vrednosti bolničkih testova obavljenih u bolnicama provincije Sečuan 2019. godine, koji su kasnije upoređeni sa podacima iz 2020. da bismo odredili ciklus FOCUS-PDCA.
Rezultati: Udeo kritičnih vrednosti u celoj bolnici smanjen je sa 3,5% pre optimizacije na 2,5% do 3% nakon optimizacije. Kritične vrednosti odeljenja intenzivne intenzivne nege, hematologije, nefrologije, urologije i neonatalnog odeljenja posle optimizacije su značajno smanjene u odnosu na one pre optimizacije, dok kritične vrednosti kardiohirurgije,

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gery, emergency ICU, cardiology, and neurosurgery ICU showed no significant difference before and after optimization. Contrary, the critical values of the infection department after optimization significantly increased before optimization.

Conclusions: FOCUS-PDCA can effectively optimize the critical value of test items, which is beneficial for rational utilization of medical resources.

Keywords: critical value, optimization, FOCUS-PDCA

urgentne intenzivne nege, kardiologije i neurohirurgije ICU nisu pokazale značajnu razliku pre i posle optimizacije. Naprotiv, kritične vrednosti infektivnog odeljenja nakon optimizacije su značajno porasle pre optimizacije.

Zaključak: FOCUS-PDCA može efikasno optimizovati kritičnu vrednost ispitivanja, što je korisno za racionalno korišćenje medicinskih resursa.

Ključne reči: kritična vrednost, optimizacija, FOCUS-PDCA

Introduction

The term critical value was first proposed by Lundberg in 1972 (1), referring to the laboratory test value that is life-threatening to a patient without timely clinical intervention (2). The item with critical value is called critical value item, while the critical value threshold or critical value boundary is called critical value reporting limit, which refers to the analyte-specific set limits that define a test result as a »critical value (3). The concept of critical values was endorsed by many countries including China. For example, in 2012 China developed a »medical laboratory quality and criteria for recognition, »which requires that the critical value of clinical laboratory represents a standardized reporting system (4); yet, at present, no unified critical value of the project and the threshold value has been proposed (5).

Optimizing the critical value reporting process and improving the critical value reporting rate and timely rate of critical value reporting have been explored worldwide. PDCA is a popular iterative methodology that can fix a problem or improve a process and reduce the failure rate of critical value in the laboratory department (6–7). The four processes of the PDCA cycle (Plan-Do-Check-Act) are not completed once after running and are carried out repeatedly. Some researchers have applied the PDCA cycle method to test critical value management, effectively reducing the return time of critical value management and medical intervention and improving the critical value registration rate and the qualified rate of registration (8).

FOCUS-PDCA is a novel management mode of continuous quality improvement proposed by American hospital organizations based on the PDCA cycle. It creatively combines FOCUS and continuous cycle improvement (PDCA) and produces a management improvement mode (9–12). The characteristics of FOCUS-PDCA are big ring with small ring, step rise, and scientific statistics (13), which are more widely used in patient care, drug management, and medical record management.

This study aimed to optimize the critical value of the test by FOCUS-PDCA and set the personalized critical value of the test for different departments.

Methods

Literature Research

We searched for literature reporting on the critical value and FOCUS-PDCA published over recent 5 years in order to understand the significance and status quo of critical value and FOCUS-PDCA. Then, we collected and analyzed the critical value data of hospital tests performed in Sichuan province hospitals in 2019, which was later compared to data from 2020to determine the FOCUS-PDCA cycle.

Find Improvement Items

The following data were then analyzed: clinical laboratory specimen, critical value of specimen. After the critical value ratio of the whole hospital and all departments in each month was analyzed, the critical value ratio of some departments was too high.

Taking the optimization of critical value as the goal of this improvement project, we expected the project target to be »SMART«, i.e., the target belongs to the specific field of »critical value management«. The proportion of critical value can be used to measure the target situation.

Organize Improvement Team

An improvement group, which was set up according to the optimized critical value, included those affected by the excessively high proportion of critical value and those who will be affected by the critical value reform into the group.

Clarify the Current Process

According to the current test critical value version, when the LIS system detected the critical value, the test ends are automatically sent to the doctor, and the test staff informs the department and registers the value within the effective time. The critical value items and threshold values for the whole hospital are the same versions and include: blood biochemistry project, blood gas project, coagulation project, and blood routine project.

Understand Analyze the Root Cause

We hypothesized three fundamental reasons that could lead to a high proportion of critical values: (1) laboratory staff did not know how to optimize critical values on the new system; (2) there were no rules and regulations on the regular optimization of critical values, and there was little communication between clinical departments and clinical laboratory departments on critical values; (3) there was no personalized critical value, and medical staff adopted different clinical treatment methods for patients in different departments.

Select the Improvement Plan

We selected the problem points that needed to be improved and set the personalized critical value. The improvement team reviewed relevant literature in the Medical Department and clinical laboratory in early February 2020 and selected the way of »clinical critical value communication meeting« to establish personalized critical value in clinical departments.

PDCA (Plan-Do-Check-Act)

Plan

It was necessary to improve team planning critical value communication to implement specific matters. The project improvement team leader held a critical value clinical communication meeting in the medical department conference room (February 2020) regarding the critical value items and reporting limits. The improvement team members then summarized the critical value communication and informed the medical department (within one week after the meeting). The new critical values were released to the whole hospital after being confirmed by the medical department (March 2020). Soon after that, clinical laboratory and clinical departments held department meetings, respectively, to inform all the staff regarding the new critical value.

Do

From February to March 2020, the improvement team successfully implemented the action as planned. In April 2020, the whole hospital began to apply the new critical value. After that, the clinical laboratory regularly communicated the critical value to the clinical department.

Check

It was necessary to periodically check whether the LIS system missed the critical value and the understanding degree of laboratory staff to the current critical value version, and to find the following problems: At the beginning of the revision of the critical value version, some staff were not familiar with the new critical value; thus, it was necessary to further adjust the personalized critical value for some departments.

The improvement team collected the critical value specimen information and total specimen information of the whole hospital from May 2020 to March 2021.The proportion of critical value in each month after optimized critical value in the whole hospital and all clinical departments was then counted and compared with the data in 2019. Next, a table was created to observe the difference in the proportion of critical values before and after optimization.

Act

After this critical value optimization, the hospital formed a »new version of critical value with personalized critical value«, and the clinical laboratory applied continuous improvement measures of critical value, including the improvement team to evaluate the hospital's critical value periodically every year.

Results

Changes of critical value before and after optimization (monthly data)

Data from January to March 2019 (before optimization) and 2021 (after optimization) were analyzed and compared. The data from January to March 2020 were actually data from January to March 2021 in order to exclude the impact of the epidemic in 2020. Data from May to December 2019 and 2020 were analyzed and compared. As shown in *Figure 1*, the proportion of critical values in each month of the hospital decreased from 2.7%–4.1% before optimization to 2.6%–3.1% after optimization.

Proportion change of critical values in departments before and after optimization

The proportion of critical value in each month before and after the optimization of critical value in each department was counted. The proportion of critical value in some departments decreased significantly, as shown in *Table I* and *Table II*. The critical values of ICU, hematology, nephrology, urology, and neonatal departments after optimization decreased significantly compared with those before optimization, while the critical values of cardiac surgery, emergency ICU, cardiology, and neurosurgery ICU showed no significant difference before and after optimization. Contrary, the critical values of the infection department after optimization significantly increased before optimization.

The proportion of critical values after ICU optimization was only half of that before optimization. The data from

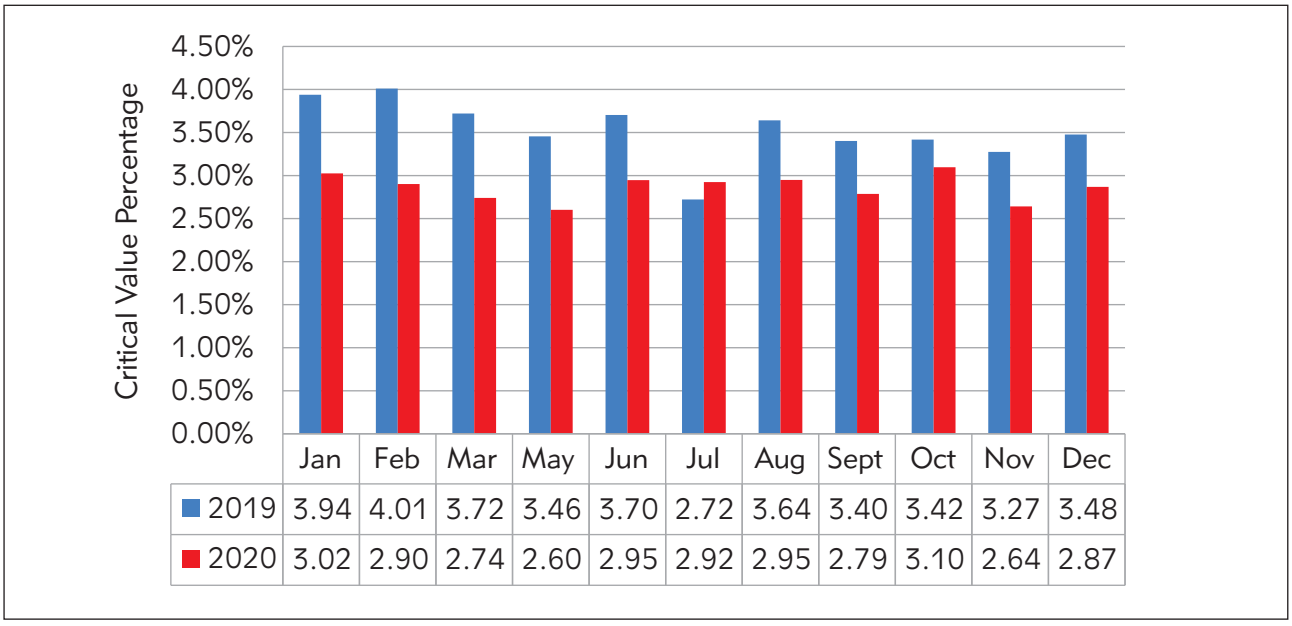

Figure 1 Proportion of critical values of the hospital during 2019 (before optimization) and 2020 (after optimization).

Table I Change of critical value rate before and after optimization of critical value in each department.

Month	ICU		Hematology department		Nephrology department		Organ transplantation center		New pediatric	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Jan	13.15%	5.54%	15.75%	2.51%	7.65%	3.34%	4.64%	1.77%	7.67%	5.04%
Feb	11.69%	4.89%	16.75%	1.85%	7.89%	3.79%	5.64%	1.73%	8.52%	6.28%
Mar	12.17%	4.53%	19.76%	2.35%	5.93%	3.31%	7.03%	2.21%	7.80%	5.69%
May	12.46%	4.28%	19.65%	2.43%	5.84%	4.08%	6.16%	1.53%	6.91%	7.61%
Jun	12.24%	4.98%	18.94%	3.04%	7.29%	4.12%	4.87%	2.33%	10.98%	8.35%
Jul	11.87%	5.80%	17.50%	3.15%	7.00%	2.93%	2.90%	1.98%	11.83%	8.33%
Aug	13.68%	6.14%	21.19%	2.13%	6.47%	2.98%	7.79%	2.26%	10.23%	6.05%
Sept	12.83%	4.21%	15.74%	2.70%	7.05%	3.96%	5.67%	3.02%	9.85%	6.35%
Oct	11.31%	3.60%	16.96%	3.59%	7.42%	4.08%	4.68%	4.63%	10.59%	6.92%
Nov	11.48%	4.01%	16.66%	2.20%	5.98%	3.65%	6.60%	2.68%	13.29%	8.11%
Dec	13.65%	3.66%	16.71%	2.97%	6.28%	3.41%	7.33%	1.05%	8.89%	7.30%

January to March 2020 were actually the data from January to March 2021, as shown in *Figure 2*. The proportion of critical value after optimization in the Department of Hematology was less than 1/3 of that before optimization (*Figure 3*), while the proportion of critical value after optimization in the Department of Nephrology was about 1/2 to 2/3 of that before optimization (*Figure 4*).

The proportion of critical value between the adjacent months of external urology in 2019 was significantly different, with the lowest value being 2.90% in July and the highest value being 7.79% in August. However, the optimized data for 2020 and 2021 showed a small difference from month to month that was stable at about 2%. October was an exception, with the critical value ratio reaching 4.63% (*Figure 5*).

Different from the above departments, the proportion of critical values in the Infection Department after optimization could be as low as 1.5 times and as high as 5 times before optimization (*Figure 6*).

Table II Change of critical value rate before and after optimization of critical value in each department.

Month	Cardiac surgery		Emergency ICU		Infectious department		Cardiology department		Neurosurgical ICU	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Jan	2.15%	0.90%	11.27%	10.42%	0.91%	4.99%	5.04%	6.76%	6.20%	4.59%
Feb	1.64%	0.64%	11.02%	10.02%	1.44%	4.33%	5.01%	6.42%	5.16%	3.68%
Mar	2.41%	1.86%	9.31%	11.36%	1.83%	4.46%	4.80%	5.46%	3.89%	4.54%
May	2.44%	2.10%	9.20%	7.25%	0.86%	4.93%	4.70%	4.21%	3.86%	2.84%
Jun	2.60%	1.87%	8.03%	9.76%	1.29%	4.81%	4.89%	5.36%	4.99%	6.02%
Jul	2.05%	1.55%	7.97%	10.46%	1.88%	3.89%	4.61%	5.23%	4.32%	4.43%
Aug	1.92%	2.44%	8.11%	8.71%	1.76%	3.23%	5.31%	5.85%	3.15%	4.96%
Sept	2.31%	2.74%	7.57%	9.22%	1.85%	3.21%	5.22%	6.34%	3.11%	4.58%
Oct	2.61%	2.04%	8.06%	8.32%	1.37%	5.85%	5.25%	6.82%	4.09%	4.10%
Nov	1.54%	1.28%	8.34%	8.52%	1.54%	5.36%	5.06%	5.79%	4.92%	2.70%
Dec	1.63%	1.87%	10.23%	10.00%	1.36%	4.79%	5.23%	6.29%	3.32%	2.14%

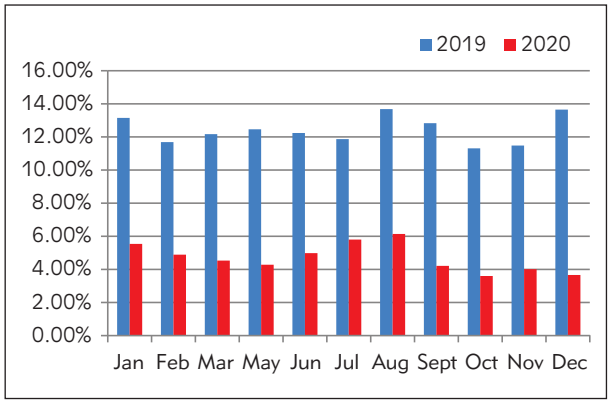

Figure 2 Proportion of critical values of ICU test items before and after optimization.

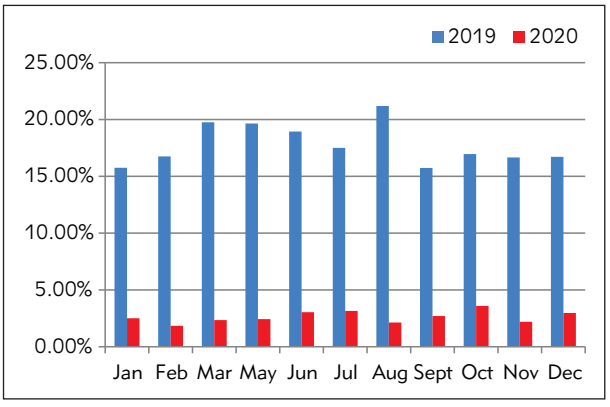

Figure 3 Proportion of critical value before (2019) and after (2020) optimization in Department of Hematology.

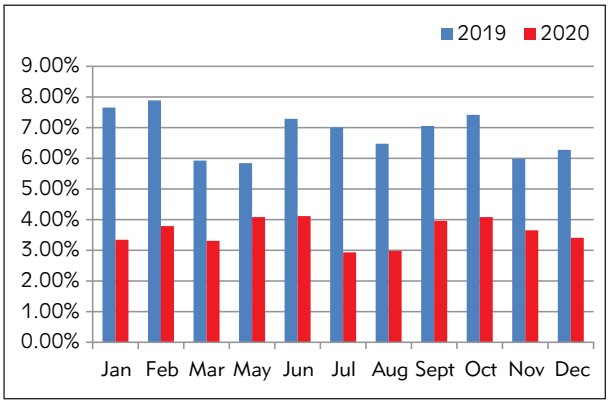

Figure 4 Proportion of critical value before (2019) and after (2020) optimization in Department of Nephrology.

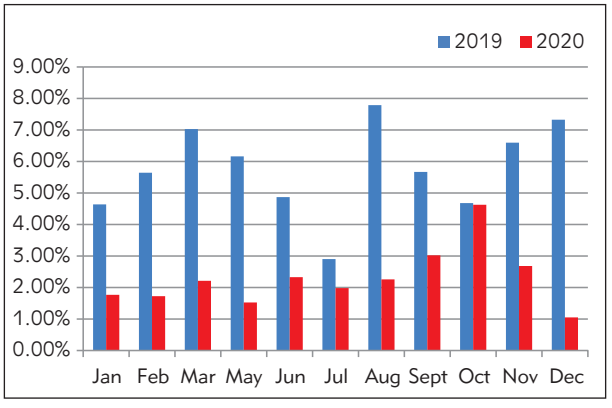

Figure 5 Proportion of critical value before (2019) and after (2020) optimization in Organ Transplantation Center.

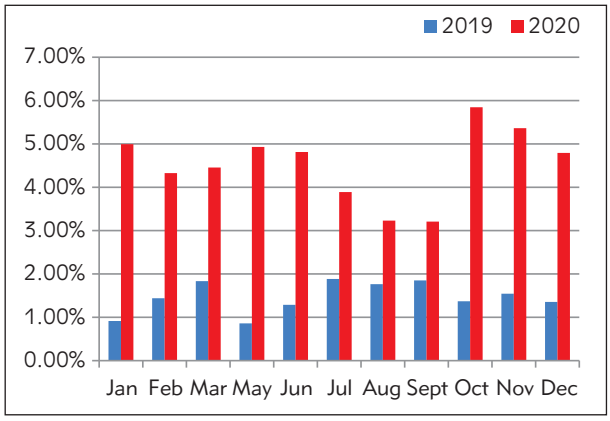

Figure 6 Proportion of critical value before (2019) and after (2020) optimization in Infection Department.

Discussion

»Critical value« result is an abnormal laboratory test value that is life-threatening to a patient and is reported by laboratory staff based on preset critical limits. It also refers to closely related disease outcomes of test results or to the national major communicable diseases (14, 15).

In China, the concept of critical values has been defined in the Patient Safety Objectives (2014–2015) issued by China Hospital Association in 2014 (16) and Medical Quality Control Indicators for Clinical Laboratory Professionals issued by National Health and Family Planning Commission in 2015. However, standardized optimization of critical values remains a challenge. For example, the effective critical value of auxiliary departments is not consistent with that of clinical identification. Different departments treat the same critical value items differently, and the selection of critical value items and the determination of critical value limits have not been standardized at home and abroad. So far, only a few studies have reported on critical value optimization (17).

AT present, PDCA is the most common method used for critical value optimization. PDCA cycle was first proposed by Hughart, then perfected and popularized by Dr. Deming in 1950 (18, 19). Many researchers have applied the PDCA cycle to improve management quality (20–22). However, PDCA is mainly used to establish critical value reporting procedures or improve the timely rate and qualified rate of critical value in medicine, while it is rarely used to optimize critical value testing projects. Some researchers used the PDCA cycle to improve the registration rate, registration pass rate, and rescue success rate (23), while others used it to analyze the critical value management of hospital clinical laboratory, improve existing problems, and finally improve the standardization of critical value management of hospital clinical laboratory (24). After applying PDCA to manage critical value, laboratory staff's working attitude, test quality, safety awareness, and operation standardization score have been improved (25).

FOCUS-PDCA is relatively a new approach developed by Hospital Corporation of America (HCA) and used to improve processes. It is mainly used in drug management, patient care, and medical record management, while it is rarely applied for critical value optimization management. Some researchers applied FOCUS-PDCA to solve drug management problems after investigating the procurement, allocation, and use of essential national drugs in the Affiliated Hospital of Nantong University (26). Also, the application of FOCUS-PDCA significantly reduced the dispensing error rate in pharmacies (27). Moreover, some studies applied the FOCUS-PDCA cycle method to effectively reduce the incidence of drug proximity error (28).

In terms of patient care, FOCUS-PDCA has been used to effectively solve the difficulties in moisture management of maintenance hemodialysis patients during dialysis (29). Some researchers also applied FOCUS-PDCA to treat trauma patients, effectively improving the treatment rate of patients (30).

Furthermore, FOCUS-PDCA can reduce the incidence of unreasonable medical orders of parenteral nutrition (31). In addition, the application of this model effectively improves the completion rate of the first page of inpatient medical records (32). This circulation can also improve the overall management of blood (33) and help medical institutions achieve significant process improvement (10).

Other researchers adopted FOCUS-PDCA during the epidemic to improve the capacity of hospitals dealing with COVID-19 outbreaks (34–36). Previous studies have also suggested that FOCUS-PDCA effectively improves the conversion rate of intravenous drug preparations and reduces medical costs (10).

FOCUS-PDCA has been rarely applied for critical value optimization management. The aim of this study was to optimize the critical value of test items using FOCUS-PDCA (find, organize, clarify, understand, select, plan, do, check and act) and to set the personalized critical value of the test for different departments. Our improvement team collected the total number of clinical specimens and critical value specimen information of each department in Sichuan Provincial People's Hospital in January 2020 and analyzed the change of critical value proportion of each department in the whole year and each month in 2019. The new version of the critical value was implemented in April 2020. The critical value and total specimen data from May 2020 to March 2021 were collected in April 2021 and then compared to data from 2019. In 2020–2021, the hospital's critical values (each month) were 2.5%–3%, while in 2019, the proportion was 3.5%, indicating a significant decrease in the value.

In some representative departments, such as ICU, hematology, nephrology, external urology, and neonatology, critical values decreased significantly after optimization. A particular decrease has been observed in the ICU and the department of hematology (ICU: 12% before optimization to about 4% after optimization; department of hematology from 15% ~ 22% to 1.5% ~ 3.6%).

The optimization of critical value items and their threshold values is not to reduce the proportion of critical value, but to set the critical value according to specimen

items, item values, and department personalization. For example, no significant change in critical values was found in the cardiac surgery and emergency ICU department before and after optimization. Interestingly, the critical value in the infection department increased after optimization (less than 2% before and 3%-6% after optimization).Because the infection department has narrowed the range of critical coagulation values.

To sum up, a new version of critical value with personalized critical value has been formed in the hospital using FOCUS-PDCA. The clinical laboratory has effectively optimized the critical value items and their boundary values, screened out the critical value that can truly reflect the critical state of patients in each clinical department, and established continuous improvement measures for the critical value.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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GABPA PROTECTS AGAINST GASTRIC CANCER DETERIORATION VIA NEGATIVELY REGULATING GPX1

GABPA ŠTITI OD POGORŠANJA KARCINOMA ŽELUCA NEGATIVNIM REGULISANJEM GPX1

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Summary

Background: To explore the anti-cancer role of GABPA in the progression of gastric cancer (GC), and the underlying mechanism.
Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to detect the expression pattern of GABPA in 45 pairs of GC and non-tumoral tissues. The relationship between GABPA expression and clinic pathological indicators of GC patients was analyzed. In AGS and SGC-7901 cells overexpressing GABPA, their migratory ability was determined by trans well and wound healing assay. The interaction between GABPA and its downstream target GPX1 was explored by dual-luciferase reporter assay, and their synergistical regulation on GC cell migration was finally elucidated.
Results: GABPA was downregulated in GC tissues in comparison to normal ones. Low level of GABPA predicted high incidences of lymphatic and distant metastasis in GC. Overexpression of GABPA blocked AGS and SGC-7901 cells to migrate. GABPA could target GPX1 *via* the predicted binding site. GPX1 was upregulated in clinical samples of GC, and negatively correlated to GABPA level. The anticancer effect of GABPA on GC relied on the involvement of GPX1.

Kratak sadržaj

Uvod: Cilj je bio da se istraži antikarcenogena uloga GABPA u progresiji raka želuca (GC) i osnovni mehanizam.
Metode: Kvantitativna lančana reakcija polimeraze u realnom vremenu (kRT-PCR) je sprovedena da bi se otkrio obrazac ekspresije GABPA u 45 parova GC i netumorskih tkiva. Analiziran je odnos između ekspresije GABPA i kliničkopatoloških pokazatelja pacijenata sa GC. U ćelijama AGS i SGC-7901 koje prekomerno eksprimiraju GABPA, njihova migraciona sposobnost je određena testom transvella i zarastanja rana. Interakcija između GABPA i njegovog nizvodnog ciljanog GPKS1 istražena je testom sa dvostrukom luciferazom, a njihova sinergistička regulacija migracije GC ćelija je konačno razjašnjena.
Rezultati: GABPA je smanjena u GC tkivima u poređenju sa normalnim. Nizak nivo GABPA predviđa visoku incidencu limfnih i udaljenih metastaza u GC. Prekomerna ekspresija GABPA je blokirala AGS i SGC-7901 ćelije da migriraju. GABPA bi mogao da cilja GPKS1 preko predviđenog mesta vezivanja. GPKS1 je bio pojačan u kliničkim uzorcima GC i imao je negativnu korelaciju sa nivoom GABPA. Efekat GABPA protiv raka na GC oslanjao se na učešće GPKS1.

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List of Abbreviations: Gastric cancer (GC); GA-binding protein alpha (GABPA); telomerase reverse transcriptase (TERT); Helicobacter Pylori (HP); gastric mucosal epithelial cell line (GES-1); American Type Culture Collection (ATCC); Dulbecco's Modified Eagle's Medium (DMEM); fetal bovine serum (FBS); ethylenediaminetetraacetic acid (EDTA); phosphate-buffered saline (PBS); Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR); complementary deoxyribose nucleic acids (cDNAs); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); radio immunoprecipitation assay (RIPA); bicinchoninic acid (BCA); sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); polyvinylidene difluoride (PVDF); glutathione peroxides (GPXs).

Conclusions: GABPA is downregulated in GC samples, which can be utilized to predict GC metastasis. Serving as a tumor suppressor, GABPA blocks GC cells to migrate by targeting GPX1.

Keywords: GABPA, GPX1, gastric cancer, migration

Zaključak: GABPA je smanjena u GC uzorcima, što se može koristiti za predviđanje GC metastaza. Služeći kao supresor tumora, GABPA blokira GC ćelije da migriraju ciljajući GPKS1.

Ključne reči: GABPA, GPKS1, rak želuca, migracija

Introduction

Gastric cancer (GC) is the number two killer of cancer death (1, 2). Recently, a growing number of therapeutic strategies for GC have been developed and improved, especially novel targeted therapy. However, the 5-year survival of metastatic GC is not ideal (3, 4). The study results demonstrated that GC cells are prone to metastasize through lymph nodes (4). A large number of GC patients are in the progressive stage at the first time of clinical diagnosis, thus losing the best surgical opportunity (4-6). Therefore, to clarify the mechanism of GC metastasis and actively explore new biomarkers are urgently to be solved (7–9).

GA-binding protein alpha (GABPA) encodes one of the three GA-binding protein transcription factor subunits, serving as a DNA-binding subunit. Since this subunit has a common feature to that encoding the nuclear respiratory factor 2 gene, it may participate in the activation of cytochrome oxidase expression and nuclear control of mitochondrial function (10, 11). GABPA also has a similar structure to that of the transcription factor E4TF1. Hence, it is involved in the expression of the adenovirus E4 gene (12). Current evidences have proven the interaction between GABPA and several important transcription factors (i.e. HCFC1, SP1 and SP3), and its vital function in affecting pathways of interactions at neuromuscular junction and mitochondrial gene expression (13). In multiple types of tumor cells, GABPA can selectively bind to the ETSdomain of the mutated telomerase reverse transcriptase (TERT) promoter and activate its transcription (14, 15). Previous studies have already reported the involvement of GABPA in the progression of bladder cancer and breast cancer (16, 17). Its potential influence in GC, however, is unclear.

