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THE ROLE OF DIFFERENT EXERCISES IN IRISIN, HEAT SHOCK PROTEIN 70 AND SOME BIOCHEMICAL PARAMETERS

ULOGA RAZLIČITIH VEŽBANJA NA IRISIN, PROTEIN TOPLOTNOG ŠOKA 70 I NEKE BIOHEMIJSKE PARAMETRE

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Summary

Background: The aim of the study was to determine the effects of different and regularly applied exercise programs on irisin, heat shock protein 70 and some biochemical parameters.
Methods: 120 male university students participated in the study. Participants were divided into 4 equal groups as control (C), resistance exercise group (RE), high intensity interval (HIIT) and aerobic exercise group (AE). While the control group did not perform any exercise, the pre-determined exercise programs were applied to the other groups for 8 weeks and 3 days in a week. Blood samples were taken from all participants before and after the exercise program. Cholesterol, High-density Lipoprotein (HDL) and Low-density Lipoprotein (LDL) cholesterol, triglyceride (TG), Creatine kinase (CK), Lactate dehydrogenase (LDH), Irisin and Heat shock protein 70 (HSP70) levels were analyzed in blood samples.
Results: It is determined that there are significant differences in pre-posttest values of the AE group's LDH, cholesterol, HDL-cholesterol, TG and HSP 70 levels, HIIT group's CK, LDH, Cholesterol, HDL-cholesterol, TG, Irisin and HSP70 levels and RE group's CK, LDH, Cholesterol, LDL-cholesterol, TG and Irisin levels ($p<0.05$).
Conclusions: It can be said that exercise can provide improvements in lipid profile, changes in HSP70 levels may vary depending on muscle damage, the increase of irisin due to exercise.

Keywords: biochemistry, exercise, irisin, lipid profile, muscle damage

Kratak sadržaj

Uvod: Cilj ove studije je bio sa se utvrde efekti različitih i redovno primenjivanih programa vežbanja na irisin, protein toplotnog šoka 70 i neke biohemijske parametre.
Metode: U studiji je učestvovalo 120 univerzitetskih studenata. Učesnici su podeljeni u 4 jednake grupe kao kontrola (K), grupa vežbi otpora (RE), interval visokog intenziteta (HIIT) i grupa aerobnih vežbi (AE). Dok kontrolna grupa nije izvela nijednu vežbu, unapred određeni programi vežbanja primenjeni su na ostale grupe 8 nedelja i 3 dana u nedelji. Uzorci krvi uzeti su od svih učesnika pre i posle programa vežbanja. U uzorcima krvi je analiziran nivo holesterola, lipoproteina visoke gustine (HDL) i lipoproteina niske gustine (LDL), triglicerida (TG), kreatin kinaze (CK), laktat dehidrogenaze (LDH), irisina i proteina toplotnog šoka 70 (HSP70).
Rezultati: Utvrđeno je da postoje značajne razlike u vrednostima pre-posttesta nivoa LDH, holesterola, HDL-holesterola, HDL-holesterola, TG i HSP 70 u AE grupi, CK, LDH, holesterola, HDL-holesterola, TG, irisina i HSP70 u grupi HIIT nivoi CK, LDH, holesterola, LDL-holesterola, TG i irisina u grupi RE ($p<0,05$).
Zaključak: Može se reći da se vežbanjem može poboljšati profil lipida, promene nivoa HSP70 mogu varirati u zavisnosti od oštećenja mišića i povećanja irisina usled vežbanja.

Ključne reči: biohemija, vežbe, irisin, lipidni profil, oštećenje mišića

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Introduction

Exercise is defined as planned, structured, repetitive, continuous activities that aiming to improve one or more elements of physical fitness (1). This development occurs mostly by the activation of skeletal muscles in the organism. Depending on the characteristics and content of the training, the exercises can be performed in various types as aerobic or anaerobic (2). In general, it is known that exercise has many effects on the human organism. Exercise causes an increase or decrease in the levels of some hormones. These changes occur with the activation of the glands (3). The recently discovered protein that causes white adipose tissue to turn into brown adipose tissue and enables it to be effective in energy metabolism is called "irisin". Boström et al. (4) have reported that there is an increase in irisin values due to the overexpression of Fibronectin Type III Domain Containing 5 (FNDC5) in the liver of overweight (obese) rats. It has been stated that this increase causes browning of white adipose tissue and decreases in body weight. They also revealed that better oxygen consumption leads to glucose tolerance and insulin sensitivity. It is known that irisin is released into the circulation from many different parts and these are mainly skeletal and cardiac muscle, adipose tissue, brain, kidney, lung, liver and stomach (5). However, it is stated that the synthesis of the irisin mainly takes place in the skeletal and cardiac muscles and adipose tissue (6). In a study conducted on this field, it has been shown that resistance training applied 3 days a week for 12 weeks improves muscle functions and increases the level of irisin (7). There are Heat shock proteins that have different roles in living organisms. It is known that these proteins are located in different sections of the cell. Since cells have common structural and functional properties, they contain members of different HSP families with distinct stress excitability. One of them is HSP70. HSP70 is known to be generally highly induced by stress such as oxidative stress and elevated temparature (8). In an important study conducted in this topic, Cumming et al. (9) revealed that increases in HSP 70 level occur 48 hours after resistance exercises. It is stated that exercise has biochemical effects besides these effects, and it can have significant effects on muscle damage (10, 11) and blood lipid profiles depending on the type and intensity of the exercise (12). It is known that physical activity and exercises have many positive effects on the human organism. Regular exercise helps weight control, supports body composition and reduces cardiovascular risks. The number of studies supporting these positive effects is increasing day by day. While the level of the irisin increases with the exercise factor, it is important how heat shock proteins will respond to this. The aim of this study is to examine the changes of irisin, HSP 70 and some biochemical parameters (blood lipid profile and muscle damage markers) of different exercises for eight weeks.

Materials and Methods

Participants

120 healthy students between the ages of 19–24 who study at Firat University, do not exercise regularly, stay in the same dormitory and are subject to the same nutrition program participated in the study. The research was conducted on a voluntary basis. Therefore, individuals who wanted to participate voluntarily and did not have any disability for exercise practices were included in the study. The research was explained to the participants as detail and approval was obtained from Firat University Non-Invasive Research Ethics Committee before starting the study (09/20-08.06.2017).

Research Groups and Exercise Programs

Control Group (n: 30)

The group that did not have any exercise program.

Aerobic Exercise Group (n: 30)

An exercise program with 60–75% intensity (maximal heart rate) of the Karvonen method was applied to the participants for eight weeks, 3 days a week with continuous running method. Each exercise unit consisted of 10 minutes of warm-up, 40 minutes of main phase and 10 minutes of cool-down exercises. The training intensity of the group was designed according to the karvonen method by checking the maximal heart rate every week regularly.

High Intensity Interval Training Group (n: 30)

Participants were given an exercise program with the HIIT (High-intensity interval training) method 3 days a week for eight weeks. According to the principle of Tabata Type High Intensity Interval Training, each exercise unit was performed in 8 repetitions, with 15 minutes of warm-up, 20 seconds of ultra-intensive loading, followed by 10 seconds cool-down (13).

Resistance Exercise Group (n: 30)

The trainings were carried out for eight weeks with 65–75% of the 1RM (Maximum repetition) values of the participants. Exercises were performed 3 days a week in a circular training format and exercise program consisting of 10 exercises covering both upper and lower extremity muscle groups. Each exercise unit was applied for 15 minutes of warm-up, each movement 3 sets and 8–10 repetitions. The weights used were arranged by checking each week according to the 1 maximum repetitions of the participants.

Analysis of Biochemical Parameters

Blood samples taken for blood lipids (Cholesterol, HDL-cholesterol, LDL-cholesterol, Triglyceride), CK (Creatine Kinase), LDH (Lactate dehydrogenase), values of the participants in the study were centrifuged at 4000 rpm for 5 minutes. Blood samples were stored at -80 °C until the day of analysis. Serum samples were analyzed with Olympus autoanalyzer (AU 2700; Hamburg, Germany).

Analysis of Irisin and HSP 70

Irisin (Catalog no: 201-12-5328 Sunred, Biological Technology Co., Ltd., Shanghai, CHINA) and HSP70 levels (Catalog no: 201-12-1814, Sunred, Biological Technology Co., Ltd., Shanghai, CHINA) were analyzed by ELISA method in accordance with the working procedures specified in the kit catalogs. Irisin measuring range of ELISA kit: 0.2-60 ng/mL sensitivity was 0.157 ng/mL, HSP 70; 0.5–150 ng/mL sensitivity was 0.458 ng/mL. Irisin and HSP 70 Intra-Assay: CV <10%, Inter-Assay: CV was <12%. Automatic washer Bio-Tek ELX50 (BioTek Instruments, USA) was used for plate washing, ChroMate for absorbance readings, Microplate Reader P4300 devices (Awareness Technology Instruments, USA) and test results were reported as ng/mL.

Statistical analysis

SPSS 22.0 package program was used to analyze the data obtained from the research. Data distribution was checked Kolmogorov Smirnov test. The distribution was normal. Thus Paired Samples t test was performed to determine the intra-group differences that occurred before and after the exercise protocol and One Way ANOVA analysis was performed to reveal the differences between groups. Tukey test, one of the Post Hoc tests, was applied to determine which group caused the difference. Statistical significance was accepted as $p < 0.05$ for all tests. The data were presented in *Tables*.

Results

In *Table I*, it was determined that there was significant differences between the pre-test post-test values of LDL-cholesterol in the control group. There were significant differences cholesterol, HDL-cholesterol, triglyceride levels of aerobic exercise group and HIIT group. In addition significant differences were found in cholesterol, LDL-cholesterol, triglyceride levels of the resistance exercise group ($p<0.05$). When the percentage change of these differences is examined; it was observed that the greatest change in cholesterol and triglyceride levels occurred in the aerobic exercise group, HDL-cholesterol in the HIIT group, and LDL-cholesterol in the resistance exercise group.

When *Table II* is evaluated; It was determined that there were significant differences between pre-test post-test values of CK in control group, LDH level in aerobic exercise group and both CK and LDH levels in HIIT group and resistance exercise group ($p <0.05$). The biggest change was seen in the Resistance exercise group in both parameters as a percentage. A statistically significant difference was

Table I Blood Lipids Levels of the Research Groups.

Parameters	Groups	Pretest	Posttest	Changes (%)	Intra-group	Inter-groups
		Mean±Sd	Mean±Sd		p	p
Cholesterol (mmol/L)	Control	4.2030±0.80	4.2056±0.80	0.06	0.620	0.089
	Aerobic	4.5255±0.71	4.1867±1.03	-7.48	0.020*	
	HIIT	4.1996±0.80	3.9927±0.67	-4.92	0.010**	
	Resistance	3.9979±0.70	3.7411±0.59	-6.42	0.022*	
HDL-Cholesterol (mmol/L)	Control	1.0868±0.20	1.0843±0.20	-0.23	0.083	0.219
	Aerobic	1.0912±0.20	1.1732±0.21	7.50	0.001**	
	HIIT	0.9852±0.15	1.1637±0.17	18.11	0.000**	
	Resistance	1.0879±0.14	1.1150±0.15	2.51	0.310	
LDL-Cholesterol (mmol/L)	Control[b]	2.4238±0.86	2.4326±0.86	0.36	0.002**	0.009**
	Aerobic[b]	2.5757±0.54	2.5808±0.88	0.20	0.965	
	HIIT[b]	2.4334±0.86	2.2886±0.57	-5.60	0..236	
	Resistance[a]	2.2014±0.61	1.9309±0.66	-12.28	0.004**	
Triglyceride (mmol/L)	Control[b]	1.7518±0.75	1.7310±0.77	-1.18	0.084	0.004**
	Aerobic[a]	1.7496±0.77	1.2174±0.40	-30.41	0.000**	
	HIIT[a]	1.8301±0.85	1.4067±0.44	-23.13	0.021*	
	Resistance[a]	1.6249±0.48	1.4368±0.43	-11.57	0.041*	

**There is a significant difference ($P<0.01$). The difference between groups with different letters in the same column is significant (a,b,c,d)

Table II Muscle Damage Markers of the Research Groups.

Parameters	Groups	Pretest	Posttest	Changes (%)	Intra-group	Inter-groups
		Mean±Sd	Mean±Sd		p	p
CK (U/L)	Control[b]	235.10±136.93	237.23±135.85	0.90	0.000**	0.004**
	Aerobic[b]	234.70±137.80	264.90±165.01	12.86	0.195	
	HIIT[b]	237.33±90.08	387.30±110.06	63.19	0.000**	
	Resistance[a]	231.17±121.6	417.87±185.59	80.76	0.007**	
LDH (U/L)	Control[d]	173.57±10.23	176.17±8.89	1.49	0.058	0.000**
	Aerobic[d]	170.87±8.39	178.30±16.66	4.34	0.011*	
	HIIT[c]	176.00±12.76	202.23±17.41	14.90	0.000**	
	Resistance[ab]	171.50±12.47	239.47±30.17	39.63	0.000**	

**There is a significant difference (P<0.01), The difference between groups with different letters in the same column is significant (a,b,c,d)

Table III Irisin and HSP70 Levels of the Research Groups.

Parameters	Groups	Pretest	Posttest	Changes (%)	Intra-group	Inter-groups
		Mean±Sd	Mean±Sd		p	p
Irisin (ng/mL)	Control[a]	13.91±3.05	14.39±4.90	3.45	0.464	0.000**
	Aerobic[a]	8.36±2.41	11.41±4.42	36.48	0.000**	
	HIIT[b]	17.32±4.54	26.78±3.49	54.61	0.000**	
	Resistance[b]	19.82±6.57	26.02±7.65	31.28	0.000**	
HSP70 (ng/mL)	Control[a]	25.70±4.25	24.83±4,41	-3.38	0.000**	0.000**
	Aerobic[b]	39.44±8.74	7.66±3.26	-80.57	0.000**	
	HIIT[b]	20.03±6.98	5.54±1.93	-72.34	0.000**	
	Resistance[c]	16.40±5.25	15.09±2.19	-7.98	0.156	

**There is a significant difference (P<0.01), The difference between groups with different letters in the same column is significant (a,b,c,d)

found between the posttest CK and LDH levels between the groups (p <0.05). While this difference is due to the resistance exercise group at the CK level (p: 0.01), it is due to the resistance exercise group and interval group at the LDH level (p: 0.00). There was also a difference in LDH level between resistance exercise and HIIT group (p: 0.00).

When *Table III* is examined, it is determined that there was a difference between the pre-test post-test values of Irisin in all exercise groups (p <0.05), and in the HSP70 level, there was a significant difference in all groups except the resistance exercise group (p <0.05). When the differences was calculated as percentage, it was determined that the greatest change in irisin level occurred in the HIIT group and at the HSP70 level in the aerobic exercise group. In addition, it was found that there was a significant difference between the groups in both irisin (p: 0.00) and HSP 70 posttest levels (p: 0.00).

Discussion

In the present study, it was determined that exercises performed in different types (resistance, high intensity interval and aerobic) during eight weeks caused significant changes in blood lipid profile, enzymes associated with muscle damage, irisin and HSP 70 levels. When the changes in the lipid profile are examined; It was determined that HDL-cholesterol levels increased in all exercise groups. As with the change in cholesterol levels, the change in HDL-cholesterol is thought to be due to the positive effects of exercise. When LDL-cholesterol values were examined, it was observed that there was a significant decrease in the resistance exercise group. In addition triglyceride levels changed significantly in all exercise groups. It can be said that this situation is due to the positive effects that exercise can create on triglycerides. When the effects of exercise on lipid profile are investigated, it is seen that there are many studies. 54 healthy men voluntarily participated in one of these studies, it was stated that aerobic and anaerobic training programs applied 3 days a week for 8 weeks caused an increase in HDL-cholesterol, however it led to a decrease in LDL-cholesterol and consequently had positive effects on lipid profile (14). In another study, Wang and Xu (15) concluded that aerobic exercise led to an improvement in lipid profile in a review study aimed at evaluating the effect of aer-

obic exercise on lipids and lipoproteins. Similarly, Yao et al. (16) examined the effects of aerobic and resistance exercises on liver enzymes and blood lipids in non-alcoholic fatty liver patients. As a result of the research, they stated that similar to current study results, there was a change in HDL-cholesterol level in the resistance exercise group, and there were changes on both HDL-cholesterol and triglyceride levels in the aerobic exercise group. The other study, the effects of traditional resistance training and high-intensity resistance training were investigated on lipid profile in the elderly population. As a result of the research, it has been determined that both resistance exercise groups have positive effects on blood lipids just as in the present study (17). When the effects of exercise on markers of muscle damage are evaluated; it was observed that CK values increased significantly in the resistance exercise group and high-intensity interval group. When the differences between the groups were determined, it was determined that the resistance exercise group reached the highest value. It is thought that this situation is caused by the intensity of the exercise and the use of extra weights in the resistance exercise group. There were also increases in LDH values in all exercise groups. The greatest increase was determined in the resistance exercise group, just as in CK. This result suggests that exercise may have negative effects in the transition to regular exercise in individuals who have not exercised regularly before. As a result, it is seen that the effects of different exercise types on muscle damage markers differ according to their intensity. When studies on muscle damage are analyzed; as in the resistance exercise group in current study, it is also seen as a result of a study that was applied acutely that strength training applied maximally caused muscle damage significantly (18). In another study, Penailillo et al. (19) have reported that both concentric and eccentric cycling exercises caused increases at a low level in CK levels. In a study conducted by Brentano et al. (20) it was stated that strength training with 5 sets and 8–10 repetitions prepared according to the super set method significantly increased the CK level. Similarly, in a study involving 16 volunteer participants who did not do sports, a resistance exercise program consisting of 3 sets of 10 repetitions and 5 exercises was applied to the participants. As a result of this research applied acutely, it has been determined that resistance exercises cause muscle damage and cause significant increases in CK and LDH values (21). Pal et al. (22) have examined oxidative stress and muscle damage that occur in response to high intensity exercise in young people. CK and LDH levels were analyzed in the blood samples taken 24 and 48 hours after the applied high intensity treadmill run. As a result, they found that an increase in enzyme levels associated with muscle damage after high intensity exercise. Similar results were obtained in present study, although it was applied for a long time. Current study shows that it was determined that irisin level increased in all exercise groups. In comparisons between groups, it was determined that the lowest increase was in the control group. From this point of view, it is thought that the effect of exercise on the irisin level is closely related to the type, intensity, frequency, scope of the exercise, and the age and training level of the participants. It can be said that these factors can seriously affect the results of the studies on irisin. Previous studies are explain the relationship and interaction between exercise and irisin. In one of these studies, it was stated that regular exercise led to the activity of the irisin, although the effects of exercise and physical activity at the plasma irisin level were contradictory (23). In addition, it is stated that the level of irisin is higher in young people than in the elderly, and higher in physically active individuals than in sedentary individuals (24). Kim et al. (7) examined the effects of exercise on the irisin level on both rats and humans in their study. For this purpose, resistance (climbing exercise) applied to 19-week old rats 3 days a week for 12 weeks, and resistance (elastic band) exercises were applied to people over 65 years old 2 days a week for 12 weeks. As a result of the research, both animals and humans revealed that there was an increase in strength and an irisin levels. In another study, Reisi et al. (25) do resistance exercises change the level of the irisin? Based on the question, they have investigated that the effect of 3 sets with 5 repetitive resistance exercises applied for 8 weeks, 3 days a week on the level of irisin in rats. As a result of the study, they concluded that eight-week resistance exercises increased the level of irisin. The present study shows that HSP70 level did not change in the Resistance exercise group, but decreased in all other groups. This result shows that especially the aerobic and high-intensity interval groups adapt to the exercises more quickly. Depending on this adaptation, decreases in HSP70 levels are observed. In addition, it is thought that the use of additional weights in the resistance exercise group causes a stress in the human organism and causes this decrease to be less. As a result of the literature review, it is seen that the number of studies investigating the effect of exercise on HSP70 level is limited. Previous studies show that different results are obtained regarding the relationship between regular exercise and HSP70. Although there are still conflicting results, the general opinion is that regular exercise reduces or does not change the level of HSP70. This situation reveals the importance of the type, duration, frequency, intensity and scope of the exercise. The research group is also important. Considering the studies on HSP 70, it was concluded that 11-week strength training did not cause an increase in the trapezius muscle while there was a significant increase on HSP70 level at the vastus lateralis muscle in the biopsy samples (26). Considering the muscle groups with and without an increase, the part of body included in the resistance exercise program, the number of repetitions and the exercise intensity

could gain importance. In addition, the resting period is another important issue (27). In another study, Krause et al. (28) investigated the HSP70 level in healthy elderly individuals with combined resistance exercises using body weight and elastic bands. Within the scope of the research, participants were divided into four groups: placebo, protein supplement, placebo + exercise, and protein supplement + exercise. As a result of exercises that were continued for 12 weeks and 3 days a week, it was stated that there was no statistically significant difference in the group that exercised and was given placebo. This result is similar to the resistance exercise group of the present study.

Conclusion

As a result, considering all exercise groups; in parallel with the literature, it can be stated that regular exercise has positive positive effects in human organism. Within the scope of the current study, it was said that regular exercise programs produced positive results in blood lipids, which are considered as cardiovascular risk markers. In addition, it has been observed that irisin levels increase with exercise. It could be said that HSP 70 levels decrease especially with aerobic exercise and high-intensity interval exercise, and that stress decreases in response to exercise and hence the release of HSP 70 level decreases

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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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INCREASED MATERNAL LEPTIN LEVELS MAY BE AN INDICATOR OF SUBCLINICAL HYPOTHYROIDISM IN A NEWBORN

POVEĆANI NIVO MAJČINSKOG LEPTINA MOŽE BITI POKAZATELJ SUBKLINIČKOG HIPOTIROIDIZMA KOD NOVOROĐENČADI

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Summary

Background: Several factors may influence newborn thyroid-stimulating hormone (TSH) concentrations and cause subclinical hypothyroidism in a newborn. A sufficient level of leptin signalling is needed for the normal production of TSH and thyroid hormones by the thyroid gland. Our study aimed to investigate the correlation between maternal serum leptin concentration during the third trimester of pregnancy and newborn screening-TSH levels.
Methods: This prospective cross-sectional study was conducted in obstetrics and gynaecology clinics of a state hospital between June and August 2013. Maternal venous blood samples were collected from 270 healthy pregnant women in the third trimester just before delivery. Measurements of maternal fT3, fT4, TSH, anti-thyroid peroxidase (TPO), and anti-thyroglobulin (anti-Tg) antibodies from serum samples were performed by chemiluminescence immunoassay. Maternal serum leptin levels were determined by ELISA. Dried capillary blood spots were used to measure newborn TSH levels.
Results: Subjects were divided into two groups according to the neonatal TSH levels using a cut-point of 5.5 mIU/L. Median maternal serum leptin levels were significantly higher in newborns whose TSH levels were higher than >5.5 mIU/L [13.2 μg/L (1.3–46.5) vs 19.7 μg/L (2.4–48.5), $p<0.05$]. Serum leptin levels showed a negative

Kratak sadržaj

Uvod: Postoji nekoliko faktora koji mogu uticati na koncentraciju tiroidno-stimulišućeg hormona (TSH) kod novorođenčadi i na izazivanje subkliničkog hipotiroidizma. Da bi štitna žlezda normalno proizvodila TSH i tiroidne hormone potreban je dovoljan nivo signalizacije leptina. U našoj studiji smo želeli da istražimo korelaciju između koncentracije serumskog majčinskog leptina tokom trećeg trimestra trudnoće i nivo TSH na skriningu kod novorođenčadi.
Metode: Ova prospektivna studija preseka sprovedena je na akušersko-ginekološkim klinikama jedne državne bolnice u periodu od juna do avgusta 2013. Uzorci venske krvi majki prikupljeni su od 270 zdravih trudnica u trećem tromesečju neposredno pre porođaja. Merenje majčinskih fT3, fT4, TSH, antitireoidnih peroksidaza (TPO) i antitireoglobulinskih (anti-Tg) antitela iz serumskih uzoraka izvedeno je hemiluminiscentnim imunološkim testom. Nivo leptina u serumu kod majki je određen ELISA testom. Za merenje nivoa TSH kod novorođenčadi korišćene su suve kapilarne krvne tačke.
Rezultati: Ispitanici su podeljeni u dve grupe prema neonatalnim nivoima TSH sa graničnom vrednošću od 5,5 mIU/L. Srednji nivo serumskog leptina je bio značajno veći kod novorođenčadi čiji su nivoi TSH bili veći od > 5,5 mIU/L [13,2 μg/L (1,3–46,5) naspram 19,7 μg/L (2,4–48,5), $p < 0,05$]. Nivo leptina u serumu je pokazao nega-

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correlation with maternal fT4 (r=0.32, p<0.05), fT3 (r=0.23, p<0.05), and a positive correlation with BMI (r=0.30, p<0.05).

Conclusions: Our results suggest that high leptin levels in the third trimester of pregnancy influence maternal thyroid functions and might cause an increase in newborn TSH levels. Detection of high maternal serum leptin levels may be a reason for subclinical hypothyroidism.

Keywords: leptin, congenital hypothyroidism, maternal-fetal relations, newborn TSH (Thyroid Stimulating Hormone), maternal thyroid hormones

tivnu korelaciju sa majčinim fT4 (r = 0,32, p < 0,05), fT3 (r = 0,23, p < 0,05) i pozitivnu korelaciju sa BMI (r = 0,30 p < 0,05).

Zaključak: Naši rezultati ukazuju da visoki nivoi leptina u trećem tromesečju trudnoće utiču na funkcije štitne žlezde majke i mogu izazvati i povećati nivo TSH kod novorođenčadi. Otkrivanje visokog nivoa majčinskog leptina u serumu može biti razlog za subklinički hipotiroidizam.

Ključne reči: leptin, urođeni hipotiroidizam, odnosi majke i fetusa, TSH (hormon za stimulaciju štitne žlezde) novorođenčeta, hormoni štitne žlezde majke

Introduction

Adipose tissue is an active endocrine organ that secretes various bioactive hormones called adipokines with multiple metabolic, neuroendocrine, cardiovascular, and inflammatory functions (1). Leptin is a hormone that is exclusively secreted by adipose tissue and encoded by the *ob* gene. The primary role of leptin is to regulate energy homeostasis and suppress food intake, thereby inducing weight loss (2). In addition, leptin has a vital role in the regulation and synthesis of thyroid hormones. In the paraventricular nucleus of the hypothalamus, leptin has a regulatory role in the expression and secretion of thyrotropin-releasing hormone (TRH); thus, it has a regulatory effect on thyroid-stimulating hormone (TSH) and thyroid hormone production (3).

One of the most common preventable causes of mental retardation among newborns is congenital hypothyroidism (CH). It is possible to diagnose newborn CH at early stages with newborn CH screening programs. Agenesis or dysgenesis of the thyroid gland and thyroid hormone production deficiency are the most common causes of permanent CH (4). However, several other factors may influence newborn TSH concentrations and cause mild hypothyroidism. Iodine deficiency, maternal hypothyroidism (5), maternal medications, blocking antibodies (6), body mass index (BMI), and smoking (7), as well as weight gain during pregnancy (8), are well-known factors that may affect newborn thyroid function. A number of physiological and hormonal changes occur in pregnant women, such as changes in leptin concentrations. Increasing adipose tissue stores and secretion from the placenta results in elevated concentrations of leptin (9), which peaks at the end of the second or beginning of the third trimester and remains stable after that until delivery (10). Leptin was suggested to play the primary role as a regulator of fetal growth and development (11).

The mechanisms of how leptin influences newborn thyroid function are not known. This study investigated the correlation between screening TSH levels in the newborn and serum leptin concentration of the mother at the third trimester to test whether increased leptin levels affect newborn thyroid function.

Patients and Methods

Patients

A total of three hundred healthy full-term pregnant women (37–41 weeks of gestational age) were included in this prospective cross-sectional study. This study did not include pregnant women with multiple gestations, abnormal ultrasound findings, and metabolic diseases. In addition, 30 out of 300 participants were excluded due to missing data (n=6), premature births (<37 weeks) (n=10), and thyroid hormone abnormalities during pregnancy (n=14). All infants were born by spontaneous delivery (n=270). There was no evidence of fetal distress during labour.

Sample Collection and Laboratory Measurements

Maternal venous fasting blood (8 hours fasting) samples were collected in the 38th week of pregnancy in the morning and were kept at -80 °C until analyses. Free T4 (fT4), free T3 (fT3), TSH, anti-thyroglobulin (anti-Tg) antibody, and anti-thyroid peroxidase (TPO) antibody measurements were made using chemiluminescence immunoassay (CLIA) with Advia Chemistry XPT System (Siemens Diagnostics, Germany). Enzyme-linked immunosorbent assay (ELISA) was used to measure leptin levels (DIA source Europe SA; Nivelles, Belgium). Inter-assay coefficient variation for fT4, fT3, TSH, anti-Tg antibody, and anti-TPO antibody at low levels were 1.21, 2.35, 2.28, 9.06, and 6.43%, and at high levels were 4.55, 1.61, 2.71, 8.14, and 1.74, respectively.

Heel-prick samples of whole blood were collected on filter paper cards from all newborns within 3 to 5 days after delivery. Dried capillary blood spots were used for TSH measurements.

Statistical Analysis

Participants were divided into two groups according to newborn TSH levels using a cut-off value of 5.5 mIU/L based on the definitions of the Turkish national newborn screening program for congenital hypothyroidism (12): group 1: TSH 5.5 mIU/L,

group 2: TSH >5.5 mIU/L. Leptin levels and hormone concentrations showed a non-normal distribution; therefore, they are expressed as median and first and third quartiles, and intergroup comparisons were made using the Mann-Whitney U test. Pearson's correlation analysis was used to examine the correlation between newborn TSH and maternal leptin levels. Spearman's test was used to examine the correlations between leptin concentrations, thyroid hormones, and other clinical parameters in mothers. A p-value of <0.05 was considered statistically significant.

Results

According to newborn TSH levels, *Table I* compares demographical characteristics such as gestational and maternal age, body mass index, birth weight, and intrapartum laboratory findings. Gestational age, maternal age, body mass index, and birth weight were similar across the two groups (p>0.05). In addition, the groups showed similar findings for TSH, fT4, anti-TG, anti-TPO levels (p>0.05). On the other hand, maternal leptin levels were significantly higher [13.2 (1.3–46.5) vs 19.7 (2.4–48.5) µg/L, p<0.05] and fT3 levels were significantly lower [4.5 (3.3–5.9) vs 4.7 (3.5–6.36)

Table I Demographic and laboratory characteristics of the studied population according to the newborn TSH levels. Maternal and newborn characteristics were compared with the newborn using a TSH cut-point of 5.5 mIU/L.

Newborn TSH (mIU/L)					
	Group I n=140 TSH 5.5 (mIU/L)		Group II n=130 >5.5 (mIU/L)		
	Median	2.5–97.5 P	Median	2.5–97.5 P	p-value
Maternal age	26	19–40	26	18.9–37.2	p>0.05
Pregnancy period	38	35–42	40	32–42	p>0.05
BMI	28.5	21.1–38.2	28.4	21.2–39.2	p>0.05
Intrapartum TSH (mIU/L)	2.2	0.59–8.65	2.3	0.6–6.1	p>0.05
Intrapartum FT4 (pmol/L)	11.9	8.31–16.63	11.7	8.7–16.2	p>0.05
Intrapartum FT3 (pmol/L)	4.7	3.5–6.36	4.5	3.3–5.9	p*<0.05
Intrapartum anti TG (kIU/L)	15.8	10–122.6	26.8	10–86.2	p>0.05
Intrapartum anti TPO (kIU/L)	5.6	5–110	5.9	5–71.6	p>0.05
Intrapartum Leptin (µg/L)	13.2	1.3–46.5	19.7	2.4–48.5	p*<0.05
Baby Weight (gram)	3360	2042–4253	3280	2647–3923	p>0.05

Data arepresented as median, first and third interquertile ranges (IQR).
p<0.05; Statistical significance of the difference between group I and II.

Table II Spearman's ρ coefficients of correlations between maternal serum leptin levels and other parameters.

	Correlation (r)	*p-value*
Maternal age	-0.2	>0.05
Pregnancy period	0.014	>0.05
Intrapartum		
BMI	0.30	**<0.001**
TSH (mIU/L)	-0.03	>0.05
fT4 (pmol/L)	-0.32	**<0.001**
fT3 (pmol/L)	-0.23	<0.05
Anti-TG (kIU/L)	-0.049	**>0.05**
Anti-TPO (kIU/L)	-0.077	>0.05
Newborn		
Weight (kg)	0.049	>0.05
TSH (mIU/L)	0.16	**<0.05**

Bolded values indicate statistical significance.
leptın as a novel placenta-derıved hormone ın humans

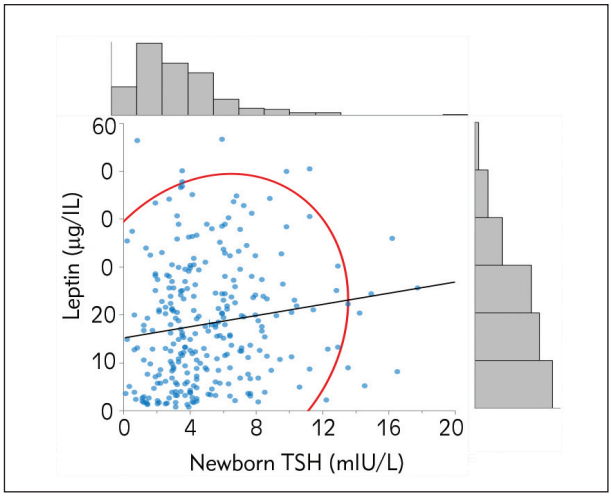

Figure 1 Correlation between maternal leptin and newborn TSH levels. Pearson's correlation coefficient was determined as 0.16 (p<0.05). The frequency histogram shows the number of values (n) in the corresponding axis. The red curve represents 95% CI of the distribution.

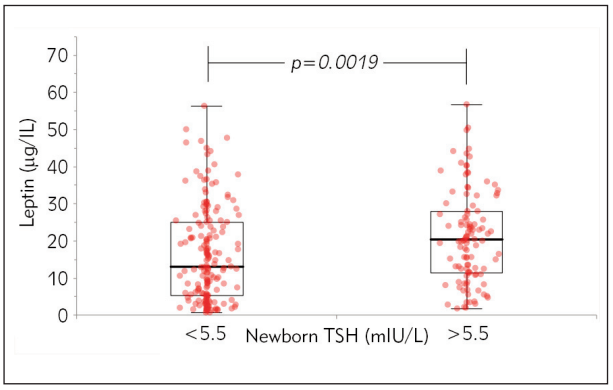

Figure 2 Relationship between newborn TSH and maternal serum leptin levels. p<0.05 was considered as a significant difference.

pmol/L, p<0.05] in group 2 when compared to group 1 (*Table I*).

Correlations with leptin levels and other parameters are shown in *Table II*. Serum leptin levels had negative correlations with intrapartum fT4, fT3, and positive correlations with BMI and newborn TSH levels. *Figure 1* demonstrates the positive correlation between maternal serum leptin concentrations and newborn TSH levels (r=0.16, p<0.05).

Discussion

This study examined the relations between maternal serum leptin and thyroid hormone levels in the third trimester and TSH levels of the newborn measured from capillary blood samples and found a positive correlation between newborn TSH levels and maternal leptin levels. This is the first study to focus on the relation between third-trimester maternal serum leptin levels and the thyroid function of the newborn.

T3 and T4 circulating in the fetus are of maternal origin during the first trimester; whereas, developing the fetal thyroid gland increasingly contributes to the levels of the thyroid hormones from the beginning of the second trimester. Thyroid hormones have crucial roles in healthy fetal growth and development (13). Several environmental factors may affect the thyroid function of the mother and the newborn (14). Iodine deficiency (15), maternal thyroid hormones (5), low birth weight infants (16), pregnancy duration, maternal weight gain during pregnancy (8), high BMI, and lifetime smoking behaviour (7) are among the known factors associated with subclinical hypothyroidism in the newborn. One of the well-established causes of subclinical hypothyroidism during infancy is iodine deficiency; such that world Health Organization (WHO) recommended the use of a percentage of newborns with a TSH >5 mIU/L as a marker for population iodine deficiency (17). The Turkish Newborn Screening Program recommends spot TSH levels of 5.5 mIU/L as a threshold for detecting congenital hypothyroidism (12, 15). In our study, leptin levels were significantly higher in mothers of newborns whose TSH levels were higher than >5.5 mIU/L. Our results showed that increased maternal leptin levels could influence newborn TSH levels, which can be one of the main reasons for subclinical hypothyroidism in the newborn.

Leptin has modulatory roles in critical processes such as invasion, proliferation, protein synthesis, and placental cell apoptosis during early pregnancy (18). In the later stages of a healthy pregnancy, increasing nutrient availability and regulating fetal growth is required. However, elevated leptin concentrations may represent a state of leptin resistance, which may be due to reduced bioactivity or reduced sensitivity at the hypothalamic level (19). On the other hand, leptin overproduction by the placenta is associated with diabetes mellitus (20), hypertension (21), high BMI (7), and weight gain during pregnancy (8, 22). Diabetes, obesity, and inflammation seem to be associated with the development of peripheral leptin resistance, which causes impaired leptin signalling in the brain (19). Therefore, it is crucial to understand both the physiological and pathological effects of increased leptin levels during pregnancy on the mother and the newborn. In the present study, third-trimester leptin levels were positively correlated with BMI and maternal weight. These results are consistent with the findings of Sattar et al. (23), who found a positive correlation between BMI and third-trimester leptin levels. An increase in leptin levels is expected during pregnancy due to fat tissue accumulation (23).

Additionally, Shaarawy et al. (24) reported a positive correlation between weight gain and BMI as well as third-trimester leptin levels in pregnant women. However, in contrast with our findings, they did not find a significant difference between pregnant women with high and normal BMI in terms of leptin levels. The results of that study supported the suggestion that leptin release is mainly placenta-based during pregnancy (25).

Increased weight gain during pregnancy results in higher fetal weight gain (26). Although leptin levels are known to increase with increasing fat tissue, we were not able to find a correlation between maternal leptin levels and the birthweight of the newborn. Similarly, Serapio et al. (27) found no correlation between maternal leptin levels and weight at birth. However, Manderson et al. (28) found an association between birth weight and cord leptin levels. On the other hand, Stefaniak et al. (29) found an association between birth weight and cord leptin levels (r=0.23; p=0.00), but not between the birthweight and maternal leptin levels. These studies support that cord leptin may increase fetal adipose tissue.

Various factors such as autoimmunity, fertility, hormones like estrogen, gender, insulin resistance, and high BMI affect the relationship between thyroid function and leptin (30). The relation between leptin levels and thyroid function were examined in many studies. In our study, we found inverse relations between fT4/fT3 and leptin levels measured in the third trimester. However, we could not find correlations between maternal TSH hormone levels, levels of anti-TG and TPO, and leptin hormone. Pop et al. (22) recently examined the adverse effects of high BMI during pregnancy on thyroid function. In that study, pregnant women who gained much weight were found to have higher TSH levels and lower FT4 levels than pregnant women with a healthy weight increase. Their study speculated that the excessive leptin released from fat tissue might have affected the thyroid function of pregnant women (22). Our results are in line with that study.

In the study by Iacobellis et al. (31), a positive correlation was found between TSH and leptin levels adjusted for BMI in euthyroid obese women ($r=0.33$ $p=0.03$). On the contrary, Betry et al. (32) showed a positive association between leptin and TSH levels independent of BMI in healthy individuals ($p<0.001$). Several studies showed conflicting results; some researchers showed a negative correlation, whereas others could not show a significant modulatory role for leptin on thyroid function (30).

In our study, the median leptin level was 19 μg/L (IQR: 1.546) in the third trimester, which was similar to the distribution of leptin levels in the study by Okdemir et al. (33). In the cited study, the median leptin level was 7.32 μg/L (range, 1.00–33.19) and 12.54 μg/L (range, 1.07–45.75) in pregnant women with healthy and excess weight gain, respectively. On the other hand, Mazaki-Yovi's research found higher leptin levels at the third trimester: 30.2 μg/L (range, 16.9–43.5) (34). These suggest that BMI and weight gain, as well as ethnicity, may affect leptin levels.

To the best of our knowledge, this is the first study revealing that maternal leptin levels may be correlated with maternal thyroid functions and increased newborn TSH levels and subclinical hypothyroidism. This study was limited in such a way that it was a cross-sectional study in which maternal blood was obtained only in the third trimester, and pregnancies were not regularly followed up. Larger prospective studies are warranted to elucidate the clinical relevance of our findings, focusing on the maternal thyroid functions, leptin levels, and weight gain during the first and second trimesters.

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Ethical approval. The study protocol was approved by the Acibadem University Ethics Committee (ATADEK 2013-507), and the study was conducted following the Declaration of Helsinki and its later amendments. All subjects gave informed consent before enrolment into the study.

Consent to participate. All participants received informed consent, and a signed copy was filed.

Consent for publication. All authors read and approved the final manuscript.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Data deposition. The data will not be deposited.

Geolocation information. Not available.

Supplemental online material. Not available.

Health and safety. Not available.

Conflict of interest statement

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

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IS SERUM FIBROBLAST GROWTH FACTOR 21 ASSOCIATED WITH THE SEVERITY OR PRESENCE OF CORONARY ARTERY DISEASE?

DA LI JE RAST FIBROBLASTNOG SERUMSKOG FAKTORA 21 POVEZAN SA TEŽINOM ILI PRISUSTVOM KORONARNOG ARTERIJSKOG OBOLJENJA

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Summary

Background: Recent studies have shown that increased circulating concentrations of fibroblast growth factor 21 (FGF21) are associated with obesity, metabolic disorder, and atherosclerosis. However the relationship between FGF21 and coronary artery disease (CAD) is controversial This study was planned to investigate the role of FGF21 in CAD development and CAD severity.
Methods: Seventy-eight patients with stable angina pectoris (SAP) (lesion positive) and 40 control patients (lesion negative) with similar cardiovascular risk factors were included in the study. Serum FGF21 levels were measured by ELISA method. CAD severity was evaluated by using SYNTAX and GENSINI risk scores.
Results: FGF21 concentrations were found significantly higher in the SAP group than in the control group. [101.18 ± 141.62 vs. 47.93 ± 58.74 pg/mL; p = 0.03], no correlation was found between the SYNTAX (r = 0.146 and p = 0.134) and GENSINI (r = 0.211 and p = 0.084) scores with serum FGF21 levels. There was a negative relationship between serum FGF21 and serum HDL-C levels in correlation analysis (r = - 0.272; p = 0.026).
Conclusions: The serum FGF21 levels are different between SAP and control patients. FGF21 is a marker for CAD diagnosis, but not for the evaluation of CAD severity.

Keywords: FGF21, stable angina pectoris, coronary artery disease

Kratak sadržaj

Uvod: Poslednja istraživanja su pokazala da je povećanje koncentracija u cirkulaciji fibroblastnog faktora rasta 21 (FGF21) praćeno sa gojaznošću, metaboličkim poremećajem i aterosklerzom. Međutim, odnos između FGF21 i koronarnog arterijskog oboljenja (KAO) je kontraverzan. U ovom izučavanju je planirano da se ispita uloga FGF21 u razvoju KAO kao i težina samog oboljenja.
Metode: U izučavanje je uključeno sedamdeset osam pacijenata sa stabilnom anginom pektoris (SAP) (pozitivna lezija) i 40 kontrolnih pacijenata (lezija negativna) sa sličnim faktorima rizika. Nivoi serumskog FGF21 mereni su ELISA metodom. Težina KAO procenjivana je SYNTAX i GENSI faktorima rizika.
Rezultati: Koncentracije FGF21 bile su značajno više u grupi sa SAP nego u kontrolnoj grupi. [101,18 ± 141,62 vs. 47,93 ± 58,74 pg/mL; p = 0,03]. Nije utvrđena korelacija između SYNTAX (r = 0,146 i p = 0,134) i GENSI (r = 0,211 i p = 9,084) skorova sa nivoima serumskog FGF21. Nađen je negativan odnos između serumskog FGF21 i nivoa HDL-C u serumu primenom korelacione analize (r = - 0,272; p = 0,026).
Zaključak: Nivoi FGF21 u serumu su različiti kod SAP i kontrolnih pacijenata. FGF21 je marker za dijagnostikovanje KAO, ali ne i za procenu težine KAO.

Ključne reči: FGF21, stabilna angina pektoris, koronarno arterijsko oboljenje

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Introduction

Cardiovascular diseases (CVDs) are among the most common causes of morbidity and mortality (1). Despite the developments in evidence-based medical treatments and revascularisation strategies, CVD still continues to be a global health problem and leads to a significant burden on the health system (2, 3). Atherosclerosis has been assumed to be a chronic inflammatory process, and the immune system's response to oxidised lipoproteins, in particular, initiates this process (4, 5). Many inflammatory biomarkers, especially cytokines, have been investigated for the prevention and early detection of CVD (2, 6).

Adipose tissue secretes many bioactive adipokines. These adipokines not only affect the metabolism, but also have many effects on the cardiovascular system (3). Fibroblast growth factor (FGF) is a hormone-like structure that plays a role in cell proliferation, hyperplasia, differentiation, and angiogenesis (7). FGF21 is a member of the FGF endocrine subfamily.

High serum FGF21 levels have been shown in obese patients with type 2 diabetes mellitus and metabolic syndrome (10). Although the relationship between FGF21 and CVD has been shown in some studies, its role in the pathophysiology of CAD is still not fully understood (11). We aimed to investigate the role of FGF21 in CAD development and CAD severity in patients with SAP.

Materials and Method

Patient population

In this cross-sectional case-control study, the patients who underwent coronary angiography with suspicion of CAD at the Pamukkale University Cardiology Department between January 1, 2018 and January 5, 2018 were included. All patients were evaluated in terms of age, gender, history, family history, smoking, presence of comorbidities, and drug use before coronary angiography. The physical examinations were performed. A comprehensive, two-dimensional transthoracic echocardiography (2D-TTE) examination (GE vivid S5) was performed for all patients. The left ventricular ejection fraction (LVEF) was calculated by the bi-planar Simpson method.

Previous acute coronary syndrome (ACS), documented CAD, chronic or inflammatory systemic diseases, autoimmune diseases, malignancies, severe heart valve diseases, a left ventricular ejection fraction <50%, chronic kidney failure [Cockcroft-Gault formula calculated glomerular filtration rate 90 mL/min1.73 m^2)], haematological disorders, thyroid dysfunctions, pregnancy, and suspicious pericarditis or myocarditis were determined as exclusion criteria. Stable angina pectoris (SAP) was defined as the presence of typical chest pain or equivalent symptoms during exercise and a positive treadmill test or visualisation of ischaemia on stress echocardiography and myocardial perfusion scintigraphy. Patients who did not have any atherosclerotic lesions on coronary angiography were included in the control group. This study was carried out with the approval of the Institutional Review Board of the Pamukkale University Medical Faculty, and informed consent was obtained from all registered patients.

Blood samples

Peripheral venous blood samples of the patients were collected after eight to 12 hours of fasting. Fasting serum glucose (FSG), creatinine, triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and C-reactive protein (CRP) levels were analysed with the electrochemiluminescence method on a Cobas 702 autoanalyser (Roche Diagnostics GmbH, Mannheim, Germany). The hemogram parameters were analysed with the Mindray BC-6800 system through the electrical impedance method.

FGF21 level measurement

Blood samples were centrifuged at 3,000 x g for 10 minutes, and the serum was stored at -80 degrees Celsius (°C) until the analysis. Serum FGF21 concentrations were measured in accordance with the manufacturer's protocol with a commercial ELISA kit (YLA0238HU, Shanghai YL Biotech Co., Ltd). The concentration range of the assay was 5 to 1,500 pg/mL. The absorbance was measured at 450 nm with a Biotek Elx800 microplate reader (BioTek Instruments Inc., U.S.A.). The data were processed with the Gen5 Data Analysis software (BioTek Instruments Inc., U.S.A.). The variation coefficients within and between experiments were determined as < (8%) and < (12%), respectively.

Coronary angiography

Coronary angiography was performed using standard tech-nique (GE Innova 2100) at the Pamukkale University Cardiology Department Selective cine angiographic images of the coronaries were recorded with a digital angiographic system. The significant CAD was defined as narrowing of the vessel lumen diameter> 50%, including the three major coronary arteries and the first branches of the left anterior descending artery or circumflex artery. Diagnostic angiograms were recorded using a digital media viewer and their analysis was randomly performed by two experienced cardiologists, blinded to the study protocol.

Angiographic risk scoring

Atherosclerotic lesion severity was evaluated by using SYNTAX and GENSINI scores. SYNTAX score is a scoring system developed based on the number, location, function and complexity of the coronary lesions, calculated for stenosis diameter of 50% or greater in vessels of 1.5 mm or more in diameter. The final online updated version [2.11] was used (www.syntaxscore.com) (12). In the GENSINI scoring system, the lesions were classified as 0–25%, 26–50%, 51–75%, 76–90% according to the degree of angiographic stenosis, and were scored 1,2,4,8,16 and 32 points, respectively. Then the score was multiplied by the coefficient defined according to the localization of the lesion. (Left main coronary artery, 5; proximal segment of left anterior descending (LAD) coronary artery or left circumflex (LCx) artery, 2.5; middle segment of LAD or LCx coronary artery, 1.5; distal segment of LAD and LCx, first diagonal branch, first marginal branch, right coronary artery, posterior descending artery, 1; and intermediate artery and second diagonal and second large marginal branches, 0.5) (13).

Statistical analysis

Statistical analysis were performed on SPSS version 20.0. Normality check for continuous variables were performed by Kolmogorov-Smirnov test and normality assumption was proven for variables and subgroups. Chi-square test was used for categorical variables and independent sample t test was used for continuous variables. Pearson correlation was used to determine relationship between continuous variables. Statistical significance level (alpha) was determined as 0.05.

Results

A total of 118 consecutive patients were enrolled in the study. The baseline demographic, clinical features, laboratory test values and other parameters of the groups are shown in *Table I*. The patients in the SAP group were significantly older than those in the control group (63.29 ± 10.79 vs 58.88 ± 11.49; p= 0.04), and there were more male patients in the SAP group (74% vs. 58%; p=0.015). The incidences of cardiovascular risk factors, such as hypertension, diabetes and smoking, were similar in both

Table I Baseline demographics – clinical characteristics, laboratory and angiographic parameters.

Baseline demographics and clinical characteristics	SAP group (lesion +) (n=78)	Control group (lesion -) (n=40)	p value
Age (y)	63.29 ± 10.79	58.88 ± 11.49	0.04
Males, n (%)	58 (74%)	21 (53%)	0.015
Hypertension, n (%)	46 (58%)	24 (60%)	0.915
Diabetes Mellitus, n (%)	29 (37%)	12 (30%)	0.438
Smoking, n (%)	17 (21%)	7 (18%)	0.583
LVEF (%)	53.45 ± 9.16	57.03 ± 6.23	0.03
Laboratory data			
FSG (mmol/L)	7.04 ± 2.73	6.64 ± 1.92	0.41
Creatinine (mmol/L)	88.4 ± 75.14	87.51 ± 27.40	0.51
Haemoglobin (g/L)	133.2 ± 19.0	135.3 ± 17.3	0.56
WBC ($\times 10^9$/L)	9.34 ± 2.86	9.21 ± 2.89	0.82
TChol (mmol/L)	4.62 ± 1.32	4.91 ± 1.60	0.29
LDL-C (mmol/L)	2.74 ± 1.15	2.79 ± 1.29	0.85
HDL-C (mmol/L)	1.08 ± 0.30	1.42 ± 0.89	<0.00
TG (mmol/L)	1.79 ± 1.24	1.65 ± 0.99	0.54
CRP (mg/mL)	12.9 ± 27.1	5.8 ± 5.2	0.12
FGF21 (pg/mL)	101.18 ± 141.62	47.93 ± 58.74	0.03
Angiographic data			
SYNTAX score	23.80 ± 9.53		
GENSINI score	50.01 ± 32.15		

Abbreviations: FSG, fasting serum glucose; WBC, white blood cells; TChol, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; CRP, C-reactive protein; FGF21, fibroblast growth factor 21; LVEF, left ventricular ejection fraction, SAP, Stable angina pectoris.

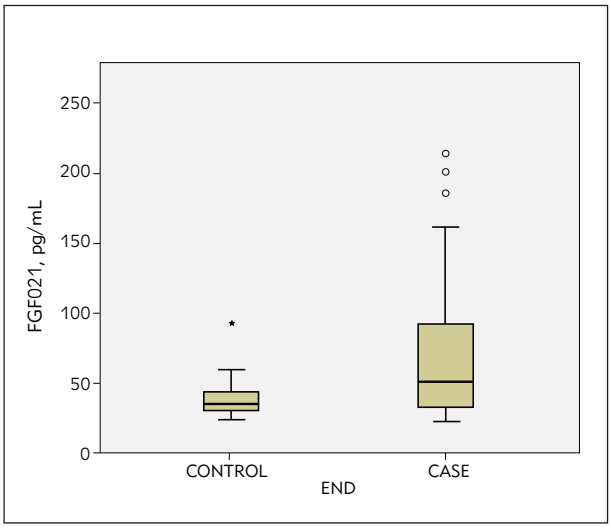

Figure 1 The comparison of serum FGF21 levels in both groups.

Table II The correlation of FGF21 levels with baseline clinical, biochemical, hemorheological and other parameters.

FGF21	Correlation	Sig. (2-tailed)
Age	0.147	0.232
LVEF	-0.075	0.544
WBC	0.129	0.293
Hemoglobin	0.046	0.707
FSG	-0.074	0.547
Creatinin	-0.103	0.402
TChol	0.046	0.712
LDL-C	0.007	0.956
HDL-C	-0.272*	0.026
TG	-0.082	0.510
CRP	-0.081	0.512
SYNTAX	0.146	0.234
GENSİNİ	0.211	0.084

Abbreviations: FSG, fasting serum glucose; WBC, white blood cells; TChol, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; CRP, C-reactive protein; FGF21, fibroblast growth factor 21; LVEF, left ventricular ejection fraction

groups (p> 0.05). LVEF was different between the SAP and control groups (53.45 ± 9.16% vs. 57.03 ± 6.23%, respectively; p = 0.03), and the SAP group had lower serum HDL-C levels (41.57 ± 11.40 vs. 54.90 ± 34.47; p = 0.02).

Serum FGF21 levels of the SAP and control groups were measured as 101.18 ±141.62 pg /mL and 47.93 ± 58.74 pg/mL, respectively, and showing significantly higher levels in the SAP group (p= 0.03), (*Figure 1*). Levels of another inflammatory biomarker, CRP, were similar in both groups (12.9± 27.1 vs 5.8 ± 5.2 mg/mL; p= 0.12). There was a significant and negative relationship between serum FGF21 and serum HDL-C levels based on correlation analysis (r = -0.272, p = 0.026), (*Table II*). The SYNTAX and GENSINI scores of the SAP group were 23.80 ± 9.53 and 50.01 ± 32.15, respectively. However, there was no correlation between the CAD severity risk scores and serum FGF21 levels (r = 0.146, p = 0.134 and r = 0.211 and p = 0.084, respectively), (*Figures 2* and *3*).

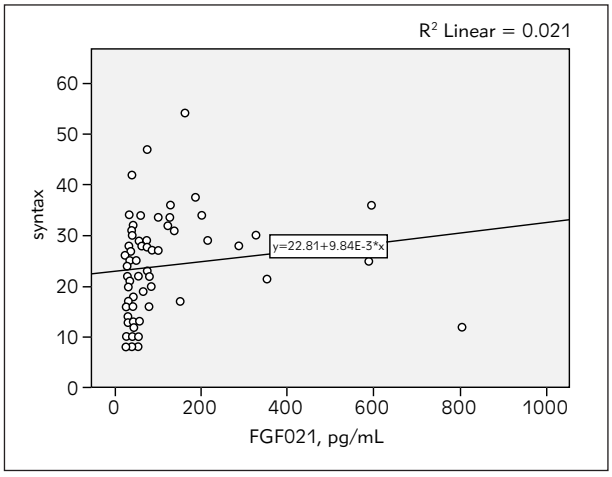

Figure 2 The correlation between FGF21 levels and SYNTAX score.

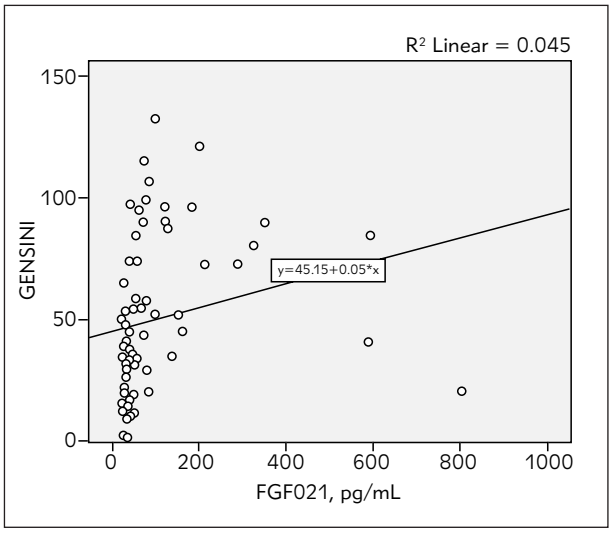

Figure 3 The correlation between FGF21 levels and GENSINI Risk score.

Discussion

In this study, we evaluated the role of FGF21 in the diagnosis and severity of CAD. Although, we found a significant difference between the serum FGF21 levels and the diagnosis of CAD in the SAP

and control groups, there was no correlation between the serum FGF21 levels and the SYNTAX and GENSINI scores.

Several studies have shown that serum FGF21 levels may increase in cardiovascular risk factors such as obesity, hypertension and type 2 diabetes. However, the results of human studies investigating the physiological functions of FGF21 have been inconsistent and contradictory (14, 15). In a study involving 253 Chinese patients, high serum FGF21 levels were an independent risk factor for CAD (8). An et al. (16) found that FGF21 levels increased in diabetic patients and high levels were associated with diabetic complications, including carotid plaques. In another study with 235 patients, the CAD group had higher serum FGF21 compared to non-CAD group (8). Consistent with these studies, we found a statistically significant difference between the serum FGF21 levels of the SAP and control groups. However, Lee et al. (17) did not find significant differences between serum FGF21 levels in patients with or without CAD, using coronary CT angiography to diagnose CAD. The authors explained that this result was due to group matchings in terms of body mass index, age, inflammatory, and lipid markers, and low serum FGF21 levels (17). A study that investigated the role of FGF21 in SAP found higher FGF21 levels (SAP: 323.16 ± 434.66 vs. control: 266.46 ± 417.13 pg/mL; $p = 0.039$), similar to our study; however, multiple regression analyses showed that FGF21 levels could not be used as a marker for SAP (18). In another study, serum FGF21 levels were found higher in patients with unstable angina pectoris than in the SAP and control group, and there was no difference between serum FGF21 levels in SAP and control subjects unlike to our study (19). This may be due to study design differences, heterogeneities in patients' cardiovascular risk profiles, insulin resistance, drug use, body mass index, visceral fat distribution, and gender distribution.

A few studies have investigated the relationship between CAD severity and FGF21. In a study of 417 patients, the serum FGF21 levels of the patients with CAD and without CAD were similar regardless of the presence of diabetes, and there was no association between the serum FGF21 levels and the severity of CAD defined by the number of stenotic vessels and segments (20). In Park et al. (21) study, no relationship was found between serum FGF21 levels and the severity of CAD determined by SYNTAX scores. In another study, Kim et al. (22) initially observed a significant correlation between serum FGF21 levels and GENSINI and EXTENT scores of 120 patients; however, in the final analysis, they found that there was no relationship between FGF21 levels and the risk scores in diabetic patients ($r = 0.332$, $p = 0.055$; and $r = 0.296$, $p = 0.084$, respectively). Similar to these studies, we did not find any relationship between serum FGF21 levels and SYNTAX and GENSINI scores.

In this study, we found a negative correlation between FGF21 levels and only HDL-C. No relationship was observed between FGF21 and parameters such as FSG, CRP, non-HDL-C lipid values, creatinine, and LVEF. Indeed, Matuszek et al. (23) confirmed that circulating FGF21 levels had a negative correlation with HDL-C. Similar to our study, Lee et al. (17) failed to demonstrate an association of serum FGF21 levels with FSG and CRP values, and suggested that higher FGF21 levels may be a reflection of hyperinsulinemia rather than high serum glucose levels.

Limitations of the study

The main limitations of our study include its cross-sectional design, small sample, and failure to determine long-term prognoses. The correlation analysis results may also be affected by various uncorrected confounding factors of life. The study was also limited by the collection of blood samples during the stable period before angiography and the inability to measure FGF21 levels simultaneously with the occurrence of chest pain, as acute angina may increase serum FGF21 levels.

Conclusion

Although the serum FGF21 levels were different between patients with and without SAP in this study, there was no significant relationship between angiographic scores of CAD severity and FGF21 levels. One of the strengths of the study is that FGF21 levels, lipid profile, and coronary artery disease severity were evaluated simultaneously. However, larger studies are needed to determine the role of FGF21 in CAD development and progression.

Conflict of interest statement

The authors reported no conflict of interest regarding the publication of this article.

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VITAMIN D IN HEALTHY TUNISIAN POPULATION: PRELIMINARY RESULTS

VITAMIN D U ZDRAVOJ POPULACIJI TUNISA: PRELIMINARNI REZULTATI

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Summary

Background: Vitamin D deficiency is one of the most common medical conditions worldwide. In Tunisia, several studies evaluated Vitamin D status, but this was concerning specific populations (pregnant women, obese or diabetic patients and children with asthma). The only study that evaluated Vitamin D status in a healthy Tunisian population was conducted by Meddeb and associeties in 2002. The update of data available, based on the currently recommended limits, is necessary. This study aimed to estimate the prevalence of hypovitaminosis D in a healthy Tunisian population, and correlate the values with potential risk factors.
Methods: It was conducted on 209 Tunisian healthy subjects. Data collected included clinical characteristics and dietary intakes. We measured 25-hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), glycemia, creatinine, calcium, phosphorus, and alkaline phosphatase concentrations. Hypovitaminosis D was retained for 25(OH)D concentrations <75 nmol/L. Vitamin D deficiency was defined by 25(OH)D concentrations <25 nmol/L.
Results: The prevalence of hypovitaminosis D and vitamin D deficiency were respectively 92.3% and 47.6%. The main factors that were significantly associated with low vitamin D

Kratak sadržaj

Uvod: Deficijencija vitamina D je jedno od najčešćih medicinskih stanja širom sveta. U Tunisu je bilo nekoliko proučavanja statusa vitamina D, koja su obuhvatila specifične populacije (trudnice, gojazne ili dijabetične osobe i decu sa astmom). Jedino je Meddab sa saradnicima 2002. godine proučavao status vitamina D kod zdravih osoba. Da bi se dopunili raspoloživi podaci o stanju vitamina D neophodna su dodatna istraživanja. Ovo izučavanje je imalo za cilj da proceni prevalenciju hipovitaminoze D u zdrave populacije u Tunisu, kao i da se vrednosti procene u odnosu na potencijalne faktore rizika.
Metode: Ispitivano je 209 zdravih osoba u Tunisu. Objedinjeni su podaci kliničkih karakteristika kao i načina ishrane. Mereni su 25-hidroksivitamin D (25(OH)D), paratireoidni hormon (PTH), glikemija, kreatinin, kalcijum, fosfor i alkalna fosfataza. Hipovitaminoza 25(OH)D je definisana kao koncentracija < 75 nmol/L, a deficijencija kao koncentracija < 25 nmol/L.
Rezultati: Prevalencija hipovitaminoze D i deficijencija vitamina D iznosile su 92,3% i 47,6%. Glavni faktori koji su bili pridruženi sa nivoima vitamina D u našoj mulitivarijantnoj analizi bili su način života, život u ruralnim sredinama i

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List of abbreviations: 25(OH)D: 25-hydroxyvitamin D; BMI: Bone Mass Index; GRIO: Group of Research and information on osteoporosis; PTH: Parathyroid hormone; OR: Odds Ratio

levels in our multivariate analysis were veiling, living in rural areas and sunscreen use. However, sex, age, socioeconomic level, phototype, solar exposure score, smoking and bone mass index, were not statistically associated with hypovitaminosis D. The study of relationship between vitamin D status and serum PTH levels showed a significative and negative correlation ($P < 0.005$).

Conclusions: Given the high prevalence of vitamin D, an adapted health policy is essential. A widespread vitamin D supplementation and food fortification seems to be necessary in Tunisia.

Keywords: healthy volunteers, parathyroid hormone, prevalence, Tunisia, vitamin D deficiency

zaštita od sunca. Međutim, pol, starost, socioekonomski status, fototip, izlaganje suncu i indeks koštane mase nisu bili statistički značajni za hipovitaminozu D. Odnos vitamina D i nivoa PTH je bio u značajno negativnoj korelaciji ($P < 0,005$).

Zaključak: Značajno je praćenje vitamina D, kao i davanje vitamina D kao suplementa populaciji u Tunisu.

Ključne reči: zdravi volonteri, paratireoidni hormon, prevalencija, Tunis, deficit vitamina D

Introduction

The importance of vitamin D in phosphocalcic metabolism has been clearly established. It helps prevent the risk of rickets, fractures, osteopenia and osteoporosis (1).

The increased interest in this hormone is due to the discovery of the ubiquitous tissue distribution of vitamin D receptors (2). Its involvement goes beyond bone metabolism to act at different levels of human physiology: preventing cancers (3), cardiovascular protection (4), regulation of the immune system (5). In many recent studies, vitamin D status has been also correlated with the severity of Corona Virus Disease 2019 (6, 7).

Vitamin D deficiency is considered to be one of the most common medical conditions worldwide (8). Numerous studies around the world have shown a high prevalence of vitamin D deficiency (1) in different degrees, going up to 100% in certain populations (9).

In Tunisia, several studies have evaluated Vitamin D status, but this has concerned specific populations such as pregnant women, obese or diabetic patients, intensive care unit patients and children with asthma. The only study that evaluated Vitamin D deficiency in a healthy Tunisian population was conducted by Meddeb et al. (10) in 2002. In this study, hypovitaminosis D was defined by serum 25-hydroxyvitamin D concentrations under the limit of 38 nmol/L, which has been considered, since many years, insufficient to ensure optimal effect of vitamin D on health (11). A wide optimal range for vitamin D is reported, and differences of opinion exist as to the definition of vitamin deficiency. Evidence suggests that low serum vitamin D concentrations are associated with an increase in parathyroid hormone (PTH). Yet, the normal vitamin concentrations could be considered as concentrations below which serum PTH increases. These levels are still a matter of debate. The update of data available about vitamin D status in a healthy Tunisian population based on the currently recommended limits is necessary.

This study aimed to estimate the prevalence of hypovitaminosis D in a healthy Tunisian population, and correlate the values with potential risk factors, and PTH levels.

Materials and Methods

Subjects

A transversal descriptive study was conducted during the spring of 2017. A total of 209 volunteer subjects aged between 18 and 65 years were selected from the paramedical staff of the Pasteur Institute of Tunis, their relatives, and the employees followed-up by the occupational doctors of the Charles Nicolle Hospital in Tunis.

Non-inclusion criteria were pregnancy, breastfeeding, renal failure, liver, digestive or endocrine illness, high blood pressure, patients with granulomatosis, mycobacterial infection or neoplasia, intake of medication which could possibly interfere with phosphate and calcium metabolism.

Exclusion criteria were the presence of high blood pressure on somatic examination and the abnormalities in blood test results such as renal failure, glycoregulatory disorders or hyperparathyroidism.

Clinical Characteristics

The data collected included age, sex, socio-economic level (classified as low, medium or high depending on whether the participant was a worker, middle manager or senior manager), habitat (urban or rural), habits (smoking, veiling, sunscreen use), solar exposure level: evaluated by a simplified score (12), a solar exposure score ≥ 3 reflects a sufficient solar exposure.

Physical examination included determination of subjects' phototypes according to Fitzpatrick classification (13), weight, high, size, calculation of the body mass index, waist size and blood pressure measurement.

Dietary Interview

A dietary interview based on the frequency of consumption of vitamin D and calcium was conducted to evaluate nutritional intakes. The results were interpreted according to the daily intake recommended by the national health security agency.

Laboratory Studies

The participants were asked to fast for at least 12 h, whereupon morning fasting venous blood samples were drawn for biochemical analyses.

Routine biochemical measurements (glycemia, creatinine, calcium, phosphorus, proteins and alkaline phosphatase) were realized on Cobas Integra 400® analyzer (Roche Diagnostics, Swiss).

Serum 25(OH)D levels and serum parathyroid hormone levels (PTH) were both measured by Electrochemiluminescence Technology (Cobas e411 analyzer, Germany, Roche Diagnostics®). This method is an immunological test for a quantitative determination of 25(OH)D and PTH concentrations based on a competition principle for 25(OH)D and a sandwich principle for the PTH. The inter-assay coefficient of variation for 25(OH)D and PTH were lower than 11% and 4% respectively.

Serum PTH concentrations between 1.6 and 6.9 pmol/L were considered normal. PTH concentrations above or below these ranges were classified as being high and low, respectively.

Vitamin D concentrations were interpreted according to the standards recommended by the research and information group on osteoporosis (*Table I*) (14).

The choice of 75 nmol/L as a cutoff value agrees with previous studies that demonstrated secondary hyperparathyroidism, increased bone turnover, and decreased bone density at the hip at 25(OH)D serum concentrations below this level (15, 16).

Table I Recommended 25-hydroxyvitamin D concentrations according to the research and information group on osteoporosis.

	25 (OH) D levels	
UNIT	ng/mL	nmol/L
Hypovitaminosis D		
– Deficiency	< 10	< 25
– Insufficiency	10–29	25–74
Optimal level	≥ 30	≥ 75

Statistical Analysis

All statistical analyses were performed using the IBM SPSS STATISTICS Version 20 (SPSS Inc., Chicago, USA).

The comparison of two means on independent series was carried out by Student's t-test, and in the event of a small number, by the non-parametric test of Mann and Whitney.

The comparison of several means on independent series was carried out by Snedecor's F test of parametric variance analysis, and in the event of a small number, by Kruskall-Wallis' H-test of non-parametric variance analysis.

Pearson's Chi-square test was used for comparing the frequencies on independent series.

Correlations between two quantitative variables have been studied by Pearson's correlation coefficient, and in case of invalidity, by the ranks' correlation coefficient for Spearman.

Risk factors research was carried out by calculating the Odds Ratio (OR).

A multivariate analysis in logistic regression (backward stepwise method) was carried out to identify risk factors independently with the event.

A statistical significance level was fixed at 0.05.

Ethical considerations

All participants gave informed consent. Participation was voluntary, confidential ant not remunerated. The study protocol was approved by the ethics committee of the Pasteur Institute of Tunis.

Results

Two hundred and nine subjects were initially recruited. Thirteen were excluded for different reasons: one subject had high blood pressure, eight subjects had glucoregulatory disorders and four subjects had hyperparathyroidism. The study population thus consisted of 196 subjects whose main sociodemographic and biochemical characteristics are summarized in *Table II*.

The accumulated prevalence of hypovitaminosis D (25(OH)D < 75 nmol/L) and vitamin D deficiency (25(OH)D < 25 nmol/L) were respectively 92.3% and 47.6. Only 15 subjects had optimal concentrations of serum 25(OH)D.

The prevalence of vitamin D deficiency was significantly higher in women ($P < 0.001$) with an OR of 3.26 in univariate study. Multivariate analysis showed that female was not independently related to the risk of vitamin D deficiency.

Table II Sociodemographic and biochemical characteristics of the sample (N=196).

VARIABLE	VALUE
Mean age ± SD (years)	36.9 ± 6.4
Extreme ages (years)	22–56
Sex (F/M)	103/ 93
Female (%)	52
Habitat (urban/rural)	174/22
Urban habitat (%)	88.8
Socioeconomic level – Low (%) – Medium (%) – High (%)	 7.1 88.8 4.1
Phototype – II – III – IV	 6 189 1
Solar exposure score ≥ 3 (%)	10.2%
Sunscreen use (%)	43.4%
Veiled women (N=103) (%)	35%
Smoking Average pack-year ± SD	18.9% 7.6 ± 5.2
Average BMI ± SD (kg/m^2) – Normal weight (%) – Overweight (%) – Obese (%)	25.91 ± 3.93 45 41 14
Average systolic blood pressure ± SD (mmHg)	109.6 ± 11.7
Average diastolic blood pressure ± SD (mmHg)	69.11 ± 10.45
Vitamin D nutritional intakes – Average intakes ± SD (µg/day) – Insufficient intakes (%)	 2.88 ± 1.73 89.3
Calcium nutritional intakes – Average intakes ± SD (mg/day) – Insufficient intakes (%)	602.12 ± 26991.3
Mean glycemia level ± SD (mmol/L)	5 ± 0.3
Mean serum creatinine level ± SD (µmol/L)	61.6 ± 12.9
Mean serum calcium level ± SD (mmol/L)	2.46 ± 0.07
Mean serum phosphate level ± SD (mmol/L)	1.06 ± 0.15
Mean serum alkaline phosphatase level ± SD (IU/L)	67.56 ± 20.3
Mean serum PTH level ± SD (pmol/L)	4.6 ± 1.7
Mean serum 25 (OH)D level ± SD (nmol/L)	31.8 ± 25.7

SD: Standard deviation, BMI: Body mass index, PTH: Parathormone, 25 (OH)D: 25 hydroxyvitamin D

Table III Parathyroid hormone concentrations according to vitamin D range.