This study aims to elucidate the role of GABPA in the malignant progression of GC and the molecular mechanism, which provides a novel target for individualized therapy.

Materials and Methods

GC Samples

Forty-five GC patients with surgical resection in our hospital were retrospectively analyzed, and these patients did not have preoperative anti-cancer treatment, previous infection of Helicobacter Pylori (HP) was all positive as well as pathologically was confirmed as GC. Invasive GC tissues and adjacent normal tissues were harvested during subtotal gastrectomy and stored in liquid nitrogen. Follow-up of each GC patient through telephone and outpatient review was conducted after discharge, including physical conditions, clinical symptoms and signs, and imaging examinations. In addition, the patients with other malignancies; mental disease; myocardial infarction; heart failure or other chronic diseases, or those previously exposed to radioactive rays were excluded. This investigation was approved by the research Ethics Committee of Handan Central Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients.

Cell Lines and Reagents

GC cell lines (AGS, BGC-823, SGC-7901) and the human gastric mucosal epithelial cell line (GES-1) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cell passage was conducted at 90% confluence using 1×tyrpsin containing EDTA (ethylenediaminetetraacetic acid).

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were cultured to 30–50% density in a 6-well plate, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Transwell Migration Assay

Cell suspension was prepared at 5×10^5 cells/mL. 200 μL of suspension and 700 μL of medium containing 20% FBS was respectively added on the top and bottom of a transwell insert and cultured for 48 h. Migratory cells on the bottom were induced with methanol for 15 min, 0.2% crystal violet for 20 min and captured using a microscope. Five random fields per sample were selected for capturing and counting migratory cells.

Wound Healing Assay

Cell suspension in serum-free medium was prepared at 5×10^5 /mL and implanted in 6-well plates. Cells were cultivated to 90% density, followed by cre-

ating an artificial scratch using a sterilized pipette tip. Cells were washed in phosphate-buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS. 24 hours later, wound closure percentage was calculated.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Shiga, Japan), followed by qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences were as follows. GABPA: Forward: 5′-GGAGGAAGTGGAGGGACTGA-3′, reverse: 5′-GCTTACACATTCAGCTGGCG-3′; GPX1: Forward: 5′-TATCGAGAATGTGGCGTCCC-3′, reverse: 5′-TCTTGGCGTTCTCCTGATGC-3′; GAPDH: forward: 5′-CCTGGCACCCAGCACAAT-3′, reverse: 5′-TGCCGTAGGTGTCCCTTTG-3′.

Western Blot

Cells were lysed in radio immunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) on ice for 15 min, and the mixture was centrifuged at 14000×g, 4 for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

Dual-Luciferase Reporter Assay

Wild-type and mutant-type GABPA vectors were synthesized based on bioinformatics screening on the binding site to GPX1. They were co-transfected in HEK293T cells with either pcDNA-NC or pcDNA-GPX1 for 48 h. Luciferase activity was finally measured in a standard method (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for statistical analyses and data were expressed as mean ± standard deviation. Differences between groups were compared by the *t*-test. The relationship between GABPA expression and clinicopathological indicators of GC patients was analyzed by Chi-square test. $P<0.05$ was considered as statistically significant.

Results

GABPA Was Lowly Expressed in GC

Forty-five cases of GC and paired adjacent normal tissues were collected in our center. It is shown that GABPA was downregulated in GC tissues (*Figure 1A*).

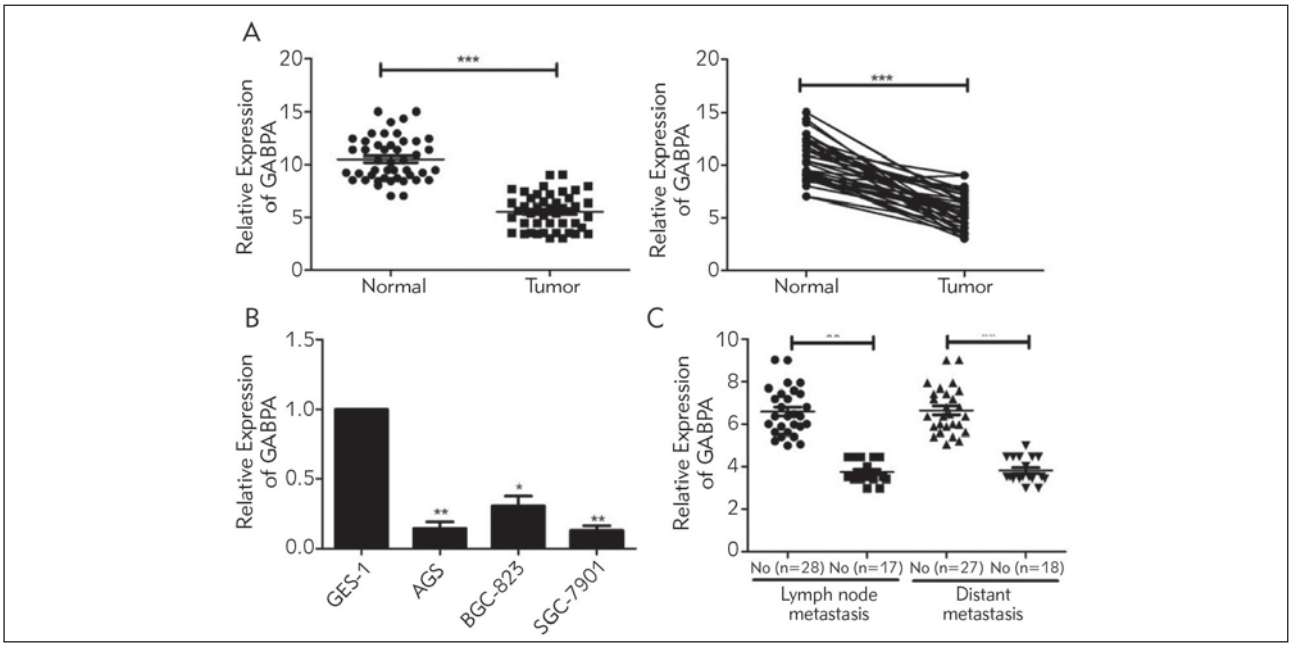

Figure 1 Proportion of critical values of the hospital during 2019 (before optimization) and 2020 (after optimization).

Table I Association of GABPA expression with clinicopathologic characteristics of gastric cancer.

Parameters	Number of cases	GABPA expression		P-value
		High (n=23)	Low (n=22)	
Age (years)				0.181
<60	23	14	9	
≥60	22	9	13	
Gender				0.295
Male	22	13	9	
Female	23	10	13	
T stage				0.182
T1-T2	25	15	10	
T3-T4	20	8	12	
Lymph node metastasis				0.023
No	28	18	10	
Yes	17	5	12	
Distance metastasis				0.011
No	27	18	9	
Yes	18	5	13	

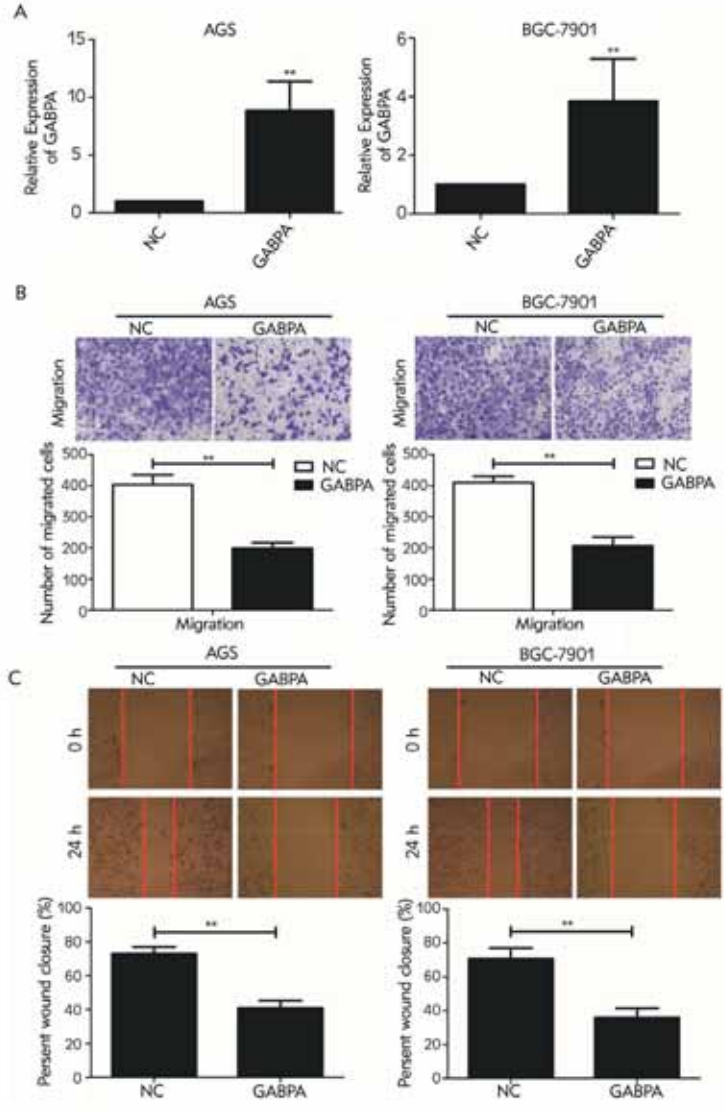

Figure 2 Proportion of critical values of the hospital during 2019 (before optimization) and 2020 (after optimization).

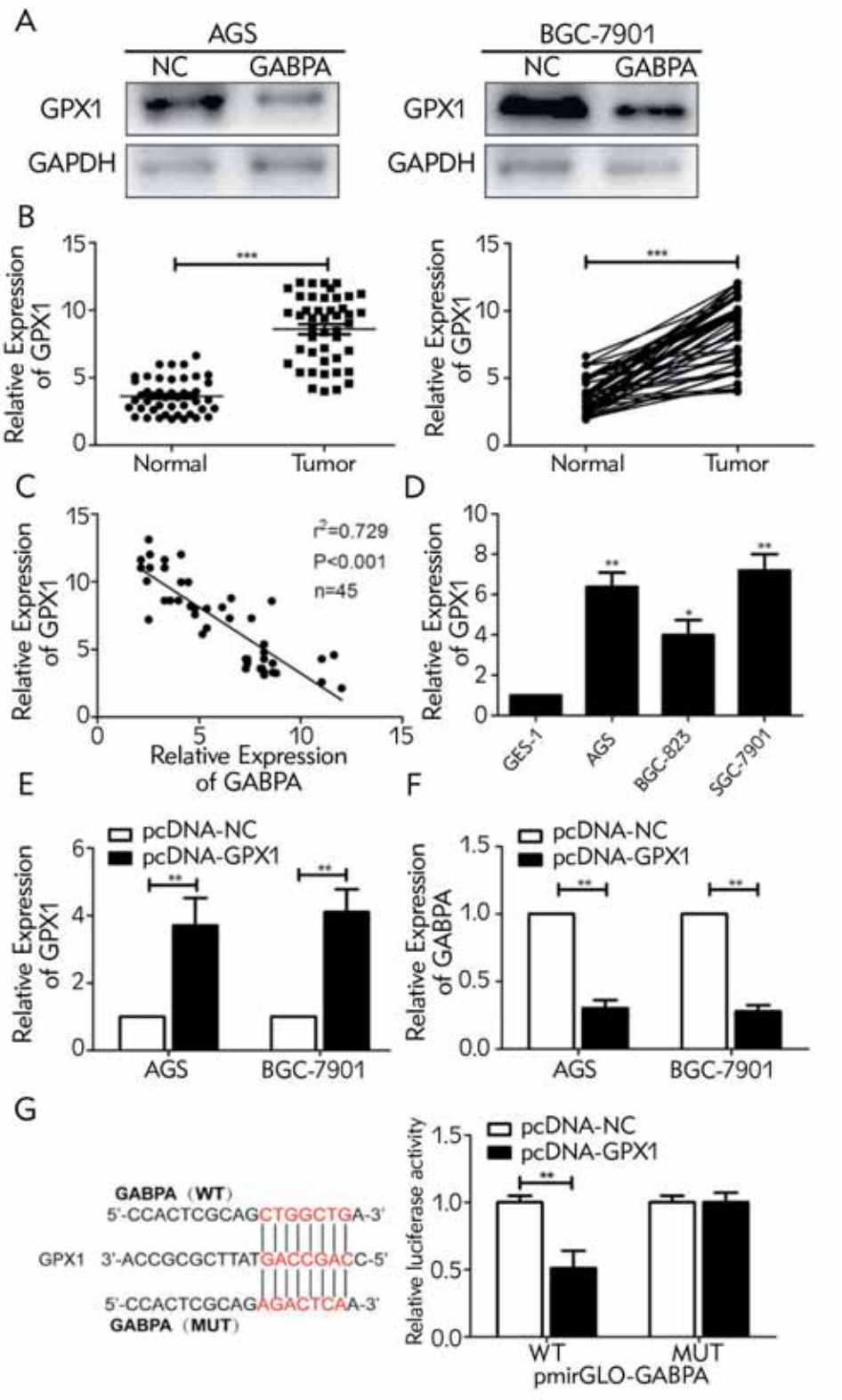

Figure 3 3GABPA was bound to GPX1. (A) Protein level of GPX1 in AGS and SGC-7901 cells overexpressing GABPA; (B) Differential levels of GPX1 in GC and adjacent normal tissues; (C) A negative correlation between mRNA levels of GPX1 and GABPA in GC tissues; (D) GPX1 levels in GC cell lines; (E) Transfection efficacy of pcDNA-GPX1; (F) GABPA level in AGS and SGC-7901 cells overexpressing GPX1; (G) Binding relationship between GABPA and GPX1. *P< 0.05, ***P< 0.001.

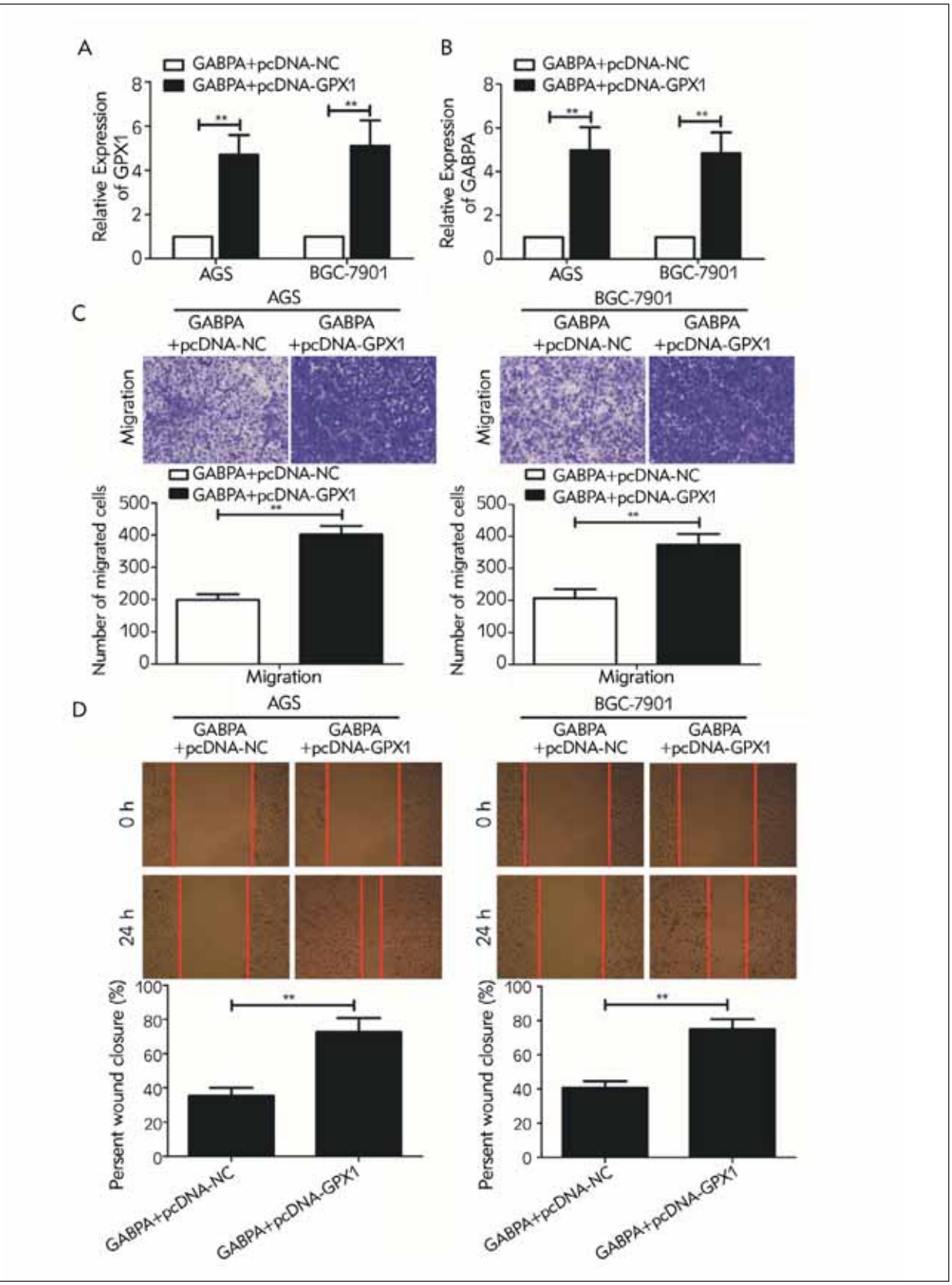

Figure 4 GABPA and GPX1 synergistically regulated GC migration. (A) GPX1 level in AGS and SGC-7901 cells co-overexpressing GABPA and GPX1; (B) GABPA level in AGS and SGC-7901 cells co-overexpressing GABPA and GPX1; (C) Migration in AGS and SGC-7901 cells co-overexpressing GABPA and GPX1 (magnification 20×); (D) Wound closure in AGS and SGC-7901 cells co-overexpressing GABPA and GPX1 (magnification 20×). *P< 0.05, **P< 0.01.

In addition, GABPA was lowly expressed in GC cells in comparison to the human gastric mucosal epithelial cells (*Figure 1B*). It is speculated that GABPA may be a tumor suppressor gene involved in GC. We subsequently analyzed clinicopathological indicators of GC patients based on their GABPA levels. It is demonstrated that GABPA was closely linked to the incidences of lymphatic and distant metastasis in GC patients (*Table I*). As expected, GC cases with lymphatic or distant metastasis expressed lower level of GABPA than non-metastatic ones (*Figure 1C*).

Knockdown of GABPA Blocked GC to Migrate

To explore the biological functions of GABPA in GC, pcDNA-GABPA was generated and transfection of pcDNA-GABPA effectively upregulated GABPA in AGS and SGC-7901 cells (*Figure 2A*). Later, overexpression of GABPA was identified to reduce migratory cell number and wound closure percentage in GC cells, suggesting the attenuated migratory potential (*Figure 2B*, *2C*).

GABPA Was Bound to GPX1

Using online bioinformatics tools, it is predicted that GABPA may bind to GPX1. Western blot analysis showed that protein level of GPX1 was markedly downregulated in AGS and SGC-7901 cells overexpressing GABPA (*Figure 3A*). GPX1 was detected to be highly expressed in GC tissues and cell lines (*Figure 3B*, *3D*). It was negatively correlated to mRNA level of GABPA in clinical samples of GC (*Figure 3C*). Subsequently, transfection efficacy of pcDNA-GPX1 was examined (*Figure 3E*). Overexpression of GPX1 downregulated GABPA in GC cells, further supporting their negative correlation (*Figure 3F*). Dual-luciferase reporter assay revealed a decline of luciferase activity in the wild-type GABPA vector after overexpression of GPX1. Nevertheless, GPX1 could not affect luciferase activity in the mutant-type one (*Figure 3G*). As a result, we have proven that GPX1 could be targeted by GABPA through the predicted binding site.

GABPA and GPX1 Synergistically Regulated GC Migration

To further uncover the synergistical regulation of GABPA and GPX1 on GC, we co-transfected pcDNA-GABPA and pcDNA-GPX1 in AGS and SGC-7901 cells. Compared with those overexpressing GABPA, both GPX1 and GABPA levels were much higher in AGS and SGC-7901 cells co-overexpressing GABPA and GPX1 (*Figure 4A*, *4B*). Co-overexpression of GABPA and GPX1 enhanced migratory potential in GC cells with solely overexpression of GABPA (*Figure 4C*, *4D*).

Discussion

Globally, GC is a severe cancer that has high incidence and mortality, and is also one of the digestive system in our country (1–3). Epidemiological investigations have proposed that the carcinogenesis of GC involves environmental, biological, genetic and epigenetic factors (4, 5). Microarray analysis is an effective technology to identify differentially expressed genes in the profile of cancer. These abnormally activated or inactivated genes are potential molecular biomarkers for screening or predicting the progression of human cancers (6–8).

Previous study reported that GABPA could inhibit the metastasis of papillary thyroid carcinoma through regulating DICER1 (18). In addition, another study revealed that it served as a novel biomarker for the prognosis of hepatocellular carcinoma since GABPA was able to block the migration of cancer cells by regulating E-cadherin (19). However, the function of GABPA in HCC is not clear. Our findings showed that GABPA was downregulated in GC tissues, compared to adjacent normal ones. Low level of GABPA predicted high incidences of lymphatic and distant metastasis in GC patients. Thus, it is speculated that GABPA could be a tumor suppressor involved in the progression of GC. Later, a series of functional experiments identified that overexpressing GABPA, the migratory potential was markedly inhibited in AGS and SGC-7901 cells.

We thereafter verified that GPX1 was the downstream target binding GABPA by miRDB starbase. GPXs (glutathione peroxides) are vital antioxidant enzymes in the body, which are responsible for eliminating ROS (20). GPX1 is the most common antioxidant enzyme in the GPX family. It is widely present in human cells, and has antioxidant and detoxifying effects (21, 22). Protein level of GPX1 was found to be downregulated by overexpression of GABPA in GC cell lines. In addition, we also found that GPX1 was upregulated and negatively correlated to GABPA level in GC tissues. Furthermore, the rescue experiments identified the migration ability of GPX1 to reverse the regulatory effect of GABPA on GC cell lines. Taken together, GABPA was a tumor suppressor that inhibited migratory potential in GC through negatively regulating GPX1, which could be utilized in the clinical targeted therapy of GC.

Conclusions

GABPA is downregulated in GC samples, which can be utilized to predict GC metastasis. Serving as a tumor suppressor, GABPA blocks GC cells to migrate by targeting GPX1.

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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Plenarne sekcije/Plenary Sessions
Apstrakti/Abstracts

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Plenarno predavanje
Opening lecture

LABORATORY MEDICINE IN THE ERA OF COVID-19: LESSONS FOR THE FUTURE

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The lockdown due to the coronavirus disease 2019 (COVID-19), a major healthcare challenge, is a worldwide threat to public health, social stability, and economic development. The pandemic has affected all aspects of society, dramatically changing our day-to-day lives and habits. It has also changed clinical practice, including practices of clinical laboratories. After two years, it is time to rethink what has happened, and is still happening, in order to learn lessons for the future of laboratory medicine and its professionals. The first issue is the increased visibility of the central role of clinical laboratories in modern healthcare. Before the pandemic, several documents, papers and initiatives emphasized the importance of laboratory testing in numerous clinical pathways, but the pandemic further raised awareness of the essential contribution made by clinical laboratories to diagnostic reasoning and the management of cases of suspected or confirmed SARS-CoV-2 infection. These include etiological diagnosis, patient monitoring, and epidemiological surveillance. Further evidence of the importance of laboratory medicine is being gained thanks to serological testing for SARS-CoV-2 antibodies in vaccine(s) clinical trials, in properly monitoring vaccinated subjects (eventually with different vaccines and different clinical histories), and in better understanding the effects of virus variants from both diagnostic and clinical viewpoints. The second lesson is that »speed must never compromise quality, and a marriage between accuracy, reliability and quickness should be assured«. A thrid lesson is that we have to »assure and monitor quality in all phases of the testing process and measure clinical and economical outcomes to provide evidence of the effectiveness of laboratory services. The IFCC Model of Quality Indicators (MQI) is a valuable tool for achieving this goal«. A fourth lesson is that laboratory professionals have to evaluate all well-developed and promising technologies, validate and deploy them according to established guidelines and recommendations focusing on patient needs. And we have to integrate different diagnostic approaches in a clear and reliable report so that the information is conducive to diagnostic accuracy, effective therapy and the best possible clinical outcome. However, the most important lesson is to move slinical laboratories out of the silo, avoid isolation and integrate laboratory testing in diagnostic and clinical pathways that effectively prevent disease, and provide early diagnosis, valuable monitoring, personalized therapy and epidemiological surveillance. The ultimate goal is, in fact, effectiveness, not just efficiency.

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Plenarno predavanje
Plenary Lecture

LABORATORY MANAGEMENT IN THE NEW NORMAL

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Laboratory leadership is a skill mostly learned by experience and intuition (often referred to as emotional inteligence). The art of leadership is akin to the art of living – we learn as we go along. Preanalytical, analytical and postanalytical processes need to be constantly monitored and optimized, with no visible disruptions in the service. It is often presumed that the most prominent sign of a smoothly operated laboratory are happily oblivious customers who never give a laboratory a second thought, which means that results are always delivered as requested, in an efficient and timely manner. This notion might be true in a fee for service oriented laboratory, but not neccessarily in the one which aspires to provide an added value. An "added value" laboratory is the one where not all requests are uniformely received as unquestionable orders to be fullfilled, where results are not only numbers but may and should contain meaningful and informative comments and where laboratory people bear names and faces known to their users.This presentation therefore deals with all the additional chalenges faced by hospital laboratory leaders during the two years of the Covid 19 pandemic. All the hospital laboratories needed to promptly adapt to the sudden unprecedented demands, not only related to SARS CoV-2 diagnostic, but also to all the other challenges connected to swift and often chaotic hospital workflow and case mix changes. Staff routines were adapted to accomodate the highest priority – no disruption of laboratory services due to within laboratory infection spread. Preanalytical workflow suddenly became crucial in terms of timely and safe specimen delivery while minimising human contact, both from emergency departements and intensive care units dealing with covid patients. Any process changes which were not carefully talked through have resulted in serious TAT delays, as will be discussed in detail with practical examples. New tests (SARS CoV2 PCR and antibodies, IL-6) needed to be quickly added to the existing STAT routine. But, above all, throughout this period laboratory technicians and other employees had to feel safe and cared for. As in any emergency situation, people's individual vulnerabilities became visible and needed immediate attention. All this had to be done by the laboratory leader, who simultaneously held an 24 hours' open hotline with hospital administration and their various questions and demands. Finally, the laboratory remains as usual the only voice of reason to be heard regarding excessive unnecessary testing conected with Covid and suspected post Covid sydromes.

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Plenarne sekcije
Plenary sessions

MEETING THE LEADERSHIP CHALLENGE OF DISRUPTIVE INNOVATION

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The 20th century digital revolution has seen the introduction of faster, innovative and easier to use technologies that have taken laboratory medicine services closer to patients in primary and community care. For the 21st century artificial intelligence driven algorithms are increasingly supporting evidence-based decision making that is reducing the need for expert human resource and opening opportunities for global, information technology providers to disrupt conventional ways of working at the point of care. A new leadership challenge emerges for specialists in laboratory medicine. Perhaps no longer laboratory-based specialists will extend their knowledge, skills and competence to a) guiding appropriate services for local environments based on clinical need, b) ensuring technology solutions are cost-effective, safe and reliable, c) developing the business acumen to market, negotiate and manage change d) getting a better understanding of imaging technologies, genomics, and health information science (data mining and health economics) that also drive the changing landscape. In providing examples of the new ways of working this talk will also emphasise the potential to exploit specialist leadership to ensure effective use of resource across the diagnostics and information technology industries, service commissioners, academia and policy related healthcare organisations.