Vitamin D (nmol/L)	PTH (pmol/L) mean ± standard deviation
< 25	4.9 ± 1.9
25–74	4.42 ± 1.38
> 75	3.7 ± 1.1

Age of subjects with vitamin deficiency was close to those with normal vitamin D levels (36.87 ± 6.61 versus 37.63 ± 3.86 years, P = 0.65). Age was not a risk factor of vitamin D deficiency.

Subjects living in rural areas had a significantly higher prevalence of vitamin D deficiency than subjects living in urban areas (P = 0.006) with an OR of 3.995. Multivariate analysis showed that rural habitats was a risk factor independently related to vitamin D deficiency.

Among veiled women, 77.8% had a vitamin D deficiency, compared to 55.2% among unveiled women. Thus, vitamin D deficiency was significantly higher in veiled women (P=0.024) with an OR of 2.838. Multivariate analysis showed that veiling was a risk factor independently related to vitamin D deficiency. The prevalence of vitamin D deficiency was significantly higher in subject using regularly sunscreen, with an OR of 3.012.

Similarly, regular sunscreen use was considered as a risk factor independently related to vitamin D deficiency by a multivariate analysis.

Concerning influence of dietetics, univariate analysis showed there was no significant difference in serum 25(OH)D concentrations between subjects with optimal and those with insufficient intake of vitamin D (P= 0.269) and calcium (P=0.46).

In our study, socioeconomic level, phototype, solar exposure score, smoking and BMI were not risk factors of vitamin D deficiency: no statistically significant difference was noticed between subjects according to those factors.

The study of relationship between vitamin D status and serum PTH levels showed a significative and negative correlation between concentrations of 25(OH)D and PTH (P<0.005).

These data suggest that individuals with vitamin D deficiency and insufficiency had high PTH concentrations (4.9 and 4.42 pmol/L, respectively), while those with vitamin D concentrations above 75 nmol/L had lower levels of PTH (3.7 pmol/L) (*Table III*).

Discussion

Vitamin D deficiency is one of the globally alarming health problems (17).

In our study, the prevalence of hypovitaminosis D was high in healthy Tunisian subjects affecting 92.3% of the study population (81.1% had a rate of 25 (OH) D <50 nmol/L and 49.5% had a deficiency in vitamin D with 25 (OH) D level < 25 nmol/L). The mean level of 25 (OH) D levels was 31.8 ± 25.7 nmol/L.

Previous studies in Tunisia revealed various rates of vitamin D deficiency but the populations studied had specific diseases such as obesity in the study of Ben Othman et al. (18) and diabetes in the study of Abdessalem et al. (19). The only evaluation of vitamin D status in a healthy Tunisian population was published in 2005 in the study of Meddeb et al. (10). For a 38 nmol/L cut-off, the prevalence of hypovitaminosis D was 47.6. This prevalence was lower than in our study. The difference could be explained by many factors. Using different assays for 25(OH)D measurement, and different cut-offs for hypovitaminosis D interpretation may be the main reasons. Actually, a higher cut-off currently adopted may explain this increase in hypovitaminosis D prevalence in Tunisia and worldwide.

Many studies have estimated vitamin D deficiency throughout the world. Closely to our results, high prevalence rates of hypovitaminosis D were reported in other countries. A Moroccan study (20) conducted on healthy women found hypovitaminosis D in 91% of cases. In India, several studies on healthy subjects (9, 21, 22) have shown that more than 90% of the Indian population has hypovitaminosis D. In Africa, despite the significant amount of sun exposure, 92% of subjects from East Africa have a 25(OH)D levels < 50 nmol/L according to the study of SA Skull et al. (23). In Europe, the prevalence of hypovitaminosis D is different depending on the country, the population studied and the limits of interpretation. Indeed, a North-South gradient was noticed, with a lower prevalence of hypovitaminosis D in the Scandinavian countries, where there is a significant consumption of fatty fish and a fortification of certain foods in vitamin D: 80% of healthy French people had a vitamin D level < 75 nmol/L (24) compared to 53% of Danes (25).

The main factors that were significantly associated with low levels of vitamin D in our multivariate analysis were veiling, living in rural areas and sunscreen use.

In our study, age was not a risk factor of vitamin D deficiency. Studies conducted in East Africa (26), the United Arab Emirates (27), and Germany (28) did not also find a significant relationship between age and vitamin D status. However, other studies highlighted the negative correlation between age and serum vitamin D levels (24, 29). The effect of age

seems to be related to different factors. Actually, cutaneous production of vitamin D in elderly is reduced compared to young adults: a 70-year-old subject produces four times less vitamin D3 than a 20-year-old subject. Also, the food intake of the elderly is generally less, leading to a lower food intake of vitamin D (30). The effect of age is more pronounced in postmenopausal women, most probably due to the conversion of 25(OH)D into 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) under the effect of estrogen contained in hormonal substitution therapy for menopause (31).

Female gender was not independently related to the risk of vitamin D deficiency in our study. Other studies carried out in European countries such as France (24) or Finland (32) have, also, not found any difference between the two sexes. However, several international (22, 25, 26) and Tunisian (10, 34, 35) studies confirm the predominance of hypovitaminosis D in women. This difference between men and women could be explained mainly by the effect of endogenous estrogens which increase the conversion of 25(OH)D to $1,25(OH)_2D$ (31). It could be also explained by clothing habits in some countries with covered clothes for women (1, 36).

Our multivariate study found that veiling was independently linked to the risk of vitamin D deficiency. These results agree with those of Meddeb and al. that found hypovitaminosis D in 70.5% of veiled women and 48.9% in unveiled women ($P = 0.006$) (10). Various studies have already shown that veiled women have a two to five times higher risk of vitamin D deficiency (20, 28). This deficiency increases proportionally to the extent of covered skin (37).

Although living in rural areas was usually described as a protective factor against hypovitaminosis D (38), the findings of our multivariate study showed that living in rural areas was an independent risk factor associated to vitamin D deficiency. Indeed, in our study, 59% of subjects from rural areas were women. Among rural women, 61% were veiled. We mentioned this disparity between the gender of participants of rural and urban origin and their clothing habits to explain the higher prevalence observed in rural areas in our study, but the multivariate study rejected this hypothesis. Certain parameters described to influence circulating levels of 25(OH)D, such as altitude (39), number of children (28), or month of birth (40), that have not been considered in our study, may also have affected our results. In addition, despite of living in rural areas, the participants in our study were city workers, thus their lifestyle was mainly urban.

Since 1988, the study of Matsuoka et al. (41) in the United States had noticed the decline in circulating levels of 25(OH)D among long-term sunscreen users. The use of sunscreens protects the skin from erythematous radiation and skin cancers, but at the same time, it prevents the synthesis of vitamin D3 (42). In our study, 64.7% of participants who used sunscreen regularly had vitamin D deficiency, compared to 37.8% of those who did not.

Several factors have been described as influencing the circulating levels of vitamin D such age, sex, wearing of the veil, phototype, socio-economic level, rural or urban residence, anthropometric parameters and dietetics. Our study confirms only the harmful effect of veiling and sunscreen use on vitamin D levels.

The association between PTH and vitamin D may be an important determinant of bone remodeling. A negative and significant correlation was found between PTH and 25(OH)D concentrations in the present study. Individuals with low vitamin D concentrations were those who had higher values of PTH, while individuals with high values of vitamin D showed low values of PTH. Similar results were observed in healthy individuals in Australia and Riga, and a value of 38 ng/mL was suggested as sufficient to avoid an increase in PTH (43, 44). Other studies reinforce the negative correlation between vitamin D and PTH (45, 46).

Although our study is the first, since 2005, to update the national data concerning the vitamin D status in a healthy adult Tunisian population, certain limits should be noticed. Indeed, the population studied is not representative since most participants were living in urban areas with an average socio-economic level. Random sampling at the national level is necessary to overcome selection biases and ensure the representativeness of the population studied. Moreover, the number of participants could be increased to improve the strength of statistical tests.

Conclusion

In conclusion, given the importance of vitamin D for health and the very high prevalence of this hormone deficiency, an adapted health policy is essential. A more widespread vitamin D supplementation and food fortification, especially during the winter months seems to be necessary in Tunisia.

Conflict of interest statement

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

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RELATIONSHIP OF SERUM ADIPONECTIN AND RESISTIN LEVELS WITH THE SEVERITY OF LIVER FIBROSIS IN PATIENTS WITH CHRONIC HEPATITIS B

ODNOS SERUMSKIH NIVOA ADIPONEKTINA I REZISTINA KOD PACIJENATA SA HRONIČNIM HEPATITISOM B I TEŠKOM FIBROZOM JETRE

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Summary

Background: Recent research has closely linked adipocytokines to liver inflammation and fibrosis progression in patients with non-alcoholic liver disease. This study aimed to determine the relationship of serum adiponectin and resistin levels with the severity of liver fibrosis in patients with chronic hepatitis B (CHB), depending on the duration of antiviral therapy.
Methods: The cross-sectional study included 75 patients with CHB divided into two groups: the T1 group (undergoing antiviral therapy for up to 2 years) and the T2 group (undergoing antiviral therapy over 2 years). The control group consisted of 40 healthy people. Serum concentrations of adiponectin and resistin were estimated with the ELISA method, while the degree of liver fibrosis was determined using FIB-4 and APRI score.
Results: There were no statistically significant differences in the mean serum adiponectin levels in relation to the duration of antiviral therapy. Higher values of serum resistin concentration were confirmed in patients of the T1 group compared to healthy controls (p=0.001) and to the T2 group (p=0.031). The mean level of serum resistin concentration was significantly higher in the group of patients with a higher FIB-4 score (9.12±3.39 vs 5.58±3.36 ng/mL, p=0.001) and higher APRI score (17.45±3.96 ng/mL vs 4.82±1.11 ng/mL, p=0.001). A positive correlation was found between serum resistin levels and the

Kratak sadržaj

Uvod: Novija istraživanja su usko povezala adipocitokine sa progresijom upale i fibroze jetre kod bolesnika koji imaju bolesti jetre koje nisu povezane sa konzumiranjem alkohola. Cilj ovog istraživanja bio je da se utvrdi odnos serumskog nivoa adiponektina i rezistina sa težinom fibroze jetre kod pacijenata sa hroničnim hepatitisom B (CHB), u zavisnosti od trajanja antivirusne terapije.
Metode: Studija preseka je obuhvatila 75 pacijenata sa HHB koji su bili podeljeni u dve grupe: T1 grupa (na antivirusnoj terapiji do 2 godine) i T2 grupa (na antivirusnoj terapiji preko 2 godine). Kontrolnu grupu je činilo 40 zdravih osoba. Serumske koncentracije adiponektina i rezistina su procenjene uz pomoć ELISA metode, dok je stepen fibroze jetre određen pomoću FIB-4 i APRI skora.
Rezultati: Nije bilo statistički značajnih razlika u srednjim nivoima adiponektina u serumu u odnosu na trajanje antivirusne terapije. Veće vrednosti koncentracije rezistina u serumu su potvrđene kod pacijenata iz T1 grupe u poređenju sa zdravim pacijentima iz kontrolne grupe (p = 0,001) i pacijentima iz grupe T2 (p=0,031). Prosečan nivo koncentracije rezistina u serumu bio je značajno veći u grupi pacijenata sa većim skorom FIB-4 (9,12 ± 3,39 naspram 5,58 ± 3,36 ng/mL, p = 0,001) i većim skorom APRI (17,45 ± 3,96 ng/mL naspram 4,82 ± 1,11 ng/mL, p=0,001). Utvrđena je pozitivna korelacija između serumskog nivoa rezistina i stepena fibroze jetre (p < 0,001).

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List of abbreviations: CHB, chronic hepatitis B; FIB-4, Fibrosis-4; APRI, Aspartate Aminotransferase-to-Platelet Ratio Index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; CG, control group; T1 group, treated patients with antiviral therapy for up to 2 years; T2 group, treated patients with antiviral therapy over 2 years.

degree of liver fibrosis ($p<0.001$). There was no significant difference between mean serum adiponectin levels according to the values of FIB-4 and APRI scores.
Conclusions: Progression of liver fibrosis estimated by FIB-4 and APRI scores as well as the length of antiviral treatment had a significant effect on serum resistin values in CHB patients on antiviral therapy.

Keywords: adipocytokines, APRI, FIB-4, hepatitis B

Nije bilo značajne razlike između srednjih nivoa adiponektina u serumu prema vrednostima FIB-4 i APRI skoru.
Zaključak: Napredovanje fibroze jetre procenjeno rezultatima FIB-4 i APRI, kao i dužina antivirusnog lečenja su imali značajan uticaj na vrednosti rezistina u serumu kod pacijenata sa hroničnim hepatitisom B na antivirusnoj terapiji.

Ključne reči: adipocitokini, APRI, FIB-4, hepatitis B

Introduction

Adipose tissue is an important endocrine organ, which regulates a wide variety of physiological functions through the secretion of adipocytokines (1, 2). Adipose tissue and the liver cooperatively regulate energy homeostasis. A recent study by Chang et al. (3) confirmed that non-alcoholic fatty liver disease and viral hepatitis cause specific alterations in adipocytokine profiles. Proinflammatory effects of resistin and anti-inflammatory effects of adiponectin have been shown in various metabolic and inflammatory diseases (atherosclerosis, diabetes mellitus, fatty liver disease, viral hepatitis), including patients with chronic hepatitis B (4, 5). Research by Hsu et al. 6 has shown that serum adipocytokine levels independently correlate with liver fibrosis stages in patients with chronic hepatitis B (CHB), which classifies them as prognostic markers.

Improved non-invasive tests, Fibrosis-4 score (FIB-4) and Aspartate Aminotransferase-to-Platelet Ratio Index (APRI score) during antiviral therapy are a reflection of treatment-regression in liver histopathology (7, 8). Considering that CHB requires long-term antiviral therapy, monitoring the effect of therapy on the regression of liver fibrosis using non-invasive methods and parameters is of great importance, especially nowadays when the rate of hepatocellular carcinoma is extremely high, presenting the third leading cause of cancer death among the cancers of digestive system worldwide (9).

The role of adipocytokines in non-alcoholic liver disease has been extensively investigated (10, 11), but their role in patients with CHB is not sufficiently clarified through research to date. Therefore, this study aimed to determine the importance of serum adiponectin and resistin levels as prognostic markers and indicators of the liver fibrosis stages in patients with CHB, depending on the duration of antiviral therapy.

Materials and Methods

Study population

Seventy-five patients (42 men and 33 women, mean age 52.5 years) with a confirmed diagnosis of CHB, treated with antiviral therapy by nucleoside analog tenofovir, were included in the cross-sectional study. The clinical criteria for the diagnosis of CHB were: positive hepatitis B surface antigen (HBsAg) for at least 6 months, increased alanine aminotransferase (ALAT) and detectable serum hepatitis B virus (HBV) DNA by PCR test. The study was conducted at the Clinic for Gastroenterohepatology, Clinical Center of the University of Sarajevo, from January 2020 to December 2020.

All patients were divided into two groups: the T1 group included 37 patients on antiviral therapy for up to 2 years, while the T2 group included 38 patients on antiviral therapy for more than 2 years. Antiviral therapy with tenofovir was administered at a dose of 245 mg *per os* once daily for a long time, according to defined guidelines for treating viral hepatitis B (12). The study did not include patients with radiologic evidence of hepatocellular carcinoma, hepatitis C virus coinfection, autoimmune liver disease, liver cirrhosis, diabetes mellitus and previous history of alcohol consumption, patients with a BMI>25 as well as the patients with hepatosplenomegaly, ascites, peripheral edema, jaundice, and signs of liver cirrhosis.

The control group (CG) consisted of 40 healthy persons (22 male and 18 female), mean age 52.0 years, recruited from subjects who underwent preventive examination at the Counseling Centre for Gastroenterohepatology, at the Clinical Center of the University of Sarajevo. They had no clinical and laboratory signs of liver and metabolic disease. Additionally, patients and the control group who took hepatotoxic or fatty liver-inducing medicines (estrogen, amiodarone, methotrexate, tamoxifen) in the past three months were not included in the study.

All respondents gave informed consent to participate in the study. The study protocol was approved by the local Ethical Committee (No: 03-02-3083/2019). The study was conducted according to ethical standards of medical research and the Declaration of Helsinki.

Methods

A detailed history was taken from patients treated for chronic hepatitis B on the day of enrollment in the study and during a follow-up examination. Abdominal examination was performed in all participants in the study as an essential part of all routine

physical examinations. Body height was measured without shoes on a height measuring scale, while the body weight was measured using a digital scale. Body mass index (BMI) was calculated as the ratio of body weight in kilograms (kg) to the square of body height in meters (m^2).

The presence of liver fibrosis and the degree of its severity was determined using the FIB-4 score, ranging from <1.45 (negative predictive value for advanced fibrosis) to >3.25 (positive predictive value for advanced fibrosis) and also APRI score ranging from <0.5 (negative predictive value for advanced fibrosis) to >1.45 (positive predictive value for advanced fibrosis). The values of FIB-4 and APRI scores were calculated using formulas, which included serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values, as well as platelet count and age values (13, 14).

Blood sampling and measurement

Blood samples were taken on the day of inclusion in the study from the cubital vein of fasting patients in non-heparinized tubes and then centrifuged at 4000 rpm for 10 minutes. All separated serum samples for determination of adiponectin and resistin concentration were stored at -70 °C until laboratory analysis, while serum levels of AST and ALT were determined on the day of the blood sampling as well as the platelet count from the full blood.

Serum adiponectin concentration was assessed using a commercial serum adiponectin level kit (Demeditec Diagnostics GmbH, Kiel, Germany), while serum resistin concentration was assessed using a commercial resistin concentration kit (Demeditec Diagnostics GmbH, Kiel, Germany). Both biomarkers were analyzed by immunoenzymometric assays (ELISA sandwich-assays) with two specific antibodies. The adiponectin and resistin in the samples first binded to the antibody coated on the microtiter plate. In the next step, the second specific anti-adiponectin-antibody and the anti-resistin-antibody binded to the immobilized adiponectin and resistin. The second antibody was biotinylated and administered in a mixture with the peroxidase-enzyme conjugate. The enzymatic reaction led to the appearance of colour in proportion to the concentration of adipocytokine levels of the samples. After antibody conjugation, stop solution was added to the plate, followed by incubation at room temperature in a plate shaker with a rotation frequency of 350 rpm. The results were read spectrophotometrically at a wavelength of 450 nm on a plate reader BioTek ELX50 (Winooski, Vermont, USA). The measured adiponectin concentration was expressed in micrograms per millilitre (μg/mL), while the resistin concentration was expressed in nanograms per millilitre (ng/mL).

Serum levels of liver transaminases (AST, ALT) were determined by enzyme analysis on COBAS 6000 (Roche, Basel, Switzerland), while the platelet count was determined on the counter CELL-DYN Ruby (Abbott, Illinois, United States). In addition, serum HBV DNA levels were determined with a commercially available quantitative polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche Molecular Diagnostics Systems, Branchburg, N.J., USA).

Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Science (SPSS) software, version 22 for Windows (SPSS Inc, Chicago, Illinois, SAD). The normality of data distribution was determined by the Kolmogorov-Smirnov test. Variables with normal distribution were presented as mean ± standard deviation (SD) and compared using the t-test for independent samples. ANOVA test was used for statistical evaluation of variables of more than two groups. The post-hoc Scheffe test was used to uncover specific differences between three or more group means when an analysis of variance (ANOVA) was significant. A univariate two-way ANOVA test was performed to examine the effects of independent variables on the dependent variable and their interaction effects. The Spearman correlation coefficient was used to analyze the relationships of the monitored variables. The significance level was set at $p<0.05$.

Results

Clinical characteristics

There was no statistically significant difference between the study groups concerning the mean age of patients and gender, while a significant difference was confirmed in the degree of liver fibrosis between two groups of patients with different duration of disease treatment. Patients on antiviral treatment up to 2 years had a higher degree of liver fibrosis based on values of APRI and FIB-4 score in comparison with patients who have been on therapy for more than 2 years ($p<0.001$). The basic characteristics of the study groups are presented in *Table I*.

Serum adiponectin and resistin levels

Serum adiponectin values were significantly lower in the group of patients on antiviral therapy longer than 2 years compared to the control group (13.35±7.82 vs 17.67±7.16 μg/mL, $p=0.047$), while there were no statistically significant differences between the control group and group of patients on antiviral therapy up to 2 years ($p=0.50$), as well as between two groups of patient with duration of antiviral treatment up to and over 2 years (15.44±8.06 vs 13.35±7.82 μg/mL $p=0.448$), *Table II*.

Table I Basic characteristics of patients with chronic viral hepatitis and control group.

Variables	CG (n= 40)	T1 group (n=37)	T2 group (n=38)	p
Age (mean)	52.0±3.0	54.0±2.0	51.0±5.0	0.541
Male (n, %) Female (n, %)	22/55% 18/45%	20/54% 17/46%	22/57% 16/43%	0.443 0.608
BMI (kg/m^2)	22.76±1.91	22.72±1.97	22.79±1.89	0.885
Treatment length (years)		1.8±0.1	4.3±0.2	0.001
FIB-4 score		1.84±0.72	1.28±0.71	0.001
APRI score		0.75±0.44	0.39±0.25	0.001

CG – control group; T1 – patients on antiviral therapy for up to 2 years; T2 – patients on antiviral therapy for more than 2 years.

Table II The values of adiponectin and resistin serum concentration in treated patients with chronic viral hepatitis B and control group.

Variables	CG	T1 group	T2 group	p
Adiponectin (µg/mL)	17.67±7.16	15.44±8.06	13.35±7.82*	0.049
Resistin (ng/mL)	4.47±1.61	8.68±4.09‡	6.75±3.23$^{\perp\mp}$	0.001

Data are presented as mean ± SD.
CG – control group; T1 – CHB patients on antiviral therapy for up to 2 years; T2 – CHB patients on antiviral therapy for more than 2 years; *p<0.05 comparison of serum adiponectin level in the CG and T2 groups; ‡p<0.01 – serum resistin level between T1 and CG group; $^{\perp}$p<0.05 – serum resistin level between T1 and T2 group; $^{\mp}$p<0.01 – serum resistin level between T2 and CG group

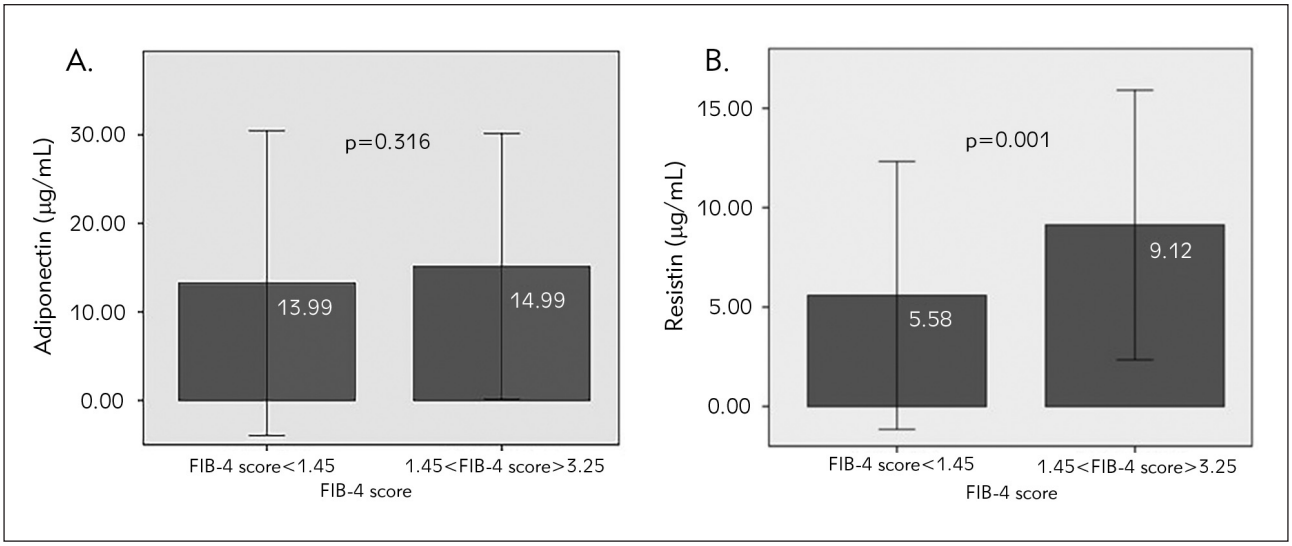

Figure 1 Serum levels of adipokines (A. adiponectin; B. resistin) according to FIB-4 score values in patients with CHB on antiviral therapy.

Bar chart express the serum adiponectin (*Figure 1A*) and resistin levels (*Figure 1B*) in the in chronic hepatitis B virus patients on antiviral therapy with different degree of liver fibrosis according to the values of FIB-4 score (<1.45, 1.45–3.25). The top of the bar represents mean value and the error bars represent standard deviation (±2SD); p<0.001 significant difference.

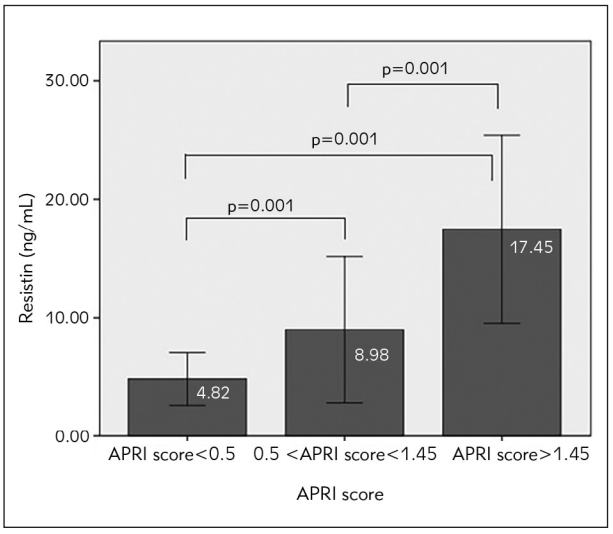

Figure 2 Serum resistin levels according to the APRI score values in patients with CHB.

Bar charts of serum resistin levels (μg/mL) in the CHB patients with APRI score of different degree of liver fibrosis (<0.5, 0.5–1.45 and >1.45). The top of the bar represents mean value and the error bars represent standard deviation (±2SD); p<0.001 significant difference in mean serum resistin levels between the different degrees of liver fibrosis according to the values of APRI score.

The mean values of serum resistin concentration were statistically significantly different between subjects of all groups (p=0.001). A higher serum resistin concentration was confirmed in the group of patients on antiviral therapy for up to 2 years compared to the control group (8.68±4.09 vs 4.47±1.61 ng/mL, p=0.001). In the group of patients who were on antiviral therapy for more than 2 years, the level of serum resistin was significantly lower compared to the group of patients on antiviral therapy for up to 2 years (6.75± 3.23 vs 8.68±4.09 ng/mL, p=0.031). *Table II* shows a significant difference between T2 and the control group (6.75±3.23 vs 4.47±1.61 ng/mL, p=0.007).

Relationship of serum adiponectin and resistin levels to the liver fibrosis parameters

There was no significant difference between mean serum adiponectin levels in patients with a higher FIB-4 score (1.45–3.25) compared to a score lower than 1.45 (15.14±7.50 μg/mL vs 13.25± 8.60 μg/mL, p=0.316), *Figure 1A*. Additionally, the mean serum resistin level was significantly higher in the group of patients with a higher FIB-4 score (9.12±3.39 ng/mL vs 5.58±3.36 ng/mL, p=0.001), *Figure 1B*.

Table III Correlation of serum adiponectin and resistin levels with FIB-4 and APRI score of liver fibrosis in CHB patients on antiviral treatment.

	T1 group				T2 group			
	FIB-4 score		APRI score		FIB-4 score		APRI score	
	rho	p	rho	p	rho	p	rho	p
Adiponectin (μg/mL)	0.152	0.368	0.088	0.604	0.131	0.435	0.139	0.406
Resistin (ng/mL)	0.699	0.001	0.860	0.001	0.784	0.001	0.831	0.001

T1 – CHB patients on antiviral therapy for up to 2 years; T2 – CHB patients on antiviral therapy for more than 2 years

Table IV Combined effects of severity disease (estimated by FIB-4 or APRI score) and duration of antiviral therapy on serum resistin and adiponectin concentrations.

Dependent variable:	Resistin (ng/mL)		Adiponectin (μg/mL)	
Independent variables	F	p	F	p
FIB-4 score	17.422	0.001	0.755	0.316
length of antiviral treatment	8.109	0.031	0.196	0.659
FIB-4 score* length of antiviral treatment	4.835	0.048	1.634	0.205
APRI score	40.092	0.001	0.940	0.378
APRI score* length of antiviral treatment	4.483	0.051	1.651	0.203

F value – the ratio of the mean-square value for the source of variation to the residual mean square; FIB-4 score* length of antiviral treatment – effects of these two factors interaction on values of the dependent variable; APRI score* length of antiviral treatment – effects of these two factors interaction on values of the dependent variable; the significance of results at p<0.05.

Serum adiponectin concentrations in patients with APRI score <0.5 (12.87±8.73 μg/mL), APRI score 0.5–1.45 (15.50±7.36 μg/mL) and APRI score >1.45 (13.09±8.64) did not differ significantly (p=0.378). As opposite of that, there was a significant difference between serum resistin levels depending on the degree of liver fibrosis, according to the values of APRI score (4.82±1.11 ng/mL in APRI score <0.5 vs 8.98±3.08 ng/mL in APRI score 0.5–1.45 vs 17.45±3.96 in APRI score >1.45, p=0.001). The post-hoc Scheffe test confirmed a significant difference in the mean serum resistin levels between each pair of groups (p=0.001), *Figure 2*.

A significant positive correlation was found between serum concentrations of resistin and liver fibrosis estimated based on the values of FIB-4 (r=0.741, p=0.001) and APRI score (r=0.839, p=0.001). The higher values of serum resistin concentration are associated with higher values of fibrosis score (*Table III*). Serum adiponectin levels did not significantly correlate with FIB-4 and APRI score of liver fibrosis in CHB patients, regardless of the duration of antiviral treatment (p>0.05).

Univariate two-way ANOVA test showed that progression of liver fibrosis estimated by FIB-4 and APRI scores had a significant effect on resistin values (F 17.42, p=0.001; F 40.09, p=0.001), as well as the length of antiviral treatment (F 8.109, p=0.031), *Table IV*. Progression of liver fibrosis expressed by values of FIB-4 score and length of treatment interaction had a significant effect on serum resistin values (F 4.835, p=0.048), while APRI score and length of treatment interaction did not have a significant effect on serum resistin levels (F 4.483, p=0.051). These scores and the length of antiviral treatment did not significantly affect the values of serum adiponectin (*Table IV*).

Discussion

Treatment and control of CHB may become complicated due to the poor awareness of the disease and lack of screening programs and adequately monitoring of complications (15). Considering that 15–40% of patients with CHB will progress to cirrhosis and hepatocellular carcinoma (HCC), even with sustained viral suppression during therapy, the use of appropriate serum biomarkers as indicators of disease progression is crucial. Durazzo et al. (16) pointed out the role of serum adiponectin and resistin in the course of CHB and verified a significant decrease in resistin with non-significant reduction in adiponectin after treatment of CHB. Our study examined the variations of serum adipocytokine values in patients with CHB depending on the length of antiviral therapy to determine if they could be appropriate parameters to assess the degree of liver fibrosis and indirectly on the effectiveness of antiviral therapy in controlling disease progression.

The study results showed that mean serum resistin concentration was significantly higher in patients with CHB on antiviral therapy compared to healthy control. We also found a significantly higher value of serum resistin concentration in the early period of antiviral treatment of CHB patients, and the two-way analysis of variance (ANOVA) showed that the combined effects of disease severity assessed by FIB-4 score and duration of antiviral therapy had a significant effect on serum resistin values. Our results are in accordance with the study of Meng et al. (17), which showed higher values of resistin in patients with advanced intrahepatic inflammation and higher stage of fibrosis in three groups of patients – patients with CHB, patients with liver cirrhosis and patients with liver failure as a consequence of CHB. Significantly higher serum resistin levels in that study were found in the latter two groups, considering resistin as a prognostic factor in disease severity. Contrary to these results, the study of Tsochatzis et al. (18) showed that serum resistin levels were lower in patients with severe liver fibrosis, as well as that resistin levels are independently associated with fibrosis severity in patients with chronic hepatitis B and C infection, but it remained unclear to the authors whether lower levels of resistin in advanced fibrosis represent a marker of disease severity or whether resistin is directly implicated in disease progression.

Our results indicated a decrease in adiponectin values in patients with CHB compared to healthy controls, with verified statistically significant difference only between the group of patients on longer antiviral therapy (for more than two years) and the control group. We did not find a significant difference in adiponectin values in patients with CHB on antiviral therapy according to the different degree of liver fibrosis, based on the results of FIB-4 and APRI score, although some discrepancies were observed in the ratio of serum adiponectin levels to higher degrees of liver fibrosis assessed based on these scores. Higher values of adiponectin were observed with a FIB-4 score greater than 1.45. Additionally, in 43 patients with APRI score values between 0.5 and 1.45, serum adiponectin levels showed a tendency to increase insignificantly compared to 29 patients with APRI score lower than 0.5, while in the group of 3 patients with APRI score greater than 1.45, these values were lower. If we take into consideration that only three patients had an APRI score greater than 1.45, the difference found can be taken with caution, and it can be concluded that an increase in adiponectin levels might indicate a higher degree of fibrosis according to the APRI score. These results are partially in accordance with results of some recently published studies, which have shown higher levels of adiponectin in CHB patients with advanced liver fibrosis and inflammation (19, 20), although the question of its elevated values in advanced liver disease remains open because adiponectin is an anti-inflammatory factor

that contributes to the reduction of inflammation in the liver parenchyma (21). A possible explanation for higher adiponectin values in patients with CHB compared to healthy controls lies in the fact that adiponectin is a potent inhibitor of hepatic stellate cells activation, and consequently, its deficiency leads to greater fibrosis (22). The study investigating the adiponectin – FGF 15/19 axis as an essential axis in the development of alcoholic steatohepatitis determined that ethanol dysregulates adiponectin production, reduces hepatic adiponectin receptors disrupts adiponectin signalling (23). A higher degree of steatohepatitis is consequently associated with lower levels of adiponectin. Our study showed that the concentration of serum resistin in patients with chronic hepatitis B corresponds to the severity of liver fibrosis and depends on the duration of antiviral therapy.

Conclusions

This study observed a significantly higher serum resistin level in patients with CHB on the different duration of antiviral therapy compared to healthy control subjects. A higher degree of liver fibrosis in patients with viral hepatitis B was associated with significantly higher serum resistin concentrations. These results suggest that the serum resistin could be a potential non-invasive biomarker of liver fibrosis and its severity in the patients with hepatitis B infection and an indicator of the effects of antiviral therapy on liver histology.

There was no significant difference between the mean serum adiponectin levels in patients with CHB on antiviral therapy concerning the degree of liver fibrosis determined using the FIB-4 and APRI score. Therefore, the role of adiponectin in CHB infection needs to be further studied.

Conflict of interest statement

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

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IDENTIFICATION OF SERUM miR-378 AND miR-575 AS DIAGNOSTIC INDICATORS AND PREDICTING SURGICAL PROGNOSIS IN HUMAN EPILEPSY

IDENTIFIKACIJA U SERUMU miR-378 I miR-575 KAO DIJAGNOSTIČKIH POKAZATELJA I PREDVIĐANJA HIRURŠKE PROGNOZE U HUMANOJ EPILEPSIJI

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Summary

Background: Epilepsy (EP) is a common neurological disorder which is characterized by excessive abnormal synchronization of neuronal discharges in the brain due to chronic recurrent seizures of multiple etiologies. Variety of microRNAs have been associated with the occurrence and development of EP. This study aimed to determine the aberrant expression of miR-378 and miR-575 in EP patients to validate their potential to distinguish EP from healthy patients.
Methods: RT-qPCR was used to determine the expressions of miR-378 and miR-575 from serum specimens of 106 EP and 103 control individuals. Clinical indicators between EP patients and controls were assessed. Based on surgical outcome, EP patients were further divided into Engel I–IV EP. The potentials of miR-378 and miR-575 in discriminating EP from healthy participants and predicting surgical prognosis were calculated by receiver operating characteristic (ROC) analysis.
Results: We found the miR-378 and miR-575 were significantly declined ($P<0.001$) in Engel I–II and III–IV EP patients with no difference in clinical parameters compared. Moreover, miR-378 and miR-575 displayed high sensitivity, specificity, and accuracy in distinguishing EP patients and predicting surgical outcomes. Moreover, after surgical treatment, miR-378 and miR-575 levels were increased compared with those at admission, suggesting their potentials in treatment response.
Conclusions: miR-378 and miR-575 could be utilized as novel and non-invasive serum biomarkers in discriminating EP from healthy controls and predicting surgical outcome, shedding new insights on epileptogenesis and EP treatment.