ADDED VALUE BY INTERPRETATIVE COMMENTING

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Consultation by adding interpretative comments to reports has long been recognized in laboratory medicine as one of the activities that can support physicians and help to improve patient treatment outcomes. Interpretation of laboratory test results might in some cases considerably be supported when additional tests are performed on the available samples. This activity was named reflective testing-where the reflection is done by the laboratory specialist - and that can improve the diagnostic efficiencyconsiderably. Both the need, clinical value and appreciation by stakeholders of these forms of consultation have been proven by a diversity of studies. Both general practitioners and medical specialists have been shown to value interpretative commenting. Other forms of consultation are emerging: reporting of laboratory results to patients is becoming the rule. Most patients have little understanding of these results, and consultation of patients could add a new dimension to the service of the laboratory. These developments have been recognized by the European Federation of Clinical Chemistry and Laboratory Medicine, which has established the working group on Patient Focused Laboratory Medicine for work on the matter. Providing proper interpretative comments is, however, labor intensive. Harmonization is necessary to maintain quality between individual specialists. In present-day high-volume laboratories, there are few options on how to generate high-quality, patient-specific comments for all the relevant results without overwhelming the laboratory specialists. Automation and application of expert systems could be to only solution.

SMARTPHONE APPLICATIONS USING LABORATORY MEDICINE DATA – RELIABILITY AND BENCHMARKING

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Thanks to their accessibility, many of smartphone mobile applications (apps) are used for delivering health interventions to clinicians and patients. However, the burning issue is the quality of health related apps and how to evaluate it. The content of most of them is not officially regulated, unless they function as part of a medical device. Mobile App Rating Scale (MARS) is a multidimensional tool for classifying and rating the quality of mobile health apps. Its quality criteria consider engagement, functionality, aesthetics, and information quality of the app content. The project of EFLM Patient Focused Laboratory Medicine Working Group was to analyze the number and quality of smartphone apps available on the market using in any way laboratory medicine data. Seven categories were distinguished: 1) apps that offer medical advice about symptoms and health queries with the possibility to upload laboratory test results, which can be seen, stored and shared; 2) reference ranges of selected analysis with basic information about the causes of increase or decrease designed for patients; 3) quick reference for laboratory tests for medical students and doctors; 4) apps for monitoring the state of user's health through a wide range of health parameters, including glucose and/or cholesterol as laboratory data, 5) apps that provide access to patients' laboratory results to physicians; 6) apps that enable patients to access their laboratory test results directly from the diagnostic center; and 7) electronic health records apps that include laboratory test results. MARS score values revealed the poorest performance and quality of the apps intended for patients, with significant issues of security of personal information used by the apps, and the questionable affiliation of developers, without referencing the source of information cited. The working group also analyzed the users' (i.e. patients') opinion on selected apps, which pointed out the trustworthiness, the adequate style of presenting information the graphics, and the appearance of the app as the key issues of the app quality.

THE PROFESSIONAL DEVELOPMENT OF LABORATORY MEDICINE PROFESSIONALS IN/THROUGH EFLM COUNTRIES: WHAT TOOLS AND OPPORTUNITIES DO WE HAVE?

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EFLM Professional Committee, Chair

University Clinical Centre Maribor, Slovenia

The International Federation of Clinical Chemistry and Laboratory medicine (IFCC), and the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) are two essential professional organizations in a field of Clinical Chemistry and Laboratory Medicine (CCLM), that join and linking together Professional National societies (NSs) and their members through Europe and all over the world. In a scope of both organizations, professionals as NSs representatives are actively involved through different roles and issues in various professional working groups and committees. EFLM has several essential Committees: for Education and Training (C-ET), Communication (C-C), Quality and Regulation (C-QR), Science (C-S) and Professional Committee (C-P). C-P is responsible for several important issues, to represent the professional interests of specialists in laboratory medicine across Europe. One of the most important is the effort to achieve recognition of professional qualifications under European Union (EU) legislation based on the principles of free movement of professionals within Europe. According to the »EU Directive 2013/55/EU on recognition of professional Qualifications«, this effort could be achieved with the harmonization of our profession on EFLM level through Common Training Framework (CTF) and confirmed exit qualifications. This process/approach is started almost 20 years ago, with the established European Communities Confederation of Clinical Chemistry and Laboratory Medicine (EC4) who put the base for rules and minimal criteria for harmonization of our diverse education system through EU Countries. Since 2016 EC4 has been transferred to the EFLM C-P. Through these years, several (5) versions of the Syllabus for post-graduate training in CCLM was prepared, which present the education/training program, with important areas of knowledge and essential competencies for our profession. It represents the cornerstone for later established Equivalents of standards (EoS) for European Specialist in Laboratory Medicine. According to determined criteria, first EoS have been delivered to the Countries whose education program/system for specialization post-graduate training fulfil and include all important parts of these established rules for EoS: education/training duration, program (polyvalent), final exam/exit qualification.

Equivalence of Standards in Education and Training (EoS)

- Minimum 9 years (ideally 10): years academic (4 or 5 years) and specialist training (5 years);
- Education and training to standards set in the EFLM syllabus version 5;
- A Master's degree in Medicine, Pharmacy or Science;
- An EFLM Profession Committee recognised 'Equivalence of Standards' exit qualification;
- Evidence of participation in continuous professional development (CPD).

It requires curriculum content to include:

- General chemistry of at least 35%;
- General chemistry plus haematology of at least 65%;
- Flexibility as to the remaining 35%, including general chemistry, haematology, microbiology, and genetics and IVF in a proportion consistent with the requirements in the country of destination.

For the recognition and legislation of our profession, on the EU level through Directive, it is essential to obtain EoS of post-graduate education and training and confirmation of CTF on government level at least in 33% of EU Member States. Once the recognition and legislation of our profession would be accepted there is an EU requirement for all Member States to implement it. This is important especially for non-medical specialists since the specialists who are medical doctors have been already recognised on EU level. Now we have already 15 countries with achieved/confirmed EoS and the number still growing. To support the Profession Committee in its strategy to achieve recognition of Specialists in Laboratory Medicine, EFLM has established the EFLM Register of European Specialists in Laboratory Medicine (EuSpLM). It was first established in 1997 by EC4, the merger of EC4 with FESCC culminated in 2016 with the transfer of the Register to EFLM. Through the standards it sets and the code of conduct it expects from its registrants, the Register has identified a cohort of nearly 3000 individuals with unique knowledge, skills and competencies for leading/delivering high-quality laboratory medicine services. EFLM members in this register who already fulfil and have confirmed exit qualification according to the EoS criteria obtain the common title EuSpLM. However, the goal of our efforts and for our profession is to achieve EoS in all EFLM countries and raising the level of professional knowledge and skills in all field of Laboratory Medicine. To achieve this goal, EFLM through activities of their professional units have developed several tools and

opportunities. Education Committee through important efforts and work of their working groups (WG) offer several possibilities for additional education and training. WG: Congresses and Postgraduate Education has launched post-graduate courses, that NSs can apply for them, to offer their members additional knowledge in two fields: Biostatistics, How to write a good professional article. Another very important tool is EFLMLabX project of exchanging practical knowledge and skills through EFLM web-platform (https://eflmlabx.eflm.eu/en), where potential users can search and apply for practice/visiting/research in different laboratories/institutions through EFLM countries and get direct contacts. WG: Distance Education and e-Learning offer several different webinars on different topics and recordings of conferences/congresses. Lately, under Task Group: EFLM Syllabus Course it has been launched first webinars also on Syllabus topics, which could be an important additional tool for the trainees/specialists, for their professional development. Science Committee with several and different WGs provide evidence and recommendations for harmonization of knowledge and practice of our profession on different field of LM. In 2019 EFLM has, next to EuSpLM Register, established EFLM-Academy. Its memberships offer/bring a lot of benefits not only to already registered EuSpLM but also to all other (non-EuSpLM) members who want to be a part of »big EFLM family« and show the interest for Laboratory Medicine (from non- EFLM countries, other profession (medical doctors, nurses), engineers on field of LM..).

The important benefits of EFLM-Academy are:

- Free on-line subscription to CCLM, the official EFLM journal;
- Unlimited access to all documents (laboratory standards) of the CLSI (Clinical and Laboratory Standards Institute) database;
- Regular e-mail notifications of all EFLM activities, programmes and opportunities;
- Eligibility to apply for EFLM travel grants (subordinated to application's criteria of each specific EFLM initiative)
- Reduced registration fee to all EFLM conferences and courses
- Free access to EFLM webinars
- Enrollment in the EuSpLM Register for those who meet the Educational and Training EFLM Equivalence of Standards (subordinated to the evaluation of the requested documentation by the EFLM Profession Committee).
- With all the activities, efforts, opportunities and benefits that come and grow with the enthusiastic work of experts in EFLM WGs and committees and across EFLM countries, we help to create the tools for expanding our knowledge of Laboratory Medicine. By using these tools and opportunities, we also rising the strength of our profession for present and for the future.

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ACCREDITATION OF LABORATORIES

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An actual contribution of accreditation certificate to day to day laboratory routine is not easily measurable. Does it represent an objective, independent confirmation of the quality of laboratory practice and reliability of laboratory test reports, or is it a mere formality to be fulfilled and carefully followed? How does accreditation contribute towards minimizing error occurences and risks? In the past, the quality of laboratory practice was traditionally and exclusively demonstrated by means of external quality assessment (EQA) results. When considering eligibility of a laboratory for participation in clinical studies, EQA certificates are even today still the most often required and sufficient proof of quality.Increasingly, however, this approach does not allways suffice as EQA covers only some (mostly analytical) aspects of laboratory practice.Therefore, in 2003 the first version of the international standard for implementation of the quality management system in medical laboratories was adopted and published, i.e. ISO 15189: Medical laboratories – Requirements for quality and competence. A new and somewhat altered and updated version of this standard was published in 2012.This specific standard consists of a number of regulations and requirements to be met by an accredited laboratory, and is intended for all medical laboratories that perform biological, microbiological, immunological, chemical, immunohematological, hematological, biophysical, cytological, pathological and other examinations of human material. ISO 15189 standard was adopted as a national standard in the Republic of Croatia in 2006, and so far 13 medical diagnostic laboratories have been accredited (domains of work: medical biochemistry, microbiology and transfusion). Accreditation of laboratories in Croatia (and of one laboratory in Slovenia) has been carried out by the Croatian Accreditation Agency, an independent and non-profit public institution founded according to the Croatian Governmentdecree based on the Accreditation Act. Accreditation of Croatian laboratories is voluntary and for the time being it does not confer any particular privileges to accredited as compared to unaccredited laboratories in public healthcare system. The only advantage worth mentioning is a comparatively simpler acceptance of laboratories for participation in clinical studies, however laboratories with acceptable EQA results are included without problems as well. Accreditation of medical biochemistry laboratories according to ISO 15189 standard requires continuous monitoring, surveillance and improvements of all laboratory processes (preanalytical, analytical, postanalytical), active interpretation of laboratory test results, and establishment of full laboratory users' trust in the quality of reported results. It should be stated that the aim of an accredited laboratory is not only to issue accurate results based on physician's request, but also to participate in correct test selection and in interpretation of results, to respect patients' rights to privacy and to focus its attention on patient safety and on laboratory practice according to ethical principles. All these requirements are put in place in order to elevate the role of the laboratory from mere anonymus service towards diagnostic partnership. A question whether a fully accredited medical laboratory achieves this goal, remains to be answered.

IMPORTANCE OF LABORATORY GUIDELINES FOR MEDICAL LABORATORY PRACTICAL WORK

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Despite recent progress towards laboratory test standardization and harmonization, there are still huge problems that have to be addressed. Since in clinical guidelines only the laboratory parameters are defined, in different countries are useddiverse laboratory methods for the same diagnoses. In order to improve the quality of treatment and achieve standardization in treatment along with using of resources properly, there is a need for formulation of common laboratory guidelines for European countries and beyond. On this way, problems which can be found in developing laboratory guidelines on national or hospital level can be avoided and update of the new scientific advances would be faster. One of the first steps towards laboratory guidelines development is creation of guideline's frame that incorporate essential information related preanalytical, analytical and postanalytical process, clinical benefit and cost-effectiveness data. The most guidelines enclose data related to sample collection, biological variations, sample type, transport and storage of the sample. For most methods, analytical information (reference material, total error, bias, inaccuracies and interferences) is also known. From the postanalytical information, measurement units, reference interval, maximum allowed TAT is available. Future laboratory guidelines should focus on laboratory tests that may influence the decision-making process, treatment optimization, disease prediction and improvement of patient outcome while also be cost – effective. Laboratory results are critical to ensuring the treatment of most patients. A number of locally accepted laboratory guidelines remain too vague with respect to new scientific information and optimal analytical approaches. In order to develop a laboratory guideline, many obstacles need to be overcome. It should consider different patients' needs and reimbursement systemsin different countries. Also, laboratory guidelines should be synchronized with clinical guidelines. Guidelines should be translated into national languages and be accepted by the most European countries.

MANAGING THINGS AND LEADING PEOPLE

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What is a Leadership?

It is the process through which leaders influence the values, behavior and attitude of others. Leadership qualities can either be innate or also can be acquired. A Leader is someone who shows a direction, influences, motivates and inspires. A Leader is a person who can bring constructive change. Core values of a Leader are moral courage, integrity, decisiveness and assertiveness. Good Leader has to have: knowledge and skills, sense of priority, focus, vision, judgment, charisma, trust and emotional intelligence. A Leader motivates the team members in any situation, if they perform well or in case of failure. A good Leader applies the following approaches to lead: strategic approach, human assets approach, expertise approach, unbox approach, change approach. A Leader does the right thing at the right time, in right place. Manager does things right. A Manager is someone who plans, organizes and allocates resources, controls and solves problems. Manager administers while Leader innovates. Manager focuses on system processes, a Leader focuses on people; Manager relies on control, a Leader inspire trust; Manager has a short range view; a Leader has a long-range perspective. A Leader knows the way, goes the way and shows the way. All Managers are not Leaders but all Leaders can be Managers.

PRIMENA TEHNIKA KONTROLE KVALITETA RADA U REALNOM VREMENU KOJE KORISTE REZULTATE PACIJENATA U MEDICINSKIM LABORATORIJAMA

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Tehnike kontrole kvaliteta u realnom vremenu (Patient-Based Real Time Quality Control, PBRTQC) koje koriste rezultate pacijenata u medicinskim laboratorijama koriste izračunate parametre iz uzoraka pacijenata u realnom vremenu kao oblik kontrole kvaliteta (QC). Upotreba uzoraka pacijenata široko se koristi u hematologiji kao QC alat vec četrdeset godina. U medicinskim laboratorijama, mada se QC tehnike koje koriste rezultate pacijenata opisane pre više od pedeset godina, ovaj koncept se smatra zanimljivim, ali zbog praktičnih problema nije široko korišcen. Strategije QC koje koriste rezultate pacijenata, kao što su delta provere (delta check), prosečna vrednost normala (average of normals, AON), pokretni prosek (moving average, MA) i prosečna delta vrednost (average of delta, AOD) su sve češće zasupljeni u medicinskim laboratorijama zbog dostupnosti laboratorijskog informacionog sistema (LIS)/middleware. U poređenju sa »konvencionalnim« QC strategijama (interna kontrola kvaliteta, IQC), PBRTQC ima više prednosti i postoji sve veća zabrinutost da IQC nije dovoljna za brzo otkrivanje analitičke greške. Tradicionalni QC materijali nisu komutabilni, neki IQC materijali navode samo takozvane »ciljne« vrednosti koje su specifične za anlizatore umesto prave koncentracije analita. Danas su analizatori pouzdaniji i medicinske laboratorije određuju manji broj QC uzoraka što povecava broj rezultata iz uzoraka pacijenata koji se izveštavaju pre nego što se otkrije sistematska greška ili »odstupanje« (bias) se otkrije tek naknadnim neodgovarajućim rezultatom QC. AON i MA tehnike kontinuirano prate performanse određivanja u medicinskim laboratorijama. Srednja vrednost ili medijana za grupe rezultata pacijenata koje se prate u određenim vremenskim intervalima mogu da se koriste u statističkoj QC. AoN ili MA pristup je više zasnovan na riziku koji koristi karakteristike populacije pacijenta da otkrije pomeranje u izmerenoj prosečnoj vrednosti za populaciju. U medicinskim laboratorijama PBRTQC predstavlja QC nove generacije, ali njena implemntacija nije jednostavna kao što je to za »konvencionalnu« QC. Postoje određeni zahtevi za LIS/middlware-a koji su ključni za primenu PBRTQC. Potrebne su sledeće neophodne karakteristike LIS/middlware softvera za uspešnu

IMPLEMENTATION OF PATIENT-BASED REAL TIME QUALITY CONTROL TECHNIQUES IN MEDICAL LABORATORIES

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Patient-Based Real Time Quality Control (PBRTQC) techniques in medical laboratories use parameters calculated from patient samples in real time as a form of quality control (QC). The use of patients samples have been widely used in haematology as QC tool for over forty years. In medical laboratories, even patient-based QC techniques have been described for more than fifty years, the concept were seen as interesting, however, because of practical issues it has not been widely utilized. Patient based QC strategies such as the delta check, average of normal (AON), moving average (MA) and average of delta (AOD) are becoming more commonplace in medical laboratories because of availability of laboratory information system (LIS)/middleware programs. There are many advantages of PBRTQC in comparison with »conventional« QC strategies (internal quality control, IQC) and there is growing concerns that IQC alone is not sufficient to rapidly detect analytical error. Traditional QC materials may be non-commutable, some IQC materials state only so-called assay targets that are specific to anlyzers instead of the true analyte concentration. Today analyzers are more reliable and medical laboratories run fewer QC samples which increases the number of patients samples reported before systematic error or bias event is detected by a subsequent QC failure. The AON and MA techniques continuously monitor assay performance in medical laboratories. The mean or median for groups of patient results is tracked over sequential time intervals can be used in a statistical QC process. An AoN or MA approach is more of risk based approach using the patient population characteristics to detect a shift in the measured population mean. PBRTQC is next generation medical laboratory QC, but is not as simple to implement as conventional QC. There are some requirements of LIS/middleware that are crucial for adoption of PBRTQC. The essential features of LIS/middleware for successful implantation and operational application of PBRTQC are: data capture and storage, data extraction, analysis, visualization, exploration and transformation, statistical analysis and testing environment, live application and reporting. Also, a software has to have some additional features: advanced data visualization, for-

implantaciju i operativnu primenu PBRTQC-a: prikupljanje i čuvanje podataka, ekstrakcija podataka, analiza, vizualizacija, istraživanje i transformacija, statistička analiza i okruženje za testiranje, aplikacija u realnom vremenu i izveštavanje. Takođe, softver mora da poseduje neke dodatne karakteristike: naprednu vizualizaciju podataka, formalnu statističku analizu i mogucnost da se inkorporiraju podaci unutrašnje kontrole kvaliteta rada. QC programi koji koriste rezultate pacijenata trenutno se široko koriste i primena PBRTQC je u fazi brzog rasta. Međutim, softverska podrška je ograničena, a za sprovođenje programa PBRTQC potrebno je dosta vremena i statističkih veština. PBRTQC tehnike ne mogu u potpunosti da zamene tradicionalnu IQC. One su superiornije u odnosu na klasičnu QC za veliki broj određivanja, ali za određivanja u malim serijama, konvencionalna QC je pogodnija. Kombinacija tehnika QC omogućava najbolju zaštitu protiv pogrešnih rezultata koji se izveštavaju.

DA LI SE *MOVING AVERAGE* PROCEDURE MOGU KORISTITI KAO KONTINUIRANA KONTROLA KVALITETA RADA U MEDICINSKIM LABORATORIJAMA?

Vera Lukić

Odeljenje za laboratorijska ispitivanja,
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Tradicionalno se kontrola kvaliteta analitičkog rada u medicinskim laboratorijama sprovodi analiziranjem komercijalno dostupnih kontrolnih uzoraka u određenim vremenskim intervalima, kao i učešćem u spoljašnjim programima kontrole kvaliteta. Nedostaci tradicionalne kontrole su intermitentnost i nekomutabilnost. Zbog toga se javlja potreba za uvođenjem dodatnih kontrolnih mehanizama koji bi mogli prevazići ove nedostatke i obezbediti kontinuirani nadzor nad analitičkim procesom. U tu svrhu u savremenoj laboratorijskoj praksi se razmatra ideja kontrole kvaliteta zasnovane na rezultatima pacijenata. Jedan od mogućih načina korišćenja rezultata pacijenata u svrhu kontrole kvaliteta analitičkog rada jeste pokretni prosek (eng. moving average, MA). MA podrazumeva izračunavanje prosečne vrednosti iz dobijenog seta rezultata pacijenata i dalje korišćenje te vrednosti u kontrolne svrhe. Naziva se pokretnim, jer se vrši rekalkulacija MA vrednosti svaki put kada se primi novi rezultat, odnosno podaci se kontinuirano ažuriraju i evaluiraju, kako se uzorci pacijenata analiziraju. Najčešće korišćeni algoritmi za izračunavanje MA vrednosti su: prosti MA, eksponencijalno ponderisani MA i Bulov algoritam. Iako je konmal statistical analysis and possibility to incorporate internal quality data. Patient-based QC programs are currently widely used and PBRTQC is in rapid growth phase. However, there is only limited software support, and to implement a PBRTQC program requires considerable time and statistical skills. PBRTQC techniques cannot totally replace traditional IQC. For high volume assays they are superior to conventional QC, but for small batch type assays, conventional QC has a place. A combination of techniques QC will provide the best protection against erroneous results being reported.

CAN *MOVING AVERAGE* PROCEDURES BE USED AS A CONTINUOUS QUALITY CONTROL IN MEDICAL LABORATORIES?

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Traditionally, the analytical quality control in medical laboratories is conducted by analysing commercially available control materials in certain time intervals, as well as by participating in external quality control programs. The disadvantages of traditional control are intermittency and non-commutability. Therefore, there is a need to introduce additional control tools that can overcome these shortcomings and ensure continuous control of the analytical process. In this light, the idea of quality control based on patient results is being considered in modern laboratory practice. One of possible methods of using patient results for control purposes is the moving average (MA). MA involves calculating the average value from the obtained set of patient results and further using that value for the purpose of continuous quality control. It is called «moving« because the MA value is recalculated every time a new result is received, that is, the data is continuously updated and evaluated as patient samples are analysed. The most commonly used algorithms for calculating MA values are: simple MA, exponentially weighted MA and Bull's algorithm. Although the concept of MA has been known for decades, it has never been wide-

cept MA poznat već decenijama, on nikada nije ušao u širu primenu u medicinskim laboratorijama iz više razloga. Jedan je složenost definisanja optimalnih MA procedura koje su specifične za svaki test i svaku laboratoriju i stoga ne mogu biti generalizovane niti preuzete iz nekog drugog izvora, već zahtevaju pojedinačni izbor, optimizaciju i validaciju. Drugi razlog koji ograničava primenu MA procedura jeste nepoznavanje detalja sposobnosti odabrane MA procedure da otkriva klinički značajan bias, pri čemu se posebno nameće pitanje da li je ovim procedurama moguće otkriti pojavu bias-a za ređe zahtevane testove i u laboratorijama koje imaju mali dnevni broj uzoraka i urađenih testova. Poslednjih godina ponovo raste interesovanje istraživača za ovu temu, sa novim predlozima za načine na koje bi se MA procedure mogle optimizovati za rutinsku upotrebu.

ly adopted in medical laboratories for many reasons. One is the complexity of defining optimal MA procedures that are specific to each test and each laboratory and therefore cannot be generalized or downloaded from another source, but require individual selection, optimization, and validation. Another reason limiting the use of MA procedures is the lack of insight in the ability of selected MA procedures to detect clinically significant bias, especially their ability to detect the occurrence of bias for less frequently required tests and in laboratories with a small daily number of samples and tests performed. In recent years, there has been a renewed interest of researchers in this topic, with new suggestions for ways in which MA procedures could be optimized for routine laboratory use.

KORIŠĆENJE REZULTATA PACIJENATA ZA IZRAČUNAVANJE REFERENTNIH VREDNOSTI

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U cilju interpretacije rezultata u medicinsko biohemijskim laboratorijama, najčešće se kvantitativni rezultat posmatra u odnosu na referentni interval (RI), koji predstavlja fiksni procenat referentne populacije u intervalu koje opisuju donje i gornje referentne granice. Preporučeno određivanje RI ili direktno određivanje, podrazumeva predhodan odabir referentne populacije po tačno definisanim kriterijumima, uzorkovanje biološkog materijala i analiziranje uzoraka. Alternativni pristup određivanju RI ili indirektno određivanje podrazumeva korišćenje velikog broja postojećih, odrađenih rezultata iz uzoraka koji se sakupljaju u rutinske svrhe, iz kojih se biohemijski parametri određuju u svrhu skrininga, postavljanja dijagnoze ili praćenja, i koji se čuvaju u bazama laboratorijskih informacionih sistema. Veliki broj objavljenih radova ukazuje na prednost indirektno određenih RI uz uslov pravilne selekcije »nezdravih« osoba, kao i razlikovanja hospitalizovanih od ambulantnih pacijenata, ali i korišćenje složenih statističkih algoritama za dobijanje krajnjeg RI. Trenutne smernice i vodiči ne podržavaju indirektni metod kao primarni zbog činjenice da većina podataka možda ne potiče od zdravih osoba. Takođe, statistička analiza koja se koristi za obradu velikog broja podataka je primarno namenjena za direktno određivanje RI, tj. analizu preporučenih 120 rezultata referentne popu-

THE USE OF PATIENT RESULTS FOR CALCULATION OF REFERENCE INTERVALS

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In order to interpret the results in the medical biochemical laboratories, the quantitative result is most often viewed in relation to the reference interval (RI), which represents a fixed percentage of the reference population at the interval described by the lower and upper reference limits. Recommended RI determination, or direct determination, involves preselecting a reference population according to well-defined criteria, sampling biological material and analyzing samples. An alternative approach to RI determination, or indirect determination, involves the use of a large number of existing, extracted results from samples collected for routine purposes, from which biochemical parameters are determined for screening, diagnosis or monitoring purposes, and stored in the laboratory information system databases. The large number of published papers points to the advantage of indirectly determined RIs with the condition of proper selection of "unhealthy" persons, as well as separation of hospitalized from outpatients, as well as the use of complex statistical algorithms to obtain the ultimate RI. Current guidelines and documents do not support the indirect method as primary, due to the fact that most data may not come from healthy individuals. Also, the statistical analysis used to process large amounts of data is primarily intended to directly determine RI, i.e. analysis of the recom-

lacije. Međutim, postoje posebni staistički programi i tehnike kojima je omogućena pravilna statistička analiza velikog broja podataka za indirektno određivanje RI. Preporuka da svaka laboratorija definiše sopstvene RI, a da to bude ekonomično i minimalno kompleksno, daje prednost indirektnom određivanju RI, u smislu korišćenja postojećih baza podataka. Takođe, nijedan RI nije apsolutno tačan i predstavlja samo procenu. Buduća ispitivanja bi trebalo da regulišu etičke aspekte indirektnog određivanja, kao i pravilnu verifikaciju na ovaj način definisanih RI.

mended 120 results of the reference population. However, there are special statistical programs and techniques that allow proper statistical analysis of a large amount of data for indirect RI determination. The recommendation that each laboratory defines its own RIs, while being cost-effective and minimally complex, favors indirectly determining RIs, in terms of using existing databases. Also, no RI is absolutely correct and is only an estimate. Future trials should regulate the ethical aspects of indirect determination as well as the proper verification of RIs defined in this way.

NOVI BIOMARKERI AKUTNOG OŠTEĆENJA BUBREGA

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Akutno oštećenje bubrega (AOB) je često i po život opasno stanje. AOB se u detinjstvu najčešće javlja tokom prve godine života. Deca sa epizodom AOB imaju povecan rizik od razvoja hronične bolesti bubrega. AOB je reverzibilno ako se prepozna u ranoj fazi i ako se brzo preduzmu terapijske mere. Kreatinin u serumu kao tradicionalni biomarker koji se koristi za definisanje i ocenjivanje stepena AOB nije dovoljno senzitivan biomarker. Naime, potrebno je neko vreme nakon oštećenja bubrega odnosno smanjenja diureze, da bi se nivo kreatinina u serumu povisio, pa je kreatinin kasni marker AOB. Pored toga, kreatinin u serumu zavisi od uzrasta, pola, mišicne mase i upotrebe lekova. Potrebni su nam dakle bolji biomarkeri od kreatinina, koji bi trebali biti senzitivniji i specifičniji, omogucujuci bržu dijagnozu AOB i upotrebu odgovarajuce kliničke intervencije koja može zaustaviti ili preokrenuti AOB. Ograničenja serumskog kreatinina podstakla su istraživanja koja su razvila biomarkere AOB, uključujuci cistatin C, lipokalin povezan sa želatinazom neutrofila, molekul oštećenja bubrega 1, interleukin-18, protein koji veže masne kiseline u jetri, inhibitor tkiva metaloproteinaza-2 i protein 7-vezujuci protein. U poređenju sa kardiologijom, klinička upotreba novih biomarkera u nefrologiji je ograničena. Iako je intenzivna istraživačka aktivnost otkrila nekoliko novih biomarkera AOB, potrebna su dodatna istraživanja kako bi se odredila njihova klinička uloga. Činioci koji utiču na upotrebu biomarkera AOB uključuju cenu, dostupnost u lokalnoj laboratoriji ili na mestu lečenja i jednostavnu upotrebu. Primena inovativnog učenja i veštačke inteligencije može omoguciti brže otkrivanje

NEW BIOMARKERS OF ACUTE KIDNEY INJURY

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Acute kidney injury (AKI) is a common and life-threatening condition. AKI in childhood is most common during the first year of life. Infants with an episode of AKI have increased risk of developing chronic kidney disease. AKI is reversible when recognized early and promptly treated. Serum creatinine as traditional biomarker used to define and grade AKI is not sensitive enough. Furthermore, it takes some time after kidney injury or decrease in urine output until serum creatinine level rises, so creatinine is a late marker of AKI. In addition, serum creatinine depends on age, gender, muscular mass and medication. Better biomarkers than creatinine should be more sensitive and specific, allowing faster diagnosis of AKI, and use of appropriate clinical intervention that may stop or reverse AKI. Limitations of serum creatinine stimulated research that developed biomarkers of damage in AKI including cystatin C, neutrophil gelatinase–associated lipocalin, kidney injury molecule 1, interleukin-18, liver type fatty acid–binding protein, tissue inhibitor of metalloproteinase-2, and IGF-binding protein 7. In comparison to cardiology, clinical use of novel biomarkers in nephrology has been limited. Although intensive research activity identified several new AKI biomarkers further investigation is needed to define their clinical role. Factors influencing use of AKI biomarkers include price, availability at the local lab or point of care, and simple use. Application of innovative learning and artificial intelligence may allow faster detection and earlier treatment of AKI.

ZAKASNELI PUBERTET – IZAZOVI U DIJAGNOSTICI

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Zakasneli pubertet se viđa kod približno 5% adolescenata oba pola, a u diferencijalnoj dijagnozi, pored konstitucionalnog zakasnelog puberteta kao najčešćeg uzroka, na drugom mestu se nalazi hipogonadotropni hipogonadizam. Veliki broj urođenih i stečenih uzroka mogu dovesti do hipogonadotropnog hipogonadizma, koji takođe može nastati i usled intenzivnih sportskih treninga, poremećaja u ishrani ili u sklopu kliničke slike hroničnih sistemskih bolesti. Razlikovanje izolovanog hipogonadotropnog hipogonadizma od konstitucionalnog zakasnelog puberteta predstavlja veliki dijagnostički izazov u pubertetskom uzrastu i zasniva se na kliničkom nadzoru, izuzev kada postoje jasne udružene fenotipske odlike hipogonadotropnog hipogonadizma, poput anosmije ili hiposmije u sklopu Kallmannovog sindroma. Poslednjih godina sve značajniju ulogu u diferenciranju konstitucionalnog zakasnelog puberteta u odnosu na hipogonadotropni hipogonadizam ima određivanje koncentracija inhibina B, a sa sve dostupnijim genetskim analizama metode molekularne dijagnostike dobijaju sve veću ulogu ne samo u istraživačkom, već i u kliničkom kontekstu. U terapijskom pogledu, bez obzira da li se radi o izolovanom hipogonadotropnom hipogonadizmu ili konstitucionalnom zakasnelom pubertetu, kod dečaka koji imaju psihološke tegobe zbog kašnjenja ili zastoja u pubertetskom razvoju treba razmotriti kratkoročnu primenu depo preparata testosterona. Kod najvećeg broja dečaka sa hipogonadotropnim hipogonadizmom je moguća dalja indukcija pubertetskog razvoja i fertiliteta, uključujući razvoj i porast testisa uz uspostavljanje spermatogeneze u periodu adolescencije upotrebom humanog horionskog gonadotropina i rekombinantnog folikulo-stimulišućeg hormona.

DELAYED PUBERTY – DIAGNOSTIC CHALLENGES

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Delayed puberty can be observed in approximately 5% of the adolescent population, with majority of the affected youth having a benign variant of pubertal development - constitutional delay of puberty, with hypogonadotropic hypogonadism being the most frequent differential diagnosis. Multitude of congenital and acquired etiologies can lead to hypogonadotropic hypogonadism, including the development of »functional« hypogonadotropic hypogonadism due to secondary causes, such as vigorous exercise, eating disorders or systemic chronic illnesses. Distinguishing between constitutional delay of puberty and isolated hypogonadotropic hypogonadism remains a major clinical challenge during adolescence, and is mainly based on watchful waiting, unless specific features suggestive of hypogonadotropic hypogonadism are present, such as anosmia or hyposmia in Kallmann syndrome. During the recent years, inhibin B levels are proving more useful in distinguishing between constitutional delay of puberty and isolated hypogonadotropic hypogonadism, and with the genetic analyses becoming more available, molecular diagnostics are becoming increasingly important in both research and clinical practice. Regarding treatment approach, whether the aetiology is constitutional delay of puberty or hypogonadotropic hypogonadism, in boys with psychosocial complaints resulting from delayed or arrested pubertal development, short-term treatment with testosterone should be considered. In most of the boys with hypogonadotropic hypogonadism, complete pubertal development including the testicular growth and spermatogenesis during adolescence can be acquired by the use of human chorionic gonadotropin and follicle-stimulating hormone subcutaneous injections.

NOVI MARKERI SEPSE U NEONATOLOGIJI

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Morbiditet i mortalitet u neonatalnoj sepsi su značajni uprkos kontinuiranom napretku u neonatologiji i izboru novih generacija antibiotika. Preterminska novorođenčad veoma niske telesne mase su posebno osetljiva zbog imunske nezrelosti, teškog opšteg stanja i česte potrebe za primenom invazivnih procedura u toku lečenja. Kod ovih pacijenata je povećan rizik od razvoja sepse, antibiotske toksičnosti i lošeg ishoda lečenja. Hemokultura je »zlatni standard« u dijagnostici bakterijske sepse, ali na rezultate treba čekati 24–48 sati. Rezultati mogu biti lažno negativni u slučaju postojanja pneumonije ili meningitisa. Tradicionalni laboratorijski pokazatelji sepse pokazuju nedostatke, kao što je širok opseg referentnih vrednosti za hematološke testove, zbog čega su nekad teški za interpretaciju, ili spadaju u kasne markere sepse, kao što je C-reaktivni protein. Razvoj novih tehnologija je omogućio bolje upoznavanje neonatalnog imuniteta i odgovora na infekciju kao i otkrivanje novih biomarkera koji bi mogli da poboljšaju rano otkrivanje infekcije i pravovremeno započinjanje terapije. Pored biomarkera koji su već u upotrebi, kao što su C-reaktivni protein i prokalcitonin, u fazi ispitivanja ili u početnim fazama primene su presepsin, neki citokini, serumski amiloid A, lipopolisaharid-vezujući protein i površinski leukocitni antigeni. Za novorođenče bi bila veća šteta da infekcija nije dijagnostikovana i lečena, nego lažno dijagnostikovana i nepotrebno lečena. Zato je važnija osobina dijagnostičkog testa za neonatalnu infekciju visoka osetljivost i negativna prediktivna vrednost blizu 100%, nego visoka specifičnost. Mnogi autori smatraju da kombinacija serumskih markera infekcije pokazuje bolju dijagnostičku specifičnost i osetljivost nego pojedinačni markeri. Kad je u pitanju neonatalna populacija pri izboru biomarkera sepse se vodi računa o fiziološkim varijacijama njihovih koncentracija u prvim danima života, kao i o vrsti i zapremini uzorka koji se koristi za analizu.

NEW MARKERS FOR SEPSIS IN NEONATOLOGY

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Morbidity and mortality in neonatal sepsis are significant, regardless of the continuous advancement in neonatology and emerging new-generation antibiotics. Preterm infants of very low weight are particularly sensitive due to immune immaturity, serious general condition and frequent necessary application of invasive procedures in the course of treatment. The risks of development of sepsis, antibiotic toxicity and poor treatment outcomes are increased in these patients. Blood culture is considered the gold standard for diagnosis of bacterial sepsis, but the results are available after 24–48 hours. Additionally, they can be false-negative in the case of pneumonia or meningitis. Traditional laboratory indices of sepsis have certain shortcomings, such as wide reference intervals for haematological tests, which make them difficult to interpret, or belong to late sepsis markers, such as C-reactive protein. The development of new technologies has enabled better understanding of neonatal immunity and response to infection, as well as the discovery of new biomarkers which could improve early detection of infection and timely initiation of therapy. In addition to biomarkers already in use, such as C-reactive protein and procalcitonin, presepsin, some cytokines, serum amyloid A, lipopolysaccharide-binding protein and surface leukocyte antigens are in the phase of investigation or initial application phases. It would be more harmful for the newborn if the infection was not diagnosed and treated, than to have false diagnosis and unnecessary treatment. Therefore, a more important feature of the diagnostic test for neonatal infection is the high sensitivity and negative predictive value near 100%, than the high specificity. Many authors consider that the combination of serum markers of infection shows better diagnostic specificity and sensitivity than individual markers. When it comes to the selection of sepsis biomarkers in neonatal population, physiological variations in their levels in the first days of life and the types and volume of the sample for analysis have to be taken into account.

ULOGA LABORATORIJE U DIJAGNOSTICI I PRAĆENJU KOMORBIDITETA TIP 1 DIJABETES MELITUSA

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Deca i adolescenti, oboleli od tip 1 dijabetes melitusa (T1DM), imaju povećan rizik za razvoj jedne ili više pridruženih autoimunskih bolesti. Autoimunski tireoiditis i celijačna bolest imaju najveću prevalencu, a slede autoimunske bolesti vezivnih tkiva, gastrointestinalnog sistema, kože i primarna adrenalna insuficijencija. Laboratorijski skrining na funkciju tiroidne žlezde i celijačnu bolest je neophodan pri postavljanju dijagnoze T1DM i kasnije u redovnim intervalima, u cilju ranog otkrivanja i lečenja bolesti. Prema preporukama, skrining na autoimunski tireoiditis uključuje određivanje tireostimulirajućeg hormona i antitela na tiroidnu peroksidazu. Kod asimptomatskih pacijenata, skrining se ponavlja svake druge godine posle dijagnostikovanja dijabetesa, odnosno češće u prisustvu karakterističnih simptoma bolesti. Određivanje antitela na tkivnu transglutaminazu (anti-tTG IgA i/ili anti-tTG IgG) je primarno u dijagnostici celijačne bolesti kod asimptomatskih pacijenata sa T1DM. Laboratorijski skrining se ponavlja svake druge, odnosno svake pete godine, posle dijagnostikovanja dijabetesa. Frekventnost analiziranja zavisi od ispoljenih simptoma, uzrasta i genetske predispozicije pacijenta. Preporučuje se i skrining na deficit vitamina D, posebno kod dece sa pridruženom celijačnom bolešću ili promenama na koži. Autoimunske bolesti udružene sa T1DM predstavljaju dodatni rizik za mikro- i makrovaskularne komplikacije. Hronična inflamacija, koja je pratilac autoimunskih poremećaja i inflamacija u aterosklerozi imaju slične karakteristike, pri čemu patofiziološki činioci karakteristični za autoimunsku bolest mogu da ispolje nezavisno ili sinergističko dejstvo na razvoj ateroskleroze i povećanje kardiovaskularnog rizika. Laboratorijska evaluacija potencijalnog proaterogenog efekta autoimunskog tireoiditisa i celijačne bolesti, kao udruženih autoimunskih bolesti, mogla bi da identifikuje dijabetičare sa povećanim kardiovaskularnim rizikom u detinjstvu i adolescenciji. Određivanje markera inflamacije, indeksa ateroskleroze uz standardni lipidni profil i brzine izlučivanja albumina, ukazalo bi na eventualni disbalans u proaterogenim i antiaterogenim komponentama kod obolelih

THE ROLE OF LABORATORY IN DIAGNOSIS AND MONITORING OF CO-MORBIDITIES IN TYPE 1 DIABETES MELLITUS

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Children and adolescents with type 1 diabetes mellitus are at increased risk for developing one or more associated autoimmune diseases. Autoimmune thyroiditis and celiac disease have the highest prevalence, followed by autoimmune diseases of connective tissue, gastrointestinal system and skin, and primary adrenal insufficiency. Therefore, laboratory screening for thyroid function and celiac disease is necessary at the diagnosis of T1DM, and later, at regular intervals, in order to early detect and treat the disease. According to the recommendations, screening for autoimmune thyroiditis involves determination of thyroid stimulating hormone and antithyroid peroxidase antibodies. In asymptomatic patients, screening is repeated every second year after diabetes is being diagnosed, or more frequently in the presence of characteristic symptoms of the disease. Determination of tissue transglutaminase antibodies (tTG IgA and/or tTG IgG) is primary in the diagnosis of celiac disease in asymptomatic patients with T1DM. Laboratory screening is repeated every second or every fifth year after diagnosis of diabetes. The frequency of analysis depends on clinical symptoms, age and the genetic predisposition of the patient. Screening for vitamin D deficiency is also recommended, especially in children with coexisting celiac disease or skin disorders. Autoimmune diseases associated with T1DM pose an additional risk for microvascular and macrovascular complications. Chronic inflammation, that accompanies autoimmune disorders and inflammation in atherosclerosis have similar characteristics. Pathophysiological factors related to autoimmune disease, can have independent or synergistic effect on the development of atherosclerosis and increase cardiovascular risk. A laboratory evaluation of the potential proatherogenic effect of autoimmune thyroiditis and celiac disease, as associated autoimmune diseases, could identify diabetics at increased cardiovascular risk in childhood and adolescence. Determination of inflammatory markers, atherosclerosis indexes, standard lipids profile and albumin excretory rate could indicate possible imbalance between proatherogenic and antiatherogenic components in patients.

LABORATORIJSKA DIJAGNOSTIKA ALERGIJA KOD DECE

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Alergijska reakcija predstavlja neočekivan i neadekvatan odgovor imunološkog sistema na različite faktore (alergene) iz spoljašnje sredine. Najčešće se ispoljava kao reakcija rane preosetljivosti (tip I) i posredovana je alergen-specifičnim IgE antitelima. Alergijske bolesti su danas najčešća hronična oboljenja kod dece i odraslih, posebno u razvijenim zemljama. Procenjeno je da 20% svetske populacije boluje od neke vrste alergije. Za razliku od drugih hroničnih bolesti, alergijske bolesti počinju još u najranijem detinjstvu, a prema mišljenju nekih autora čak i prenatalno. Alergije na nutritivne alergene se sreću kod 2–8% dece i to najčešće u uzrastu odojčeta i malog deteta. Sa druge strane, rezultati velike Internacionalne studije astme i alergija kod dece (ISAAC) pokazali su da je najveća prevalenca simptoma astme kod dece predškolskog i školskog uzrasta. Laboratorijska dijagnostika alergija uključuje čitav niz testova koji se koriste da potvrde alergijsku reakciju, odrede tip/mehanizam reakcije (posredovana imunoglobulinima ili ćelijama), identifikuju pokretača/uzročnika senzibilizacije (alergen), i za praćenje uspešnosti terapije. Određivanje tipa/mehanizma alergijske reakcije uključuje merenje koncentracije ukupnog serumskog IgE (skrining za atopiju, kao i razlikovanje atopijskih-alergija od neatopijskih bolesti-intolerancija), broja eozinofilnih granulocita u cirkulaciji, perifernom razmazu krvi i razmazu brisa nosa (pomoć u proceni trenutne izloženosti alergenu i fenotipizaciji astme), te bazofilnih granulocita (dodatni parameter u proceni alergijske bolesti) i serumske koncentracije eozinofilnog katjonskog proteina – ECP (marker aktivacije eozinofila, pogodan za praćenje stepena inflamacije kod astmatičara i efikasnosti terapije). Uzročnik alergijske reakcije se određuje posredno merenjem serumske koncentracije alergen-specifičnog IgE. U tu svrhu se koriste različite imunohemijske metode: semikvantitativne (imunoblot metoda, paneli koji sadrže nutritivne/inhalatorne alergene) i kvantitativne metode (imunohemijske metode koje odlikuje velika dijagnostička specifičnost i osetljivost). Određivanje alergen-specifičnog IgE u serumu se koristi i kada se ne mogu primeniti kožni testovi (atopijski dermatitis) ili testovi provokacije (opasnost od anafilakse – nutritivni alergeni), kao i za praćenje efikasnosti terapije. Iako je laboratorijsko ispitivanje

LABORATORY DIAGNOSIS OF ALLERGIES IN CHILDREN

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An allergic reaction is an unexpected and inadequate response of the immune system to various environmental factors (allergens). It is most commonly manifested as a hypersensitivity reaction (type I) and is mediated by allergen-specific IgE antibodies. Today, allergies are the most common chronic diseases in children and adults, especially in developed countries. It is estimated that 20% of the world's population has some type of allergy. Unlike other chronic diseases, allergies begin in the earliest childhood, even prenatal according to some authors. Food allergy affects 2–8% of children, most often infants and children under three years of age. Results of a major International Study of Asthma and Allergies in Children (ISAAC) have shown that the highest prevalence of asthma is determined in preschool and school-age children. Laboratory diagnosis of allergies includes a battery of tests that should verify an allergic reaction, determine the type/mechanism of reaction (mediated by immunoglobulins or cellular mediators), identify triggers of allergic reaction and follow up therapy. Assessment of the type/mechanism of allergic reaction involves measuring of total serum IgE concentration (screening for atopy, as well as differentiation of atopic disease-allergy from non-atopic disease-intolerance), the eosinophilic granulocyte count in the blood and nasal swab (assessment of current allergen exposure and asthma phenotyping), basophilic granulocyte count (additional parameter in assessment of allergic disease) and the eosinophil cationic protein – ECP concentration (eosinophil activation parameter, for monitoring the level of inflammation in asthma and the therapy). The trigger of the allergic reaction is determined indirectly by measuring the allergen-specific IgE concentration. Different immunoassays are in use: semi-quantitative (immunoblot method with panels containing food/inhalative allergens) and quantitative methods (immunoassays characterized by high diagnostic specificity and sensitivity). Determination of allergen-specific IgE in serum is also used when skin tests or provocation tests cannot be administered, as well as to monitor the effectiveness of therapy. Although laboratory testing of allergies is necessary for the diagnosis of allergic diseases, there are limitations that affect the accuracy of the test results. Preanalytical problems

alergija neophodno za postavljanje dijagnoze alergijskih bolesti, postoje ograničenja koja utiču na tačnost rezultata primenjenih testova. Preanalitičke greške uključuju vreme uzorkovanja krvi za određivanje ukupnog IgE, specifičnog IgE, ECP i broja eozinofilnih granulocita (sezonske alergije, alergije na lekove i otrove insekata, uticaj brzine koagulacije i temperature na koncentraciju ECP). Analitički problemi odnose se na primenu standardizovanih imunohemijskih metoda za određivanje ukupnog i specifičnog IgE. Post-analitički problemi odnose se na korelaciju između rezultata in vitro testova sa rezultatima in vivo testova (kožni testovi, provokacijski testovi) kao i sa kliničkim podacima. Pomenuta korelacija je najvažnija za dijagnozu i kontrolu alergijske bolesti.

include time of blood collection for the measurement of total IgE, specific IgE, ECP and eosinophilic granulocyte count (seasonal allergies, insect sting allergies, drug allergies, influence of coagulation time as well as temperature on ECP levels). Analytical problems are related to the use of standardized immunoassays for the determination of total and specific IgE. Post-analytical problems are related to the correlation between in vitro test results with the in vivo test results (skin tests, provocation tests), as well as with clinical data. This correlation is most important for the diagnosis and control of allergic disease.

ZNAČAJ BIOHEMIJSKIH MARKERA U PROCENI RIZIKA ZA RAZVOJ KOMPLIKACIJA U TRUDNOĆI

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Razvoj komplikacija u trudnoći, kao što su preeklampsija, gestacijski dijabetes, prevremeni porođaj, intrauterini zastoj u razvoju ploda, ukazuje na sve veću potrebu za što jasnijim definisanjem potencijalnih biomarkera čijim bi određivanjem u ranom prvom trimestru trudnoće bilo moguće predvideti rizičan tok Time se omogućavaju pravovremena klinička ispitivanja i intervencije kod visoko rizičnih trudnoća, u cilju prevencije ili adekvatnijeg tretmana komplikacije. Danas se u cilju predikcije određenih komplikacija trudnoće uglavnom koriste kombinacije nekoliko biomarkera čime se postiže veća dijagnostička tačnost. Kada je preeklampsija u pitanju, koriste se kombinacije placentalnog faktora rasta (PLGF), tirozin solubilnog proteina (sFlt-1) i vaskularnog endotelnog faktora rasta (VEGF), zatim PAPP-a i plazma protein 13 (PP13) i drugi proteini placentalnog porekla kao što je inhibin A i aktivin A. Procena rizika za razvoj prevremenog porođaja i intrauterinig zastoja u rastu, pored navedenih testova koriste još i free beta human chorionic gonadotropin (free β-HCG) i metaloproteazu (ADAM12). Procena rizika za razvoj aneuploidija uključuje takođe screening ranog prvog trimestra kojim se određuje PAPP-a i free β-hCG. Navedeni biohemijski parametri kombinuju se sa ultrazvučnim parametrima kao i karakteristikama i faktorima rizika majke (godine starosti, težina, pušenje i dr.), što se obrađuje adekvatnim sofwaer-om za procenu rizika.U narednom periodu trebalo bi i dalje unapređivati

BIOCHEMICAL PARAMETERS IN PREGNANCY COMPLICATIONS RISK ASSESSMENT

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Pregnancy complications development as preeclampsia, gestational diabetes, preterm birth, foetal intrauterine growth restriction, indicates necessity of definition and identification of specific early pregnancy biomarkers for pregnancy complication prediction. Those biomarkers would be used in order to preventing or treating these complications more appropriately. Nowadays, combinations of several biomarkers are generally used to predict certain complications of pregnancy, thus achieving greater diagnostic accuracy. In preeclampsia, combinations of placental growth factor (PLGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and vascular endothelial growth factor (VEGF) are used, followed by PAPP-A and plasma protein 13 (PP13) and other proteins of placental origin, including inhibin A and activin A. Risk assessment for preterm birth and intrauterine growth restriction, beside mentioned tests, use free beta human chorionic gonadotropin (free β-HCG) and metalloprotease (ADAM12). An aneuploidy risk assessment also includes early first trimester screening for PAPP-A and free β-hCG. These biochemical parameters are combined with ultrasound parameters as well as risk factors parameters associated with mother (age, BMI, smoking, etc.), which are processed by an adequate software for risk assessment. The aim of further investigations will be addressed to development of some new combinations of biomarkers with associated software that would be integrate

kombinacije biomarkera uz prateće sofwear-e koji bi objedinili i kvalitetan screening za procenu rizika za razvoj navedenih komplikacija, kao i screening za aneuploidije. Rezultati naših studija su izdvojili aterogeni indeks palzme (AIP) i markere lipidne peroksidacije kao potencijalne markere predikcije komplikacija u trudnoći (preeklampsija, gestacijski dijabet, IUGR).

DIJAGNOSTIČKA TAČNOST TESTOVA ZA PROCENU OVARIJALNE REZERVE

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Ovarijalna rezerva podrazumeva veličinu, kao i kvalitet ovarijalnog pula žene određene životne dobi. Ona predstavlja funkcionalni kapacitet jajnika, odnosno njihovu biološku starost i mogućnost jajnika da proizvedu kvalitetne jajne ćelije. Radi se o veoma kompleksnom kliničkom parametru koji je pre svega uslovljen godinama starosti žene, ali i različitim genetskim, kao i fakorima okruženja. Idealni test za procenu ovarijalne rezerve trebao bi da bude visoko osetljiv, ponovljiv, bez varijacija u rezultatima između ciklusa, kao i visoko specifičan, čime bi se izbegli lažno pozitivni, kao i lažno negativni rezultati. Testovi su bazirani na specifičnim hormonskim analizama i ultrazvučnim pregledima. Broj antralnih folikula i koncentracija anti-Mullerian (AMH) hormona se prema većini autora smatraju testovima koji pokazuju veću specifičnost i osetljivost u odnosu na ostale biohemijske testove, bazalnu koncentraciju folikostimulirajućeg hormona (FSH), koncentraciju estradiola, kao i u odnosu na dinamički test sa klomifen citratom. Većina ovih testova se međusobno dopunjuje i kombinuje, međutim analize su pokazale da upotreba više različitih testova za ispitivanje ovarijalne rezerve ne dovodi do značajnog poboljšanja dijagnostičke tačnosti. Rezultati se razlikuju u zavisnosti od upotrebljenih graničnih (cut-off) vrednosti testova, kao i u zavisnosti od toga koji ishod testa se prati (najčešće je to odgovor na stimulaciju jajnika u procesu vantelesne oplodnje). Rezultati testova koji ukazuju na oslabljenu ovarijalnu rezervu mogu pružiti ženi informacije značajne za pravovremenu intervenciju i ranije planiranje trudnoće, ali ne ukazuju i na nemogućnost začeća.