Keywords: miR-378, miR-575, human epilepsy, serum biomarkers

Kratak sadržaj

Uvod: Epilepsija (EP) je uobičajen neurološki poremećaj koji se karakteriše prekomernom sinhronizacijom pražnjenja neurona u mozgu usled hroničnih ponovljenih napada različitih etiologija. Različite mikro RNK su povezane sa pojavom i razvojem EP. Ova studija je imala za cilj da odredi aberantnu ekspresiju miR-378 i miR-575 kod pacijenata sa EP kako bi potvrdila njihov potencijal da se ustanovi razlika između pacijenata sa EP i zdravih pacijenata.
Metode: RT-qPCR je korišćen da bi se odredila ekspresija miR-378 i miR-575 iz serumskih uzoraka 106 pacijenata sa EP i 103 kontrolna ispitanika. Izvršena je procena kliničkih pokazatelja kod pacijenata sa EP i kontrolnih ispitanika. Na osnovu ishoda hirurškog lečenja, pacijenti sa EP su dalje podeljeni na Engel I–IV EP. Analizom krive operativnih karakteristika (ROC) izračunat je potencijal koji imaju miR-378 i miR-575 i koji karakteriše pacijente sa EP u odnosu na zdrave učesnike istraživanja kako bi se predvideo tok lečenja.
Rezultati: Utvrdili smo da su miR-378 i miR-575 značajno smanjeni ($P < 0,001$) kod pacijenata sa Engel I–II i III–IV EP bez razlika u upoređenim kliničkim parametrima. Štaviše, miR-378 i miR-575 su pokazali visoku osetljivost, specifičnost i tačnost u razlikovanju EP pacijenata i predviđanju ishoda. Dalje, nakon hirurškog lečenja, nivoi miR-378 i miR-575 su povećani u poređenju sa onima pri prijemu, što ukazuje na njihov potencijal u rekaciji na lečenje.
Zaključak: miR-378 i miR-575 se mogu koristiti kao novi i neinvazivni biomarkeri u serumu za diferencijaciju pacijenata sa EP od zdravih kontrolnih ispitanika i predviđanje hirurškog ishoda, otkrivajući nove uvide u epileptogenezu i lečenje EP.

Ključne reči: miR-378, miR-575, epilepsija kod ljudi, serumski biomarkeri

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Introduction

Epilepsy (EP) is a chronic brain disease with a long course that poses a severe threat to patients' physical and mental health (1). Seizures caused abnormal synchronous neuronal discharges (2), resulting in transient brain dysfunction and abnormalities in neuronal apoptosis, necrosis, and neurological deficits (3). In addition, EP was characterized by transience, unpredictability, and recurrence (4). About half of epilepsy patients had their onset in childhood and teenage years (5). So far, magnetic resonance imaging (MRI) and electroencephalography (EEG) have been able to help diagnose and differentiate epilepsy, whereas the assessment of anti-epileptic efficacy depended on EEG (6). However, EEG is expensive and time-consuming, which is not suitable for frequent and quick applications (7). Therefore, it is imperative to explore non-invasive and economic biomarkers to monitor EP development and evaluate the effectiveness of anti-epileptic treatment.

MicroRNAs (miRNAs) are endogenous single-stranded small RNA with approximately 22–24 nucleotides in length (8). These miRNAs are highly conserved and play a post-transcriptional role in regulating gene expressions (9). Several miRNAs are released from cells and circulate in the blood, show resistance to various RNA digesting enzymes, and are not affected by storage time, pH, temperature, repeat freezing, and thawing (10). Because these are less affected by changes in the peripheral environment, miRNAs are used as biomarkers for various diseases, including epilepsy (11, 12). In recent years, there has been increasing evidence indicating that multiple miRNAs regulate EP. For instance, the expression of miR-106b-5p, let-7d-5p, miR-130a-3p and miR-146a-5p are upregulated in the serum of EP patients, while miR-15a-5p and miR-194-5p levels are downregulated (13). Moreover, miR-134 and miR-21-5p are downregulated among epileptic rats (14, 15). Meanwhile, multiple miRNAs have been associated with epileptogenesis and the progression of EP (16, 17). In a previous study, miR-378 and miR-575 are dysregulated during the onset of EP and post-treatment (18). However, the potential of miR-378 and miR-575 concerning EP diagnosis and outcome prediction remained unknown.

In the present study, we aimed to determine the aberrant expression of miR-378 and miR-575 in EP patients to determine the potential in differentiating EP from healthy individuals. Moreover, the dynamic changes of miR-378 and miR-575 during seizure onset and after surgical treatment were evaluated to validate their application in predicting EP surgical outcome.

Materials and Methods

Samples collection

A total of 106 individuals diagnosed with EP but remained untreated in Linyi Central Hospital were enrolled as the EP group from November 2017 to April 2020. The control group consisted of 103 healthy individuals who underwent a physical examination at our hospital during the same period. The EP group and the healthy group were age and sex-matched. All enrolled participants signed informed consent, and the Ethics Committee of Linyi Central Hospital approved the current study protocol. Inclusion criteria for the EP: (1) those who were eligible for primary EP according to the diagnostic and classification criteria of the International League Against Epilepsy (ILAE); (2) the time since the last seizure was less than 1 week; (3) organic lesions such as traumatic brain injury, developmental malformation of the brain, intracranial occupancy, and cerebral infarction were excluded by imaging examinations such as computed tomography (CT), MRI and EEG. In addition, EP patients with neurological and other systemic disorders, traumatic brain injury, or receiving psychotropic medications in the last three months were excluded.

5 mL of blood sample was collected from EP patients and healthy controls after fasting and kept at room temperature for 3 hours. Then, the supernatant was aspirated into a sterilized EP tube. The sample was then centrifuged at 3000 r/min at room temperature for 10 min. After centrifugation, samples were stored at -80 °C until use.

RT-qPCR analysis

The frozen serum samples, stored at -80 °C, were thawed at 4 °C in a refrigerator. 1.5 mL of each sample was dispensed in a centrifuge tube. Next, 1 mL TRIzol was added to each sample at room temperature for 5 min. After lysis, 200 μL of chloroform was added for 10 min and then centrifuged at 12,000 g for 15 min at room temperature. Afterwards, 1 mL of 75% alcohol was used to precipitate RNA and then centrifuged at 4 °C for 5 min at 7,500 g. After washing, the RNA was placed for 15 min to dry RNA. Finally, 20 μL DNase/RNase-free deionized water was added to dissolve the RNA extract. The samples were analyzed for RNA purity using a NANODROP 2000 spectrophotometer. Then, the reverse transcription kit, SuperScript R Enzyme Mix, was used to prepare cDNA. The RT-qPCR was performed in AXYGEN 0.2 mL 96-well PCR system with U6 RNA as the internal reference. The CT values of miR-378 and miR-575 were derived at the end of the amplification reaction by fluorometric quantification and analyzed using the 2-ΔΔCT method. All the experiments were performed in triplicate.

Statistical analysis

The experimental data, for normal distribution and variance homogeneity, were expressed in the form of mean ± standard deviation ($\bar{x} \pm s$). SPSS 22.0 software was used to statistically analyze the data obtained from each group, and an independent t-test and chi-square test were used for quantification. $P<0.05$ was deemed statistically significant.

Results

Clinicopathological information of all participants

As shown in *Table I*, there were no significant differences in age, gender distribution, BMI, and smoking history between EP patients and healthy controls.

Down-regulated miR-378 and miR-575 expressions in serum samples in EP patients

We used RT-qPCR to analyze and measure the expression levels of miR-378 and miR-575 in serum samples from EP patients and healthy volunteers. As highlighted in *Figures 1A* and *1B*, the expression of miR-378 and miR-575 were prominently increased in the EP group as compared with the healthy control ($^{***}P<0.001$).

Engel grades were applied to discriminate EP patients regarding seizure response to surgical treatment. According to the guidelines, Engel I was defined as free of disabling EP; Engel II was rare seizure after surgery; Engel III was deemed worthwhile improvement of EP reduction after surgery; Engel IV was characterized as no improvement in seizure conditions. As depicted in *Figures 2A* and *2B*, Engel III–IV EP patients had significantly lower miR-378 and miR-575 expressions than Engel I–II ($^{***}P<0.001$), suggesting the potentials of miR-378 and miR-575 in predicting surgical outcome.

Table I Clinical information of healthy individuals and patients with epilepsy.

Parameters	EP patients (n = 106)	Healthy (n = 103)	P value
Gender			
Male	57	52	0.6342
Female	49	51	
Age	29.5 ± 7.2	31.2 ± 6.8	0.0809
EP duration (years)			
≤ 5	81	/	/
> 5	25	/	
Smoking history			
Yes	44	46	0.6456
No	62	57	
BMI (kg/m^2)	24.6 ± 0.7	25.0 ± 0.5	0.9555
miR-378 level	0.47 ± 0.17	1.01 ± 0.26	<0.0001
miR-575 level	0.49 ± 0.21	1.03 ± 0.24	<0.0001

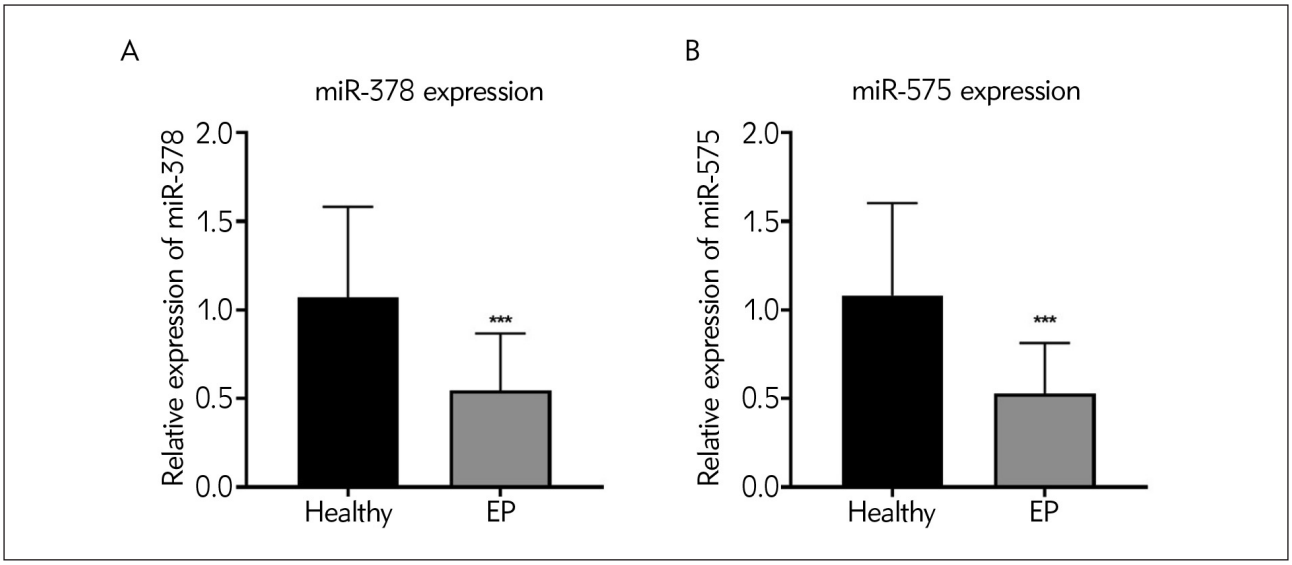

Figure 1 Aberrant expressions of miR-378 and miR-575 in serum samples from healthy controls and patients with EP. (A) Expressions of miR-378 were evaluated by qRT-PCR analysis. ***P<0.001, EP vs Healthy. (B) Expressions of miR-575 were evaluated by qRT-PCR analysis. ***P<0.001, EP vs Healthy. EP, epilepsy.

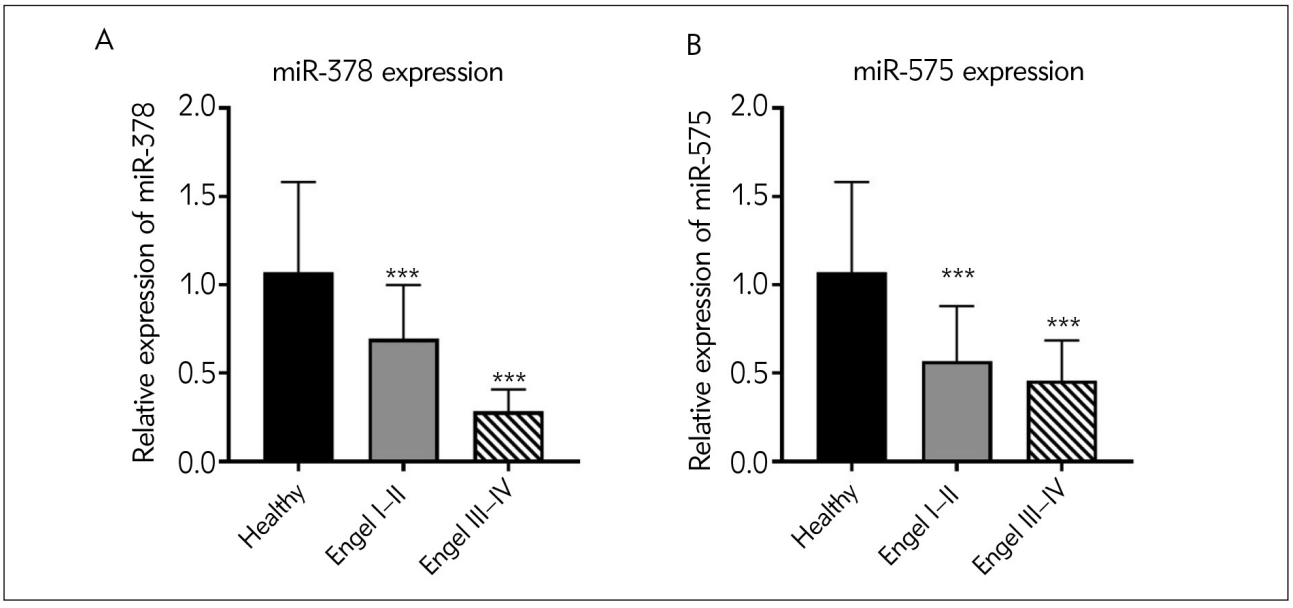

Figure 2 Different expressions of miR-378 and miR-575 in patients with EP according to Engel grades. (A) miR-378 expressions in Engel I–II and Engel III–IV EP patients. (B) miR-575 expressions in Engel I–II and Engel III–IV EP patients. ***P<0.001, Engel I–II vs. Healthy, Engel III–IV vs. Healthy. EP, epilepsy.

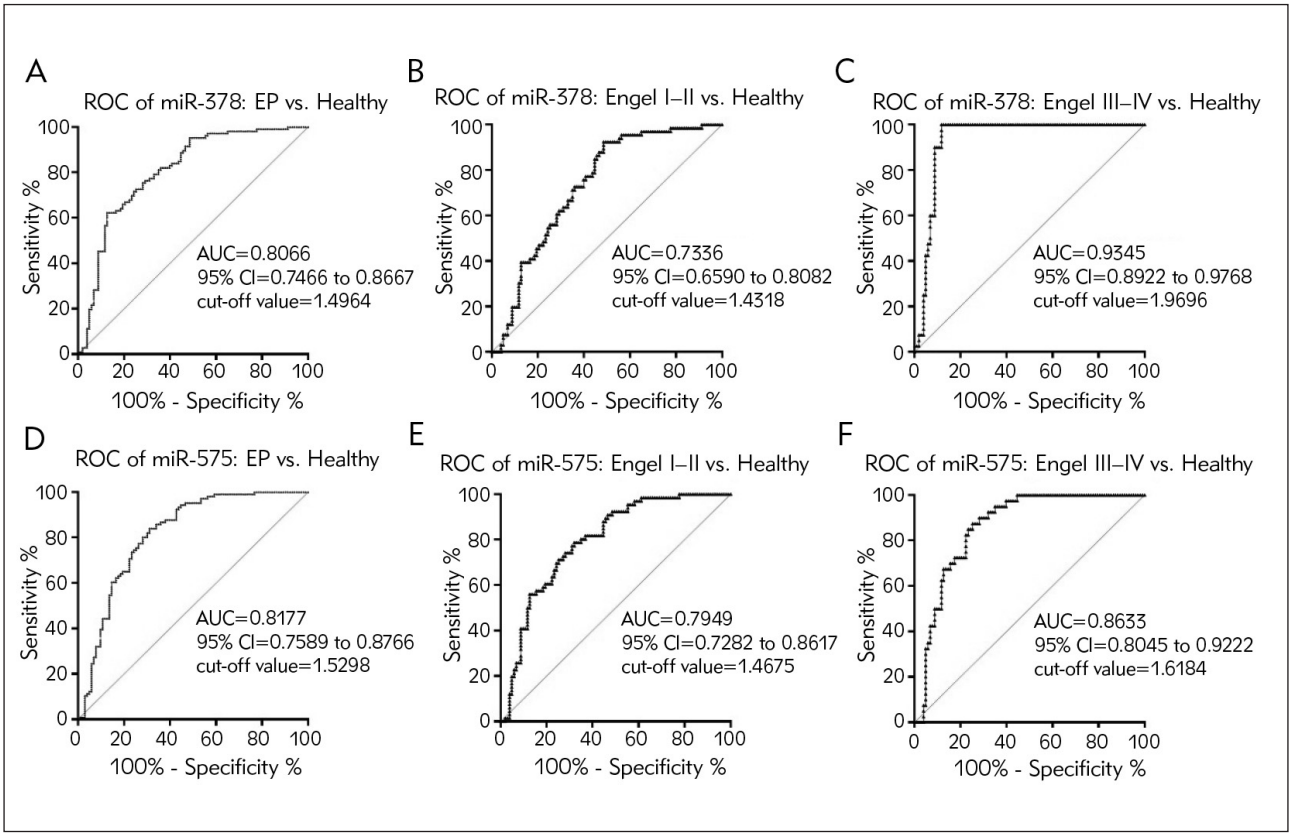

Figure 3 ROC analysis of miR-378 and miR-575 in discriminating EP patients from healthy controls. (A) AUC of miR-378 in differentiating EP patients from healthy volunteers. (B) AUC of miR-378 in differentiating Engel I–II EP patients from healthy volunteers. (C) AUC of miR-378 in differentiating Engel III–IV EP patients from healthy volunteers. (D) AUC of miR-575 in differentiating EP patients from healthy volunteers. (E) AUC of miR-575 in differentiating Engel I–II EP patients from healthy volunteers. (F) AUC of miR-575 in differentiating Engel III–IV EP patients from healthy volunteers.

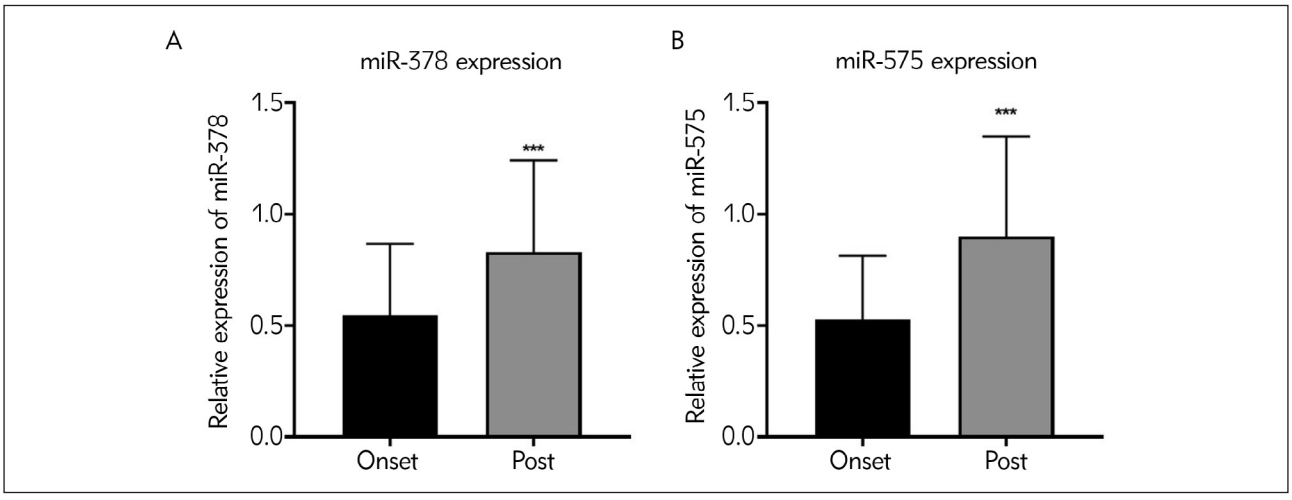

Figure 4 Dynamic expressions of miR-378 and miR-575 in EP patients before and after surgical treatment. (A) miR-378 expressions at seizure onset and post-seizure. (B) miR-575 expressions at seizure onset and post-seizure. ***P<0.001, Post vs. Onset. Post, post treatment; onset, epilepsy onset.

Diagnostic performances of miR-378 and miR-575 in distinguishing EP patients from healthy controls

ROC analysis was conducted to evaluate the capabilities of miR-378 and miR-575 abnormal expressions in discriminating EP from healthy controls. As demonstrated in *Figure 3A* and *3D*, miR-378 and miR-575 presented high accuracy, sensitivity, and accuracy in differentiating EP from healthy participants with the area under the curves (AUCs) of 0.8066 and 0.8177, respectively (cut-off value = 1.4964 and 1.5298). Moreover, the AUCs of miR-378 and miR-575 in distinguishing Engel I–II EP from healthy controls were 0.7336 and 0.7949, respectively. Additionally, the AUCs of miR-378 and miR-575 in distinguishing Engel III–IV EP from healthy controls were 0.9345 and 0.8933, with the cut-off value of 1.9696 and 1.6184. Collectively, these results suggested that miR-378 and miR-575 were implicated in epileptogenesis and monitoring EP occurrence.

Dynamic levels of miR-378 and miR-575 before and after surgical treatment

After surgical treatment, the dynamic changes of miR-378 and miR-575 in EP samples were detected using qRT-PCR assay. As shown in *Figures 4A* and *4B*, the expression of miR-378 and miR-575 was augmented after treatment compared to those at seizure onset, implying that miR-378 and miR-575 may function as feasible biomarkers for EP treatment response ($^{***}P<0.001$).

Surgical outcomes regarding miR-378 and miR-575 in EP

Giving that Engel grade could indicate the surgical outcome in EP, we further examine the AUCs of miR-378 and miR-575 between Engel I–II and Engel III–IV EP patients. As illustrated in *Figure 5A*, miR-378 could serve as an accurate and high sensitive biomarker for predicting EP surgical prognosis, with an AUC of 0.8985 and a cut-off value of 1.7326. On the contrary, miR-575 had no value in predicting EP surgery outcome with the AUC value of less than 0.8 (*Figure 5B*).

Discussion

Statistical reports showed that about 70 million people worldwide were affected by EP (19). In China, there were more than 9 million EP patients (20). The number of patients with refractory EP who have been treated regularly for more than 2 years was greater than 2 million, and the prevalence rate accounted for 7‰ of the world's population (21). The clinical manifestations of epilepsy were diverse, and the typology was complex with 4 common features, seizure, repetitive, transient, and stereotyped (22). Current treatments for EP included medication, surgery, dietary modifications, and gene therapy (23). Clinical studies have shown that 70%–80% of new EP patients can be controlled with anti-epileptic drugs (AEDs), and 60%–70% of these patients can be discontinued after 2 to 5 years of treatment with AEDs (24). Some postoperative patients with EP still needed AEDs to control their symptoms.

Epileptogenesis is a process in which the inflammatory response, the formation of new synaptic and abnormal conduction pathways, and neuronal apop-

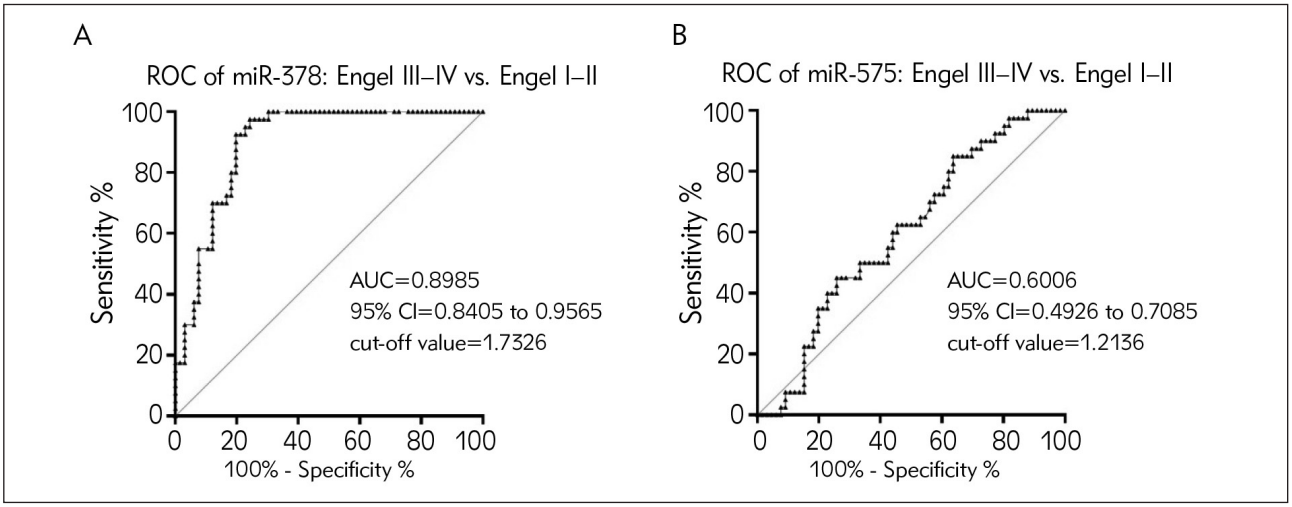

Figure 5 Potentials of aberrant miR-378 and miR-575 regarding surgical outcomes in EP. (A) AUC result of miR-378 in Engel I–II epilepsy versus Engel III–IV EP patients. (B) AUC result of miR-575 in Engel I–II epilepsy versus Engel III–IV EP patients.

tosis were jointly involved (25, 26). Current studies have shown that a variety of miRNAs are involved in the development of EP. MiR-132 was the first identified miRNA in EP, and its increase led to the development of EP (27). Furthermore, it was found that miR-34a, miR-132, miR-146a, and miR-184 miRNA expression were upregulated after the seizure, further promoting the recurrence of EP (28). Meanwhile, miR-124 is decreased in EP, inhibiting seizure activity through targeting CREB1 (29). Our study found that miR-378 and miR-575 were significantly reduced in serum samples from EP patients in relation to those in healthy controls, which was consistent with a previous study. Similar to our study, another study reported that miR-378 serves as a prognostic biomarker in cholangiocarcinoma (30).

Meanwhile, the clinical utility of microRNA-378 as an early diagnostic biomarker of human cancers has also been documented (31). Furthermore, miR-378 in serum are potential biomarkers for renal cell carcinoma (32). Additionally, it has been found that miR-378 play a role in metabolism, angiogenesis, and muscle biology (33). Similarly, miR-575 serve as a diagnostic marker for metastatic breast cancer and pancreatic cancer (34, 35).

Meanwhile, the ROC analysis validated that miR-378 and miR-575 may be highly sensitive and non-invasive candidates for EP diagnosis. More importantly, according to the Engel guidelines and dynamic changes in miR-378 and miR-575 before and after surgery treatment, we confirmed the potentials of miR-378 and miR-575 in predicting surgery outcome and treatment response in EP as well.

To sum up, miR-378 and miR-575 were decreased in EP serum samples, functioning as feasible biomarkers for EP monitoring and predicting surgical prognosis.

Conflict of interest statement

The authors reported no conflict of interest regarding the publication of this article.

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THE CLINICAL SIGNIFICANCE OF CIRCULATING miR-21, miR-142, miR-143, AND miR-146A IN PATIENTS WITH PROSTATE CANCER

KLINIČKI ZNAČAJ CIRKULIŠUĆIH miR-21, miR-142, miR-143 I miR-146A U PACIJENATA SA KARCINOMOM PROSTATE

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Summary

Background: Prostate cancer (PCa) is the most common type of solid tissue cancer among men in western countries. In this study, we determined the levels of circulating miR-21, miR-142, miR-143, miR-146a, and RNU 44 levels as controls for early diagnosis of PCa.
Methods: The circulating miRNA levels in peripheral blood samples from 43 localized PCa patients, 12 metastatic PCa (MET) patients, and a control group of, 42 benign prostate hyperplasia (BPH) patients with a total of 97 volunteers were determined the by PCR method.
Results: No differences in the ΔCT values were found among the groups. In PCa and PCaMet groups the expression of miR21 and miR142 were higher compared to the BHP group. No other differences were observed among the other groups. miR21 expression in the PCa group was 6.29 folds upregulated whereas in the PCaMet group 10.84 folds up-regulated. When the total expression of miR142 is evaluated, it showed a positive correlation with mir21 and mir 146 (both $p<0.001$). Also, the expression of miR146 shows a positive correlation with both miR21 and miR143 (both $p<0.001$). Expression of miRNAs was found to be an independent diagnostic factor in patients with Gleason score, PSA, and free PSA levels.
Conclusions: Our study showed that co-expression of miR-21, miR-142, miR-143, and miR-146a and the upregula-

Kratak sadržaj

Uvod: Karcinom prostate (PCa) je najčešći tip raka čvrstog tkiva među muškarcima u zapadnim zemljama. U ovoj studiji, odredili smo nivoe cirkulišućih miR-21, miR-142, miR-143, miR-146a i nivoe RNU 44 kao kontrole za ranu dijagnozu PCa.
Metode: Nivoi cirkulišuće miRNA u uzorcima periferne krvi određivani su PCR metodom kod 43 pacijenta sa otkrivenim PCa, 12 pacijenata sa metastatskim PCa (MET) i kao kontrolnom grupom kod 42 pacijenta sa benignom hiperplazijom prostate (BPH) u ukupno 97 dobrovoljaca.
Rezultati: Nisu pronađene razlike u vrednostima ΔCT među grupama. U grupama PCa i PCaMet ekspresija miR21 i miR-142 bila je veća u poređenju sa BHP grupom. Nisu uočene druge razlike među ostalim grupama. Ekspresija miR-21 u grupi PCa bila je 6,29 puta lošije regulisana, dok je u grupama PCaMet regulacija bila povećana 10,84 puta. Kada se proceni ukupna ekspresija miR-142, ona je pokazala pozitivnu korelaciju sa miR-21 i miR-146 (oba $p<0,001$). Takođe ekspresija miR-146 pokazuje pozitivnu korelaciju sa oba miR-21 i miR-143 (oba $p<0,001$). Utvrđeno je da je ekspresija miRNA nezavisan dijagnostički faktor kod pacijenata da Gleason skorom, i nivoima PSA i slobodne PSA.
Zaključak: Naša studija je pokazala da je ko-ekspresija miR-21, miR-142, miR-143 i miR-146a i povećana regula-

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List of abbreviations: BPH; Benign Prostate Hyperplasia, miRNA; Micro ribonucleic acid, PCa Met; Prostate Cancer Metastasis, PCa; Prostate Cancer, PSA; Prostate-Specific Antigen

tion of miR-21 resulted in increased prostate carcinoma cell growth. In the PCaMet group, miR21 is the most upregulated of all miRNAs. These markers may provide a novel diagnostic tool to help diagnose PCa with aggressive behavior.

Keywords: Prostate cancer, miR-21, miR-142, miR-143, miR-146a

cija miR-21 rezultirala povećanjem rasta ćelija karcinoma prostate. U grupi PCaMet, miR-21 je bio najbolje regulisan od svih miRNA. Ovi markeri mogu pružiti novi dijagnostički alat koji će pomoći u dijagnostici PCa sa agresivnim ponašanjem.

Ključne reči: rak prostate, miR-21, miR-142, miR-143, miR-146a

Introduction

In western countries, prostate cancer (PCa) is the most common solid tumor in men (1). In 2018, the number of newly diagnosed patients with PCa reached 1.3 million (2). As the incidence of PCa increases in aging males, especially the eighth decade of life shows malignant changes in > 70% of individuals according to autopsy reports. The annual mortality load of PCa is 220,000 deaths, making it the sixth leading cause of cancer mortality among men (3). PCa mostly arises from the peripheral zone of the gland. Epidemiological studies showed that having a first-degree relative with PCa increased risk for an individual by approximately two- to three-fold (4). The incidence of PCa in African-American men is higher when compared to White men (5).

Prostate biopsy is the gold standard for the diagnosis of PCa. Samples are taken bilaterally from apex to base, as far posterior and lateral as possible in the peripheral gland. While for small-sized prostates at least 8 systematic (~30cc) biopsies are required; for larger prostates, 10 to 12 biopsy samples are required which eventually increases the health care cost. Prostate-specific antigen (PSA), a serine protease inhibitor is produced by both malignant and non-malignant epithelial cells. Since the 1990s, it has been used as a screening test. The use of PSA as a screening test has led to the increased detection of the early stage of cancer and a fall in the incidence of metastatic disease, also a reduction in related mortality. Over detection of PCa leads to overtreatment, increased side effects, complications, patient anxiety, and high costs (6). Although PSA is prostate-specific, it increases not only in PCa but also and prostatitis. Due to these characteristics, the use of new biomarkers with high sensitivity and specificity ratios was considered for the early detection of PCa.

MicroRNAs (miRNAs) are a single-chain, endogenous, highly conserved group of small, non-coding RNA groups of about 19–25 nucleotides in length (7). miRNAs have been shown to have crucial roles in certain biological processes and pathological conditions. miRNAs appear as important cytoplasmic regulators of gene expression. miRNAs act as post-transcriptional regulators of their messenger RNA (mRNA) targets via mRNA degradation and/or translational repression (8). In recent years, miRNAs were used for defining the physiopathology of cancer and various diseases. The human genome contains more than 1000 miRNAs, and estimates indicate that some 60% of the human protein-coding genes may be regulated by miRNAs, which means they may significantly affect the expression of several proteins (9).

On the other hand, miRNAs play a role in many cellular and biological processes such as cellular differentiation, proliferation, apoptosis, erythropoiesis, fibrosis, angiogenesis, and immunity. In some cancers, some miRNAs function as oncogenes others functions as tumor suppressor genes, indicating that miRNAs regulate tumor progression, metastasis, and invasion (10). In addition to their potential as tissue-based markers for cancer classification, circulating miRNAs in the blood of cancer patients might be used as potential diagnostic and prognostic biomarkers (11). A recent study showed that miR-21 promotes hormone-dependent and hormone-independent growth in PCa (12). MiR-143 has been shown to have a strong relationship with PCa (13). It was shown that the expression of miR-146 was induced in mice with PCa (14).

Our study aimed to investigate the pattern of cancer-associated miRNAs, miR-21, -142, 143, and -146, and as a control RNU43 miRNA in the plasma of PCa patients with local/metastatic disease and patients with benign prostate hyperplasia (BPH).

Materials And Methods

Subjects

Ninety-seven men, who were admitted to the Outpatient Clinic of Urology Department, Cerrahpasa Medical Faculty of Istanbul University-Cerrahpasa were included in our study. The patients were divided into three groups as 43 patients with localized PCa, 12 patients with metastatic PCa (MET), 42 patients with BPH as a control group. All subjects gave their informed consent before participating in the study. The study design was approved by the Ethical Committee of Cerrahpasa Medical Faculty (14/07/2016-256677) and was conducted in conformity with the Declaration of Helsinki. The complete medical history, physical examination, laboratory investigation, and clinicopathological features were obtained and recorded for all patients. Tumor staging was performed in conformity with the American Joint Committee on Cancer (AJCC) system, 7th edition tumor, lymph nodes, metastasis (TNM) staging classification.