TESTS OF OVARIAN RESERVE – DIAGNOSTIC ACCURACY

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Ovarian reserve is a term that is refers to imply the capacity of the ovary to produce quality egg cells. It is a functional capacity of the ovaries that is their biological age. Ovarian reserve is a very complex clinical parameter that is primarily associated with the woman's age, but also with different genetic and environmental factors. The respectable test for the ovarian reserve evaluation should be highly sensitive, repeatable, with no variations between menstrual cycles and highly specific, to avoid false positives, as well as false negative results. Ovarian reserve tests are based on specific hormonal analyses and ultrasound examinations. According to current expert's opinions, antral follicles count (AFC) and the concentration of anti-Mullerian (AMH) hormone provides more sensitive and specific results than other biochemical tests, basal follicle stimulating hormone (FSH) concentration, estradiol concentration and dynamic clomiphene citrate test. Most of these tests are complementary and combined. However, analyses have shown that the use of multiple different tests for ovarian reserve evaluation does not significantly improve diagnostic accuracy. The results vary depending on the cut-off test used and the tests outcome (usually the response to ovarian stimulation in the process of in vitro fertilization). Test results suggesting a declining ovarian reserve might be helpful in pregnancy planning. However, these results are not reliable

ULOGA POLNIH STEROIDA KOD ŽENA I MUŠKARACA POSLE 50. GODINE

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Menopauza predstavlja period u zivotu žene koji se javlja godinu dana posle poslednje menstruacije i traje do kraja života. Involutivni hopoandrogenizam u muškaraca odlikuje se padom testosterona i pojavom tipičnih simptoma i znakova. Insuficijencija estradiola u žena i testosterona u muškaraca dovodi do valunga, promena raspolozenja, depresije, nervoze, loše koncentracije, nesanice, kardiovaskulnih bolesti, osteoporoze, metaboličkog sindroma, dijabetesa i značajno smanjuje kvaliteta života i dovodi do većeg mortaliteta. Najčešći uzrok smrti svih ljudi su kardiovaskulne bolesti. Upravo pad gonadnih steroida dovodi do dislipidemije, vazokontrikcije, promene u simpatktikusnom sistemu, hipertenzije, aritmija, uslovljavajući infarkt miokarda ili srčanu insuficijenciju. Najveći denzitet receptora za testosterone je u miokardu. Dve trećine težine mozga čine krvni sudovi, a na njima su prisutni receptori za polne steroide. Takodje, receptori su prisutni i u kardiomiocitima. Sa padom polnih steroida pojačava se inflamacija preko interleukina , citokina i brojnih faktora inflamacije. Adaptivni mehanizmi slabe, a stresori dovode do sloma adaptacije i brojnih bolesti koje predstavljaju stresogeno stanje. Osteoporoza je izazvana nedovoljnošću gonadnih steroida. Povećava se broj fraktura. Seksualnost se značajno menja odlikujući se smanjenom seksualnom željom kod žena i impotencijom kod muškaraca. U cilju prevencije bolesti neophodno je oko 50. godine uraditi sistematski pregled koji obuhvata odredjivanje hormonskog statusa, internistički nalaz, nalaz ginekologa/urologa, osteodenzitometriju, ultrasonografske preglede. Posle obavljene dijagnostike i isključivanja apsolutnih kontraindikacija neophodno je uvesti supstitcionu terapiju svih insuficijentnih hormona. Na taj način se sprečavaju brojne bolesti, invaliditeti, smanjuju morbiditet, mortalitet i poboljšava kvalitet života.

THE ROLE OF SEXUAL STEROIDS IN WOMEN AND MEN OLDER THAN FIFTY YEARS OF AGE

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Menopause represents a period in woman's life starting one year after the last menstruation and continuing all life. Involutive hypoandrogenism in male is characterized by insufficient testosterone and typical symptoms and signs. Estradiol insufficiency in women and testosterone insufficiency in male are characterized by hot flushes, changes in psychic status, depression, irritability, lack of concentration, insomnia, cardiovascular diseases, osteoporosis, metabolic syndrome, diabetes, significantly decreasing quality of life and increasing mortality rate. Cardiovascular diseases are the most frequent cause of all deaths. Gonadal steroid insufficiency induce dislipidaemia, vasocontriction, changes in symphaticus system, hypertension, arrhythmias, leading to myocardial infarction and heart insufficiency. In the myocard the greatest density of gonadal steroid receptors are found. Two thirds of brain weight are blood vessels and gonadal steroid receptors are present on them. As well, the same receptors are present on cardiomyocytes. Gonadal steroid insufficiency increase inflammation by inducing cytokines and other inflammatory factors. Adaptive mechanism are becoming more fragile, stressores brake adaptation and induce diseases, as a stress status. Low levels of gonadal steroid induce osteoporosis. Number of fractures are increasing. Sexuality changes are characterized by typical hypoactive sexual desire in women and erectile dysfunction in male. In order to do a prevention complete examination are needed about the age of 50 years of age. Hormonal analysis, internal examination, gynecological/urological examination, osteodensitometry, ultrasonography, mammography are needed. After excluding absolute contraindications hormone replacement therapy of all insufficient hormones are needed. In such an approach many diseases can be prevented, morbidity and mortality rate reduced and better quality of life obtained.

METABOLIZAM GVOŽĐA I ISHOD TRUDNOĆE

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Poremećaj homeostaze gvožđa se često javlja u trudnoći, kada su potrebe za gvožđem povećane. Nivo peptidnog hormona hepcidina, koji je ključni regulator metabolizma gvožđa, opada tokom trudnoće, čime se omogućavaju optimalna apsorpcija i bioraspoloživost gvožđa. Nedostatak gvožđa je najčešći tip nutritivne deficijencije i uglavnom se manifestuje anemijom. Anemija usled deficijencije gvožđa je česta kod žena u reproduktivnom periodu: između 30 i 70% žena nema dovoljno gvožđa u depoima pre začeća, dok je oko polovine trudnica anemično. Fetus dobija gvožđe isključivo iz krvi majke, te svaki poremećaj homeostaze gvožđa kod majke ima posledice i po fetus. Nedostatak gvožđa povezan je sa povećanim rizikom za prevremeni porođaj, poremećajima u rastu i razviću fetusa, povećanim rizikom ka nastanku infekcija, anemijom, ali i komplikacijama u odraslom dobu. Primena suplemenata gvožđa je uobičajena u praksi, ali je veoma važno utvrditi da li je zaista potrebna, proceniti odnos koristi i eventualnih rizika, odnosno pravilno utvrditi dozu koja se primenjuje. Iako je neophodno za pravilno funkcionisanje organizma, višak gvožđa može biti štetan. Povećan sadržaj gvožđa doveden je u vezu sa poremećajima u metabolizmu glukoze i mogao bi imati veze sa razvojem gestacijskog dijabetes melitusa. Povišene vrednosti gvožđa u serumu majke imaju veze sa preeklampsijom (PE), stanjem koje je specifično za trudnoću, a ima za posledicu visok morbiditet i mortalitet i majke i novorođenčeta. Slobodni radikali, nastali usled viška gvožđa, mogu indukovati oksidativni stres, strukturno i funkcionalno oštećenje endotela, što može doprineti razvoju PE. U odnosu na zdrave trudnice, povećane vrednosti hepcidina su izmerene tokom infekcije, kao i kod gojaznih trudnica, što može imati negativan uticaj na dostupnost gvožđa. Deo istraživanja Laboratorije za biologiju reprodukcije usmeren je na ispitivanje uloge metabolizma gvožđa na ishod trudnoće, kao i na eventualni značaj hepcidina kao biomarkera u komplikacijama vezanim za trudnoću.

IRON METABOLISM AND PREGNANCY OUTCOME

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Dysregulation of iron homeostasis is commonly seen during pregnancy, when iron needs are increased. In non-complicated pregnancy, levels of peptide hormone hepcidin, a key regulator of iron homeostasis, decline through the pregnancy course, in order to ensure optimal iron absorption and bioavailability. Iron deficiency, the most common nutritional deficiency, is often manifested by anaemia. Iron deficiency anaemia is frequent in women of reproductive age: 30-70% of women have insufficient iron reserves before conception and around 50% of pregnant women are anaemic. Foetus is completely dependent on mother's serum iron, so any disturbance of maternal iron homeostasis, reflects on the developing foetus. Iron deficiency is related to increased risk for preterm birth, inadequate foetal growth and development, anaemia, increased risk for infection, but also to complications in adult life. Iron supplementation is common in routine practice, but it is important to assess is it really necessary, to estimate potential benefits and possible risks and to optimise dosage. While iron is essential for optimal organism functioning, its excess might be deleterious. Higher body iron content is linked to impairment of glucose metabolism and might be involved in development of gestational diabetes mellitus. Elevated iron levels were reported in preeclampsia (PE), pregnancy specific condition, related to high mortality and morbidity of both mother and the newborn. The free radicals, released in the presence of iron excess, might lead to oxidative stress and endothelial damage and dysfunction, contributing to the PE development. Higher hepcidin concentrations in maternal serum, compared to healthy controls, are seen in infection and obesity and might adversely affect the iron bioavailability. One of the topics addressed by the Laboratory for Biology of Reproduction is impact of iron metabolism on pregnancy outcome, and the potential role of maternal hepcidin as biomarker of pregnancy-related complications.

FARMAKOGENETIKA U ONKOLOGIJI – MOĆNO ORUĐE PRECIZNE MEDICINE

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Moderna farmakogenetika je u potpunosti transformisala lečenje onkoloških pacijenata omogućavajući preciznu upotrebu molekularno-ciljanih terapija u odabranim pacijentima i u specifičnom trenutku evolucije njihovih tumora. Centralizovani farmakogenomski Servis je oformljen naInstitutu za onkologiju i radiologiju Srbije sa ciljem da se omogući personalizovani molekularni pristup svakom srpskom onkološkom pacijentu. Testiranje pojedinačnih gena je započeto 2008. godine i prošireno na NGS panele i testiranje tečnih biopsija 2016. godine. Trenutno, u Servisu se uspešno obavljaju analize KRAS/NRAS mutacija u metastatskom karcinomu kolorektuma (osetljivost na anti-EGFR monoklonska antitela), primarnih i stečenih EGFR mutacija u uznapredovalom adenokarcinomu pluća iz FFPE i tečnih biopsija (osetljivost na prvu i treću generaciju tirozin kinaznih inhibitora), BRAF mutacija u metastatskom melanomu (osetljivost na tirozin kinazne inhibitore) i BRCA1/2 mutacija u karcinomu ovarijuma (osetljivost na PARP inhibitore). Budući planovi uključuju uvođenje prediktivnog NGS testiranja za imunoterapiju određivanjem nivoa mutacionog opterećenja, mikrosatelitske nestabilnosti i deficijencije mismatch mehanizma popravke DNK, kao idetekciju klinički značajnih onkogenih genetičkih rearanžmana kao što su ALK, NTRK, RET, ROS1 fuzije i sl. Prateća farmakogenetička dijagnostika je najvišepomogla pacijentima čiji tumori imaju onkogene vodič-promene kada se uzmu uobzir preživljavanje i kvalitet života. Dalji razvoj eksperimentalnih tehnika i bioinformatičkih analiza doprineće boljoj nezi onkoloških pacijenata i smanjenju troškova lečenja.

CANCER PHARMACOGENETICS – A MIGHTY PRECISION MEDICINE TOOL

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Modern pharmacogenetics has completely transformed oncological patient care enabling the precise use of molecularly targeted therapies in selected patients and in a specific evolution point of their tumors. A centralized pharmacogenetics Service was formed at the Institute for Oncology and Radiology of Serbia (IORS) with the purpose of providing a personalized molecular approach to each Serbian cancer patient. Single-gene testing was initiated in 2008 and expanded to NGS panels and liquid biopsy testing in 2016. Currently, metastatic colorectal cancer samples are successfully tested for the presence of KRAS/NRAS mutations (sensitivity to anti-EGFR monoclonal antibodies), as well as advanced lung adenocarcinoma patients for the presence of primary and acquired EGFR mutations from FFPE biopsies and/or liquid biopsy (sensitivity to first and third generation tyrosine kinase inhibitors), metastatic melanoma patients for the presence of BRAF mutations (sensitivity to tyrosine kinase inhibitors), and ovarian cancer patients for the presence of somatic BRCA1/2 mutations (sensitivity to PARP inhibitors). Future plans include the introduction of immunotherapy predictive NGS tests for the level of tumor mutational burden, microsatellite instability and mismatch repair deficiency, and also for clinically actionable oncogenic gene rearrangements as ALK, NTRK, RET, ROS1 fusions etc. Patients with oncogenically driven cancers benefit strongly from this companion diagnostic approach when both survival and quality of life are taken into account. Further development of experimental techniques and bioinformatics data analyses will improve overall cancer patient care management and lower treatment costs.

BIOMARKERI PROŠIRENOG LIPIDNOG STATUSA U KOLOREKTALNOM KARCINOMU

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Kolorektalni karcinom se svrstava među maligne bolesti sa najvećom učestalošću u savremenom svetu, te su brojna biomedicinska istraživanja posvećena otkrivanju i evaluaciji prediktivnih i dijagnostičkih biomarkera za ovo oboljenje. S obzirom da bolest ima kompleksnu etiopatogenezu, koja uključuje širok spektar metaboličkih poremećaja, parametri lipidnog statusa bi mogli imati značajnu ulogu u dijagnostici i predikciji nastanka kolorektalnog karcinoma. Rutinsko određivanje serumskih lipidnih parametara kod ovih pacijenata najčešće pokazuje tipičan profil koji se karakteriše sniženim koncentracijama ukupnog holesterola, triglicerida, te holesterola sadržanog u česticama lipoproteina niske (LDL) i visoke gustine (HDL). Ovakav nalaz se objašnjava stanjem kaheksije i anoreksije, ali i povećanim preuzimanjem holesterola iz cirkulacije u maligno izmenjene ćelije. Osim toga, opsežna ispitivanja markera proširenog lipidnog statusa u kolorektalnom karcinomu ukazala su na prisustvo specifičnih promena metabolizma holesterola i lipoproteinskih čestica. U našim istraživanjima u ovoj oblasti izdvojio se niz parametara sa potencijalno značajnim prediktivnim ili dijagnostičkim kapacitetom u koje se ubrajaju: markeri sinteze i apsorpcije holesterola, markeri metabolizma HDL čestica i pojedini metaboliti vitamina D. Ipak, pojedinačni lipidni parametri u pravilu ne zadovoljavaju sve kriterijume koji se podrazumevaju za pouzdane i efikasne biomarkere. U tom smislu, predložen je »multimarkerski pristup«, odnosno formiranje adekvatnih kombinacija individualnih biomarkera, čijim bi se određivanjem unapredila postojeća dijagnostika i predviđanje nastanka bolesti. Ovakav pristup u analizi parametara proširenog lipidnog statusa otvara brojne mogućnosti za definisanje panela lako dostupnih analita, čijom bi se primenom mogla poboljšati kako dijagnostika, tako i skrining. Osim toga, integrativni »multimarkerski pristup« u istraživanjima ukazuje na kritične tačke lipidnog metabolizma značajne za nastanak kolorektalnog karcinoma, što unapređuje razumevanje samog patofiziološkog procesa i omogućava bolju prevenciju nastanka ove bolesti.

ADVANCED LIPID STATUS BIOMARKERS IN COLORECTAL CANCER

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Colorectal cancer is among the most prevalent malignant diseases worldwide. Therefore, numerous biomedical researches are focused to identification and evaluation of predictive and diagnostic markers of this disease. Given the fact that colorectal cancer has complex aetiology, which includes a wide spectrum of metabolic disturbances, lipid status parameters might have a role in its prediction and diagnosis. Routine determination of serum lipid parameters in these patients usually reveals a typical profile, characterized by decreased levels of total cholesterol, triglycerides, low density lipoprotein (LDL) – cholesterol and high density lipoprotein (HDL) – cholesterol. Such findings could be explained by the cachexia – anorexia syndrome, which is frequently seen in these subjects. However, decreased cholesterol concentration might as well develop as a consequence of its increased uptake by malignant cells. Moreover, detailed investigations of advanced lipid status parameters in colorectal cancer pointed towards characteristic changes in cholesterol and lipoprotein metabolism. Our research in this area revealed a range of parameters with possibly significant predictive and diagnostic capacity, including markers of cholesterol synthesis and absorption, markers of HDL particles metabolism and several vitamin D metabolites. Yet, single lipid parameters usually do not meet the criteria for reliable and efficient biomarkers of colorectal cancer. Therefore, a novel multimarker approach is proposed, which comprises clustering and simultaneous determination of several individual biomarkers. It is considered that such approach might significantly improve diagnostics and prediction of various diseases. Namely, determination of selected lipid status parameters, within carefully designed diagnostic panels, could enhance both diagnosis and screening of colorectal cancer. In addition, integrative multimarker approach in biomedical investigations could shed light on critical points of lipid metabolism during cancerogenesis, thereby enhancing the understanding of its pathophysiological basis and consequently, improving the prevention of this disease.

GALEKTINI KAO BIOMARKERI: POTENCIJAL I PERSPEKTIVE

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Galektini pripadaju široko rasprostranjenoj familiji proteina koju karakteriše prisustvo očuvanog domena (engl. carbohydrate recognition domain – CRD) odgovornog za vezivanje glikana koji sadrže β-galaktozidne strukture. U ćeliji mogu biti prisutni u jedru, citoplazmi, na površini ćelije kao i u vanćelijskom matriksu. Galektini mogu delovati unutar ćelije kroz interakcije sa drugim proteinima (protein-protein) i izvan ćelije, pokazujući lektinsku aktivnost. Na taj način učestvuju u regulaciji i modulaciji ćelijskih događaja i bioloških procesa. U skladu sa učešćem u raznovrsnim biološkim funkcijama, promenjena ekspresija i/ili funkcija se često povezuje sa različitim patološkim stanjima. Veliki broj studija identifikovao je članove ove familije proteina kao relevanne biomarkere u karcinomima, bolestima srca, bubrega, jetre i infekcijama. Promene u ekspresiji galektina mogu pomoći u dijagnozi različitih bolesti, mogu se povezati sa ishodom lečenja i terapijskim pristupima.Veliki broj studija je ispitivao promene galektina-1 i galektina-3 u tkivnoj ekspresiji ili u cirkulaciji kod različitih bolesti. Većina istraživanja je izvedena na uzorcima pacijenata obolelih od karcinoma i često je ukazivala na postojanje veze između uočenih promena u cirkulaciji i tkivnoj ekspresiji galektina. Takođe, akumulirani podaci ukazuju na sve veći značaj otkrivanja i određivanja galektina kod koronarnih bolesti, reproduktivnih poremećaja, bubrežne insuficijencije i drugih oboljenja. Uprkos nekim odstupanjima, postoji dovoljno dokaza koji pokazuju značaj galektina kao biomarkera. Međutim, ispitivanje galektina kod raznih bolesti je ukazalo na potrebu razvijanja adekvatnih metoda koje bi osigurale poboljšanu osetljivost i tačnost kod detekcije galektina, konsenzus između laboratorija i uspostavljanje referentnog opsega. Dalji napredak na ovom polju zahteva integrativni i sistematski pristup, kojim bi se dodatno potkrepio klinički značaj određivanja galektina.

GALECTINS AS BIOMARKERS: POTENTIAL AND PERSPECTIVES

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Galectins are members of a widely distributed protein family defined by specificity for β-galactoside containing glycans and presence of conserved carbohydrate recognition domain (CRD). They can be found in the nucleus, cytoplasm, at the cell surface as well as in the extracellular matrix. Galectins can act inside the cell mainly through protein-protein interactions and outside the cell displaying lectin activity, thereby regulating and modulating cellular events in biological processes. In line with this multifunctionality, altered expression and/or function of galectins has often been associated with various pathologies. An increasing number of studies have identified members of this protein family as relevant biomarkers in cancer, heart, renal and liver disease, as well as in infections. To date, detected changes in galectin expression may aid in diagnosis of various diseases, can be linked to patient outcome, and galectin-based therapeutic approaches. Increasing number of studies are mainly focused on galectin-1 and galectin-3 in different diseases, whether expressed in tissue or present in circulation. The majority were performed on cancer patients, often showing correlation between changes in circulating galectin and altered tissue expression. Accumulated data also indicates increasing importance of galectin detection and measurement in coronary diseases, reproductive disorders, renal failure and others. Despite some discrepancies, there is ample evidence showing significance of galectins as biomarkers. However, screening for galectin family members in various diseases has pointed out a need for additional studies in order to develop adequate galectin detection methods insuring improved sensitivity and accuracy, enabling consensus between laboratories and establishment of normal reference range. Further progress in the field requires integrative and systematic approach in support of galectin determination clinical utility.

IZAZOVI U LABORATORIJSKOJ DIJAGNOSTICI BOLESTI TIROIDNE ŽLEZDE

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Laboratorijski testovi imaju ključnu ulogu u postavljanju dijagnoze i lečenju bolesti tiroidne žlezde. Imunohemijske metode su danas metoda izbora za određivanje koncentracije hormona u krvi, zahvaljujući potpunoj automatizaciji, kratkom vremenu obrade i visokoj specifičnosti i osetljivosti prema velikom panelu heterogenih molekula. Pri svakodnevnom korišćenju ovih, naizgled jednostavnih, testova javljaju se brojne interferencije koje ometaju dobijanje tačnih rezultata, te zahtevaju veliko poznavanje interferencija od strane biohemičara, kako bi se broj netačnih rezultata sveo na minimum. Tačni rezultati testiranja su neophodni za uspešnu dijagnostiku i uspešno lečenje pacijenata. Pored obezbeđivanja tačnih rezultata postoje i drugi ključni izazovi koji se javljaju u laboratorijskoj endokrinologiji, a svakako danas su najveći izazovi standardizacija i harmonizacija imunohemijskih testova koje, bez obzira na ogromne napore koji se ulažu, nisu još uvek potpune. Za uspešnu dijagnostiku i uspešno lečenje pacijenata neophodno je izrada referentnih vrednosti za hormone štitne žlezde u sopstvenoj populaciji, koja se uglavnom ne sprovodi u našem okruženju, nego se koriste referentne vrednosti po preporuci proizvođača reagenasa. Takođe, potrebno je analizirati i nivoe TSH koje se koriste kao granice kliničkih odluka za hipotireozu, a koje su ovisne između ostalog, o metodi koju laboratorija koristi za merenje TSH. I na kraju, pri interpretaciji rezultata moralo bi se uzeti u obzir i vreme uzimanja krvi za analizu TSH, imajući u vidu da je nivo TSH najviši u vreme sna, a najniži u kasnim poslepodnevnim satima. Sve gore pomenuto, bez dovoljnog razumevanja i dovoljne pažnje biohemičara, moglo bi dovesti do pogrešne procene funkcionalnog stanja štitne žlezde sa velikim posledicama za pacijenta.

CHALLENGES IN LABORATORY DIAGNOSTICS OF THYROID DISORDERS

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Laboratory testing plays a key role in the diagnosis and treatment of thyroid disease. Today, immunochemical methods are methods of choice for determining the level of hormones in the blood, due to complete automatization, short processing time, and high specificity and sensitivity to a large panel of heterogeneous molecules. When using these, seemingly simple tests on a daily basis, numerous interferences occur, interfering with the obtaining accurate results and requiring a high level of knowledge in order to minimize inaccuracy. Accurate testing results are essential for successful diagnosis and successful treatment of patients. In addition, there are other key challenges that arise in laboratory endocrinology. Today, it is certainly the greatest challenge to standardize and harmonize immunochemical assays, which is still uncompleted task, regardless the enormous efforts. For successful diagnosis and treatment of patients, it is necessary to determine reference values for thyroid hormones, which is rarely done in our country. Instead, we use the reference values obtained by the reagents manufacturers. It is also necessary to reconsider the levels of TSH which are significant for clinical decisions in case of hypothyroidism and which strongly depend on the method used by the laboratory to measure TSH. Finally, when interpreting the results, blood sampling time for TSH analysis should be considered, because TSH levels are the highest during sleep and the lowest in the late afternoon. All of the above mentioned, without enough understanding and enough attention of biochemists, could lead to the wrong assessment of thyroid functional condition, with substantial consequences for the patient.

DOKTORI NAUKA U IVD INDUSTRIJI – OČEKIVANJA I MOGUĆNOSTI

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Poslednjih nekoliko decenija zapaža se značajan rast i razvoj u oblasti industrije koja se bavi medicinskim sredstvima. Prema definiciji, *in-vitro* dijagnostička sredstva (IVD) obuhvataju neinvazivne testove koji se izvode na različitom biološkom materijalu sa ciljem postavljanja dijagnoze, radi skrininga pacijenata ili praćenja terapije. IVD industrija nudi širok opseg različitih rešenja počevši od onih najjednostavnijih poput *point-of-*care testova za praćenje nivoa glukoze, preko uređaja namenjenih za rutinski rad kliničkih laboratorija do sofisticiranih tehnologija za molekularna testiranja poput *real-time* PCR tehnologije. Paralelno sa intenzivnim razvojem ove industrijske grane raste i potreba za stručnim kadrovima unutar kompanija. Poseban akcenat se stavlja upravo na visokokvalifikovano stručno osoblje koje može pružiti adekvatnu stručnu podršku kliničkim laboratorijama i pomoći kliničarima u odabiru i primeni adekvatnih testova sa ciljem postavljanja brze i tačne dijagnoze. Stoga je veoma važno dati prave smernice doktorantima o mogućnostima koje im se otvaraju u ovom segmentu, po završenom doktoratu, i pružiti informacije na koji način znanja i veštine stečene tokom doktorskih studija mogu biti prepoznate od strane budućih poslodavaca i implementirane u svakodnevni rad. Ne samo da mogu da rade u sektoru za istraživanje i razvoj, već mogu konkurisati za pozicije u okviru naučnog marketinga i stručne podrške. Rad u IVD kompanijama, bilo da su u pitanju multinacionalne kompanije ili kompanije lokalnog tipa, pred mlade ljude postavlja izazove sa kojima se nisu susretali ranije. Pored stručnog znanja iz oblasti za koje su se školovali, zahteva se posedovanje dodatnih veština. Neke od njih, poput upravljanja projektima i vremenom, su veštine kojima su doktori nauka već ovladali. Osim njih posebno značajne za rad u IVD industriji su komunikacione veštine važne za komunikaciju kako sa krajnjim korisnicima tako i sa menadžmentom, sposobnost upravljanja finansijama i budžetima, kao i sposobnost primene marketinških principa za pravilno i uspešno pozicioniranje proizvoda na tržištu.

PHD IN IVD INDUSTRY – EXPECTATIONS AND POSSIBILITIES

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Over the last few decades there have been noticed considerable rise and development in medical devices industrial segment. According to definition, *in-vitro* diagnostics (IVD) are non-invasive tests performed on biological samples used for diagnose, screening or therapy monitoring. IVD industry offers wide range of different solutions, from the simplest ones like the *point-of-care* tests for glucose monitoring, over the analyzers intended for routine clinical laboratories, up to the sophisticated solutions for molecular testing like the *real-time* PCR technology. Together with the intensive development of this industry area, there is a growing need for professionals within the companies. There is a special demand for highly qualified employees who could offer adequate professional support to clinical laboratories and help clinicians to choose and implement appropriate assays in order to get timely and accurate diagnose. Therefore, it is of the great importance to give the right guidelines to PhD students about employment possibilities in this segment after graduation, as well to inform them how skills and knowledge, that they have already gained during studies, can be recognized by employers and implemented into their everyday work routine. Not only can they work in research and development department, but they can also apply to positions in scientific marketing and scientific support. The work in both multinational and local IVD companies can put in front of the young people great challenges they have not faced before. Parallel to the professional knowledge for the area they have been educated for, it demands certain additional skills. Some of them, like project and time management, are skills PhDs have already acquired. Except them, the skills that are of special value for the work in IVD industry are communication skills, which are necessary for effective communication both with end-users and management, financial and budget management skills, as well marketing skills necessary for appropriate and successful positioning of the final product at the market.

DOKTORSKE STUDIJE KAO SVEOBUHVATNA NADOGRADNJA ZA USPEŠAN RAZVOJ KARIJERE U IN VITRO DIJAGNOSTICI

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Globalni strateški prioriteti u in vitro dijagnostici su transformacija zdravstvene zaštite na globalnom nivou i bolji rezultati kroz inovacije u efikasnosti testiranja, kliničkoj vrednosti i digitalnim rešenjima. Inovacije u kliničkoj vrednosti podrazumevaju razvoj kliničkih rešenja, koja se odnose na medicinske potrebe koje još uvek nisu rešene i koja obezbeđuju pravi benefit za pacijenta. Inovacije u kliničkim rešenjima, bilo da su novi biomarkeri, nove indikacije postojećih biomarkera ili digitalna rešenja, ostvaruju se kroz klinicke studije. U ulozi globalnog menadžera za medicinske poslove, kandidat mora da bude sposoban da prepozna, kombinuje, analizira i interpretira različite grupe kliničkih podataka, uključujući podatke iz randomizovanih kliničkih studija kao i iz opservacionih studija, elektronskih kartona, i novijih tipova podataka, kao sto su genomika, u kombinaciji sa inovativnim načinima interpretacije tih podataka. Doktorat je esencijalan za uspešno kreiranje novih podataka kao i za analizu interpretaciju već postojećih. On omogućava odgovarajuće implementiranje medicinskog znanja kao i znanja iz translacionog istraživanja, epidemiologije i biostatistike. Doktorat bi dodatno trebalo da omogući kandidatu fokus na inovativan način kreiranja novih kliničkih dokaza (maksimalno iskoristiti opservacione studije, korisiti alternativne robusne kredibilne izvore preko digitalnih kanala za publikacije) i fokus na transformaciju medicinskog angažovanja za odgovarajuće prenosenje kliničkih informacija (globalni pristup medicinskom angažovanju sa sofisticiranom kvalitetnom funkcijom, rigorozni analitički pristup u identifikaciji i prioritizaciji ključnih uticajnih osoba na globalnom nivou i prioritizacija između država, pravilna upotreba digitalnih sredstava, saradnja sa nezavisnim edukativnim platformama i digitalnim programima). Menadžer medicinskih poslova sa doktoratom bi trebalo da ima jasno razumevanje potreba korisnika I da bude u stanju da generiše i dalje promoviše solidan sadržaj koji kreira. To može biti pokretačka snaga jedinstvenog pristupa kompanije u IVD u predstavljanju vrednosti svojim korisnicima.

PHD AS A COMPREHENSIVE UPGRADE FOR SUCCESSFUL CARRIER DEVELOPMENT IN IVD

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Global IVD strategic priorities are transformation of global healthcare and improvement of outcomes through innovation in testing efficiency, medical value and digital insights. Innovation in medical value considers development of medically differentiated solutions, which address unmet needs and provide a real and superior benefit for patients. Medically differentiated solutions (either new biomarkers, new indications of existing biomarkers or clinical algorithms) bring the innovation with breakthrough clinical studies. In the role of Global Medical Affairs Manager, candidate needs to be able to select, combine, analyze and interpret the data from different data sets, including randomized clinical trials as well as real-world evidence (RWE), electronic medical records, and novel sources of data, such as genomics in combination with innovative ways of mining and interpreting that data. PhD background is essential for the generation of new data as well as analysis and interpretation of already existing data. It allows person to implement properly medical knowledge together with knowledge in translational sciences and most importantly biostatistics and epidemiology. PhD should furthermore enable candidate to focus on innovative evidence generation (leverage on RWE generation, use of other robust, credible evidence available on digital channels for publications) and transformation of medical engagement for proper dissemination of medical information (global approach to medical stakeholder engagement with robust medical excellence function, rigorous analytical approach for identification and prioritization of the key opinion leaders globally and coordination across countries, proper use of digital tools, e-congresses and other innovative methods, collaboration with independent medical education platforms and digital programs). Medical Affairs Manager with PhD should have clear understanding of stakeholder needs and be able to generate and disseminate strong value story to support it. It can be the driving force behind IVD Company's one unified collaborative approach to delivering value to its stakeholders.

GENSKA EKSPRESIJA ADIPONEKTINSKIH RECEPTORA KOD PACIJENATA SA KOLOREKTALNIM KARCINOMOM

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Neadekvatan imuni odgovor je prepoznat kao jedan od faktora koji doprinose složenoj etiopatogenezi kolorektalnog karcinoma (CRC). Nova istraživanja ukazuju na značaj različitih adipocitokina u imunološkom odgovoru. Adiponektin se ovde posebno izdvaja, s obzirom na njegovu ulogu u plastičnosti i polarizaciji makrofaga, pri čemu istovremeno utiče i na nivo cirkulišućeg faktora nekroze tumora alfa (TNF-alfa). Cilj naše studije je bio ispitivanje nivoa informacione ribonukleinske kiseline (iRNK), TNF-alfa i adiponektinskih receptora 1 i 2 (adipor 1 i 2) u mononuklearnim ćelijama periferne krvi (MĆPK). Pored toga smo ispitali i njihovu povezanost sa lipidima kao pokazateljima narušene metaboličke kontrole.U istraživanje su bile uključene dve grupe: pacijenti sa CRC-om (N= 73) i kontrolna grupa (KG) (N = 80). Za procenu relativnih nivoa ekspresije iRNK upotrebljena je lančana reakcija polimeraze (PCR), dok je beta aktin korišćen kao konstitutivno eksprimiran gen za normalizaciju podataka. Parametri lipidnog statusa određeni su korišćenjem komercijalno dostupnih enzimskih metoda na automatskom analizatoru ILAB 300+. Normalizovani nivoi adipor 1 i TNF-alfa iRNK su bili sniženi u CRC (p <0,001; p<0,050), dok se nivo adipor 2 iRNK-a nije značajno razlikovao između grupa (p = 0,442). Uočena je značajna pozitivna korelacija između TNF-alfa iRNK i koncentracije holesterola lipoproteina visoke gustine (HDL-H) u CRC (ρ = 0,242; p <0, 05), dok je koncentracija HDL-H negativno korelirala sa adipor 1 iRNK (ρ = -0,262; p <0, 05). Osim toga, nivo adipor 1 iRNK je pozitivno korelirao sa adipor 2, kako u CRC tako i u KG (ρ = 0,268; p <0, 05, ρ = 0,498; p <0,001). U KG ukupni holesterol je negativno korelirao sa TNF-alfa i sa adipor 1 iRNK (ρ = -0.228; p <0, 05; ρ = -0,230; p<0,05). Naši rezultati ukazuju da je narušena metabolička kontrola povezana sa složenom genetskom deregulacijom imuniteta. Dobijeni rezultati mogli bi predstavljati novu, važnu informaciju u istraživanjima karcinoma, koja bi mogla biti posebno značajna u razvijanju individualizovanog pristupa u dijagnozi i prognozi bolesti. Stoga dobijeni rezultati mogu predstavljati temelj za buduća istraživanja.

GENE EXPRESSION OF ADIPONECTIN RECEPTORS IN PATIENTS WITH COLORECTAL CANCER

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Immune irregularity is recognized as one of the factors that contribute to complex etiopathogenesis of colorectal cancer (CRC). Novel studies imply significance of different adipocytokines in immune response. Adiponectin can be singled out, considering its role in macrophages' plasticity and polarization, while also influencing tumor necrosis factor alpha (TNF alpha) circulating levels. The aim of our study was to investigate messenger ribonucleic acid (mRNA) levels of TNF alpha and adiponectin's receptors 1 and 2 (adipor 1 and adipor 2), in peripheral blood mononuclear cells (PBMCs). Additionally, we explored their association with lipids as indicators of impaired metabolic control. This study included two cohorts: CRC patients (N=73) and control group (CG) (N=80). Polymerase chain reaction (PCR) was employed for evaluation of relative mRNA expression levels, while beta actin was used as a constitutively expressed gene for normalization of data. Lipid status parameters were obtained by using commercially available enzymatic methods on automated analyzer ILAB 300+. Normalized adipor1 and TNF alpha mRNA levels were reduced in CRC ($p<0.001$; $p<0.050$; respectively), while adipor 2 mRNA didn't differ between our two groups ($p=0.442$). Significant positive correlation was observed between TNF alpha mRNA and high density lipoprotein cholesterol (HDL-C) in CRC ($\rho = 0.242$; $p<0.05$), while HDL-C levels negatively correlated with adipor 1 mRNA ($\rho = -0.262$; $p<0.05$). Furthermore, adipor 1 mRNA positively correlated with adipor 2 in CRC, as well as in CG ($\rho = 0.268$; $p<0.05$, $\rho = 0.498$; $p<0.001$; respectively). In CG total cholesterol correlated negatively with TNF alpha and adipor1 mRNA ($\rho = -0.228$; $p<0.05$; $\rho = -0.230$; $p<0.05$, respectively). Our results suggest that disrupted metabolic control is associated with complex genetic dysregulation of immunity. The observed relationship could represent important novel information for cancer research, which could be especially significant for more individualized approach in patients' diagnosis and prognosis. Therefore our results warrant future studies.

MARKERI HOMEOSTAZE HOLESTEROLA U VISOKORIZIČNOJ TRUDNOĆI

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Dislipidemija koja se razvija kod žena sa pre-eklampsijom (PEK) po nekim autorima se smatra značajnim činiocem razvoja endotelne disfunkcije i ne-potpune invazije trofoblasta koje se nalaze u osnovi razvoja ove komplikacije trudnoće. Molekularni mehanizmi nastanka ove dislipidemije nisu u potpunosti razjašnjeni, tako da je cilj naše studije bio da ispitamo promjene u koncentraciji ne-holesterolskih sterola (NHS), surogat markera sinteze i apsorpcije holesterola, i da definišemo metabolički profil holesterola kod žena sa visoko-rizičnom trudnoćom. Devedeset trudnica koje su na osnovu važećih preporuka bile u riziku da razviju PEK uključeno je u studiju i praćeno longitudinalno u 4 tačke tokom trudnoće. Dvadeset trudnica je razvilo PEK. Uzorci krvi su uzimani jednom u svakom trimestru i ne-posredno prije porođaja. Tečnom hromatografijom sa tandem masenom spektrometrijom (LC-MS/MS) određena je koncentracija NHS. U grupi sa visokim rizikom od 2. trimestra je uočen značajan porast dezmosterola ($p<0,001$), 7-dehidroholesterola ($p<0,05$) i latosterola ($p<0,001$), tj. markera sinteze holesterola, a vrijednosti su ostale visoke do kraja trudnoće. Kod trudnoće komplikovane PEK-om značajan porast je uočen samo za latosterol ($p<0.05$) i to od 3. trimestra. Latosterol u 1. trimestru je bio viši u grupi žena sa PEK-om u poređenju sa grupom sa visokim rizikom ($p<0,05$), ukazujući da je sinteza holesterola bila povećana od samog početka trudnoće komplikovane PEK-om. Značajan pad β-sitosterola, markera apsorpcije holesterola, je uočen samo u grupi sa visokim rizikom u tački prije porođaja ($p<0,05$). β-sitosterol u 1. trimestru je bio niži u grupi sa PEK-om u poređenju sa grupom sa visokim rizikom ($p<0,05$). Međutim, pozitivna korelacija između dezmosterola i β-sitosterola u 1. trimestru u grupi sa PEK-om ($\rho=0,459$; $p<0,05$) ukazuje da je a-psorpcija holesterola bila povišena u prisustvu povišene sinteze holesterola, tj. da je homeostaza holesterola bila narušena rano u trudnoći komplikovanoj PEK-om. Dakle, dobijeni metabolički profil holesterola sugeriše da je sinteza holesterola povišena, a homeostaza holesterola narušena već u prvom trimestru kod žena sa PEK-om.

CHOLESTEROL HOMEOSTASIS MARKERS IN HIGH-RISK PREGNANCY

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Dyslipidemia observed in women with pre-eclampsia (PEC) is considered to be a significant factor in the development of endothelial dys-function and incomplete trophoblast invasion that underlie the development of PEC. Molecular mechanisms of this dyslipidemia have not been fully elucidated so our study aimed to investigate changes in non-cholesterol sterols (NCSs), cholesterol synthesis and absorption surrogate markers, and to define the cholesterol metabolic profile in a high-risk pregnancy. Ninety pregnant women who were at risk of developing PEC based on valid recommendations were included in the study and monitored longitudinally. Twenty pregnant women developed PEC. Blood samples were taken once in each trimester and before delivery. Circulating profiles of NCSs were determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS). In a high-risk group a significant increase in desmosterol ($p<0.001$), 7-de-hydrocholesterol ($p<0.05$) and lathosterol ($p<0.001$), i.e. cholesterol synthesis markers, were observed from the 2nd trimester. In PEC complicated pregnancy, a significant increase was observed only for lathosterol ($p<0.05$) from the 3rd trimester. First trimester latosterol was higher in the PEC compared to the high-risk group ($p<0.05$), indicating cholesterol synthesis was higher from the onset of PEC complicated pregnancy. A significant decrease in β-sitosterol, cholesterol absorption marker, was observed only in the high-risk group before delivery ($p<0.05$). First trimester β-sito-sterol was lower in the PEC group compared to the high-risk group ($p<0.05$). However, a positive corre-lation between desmosterol and β-sitosterol in the PEC group in the 1st trimester ($\rho=0.459$, $p<0.05$), implied cholesterol absorption was increased in presence of increased cholesterol synthesis, i.e. cholesterol homeostasis was disrupted early in PEC complicated pregnancy. In conclusion, the cholesterol metabolic profile obtained suggests increased cholesterol synthesis and impaired cholesterol homeostasis in women with PEC as early as the first trimester.

LONGITUDINALNE PROMENE LIPOPROTEINSKIH ČESTICA VISOKE GUSTINE I ENZIMA PARAOKSONAZE 1 TOKOM RIZIČNE TRUDNOĆE

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Preeklampsija (PE) je komplikacija trudnoće koja se karakteriše de novo razvojem hipertenzije i proteinurije posle 20-te nedelje gestacije kao i prestankom svih simptoma do 6-te nedelje nakon porođaja. Incidenca PE je od 3% do 7% i predstavlja jedan od glavnih uzročnika smrtnosti kako fetusa tako i majke tokom trudnoće i porođaja. Osnovni patogenetski mehanizam nastanka PE je neadekvatno vaskularno remodelovanje koje je neophodno za adekvatnu perfuziju placente i razvoj fetusa. Posledično dolazi do neadekvatne perfuzije placente, oksidativnog stresa, inflmacije i disfunkcije majčinog endotela. U PE kod trudnica se razvija dislipidemija, pri čemu lipoproteinske čestice visoke gustine (HDL) gube svoja antiaterogena i antioksidativna svojstva, doprinoseći progresiji endotelne disfunkcije. Cilj ovog rada bio je praćenje raspodele subfrakcija HDL čestica i antioksidativnog kapaciteta ovih čestica preko aktivnosti enzima paraoksonaze 1 (PON1), trudnica koje su u visokom riziku da razviju PE. Studija je uključila 91 trudnicu sa jednim ili više faktora rizika za nastanak PE. Krv je uzorkovana u četiri tačke, od prvog do trećeg trimestra (T1, T2, T3), kao i u 37-oj nedelji gestacije pre porođaja (T4). Aktivnost PON1 je određena preko brzine razgradnje supstrata paraoksona, dok je razdvajanje HDL subfrakcija vrseno metodom vertikalne elektroforeze na poliakrilamidnom gradijent gelu. Rezultati su pokazali da je relativni udeo HDL 2b subfrakcije statistički značajno veći u T2 u odnosu na T1 kod obe grupe ispitanica, trudnica koje su razvile PE i kod trudnica koje su bile u riziku da razviju PE ($p<0.05$). Relativni udeo velikih HDL 2a čestica se smanjivao tokom trudnoće, sa značajnom razlikom u T2 u odnosu na T1 ($p<0.05$). Takođe, relativni udeo HDL 3a čestica je bio manji u T2 u odnosu na T1 ($p<0.001$). Aktivnost enzima PON1 od početka trudnoće kreće da raste i statistički je značajno veća u T2 u odnosu na T1 kada posmatramo sve ispitanice zajedno ($p<0.05$), s tim da trudnice koje su razvile PE, u startu imaju znatno veću

LONGITUDINAL CHANGES OF HIGH DENSITY LIPOPROTEIN PARTICLES AND PARAOXONASE 1 ENZYME DURING RISK PREGNANCY

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Preeclampsia (PE) is a pregnancy complication, characterized by de novo development of hypertension and proteinuria after 20th weeks of gestation and disappearance of all symptoms by the 6th week postpartum. The incidence of PE ranges from 3% to 7% and is one of the leading causes of mortality for both the fetus and the mother during pregnancy and childbirth. The pathogenetic mechanism of PE formation is inadequate vascular remodeling, which is necessary for adequate placental perfusion and fetal development. Consequently, inadequate placental perfusion, oxidative stress, inflammation and dysfunction of the maternal endothelium occur. Dyslipidemia develops in PE, with high density lipoprotein (HDL) particles losing their antiatherogenic and antioxidant properties, progressing endothelial dysfunction. The aim of this study was to monitor the distribution of HDL particle subfractions and antioxidant capacity of these particles via the activity of the enzyme paraoxonase1 (PON1), pregnant women at risk of developing PE. The study included 91 pregnant women with one or more risk factors for PE. Blood was sampled sequentially in four points, from the first to third trimesters (T1, T2, T3) as well as at the 37th week of gestation before birth (T4). PON1 activity was determined by the rate of degradation of the paraoxonase substrate, while the separation of HDL subfractions was performed by vertical electrophoresis on a polyacrylamide gradient gel. The results showed that relative proportion of HDL2b subfractions was significantly higher in T2 compared to T1 in both groups of pregnant women who developed PE as well as in high-risk group ($p<0.05$). The relative proportion of large HDL2a particles was lower in T2 compared to T1 ($p<0.05$). Also, relative proportion of HDL3a particles was lower in T2 compered to T1 ($p<0.05$). The activity of PON1 enzyme from the beginning of pregnancy starts to grow and it is significantly higher in T2 compered to T1 when we look at all subject together, but

aktivnost ovog enzima kroz T1, T2 i T4 tačku u odnosu na trudnice koje su bile u visokom riziku od razvoja PE ($p<0.05$). Na osnovu rezultata ove studije može se zaključiti da su trudnoće komplikovane PE praćene kvalitativnim i kvantitativnim promenama HDL čestica.

pregnant women who developed PE, at start having significantly higher activity of this enzyme trough T1, T2 and T4 point compered to pregnant women who were in high risk. Based on the results of this study, it can be concluded that the pregnancies complicated with PE are accompanied by qualitative and quantitative changes in HDL particles.

GAMA-GLUTAMIL TRANSFERAZA KAO MARKER EKSTRAĆELIJSKIH VEZIKULA U SEMENOJ PLAZMI ČOVEKA

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Ekstraćelijske vezikule (EV) su membranske strukture nano veličine, koje se sastoje od proteina, lipida i nukleinskih kiselina. Njihov složen sastav u velikoj meri odslikava ćeliju od koje vode poreklo, a dodatno može biti promenjen u zavisnosti od fizioloških i patoloških procesa. Upotreba EV u kliničkoj dijagnostici kao oruđa »tečne biopsije« je, stoga, sve više u porastu. Naše istraživanje se bavi EV, poznatim kao prostazomi, koje su izolovane iz semene plazme muškaraca sa normospermijom i oligospermijom. Uporedna analiza molekulskih svojstava površine prostazoma sa aspekta glikanskog sastava, bila je usmerena na moguće razlike u manozilovanim i sijalinizovanim glikoproteinima. Posebna pažnja je bila posvećena praćenju membranskog glikoproteina, gama-glutamil transferaze (GGT; EC 2.3.2.2.). U oblasti reproduktivne fiziologije, GGT se, do sada, nije ispitivala kao marker prostazoma. Sticanjem uvida u obrasce distribucije GGT na različitim supopulacijama ili domenima EV, može se povećati njen biomarkerski potencijal u rutinskoj laboratorijskoj dijagnostici. Intaktni ili solubilizovani prostazomi, izolovani diferencijalnim centrifugiranjem i gel filtracijom, okarakterisani su pomoću elektronske mikroskopije, afinitetne hromatografije, određivanjem aktivnosti GGT i blota korišćenjem lektina konkavalina A, lektina iz pšeničnih klica i antitela na tetraspanine. Dobijeni rezultati su pokazali da distribucija GGT, generalno, prati distribuciju CD63 i N-glikana. U odnosu na ko-distribuciju ostalih ispitivanih membranskih glikoproteina, molekulski obrasci povezani sa GGT su odražavali razlike u prostazomima muškaraca sa normospermijom i oligospermijom. Dobijeni rezultati su otkrili GGT na EVs kao analit i referentni parametar.

GAMMA-GLUTAMYL TRANSFERASE AS A MARKER OF EXTRACELLULAR VESICLES IN HUMAN SEMINAL PLASMA

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Extracellular vesicles (EVs) are nano-sized membranous structures, carrying diverse cargoes including proteins, lipids and nucleic acids. Their complex composition largely reflects the cells which they originated from and can be changed in physiological and pathological processes. With the emerging interest in the use of EVs for clinical and diagnostic purposes, its application as a 'liquid biopsy' tool have exponentially growed. Our research deals with EVs, known as prostasomes, isolated from human seminal plasma of normozoo- and oligozoospermic men. Comparative examination of molecular properties of prostasomal surface, exemplified by glycan compositions as possible distinction factor, was focused on mannosylated and sialylated glycoproteins. Specifically, membranous glycoprotein gamma-glutamyl transferase (GGT; EC 2.3.2.2.) was monitored. So far, GGT was not studied as a prostasomal marker in relation to reproductive physiology. Getting insight into distribution patterns of GGT on different EVs subpopulation or domains can add new value to its common use as a biomarker by raising its laboratory diagnostic potential. Intact or detergent-treated prostasomes, isolated by differential centrifugation and gel filtration, were characterized by electron microscopy, affinity chromatography, GGT activity and blotting using concanavalin A, wheat germ lectin and antibodies to tetraspanins. The results obtained indicated that GGT distribution generally overlapped distribution of CD63 and N-glycans. In relation to co-distribution of individual membrane glycoproteins studied, distinct GGT-associated molecular patterns were found to reflect differences in prostasomes from normozoo- and oligozoospermic men. Consequently, GGT on EVs as an analyte and new reference parameter emerged.

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P001
PRESENTATION OF THE RESULTS OF PREGNANCY CHROMOSOMAL ABNORMALITIES IN PHO CLINICAL HOSPITAL BITOLA FOR 2020 YEAR

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Genetic screening for chromosomopaty is performed in the first trimester of pregnancy by determining fetal nuchal translucency and pregnancy associated plasma protein A and free human chorionic gonadotropin hormone in maternal serum. This study was performed in 2020 year in Clinical Hospital Bitola. A total of 503 pregnant women were screened during the first trimester. The serum was separated and pregnancy associated plasma protein-A and free beta human chorionic gonadotrophin hormone were measured. The ultrasound scan included a full structural survey, and nuchal translucency. Risks for chromosomal abnormalities were calculated using the software Prisca - mathematical model which gives individual risks for trisomy 21, 18 and 13.Screening was carried out in 503 pregnancies. Median maternal age was 22,98 ±0.37 years (range: 16 to 42 years). Among the 503 pregnant women overall, 63 (13%) fetuses had an estimated risk for trisomy 21 and trisomy 13/18. Of the 440 (87%) cases chromosomal abnormality was not found.Of utmost importance for pregnant woman and for the society is screened for chromosomal abnormalities in pregnancy and assessed the risk of Down syndrome, Edward syndrome and Patay. With this screening we are going to prevent their occurrence and we'll reduce the psychological and physical suffering of parents and society, especially in today's modern society, where there are the most developed technologies in the industry and prevention is really possible!

P001
PREZENTACIJA REZULTATA HROMOZOMSKIH ABNORMALNOSTI TRUDNOĆE U PHO KLINIČKOJ BOLNICI BITOLJA ZA 2020. GOD.

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Genetski skrining za hromozomopatiju se sprovodi u prvom tromesečju trudnoće određivanjem fetalne nuhalne translucencije i plazma proteina A povezane sa trudnoćom i slobodnog humanog horionskog gonadotropina hormona u serumu majke. Studija je urađena 2020. godine u Kliničkoj bolnici Bitola. Ukupno 503 trudnice su pregledane tokom prvog trimestra. Serum je odvojen i izmereni su proteini plazme-A povezani sa trudnoćom i slobodni beta humani horionski gonadotropin hormon. Ultrazvučni pregled je uključivao potpuni strukturalni pregled i nuhalnu translucenciju. Rizici za hromozomske abnormalnosti su izračunati korišćenjem softvera Prisca – matematički model koji daje individualne rizike za trizomiju 21, 18 i 13. Skrining je sproveden u 503 trudnoće. Srednja starost majke bila je 22,98 ±0,37 godina (raspon: 16 do 42 godine). Među ukupno 503 trudnice, 63 (13%) fetusa je imalo procenjeni rizik za trizomiju 21 i trizomiju 13/18. Od 440 (87%) slučajeva hromozomska abnormalnost nije pronađena. Od najveće važnosti za trudnicu i društvo je skrining na hromozomske abnormalnosti u trudnoći i procenjen rizik od Daunovog sindroma, Edvardovog sindroma i Pataiovog sindroma. Ovim skriningom ćemo sprečiti njihovu pojavu i umanjiti psihičku i fizičku patnju roditelja i društva, posebno u današnjem savremenom društvu, gde postoje najrazvijenije tehnologije u industriji i prevencija je zaista moguća!

P002
THE INFLUENCE OF OBESITY TO INFLAMMATORY AND ANTIOXIDATIVE MARKERS IN UNIVERSITY STUDENTS WITH INCREASED CARDIOVASCULAR RISK

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The aim of this study was to assess the oxidative stress status through the values of inflammatory (CRP) and antioxidative parameters (SOD, GPx, GR and TAS), together with cardiovascular risk factors and anthropometric parameters in a group of obese University students. Study included 238 students (126 men and 112 women), with a mean age of 22.32±1.85 years. According to the body mass index (BMI) lower and higher than 25 kg/m^2 and waist circumference (WC) of less and more than 94cm (80cm for females) the whole group of 238 students was divided into 2 subgroups: the group at increased risk for CVD (n=164) and the group at lower risk for CVD (n=74). Reduced SOD and GPx and increased GR and TAS, inflammatory and lipoprotein parameters were obtained in the high risk group compared to the controls ($p<0.05$). Positive association of CRP, TAS and GR and negative association of GPx and HDL-cholesterol with cardiovascular risk were found in obese students. According to the ROC analysis, GR>44.8 U/L, TAS>1.35 mmol/L, CRP>1.71 mg/L and HDL-cholesterol<1.13 mmol/L had sufficient predictive ability for cardiovascular disease in obese students. Significant association of antioxidant defense parameters, anthropometric, lipid and inflammatory markers with increased cardiovascular risk suggest that screening of these parameters is necessary and highly recommended.

P002
UTICAJ GOJAZNOSTI NA INFLAMATORNE I ANTIOKSIDANTNE MARKERE KOD STUDENATA SA POVEĆANIM KARDIOVASKULARNIM RIZIKOM

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Cilj ovog rada je bio da se utvrdi status oksidativnog stresa kroz vrednosti inflamatornih (CRP) i antioksidantnih parametara (SOD, GPx, GR i TAS), zajedno sa faktorima rizika za kardiovaskularne bolesti i antropometrijskim parametrima u grupi gojaznih studenata. U ovoj studiji je bilo uključeno 238 studenata (126 muškarac i 112 žena), prosečne starosti 22,32±1,85 godina. U odnosu na indeks telesne mase (BMI) manjeg ili većeg od 25 kg/m^2 i obima struka (WC) većeg ili manjed od 94 cm (za muškarce), odnosno 80 cm (za žene), celokupna grupa od 238 studenata je podeljena na 2 podgrupe: grupa sa povećanim kardiovaskularnim rizikom (n=164) i grupa sa snižem kardiovaskularnim rizikom (n=74). Značajno snižene vrednosti SOD-a i GPx-a a povećane vrednosti GR i TAS-a zajedno sa inflamatornim (CRP) i lipoproteinskim parametrima su dobijene u grupi gojaznih studenata u odnosu na kontrolnu grupu ($p<0,05$). Pozitivna asocijacija je dobijena za CRP, TAS i GR, a negativna za GPx i HDL-holesterol sa faktorima rizika za kardiovaskulane bolesti u grupi gojaznih studenata. ROC analiza je pokazala da su GR>44,8 U/L, TAS>1,35 mmol/L, CRP>1,71 mg/L and HDL-cholesterol<1,13 mmol/L značajni prediktori kardiovaskularnih bolesti kod gojaznih studenata. Značajna asocijacija koja je dobijena između parametara oksidativnog stresa, inflmacije, antropometrijskih i lipoproteinskih parametara, ukazuje na to da je screening ovih gojaznih ososba zaista potreban i preporučljiv.