According to the result of prostate biopsy, patients diagnosed with PCa and BPH were enrolled in our study. Patients who had undergone surgery, chemo/radiotherapy, and patients with secondary malignancy, acute infections, diabetes mellitus, hypertension, kidney diseases, and rheumatologic diseases were excluded from the study.

All patients underwent at least a 12-core biopsy at our institution due to increased prostate-specific antigen serum levels (>4 ng/mL) and/or suspicious findings on digital rectal examination. Samples are taken bilaterally from apex to base, as far posterior and lateral as possible in the peripheral gland. The tumors were graded according to the modified Gleason grading system (15) and staged according to the guidelines (16).

Collection of blood samples

Venous blood samples were collected into plain tubes and tubes which were coated inside with ethylenediaminetetraacetic acid (EDTA), in the morning after overnight fasting (10–12 h). The plain tubes were centrifuged for 10 minutes at 4000 rpm at 4 °C.

Biochemical analysis

Extraction of small RNA molecules from human blood serum was performed by the EXTRACTME miRNA KIT (BLIRT, Poland) according to the manufacturer's instructions. The RNA samples were immediately stored at -80 °C until they were reverse transcribed into cDNA. Firstly, the miRNA samples were transcribed into complementary DNA (cDNA) using a cDNA Synthesis Kit (High Capacity) (Wizbio Solutions, Korea). The reaction was carried out at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min on a SimpliAmp Thermal Cycler (Thermofisher, USA). The cDNA samples were stored at -20 °C until used.

Quantitative Real-Time PCR kits made specifically for accurate miRNA analysis were used to evaluate the expression of the following miRNAs from serum samples: miR-21, miR-142, miR-143, miR-146a, and RNU44 (endogenous control). For Real-Time PCR, Amplifyme Sg Universal Mix (Blirt, Poland) was used. The primers used in the study were obtained from Suarge Biyoteknoloji (Istanbul, Turkey) (*Table I*). Each sample was tested in triplicate on a real-time PCR system (Step One Plus real-time PCR system, Applied Biosystems, Carlsbad, CA, USA). The RT-PCR reaction was performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 1 min. The expression levels of miR-21, miR-142, miR-143, miR-146a were normalized to RNU44 were calculated using the 2-ΔΔCt method (17).

Table I Reverse transcription oligos specific to miRNAs.

Primers	Sequence
miR-142 RT	GAAAGAAGGCGAGGAGCAGATC-GAGGAAGAAGACGGAAGAATGT-GCGTCTCGCCTTCTTTCTCCATAAA
miR-143 RT	GAAAGAAGGCGAGGAGCAGATC-GAGGAAGAAGACGGAAGAATGT-GCGTCTCGCCTTCTTTCACCAGA-GA
miR-146a RT	GAAAGAAGGCGAGGAGCAGATC-GAGGAAGAAGACGGAAGAATGT-GCGTCTCGCCTTCTTTCAACCCATG
miR-21 RT	GAAAGAAGGCGAGGAGCAGATC-GAGGAAGAAGACGGAAGAATGT-GCGTCTCGCCTTCTTTCTCAACATC
RNU44 RT	GAAAGAAGGCGAGGAGCAGATC-GAGGAAGAAGACGGAAGAATGT-GCGTCTCGCCTTCTTTCAGTCAGTT
miR-142 Forward	GCGGTGTAGTGTTTCCTACT
miR-143 Forward	GGTGCAGTGCTGCATCT
miR-146a Forward	GGCCTGAGAACAGAATTCCAT
miR-21 Forward	GCGGTAGCTTATCAGACTGATGT
RNU44 Forward	CCTGGATGATGATAAGCAAATG
Universal Reverse	CGAGGAAGAAGACGGAAGAAT

Serum PSA levels were determined by electrochemiluminescence immunoassay on the Roche Modular Analytics E 601 immunoassay analyzers. For PSA, inter-and intra-assay coefficients of variation (CV) values were <10%.

Statistical Analysis

For statistical analysis, SPSS 21.0 software package (SPSS Inc., Chicago, IL, USA) was used. Descriptive statistics such as age, free PSA, PSA levels were given in mean and median. For expression test of normality was followed by the Kruskal Wallis test for comparison. For post hoc comparison within significantly different groups, Mann Whitney U test was performed. Stepwise regression analysis was applied to determine the independent effects of expressions and to reveal the relation Spearman's test was used. The statistical significance level was $p<0.05$.

Results

Age, free PSA, and total PSA levels of the groups are given in *Table II*. No differences in age

Table II Demographic characteristics and pathological findings of the BPH, PCa, PCaMet groups.

	Age (years)	FreePSA (ng/mL)*	PSA*	Gleason's Score**
BPH (n: 42)	64.33±7.04	0.89±0.60	4.99±2.66	
PCa (n: 43)	65.55±6.69	1.52±0.91[a]	8.68±6.21[a]	6.37±0.61
PcaMet (n: 11)	66.45±4.69	1.97±0.53[a,b1]	16.04±8.48[a,b2]	7.00±0.10[b]

BPH, benign prostate hyperplasia; PCa, prostate cancer; MET, metastatic PCa.
Comparison with BPH a= p<0,001 ; a1=p<0.01
Comparison with PCa,b=p<0,001; b1=p<0,05 b2=p<0,01
*Kruskal-Wallis; **Mann Whitney U

Table III Analysis of ΔCT values of Benign Prostate Hyperplasia; Prostate Cancer; and Metastatic PCa groups.

	BPH*		PCA*		MET*	
miR-21	32.18	±2.78	32.54	±2.54	31.65	±3.05
miR-142	32.83	±3.31	30.86	±5.74	32.15	±3.06
miR-143	24.45	±12.92	25.58	±12.34	28.21	±11.91
miR-146	31.37	±9.37	32.36	±8.07	34.40	±2.07

BPH, benign prostate hyperplasia; PCa, prostate cancer; MET, metastatic PCa.
*Kruskal Wallis

Table IV Comparison of ΔΔCT Values of Benign Prostate Hyperplasia; Prostate Cancer; and Metastatic PCa groups.

	BPH	PCa	PCaMet
miR-21	-2.42±0.37	0.95±3.95[a]	0.02±4.41[a]
miR-142	-1.96±0.13	-0.02±3.30[a]	0.07±3.80[a]
miR-143	1.05±0.05	0.70±2.75	2.27±1.98
miR-146	1.46±0.23	-0.19±1.97	-0.35±2.79

BPH, benign prostate hyperplasia; PCa, prostate cancer; MET, metastatic PCa.
Comparison with BPH a= p<0.005
Comparison with PCa b=p<0.005

Table V Comparison of the fold changes of miRNA-143, 146, 142 and miR-21.

Group	miRNA143	miRNA146	miRNA142	miR21
PCa (n=45)	2.92[a]	3.8489[a]	10.0838[a]	6.294[b]
PcaMet (11)	0.37[a]	5.5250[a]	9.2418[a]	10.8464[b]

PCa, prostate cancer; MET, metastatic PCa.
Comparison with BPH group a=p<0.005
Comparison with RNU44 b=p<0.005

among the groups were found. Free PSA levels in the PCaMet group were found significantly higher than PBH (p<0.001) and PCa groups (p<0.05).

PSA levels in the PCaMet group were also higher than BPH (p<0.001) and PCA groups (p<0.01). According to the Gleason score, PCaMet also showed a higher mean compared to the PCa group (p<0.05).

When the average CT value of RNU44, which is the reference miRNA, is subtracted from the CT values of the groups, the ΔCT values are obtained and these values are given in *Table III*. No differences in the ΔCT values (*Table II*) were found among the groups (p> 0.05)

In *Table IV*, the mean of ΔΔCT values is given. In PCa and PCaMet groups the expression of miR-21 and miR-142 were higher compared to the BHP group. No other differences were observed among the other groups. In *Table V* data are presented as fold changes derived in terms of the mean $2^{-\Delta\Delta CT}$ method. According to *Table V*; miR-21 expression in the PCa group was 6.29 folds upregulated whereas in the PCaMet group 10.84 folds up regulated. miR-142 expression was 10.98-fold up-regulated in the PCa group and 9.24-fold upregulated in the PCaMet group. In *Table VI*, correlations of the miRNAs were given. When the total expression of miR-142 is evaluated, it showed a positive correlation with miR-21

Table VI Correlation table for the miRNA expressions.

	FreePSA	PSA	ΔCtmiR142	ΔCtmiR21	ΔCtmiR146	ΔCtmiR143
FreePSA		0.843**	-0.134	0.022	-0.172	-0.142
PSA	0.843**		-0.088	0.021	-0.156	-0.077
ΔCt mir142	-0.134	-0.088		0.751**	0.685**	0.530
ΔCt mir21	0.022	0.021	0.751**		0.780**	0.103
ΔCtmir146	-0.172	-0.156	0.685**	0.780**		0.645**
ΔCtmir143	-0.142	-0.077	0.530	0.103	0.645**	

PSA, Prostate specific antigen
**p<0.001 *p<0.05

and mir 146 (both p<0.001). Also, expression of miR-146 shows a positive correlation with both miR-21 and miR-143 (both p<0.001). The stepwise analysis results show that none of these markers are good at distinguishingly predicting the PSA level. And age, PSA, free PSA levels are independent of miR-21, miR-142, and miR-143 expression.

Discussion

In recent studies, miRNAs have been used as a predictive tool in many cancer types such as prostate, breast, ovarian, colon, pancreas, and lung. The serum expression level of miRNAs varies according to the cancer type, the diagnosis of the disease can be made early and treatment can be started and important information can be obtained about the prognosis (18–28). In the current study, we have found a significant difference between BPH and PCa in the expression of miR-21. In addition, when PSA levels were divided into groups, the PSA value of the groups (approximately 76%) had 2.5–9.9 ng/mL miR-21 and miR-143 expressions were significantly different among the groups. A negative correlation was found between miR-21 with oncogenic function and free PSA only in the PCa group. However, the expression of this miRNA was found to be an independent diagnostic factor in patients with Gleason scores. These results showed that changes (especially increases) in miR-21 expression can be effective in the transition to invasive potential. miR-21 can contribute to prostate cell transformation in PCa.

Although the current gold standard of PCa diagnosis is the prostate biopsy, this sampling technique is susceptible to misdiagnosis and a negative biopsy cannot fully rule out cancer (29). Circulating PSA is currently the most common non-invasive biomarker used to detect PCa, despite the controversies around its use as a screening tool (18). On the way to better tools, miRNAs can be a non-invasive marker. Zhang et al. (28) reported that serum miR-21 expression is upregulated in patients with hormone-refractory PCa, which is resistant to docetaxel-based chemotherapy. Serum miR-21 levels have been also shown to correlate with serum PSA. Upregulation of miR-21 is a critical event in the development of metastasis and invasion of PCa in immunocompromised mice (30). Bertoli et al. (31) reported a group of miR-21 is commonly deregulated in extracellular fluids of PCa patients. Kurul et al. (20) observed that miR-21 was overexpressed in patients with low-risk PCa. Unlike other studies, Folini et al. (32) found no difference in terms of miR-21 expression between PCa and normal tissue. Liu et al. (33) demonstrate that miR-21 induces tumor angiogenesis through targeting PTEN, leading to activate AKT (also known as Protein Kinase B) and extracellular signal-regulating kinase 1/2 (ERK1/2) signaling pathways, and thereby enhancing hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression; HIF-1α is a key downstream target of miR-21 in regulating tumor angiogenesis in human PCa cells. Li et al. (34) suggested that miR-21 could promote apoptosis resistance, motility, and invasion in prostate cancer cells and these effects of miR-21 may be partly due to its regulation of PDCD4, TPM1, and MARCKS. Gene therapy using miR-21 inhibition strategy may therefore be useful as a PCa therapy. In our study, overexpression of miRNA-21 was approximately observed in half of the patients. miR-21 expression of group C (PSA=10–19.9 ng/mL) is significantly higher than group A (PSA <2.5 ng/mL).

MiR-21 expression of group D (PSA>20 ng/mL) is significantly higher than group C (PSA=10–19.9 ng/mL). High levels of expression of miR-21, which was found to function as an oncogene, have been observed in hematological malignancies such as AML, CLL, and glioblastoma, and in many cancer types of solid tumors such as prostate and thus, miR-21 is transcriptionally activated by Stat3 in the IL-6 signaling pathway (35). miR-21 has been well characterized in invasion and metastasis events. miR-21 promotes cell movement and invasion by targeting the mRNA of PTEN, a tumor suppressor protein (32, 36) miR-21 expression in PCa tissue samples was significantly associated with pathological stage, lymph node metastasis, capsular invasion, organ-confined disease, Gleason score, biochemical recurrence, and patient follow-up.

The miR-21 expression could also be an independent predictor of biochemical recurrence (25). In our study, miR-21 was shown to be the most common of the over-expressed oncomiRs in PCa as in other cancers. In the PCaMet group, miR-21 is also the most upregulated of all miRNAs. Expression of miR-21 showed a positive correlation with both miR-146 and miR-143. Accordingly, gene therapy using miR-21 inhibition strategies may prove useful for PCa therapy (25). miR-21 is also helpful as a biomarker to predict cancer progression and miR-21 promotes tumor invasiveness and induces castration-resistance phenotype in PCa (37).

MiR-142-3p plays multiple roles in human cancers. miR-142-3p was upregulated in PCa tissues and cell lines relative to non-tumor samples and normal prostate cells. miR-142-3p levels were negatively correlated with forkhead box transcription factor O1 (FOXO1) in PCa and confirmed that miR-142-3p repressed FOXO1 expression through binding to the 3 UTR of FOXO1 mRNA (38). Barceló et al. (39) by using miRNA-based models states that evaluation of miRNA expression and PSA levels together, might increase the classification function of the PSA screening test with diagnostic and/or prognostic potential: (PSA + miR-142-3p + miR-142-5p + miR-223-3p) model to discriminate PCa from BPH; and (PSA + miR-342-3p + miR-374b-5p) model to discriminate between GS 7 tumors and men presenting PSA 4 ng/mL with no cancer or GS6 tumors.

The pathway analysis of predicted miRNA target genes supports a role for these miRNAs in the etiology and/or progression of PCa diagnostic biomarkers in semen exosomes. Baffa et al. (40) show that when the results obtained from primary and metastatic bladder tumors were compared, it was determined that miR-143 expression decreased, however, miR-142-5p expression level increased.

In another study conducted with serum samples of 25 metastatic PCa patients, an increase in expression levels of miR-143 was found (41). Another study by Barceló et al. (42) obtained that a clinically useful semen plasma miRNA-based combined model (PSA+miR-142-3p+miR-223-3p+miR-93-5p), which improves PCa specificity of the PSA test, for, firstly, predicting the presence of malignant tumors in a sample from the total population and secondly, and more interestingly for clinicians, for predicting PCa in samples from the positive PSA screening test (PSA>4 ng/mL). miR-143 expression in PCa tissue is significantly higher compared with expression in adjacent non-cancerous tissue, indicating an association between the molecule and the development of the disease (43, 44). Transfection of miR-143 induces the apoptosis of PCa LNCap cells by down-regulating Bcl-2 expression (45). In our study, miR-142 expression was suppressed by 53.3% in the BPH group and 33.3% in the PCa group. A positive correlation between miR-142 with miR-21 and miR-146 was found in all groups. When stepwise regression analysis was performed, the expression levels were affected by miR-142, and miR-146a was found to be independent of miR-21. Regression analysis showed that when the dependent variable miR-21 was taken, mir-142 and miR-146 were dependent on expression levels. There are also some regulatory roles attributed to miR-146a in PCa (46, 47).

Lin et al. (48) determined that the conditions with Gensini score> 7 were accompanied by the decreasing expression of miR-146a. When androgen-independent PCa cell lines (LNCaP-C81, LNCaP C4-2B, and PC3) were compared with androgen-sensitive cell lines (LNCaP and PC3-AR9), a decrease in miR-146 gene expression was observed in androgen-independent PCa cell lines. Moreover, the miR-146 loss has also been found to cause an aggressive course of PCa with an increase in multiple pro-metastatic proteins (ROCK1 and CXCR4)(48). Hsa-miR-146a expression was decreased in Ta tumors of urothelial carcinomas (49). It has been shown that miR-146a down-regulated may be due to increased EGFR signaling and may lead to aggressive PCa progression (50). In the study of Mihelich et al. (51), 50 men with 100% Gleason grade 3 (low grade) PCa, 50 men with 30–90% Gleason grade 4 and/or 5 (high grade) PCa, and 50 patients with BPH, 16 miRNAs were detected in serum. miR-146a, one of which was detected at low levels in the high-grade PCa group. However, higher, and more heterogeneous levels were significantly detected in patients with low-grade PCa or BPH. It seems that our study is compatible with the existing studies in the literature. In PCa, miR-146a acts as a tumor suppressor gene.

As with many diseases, accurate and timely diagnosis of the disease is as vital as the application of appropriate and effective treatment in PCa. Although overexpression of miR-21 does not allow it to be used in the differential diagnosis of BPH, metastasis, and PCa, anti-miR-21 developed against miR-21 overexpressed in tissues and cells may be an effective treatment option for this disease. Therefore, it is obvious that the free and total PSA test used in diagnosis will be used to distinguish between PCa and BPH for a while. In terms of PCa, miR-21, miR-142, miR-143, miR-146a can contribute to the literature as well as guide developing PCa treatment strategies. But it has been shown that accurate and rapid diagnoses of PCa patients staying in the gray area cannot be possible with these miRNAs. These miRNAs should be investigated more widely to be used in screening for malignant-benign differentiation, to increase the diagnostic power of miRNAs, or to be used as a stronger marker alone or as a panel.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, I. Murat Bolayırlı upon request.

Conflict of interest statement

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

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TOTAL ANTI-SARS-COV-2 ANTIBODIES MEASURED 6 MONTHS AFTER PFIZER-BIONTECH COVID-19 VACCINATION IN HEALTHCARE WORKERS

UKUPNA ANTI-SARS-COV-2 ANTITELA IZMERENA 6 MESECI NAKON PHIZER-BIONTECH COVID-19 VAKCINACIJE KOD ZDRAVSTVENIH RADNIKA

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Summary

Background: This study aimed at monitoring the kinetics of serum total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) antibodies in a cohort of healthcare workers after voluntary vaccination with Pfizer-BioNTech coronavirus disease 2019 (COVID-19) mRNA-based vaccine.
Methods: The study population consisted of 787 healthcare workers (mean age 44±12 years; 66% females), who received two 30 μg doses of Pfizer-BioNTech COVID-19 vaccine, 3 weeks apart. Venous blood was drawn before the first vaccine dose, immediately before the second vaccine dose, and then at 1, 3 and 6 months after the second vaccine dose. Serological testing employed the total anti-SARS-CoV-2 antibodies measurement with Roche Elecsys Anti-SARS-CoV-2 S chemiluminescent immunoassay.
Results: The median serum levels of total anti-SARS-CoV-2 antibodies reached the peak (1762 kU/L) 1 month after the second vaccine dose, but tended to progressively decline at the 3-month (1086 kU/L) and 6-month (802 kU/L) follow-up points. Overall, the values after 3- and 6-months were 37% and 57% lower than the corresponding concentrations measured at the peak. No healthcare worker had total anti-SARS-CoV-2 antibodies below the method-dependent cut-off after 6 months. The decline compared to the peak was more accentuated in baseline seropositive persons than in those who were baseline

Kratak sadržaj

Uvod: Ova studija je imala za cilj praćenje kinetike ukupnih serumskih antitela na SARS-CoV-2 (teški akutni respiratorni sindrom koronavirus 2) u kohorti zdravstvenih radnika nakon dobrovoljne vakcinacije sa Pfizer-BioNTech koronavirusnom bolešću 2019 (COVID-19) na bazi mRNA-vakcine.
Metode: Ispitivanu populaciju činilo je 787 zdravstvenih radnika (prosečna starost 44 ± 12 godina; 66% žena), koji su primili dve doze od 30 μg vakcine Pfizer-BioNTech protiv COVID-19, u razmaku od 3 nedelje. Venska krv je uzeta pre prve doze vakcine, neposredno pre druge doze vakcine, a zatim 1, 3 i 6 meseci nakon druge doze vakcine. Serološko testiranje je koristilo merenje ukupnih anti-SARS-CoV-2 antitela pomoću Roche Elecsis Anti-SARS-CoV-2 S hemiluminiscentnog imunološkog testa.
Rezultati: Srednji serumski nivo ukupnih antitela na SARS-CoV-2 dostigao je vrhunac (1762 kU/L) 1 mesec nakon druge doze vakcine, ali je imao tendenciju progresivnog opadanja tokom 3 meseca (1086 kU/L) i 6-mesečni (802 kU/L) praćenja. Sve u svemu, vrednosti nakon 3- i 6 meseci bile su 37% i 57% niže od odgovarajućih koncentracija izmerenih na vrhuncu. Nijedan zdravstveni radnik nije imao ukupna antitela na SARS-CoV-2 ispod granične vrednosti zavisno od metode. Pad u odnosu na vrhunac bio je više naglašen kod osnovnih seropozitivnih osoba nego kod onih koje su bile osnovno seronegativne (74% naspram 52%) kohorte. Šestomesečna post-vakcinalna antitela na SARS-

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seronegative (74% vs. 52%) cohort. The 6-month post-vaccination anti-SARS-CoV-2 antibodies in subjects aged <65 years remained over 2-fold higher than in those aged ≥65 years (813 vs. 343 kU/L) and also remained consistently higher in women than in men.

Conclusions: Gradual decline of total anti-SARS-CoV-2 antibodies occurred 6 months after Pfizer-BioNTech COVID-19 vaccination, though values remained higher than the method-dependent cut-off, with no case of seronegativization.

Keywords: COVID-19, SARS-CoV-2, vaccination, antibodies, immune response

CoV-2 kod ispitanika mlađih od 65 godina ostala su preko dva puta veća od onih izmerenih kod osoba starijih od 65 godina (813 naspram 343 kU/L) i takođe su konstantno veća kod žena nego kod muškaraca.

Zaključak: Postepeno opadanje ukupnih antitela na SARS-CoV-2 dogodilo se 6 meseci nakon vakcinacije protiv Pfizer-BioNTech-a protiv COVID-19, iako su vrednosti ostale veće od preseka zavisnog od metode, bez slučaja seronegativizacije.

Ključne reči: COVID-19, SARS-CoV-2, vakcinacija, antitela, imuni odgovor

Introduction

There is now consolidated evidence that the onset of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection in healthcare facilities is associated with an enhanced risk of morbidity and mortality, both in hospitalized patients as well as in healthcare workers. Therefore, regular SARS-CoV-2 diagnostic testing and coronavirus disease 2019 (COVID-19) vaccination are now regarded as major cornerstones for preventing and/or limiting the burden of SARS-CoV-2 inside and outside healthcare facilities (1). A recent study published by Yoshimura et al. (2) evidenced a fairly good response in terms of anti-SARS-CoV-2 IgG level after a complete cycle of Pfizer BNT162b2 mRNA-based COVID-19 vaccine. Since it is now acknowledged that large part of vaccine effectiveness is attributable to the generation of anti-SARS-CoV-2 antibodies of different classes and with different antigenic targets (3), but capable to quickly and efficiently neutralize viral particles inside the host, regular assessment of these antibodies seems essential for monitoring immune protection among healthcare workers, especially given that serum levels of most vaccine-induced antibodies are observed to decline over time (4). This study was hence aimed at monitoring the kinetics of serum total anti-SARS-CoV-2 antibodies in a cohort of healthcare workers who underwent voluntary administration of Pfizer-BioNTech COVID-19 mRNA-based vaccine.

Materials and Methods

The study population consisted of 787 healthcare workers of Peschiera del Garda hospital in Italy (mean age 44±12 years; 66% females), who received two 30 μg doses of Pfizer-BioNTech COVID-19 vaccine, 3 weeks apart. Venous blood was drawn before the first vaccine dose, immediately before the second vaccine dose (i.e., 21 days after the first dose), and then at 1, 3 and 6 months after the second vaccine dose (51, 111 and 201 days after the first vaccine dose). Serological testing was based on total anti-SARS-CoV-2 antibodies measurement with Roche Elecsys Anti-SARS-CoV-2 S chemiluminescent immunoassay, on Roche Cobas 6000 (Roche Diagnostics, Basel, Switzerland; positive result: ≥0.8 kU/L). Recent evidence suggests that this method reliably mirrors the overall SARS-CoV-2 neutralizing potential developed after COVID-19 vaccination, with sensitivity as high as 98% compared to a pseudovirus neutralization test (5). Test results were reported as median and interquartile range (IQR), and the statistical analysis was performed using Analyse-it (Analyse-it Software Ltd, Leeds, UK). Between-group comparisons were carried out with Mann-Whitney test. All study participants gave informed consents for vaccination and undergoing serial anti-SARS-CoV-2 antibodies monitoring. This observational study was reviewed and cleared by the Ethics Committee of Verona and Rovigo provinces (3246CESC).

Results

The main results of this study are shown in *Figure 1*. The median serum levels of total anti-SARS-CoV-2 antibodies reached the peak (1762 kU/L; IQR, 933–3761 kU/L) 1 month after the second vaccine dose (i.e., 51 days after the first vaccine dose), but then tended to progressively decline at the 3-month (i.e., 111 days after the first vaccine dose: 1086 kU/L; IQR, 629–2155 kU/L) and 6-month (i.e., 201 days after the first vaccine dose: 802 kU/L; IQR, 447–1487 kU/L) follow-up points. Overall, the serum values after 3- and 6-months were 37% (IQR, 15–53%; $p<0.001$) and 57% (IQR, 35–71%; $p<0.001$) lower than the corresponding concentrations measured at the peak, respectively. Notably, no healthcare worker had total anti-SARS-CoV-2 antibodies below the method-dependent cut-off (i.e., 0.8 kU/L) at either the 3- or 6-month follow-up points. Notably, the relative decline of serum antibodies values compared to the peak was more accentuated in baseline seropositive (74%; IQR, 62–82%) persons than in those who were baseline seronegative (52%; IQR, 31–67%) cohort. A decrease of serum total anti-SARS-CoV-2 antibodies levels occurred in 576/624 (92.3%) baseline seronegative subjects and in 160/163 (98.1%) seropositive subjects. The 6-month post-vaccination serum level of total anti-SARS-CoV-2 antibodies in subjects aged <65 years (n=754; 813

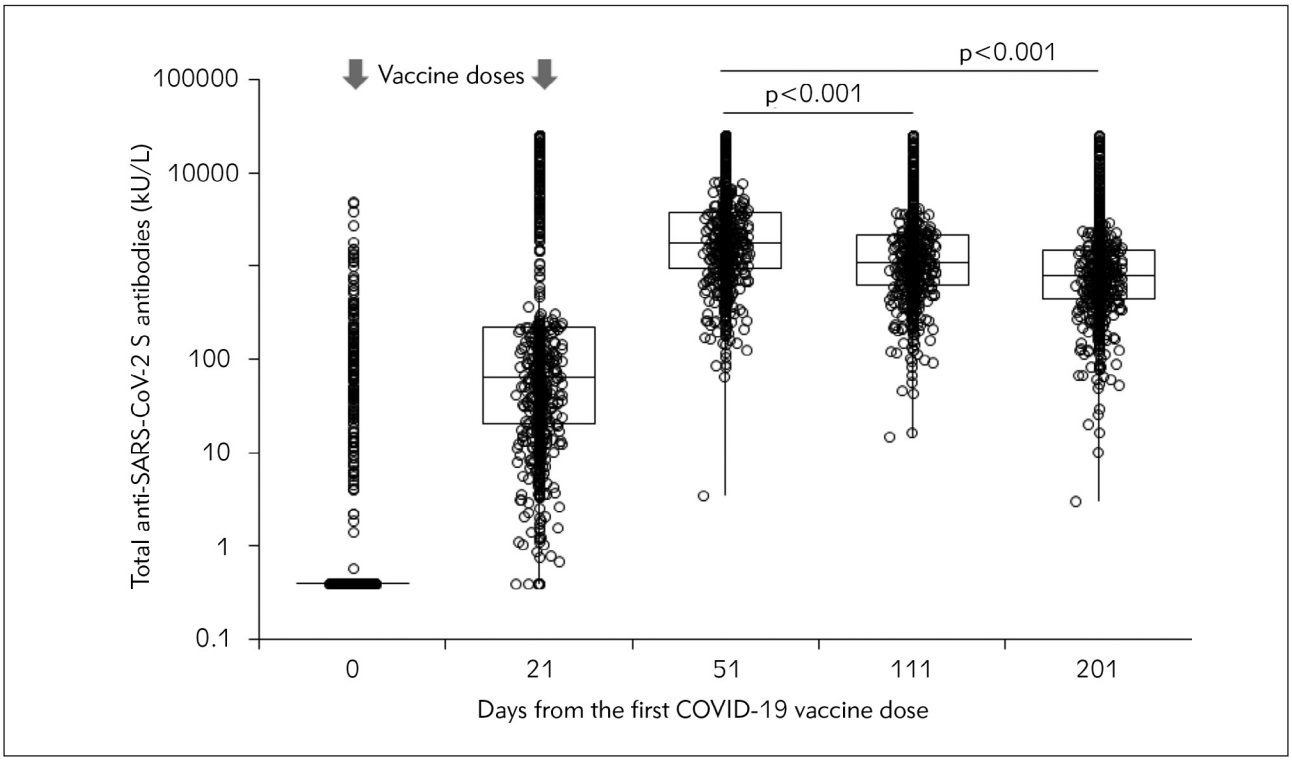

Figure 1 Kinetics of total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) serum antibodies in a cohort of healthcare workers who underwent voluntary administration of two doses of Pfizer-BioNTech COVID-19 mRNA-based vaccine.

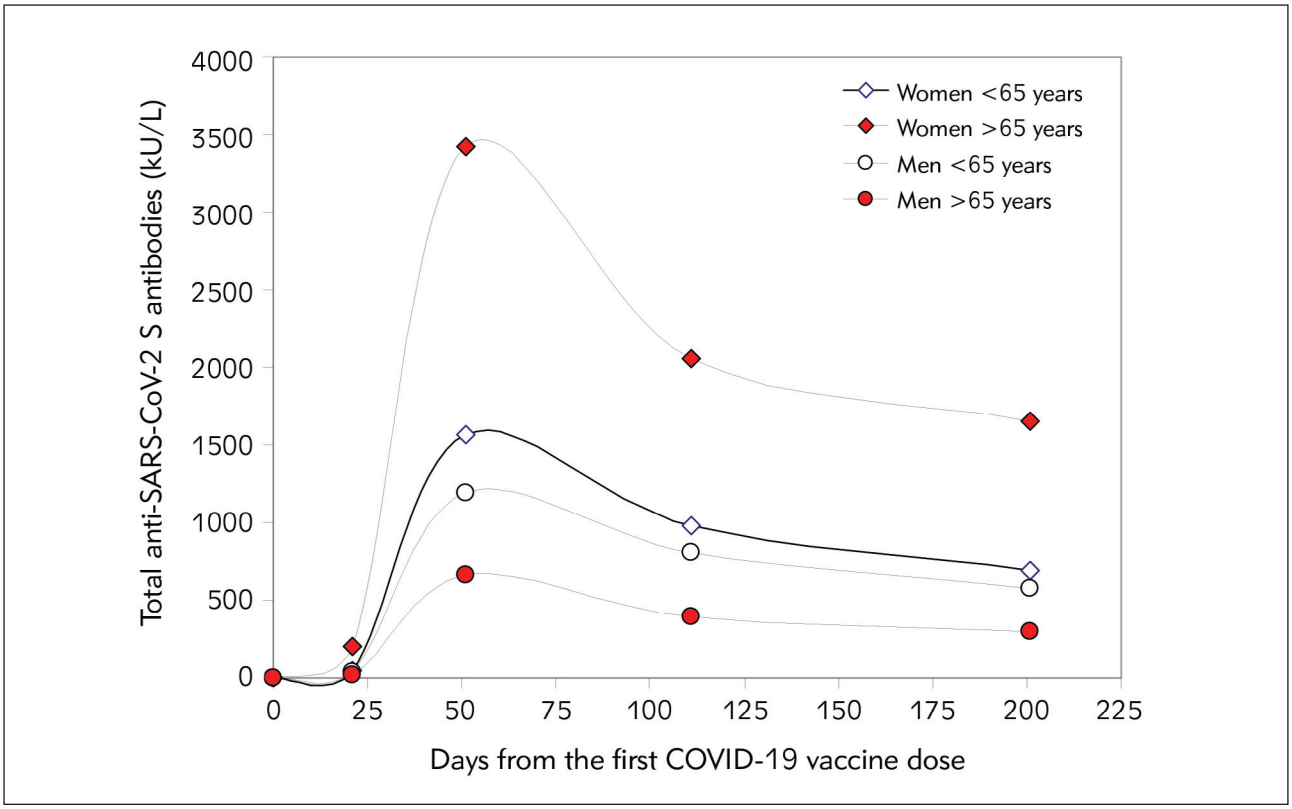

Figure 2 Kinetics of total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) serum antibodies in a cohort of healthcare workers who underwent voluntary administration of two doses of Pfizer-BioNTech COVID-19 mRNA-based vaccine, stratified by age and sex.

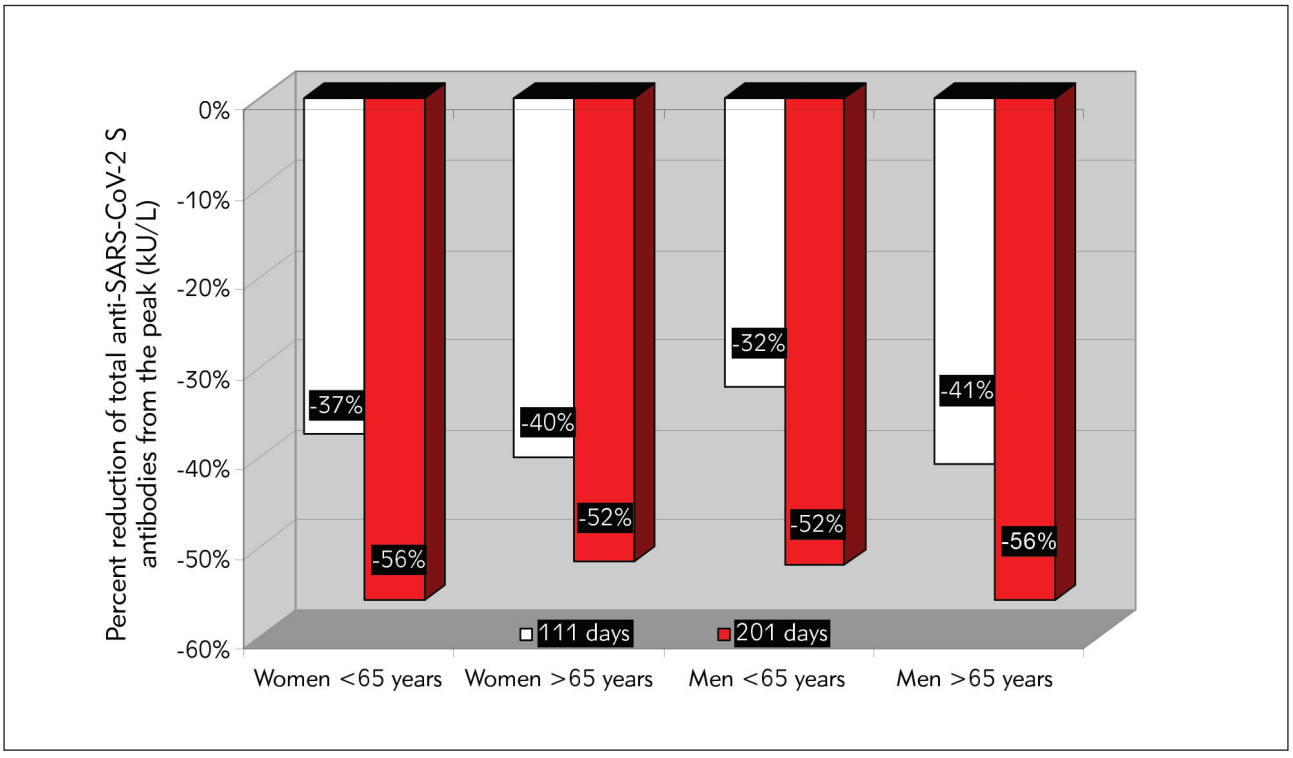

Figure 3 The relative decrease of the serum level of total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) antibodies compared to the peak after 3 and 6 months from coronavirus disease 2019 (COVID-19) vaccination, stratified by age and sex.