P003
ABNORMALITIES IN LABORATORY PARAMETERS IN PATIENTS WITH COVID-19 – CASE STUDY

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Objective: COVID-19 (CoronaVIrus Disease 2019) is a respiratory and multiple organ disease caused by SARS-CoV-2. Virus member of the Coronaviridae. Immunocompromised patients, older people and people with chronic medical conditions/underlying conditions are at a higher risk of developing severe form. The objective of this paper is to present the Abnormalities in laboratory parameters in survived and in non-survived patients.
Material and methods: Average values of several serum biomarkers are shown in this paper: CRP, LDH, CK, ALT, AST, Urea, Creatinine, Total Bilirubin, Total proteins, Albumin, differential blood counts for 20 hospitalized patients: 10 recovered and 10 non-survivors. Biochemical analysis and blood tests were performed several times in different time intervals depending on the clinical course of the patients.
Results: Average results in recovered patients: Complete blood count: Hbg 132 g/L; Er 4.533×10 3 /μL; Leuk 7,1×10 3 /μL; Tromb 245×10 3 /μL; Hct 0,39%; Neutr 0,58%; Limf 0,30%; Mono 0,10%; Eoz 0,02%; Biochemical parameters: TBIL 9 μmol/L; UREA 4,2 mmol/L; CREA 50 μmol/L; GLUC 6 mmol/L; ALT 58 IU/L; AST 45 IU/L; LDH 273 IU/L; CK 41 IU/L; TP 68 g/L; Alb 36 g/L; CRP 22 mg/L. Average results in patients with fatal outcome: Complete blood count: Hbg 127 g/L; Er 4.422× 10^3/μL; Leuk 12,7×10^3 /μL; Tromb 256×10^3 /μL; Hct 0,38%; Neutr 0,84%; Limf 0,11%; Mono 0,05%; Eoz 0,01%. Biochemical parameters: TBIL 10 μmol/L; UREA 18,0 mmol/L; CREA 171 μmol/L; GLUC 8,5 mmol/L; ALT 79 IU/L; AST 84 IU/L; LDH 847 IU/L; CK 506 IU/L; TP 64 g/L; Alb 32 g/L; CRP 220 mg/L.
Conclusion: No significant changes/abnormalities were noticed in the blood count of recovered patients; serum biomarkers ALT, LDH, CRP were slightly elevated. In non-survived patients significant laboratory abnormalities were noticed; neutrophilia with lymphopenia, multiple elevated levels of LDH (4x), CK (3x) and CRP (20x).
Key words: COVID-19, blood count test, biochemical parameters.

P004
LIPID PROFILE IN GERIATRIC PATIENTS WITH VASCULAR DEMENTIA AND ALZHEIMER DISEASE

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Lipids are part of the dry mass of the brain and are associated with healthy and pathologic functions of the brain. It is found that most common genetic risk factor of Alzheimer disease is ApoE e4 variant, lipids are also involved in blood-brain barrier function and processing of Amyloid precursor protein, inflammation, and energy balance. Most common types of dementia in our facility are Alzheimer and Vascular dementia. For this study we analyze lipid profile of 67 patients, 37 patients with Alzheimer disease and 30 patients with vascular dementia. 15 patients were male or 5 with AD and 10 with VD, and 52 female patients or 32 with AD and 20 with VD. Age range of patients was from 68 to 98 years. Serum samples were collected and we analyzed samples on Cobas Integra 400 automated clinical chemistry analyzer for total cholesterol, triglycerides, and high and low density lipoprotein. Results in two groups have lower levels for cholesterol, triglycerides and LDL, also was found that patients with Alzheimer disease have lower triglyceride levels from those with vascular dementia. Mean median for triglycerides in AD group was 1.12 mmol/L IQR=0.5 different from those with VD 1.41 mmol/L IQR=1.0. High density lipoprotein was significantly lower in patients with VD Mean Median 0.913 mmol/L IQR=0.6 and normal in AD patients 1.21 mmol/L IQR=0.3. From those results we can propose that high triglyceride levels are characteristic for vascular dementia and must consider the link between Stable levels of high density lipoprotein and Alzheimer Disease.

	Alzheimer disease	Vascular Dementia
Cholesterol	(4.1)	(3.79)
Triglycerides	(1.12) IQR=0.5	(1.41) IQR=1
HDL	(1.21) IQR=0.3	(0.913) IQR=0.6

P005
POREĐENJE KONCENTACIJA KALCIJUMA I MAGNEZIJUMA ODREĐENO U UZORCIMA HEPARINIZIRANE PUNE KRVI I PLAZME

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Kalcijum (Ca) i magnezijum (Mg) su minerali od velikog značaja za regulaciju mnogih procesa u organizmu. Tradicionalno se koncentracije ukupnog Ca (uCa) i ukupnog Mg (uMg) određuju u serumu, što je i najčešći tip uzorka u medicinsko biohemijskoj laboratoriji. Jonizovani oblici ovih minerala (jCa i jMg) se određuju u uzorcima pune krvi. Iako je serum najčešći i osnovni uzorak za određivanje većine biohemijskih analita i dalje postoji kontinuirana naučna debata o tome koji tip uzorka može biti uzorak izbora. Cilj ovog ispitivanja je da se analiziraju parametri statusa Ca i Mg u uzorcima pune krvi i heparinizirane plazme. Istraživanjem je obuhvaćeno 87 uzoraka populacije zdravih studenata prosečne starosti od 23 godine. Ukupne koncentracije Ca i Mg određene su na biohemijskom analizatoru Olympus AU400 (Beckman Coulter Diagnostics, Hamburg, Nemačka) u uzorcima heparinizirane plazme. Koncentracije jCa i jMg izmerene su na Stat Profile Prime Critical Care Analyzer (New Biomedical Corporation, Waltham, MA, SAD) iz heparinizirane pune krvi. Indeksi uMg/uCa i jMg/jCa su izračunati računski. Izmerene koncentracije ispitivanih parametara, izuzev uCa, pratile su normalnu raspodelu podataka ($P > 0,05$). Dobijena je statistički značajna korelaciju između uCa vs jCa i uMg vs jMg (rho = 0,307; P = 0,004 i rho = 0,281; P = 0,008, redom). Utvrđeno je i da između vrednosti uMg vs jMg/jCa i jMg vs uMg/uCa postoji jaka pozitivna korelacija (rho = 0,286; P = 0,007 i rho = 0,267; P = 0,013, redom). Takođe je utvrđena značajna pozitivna korelacija između indeksa (rho = 0,312; P = 0,003). Međutim, konačno slaganje između ispitivanih parametara nije utvrđeno. Iako rezultati ovog ispitivanja ukazuju da postoji

P005
COMPARISON OF CALCIUM AND MAGNESIUM CONCENTRATIONS DETERMINED IN HEPARINIZED WHOLE BLOOD AND PLASMA SAMPLES

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Calcium (Ca) and magnesium (Mg) are minerals of great importance for the regulation of many processes in the body. Traditionally, the concentrations of total calcium (tCa) and total magnesium (tMg) are determined in serum, which is the most common type of sample in the medical biochemical laboratory. Ionized forms of these minerals (iCa and iMg) are determined in whole blood samples. Although serum is the most common and basic sample for determining most biochemical analytes, there is still an ongoing scientific debate about which type of sample can be the sample of choice. The aim of this study was to analyze the parameters of Ca and Mg status in whole blood and heparinized plasma samples. The study included 87 samples of the population of healthy students with an average age of 23 years. Total Ca and Mg concentrations were determined on an Olympus AU400 biochemical analyzer (Beckman Coulter Diagnostics, Hamburg, Germany) in heparinized plasma samples. Concentrations of iCa and iMg were measured on a Stat Profile Prime Critical Care Analyzer (New Biomedical Corporation, Waltham, MA, USA) from heparinized whole blood. Also, tMg/tCa and iMg/iCa indices were calculated. The indices tMg/tCa and iMg/iCa were calculated. The measured concentrations of the examined parameters, except for tCa, followed the normal distribution of data ($P > 0.05$). A statistically significant correlation was obtained between tCa vs iCa and tMg vs iMg (rho = 0.307; P = 0.004 and rho = 0.281; P = 0.008, respectively). It was also found that there is a strong positive correlation between the values of tMg vs iMg/iCa and iMg vs tMg/tCa (rho = 0.286; P = 0.007 and rho = 0.267; P = 0.013, respectively). A

značajna korelacija između parametara statusa Ca i Mg izmereno u različitom tipu uzoraka, potrebne su buduće prospektivne, dobro kontrolisane studije, i na specifičnim populacijama ispitanika, kako bi se potvrdila značajna povezanost i slaganje i moguća predikcija vrednosti između ukupnih i jonizovanih oblika ovih minerala.

significant positive correlation was also found between the indices (rho = 0.312; P = 0.003). However, the final agreement between the examined parameters was not determined. Although the results of this study indicate that there is a significant correlation between Ca and Mg status parameters measured in different sample types, future prospective, well-controlled studies, and examination on specific patient populations, are needed to confirm significant correlation and agreement and possible prediction of values between total and ionized forms of these minerals.

P006
UTICAJ DUŽINE DIJALIZIRANJA NA PARAMETRE MINERALNO KOŠTANOG METABOLIZMA KOD BOLESNIKA NA HRONIČNOJ HEMODIJALIZI

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Hronična bubrežna insuficijencija (HBI) je progresivno i ireverzibilno oštećenje bubrega uz smanjenje broja funkcionalnih nefrona koje dovodi do potupnog gubitka bubrežne funkcije i potrebe za liječenjem hemodijalizom. Jedna od komplikacija koja se javlja u sklopu HBI je i poremećaj mineralno-koštanog metabolizma što vremenom dovodi do koštane bolesti. Cilj istraživanja je bio procjena uticaja dužine dijaliznog staža na biohemijske parametre mineralno koštanog metabolizma kod pacijenata na hemodijalizi. Istraživanje je obuhvatilo 35 pacijenata prosječne starosti 62.94±14.85 godina, podjeljenih u 3 grupe u odnosu na dužinu dijaliznog staža (I grupa-do 5 godina, II grupa-5 do 10 godina; III grupa-preko 10 godina). Našim istraživanjem smo pokazali da su vrijednosti fosfora povišene kod gotovo svih pacijenata u svim grupama. Vrijednosti kalcijuma su približno iste u svim grupama. Vrijednosti PTH su niže kod pacijenata u I grupi u donosu na pacijente u II i III. Dok su vrijednosti ALP nešto visočije u I u odnosu na II i III grupu.

P006
INFLUENCE OF DIALYSIS LENGTH ON PARAMETERS OF MINERAL BONE METABOLISM IN PATIENTS ON CHRONIC HEMODIALYSIS

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Chronic renal failure (HBI) is a progressive and irreversible damage to the kidneys with a decrease in the number of functional nephrons, which leads to a complete loss of kidney function and the need for hemodialysis treatment. One of the complications that occurs within HBI is a disorder of mineral-bone metabolism, which eventually leads to bone disease. The aim of the study was to assess the influence of the length of dialysis on the biochemical parameters of mineral and bone metabolism in patients on hemodialysis. The study included 35 patients with an average age of 62.94 ± 14.85 years, divided into 3 groups according to the length of dialysis (I group-up to 5 years, II group-5 to 10 years; III group-over 10 years). Our research has shown that phosphorus values are elevated in almost all patients in all groups. Calcium values are approximately the same in all groups. PTH values were lower in patients in group I compared to patients in groups II and III.

P007
STATUS VITAMINA D KOD BOLESNIKA SA KOVID-19 I UTICAJ NA TEŽINU BOLESTI

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Vitamin D je uključen u modulaciju urođenog i stečenog imunog sistema, proizvodnju antimikrobnih peptida, kao i u ekspresiji gena uključenih u intracelularno uništavanje patogena. Nizak nivo 25-OHD u serumu se često nalazi kod starijih osoba ili kod onih sa hroničnim stanjima, koji su takođe prijavljeni kao loši prognostički faktori za COVID-19. Smanjenje ACE2 od strane SARS-CoV-2 dovodi do disregulacije sistema renin-angiotenzin, što doprinosi »oluji citokina« koja prethodi sindromu akutnog respiratornog distresa karakterističnom za teški oblik COVID-19. U tom smislu, vitamin D može inhibirati proizvodnju proinflamatornih citokina u ljudskim monocitima/makrofagima, a hronični nedostatak vitamina D može izazvati aktivaciju RAS, što dovodi do proizvodnje fibroznih faktora i, prema tome, oštećenja pluća. S obzirom na razlike u težini i fatalnosti COVID-19 u svetu, važno je razumeti razloge koji stoje iza toga. Poboljšanje imuniteta kroz bolju ishranu može biti značajan faktor. Ova studija je procenila korelaciju koncentracija vitamina D sa slučajevima COVID-19 i njegov uticaj na težinu i smrtnost od COVID-19. U studiju su uključena 83 pacijenta (55,2% muškaraca, prosečne starosti 57 godina i 45,8% žena prosečne starosti 56 godina) sa potvrđenom COVID-19 pneumonijom, dijagnostikovan i lečen, između 1. juna i 12. avgusta 2020. godine u Gradskoj opštoj bolnici »8. Septembra«-Skoplje. U celoj studiji primećen je veoma nizak nivo vitamina D kod oba pola. Medijan VitD je bio značajno niži kod žena (28,01 nmol/L) u odnosu na podgrupu muškaraca (43,96 nmol/L). Uočeno je da kod žena, iako je manji procenat hospitalizovanih od COVID-19, one imaju veću stopu mortaliteta koja iznosi 18,42%, u poređenju sa muškarcima kod kojih iako imamo veći procenat hospitalizovanih, mortalitet je manji i iznosi 8,9 % . Takođe, dužina hospitalizacije kod žena je duža, 19 dana, u odnosu na muškarce koja iznosi 15,5 dana. Ukratko, ova opservaciona studija među pacijentima sa COVID-19 koji su doživeli definitivan ishod, pokazuje povezanost između VitD statusa i težine i mortaliteta od COVID-19.

P007
VITAMIN D STATUS IN COVID-19 PATIENTS AND IT'S INFLUENCE ON DISEASE SEVERITY

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Vitamin D is involve in the modulation of the innate and acquired immune system and also in the production of antimicrobial peptides, as well as in the expression of genes involved in the intracellular destruction of pathogens. Low serum 25OHD levels are frequently found in elderly individuals or in those with chronic conditions, which have also been reported as poor prognostic factors for COVID-19. The downregulation of ACE2 by SARS-CoV-2 leads to a dysregulation of the renin–angiotensin system, which contributes to the »cytokine storm« that precedes the acute respiratory distress syndrome characteristic of the severe form of COVID19. In this sense, vitamin D can inhibit proinflammatory cytokine production in human monocytes /macrophages, and chronic vitamin D deficiency may induce RAS activation, leading to the production of fibrotic factors and, therefore, lung damage. Considering the differences in the severity and fatality of COVID-19 in the globe, it is important to understand the reasons behind it. Improvement of immunity through better nutrition might be a considerable factor. This study evaluated the correlation of vitamin D concentrations with COVID-19 cases and its impact on the severity and mortality of COVID-19. Included in the study were 83 patients *(*55.2% men, mean age was 57 years and 45.8% women mean age 56 years*)* with confirmed COVID-19 pneoumonia, diagnosed and treated , between 1 June and 12 August 2020 in City General Hospital »8th of September«-Skopje. In the entire study, very low vitamin D levels are observed in both genders. Median VitD level was significantly lower in the female (28.01 nmol/L) versus the male subgroup (43.96 nmol/L). It has been noticed that in women, althought the percentage of hospitalized from COVID-19 is lower, they have a higher mortality rate which is 18.42%, compared to men where althought we have a higher percentage of hospitalized, mortality is lower and is 8.9%. Also the length of hospitalization among women is longer, 19 days, compared to men which is 15.5 days. In summary, this observational study among patients with COVID-19 who have experienced a definite outcome, shows an association between VitD status and severity of and mortality from COVID-19.

P008
NEUTRALIZING ANTIBODIES (NABS) AFTER IMMUNIZATION WITH GAM-COVID-VAC VACCINE IN A SAMPLE OF HEALTHCARE WORKERS

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Background: The process of conferring immunity after vaccination for any virus can be best estimated by determining the levels of specific neutralizing antibodies (NAbs). The specific immune response can diminish over the course of few months, leading to uncertainty about how long the patients are protected. The aim of the present study is to evaluate the presence of NAbs after vaccination with the Gam-COVID-Vac vaccine (viral vector vaccine).
Material and Methods: The sample consisted of 131 healthcare workers (HCW), out of which 66 patients were female, while the median age of the sample was 36 years (IQR 32-42). At enrollment, patients provided baseline data and information about previous SARS-CoV-2 infections. Patients were examined 6 months after 2nd dose of vaccine for the presence of NAbs. The blood samples were analyzed by the CLIA method with the MAGLUMI 800 analyzer.
Results: From the whole sample, 38 patients reported previous known SARS-CoV-2 infection. Six months after the vaccination, all patients with previous infection achieved NAbs above the threshold value of 0.3 , while from the other group only two patients had NAbs levels below 0.3. The median value of the NAbs in the whole sample was 1.56 (IQR 0.42 – 5.73), while patients with previous SARS-CoV-2 infection had median value of 6.45 (IQR 4.16 – 9.03), reaching striking difference when compared to patients without previous infection ($p < 0.001$).
Conclusion: The immunization with the Gam-COVID-Vac produced NAbs titer above the threshold value in 98.5% of the participants, six months after the second dose. Participants with previous documented infection had substantially higher titer of NAbs, leaving room for further exploration on the best practice for immunization in this group of participants.
Keywords: MAGLUMI 800; neutralizing antibodies; SARS-CoV-2; Gam-COVID-Vac; immunization; vaccine

P009
EVALUATION OF ANTI-SARS-COV-2 ANTIBODY RESPONSES IN MACEDONIAN HEALTHCARE WORKERS: AN INTERIM ANALYSIS

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Amidst the COVID-19 pandemic, healthcare workers are exposed to an anticipated higher risk of infection with SARS-CoV-2 considering their work environment, than other members of society. Antibody testing and seroprevalence of COVID-19 antibodies can be a beneficial tool for comprehension of the incidence of disease exposure in this population. This study aims to examine and evaluate the level of SARS-CoV-2 antibodies among HCW in North Macedonia during the period from 28/05/2020 to 20/08/2020. A total of 2334 HCW have been tested for SARS-Cov-2 IgM and IgG antibody assays, using the Chemiluminescent (CLIA) method on the Maglumi 800 platform. Out of the 2334 HCW tested, 1676 (71.87%) were women and 656 (28.13%) were men. The age range was between 19 and 70 years old with the mean age being 45.23. A total of 195 HCWs tested positive for either IgM or IgG anti-SARS-CoV-2 serum antibodies. Of them, 167 individuals tested positive for IgM antibodies and 54 tested positive for IgG antibodies. The cumulative incidence during the period from 28/05/2020 to 20/08/2020 of anti-SARS-CoV-2 antibody response in HCWs was estimated at 8.355% (95% CI = 7.279–9.57%). HCWs represent a population predisposed to getting infected with COVID-19. We report a relatively low seroprevalence rate in the tested group among HCW, in the set period, which can be due either to an early test request by the participants or increased perception of risk and proper preventative behavior.
Keywords: SARS-CoV-2, health care workers, anti SARS CoV-2 IgM/IgG antibodies, CLIA, COVID-19

P010
VITAMIN D STATUS KOD PREDGOJAZNE I GOJAZNE ŠKOLSKE DJECE U PODGORICI

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Brojne studije sugerišu udruženost gojaznosti i nedostatka vitamina D kod djece i odraslih. Kao objašnjenja se često navode pojačana akumulacija i izmijenjen metabolizam vitamina D u hipertrofičnom adipoznom tkivu. Cilj: ispitivanje serumskih vrijednosti vitamina D kod predgojazne i gojazne djece školskog uzrasta u odnosu na njihove normalno uhranjene vršnjake. Istraživanje je obuhvatilo 202 školske djece uzrasta 7–15 godina (63,9% dječaka, 36,1% djevojčica) iz Podgorice, Crna Gora. Učesnici su podijeljeni u 3 grupe prema nutritivnom statusu (kriterijumi International Obesity Task Force): normalno uhranjeni (42,1%), predgojazni (40,6%) i gojazni (17.3%). Antropometrijska mjerenja obuhvatila su tjelesnu masu i visinu, indeks tjelesne mase (BMI, kg/m^2), i obim struka. Ukupna količina tjelesne masti određivana je metodom bioelektrične impedance (Tanita BC-418, Tokio, Japan). Vrijednost 25(OH) vitamina D (nmol/L) određivana je iz seruma kod 176 djece (imunohemija, Cobas 6000, Roche, Manhajm, Njemačka). Deficijencijom su smatrane vrijednosti vitamina D ≤50 nmol/L. Medijana vrijednosti vitamina D za normalno uhranjenu djecu iznosila je 77,2 (interkvartilni rang (IR) 67,70–95,10), za predgojaznu 70,1 (IR 56,00–86,60) i gojaznu 69,6 (59,30–85,87); ova razlika je bila granično statistički značajna ($p < 0,046$). U grupi gojaznih, vrijednost vitamina D je negativno korelirala sa vrijednošću obima struka ($r = - 0,403$). Deficijencija vitamina D je utvrđena kod 4,3% normalno uhranjene, 16,0% predgojazne i 12,5% gojazne djece. Nije bilo statistički značajne razlike u učestalosti deficijencije vitamina D u odnosu na nutritivni status kod ispitivane djece ($\chi^2 = 5,185$, $p = 0,075$). Takođe, nije utvrđena statistički značajna korelacija između ukupne količine tjelesne masti kod predgojaznih i gojaznih, i vrijednosti vitamina D ($r = 0,133$. $r = - 264$). U zaključku, vrijednost vitamina D bila je niža u serumu predgojaznih i gojaznih u odnosu na normalno uhranjenu djecu i negativno je korelirala sa centralnom gojaznošću u grupi gojazne djece. Ipak, primjenom

P010
VITAMIN D STATUS IN OVERWEIGHT AND OBESE SCHOOLCHILDREN IN PODGORICA

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Numerous studies suggest an association between obesity and vitamin D deficiency in children and adults. Increased accumulation and altered vitamin D metabolism in hypertrophic adipose tissue are often cited as explanations. Objective: to examine the serum levels of vitamin D in overweight and obese school aged children in relation to their normal weight peers. The survey included 202 schoolchildren aged 7–15 (63.9% boys, 36.1% girls) from Podgorica, Montenegro. Participants were divided into 3 groups according to nutritional status (International Obesity Task Force criteria): normal weight (42.1%), overweight (40.6%) and obese (17.3%). Anthropometric measurements performed: body weight and height, body mass index (BMI, kg/m^2), waist circumference. Total body fat was determined by bioelectrical impedance device (Tanita BC-418, Tokyo, Japan). The value of 25 (OH) vitamin D (nmol/L) was determined from the serum of 176 children (immunochemistry, Cobas 6000, Roche, Mannheim, Germany). Vitamin D values ≤50 nmol/L were considered deficient*. The median value of vitamin D for normal weight children was 77.2 (interquartile range (IR) 67.70–95.10), overweight 70.1 (IR 56.00–86.60) and obese 69.6 (59.30–85.87), this difference was borderline statistically significant ($p < 0.046$). In the obese group, the value of vitamin D was negatively correlated with the value of waist circumference ($r = - 0.403$). Vitamin D deficiency was found in 4.3% of normal weight, 16.0% of overweight and 12.5% of obese children. There was no statistically significant difference in the frequency of vitamin D deficiency in relation to the nutritional status of the examined children ($\chi^2 = 5.185$, $p = 0.075$). In addition, no statistically significant correlation was found between the total body fat in overweight and obese and the value of vitamin D ($r = 0.133$. $r = - 264$). In conclusion, the value of vitamin D was lower in the serum of overweight and obese compared to normal weight children, and negatively correlated with central obesity in

datog kriterijuma za deficijenciju vitamina D, nije utvrđena razlika u učestalosti deficijencije vitamina D između ispitivanih grupa.

*Holick MF et al. (2011). Evaluation, Treatment, and Prevention of Vitamin D Deficiency, JCEM; https://www.endocrine.org/clinical-practice-guidelines/vitamin-d-deficiency

the group of obese children. Nevertheless, by applying the given criterion for vitamin D deficiency, no difference was found in the frequency of vitamin D deficiency between the examined groups.

*Holick MF et al. (2011). Evaluation, Treatment, and Prevention of Vitamin D Deficiency, JCEM; *https://www.endocrine.org/clinical-practice-guidelines/vitamin-d-deficiency*

P011
CASE REPORT: SOLITARY MASTOCYTOMA DIAGNOSED SUCCESSFULLY WITH MEASUREMENT OF SERUM LEVELS OF TRYPTASE

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Solitary mastocytoma, a rare dermatological entity accounts for 10–15% of cutaneous mastocytosis. One of the most important blood tests in the field of allergy, mast cell tryptase has numerous diagnostic uses, particularly for anaphylactic reactions and for the diagnosis of mastocytosis. Hypertryptasemia (elevated serum tryptase levels) is present in multiple disorders like systemic mastocytosis, hematological malignancies, and chronic kidney disease. We represent a rare case of solitary bullous mastocytoma presenting at birth, diagnosed successfully with subsequent measurement of serum tryptase levels thus avoiding biopsy of the patient.

P011
PRIKAZ SLUČAJA: SOLITARNI MASTOCITOM USPEŠNO DIJAGNOSTIKOVAN MERENJEM NIVOA TRIPTAZE U SERUMU

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Solitarni mastocitom, retki dermatološki entitet, čini 10–15% kožne mastocitoze. Serumska triptaza, jedan od najvažnijih testova krvi u oblasti alergije, ima brojne dijagnostičke upotrebe, osobito u anafilaktičke reakcije i u dijagnosticiranju mastocitoze. Hipertriptazemija (povišeni nivoi triptaze u serumu) prisutna je kod višestrukih poremećaja poput sistemske mastocitoze, hematoloških maligniteta i hronične bolesti bubrega. Predstavljamo redak slučaj solitarnog buloznog mastocitoma prisutan pri rođenju, uspešno dijagnostikovan uzastopnim merenjem nivoa triptaze u serumu, čime se izbegava biopsija pacijenta.