KUL; IQR, 473-1501 kU/L) remained over 2-fold higher than that measured in those aged ≥65 years (n=33; 343 kU/L; IQR, 210-903 kU/L; p<0.001), and also remained consistently higher in women than in men (*Figure 2*). The relative decrease of the serum level of total anti-SARS-CoV-2 antibodies compared to the peak after 3 and 6 months from vaccination is also shown in *Figure 3*, where a very similar trend can be seen despite the rather different peak levels (*Figure 3*).

Discussion

The preliminary results of this ad-interim analysis of anti-SARS-CoV-2 humoral immunity in healthcare workers who received a complete (2-dose) cycle of Pfizer-BioNTech COVID-19 vaccination reveal that a gradual decline of total anti-SARS-CoV-2 antibodies (and hence of neutralizing potential) has occ urred 6 months after vaccination, though the antibodies values have remained still considerably higher than the method-dependent cut-off and no seronegativization seems to have occurred in either baseline seronegative or seropositive subjects. These results compared rather well with other preliminary data made available by Bayart and colleagues (6), who also showed that the decay of anti-SARS-CoV-2 RBD total antibodies after 6 months from Pfizer-BioNTech COVID-19 vaccine administration was around 55% in seronegative healthcare workers, thus nearly identical to the 57% decrease that we found in our cohort over an identical period of monitoring. Although better medium-term response would have been theoretically expected in subjects with double immunization (i.e., SARS-CoV-2 infection followed by full vaccination), we instead found that total anti-SARS-CoV-2 antibodies reduction appeared larger in baseline seropositive than seronegative recipients (i.e., 74% vs. 52%) (*Figure 2*). This is perhaps attributable to the fact that the former cohort also displayed higher peak values, so that the catabolism may have been relatively more pronounced in these subjects. The difference in antibody levels distribution between age and sexes is not really unexpected, since some concomitant studies showed similar trends, with lower immunogenicity been reported in men and in the elderly (7, 8).

Notably, the significantly lower antibodies values found in elderly healthcare male workers (*Figure 2*) also raises further doubts as to whether further vaccine boosters may be especially advisable in this cohort between 6 and 12 months after completing a regular Pfizer-BioNTech COVID-19 vaccine cycle, to ensure a reinforced protection (9), since this is the most vulnerable population to severe SARS-CoV-2 infection (10).

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: This observational study was reviewed and cleared by the Ethics Committee of Verona and Rovigo provinces (3246CESC).

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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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ASSOCIATION OF *KLOTHO* GENE POLYMORPHISM WITH CEREBRAL INFARCTION

VEZA POLIMORFIZMA *KLOTHO* GENA SA CEREBRALNIM INFARKTOM

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Summary

Background: We aimed to investigate the expression of *Klotho* gene in peripheral blood of patients with cerebral infarction (CI) and the association of its polymorphisms with the occurrence of CI.
Methods: A total of 60 CI patients (CI group) and 20 healthy people receiving physical examination (control group) were enrolled as the research subjects. The expression of *Klotho* gene in CI group and control group was determined using enzyme-linked immunosorbent assay kit. Single nucleotide polymorphisms (rs192031, rs200131 and rs102312) in the promoter region of the *Klotho* gene were typed *via* conformational difference gel electrophoresis. Besides, whether the distribution frequencies of *Klotho* genotypes conformed to Hardy-Weinberg equilibrium was evaluated by chi-square test. Meanwhile, the associations of *Klotho* alleles and gene polymorphisms with CI occurrence were analyzed.
Results: The protein expression level of *Klotho* in the peripheral blood was remarkably lower in patients in CI group than that in control group ($P<0.05$).Hardy-Weinberg equilibrium analysis revealed that *Klotho* gene polymorphisms (rs192031, rs200131 and rs102312) conformed to the genetic equilibrium distribution ($P>0.05$). Gene-based association analysis manifested that only rs192031 polymorphism and alleles were correlated with CI occurrence ($P<0.05$). Systolic blood pressure and high-density lipoprotein cholesterol were notably higher in CI patients with TT genotype of *Klotho* gene polymorphism rs192031 than those in control group ($P<0.05$).

Kratak sadržaj

Uvod: Cilj nam je bio da istražimo ekspresiju *Klotho* gena u perifernoj krvi pacijenata sa cerebralnim infarktom (CI) i povezanost njegovih polimorfizama sa pojavom CI.
Metode: Ukupno 60 pacijenata sa CI (CI grupa) i 20 zdravih osoba na fizikalnom pregledu (kontrolna grupa) upisano je kao subjekti istraživanja. Ekspresija *Klotho* gena u CI grupi i kontrolnoj grupi je određena korišćenjem kompleta za enzimski imunosorbentni test. Polimorfizmi pojedinačnih nukleotida (rs192031, rs200131 i rs102312) u promotorskom regionu *Klotho* gena su tipizovani putem konformacione razlike u gel elektroforezi. Osim toga, da li su frekvencije distribucije *Klotho* genotipova u skladu sa Hardy-Weinbergovom ravnotežom procenjeno je hi-kvadrat testom. U međuvremenu, analizirane su asocijacije *Klotho* alela i polimorfizama gena sa pojavom CI.
Rezultati: Nivo ekspresije proteina *Klotho*-a u perifernoj krvi bio je značajno niži kod pacijenata u CI grupi nego u kontrolnoj grupi ($P<0,05$). Hardy-Weinbergova analiza ravnoteže otkrila je da se polimorfizmi *Klotho* gena (rs192031, rs200131 i rs102312) podudaraju sa distribucija genetičke ravnoteže ($P>0,05$). Analiza asocijacije zasnovana na genima pokazala je da su samo rs192031 polimorfizam i aleli u korelaciji sa pojavom CI ($P<0,05$). Sistolni krvni pritisak i holesterol lipoproteina visoke gustine bili su značajno viši kod pacijenata sa CI sa TT genotipom polimorfizma *Klotho* gena rs192031 od onih u kontrolnoj grupi ($P<0,05$). Štaviše, nije bilo asocijacija rs200131 i rs102312 polimorfizama i alela sa pojavom CI ($P>0,05$).

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Furthermore, there were no associations of rs200131 and rs102312 polymorphisms and alleles with the occurrence of CI (P>0.05).
Conclusions: The expression level of *Klotho* is evidently reduced in the peripheral blood of CI patients. Rs192031 in the promoter region of the *Klotho* gene is associated with the occurrence of CI, while rs200131 and rs102312 have no relations with CI.

Keywords: *Klotho*, cerebral infarction, polymorphism

Zaključak: Nivo ekspresije *Klotho*-a je evidentno smanjen u perifernoj krvi pacijenata sa CI. Rs192031 u promotorskom regionu *Klotho* gena je povezan sa pojavom CI, dok rs200131 i rs102312 nemaju veze sa CI.

Ključne reči: *Klotho*, cerebralni infarkt, polimorfizam

Introduction

Cerebral infarction (CI) is an ischemic-hypoxic necrosis induced by insufficient blood supply to local brain tissues. It is characterized by high incidence and disability rates. Various factors can lead to the occurrence of CI, the main cause of which is cerebral atherosclerosis (1). The nature of atherosclerosis lies in the chronic activation of endothelial cells caused by inflammatory and fibro proliferative reactions, which can induce vascular stenosis and insufficient blood supply to the brain, and the secondary rupture of fibrous cap in atherosclerotic plaque eventually leads to CI (2, 3).The correlation of genetic factors (especially gene polymorphisms) with acute atherosclerotic CI has become a research hotspot in recent years. Cathepsin S (CTSS), a cysteine protease of the papain superfamily, plays a vital role in the degradation and reconstruction of extracellular matrix, antigen presentation, inflammation, immunity and angiogenesis (4). A study revealed that single nucleotide polymorphisms (SNPs) (rs774320676 and rs928508030) of the CTSS gene are related to the risk of acute atherosclerotic CI. The T allele of rs774320676 and the G allele of rs928508030 of CTSS are genetic susceptibility genes for acute atherosclerotic CI (5).

Klotho, as an anti-aging gene, is able to reduce oxidative stress, thus protecting the cardio-cerebrovascular system (6). A study indicated that the elevated plasma *Klotho* concentration in patients with acute ischemic stroke is correlated with a good functional prognosis (7). Basic experiment illuminated that *Klotho* is an inducer of metabolic coupling between neurons and astrocytes. *Klotho* can be released by the neuronal glutamatergic activity and insulin regulation, thereby stimulating the formation and release of lactic acid in astrocytes (8). Traditional Chinese medicine ligustilide is capable of ameliorating cerebral ischemia-reperfusion injury in mice by up-regulating *Klotho* expression (9). However, the association of *Klotho* gene polymorphism with CI has not been reported yet.

The distribution of polymorphisms (rs192031, rs200131 and rs102312) in the promoter region of the *Klotho* gene was determined in CI patients in this study, so as to provide a certain reference for further research of the genetic pathogenesis of CI.

Materials and Methods

Subjects

A total of 60 CI patients admitted to our hospital from January 2016 to January 2019 were enrolled as the research subjects, including 31 males and 29 females aged (57.41±2.34) years old. About 4 mL of venous blood was extracted, anticoagulated with sodium citrate, and stored in a refrigerator at -20 °C. Meanwhile, 20 healthy people receiving physical examination were selected as the controls, with 10 males and 10 females aged (57.13±2.19) years old. This study was approved by the Ethics Committee of Linyi Central Hospital, and all the participants signed the informed consent. The patients in the CI group met the diagnostic criteria of the *Chinese Guidelines for Diagnosis and Treatment of Acute Ischemic Stroke 2019*, without transient ischemic attack, and the diagnosis was confirmed by imaging examinations. The subjects in the control group were healthy people who underwent routine physical examination in our hospital, without a history of cardiovascular and cerebrovascular diseases and related family history.

Detection of serum Klotho *protein*

About 4 mL of venous blood was collected and anticoagulated with sodium citrate to detect serum Klotho protein using an enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, St. Louis, MO, USA) in accordance with the instructions.

Deoxyribonucleic acid (DNA) extraction

A total of 4 mL of patient's EDTA-anticoagulated blood was taken to extract genomic DNA according to the instructions of the DNA Extraction Kit (Guge Bio-Technology Co., Ltd., Wuhan, China). Subsequently, the quality of 2 μL of sample was measured in 1.5% agarose gel electrophoresis. Meanwhile, the concentration of the extracted DNA was determined using an ultraviolet spectrophotometer.

Polymerase chain reaction (PCR) amplification

Primers were designed to amplify *Klotho* gene polymorphismsrs192031, rs200131 and rs102312.

Table I Primer sequences and product sizes of different polymorphisms in the promoter region of the *Klotho* gene.

Polymorphism	Primer sequence (5′-3′)	Product (bp)
rs192031	Forward: AGCTGATGGCTATCGTAGCGACCReverse: TGGGCTAGCTAGCTAGTCGG	223
rs200131	Forward: AAGTCGATCGTTAGGGCAAReverse: GTGACTTAGGCCAATGAAA	302
rs102312	Forward: AGGCAAATTCGATCGTAGCTAGReverse: TGCTGTAGCTAGCTGATCG	381

Table II Probe sequences and product sizes of ligase reaction for different polymorphisms of the *Klotho* gene.

Polymorphism	Probe	Probe sequence (5′-3′)	Product (bp)
rs192031	rs192031 rs192031-Ars192031-T	P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTT-FAM TTTTTTTTTTTTTTACCCATTTTTTTTTAT TTTTTTTTTTTTTTGCGACGAGCATTTTTTTTTAAA	124
rs200131	rs192031 rs192031-C rs192031-T	P-AGCCATGCACCCAATTTTTTTTTTTTTTTTTTTT-FAM TTTTTTTTTTTTTTTTTTTTTTTTTCGTAGCTAAAC TTTTTTTTTTTTTTTTTTTTTTTTTTACGATCGATG	115
rs102312	rs102312 rs102312-Ars102312-C	P-ACGGGATGCCATTTTTTTTTTTTTTTTTTT-FAM TTTTTTTTTTTTTTTTTTTTTTTTTTTGCGGACGAG TTTTTTTTTTTTTTTTTTTTTTTTTGCGGCCAAA	108

Primer sequences of each polymorphism were shown in *Table I*. The reaction system of PCR (20 μL) included 2.0 μL of DNA template, 10.0 μL of 2× mix, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 7.2 μL of ddH_2O. PCR amplification was performed under the following conditions: 95 °C for 120 s, 35 cycles of 94 °C for 30 s, 57 °C for 90 s and 72 °C for 60 s, followed by extension at 72 °C for 10 min. Subsequently, agarose gel electrophoresis was utilized to detect the amplification of gene fragments.

Ligase detection reaction

The upstream and downstream probes used in this reaction were designed and synthesized by BGI. The upstream probe was modified by phosphorylation at the5′-terminal region to prepare a probe mixture with a concentration of 12.5 pmol/μL. Ligase detection reaction system (3.05 μL) was composed of 0.05 μL of ligase, 1 μL of buffer, 1 μL of PCR product, and 1 μL of probe mixture. PCR amplification was carried out under the following conditions: 95 °C for 120 s, 94 °C for 15 s and 50 °C for 25 s, 30 cycles in total. After that, the concentration was measured using the ultraviolet spectrophotometer. Subsequently, BGI was commissioned to sequence and analyze the target gene. All data were analyzed using Gene Mapper (*Table II*).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was applied for statistical analysis. Enumeration data were expressed by frequency and percentage, and measurement data were presented as mean±standard deviation. The genotype frequency in the sample was calculated and tested using the Hardy-Weinberg equilibrium formula. The chi-square test was used for multiple comparisons of enumeration data. Besides, *t*-test and analysis of variance were utilized for measurement data. $P<0.05$ indicated that the difference was statistically significant.

Results

Comparisons of clinical baseline data between CI group and control group

As shown in *Table III*, blood glucose and systolic blood pressure were obviously increased, but high-density lipoprotein cholesterol (HDL-C) significantly declined in CI group compared with those in control group ($P<0.05$).

Comparison of Klotho *protein levels in peripheral blood between CI group and control group*

As shown in *Figure 1*, the expression level of Klotho in the peripheral blood of patients was markedly reduced in CI group in comparison with that

Table III Comparisons of clinical data between CI group and control group.

Index	Control group	CI group	P
Triglyceride (mmol/L)	1.75±0.28	1.81±0.21	0.112
Total cholesterol (mmol/L)	4.88±0.48	4.91±0.25	0.341
HDL-C (mmol/L)	1.28±0.11	1.14±0.49	0.003
LDL-C (mmol/L)	2.99±0.28	3.02±0.12	0.288
Blood glucose (mmol/L)	5.22±1.28	6.29±0.94	0.001
Systolic blood pressure (mmHg)	131.92±4.02	145.03±6.82	0.000
Diastolic blood pressure (mmHg)	80.02±3.02	84.29±4.02	0.018

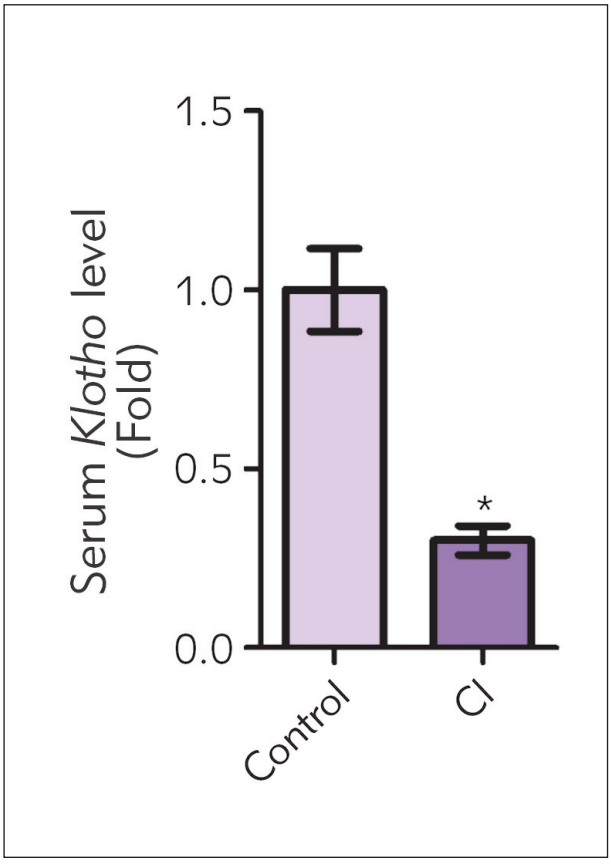

Figure 1 Comparison of *Klotho* protein levels in peripheral blood between CI group and control group. CI: cerebral infarction group. Control: healthy control group. *P<0.05: a statistical difference vs. control group.

in control group (P<0.05), indicating that Klotho might be involved in the occurrence and development of CI.

Analysis results of Klotho gene polymorphism-srs192031, rs200131 and rs102312

The *Klotho* gene polymorphisms rs192031, rs200131 and rs102312 in CI group and control group were cleaved by *BSTU I* restriction enzyme, manifesting that polymorphism rs192031 had two alleles (A and T) and three genotypes (AA, AT and TT), rs200131 had two alleles (C and T) and three genotypes (CC, CT and TT), and rs102312 had two alleles (A and C) and three genotypes (AA, AC and CC).

Results of Hardy-Weinberg equilibrium test

The linkage disequilibrium test results of different *Klotho* gene polymorphisms were tested using the Hardy-Weinberg equilibrium formula. As shown in *Table IV*, r^2<0.33 was detected between groups of polymorphisms, indicating the conformity of polymorphisms with the equilibrium test between groups.

Associations of Klotho gene polymorphisms with CI

The genotype frequencies of each gene polymorphism in the two groups were shown in *Table V*. The polymorphism rs192031 was remarkably related to the occurrence of CI (P<0.05), while rs200131 and rs102312 were not correlated with CI (P>0.05).

Table IV Results of linkage equilibrium test for the *Klotho* gene polymorphisms between groups.

Polymorphism	r^2		
	rs192031	rs200131	rs102312
rs192031	-	0.012	0.108
rs200131	0.012	-	0.221
rs102312	0.108	0.221	-

Table V Distribution of different genotypes of *Klotho* gene polymorphisms and CI.

Group	rs192031			rs200131			rs102312		
	AA	AT	TT	CC	CT	TT	AA	AC	CC
CI	10.1%	50.9%	39.0%	20.1%	48.0%	30.0%	20.1%	50.9%	29.0%
Control	24.0%	51.2%	24.8%	22.8%	46.0%	31.2%	19.3%	51.2%	29.5%
C^2	1.661			0.499			0.717		
P	0.032			0.221			0.610		

Table VI Distribution of alleles of *Klotho* gene polymorphisms and CI.

Group	rs192031		rs200131		rs102312	
	A	T	C	T	A	C
CI	30.00%	70.00%	70.21%	29.79%	45.23%	54.77%
Control	82.11%	17.89%	72.22%	27.78%	42.08%	57.92%
C^2	1.432		0.782		0.644	
P	0.001		0.114		0.412	

Table VII Correlation analysis of TT genotype of *Klotho* gene polymorphism rs192031 and clinical parameters of CI.

Index	TT genotype		P
	CI	Control	
Age	48±7	49±10	0.231
Triglyceride, (mmol/L)	1.88±0.27	1.85±0.18	0.302
Total cholesterol, (mmol/L)	4.91±0.26	4.91±0.18	0.429
HDL-C, (mmol/L)	1.33±0.28	1.16±0.15	0.019
LDL-C, (mmol/L)	3.02±0.15	2.98±0.21	0.192
Blood glucose, (mmol/L)	6.22±0.56	5.81±0.47	0.821
Systolic blood pressure, (mmHg)	142.11±4.20	134.83±5.22	0.001
Diastolic blood pressure, (mmHg)	82.11±5.92	80.01±6.92	0.231

Association of Klotho alleles with CI

According to the genotype frequencies of each polymorphism in the two groups (*Table VI*), the polymorphism rs192031 was obviously associated with occurrence of CI ($P<0.05$), while rs200131 and rs102312 had no relations with CI ($P>0.05$).

Correlations of TT genotype of Klotho gene polymorphism rs192031 with clinical parameters of CI

The further research revealed that the systolic blood pressure and HDL-C were notably higher in CI patients with TT genotype of *Klotho* gene polymorphism rs192031 than those in control group ($P<0.05$) (*Table VII*).

Discussion

CI is a cerebrovascular disease caused by cerebral blood supply disorders, which is characterized by high morbidity and high mortality, seriously threatening people's lives (10). CI can be caused by multiple factors, especially atherosclerosis. Atherosclerotic CI is a multi-source disease resulting from the combined action of genetic and environmental factors. Atherosclerosis can lead to stenosis, occlusion and thrombosis of the blood vessel cavity, or the shedding of unstable plaques can result in CI (11). Therefore, further elucidating the genetic mechanism of CI occurrence is of important significance for the early prevention and precise treatment of CI.

Previous studies have shown that the polymorphisms of multiple genes are potentially correlated with the occurrence and prognosis of CI. The allele frequency of APOE 4 is notably higher in CI patients than that in healthy controls (12). Tissue inhibitors of metalloproteinases (TIMPs), as endogenous inhibitors of matrix metalloproteinases, participate in the normal cellular processes and the occurrence and progression of atherosclerosis. A study indicated that there is a strong linkage disequilibrium between 1296T/C and -915A/G of TIMP gene, and people with TC+CC genotype are 1.8 times more likely to suffer mixed carotid plaque than those with TT genotype (13). The certain association of *Klotho* gene polymorphisms with the occurrence and progression of CI was revealed in this study.

In animal models, a broad phenotype similar to the aging phenotype will be caused by suppressing the *Klotho* gene, including atherosclerosis, ectopic calcification, infertility, skin atrophy and severe hypoglycemia, while the overexpression of *Klotho* gene increases the overall life span of guinea pigs by 20-30% (14). The human *Klotho* gene is located on chromosome 13 and can be expressed as a secretory Klotho protein by variable cleavage of the third exon. The anchored *Klotho* protein is mainly present in the distal convoluted tubules of the kidney and the choroid plexus of the brain, but it can be processed

and released into the blood after translation, dissociating outside the cells and playing a hormone-like role (15). *Klotho* gene has 6 SNPs in exon 2 and flanking regions, and such polymorphisms are closely related to the occurrence and progression of cardiocerebrovascular diseases (16). The content of serum *Klotho* is higher in patients with a history of myocardial infarction but no history of coronary artery disease or stroke, but the haplotype of *Klotho* is not correlated with the above variables (17). *Klotho* gene polymorphism may be a genetic risk factor for atherosclerotic coronary artery disease rather than vasospasm angina pectoris in the Japanese population. Specifically, a higher ratio of A genotype of the *Klotho* gene polymorphism G-395A was observed in patients with coronary heart disease than that in healthy controls (18).The correlation between polymorphisms (rs192031, rs200131 and rs102312) in the promoter region of *Klotho* and CI occurrence in the Han population was analyzed in this study. The protein was extracted from the peripheral blood of healthy people undergoing physical examination and CI patients. First, the results of ELISA revealed that the expression of *Klotho* protein was lower in the peripheral blood of patients in CI group ($P<0.05$). Subsequently, the target polymorphisms were genotyped to record the distribution of genotype and allele frequencies in different groups. The results indicated that there were significant correlations between the *Klotho* gene polymorphism rs192031 and its genotypes and the occurrence of CI ($P<0.05$). People with AT genotype were more likely to suffer CI than those with AA or TT genotype. The polymorphisms rs200131 and rs102312 and their genotypes had no remarkable associations with CI occurrence ($P>0.05$).

Conclusions

In conclusion, this study illuminates for the first time that the *Klotho* gene polymorphism rs192031 was potentially associated with the occurrence of CI, while polymorphisms rs200131 and rs102312 and their genotypes were not related to the onset of CI.

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Conflict of interest statement

The authors reported no conflict of interest regarding the publication of this article.

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SENSITIVITY OF THREE THYROTROPIN RECEPTOR ANTIBODY ASSAYS IN THYROID-ASSOCIATED ORBITOPATHY

OSETLJIVOST TRI TESTA ZA ODREĐIVANJE ANTITELA NA RECEPTOR ZA TIREOSTIMULIŠUĆI HORMON KOD PACIJENATA SA ORBITOPATIJOM UDRUŽENOM SA ŠTITNOM ŽLEZDOM

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Summary

Background: Thyrotropin receptor autoantibodies (TSH-R-Ab) are indispensable biomarkers in the laboratory assessment of thyroid-associated orbitopathy (TAO). Clinical sensitivity of three different assays for TSH-R-Ab determination was evaluated in patients with TAO.
Methods: 87 consecutive TAO patients were enrolled and their serum samples analyzed in parallel with three assays. An ECLIA competitive binding and a chemiluminescent bridge immunoassay were used to measure total and binding TSH-R-Ab concentration, while their functional activity was determined using a stimulatory TSH-R-Ab (TSAb) cell-based bioassay.
Results: Compared to the two binding assays (ECLIA $p<0.001$, bridge $p=0.003$), the TSAb bioassay was more sensitive pertaining to the positive detection of TSH-R-Ab in TAO patients. No difference ($p=0.057$) was noted between the ECLIA and bridge assays regarding sensitivity rate. All patients with active and/or moderate-to-severe TAO tested positive in the TSAb bioassay (100% and 100%, respectively), while the positivity rates for bridge and ECLIA binding assays were 89.7% and 82.1% for active

Kratak sadržaj

Uvod: Autoantitela na receptore za tireostimulišući hormon (TSH-R-Ab) su nezamenljivi biomarkeri u laboratorijskoj proceni orbitopatije udružene sa štitnom žlezdom (TAO). U radu je procenjena klinička osetljivost tri različita testa za određivanje TSH-R-Ab kod pacijenata sa TAO.
Metode: U studiju je uključeno 87 uzastopnih pacijenata sa TAO i njihovi uzorci seruma su analizirani paralelno sa tri testa. Za merenje ukupne i vezujuće koncentracije TSH-R-Ab korišćeni su ECLIA imunohemijski test kompetitivnog vezivanja i hemiluminiscentni imunohemijski »sendvič« test, dok je njihova funkcionalna aktivnost određena pomoću ćelijskog biološkog testa (bioeseja) za određivanje stimulatornih TSH-R-Ab (TSAb).
Rezultati: U poređenju sa dva imunohemijska testa vezivanja (ECLIA $P<0,001$, »sendvič test« $P=0,003$), TSAb bioesej se pokazao najosetljivijim u pogledu pozitivne detekcije TSH-R-Ab kod TAO pacijenata. Nikakva razlika nije detektovana ($P=0,057$) između ECLIA i »sendvič« testa u pogledu stope osetljivosti. TSAb bioesej je bio pozitivan kod svih pacijenata sa aktivnom i/ili umerenom do teškom TAO (redom 100% i 100%), dok su stope pozitivnosti za sendvič i ECLIA imuno-

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List of abbreviations: TAO-thyroid-associated orbitopathy, GD-Graves' disease, HT-Hashimoto's thyroiditis, ET-euthyroid, TSHR-thyrotropin receptor, TSH-R-Ab-thyrotropin receptor antibodies, TSAb-TSHR stimulating antibodies, TBAb-TSHR blocking antibodies

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TAO, and 90.2% and 86.3% for severe TAO, respectively. Negative predictive values of the bioassay, bridge, and ECLIA assays were 100%, 75%, and 71%, respectively for active TAO, and 100%, 86%, and 71%, respectively for moderate-to-severe TAO. The superiority of the bioassay was most prominent in euthyroid (ET) TAO. Positivity rates of the TSAb bioassay, bridge and ECLIA binding assays were 89.6%, 75%, and 64.6%, respectively for inactive TAO; 86.1%, 69.4%, and 52.8%, respectively for mild TAO; 87.5%, 62.5%, and 12.5%, respectively for euthyroid TAO. The bridge assay correlated better with the ECLIA binding assay ($\rho=0.893$, $p<0.001$), compared to the bioassay ($\rho=0.669$, $p<0.001$).

Conclusions: In patients with TAO of various activity and severity, the TSAb bioassay demonstrates a superior clinical performance compared to both ECLIA and bridge binding assays.

Keywords: thyroid-associated orbitopathy, thyrotropin receptor antibodies, bioassay, bridge binding assay, ECLIA binding assay

hemijski test bile 89,7% i 82,1% za aktivnu TAO, i 90,2% i 86,3% za tešku TAO, redom. Negativne prediktivne vrednosti bioeseja, »sendvič« i ECLIA testova bile su redom 100%, 75% i 71%, za aktivnu TAO, odnosno 100%, 86% i 71%, redom za umerenu do tešku TAO. Superiornost biološke analize bila je najistaknutija kod eutiroidnog oblika (ET) TAO. Stope pozitivnosti TSAb bioeseja, »sendvič« i ECLIA testova vezivanja bile su redom 89,6%, 75% i 64,6%, za neaktivnu TAO; 86,1%, 69,4% i 52,8% za blagu TAO; 87,5%, 62,5% i 12,5% za eutiroidni TAO. »Sendvič« imunohemijski test je bio u boljoj korelaciji sa ECLIA testom kompetitivnog vezivanja ($\rho=0,893$, $P<0,001$), u poređenju sa bioesejom ($\rho=0,669$, $P<0,001$).

Zaključak: Kod pacijenata sa TAO različite aktivnosti i težine TSAb bioesej pokazuje superiorne kliničke performanse u poređenju sa obe vrste imunohemijskih testova vezivanja (ECLIA i »sendvič test«).

Ključne reči: orbitopatija udružena sa štitnom žlezdom, antitela na receptor za tireostimulišući hormon, bioesej, »sendvič« imunohemijski test vezivanja, ECLIA test vezivanja

Introduction

Thyrotropin receptor autoantibodies (TSH-R-Ab) are specific biomarkers of both Graves' disease (GD) and thyroid-associated orbitopathy (TAO) that define their pathogenetic background and clinical phenotype. They represent an indispensable diagnostic tool in the clinical assessment of GD and TAO (1–6). TSH-R-Ab express variable biological activity and are accordingly classified as stimulating (TSAb), blocking (TBAb), and neutral antibodies (7). A timely and sensitive serological TSH-R-Ab testing is crucial for definitive diagnosis of GD and thyroid related orbitopathy (8). Total TSH-R-Ab concentration, usually denoted as TSH-R binding inhibitory imunoglobulins (TBII), is quantified by competitive binding immunoassays traditionally widely used in routine laboratory diagnostics. It is a net sum of TSH-R-Ab of different variety and not representative of their biological activity. Chemiluminescent or radiolabeled TSH or monoclonal antibody competes with TSH-R-Ab from patient's serum for binding to the same binding sites at TSH-R (9–10). To this day, two sorts of assays for detection of thyrotropin stimulatory antibodies have been developed and proposed as an alternative to the existing TBII testing: bioassays which measure the level of TSH-R-Ab functional activity (TSAb and TBAb) and recently developed bridge-immunoassay. The latter utilizes bridge immunoassay technology and reportedly detects TSH-R-Ab. This assay is intended for use on fully automated commercial platforms (11–13). In contrast, the exclusivity of bioassay methodology is reflected in the ability to detect the biological function of thyrotropin autoantibodies. It uses genetically engineered CHO cells (Chinese hamster ovary cells), transfected with human TSH-R and cAMP-inducible luciferase reporter gene. Bioassays have been reported as highly specific and sensitive biomarkers of GD and TAO (14). However, they are still not widely introduced into routine practice and require special laboratory conditions and well-trained staff.

Three major roles of a new diagnostic test have previously been defined: replacement (new test replaces an existing one), triage (new test is used before an existing one), and add-on (new test is used after an existing one). Thorough examination needs to preceed the implemantation of every medical test. Diagnostic accuracy assessment represents the major step when introducing new biomarkers and laboratory tests into routine clinical practice (15). The intended clinical goal of the biomarker (early and accurate diagnosis, screening, prediction, prognosis, etc.) is a clear guidance for how the test will be used. Diagnostic accuracy is often balanced with the practical aspects involved as well. Apart from the superior diagnostic properties compared to the existing methodology other features must be also taken into consideration: invasiveness, cost, feasibility, availability, turnaround time, laboriousness, etc. (16).

The aim of this study was to perform a comparative analysis of the bioassay and bridge immunoassay for quantification of TSH-R-Ab, relative to the existing, commonly used competitive binding immunoassay. We intended to evaluate the diagnostic performance of the above mentioned laboratory tests, primarily in terms of diagnostic sensitivity, and accordingly to our findings propose the most appropriate replacement for the existing binding assay.

Material and Methods

Patients

This was an observational retrospective study. A total of 87 consecutive patients with clinically manifest TAO were recruited. All patients were regularly

treated at the tertiary University Clinical Center of Serbia (Clinic for Endocrinology, Diabetes and Metabolic Diseases) according to the current guidelines and protocols (17–19). Hormone analysis was carried out in all patients and the diagnosis was classified as Graves' disease (GD), Hashimoto's thyroiditis and euthyroid TAO. TAO was categorized according to its activity as active or inactive (seven points Clinical Activity Score (CAS), cut-off 3/7) and according to its severity as mild, moderate-to-severe, and sight-threatening TAO (current 2021 EU guidelines) (17, 20).

Upon admission, a detailed medical history and demographic data were taken. All participants signed an informed consent and Ethics Committee approval was obtained prior to the start of the study (17.06.2019/944/3). Research was conducted in accordance with the guidelines of clinical and laboratory practice, the Declaration of Helsinki and applicable institutional and national regulations.

Blood draw was performed in the morning, after 12 hours fasting period. After separation of serum, samples were appropriately aliquoted and stored at -80 °C, until analysis. Samples were analyzed in parallel with all three different methods for TSH-R-Ab quantification.

Conventional binding assay

Total TSH-R-Ab concentration was measured using a commercial automated binding immunoassay (ECLIA, Elecsys Anti-TSHR Immunoassay Roche Diagnostics, GmbH, Mannheim, Germany) on the Cobas e411 analyzer (Roche, Diagnostics, GmbH) according to the manufacturer's instructions (cut-off 1.75 IU/L). This assay employs a monoclonal M22 antibody with a high affinity for TSH-R-Ab but without the ability to distinguish their functionality.

Cell-based bioassay

Serum TSAb and TBAb were measured using a commercial FDA-cleared bioassay (Thyretain, Quidel, San Die, CA, USA) and CE-marked bioassay, respectively, according to the manufacturer's instructions (21, 22). Both tests utilize Chinese hamster ovary (CHO) cells that express chimeric TSH-R (Mc4) and cAMP-inducible luciferase reporter gene. When CHO cells are exposed to TSAb, cAMP-dependent production of luciferase occurs, which is quantified after addition of luciferin. In contrast, TBAb antibodies inhibit cAMP production and consequent light signal generation. Patients' samples and controls were added to CHO-Mc4 cells, previously seeded and grown in a 96-well plate. After incubation with CO_2 and cell lysis, a chemiluminescent signal was quantified as relative light units with a luminometer. TSAb level is expressed as percentage specimen-to-reference-ratio (cut-off 140 SRR %) and TBAb as percentage inhibition (cut-off 34% inhibition).

Serum TSAb and TBAb were measured in a blinded manner at the Molecular Thyroid Research Lab of the Johannes Gutenberg-University (JGU) Medical Center, Mainz, Germany where the samples were shipped on dry ice. The samples underwent only one thawing procedure and were analyzed with one reagent lot.

Siemens bridge immunoassay

Commercial bridge immunoassay was used in this study (IMMULITE TSI 2000, Siemens Healthcare Diagnostics, UK), according to the manufacturer's instructions, on an IMMULITE 2000 analyzer. This is a fully automated, chemiluminescent immunoassay that employs the bridge assay format. It uses a pair of recombinant hTSH-R, where TSH-R-Ab from the sample binds through one arm to the immobilized capture hTSH-R and to the signal, alkaline phosphatase labeled hTSH-R, through the other arm, thus forming a bridge. The assay involves two cycles, the incubation with a capture receptor and the incubation with a signal receptor, with removal of the unwashed material in between. After addition of the chemiluminescent substrate to the reaction, the light signal is triggered and is measured by a luminometer. The signal is directly proportional to the TSH-R-Ab concentration in the sample (12, 13).

Statistical analysis

Statistical analysis was performed using SPSS Software package version 20 (SPSS Inc., Chica, IL, USA). We assessed the normality of distribution with the Kolmorov-Smirnov and Shapiro-Wilk tests, depending on the sample size. We reported categorical variables as numbers or percentages, normally distributed continuous variables as mean ± standard deviation (SD), and non-normally distributed variables as median (interquartile range, IQR). Sensitivity rates among examined TSH-R-Ab assays were compared using the McNemara's test. Statistical significance was considered at a value of $p<0.05$.