P012
OKSIDATIVNI STRES I STATUS VITAMINA D KOD COVID-19 PACIJENATA

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Koronavirusi pripadaju porodici Coronaviridae koji spadaju u jednolančane RNK viruse; SARS-CoV-2 je prvi put identifikovan u gradu Vuhanu u Kini 2019. godine nakon što je izazvao pandemiju COVID-19. Značajnu ulogu u razvoju i progresiji ove infekcije ima i razvoj oksidativnog stresa kao pratioca inflamacije, posebno u komplikovanijim oblicima ove bolesti. Ova studija je sprovedena s ciljem da ispita promene parametara oksidativnog stresa kod COVID-19 pacijenata. Parametri oksidativnog stresa su određivani u serumu 31 pacijenta, sa lakšim oblikom bolesti, lečenih ambulantno, u tri tačke (postavljanje dijagnoze, kontrola nakon 14 i nakon 21 dana). Parametri redoks statusa koji su određivani u ove tri tačke obuhvataju prooksidanse (totalni oksidativni status (TOS), superoksidni anjon radikal ($O_2^{\cdot -}$), prooksidantno -antioksidantni balans (PAB) kao i produkte njihovog delovanja: uznapredovali produkti oksidacije proteina (AOPP), malondialdehid (MDA), ishemijom modifikovan albumin (IMA)) i antioksidanse: totalni antioksidativni status (TAS), aktivnost enzima superoksid-dismutaze (SOD) i paraoksonaze 1 (PON1), odnos totalnog antioksidativnog i totalnog oksidativnog statusa (TAS/TOS), ukupne sulfhidrilne grupe (SHG). Svi prooksidansi i markeri njihovog delovanja su pokazali značajan pad tokom 14 dana trajanja studije, dok su antioksidansi istovremeno pokazali značajan rast što je ukazivalo na oporavak pacijenata. U prvoj tački ispitivanja određene su i vrednosti vitamina D kako bi se ispitala veza deficijencije vitamina D sa porastom oksidativnog stresa. Prema vrednostima vitamina D, pacijenti su podeljeni u tri grupe: ≤50 ng/mL, 51–70 ng/mL, 71 ng/mL. Primećeno je da pacijenti sa višim vrednostima vitamina D imaju i značajno veći

P012
OXIDATIVE STRESS AND VITAMIN D STATUS IN COVID PATIENTS

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Coronaviruses belong to the family Coronaviridae which are single – chain RNA viruses; SARS-CoV-2 was for the first time identified in the city Wuhan in China 2019th causing a pandemic COVID-19. The important role in the development and progression of infection also has the development of oxidative stress as an inflammation consequence, especially in complicated forms of this disease. This study was performed with the aim of testing the oxidative stress parameters changes in COVID-19 patients. Oxidative stress parameters were determined in the serum of 31 patients, with a milder form of the disease, treated on an outpatient basis, at three time-points (diagnosis, control after 14 and after 21 days). The redox status parameters determined at these three points include prooxidants (total oxidative status TOS); superoxide anion radical ($O_2^{\cdot -}$); prooxidant – antioxidant balance (PAB); as well as products of their activity: advanced oxidation protein products (AOPP), malondialdehyde (MDA), ischemia – modified albumin (IMA) and antioxidants: total antioxidant status (TAS), superoxide dismutase (SOD) and paraoxonase 1 (PON1) activity, ratio of total antioxidant and total oxidative status (TAS/TOS), total sulfhydryl groups (SHG). All prooxidants and markers of their activity showed a significant decrease during the 14 days of the study, while antioxidants at the same time showed a significant increase, which indicated the recovery of patients. In the first point of the examination, the values of vitamin D were determined in order to examine the connection between vitamin D deficiency and the increase in oxidative stress. According to the values of vitamin D, patients were divided into three groups: ≤50 ng/mL, 51–70 ng/mL, 71 ng/mL. It was noticed that patients with

ukupni broj leukocita i više vrednosti $O_2^{\cdot -}$, dok su vrednosti TAS/TOS odnosa i PAB niže ($p<0,05$). Vitamin D utiče na funkciju imunskog sistema, sintetišu ga T i B limfociti, što omogućava njegovo autokrino delovanje. Deficijencija vitamina D povezana je sa razvojem težih oblika ove bolesti. Pored antivirusne, antibiotske, kortikosteroidne, antikoagulantne terapije u COVID-19 i terapija antioksidansima, mineralima i vitaminom D je bila deo protokola lečenja ovih pacijenata, čiju opravdanost potvrđuju rezultati naše studije.

higher values of vitamin D had a significantly higher total number of leukocytes and higher values of $O_2^{\cdot -}$; while the values of TAS/TOS ratio and PAB were significantly lower ($p<0.05$). Vitamin D affects the function of the immune system, it is synthesized by T and B lymphocytes, which enables its autocrine action. Vitamin D deficiency is associated with the development of more severe forms of this disease. Besides antiviral, antibiotic, corticosteroid, anticoagulant therapy in COVID-19 also therapy with antioxidants, minerals and vitamin D were the part of the treatment protocol of these patients, the justification of which is confirmed by the results of our study.

P013
EVALUACIJA TITARA ANTI-SARS-COV-2 S1-RBD IGG ANTITJELA KOD PRELEŽANIH I VAKCINISANIH ISPITANIKA

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Cilj naše studije bio je uporediti titre anti-RBD antitjela kod preležanih, vakcinisanih i vakcinisanih ispitanika koji su preležali COVID-19. Naša studija je uključila 206 ispitanika; 73 preležanih, 71 vakcinisanih i 62 vakcinisanih sa preležanim COVID-19. Titar anti-SARS-CoV-2 S1-RBD IgG antitjela mjeren je u serumu ispitanika SARS-CoV-2 IgG Quant testom (metoda CMIA) na integrisanom imunobiohemijskom analizatoru Abbott Architect ci4100. Preležani ispitanici su imali najniži titar antitjela. BNT162b2 stvara viši titar antitjela u odnosu na preležane ($p=0,001$). BBIBP-CorV stvara niži titar antitjela u odnosu na preležane ($p=0,004$), ali znatno viši titar ukoliko su ispitanici i preležali COVID-19 ($p=0,009$). Ispitanici, vakcinisani koji su i preležali, imali su najviši titar antitjela ($p<0,001$). Titar antitjela pozitivno je korelirao sa godinama kod preležanih ($r=0,059$), negativno kod vakcinisanih ($r=-0,146$), i pozitivno kod preležanih i vakcinisanih ($r=0,146$). U odnosu na pol, u našoj studiji nije se pokazala statistički signifikantna razlika u titarima antitjela. Zaključujemo da preležani ispitanici vakcinisani protiv SARS-CoV-2 virusa imaju znatno viši titar u odnosu na sve druge grupe, nezavisno od primljene vakcine.

P013
EVALUTATION OF ANTI-SARS-COV-2 S1-RBD IGG ANTIBODY TITERS IN RECOVERED AND VACCINATED PARTICIPANTS

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The aim of our study was to compare the titers of anti-RBD antibodies in COVID-19-recovered individuals, vaccinated and in vaccinated individuals with past-COVID-19. Our study included 206 participants; 73 recovered, 71 vaccinated and 62 vaccinated with past COVID-19. The titers of nti-SARS-CoV-2 S1-RBD IgG antibodies were measured in the sera of participants using the SARS-CoV-2 IgG Quant test (CMIA method) on the integrated Abbott Architect ci4100 analyser. Recovered participants had the lowest titters. BNT162b2 elicited higher titters compared to recovered participants ($p=0.001$). BBIBP-CorV elicited lower titters compared to recovered participants ($p=0.004$), but higher titters if they also had past COVID-19 ($p=0.009$). Participants, vaccinated and with past COVID-19 had the highest titters ($p<0.001$). Antibody titters correlated positively with age in the recovered group ($r=0.059$), negatively in the vaccinated group ($r=-0.146$), and positively in the vaccinated with past COVID-19 group ($r=0.146$). There was no statistically significant difference in the titters based on gender. We conclude that COVID-19-recovered individuals that have been vaccinated against SARS-CoV-2 have significantly higher titers compared to all other groups regardless of the vaccine received.

P014
KONCENTRACIJA HEPCIDINA KOD ŽENA SA IZOSTALIM ABORTUSOM

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Iako je poznat značaj gvožđa za zdravlje majke, rast i razvoj placente, embriona i fetusa, još uvek postoje mnoge nejasnoće u razumevanju regulacije gvožđa tokom (ab)normalne trudnoće. Nije poznato da li izostali pobačaj uzrokuje poremećaj homeostaze gvožđa ili obrnuto, da li poremećena homeostaza gvožđa pre začeća može doprineti pobačaju. Postoji 11–22% rizika od pobačaja do 20. nedelje trudnoće, pri čemu je rizik od pobačaja najveći do 14. nedelje. Izostali pobačaj je abortus kod kojeg postoji asimptomatska ili »odložena«smrt embriona ili fetusa bez dovoljnih kontrakcija materice za izbacivanje fetusa. Hepcidin je peptidni hormon i glavni je regulator gvožđa koji kontroliše apsorpciju i distribuciju gvožđa u tkivu. Određivanje koncentracije hepcidina u serumu u normalnoj ranoj trudnoći i ranom izostalom pobačaju je prvi korak ka boljem razumevanju homeostaze gvožđa u ovimuslovima. U ovu studiju su uključene ukupno 72 ispitanice, od čega 36 žena u kontrolnoj grupi (normalna trudnoća) i 36 žena u studijskoj grupi (izostali spontani pobačaj), do 14. nedelje gestacije. Za ovo istraživanje pribavljena je saglasnost Etičkog povjerenstva Sveučilišne kliničke bolnice (SKB) Mostar, a svi ispitanici su prije uzimanja krvi potpisali informirani pristanak. Uzorak krvi ispitanika je uzet nakon ultrazvučno potvrđenog izostalog pobačaja ili normalnog toka trudnoće. Kriterijumi isključenja za obe grupe: krvarenje, anemija, hipertenzija, dijabetes, endokrine abnormalnosti, ultrazvučno vidljive abnormalnosti fetusa, disfunkcija štitne žlezde, infekcija urinarnog trakta, malformacije materice, onkološke bolesti, stečena i nasledna trombofilija, autoimune bolesti, veštačka oplodnja, suplementacija. Uzorci seruma su dobijeni nakon koagulacije i centrifugiranja pune krvi bez antikoagulansa u trajanju od 10 minuta na 3500 rpm i čuvani na -80 °C do analize. Koncentracija hepcidina je određena imunohemijskom ELISA metodom (engl. Enzyme Linked Immunosorbent Assay). Statistička analiza (MedCalc verzija 20.027) pokazala je da je koncentracija hepcidina statistički značajno veća od koncentracije hepcidina kod žena sa normalnom trudnoćom ($P < 0,05$). Srednja koncentracija hepcidina kod žena sa normalnom

P014
HEPCIDIN CONCENTRATION AMONG WOMEN WITH MISSED ABORTION

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Although it is well known how important iron is for maternal health, growth and development of the placenta, embryo and fetus, there are still many ambiguities in the regulation of iron during (ab)normal pregnancy. It is unknown whether missed abortion causes disorder of iron homeostasis or vice versa, whether disturbed iron homeostasis may contribute to spontaneous abortion before its onset. There is 11–22% risk of miscarriage up to the 20th week of pregnancy, with the risk of miscarriage being highest up to the 14th week. Missed abortion is an abortion inwhich there is asymptomatic or »delayed« death of the embryo or fetus without sufficient uterine contractions to expel the fetus. Hepcidin is a peptide hormone and isa major regulator of iron that controls the absorption and distribution of iron in tissue. Determining serum hepcidin concentrations in normal early pregnancy and early missed miscarriage is the first step toward a better understanding of iron homeostasis in these conditions. A total of 72 respodents were included in this study: 36 women in the control group (normal pregnancy) and 36 women in the study group (missed abortion), up to the 14th week of gestation. The consent of the Ethics Committee of the University Clinical Hospital (UCH) Mostar was obtained for infomed consent before blood sampling. The bloog sample of the respodents was sampled after ultrasound-confirmed of missed abortion or the normal course of pregnancy. Exclusion criteria for both groups: bleeding, anemia, hypertension, diabetes, endocrine abnormalities, ultrasound visible fetal abnormalities, thyroid dysfunction, urinary tract infection, uterine malformations, oncological diseases, acquired and hereditary thrombophilia, autoimmune diseases, artificial insemination, iron supplementation. Serum samples were obtained after coagulation and centrifugation of whole blood without anticoagulant for 10 minutes at 3500 rpm and stored at -80 °C until analysis. Hepcidin concentration was determined by immunochemical ELISA (*Enzyme Linked Immunosorbent Assay*). Statistical analysis (MedCalc version 20.027) showed that the concentration of hepcidin was statistically significantly higher than the

trudnoćom bila je 3,4895 (1,3825–5,1580) ng/mL, a srednja koncentracija hepcidina kod žena sa odloženim pobačajem bila je 5,0130 (3,2880–7,8265) ng/mL. Iako je poremećena homeostaza gvožđa uključena u mnoga patološka stanja i bolesti, malo se zna o ulozi poremećene homeostaze gvožđa u abnormalnim trudnoćama. Učinjeni su brojni pokušaji da se odrede prognostički markeri koji bi ukazivali na pojavu pobačaja u ranoj trudnoći kod žena. Ovde smo pronašli poremećenu homeostazu gvožđa kod žena sa izostalim pobačajem, o čemu svedoče povišene koncentracije hepcidina u serumu. Regulatorni mehanizmi homeostaze gvožđa u ovim uslovima zahtevaju dalja istraživanja.

concentration of hepcidin in women with normal pregnancy ($P < 0.05$). The median hepcidin concentration in women with normal pregnancies was 3.4895 (1.3825–5.1580) ng/mL, and the median hepcidin concentration in women with delayed miscarriage was 5.0130 (3.2880–7.8265) ng/mL. Although disturbed iron homeostasis is involved in many pathological conditions and diseases, there are limited information available about the role of disturbed iron homeostasis inabnormal pregnancies. Numerous attempts have been made to determine prognostic markers to indicate the occurrence of miscarriage during early pregnancy inwomen. In this research, we found disturbed iron homeostasis in women with missed miscarriage, which is proven by elevated serum hepcidin concentrations. The regulatory mechanisms of iron homeostasis in these conditions require further research.

P015
THE POTENTIAL OF SALIVARY PROTEOME IN LABORATORY ANALYSIS OF SJOGREN'S SYNDROME

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Detection of pathological processes at an early stage can significantly affect the clinical course and outcome of the disease, and hence the choice of appropriate therapeutic intervention. In order to achieve this and avoid invasive sampling procedures like surgical biopsy and repeated phlebotomies which introduce additional stress and safety issues in patients, research is greatly focused on identifying alternative samples and novel biomarkers for advanced laboratory analysis. Since many oral but also systemic pathological processes are being reflected in the salivary composition, it is getting increasing attention as an alternative sample to blood, especially for some population groups like children, adolescents, geriatric or psychiatric patients, where blood sampling is often compromised by insufficient cooperation from the patients or individual factors related to the patiens' health. Sjögren's syndrome (SS) is an autoimmune disease with an insidious onset, variable course and clinical presentation, so its diagnosis is usually established about 6 years after the initiation of the disease and based on the detection and quantification of circulating autoantibodies such as: anti-Ro/SSA, anti-La/SSB, anti-muscarinic receptor antibodies, anti-nuclear antibodies and rheumatoid factor. Apart from molecular diagnostics and

P016
PCOS AND ISULIN RESISTANCE

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Polycystic ovary syndrome (PCOS) is a hormonal disorder common among women of reproductive age. Women with PCOS may have infrequent or prolonged menstrual periods or excess male hormone (androgen) levels. Due to the recent research that PCOS is probably caused by insulin resistance, we are beginning to check insulin in blood in patients who have at least 2 of 3 features irregular periods, excess androgen (high levels of »male« hormones in y our body) and polycystic ovaries. We examine hormones in blood in 86 patients between ages 21 to 43 years old on t hird day of menstrual cycle, opposite to a control group of 30 patients in same conditions: LH, FSH, testosterone, DHEAS and insulin with automates the immunoassay reactions utilizing electrochemiluminescence (ECL). LH/FSH ratio was 2,6:1 with mean values in LH 14,65 mIU/mL+/ 4,2; FSH 5,6 mIU/mL +/ 1,9; the levels of testosteron 77,5 ng/dL,+/ 23,8; DHEAS 450,7 µg/dL +/ 203,0 and insulin 24,75 µU/mL +/ 10,7. Many women with PCOS 68,2% have higher levels of insulin which had not been diagnosed. There are sta tistic significant $p< 0.01$ in levels in testosterone, DHEAS and insulin. PCOS cannot be diagnosed by symptoms alone. PCOS is a very complicated endocrine disorder. Blood tests to measure hormone levels, an US of reproductive organs and genes personal and family histories should be completed before a PCOS diagnosis is confirmed. 68% of women with PCOS have insulin resistance which increases risk for type 2 diabetes.

nanotechnology, several new approaches emerge for detecting even subtle alterations of the salivary constituents, like proteins which are employed in the proteomic analysis. Numerous proteomic studies involving sophisticated methodology like LC-MS/MS have identified aberrant expression of specific proteins in saliva of SS patients, making them potential markers of the disease and indicators of progression. Beside cytokines, many other proteins involved in the inflammatory process are upregulated vs proteins associated with the salivary production which are downregulated. Further to changes in salivary concentrations of MMP9, Complement factor B, Azurocydin Kallikrein, many other proteins including proteases like Trypsin, Catepsin, and Myeloblastin, inhibitors of Cystein proteases, Calreticulin, Protein 29, -amylase precursor of carbonanhydrase VI, -2 microglobulin, enolase etc. have shown aberrant expression in saliva from SS patients and a recent study has even proposed the combination of serum anti-SSA/Ro and upregulated salivary TRIM29, as an optimal marker with high diagnostic accuracy for fast and noninvasive diagnosis of SS. But, in patients with SS, efficient saliva collection is difficult because saliva secretion is significantly reduced. Therefore, when examining the scientific potential of this diagnostic sample, stimulated saliva is mainly used. Additionally, the great heterogeneity of the results among various studies is greatly attributed to variations in the preanalytical procedures as well as the analytical methodology itself. Hence, further studies with larger cohorts applying consensual study protocols are needed to validate the currently proposed and identify new potential markers in saliva for diagnosis, monitoring of SS or be used as targets in drug design in accordance with the precise medicine initiatives.

P017
VERIFICATION OF VIDAS 3® SARS-CO-V-2 IGG II TEST USING ENZYME LINKED FLUORESCENT ASSAY (ELFA) TECHNIQUE

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COVID-19 is unfortunately still present and continues to spread worldwide. The use of serological tests is intended to determine if individuals have developed a humoral immune response to SARS-CoV-2. The verification of the SARS-CoV-2 IgG II test was conducted, as part of the laboratory's quality control procedure before introducing a new test in the scope of our lab. The precision of the test VIDAS 3® SARS-CoV-2 IgG II was performed following the CLSI EP05-A3 recommendations. Two panels of human pool serum samples (HPSs) representing negative (N) and positive (P) level indexes (i) were performed to evaluate the variability of the assay within and between day. The tests were performed into two-levels, over five days, each panel in triplicate. According to the manufacturer the acceptable level of precision is fixed at 20% CV (and in our case corresponds to 7.27% and 12.31% for P and N HPSs, respectively. Moreover, the estimated within-run precision of our lab was 5.98% which is less than the claimed one (6.10%) with SD index of 0.293 (claimed- 0.50). N/A data for N HPSs to compare. The daily variance was estimated as 1.27% and 0.51% for P and N HPSs, respectively. The findings have demonstrated that imprecision and repeatability are in compliance with the manufacture claims, therefore the test is suitable for use. The obtained data represent a prerequisite for appropriate utilization of immune response to RBD/spike viral protein.

Keywords: SARS CoV-2 IgG II, quality control, ELFA

P018
SERUMSKI NIVOI IL-1β I IL-1RA KOD PACIJENATA SA GREJVSOVOM ORBITOPATIJOM

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Grejvsova orbitopatija (GO) predstavlja inflamatorni poremećaj orbite koju karakteriše specifičan lokalni citokinski milje. Narušavanjem ravnoteže između interleukina- β (IL-1β) i njegovog prirodnog anatagoniste, antagoniste receptora interleukina-1 (IL-1RA) gubi se prirodni regulatorni mehanizam, čime se stvara proinflamatorni fenotip orbitalnih fibroblasta koji koordiniše dalje procese u osnovi ove bolesti. Cilj naučnog istraživanja je bio da se analizira povezanost IL-1β i IL-1RA sa kliničkim oblikom GO. Istraživanjem je obuhvaćeno 65 pacijenata sa klinički prisutnom GO, lečenih na Klinici za endokrinologiju, dijabetes i bolesti metabolizma, Univerzitetskog kliničkog centra Srbije. Pacijenti su klasifikovani prema aktivnosti i težini GO, kao pacijenti sa aktivnim ili neaktivnim, odnosno blagim ili umerenim do teškim oblikom GO. IL-1 β i IL-1RA su analizirani u uzorcima seruma pacijenata, primenom komercijalnih enzimskih imunohemijskih testova (ELISA), prema preporukama proizvođača. Serumska koncentracija IL-1β bila je statistički značajno viša, a IL-1RA granično niža u grupi pacijenata sa aktivnom GO u odnosu na pacijente sa neaktivnom GO (4,86 (4,25–5,66) pg/mL i 3,83 (2,96–4,83) pg/mL, p = 0,027; 487 (285–694) pg/mL i 618 (359–812) pg/mL, p = 0,059, za IL-1β i IL-1RA, redom). Dodatno je uočena i pozitivna korelacija IL-1β sa vrednostima skora kliničke aktivnosti (ρ = 0,261, p = 0,036). Težina GO nije bila značajno povezana sa vrednostima IL-1β i IL-1RA u ispitivanom uzorku pacijenata. Rezultati ovog istraživanja ukazuju na mogućnost upotrebe IL-1β kao značajnog biomarkera aktivnosti i kliničkog toka GO. Kombinovana primena IL-1β i IL-1RA, zajedno sa tradicionalnim parametrima, mogla bi unaprediti laboratorijsku dijagnostiku ove kompleksne patologije.

P018
SERUM LEVELS OF IL-1β AND IL-1RA IN GRAVES' ORBITOPATHY PATIENTS

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Graves' orbitopathy (GO) is an inflammatory disorder of the orbit characterized by a specific local cytokine milieu. By disrupting the balance between interleukin-β (IL-1β) and its natural antagonist, the interleukin-1 receptor antagonist (IL-1RA), the natural regulatory mechanism is lost, thus creating a proinflammatory phenotype of orbital fibroblasts that coordinates further processes underlying this disease. Aim of the study was to analyze the association of IL-1β and IL-1RA with the clinical form of GO. A total of 65 consecutive patients presented with GO were enrolled in the study. All patients were regularly treated at the Clinic for Endocrinology, Diabetes and Metabolic Diseases, University Clinical Center of Serbia. Patients were classified according to the activity and severity of GO, as patients with active or inactive, and mild or moderate to severe form of GO. IL-1 β and IL-1RA were analyzed in patient sera, using commercial enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's instructions. Significantly higher IL-1β and marginally lower IL-1RA serum concentration was observed in patients with active GO, compared to patients with inactive GO (4.86 (4.25–5.66) pg/mL vs. 3.83 (2.96–4.83) pg/mL, p = 0.027; 487 (285–694) pg/mL vs. 618 (359–812) pg/mL, p = 0.059, for IL-1β and IL-1RA, respectively). Additionally, IL-1β concentration positively correlated with the clinical activity score (ρ = 0.261, p = 0.036). There was no significant association between IL-1β and IL-1RA values, and the severity of GO in the analyzed patient sample. Results of this study indicate the possibility of using IL-1β as a significant biomarker of the activity and the clinical course of GO. The combined application of IL-1β and IL-1RA, along with traditional parameters, could substantially improve the laboratory diagnosis of this complex pathology.

P019
OKSIDATIVNO-STRESNI STATUS KOD PACIJENATA NA HEMODIJALIZI

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Poslednji stadijum bolesti bubrega karakteriše se brzinom glomerularne filtracije manjom od 15 mL/min/1,73 m^2 zbog čega su potrebni tretmani koji zamenjuju renalnu funkciju poput hemodijalize (HD) ili transplantacije bubrega. Dvogodišnji nivo rizika od smrtnog ishoda (engl. *mortality risk score*) se zasniva na više laboratorijskih, kliničkih i parametara HD. Zbog starosti i pojave drugih komorbiditeta, pacijenti na HD su osetljiviji na oksidativni stres. Povećan oksidativni stres može da doprinese i povećanom riziku od mortaliteta. Cilj naše studije je bio da se odrede parametri oksidativno-stresnog statusa pre i posle HD i 6 meseci nakon tretmana, kao i promena ovih parametara u zavisnosti od nivoa rizika od smrtnog ishoda. U studiji je učestvovalo 130 pacijenata na hemodijalizi. Parametri oksidativno-stresnog statusa: uznapredovali produkti oksidacije proteina (AOPP), prooksidativni-antioksidativni balans (PAB), superoksidni anjon ($O_2^{\cdot -}$), malondialdehid (MDA), ishemijom modifikovan albumin (IMA), superoksid-dizmutaza (SOD) i sulfhidrilne (SH) grupe su odre eni spektrofotometrijskim metodama na ILAB 300+ analizatoru. Vrednosti parametara $O_2^{\cdot -}$ ($P<0,05$), IMA ($P<0,01$), AOPP, SOD, SH gr, PAB i MDA ($P<0,001$) 6 meseci nakon tretmana su bile značajno različite u pore enju sa vrednostima dobijenim pre i/ili posle HD. Umereni i najviši nivo rizika od smrtnog ishoda se karakterišu značajno različitim koncentracijama SH grupa, PAB-a ($P<0,05$) i $O_2^{\cdot -}$ ($P<0,001$), u odnosu na grupu pacijenata sa najmanjim rizikom. Dobijeni rezultati pokazuju da bi hemodijaliza mogla značajno da utiče na oksidativno-stresni status pri čemu je i rizik od smrtnog ishoda povezan sa različitim nivoima oksidativnog stresa.

P019
OXIDATIVE-STRESS STATUS OF HEMODIALYSIS PATIENTS

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The last stage of kidney disease is characterized by a glomerular filtration rate less than 15 mL/min/1.73 m^2. As a consequence, treatments such as hemodialysis (HD) or kidney transplantation are necessary to substitute for the lost function. The two-year mortality risk score is based on multiple laboratory, clinical and HD parameters. Patients on HD treatment are susceptible to oxidative stress due to aging and other comorbidities. Increased oxidative stress can also contribute to an increased risk of mortality. The aim of our study was to determine the parameters of oxidative-stress status before and after HD and 6 months after treatment, as well as the changes of these parameters depending on the levels of risk of death. The data from 130 patients on HD treatment were collected in this study. The parameters of oxidative-stress status as: advanced oxidation protein products (AOPP), prooxidant antioxidant balance (PAB), superoxide anion ($O_2^{\cdot -}$), malondyaldehide (MDA), ischemia modified albumin (IMA), superoxide-dismutase (SOD), and sulfhydryl (SH) groups were determined using spectrophotometric methods on ILAB 300+ analyzer. The results of $O_2^{\cdot -}$ ($P<0.05$), IMA ($P<0.01$), AOPP, SOD, SH gr, PAB and MDA ($P<0.001$), six months after treatment, were significantly different compared to the values obtained before and/or after HD. The moderate and the highest mortality risk levels were characterized by significantly different concentrations of SH groups, PAB ($P<0.05$) and $O_2^{\cdot -}$ ($P<0.001$) compared to the group of patients with the lowest risk. The obtained results show that hemodialysis could significantly affect the oxidative-stress status and the risk of death is associated with different levels of oxidative stress.

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- Supplements:
  Williams DN. Reducing costs and hospital stay for pneumonia with home intravenous cefotaxime treatment: results with a computerized ambulatory drug delivery system. Am J Med 1994; 97: Suppl 2A: 50–5.
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- Books and Monographs:
  Kahn CR, Weir GC, editors, Joslin's diabetes mellitus, 13ed. Philadelphia: Lea and Febiger, 1994: 1068pp.
- Chapters:
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Normally distributed data should be described using a mean, SD and/or %CV and expressed as »mean (SD)« not »mean ± SD«. When data are not normally distributed, following demonstration by tests such as the Shapiro-Wilk test (3), then medians and interquartile ranges should be used in place of mean and SD. Skewed data can often be normalized by logarithmic transformation or a power transformation. The statistical analysis and calculation of summary statistics should be carried out on the transformed data and the summary statistics transformed back to the original scale for presentation. If a logarithmic scale is used, then graphs should display non-transformed data on a logarithmic scale.

Graphs showing data of comparable magnitude should be of similar size and design. All individual points should be displayed where possible by displacing overlapping points. Error bars showing the standard error of the mean (SEM) or interquartile range, as appropriate, can be used to aid the interpretation of data.

The results of significance tests such as Student's and chi-squared should be presented with descriptive statistics, degrees of freedom (if appropriate) and probability *P*. The validity of any assumptions should be checked (e.g. conventional *t*-tests assume a normal distribution and equal variance for each set of data). For 2×2 contingency table analysis by the chi-squared test the continuity correction must be applied, and for small expected frequencies Fisher's Exact Test used.

P values should be reported in full in 1 or 2 significant figures. Describing *P* values as > 0.05 or NS (not significant) should be avoided. If the results are highly significant and the calculated *P* value from the computer is e.g. 0.000, then the use of $P < 0.0005$ is acceptable. Confidence intervals should be stated, particularly for non-significant results.

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.
3. Altman DG. Practical Statistics for Medical Research. London: Chapman & Hall, 1991: 132–12.
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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