Results

Patients' demographic, serological, and clinical data are presented in *Table I*. Out of 87 TAO patients, TSH-R-Ab positivity was detected in 82 (94.3%), 71 (81.6%), and 63 (72.4%) patients by TSAb bioassay, Siemens bridge binding assay, and Roche ECLIA binding assay, respectively (*Table II*). Only one TAO patient showed both blocking and stimulating antibody activity. TSAb bioassay showed the highest sensitivity rate for detection of TAO in patients, meaning that it exerts the strongest ability to include diagnosis in patients with TAO. A significant discrepancy of the results was seen between both the TSAb bioassay and

Table I Patients' demographic, clinical and serological, data.

Parameter	All TAO patients
n	87
Age (years)	53±11
Gender (f/m)	62/25
TAO duration (years)	1.15 (0.67–3.00)
Diagnosis GD+TAO (n) HT+TAO (n) ET+TAO (n)	 67 12 8
Activity of TAO active (n) inactive (n)	 39 48
Severity of TAO moderate-to-severe (n) mild (n)	 51 36
Current smokers (n)	50
TSH (IU/L)	1.20 (0.10–3.26)
FT4 (pmol/L)	14.95 (13–19.37)
Thyretain TSAb functional bioassay	669 (298–764)
Siemens bridge binding assay	2.45 (0.71–10.52)
Roche ECLIA binding assay	3.87 (1.54–14.56)

TAO, thyroid-associated orbitopathy; GD, Graves' disease; HT, Hashimoto's thyroiditis; ET, euthyroid; TSH, thyroid stimulating hormone; FT4, free thyroxine; TSAb, TSH-R stimulating antibodies; The Thyretain TSAb functional bioassay cut-off is at 140 SRR% (specimen-to-reference ratio) and for Siemens bridge and Roche ECLIA binding assay cut-off is at 0.55 IU/L and 1.75 IU/L, respectively.

ECLIA binding assay ($p<0.001$), and between the TSAb bioassay and bridge binding assay ($p=0.003$), but not between the two binding assays ($p=0.057$) (*Table III*). Overall concordance of the results was 85.1% for the bioassay and bridge assay, 83.9% for the bridge and ECLIA binding assay, and 78.2 % for the bioassay and binding ECLIA assay. 12 TSAb bioassay positive samples were bridge assay negative and 1 TSAb bioassay negative sample was bridge assay borderline positive, what indicated the tendency of bridge assay towards a higher rate of »false« negative results. Interestingly, one GD patient with dual stimulating and blocking antibody activity tested negative in binding bridge assay, at the same time. All 19 discrepant TSAb bioassay/binding ECLIA assay results were TSAb positive and negative in the binding assay. None of TSAb negative patients were positive in the binding assay. 11 bridge binding assay positive samples were ECLIA binding assay negative, while 3 bridge assay negative samples tested positive in ECLIA binding assay.

All five TSAb bioassay negative patients were presented with mild, inactive form of TAO. Among 16 bridge binding assay negative results, 4 patients had active TAO and 5 patients showed moderate-to-severe form of disease. Out of 24 negative ECLIA binding assay results, 7 were detected in active TAO patients and 7 in moderate-to-severe TAO patients.

Next, we analyzed the clinical sensitivity of assays relative to the activity and the severity of TAO (*Table III*). All active TAO patients were TSAb bioassay positive, indicating a 100% sensitivity and respectively, 47.6% and 100% positive (PPV) and negative predictive value (NPV) for detecting an active form of TAO. A statistically significant difference regarding sensitivity, i.e. discriminating ability to detect active TAO patients was observed between the TSAb bioassay and routine binding ECLIA assay, but not between two types of immunoassays. Siemens bridge and Roche ECLIA binding assays gave PPV and NPV of 49.3%, 50.8%, 75% and 70.8%, respectively. The difference in true positive fractions (dTPF) of the results obtained with TSAb bioassay and binding ECLIA assay was 17.9%, while relative true positive fraction (rTPF) ratio was 1.22. When tested for equality of the true positive fractions, we obtained a significant difference among these tests ($p=0.016$). dTPF between binding bridge assay and binding ECLIA assay was 7.7%, and rTPF was 1.1. P value of 0.250 suggested no significant difference in their true positive fractions.

Table II Positivity crosstabs for different TSH-R-Ab assays.

	Thyretain TSAb functional bioassay		Siemens bridge binding assay			
	positive	negative	Total	positive	negative	Total
All TAO patients						
Roche ECLIA binding assay						
positive	63	0	63	60	3	63
negative	19	5	24	11	13	24
Total	82	5	87	71	16	87
TAO activity						
Active TAO						
Roche ECLIA binding assay						
positive	32	0	32	32	0	32
negative	7	0	7	3	4	7
Total	39	0	39	35	4	39
Inactive TAO						
Roche ECLIA binding assay						
positive	31	0	31	28	3	31
negative	12	5	17	8	9	17
Total	43	5	48	36	12	48
TAO severity						
Moderate to severe TAO						
Roche ECLIA binding assay						
positive	44	0	44	43	1	44
negative	7	0	7	3	4	7
Total	51	0	51	46	5	51
Mild TAO						
Roche ECLIA binding assay						
positive	19	0	19	17	2	19
negative	12	5	17	8	9	17
Total	31	5	36	25	11	36

TAO, thyroid-associated orbitopathy; TSAb, TSH-R stimulating antibodies

Table III Distribution of the sensitivity rates among different TSH-R-Ab assays.

TAO patient group	Number of subjects (n)	Thyretain TSAb functional bioassay	Siemens bridge binding assay	Roche ECLIA binding assay	p value
		sensitivity (%)	sensitivity (%)	sensitivity (%)	
All TAO patients	87	94.3	81.6	72.4	0.003[a], <0.001[b], 0.057[c]
Active TAO patients	39	100	89.7	82.1	0.125[a], 0.016[b], 0.250[c]
Moderate-to-severe	51	100	90.2	86.3	0.063[a], 0.016[b], 0.625[c]
Inactive TAO patients	48	89.6	75	64.6	0.039[a], <0.001[b], 0.227[c]
Mild TAO patients	36	86.1	69.4	52.8	0.070[a], <0.001[b], 0.109[c]
< 1 year	42	100	90.5	81	0.125[a], 0.008[b], 0.219[c]
> 1 year	42	88.1	71.4	64.3	0.039[a], 0.002[b], 0.453[c]
TSAb (SRR%) low positive	17	100	47.1	41.2	0.004[a], 0.002[b], 1.000c
TSAb (SRR%) medium positive	44	100	93.2	81.8	0.250[a], 0.008[b], 0.125[c]
TSAb (SRR%) high positive	21	100	100	95.2	1.000[a], 1.000[b], 1.000[c]

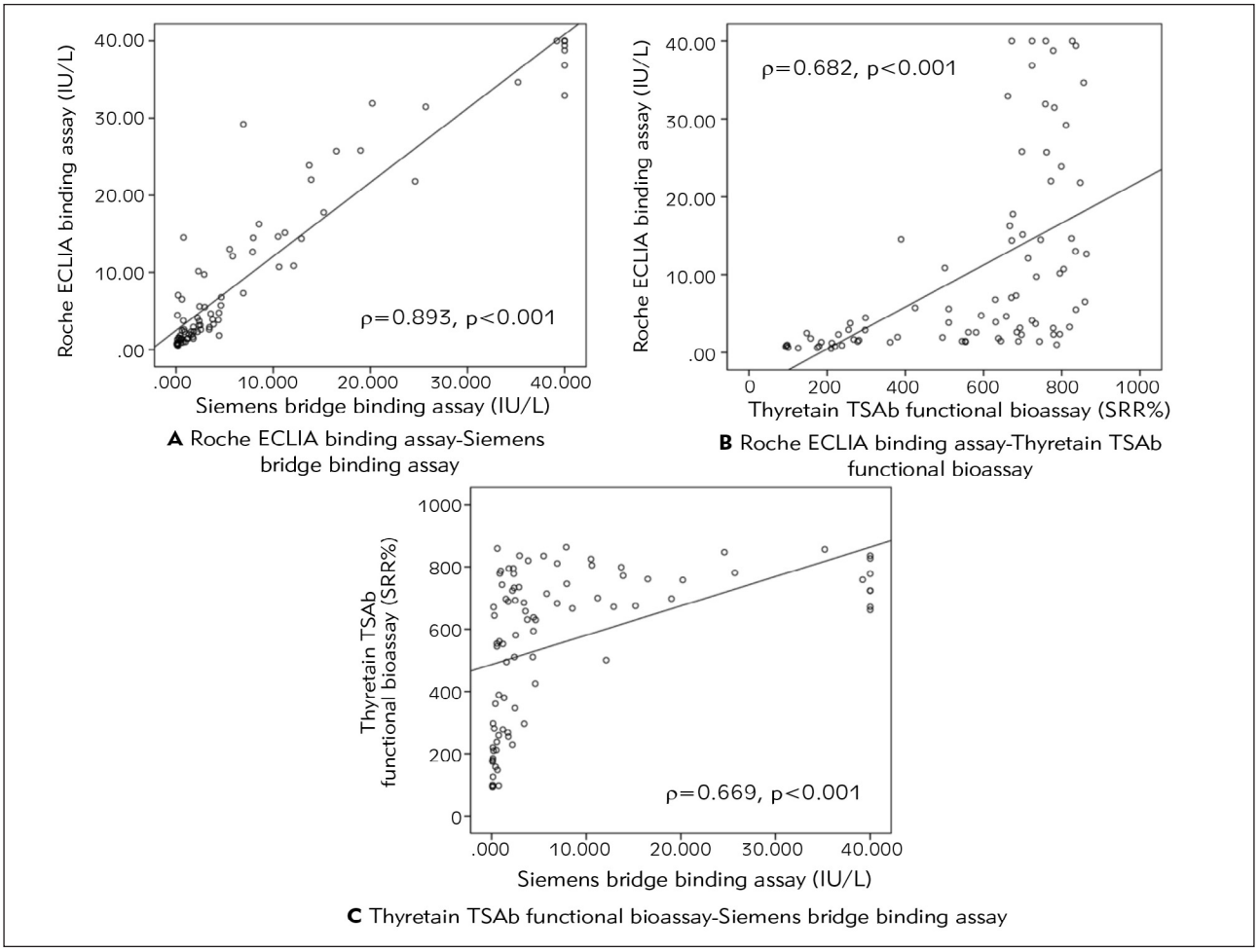
TAO, thyroid-associated orbitopathy; TSAb, TSH-R stimulating antibodies, SRR%, serum-to-reference ratio; TSAb (SRR%) low positive, TSAb level in TSAb bioassay ≤ 25th percentile (140–298 SRR%), TSAb (SRR%) medium positive, TSAb level in TSAb bioassay 25th-75th percentile (299–761 SRR%), TSAb (SRR%) high positive, TSAb level in TSAb bioassay >75th percentile (>762 SRR%); Values are presented as a number of subjects and percentage of positive results; [a]p value for difference in the sensitivity rate between Thyretain TSAb functional bioassay and Siemens bridge binding assay in different patient groups, [b]p value for difference in the sensitivity rate between Thyretain TSAb functional bioassay and Roche ECLIA binding assay, [c]p value for difference in the sensitivity rate between Siemens bridge and Roche ECLIA binding assays

Table IV Comparative presentation of TSH-R-Ab test characteristics.

Method for TSH-R-Ab determination			
Test characteristics	Thyretain TSAb functional bioassay	Siemens bridge binding assay	Roche ECLIA binding assay
Accuracy	high	Moderate-to-high	Moderate-to-high
Invasiveness	Non-invasive	Non-invasive	Non-invasive
TAT	24h	Few hours	Few hours
Laboriousness	moderate	low	low
Sample type	serum	serum, plasma	serum, plasma
Sample preparation	No pretreatment	No pretreatment	No pretreatment
Interpretation of the results	Easy interpretation except in case of dual positivity	Easy interpretation	Easy interpretation
Cost	moderate	moderate	low
Feasibility	moderate	high	high
Automation	Semi-automatic test	Fully automated test	Fully automated test
Predictive potential	yes	–	No

TSH-R-Ab, thyrotropin receptor autoantibodies; TAT, turnaround time

Figure 1 Various correlations between three different assays for TSH-R-Ab quantification in subjects with thyroid-associated orbitopathy.



A Roche ECLIA binding assay-Siemens bridge binding assay

B Roche ECLIA binding assay-Thyretain TSAb functional bioassay

C Thyretain TSAb functional bioassay-Siemens bridge binding assay

When observing the inactive TAO patient group, the TSAb bioassay was significantly more sensitive than both immunoassays examined, whereas no significant difference was shown between two types of immunoassays (*Table III*).

All moderate-to-severe TAO patients were TSAb bioassay positive, suggesting the recognition of moderate-to-severe cases of TAO with a 100% sensitivity and 62.6% PPV and 100% NPV. PPV and NPV for detecting moderate-to-severe TAO cases for bridge and ECLIA binding assays were 64.8% and 69.8%, and 86.3% and 70.8%, respectively. Relative to the bridge assay, TSAb bioassay was not significantly more sensitive in this patient group, but when compared to the routine binding ECLIA assay, bioassay was significantly more sensitive, unlike bridge assay. The difference in true positive fractions (dTPF) and relative true positive fraction (rTPF) were 13.72% and 1.16 for TSAb bioassay and ECLIA binding assay ($p=0.016$), and 3.9% and 1.05 for binding bridge and ECLIA assays, respectively ($p=0.625$).

In the mild TAO patient group no significant difference was observed in the sensitivity of bridge assay compared to neithter TSAb bioassay nor the ECLIA binding assay. However, TSAb bioassay was significantly more sensitive in relation to the commonly used ECLIA binding assay (*Table III*).

Next, when we classified TSAb levels obtained by TSAb bioassay to low, medium, and high positive, we observed the largest discrepancy of results in the low-positive level TSAb group of patients (*Table III*). In this group of patients, TSAb bioassay was the only one to express significant sensitivity rate with a median TSAb level above the defined cut-off value (TSAb bioassay 229 SRR% (182–273) vs. bridge binding assay 0.48 (0.14–1.45) IU/L vs. ECLIA binding assay 1.57 (0.85–2.68) IU/L).

Moreover, the difference in the clinical performance of the analyzed laboratory tests was particularly evident in ET TAO patients, where TSAb bioassay successfully detected 7 out of 8 (87.5%) ET TAO patients, bridge binding assay 5 out of 8 (62.5%), and ECLIA binding assay only 1 out of 8 (12.5%) ET TAO patients. Bridge assay sensitivity rate in this patient group did not significantly differ in relation to either bioassay ($p=0.500$) or ECLIA binding assay ($p=0.125$).

The bridge assay closely correlated with the ECLIA binding assay, significantly better than with the bioassay ($\rho=0.893$, $p<0.001$ vs. $\rho=0.669$, $p<0.001$). The bioassay correlated similarly with both binding immunoassays ($\rho=0.669$, $\rho=0.682$, $p<0.001$) (*Figure 1*).

Regarding the duration of TAO symptoms, superior TSAb bioassay sensitivity was the most prominent in TAO lasting over a year (*Table III*). This is in line with the natural course of TAO, where the activity of TAO coincides with the more recent onset of disease (23).

Summary characteristics of all three analyzed TSH-R-Ab methods with special focus on the practical aspects are listed in *Table IV*.

Discussion

The accuracy of a diagnostic method must be viewed from the perspective of the disease range and the context in which it is examined (24, 25). Accordingly, the study design, as well as the choice of the appropriate statistical methods used, is usually defined by the intended purpose of the examined biomarker (16). The present study focuses on the clinical value of a new replacement test relative to the standard, commonly used test for TSH-R-Ab detection. We compared the diagnostic features of the TSAb bioassay and bridge binding assay in relation to the standard competitive binding assay. We examined the variability of their performance both in all TAO patients and relative to the activity, severity, and duration of TAO. Replacement was chosen as the most suitable purpose for this assay comparison.

Superior diagnostic characteristics, primarily high sensitivity rate of the serological TSH-R-Ab methods are an imperative for accurate and timely differential diagnosis of TAO. Highly sensitive methods are necessary for the adequate recognition of a variety of TAO clinical phenotype. For clinicians, this feature is of utmost importance, since it defines further steps in patient management. 100% sensitivity and 100% NPV for detection of active and moderate-to-severe TAO means that no such patient will remain undetected and that a negative test result will certainly exclude a progressive form of disease. These patients need to receive the appropriate therapy, and are more likely to develop sight-threatening TAO that requires an urgent treatment (17). Moreover, a reliable and noninvasive serological test is a feasible first-line solution, especially if thorough clinical assessment in tertiary care units is not readily available. High sensitivity of TSH-R-Ab tests has particular clinical value in case of euthyroid TAO, a challenging clinical condition often confused with various other inflammatory disorders. Differential diagnosis of ET TAO is especially complicated and depends entirely on serological confirmation of TAO (26).

We have previously published our findings on the superior clinical performance of a TSAb cell-based bioassay versus the routine, binding assay. Bioassay showed 100% sensitivity for differentiating between active and inactive, as well as between mild and moderate-to-severe TAO patients, unlike binding assay that demonstrated significantly poorer discriminating ability (27). Distinct difference between TSAb bioassay and binding ECLIA assay ($p=0.031$) was particularly prominent in ET TAO patient group. In this paper, we complement these

previous findings in relation to the performance of the bridge assay. High analytical sensitivity of the bioassay technique was reported in a serial dilution analyses, where it demonstrated positivity at much higher serum dilution compared to the binding assays (28).

Our present findings demonstrate somewhat better diagnostic performance of the bridge binding assay compared to the traditionally used ECLIA binding test, but evidently poorer in comparison to the TSAb bioassay technique. Only in active TAO patients, the bridge assay performed similarly to the bioassay, although a small number of patients was involved. Interestingly, in neither of TAO patient groups, the bridge assay was significantly more sensitive than the ECLIA binding assay. In contrast, the functional bioassay showed markedly higher clinical sensitivity rate relative to the binding assay in all examined patient groups. The bioassay superior diagnostic sensitivity relative to both binding immunoassays was the most prominent in patients with milder clinical presentation of TAO (inactive TAO, low-positive TSAb). This suggest that the bioassay would be a better choice in management of atypical forms of TAO, without signs of inflammation and thyroid abnormalities (29). In line with this, the variability of the clinical features was especially notable in ET TAO patient group, where only bioassay demonstrated a satisfactory positivity rate, i.e. the ability to detect virtually all ET TAO patients.

Dual stimulating and blocking antibody activity was observed in one GD patient. However, this patient tested negative in the bridge binding assay. Potential explanation lies in the variable affinity and concentration of TSAb and TBAb (30), possible mutual neutralization of the antibodies, as well as the specificity issues of the chimeric TSH-R construct used. TSAb and TBAb epitopes are not completely distinct entites, that is to say, they show a high level of overlapping. Naturally the TSAb and TBAb binding sites are located physically close to each other. TBAb binds to both Epitope B that is separated from TSAb binding site (Epitope A1), and Epitope A2 that is close to Epitope A1 (31, 32). This suggests that the ability of an immunoassay to specifically measure stimulating antibody concentration and to accurately distinguish them from the blocking ones when present simultaniously in patient's sample, is a matter of debate and a problem for closer investigation (33). In line with this, it was already reported that the bridge assay was not able to differentiate between TBAb and TSAb (34).

Routine binding assays are representative of the total TSH-R-Ab concentration, i.e. a net sum of stimulating, blocking, and even neutral antibodies. Bioassay measures the biological activity of TSH-R-Ab (through the level of cAMP) that is a direct cause of the clinical course of GD and TAO. In contrast, the receptor binding techniques measure the level of antibody binding to the receptor which is highly dependent on the epitopes, antibody affinity, and concentration (33). To this day, a commercial test for measurement of neutralizing TSH-R-Ab has not yet been developed (35). According to manufacturer's claims, the bridge binding assay utilizes a recombinant human TSHR (MC4), allegedly specific for TSAb (13). However, it was shown that MC4-expressing cell lines could be used for TBAb quantification as well (36, 37). In fact the specificity of the bridge technology has never been proven and nonspecific TBAb detection was reported in multiple studies (34, 38, 39). In an animal model of GD, developed by immunizations with extracellular domain of TSHR, the bridge assay could not distinguish TSHR functionality, as it yielded positivity in both TSAb and TBAb positive samples (40). The bridge assay is therefore a purely binding assay, incapable of determining functional effect of TSH-R-Ab, meaning that bioassays provide wider information about the exact inflammatory status in TAO patients.

However, practical aspects must be kept in mind during the clinical validation of an assay, primarily the cost-benefit ratio of integrating new technologies into everyday practice (16, 41).

Long-term benefit of introducing functional biomarkers in routine clinical practice would be reflected in the reduced need for frequent hospitalization of patients and the use of expensive imaging diagnostic procedures (3, 42). This could substantially alleviate the burden on the health care system. Introduction of highly sensitive serological markers would also minimize the use of radioactive iodine in the differential diagnosis of thyrotoxicosis, which is well associated to the progression of TAO.

In addition, unlike binding assays, functional biomarkers have demonstrated remarkable predictive value as indicators of relapse/remission of GD and the clinical phenotype of TAO (27, 43, 44). This is another feature in favor of bioassays that would greatly facilitate patient monitoring and follow-up.

There are few limitations of our study: non-prospective design of the study and the impossibility of simultaneous analysis with all three laboratory tests for technical reasons. Nevertheless, this is one of very few studies to perform a comparative analysis of TSH-R-Ab assays and it was carried out at the referral, tertiary level clinic.

Conclusions

In conclusion, we demonstrated superior clinical performance of the bioassay method compared to the traditionally used competitive binding ECLIA assay and the new bridge assay technique, primarily in terms of clinical sensitivity. Bridge assay performance was positioned somewhere in the middle and as

such wouldn't be a suitable replacement for the commonly used binding method. In this way we strived to meet the clinicians' needs that are to maximize the sensitivity of the tests so as not to miss any TAO patient, especially those with mild and nonspecific presentation of the disease, as well as those who need to receive the appropriate treatment. According to these findings, as well as the clinical goals, we conclude that only the bioassay demonstrates sufficient diagnostic characteristics to replace the existing competitive binding assays where possible. Integration of bioassays into the current diagnostic algorithms of TAO could substantially improve patient management, monitoring, and prediction of clinical course of disease.

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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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PREVALENCE OF TRADITIONAL CARDIOVASCULAR RISK FACTORS FOR CORONARY ARTERY DISEASE AND ELEVATED FIBRINOGEN AMONG ACTIVE MILITARY PERSONNEL IN REPUBLIC OF SERBIA: A CROSS-SECTIONAL STUDY

PREVALENCA TRADICIONALNIH KARDIOVASKULARNIH FAKTORA RIZIKA ZA KORONARNU ARTERIJSKU BOLEST I POVIŠENOG FIBRINOGENA MEĐU AKTIVNIM VOJNIM LICIMA VOJSKE REPUBLIKE SRBIJE: STUDIJA PRESEKA

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Summary

Background: It is well known that less than 1% of the population achieves ideal cardiovascular health, and 65% of patients do not have their conventional risk biomarkers under control. Military service has its own particularities that may contribute to cardiovascular risk.
Methods: To define the preventive strategy goals, we analysed the prevalence of traditional cardiovascular risk factors for coronary artery disease and elevated fibrinogen among active military personnel in the Republic of Serbia.
Results: The cross-sectional study included 738 individuals older than 20 years, mostly between 31 and 40 years old. The mean value of SBP for the whole group was 122.39± 9.42 mmHg, and for the DBP, it was 79.94±6.56 mmHg. Among active military personnel, 72.7% (533) had prehypertension, and 13.8% (101) was hypertensive. Both body mass and BMI index among the observed age subgroups were found to increase with the age of the patients and cholesterol values. HDL cholesterol values also differed statistically significantly between age subgroups, with the proportion of individuals with HDL less than 1.5 mmol/L in all subgroups being about 85%, the only in the 41–50 age

Kratak sadržaj

Uvod: Dobro je poznato da manje od 1% populacije ima idealno kardiovaskularno zdravlje, kao i da 65% pacijenata nema kontrolu nad konvencionalnim biomarkerima rizika. Vojna služba ima svoje osobitosti koje mogu doprineti kardiovaskularnom riziku.
Metode: Da bismo definisali ciljeve preventivne strategije, analizirali smo prevalenciju tradicionalnih kardiovaskularnih faktora rizika za koronarnu arterijsku bolest i povišenog fibrinogena među aktivnim vojnim licima vojske Republike Srbije.
Rezultati: Studija preseka je obuhvatila 738 osoba starijih od 20 godina, uglavnom između 31 i 40 godina. Srednja vrednost SBP-a za celu grupu bila je 122,39 ± 9,42 mmHg, a za DBP 79,94 ± 6,56 mmHg. Među ispitivanom populacijom 72.7% (533) je imalo prehipertenziju, a 13,8% (101) hipertenziju. Utvrđeno je da se i telesna masa i indeks telesne mase među posmatranim starosnim podgrupama povećavaju sa godinama pacijenata, kao i vrednosti ukupnog holesterola. Vrednosti HDL holesterola su se takođe statistički značajno razlikovale među starosnim podgrupama, pri čemu je udeo pojedinaca sa HDL-om manjim

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List of abbreviations: TCH, cholesterol; Tg, triglyceride; LDL, low-density lipoprotein Cholesterol; BMI, body mass index; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

group was lower, 76.4%. LDL cholesterol and the proportion of individuals who had LDL 3.5 increases with the age of patients, and an identical trend was recorded with triglycerides. With ageing, fibrinogen levels increased. **Conclusions:** Those findings considering cardio and cerebrovascular risk factors would help create a new approach for primary prevention for these categories of individuals.

Keywords: traditional cardiovascular risk factors, coronary artery disease, fibrinogen, active military personnel

od 1,5 mmol/L u svim podgrupama bio oko 85%, dok je jedino u starosnoj grupi od 41–50 godina bio niži, 76,4%. LDL holesterol, kao i udeo pojedinaca koji su imali LDL ≥ 3,5, raste sa starenjem pacijenata, a identičan trend je zabeležen i kod triglicerida. Sa starenjem, nivo fibrinogena se povećava.
Zaključak: Dobijeni rezultati koji se odnose na kardio i cerebrovaskularne faktore rizika bi mogli doprineti stvaranju novog pristupa u primarnoj prevenciji kod ovih kategorija bolesnika.

Ključne reči: tradicionalni kardiovaskularni faktori rizika, koronarna arterijska bolest, fibrinogen, aktivna vojna lica

Introduction

It is well known that less than 1% of the population achieves ideal cardiovascular health, and 65% of patients do not have their conventional risk biomarkers under control (1–2). Although recent data are very suggestive for primary prevention in people with multiple risk factors, fewer than 10% of those individuals have all of them adequately controlled (2–5). The primary prevention strategy in individuals with multiple risk factors is based on the fact that the first atherosclerotic changes – fat spots and stripes, consisting mainly of macrophages filled with LDL cholesterol, appear early in life, even in childhood (6–7). These findings underline the need for lipid status screening to provide better objectivity in assessing cardiovascular risk and the rationale for the early introduction of lifestyle changes and drug therapy. Except for lipid status, cardiovascular risk evaluation includes fibrinogen levels, a widely used surrogate cardiovascular marker, which also has a predictive value (8). Halle et al. (9) underlined a clear link between higher normal fibrinogen and the expression of a more atherogenic LDL subfraction phenotype independent of body mass index, age, other serum lipids, and insulin resistance in a healthy person nonsmoking male. A meta-analysis with about 4000 coronary heart disease cases indicated that an increase in plasma fibrinogen level per 1 g/L was followed by a relative risk ratio increment of 1.8 (10). A recent meta-analysis with 246,669 otherwise healthy participants underlined the clear benefit of assessing the CRP or fibrinogen level in individuals at intermediate risk for a cardiovascular event considering prevention of an additional event over a period of 10-year follow-up (11). These findings reinforce the evidence that fibrinogen should be estimated in coronary risk assessment.

Considering the role of lipid disorders in atherosclerosis, it is important to have a screening program for the early detection of lipid disorders. Active military personnel selection should be based on »a kind of healthy worker effect« or »Healthy Warrior Effect« to provide the population that is healthier than the general one (12–13). Military service has its own particularities that may contribute to cardiovascular risk (14–16). Previous referred findings are the basis for the scientific project »Primary prevention of ischemic heart disease among active military personnel and civilian personnel in the military in the Republic of Serbia«, which aims to implement actual prevention recommendations among active-duty military personnel and military personnel. This part of the population is under systemic control and the possibility of daily health status checks. This is objectified by general medical examination yearly (younger than 40 years) or every second year (older than 40 years).

The aim of the study is to point out the prevalence of hyperlipidemia and elevated fibrinogen among active-duty military personnel in the Republic of Serbia.

Methods

Type of the study and participants

This is a cross-sectional study (2018–2019), included a sample of 738 active military personnel (20+ years) of Serbia. The study was conducted in the Military Medical Academy and Military Medical Centre Karaburma. All procedures performed in the study involving human participants were in accordance with the ethical standards of the Ethical Commission of Belgrade University of Defence. The study population included 738 males, divided into two groups. The first one consists of 289 individuals younger than 40 years, and then the second one includes 489 people older than 40.

Some epidemiological and anthropometric Characteristics were checked to assess cardiometabolic risk

Anthropometric measurements and calculations included body weight and height, as well as body mass index (BMI, calculated as weight (kg)/squared body height (m^2)). Recognised criteria were used for the assessment of overweight and obesity versus normal BMI. Cutoff value for overweight and obesity was BMI ≥ 25 kg/m^2. The systolic and diastolic blood

pressure (SBP and DBP) cardiologists measured by the traditional sphygmomanometer with a participant in a sitting position. The values of systolic and diastolic blood pressure were recorded as the arithmetic mean of three repeated measurements. In preparation for measurements, the participants were seated and rested quietly for at least five minutes before taking the first BP measurement. The right arm was used for all blood pressure measurements. All participants had BP measurements always taken by the same researcher and with the same-sized cuff for adults. Time intervals between the measurements were 5–10 minutes. The first (for systolic) and fifth (for diastolic) Korotkoff sounds were recorded for each of the 3 measurements (4, 15).

According to WHO, the examinees without antihypertensive therapy over the last 4 months were classified according to values of blood pressure into the following categories: normal blood pressure (systolic blood pressure – SBP and diastolic blood pressure – DBP: <120 and <80 mmHg); prehypertension (SBP/DBP: 120–139 and/or 80–89 mmHg); and hypertension (SBP/DBP: ≥140 and/or ≥90 mmHg). All individuals who used antihypertensive therapy over the previous 4 weeks were included in the category of hypertensive individuals (5, 17).

Biochemical data analysis

By using the Auto Analyzer HITACHI 7020 (902), Japan, the following biochemical analyses were done: high-density lipoprotein cholesterol (HDL), triglycerides (Tg), total cholesterol (TC). In addition, low-density lipoprotein cholesterol (LDL) was calculated by the Friedewald formula (LDL=TC-HDL-TG/2.2) (18). Fibrinogen was done by analysers using kits from Dade Behring Marburg GmbH.

Statistical analysis

The data were analysed using the Statistical Package for the Social Sciences IBM-SPSS, version 26.0. Categorical variables were presented as frequency and were analysed using the Chi-square test. All continuous variables are presented as median (interquartile range: 25–75th percentile) or mean ± standard deviation for the data that are not normally or normally distributed, respectively. The Shapiro-Wilk test was used to test the normality of data distribution. For intergroup comparisons, the Kruskal-Wallis test for non-parametric variables and ANOVA for parametric variables was used. Spearmen's coefficient correlation tested the relationship between variables. Also, the relationship between the fibrinogen as a dependent variable and other variables were examined using multiple linear regression analysis. Statistical significance was defined as $p<0.05$ for all comparisons.

Results

The cross-sectional study included 738 individuals older than 20 years. The median age of the participants was 38 years for the whole group. Most of the group consisted of individuals between 31 and 40 years old (*Figure 1*).

The mean value of SBP for the whole group was 122.39±9.42 mmHg, and for the DBP, 79.94±6.56 mmHg. Among active military personnel, 72.7% (533) had prehypertension, and 13.8% (101) was hypertensive.

A statistically significant difference was found in the body mass and BMI index among the observed age subgroups. Both variables gradually increased with the age of the patients, so that the highest average values were in the age group of 51–60 years; those participants had approximately body mass of 97 kg and BMI almost 29.5 kg/m^2. The proportion of patients with BMI ≥25 kg/m^2 grew with age, so in the youngest group, there were only 58.2% patients with BMI ≥25 kg/m^2, while in the group older than 51 years, there were 100% patients. Both systolic and diastolic pressure are statistically the highest in the oldest group and increase gradually with age. The proportion of hypertensive patients also increased with age; in the youngest group, there were only 6.4% of patients with hypertension, and in the oldest group, 21.4 %. Cholesterol values also increased with age; statistically, significantly higher cholesterol levels were recorded in the older group compared to the previous three subgroups. It was similar with cholesterol ≥5.2 mmol/L; the youngest group had a frequency of 36.7% and the oldest 78.6%. HDL cholesterol values also differed statistically significantly between age subgroups, with the proportion of individuals with HDL less than 1.5 mmol/L in all subgroups being about 85%, the only in the 41–50 age group was lower, 76.4%. LDL cholesterol and the proportion of individuals who had LDL ≥3.5 increased with the age of patients, and an identical trend was recorded with triglycerides (*Table I*).

With ageing, fibrinogen levels increased. *Figure 2* showed that the median fibrinogen value increased from 2.30 mmol/L in the youngest group to 3.3 mmol/L in the oldest group. There were statistically significant differences among age groups considering all observed variables of lipid status (total cholesterol, LDL and HDL cholesterols, triglycerides). With ageing, the proportion of the patients with increased lipids' levels grew (*Table II*).

The significant positive correlations among age and all other analysed parameters were recorded. With ageing, all observed parameters grew. The correlation matrix illustrated that age was positively strongly correlated with all observed variables. A mutual correlation was also found between other parameters, so it could be concluded that the values

Table I Distribution of clinical and biochemical characteristics among active-duty military personnel according to the age groups.

Characteristics	Age groups; median (IQR), number (%) or MV±SD				
	20–30 years (n=79)	31–40 years (n=410)	41–50 years (n = 235)	51–60 years (n=14)	p value
Body mass, kg	85.00 (80.00–90.00)	88.00 (80.00–96.12)	90.40 (83.17–97.50)	96.90 (89.65–106.62)	<0.001*
Body height, cm	183.00 (177.00–186.00)	181.00 (177.00–186.00)	181.00 (177.00–186.00)	181.50 (174.50–185.50)	0.524*
BMI, kg/m^2	25.08 (24.43–27.00)	26.81 (24.80–28.81)	27.20 (25.60–29.50)	29.49 (28.47–31.43)	<0.001*
<24.99	33 (41.8%)	104 (25.6%)	44 (18.8%)	–	<0.001**
≥25.00	46 (58.2%)	302 (74.4%)	190 (81.2%)	14 (100.0%)	
Systolic blood pressure, mmHg	119.60±7.69	121.43±9.13	124.81±9.94	125.36±8.65	<0.001#
Diastolic blood pressure, mmHg	78.72±5.72	79.47±6.62	81.03±6.63	82.50±5.09	0.004#
Normal bood pressure	11 (14.1%)	65 (16.0%)	23 (9.8%)	–	<0.001**
Prehypertension	63 (79.5%)	303 (74.5%)	157 (67.1%)	11 (78.6%)	
Hypertension	5 (6.4%)	39 (9.5%)	54 (23.1%)	3 (21.4%)	
Total cholesterol, mmol/L	4.87 (4.20–5.48)	5.10 (4.46–5.88)	5.61(4.93–6.29)	6.01 (5.44–6.34)	<0.001*
<5.2 mmol/L	50 (63.3%)	216 (52.8%)	82 (35.2%)	3 (21.4%)	<0.001**
≥5.2 mmol/L	29 (36.7%)	193 (47.2%)	151 (64.8%)	11 (78.6%)	
HDL cholesterol, mmol/L	1.26 (1.10–1.42)	1.18 (0.85–1.67)	1.26(1.12–1.49)	1.28 (1.04–1.48)	0.001*
≥1.5 mmol/L	12 (15.2%)	61 (15.0%)	55 (23.6%)	2 (15.4%)	0.033**
<1.5 mmol/L	67 (84.8%)	346 (85.0%)	178(76.4%)	11 (84.6%)	
LDL cholesterol, mmol/L	3.01 (2.51–3.61)	3.27 (2.75–4.00)	3.60 (3.02–4.15)	3.71 (3.31–4.21)	<0.001*
<3.5 mmol/L	58 (74.4%)	244 (60.4%)	105 (45.7%)	4 (30.8%)	<0.001**
≥3.5 mmol/L	20 (25.6%)	160 (39.6%)	125 (54.3%)	9 (69.2%)	
Triglycerides, mmol/L	0.92 (0.74–1.35)	1.18 (0.85–1.67)	1.37 (0.93–1.99)	2.03 (1.09–2.85)	<0.001*
<1.7 mmol/L	71 (89.9%)	308 (75.5%)	151 (64.8%)	6 (42.9%)	<0.001**
≥1.7 mmol/L	8 (10.1%)	100 (24.5%)	82 (35.2%)	8 (57.1%)	
Fibrinogen, mmol/L	2.30 (2.00–2.50)	2.70 (2.30–3.10)	3.20 (2.80–3.50)	3.30 (2.70–3.80)	<0.001*

* – Kruskal-Wallis test; ** – Chi-square test; # – ANOVA; TCH, Cholesterol; Tg – Triglyceride; BMI – Body Mass Index; IQR– interquartile range, MV – mean value, SD – standard deviation; BP – Blood pressure; SBP – Systolic blood pressure; DBP – Diastolic blood pressure; Normal BP (SBP<120 mmHg and DBP<80 mmHg); Prehypertension (SBP=120–139 mmHg and/or DBP=80–89 mmHg); Hypertension (SBP≥140 mmHg and/or DBP≥90 mmHg, or current treatment with antihypertensive medications).

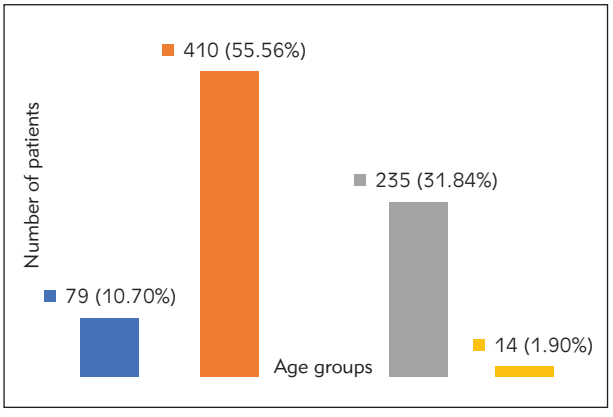

Figure 1 Distribution of the participants according to the age.

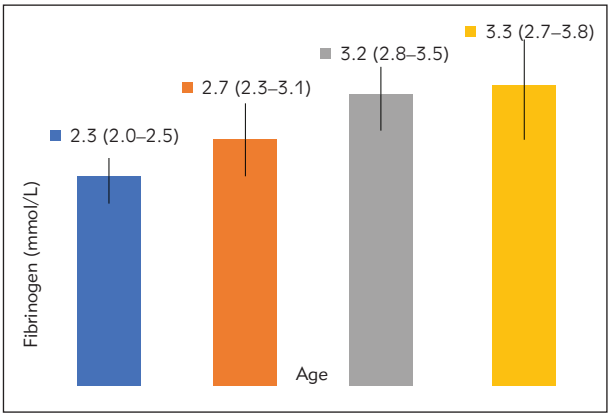

Figure 2 Fibrinogen by the age groups (Fibrinogen values were shown as median with interquartile range: 25–75. percentile).

Table II Stratified risk (low, moderate, high) within each fraction of lipid status (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides).

Characteristics	Age groups; median (IQR) or number (%)				
	20–30 years (n = 79)	31–40 years (n = 410)	41–50 years (n = 235)	51–60 years (n = 14)	p value
Total cholesterol, mmol/L					
Low <5.2	50 (63.3)	216 (52.8)	82 (35.2)	3 (21.4)	<0.001*
Moderate 5.2–6.2	26 (32.9)	127 (31.1)	84 (36.1)	6 (42.9)	
High >6.2	3 (3.8)	66 (16.1)	67 (28.8)	5 (35.7)	
LDL cholesterol, mmol/L					
Low <3.5	58 (74.4)	244 (60.4)	105 (45.7)	4 (30.8)	<0.001*
Moderate 3.5–4.1	17 (21.8)	71 (17.6)	61 (26.5)	6 (46.2)	
High >4.1	3 (3.8)	89 (22.0)	64 (27.8)	3 (23.1)	
HDL cholesterol, mmol/L					
Low >1.5	12 (15.2)	61 (15.0)	55 (23.6)	2 (15.4)	0.033*
Moderate 1.0–1.5	57 (72.2)	273 (67.1)	155 (66.5)	9 (69.2)	
High <1.0	10 (12.7)	73 (17.9)	23 (9.9)	2 (15.4)	
Triglycerides, mmol/L					
Low <1.69	71 (89.9)	308 (75.5)	151 (64.8)	6 (42.9)	<0.001*
Moderate 1.7–2.25	4 (5.1)	47 (11.5)	39 (16.7)	3 (21.4)	
High > 2.26	4 (5.1)	53 (13.0)	43 (18.5)	5 (35.7)	

* – Chi-square test

Table III The correlation matrix illustrated age was strongly positively correlated with all observed variables.

Variables		Age (years)	Total Cholesterol (mmol/L)	LDL cholesterol (mmol/L)	HDL cholesterol (mmol/L)	Triglycerides (mmol/L)	Fibrinogen (mmlo/L)	Body mass index (kg/m^2)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)
Age, years	r	1.000								
	p									
Total cholesterol, mmol/L	r	0.297	1.000							
	p	<0.001								
LDL cholesterol, mmol/L	r	0.221	0.928	0.367	0.027	1.000				
	p	<0.001	<0.001	<0.001	0.473					
HDL cholesterol, mmol/L	r	0.086	0.128	-0.442	1.000					
	p	0.019	0.001	<0.001						
Triglycerides, mmol/L	r	0.206	0.498	1.000						
	p	<0.001	<0.001							
Fibrinogen, mmol/L	r	0.465	0.231	0.137	0.054	0.201	1.000			
	p	<0.001	<0.001	0.001	0.199	<0.001				
Body mass index, kg /m^2	r	0.219	0.179	0.274	-0.204	0.166	0.139	1.000		
	p	<0.001	<0.001	<0.001	<0.001	<0.001	0.001			
Systolic blood pressure, mmHg	r	0.215	0.143	0.204	-0.068	0.115	0.184	0.288	1.000	
	p	<0.001	<0.001	<0.001	0.067	0.002	<0.001	<0.001		
Diastolic blood pressure, mmHg	r	0.149	0.086	0.142	-0.060	0.059	0.168	0.234	0.599	1.000
	p	<0.001	0.019	<0.001	0.104	0.117	<0.001	<0.001	<0.001	

r – Spearman's rho

Table IV Multi-regression analysis with fibrinogen as a dependent variable.

Variables	Unstandardised	Coefficients	Sig.	95.0% Confidence Interval for B	
	B	Std. Error		Lower Bound	Upper Bound
Age, years	0.044	0.007	0.000	0.030	0.058
Total cholesterol, mmol/L	0.010	0.099	0.916	-0.184	0.205
LDL cholesterol, mmol/L	0.057	0.106	0.594	-0.152	0.266
HDL cholesterol, mmol/L	0.001	0.007	0.940	-0.014	0.015
Triglycerides, mmol/L	0.018	0.055	0.750	-0.091	0.126
Systolic blood pressure, mmHg	-0.004	0.006	0.519	-0.016	0.008
Diastolic blood pressure, mmHg	0.007	0.008	0.428	-0.010	0.023

Table V The higher fibrinogen values significantly increased the patient's age, blood pressure, total cholesterol, LDL cholesterol and triglycerides.

Characteristics	FIBRINOGEN tertiles; median (IQR), number (%) or MV±SD			
	Low (2.0–2.5)	Mid (2.5–3.1)	High (3.1–7.0)	p value
Age, years	36.00 (30.00–38.00)	38.00 (35.00–41.00)	41.00 (37.00–45.00)	<0.001*
Body mass, kg	86.00 (80.00–95.00)	90.00 (80.90–96.00)	88.35 (81.52–97.05)	0.159*
Body height, cm	183.00 (177.00–186.00)	181.00 (177.50–186.00)	180.50 (176.50–185.00)	0.090*
Body mass index, kg/m^2	26.00 (24.52–28.05)	27.00 (25.00–28.83)	27.00 (25.12–29.41)	0.002*
Systolic blood pressure, mmHg	120.30±8.59	122.63±9.13	124.51±10.39	<0.001#
Diastolic blood pressure, mmHg	78.89±6.26	80.87±6.53	81.17±7.02	0.001#
Total cholesterol, mmol/L	5.04 (4.37–5.61)	5.04 (4.46–5.85)	5.57 (4.93–6.29)	<0.001*
LDL cholesterol, mmol/L	3.21 (2.63–3.82)	3.21 (2.67–3.97)	3.59 (3.14–4.23)	<0.001*
HDL cholesterol, mmol/L	1.23 (1.08–1.40)	1.22 (1.09–1.43)	1.22 (1.08–1.45)	0.588*
Triglycerides, mmol/L	1.12 (0.85–1.63)	1.09 (0.80–1.55)	1.37 (0.97–1.96)	0.001*

One-Way ANOVA; * Kruskal-Wallis test

of observed cardiovascular risk factors increased with age. In addition to age, fibrinogen was also seen in a strong positive correlation with cholesterol, LDL cholesterol and triglycerides (*Table III*). Also, multi-regression analysis was performed and obtained a significant model (F=7.577; p <0.001). The only significant variable that stood out was age. Ageing explains most of the variability of fibrinogen; the fibrinogen grew with increasing age (*Table IV*).

According to fibrinogen values, all patients were divided into terciles; one-third of patients from the smallest to the largest fibrinogen value. The higher fibrinogen values significantly increased the patient's age, blood pressure, total cholesterol, LDL cholesterol and triglycerides (*Table V*).

Discussion

Our study underlined the significant prevalence of traditional cardiovascular risk factors for coronary artery disease in the military population that increased with ageing. Furthermore, fibrinogen as a novel risk factor also grew with increasing age. Further analysis registered a positive correlation between fibrinogen and traditional risk factors values, but only ageing had a positive predictive value. Additional sub-analysis on the patients divided into terciles according to the fibrinogen values support previously cited results. Those finding seems to be very important, having in mind recent data considering age-related cardiovascular disease so-called« inflamm-ageing« (19). This chronic low-grade inflammation state, pathophysiologically based on the age-related increased inflammatory tone (inflamm-ageing) and nutrient excess (metaflammation) attributed to the accelerating vascular ageing and atherosclerosis per se. Except for accelerated atherosclerosis, there are also reciprocal positive interactions with traditional CV risk factors. All those findings contribute to creating novel therapeutic approaches that should promote healthy ageing and preserve health care system resources (20).

In order to define the preventive strategy goals, we analysed the prevalence of traditional cardiovascular risk factors among our specific study population. Most of our study group consisted of males younger than 40 with prehypertension in almost 75% and hypertension in 13.8% of participants. Among Serbian Armed Forces (older than 20 years of age), a significantly higher prevalence of prehypertension was identified than in the general population of the same age in the Republic of Serbia (in the age between 20 and 39 years, 67.4–54.1%; in the age between 40 and 44 years, 46.6%) (21–22). The prevalence of hypertension in the adult population of Serbia (aged ≥ 15 years) of 33.2% is significantly higher compared to hypertension among individuals in our study group (13.8%) (22).

Compared to results from the USA, the prevalence of hypertension in Serbia among the military population is more than 2.5 times higher. The proportion of hypertensive patients also increased with age; in the youngest group, there were only 6.4% of patients with hypertension, and in the oldest group, 21.4%, probably due to the so-called »lifestyle« that is characteristic for the group of upper-middle-income countries, as Serbia (21–25). Every candidate must go through a specific general medical examination and selection to become active military personnel. That may be the reason for the generally lower prevalence of hypertension in military personnel compared to the civilian population.

Besides blood pressure, both body mass and BMI index gradually increased with the age of the patients, so that the highest values were in the oldest group. Thus, our results are in accordance with the previous study considering trend, but the prevalence is a little higher, probably thanks to the specific nutrition habits and sedentary way of life among our study group (26–27).

The average Serbian solder is, at least, overweight with a non-favourable LDL trend. American Heart Association (AHA) data underline that 36% of adults and 10% of children between 9 and 12 years have elevated cholesterol (17). It seems important to consider that cumulative young adult exposures to elevated systolic BP, diastolic BP and LDL were associated with increased CVD risks in later life, independent of later adult exposures (28–29). Framingham study reported that males with total cholesterol over 8 mmol/L and females over the 6 mmol/L have almost 5 times higher risk for CVD in the next five years than the general population (18). Recent data support the previous findings that normalisation of LDL cholesterol levels may lead to almost 40 % CVD morbidity and mortality risk reduction (30–31).

In 2006, according to the survey of the Ministry of Health of the Republic of Serbia, HLP incidence was 2.7% for males and 4.2% for females, and prevalence was 7.3% for males and 8.6% for females (18). Our study pointed out that the prevalence of HLP increased with age, even in the so-called »healthy Warrior« population (13). The prevalence of dyslipidemia among military personnel in the literature was from 5.3% to 41.96%. The prevalence of hypercholesterolemia, hypertriglyceridemia and low HDL-C are respectively: between 3.12% and 5.2%, 3.9% and 28%, 31% (14–15, 21, 26–27, 31). In accordance with cited studies were our results considering all lipid fractions gradual increments with age.

Except traditional, we also analysed plasma fibrinogen levels as a novel cardiovascular risk factor for age-related cardiovascular disease and inflamm-ageing. Study data suggest that fibrinogen levels increase with ageing (32–33). There is a clear link between elevated plasma fibrinogen, cardiovascular disease and arterial and venous thrombosis (32). The Framingham study confirmed a positive correlation between fibrinogen levels and risk of cardiovascular disease, as well as with the incidence of death and/or myocardial infarction (33). Hyperfibrinogenemia is also an independent predictor of carotid thrombosis (34–35). The difference in plasma fibrinogen levels among hypertensive and normotensive patients was also registered (34). It may be important, bearing in mind that among our study population older than 20, a higher prevalence of prehypertension than the general population of the same age in the Republic of Serbia was found (21–22).

Our study analysis underlined ageing as an independent predictor influenced by the variability of fibrinogen. Fibrinogen levels were associated with traditional cardiovascular risk factors (blood pressure, total cholesterol, LDL cholesterol and triglycerides) and may not be influenced as much by body mass as CRP, supporting its usefulness as a biomarker of CVD (36). Recent meta-analysis pointed out clear associations between fibrinogen level and the risks of CHD, stroke, other vascular and nonvascular mortality in healthy middle-aged adults (37). Keeping in mind that fibrinogen levels predicted cardiovascular events independent of traditional risk factors in adults without clinical evidence of coronary heart disease at baseline, those findings may contribute to planning the strategy in primary prevention for these categories of individuals (38).

Conclusions

Our results reported that military personnel with elevated blood pressure and dyslipidemias, followed by hyperfibrinogenemia, have multiple cardiovascular and cerebrovascular disease risk factors. Higher fibrinogen level is associated with traditional cardiovascular risk factors in this population and may be a useful biomarker of CVD in this high-risk subgroup. Those findings considering cardio and cerebrovascular risk factors would help create a new approach for primary prevention for these categories of individuals.

Author declaration

Authors certify that the manuscript represents a valid workpiece. Neither this manuscript nor one with substantially similar content under named authorship has been published or is being considered for publication elsewhere. The authors have participated in the research and the shaping of the manuscript.

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Conflict of interest statement

The authors reported no conflict of interest regarding the publication of this article.

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ONE-LUNG VENTILATION PATIENTS: CLINICAL CONTEXT OF ADMINISTRATION OF DIFFERENT DOSES OF DEXMEDETOMIDINE

PACIJENTI SA VENTILACIJOM JEDNOG PLUĆNOG KRILA: KLINIČKI KONTEKST PRIMENE RAZLIČITIH DOZA DEKSMEDETOMIDINA

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Summary

Background: Open and endoscopic thoracic surgeries improve surgical exposure by One-lung ventilation (OLV). The aim of this study was to investigate the effects of different doses of dexmedetomidine on inflammatory response, oxidative stress, cerebral tissue oxygen saturation ($S_{ct}O_2$) and intrapulmonary shunt in patients undergoing one-lung ventilation (OLV).
Methods: Seventy-five patients undergoing open pulmonary lobectomy in our hospital from January 2016 to December 2017 were enrolled and randomly divided into high-dose dexmedetomidine group (group D1, 1 μg/kg, n=25), low-dose dexmedetomidine group (group D2, 0.5 μg/kg, n=25) and control group (group C, n=25). Then, arterial blood and internal jugular venous blood were taken before anesthesia induction (T0) and at 15 min after two-lung ventilation (T1) and 5 min (T2) and 30 min (T3) after OLV for later use. Next, the changes in hemodynamic parameters [mean arterial pressure (MAP), heart rate (HR) and pulse oxygen saturation (SpO_2)] of patients were observed in each group. Enzyme-linked immunosorbent assay (ELISA) was carried out to detect serum inflammatory factors such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) and oxidative stress indicators [superoxide dismutase (SOD) and malondialdehyde (MDA)]. The changes in $S_{ct}O_2$, arterial partial pressure of oxygen (PaO_2) and intrapulmonary shunt Qs/Qt (a measurement of pulmonary shunt: right-to-left shunt fraction) were observed. Additionally, the changes in lung function indicators like lung dynamic compliance (Cdyn) and airway peak pressure (Ppeak) were determined.

Kratak sadržaj

Uvod: Otvorene i endoskopske torakalne operacije poboljšavaju hirurško izlaganje pomoću ventilacije jednog plućnog krila (OLV). Cilj ove studije je bio da se ispitaju efekti različitih doza deksmedetomidina na inflamatorni odgovor, oksidativni stres, zasićenost cerebralnog tkiva kiseonikom (SctO2) i intrapulmonalni šant kod pacijenata koji su podvrgnuti ventilaciji jednog pluća (OLV).
Metode: Sedamdeset pet pacijenata koji su bili podvrgnuti otvorenoj plućnoj lobektomiji u našoj bolnici od januara 2016. do decembra 2017. godine upisani su i nasumično podeljeni u grupu sa visokim dozama deksmedetomidina (grupa D1, 1 μg/kg, n=25), niskom dozom deksmedetomidina (grupa D2), 0,5 μg/kg, n=25) i kontrolna grupa (grupa C, n=25). Zatim su uzete arterijska krv i unutrašnja jugularna venska krv pre indukcije u anesteziju (T0) i 15 min nakon ventilacije sa dva plućna krila (T1) i 5 min (T2) i 30 min (T3) nakon OLV za kasniju upotrebu. Zatim su uočene promene hemodinamskih parametara [srednji arterijski pritisak (MAP), broj otkucaja srca (HR) i pulsna zasićenost kiseonikom (SpO_2)] pacijenata u svakoj grupi. Enzimski imunosorbentni test (ELISA) je sproveden da bi se otkrili serumski inflamatorni faktori kao što su interleukin-6 (IL-6) i faktor nekroze tumora-alfa (TNF-α) i indikatori oksidativnog stresa [superoksid dismutaza (SOD) i malondialdehid (MDA)]. Uočene su promene u $S_{ct}O_2$), arterijskom parcijalnom pritisku kiseonika (PaO_2) i intrapulmonalnom šantu Ks/Kt (merenje plućnog šanta: frakcija šanta zdesna nalevo). Pored toga, određene su promene u indikatorima plućne funkcije kao što su dinamika plućne usklađenosti (Cdin) i vršni pritisak u disajnim putevima (Ppeak).

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Results: There were no statistically significant differences in the MAP, HR and SpO_2 among three groups at each observation time point (P>0.05). At T2 and T3, the levels of serum IL-6, TNF-α and IL-8 were obviously decreased in group D1 and D2 compared with those in group C (P<0.05), and the decreases in group D1 were overtly larger than those in group D2, and the decreases at T3 were markedly greater than those at T2 (P<0.05). In comparison with group C, group D1 and D2 had notably reduced levels of serum reactive oxygen species (ROS) and MDA (P<0.05) and remarkably increased SOD content (P<0.05) at T2 and T3, and the effects were markedly better in group D1 than those in group D2. Besides, they were significantly superior at T3 to those at T2 (P<0.05). The $S_{ct}O_2$ in group D1 and D2 was evidently lowered at T2 and T3 compared with that at T0, and the decrease in group D1 was distinctly smaller than that in group D2 (P<0.05). The Qs/Qt was significantly lower in group D1 and D2 than that in group C at T2 and T3 (P<0.05), while the PaO_2 content was notably raised (P<0.05), and the decrease and increase were significantly larger in group D1 than those in group D2, and they were obviously greater at T3 to those at T2 (P<0.05). At T0 and T1, no significant differences were detected in the Cdyn, Pplat and Ppeak among three groups. At T2 and T3, the Cdyn was significantly elevated, while the Pplat and Ppeak overtly declined (P<0.05), and group D1 had greater changes in comparison with group D2, and the changes were obviously more evident at T3 to those at T2 (P<0.05).
Conclusions: Dexmedetomidine effectively ameliorates inflammatory response and oxidative stress, lowers oxygenation, Qs/Qt and the decrease in $S_{ct}O_2$ and improves lung function during OLV, with good efficacy.

Keywords: dexmedetomidine, one-lung ventilation, inflammatory response, oxidative stress, cerebral tissue oxygen saturation, intrapulmonary shunt

Rezultati: Nije bilo statistički značajnih razlika u MAP, HR i SpO_2 između tri grupe u svakoj vremenskoj tački posmatranja (P>0,05). Na T2 i T3, nivoi serumskih IL-6, TNF-α i IL-8 su očigledno bili smanjeni u grupi D1 i D2 u poređenju sa onima u grupi C (P<0,05), a smanjenje u grupi D1 je bilo očigledno veće od oni u grupi D2, a smanjenja na T3 bila su značajno veća od onih u T2 (P<0,05). U poređenju sa grupom C, grupe D1 i D2 su imale značajno smanjene nivoe serumskih reaktivnih vrsta kiseonika (ROS) i MDA (P<0,05) i značajno povećan sadržaj SOD (P<0,05) na T2 i T3, a efekti su bili znatno bolji u grupi D1 od onih u grupi D2. Osim toga, oni su bili značajno bolji na T3 u odnosu na one na T2 (P<0,05). SctO2 u grupi D1 i D2 je evidentno smanjen na T2 i T3 u poređenju sa onim u T0, a smanjenje u grupi D1 je bilo znatno manje nego u grupi D2 (P<0,05). Ks/Kt je bio značajno niži u grupi D1 i D2 nego u grupi C na T2 i T3 (P<0,05), dok je sadržaj PaO_2 bio značajno povišen (P<0,05), a smanjenje i povećanje su značajno veće u grupi. D1 od onih u grupi D2, i očigledno su bili veći na T3 u odnosu na one u T2 (P<0,05). Na T0 i T1, nisu otkrivene značajne razlike u Cdin, Pplat i Ppeak između tri grupe. Na T2 i T3, Cdin je bio značajno povišen, dok su Pplat i Ppeak izrazito opali (P<0,05), a grupa D1 je imala veće promene u poređenju sa grupom D2, a promene su očigledno bile očiglednije na T3 u odnosu na one na T2 (P<0,05).
Zaključak: Deksmedetomidin efikasno ublažava inflamatorni odgovor i oksidativni stres, smanjuje oksigenaciju, Ks/Kt i smanjenje $S_{ct}O_2$ i poboljšava funkciju pluća tokom OLV, sa dobrom efikasnošću.

Ključne reči: deksmedetomidin, ventilacija jednog plućnog krila, inflamatorni odgovor, oksidativni stres, saturacija cerebralnog tkiva kiseonikom, intrapulmonalni šant

Introduction

One-lung ventilation (OLV) is commonly used for open and endoscopic thoracic surgeries to improve surgical exposure. Hypoxic pulmonary vasoconstriction (HPV) shunts blood from the non-ventilated lung to the non-surgical lung, thus maintaining adequate oxygenation (1, 2). Thoracic epidural anesthesia (TEA) is the most commonly used analgesia technique in patients receiving thoracic surgeries for lungs. It has been proved that strong inhalation anesthesia inhibits HPV in a dose-dependent manner, thereby altering the oxygenation during OLV and resulting in increased shunt and impaired oxygenation (3, 4). Studies have revealed that the development and progression of inflammatory response and oxidative stress are promoted in the peri-operative period. Dexmedetomidine is a highly selective and very potent α2-adrenergic agonist with antioxidant properties, metabolized in the liver and approved to be used in intensive care units as a sedation and anesthesia assistor, with sedative and analgesic effects (5).

A previous study demonstrated that dexmedetomidine lowers the high levels of malondialdehyde (MDA) and hypoxanthine formed after the application of tourniquets in upper-limb surgeries, with an obvious effect (6). Dexmedetomidine has analgesic, anxiolytic and sedative effects, on which many clinical and experimental studies have been conducted in recent years (7, 8). Moreover, research of the role of dexmedetomidine in ischemic and toxic inflammation models has revealed that dexmedetomidine has an anti-inflammatory effect, which evidently inhibits the production of inflammatory factors including tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), IL-6 and macrophage inflammatory protein 2, and avoids damage to organs. Furthermore, it has been reported that dexmedetomidine prominently relieves lung inflammation in a rat model of ventilator-induced lung injury. Shen et al. (9) measured the levels of TNF-α and IL-6 in the case of lung injury induced in experimental models and proved that the production of these cytokines is reduced to the utmost in lung tissues after administration with dexmedetomidine. A study pointed out that mouse

liver ischemia-reperfusion (I/R) injury results in oxidative stress, which is manifested as enhanced MDA, an oxidant and reduced superoxide dismutase (SOD), an antioxidant (10). Lung tissues are vulnerable to the harmful effects of hypovolemia, and excessive inflammation and oxidative stress response are detected in a mouse model, including SOD and MDA (11). SOD is ubiquitous, and MDA can resist the effects of SOD, with cytotoxicity. After I/R, the treatment with the antioxidant dexmedetomidine is capable of ameliorating organ oxidative stress and achieving better outcomes. Antioxidant therapy with transmembrane free radical scavengers can improve the prognosis of I/R rats (12). The above findings suggest that dexmedetomidine effectively attenuates inflammatory response and oxidative stress during surgery. The changes in cerebral tissue oxygen saturation ($S_{ct}O_2$) are measured horizontally, continuously and non-invasively in the peri-operative period through the frontal microvascular system. Additionally, the specific changes in $S_{ct}O_2$ are monitored to provide real-time oxygenation in local tissues during full-circulation arrest, venous cannula obstruction or sudden global hypoxemia in cardiac surgeries. Reduced $S_{ct}O_2$ is considered as an indication of potential hypoxia-induced injury that needs further interventions (13). Evaluating cardiac output and systemic oxygenation sufficiency may have potential value in changing the ventilation mode in the peri-operative period in patients who received the bidirectional Glenn procedure (14).

This study aims to explore the effects of different doses of dexmedetomidine on inflammatory response, oxidative stress, $S_{ct}O_2$ and intrapulmonary shunt in patients receiving OLV. Patients undergoing open pulmonary lobectomy were enrolled in this study, and then hemodynamic parameters, inflammatory factors, oxidative stress indicators, and changes in the $S_{ct}O_2$, arterial oxygen partial pressure (PaO_2) and Qs/Qt as well as lung function were observed at different time points, hoping to prove that dexmedetomidine can effectively relieve inflammatory response and oxidative stress, lower oxygenation and Qs/Qt and improve $S_{ct}O_2$ and lung function during OLV, with good effects. This study provides theoretical and experimental bases for the popularization and application of dexmedetomidine.

Materials and Methods

Clinical data

Seventy-five patients who underwent open pulmonary lobectomy in our hospital were enrolled as study subjects. Then, the patients enrolled signed the informed content and were randomly divided into high-dose dexmedetomidine group (group D1, 1 μg/kg, n=25), low-dose dexmedetomidine group (group D2, 0.5 μg/kg, n=25) and control group (group C, n=25). Inclusion criteria: Patients at America Society of Anesthesiologist (ASA) grade I and II, receiving no treatment previously, and not allergic to the drugs used in this study. Exclusion criteria: Patients allergic to the drugs, or with severe cardiovascular or cerebrovascular diseases, or secondary infection complicated with severe abnormal liver or kidney function, or those unable to communicate normally due to severe mental disorders. All clinical specimens in this study were collected with the consent of the patients and their families as per the *Declaration of Helsinki*. This clinical study protocol was carried out with approval from the Ethics Committee of our hospital. The specific clinical data of patients collected at admission included age, gender, weight, body condition and pathological grade (*Table I*).

Table I Clinical data of patients.

Parameter	Group D1	Group D2	Group C
Sample size	25	25	25
Number of male patients	12	13	12
Average age (years old)	45±11	46±12	47±11
Mean weight (kg)	49±10.5	50±10	48±10.7
BMI (kg/m^2)	21.5±3.2	22.1±3.0	21.4±2.8
ASA grade I	13	14	13
ASA grade II	12	11	12
Operation time (min)	89.9±3.5	90.5±5.0	91.4±4.7
Duration of anesthesia (min)	32.2±3.1	33.5±3.8	34.2±4.6

Therapeutic methods

Before surgery, all patients were intravenously injected with propofol (1.2 mg/kg) for anesthesia induction and then with sufentanil (0.5 μg/kg) and rocuronium (0.8 mg/kg). Next, double-lumen endotracheal intubation was conducted for smooth insertion, during which a fiberoptic bronchoscope was adopted to locate the tracheal tube and ensure good alignment. Thereafter, an anesthesia respirator was connected for mechanical ventilation (VT: 8 mL/kg, RR: 12 times/min, suction ratio: 1:2, inhaled oxygen concentration: 100%, oxygen flow rate: 1 L/min, $P_{ET}CO_2$: 40 mmHg), and the respiratory tract was kept clear for sufficient oxygen inhalation. Anesthesia was maintained by jointly injecting with propofol (5 mg/kg/h) and remifentanil (0.2 μg/kg/min), with rocuronium (0.3 mg/kg) added at intervals. Before surgery, the patients in group D1 were given dexmedetomidine at load capacity of 1 μg/kg using an infusion pump for 10 min and then at 0.5 μg/kg/h until the chest was closed. Those in group D2 were treated with dexmedetomidine at load capacity of 0.5 μg/kg using the infusion pump for 10 min and then at 0.3 μg/kg/h until the chest was closed. Those in group C were given the same amount of normal saline. The position of the patients was changed, and then the fiberoptic bronchoscope was aligned for OLV. The changes in various indexes were observed before anesthesia

induction (T0) and at 15 min after two-lung ventilation (T1) and 5 min (T2) and 30 min (T3) after OLV.

Determination of changes in hemodynamic indexes [mean arterial pressure (MAP), heart rate (HR) and pulse oxygen saturation (SpO_2)], $S_{ct}O_2$, PaO_2 and intapulmonary shunt Qs/Qt

The changes in the HR, MAP and $SpO_2$of patients were recorded in each group before thoracotomy (T1) and at 30 min (T2) after OLV. Arterial blood was sampled for blood gas analysis, the PaO_2 was recorded, the intrapulmonary shunt Qs/Qt was calculated, and the $S_{ct}O_2$ was recorded at corresponding time points. $Qs/Qt\% = (CcO_2 - CaO_2)/(CcO_2 - CvO_2)$. $CcO_2 = Hb \times 1.39 \times SaO_2 + (PaO_2 \times 0.0031)$, $PaO_2 = FiO_2 \times (Pb - PH_2O) - (PaCO_2/0.8)$, $CaO_2 = (1.34 \times Hb \times SaO_2) + (0.0031 \times PaO_2)$, $CvO_2 = (1.34 \times Hb \times SvO_2) + (0.0031 \times PaO_2)$.

Detection of serum inflammatory factors via enzyme-linked immunosorbent assay (ELISA)

After collecting venous blood (5 mL) into Eppendorf (Ep) tubes containing anticoagulant from arms, centrifugation was conducted at room temperature and 3000 g for 15 min, followed by collection of the supernatant. Next, the levels of serum inflammatory factors (IL-6, IL-8 and TNF-α) were measured according to the instruments of the ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Later, the absorbance in each group was read using a microplate reader.

Measurement of serum oxidative stress indexes through ELISA

Venous blood (5 mL) was collected into Ep tubes containing anticoagulant from arms and centrifuged at room temperature and 3500 g for 15 min. Thereafter, the supernatant was collected, and the changes in the content of serum oxidative stress indexes [MDA, SOD and reactive oxygen species (ROS)] were determined according to the instruments of the ELISA kit (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China). Lastly, the microplate reader was utilized to read the absorbance in each group.

Examination of lung function

Side stream spirometry was adopted to monitor lung function indicators [lung dynamic compliance (Cdyn), platform pressure (Pplat), and airway peak pressure (Ppeak)]. The average was taken after multiple measurements. The specific operations were performed as per the instructions of the instrument, and the obtained values were analyzed according to the instructions provided by manufacturers.

Statistical analysis

All raw experimental data recorded were processed by Statistical Product and Service Solutions (SPSS) 19.0 analysis software (SPSS Inc., Chicago, IL, USA) and subjected to multiple comparisons. The experimental results obtained were expressed as mean ± standard deviation ($\bar{x}\pm SD$), and $P<0.05$ suggested that the difference was statistically significant. Graphpad Prism 5.0 (La Jolla, CA, USA) was applied for plotting histograms.

Results

Hemodynamic indexes detected

As shown in *Table II*, the MAP, HR and SpO_2 exhibited no obvious differences at each observation time point among three groups ($P>0.05$), suggesting that there is no impact on hemodynamics during OLV.

Levels of inflammatory factors detected

At T2 and T3, the levels of serum IL-6, TNF-α and IL-8 showed marked decreases in group D1 and D2 compared with those in group C ($P<0.05$), and the decreases were overtly larger in group D1 than those in group D2, and they were evidently greater at T3 than those at T2 ($P<0.05$) (*Table III*).

Table II Hemodynamic indexes.

Group	MAP	HR	SpO_2 (%)
Group C at T0	79.9±1.0	81.5±1.1	89.1±0.8
T1	79.5±1.1	80.1±1.2	87.2±0.1
T2	79.0±1.2	81.4±1.1	85.8±0.4
T3	79.2±1.3	81.6±1.5	85.3±0.4
Group D1 at T0	78.8±1.1	80.9±1.0	85.7±0.9
T1	78.9±1.8	81.8±1.6	86.1±0.8
T2	78.5±1.9	80.5±1.7	85.1±0.6
T3	77.1±1.2	78.4±1.8	95.9±0.9
Group D2 at T0	78.8±1.3	79.5±1.2	85.3±0.8
T1	78.2±1.7	79.8±1.7	85.9±0.9
T2	80.2±3.0	79.7±2.1	84.5±0.3
T3	76.4±1.5	77.0±1.1	90.5±0.1

Note: No evident differences are detected in the MAP, HR and SpO_2 among the three groups at each observation time point ($P>0.05$). MAP: mean arterial pressure, HR: heart rate; SpO_2: pulse oxygen saturation.

Results of oxidative stress determination

According to *Table IV*, group D1 and D2 showed notably declined levels of serum ROS and MDA ($P<0.05$) and overtly raised SOD content ($P<0.05$) at T2 and T3 in comparison with group C, and the effects were markedly better in group D1 than those in group D2, and they were significantly superior at T3 to those at T2 ($P<0.05$).

$S_{ct}O_2$

Compared with that at T0, the $S_{ct}O_2$ in group D1 and D2 was evidently lowered at T2 and T3, and group D1 exhibited a smaller decrease than group D2 ($P<0.05$) (*Table V*).

PaO_2 and the intrapulmonary shunt Qs/Qt

As shown in *Table VI*, group D1 and D2 had notably lowered Qs/Qt ($P<0.05$) and overtly elevated PaO_2 content ($P<0.05$) at T2 and T3 in comparison with group C, and the decline and increase were markedly greater in group D1 than those in group D2, and they were significantly larger at T3 than those at T2 ($P<0.05$).

Lung function indexes detected

The results of lung function index detection (*Table VII*) revealed that the Cdyn, Pplat and Ppeak displayed no significant differences among three groups at T0 and T1. At T2 and T3, the Cdyn was evidently raised, while the Pplat and Ppeak overtly declined ($P<0.05$). Moreover, group D1 had better effects in comparison with group D2, and the effects were obviously superior at T3 to those at T2 ($P<0.05$).

Table III Levels of serum IL-6, TNF-α and IL-8.

Group	IL-8 (mg/L)	TNF-α	IL-6 (mg/L)
Group C at T0	58.9±1.7	38.6±1.0	46.8±1.9
T1	62.2±1.1	40.6±1.1	48.6±1.7
T2	65.7±1.9	42.5±1.3	49.4±1.6
T3	69.4±1.3	45.8±1.7	50.4±1.5
Group D1 at T0	59.9±1.5	40.3±1.1	46.9±1.7
T1	48.5 ± 1.4^{A}	38.1 ± 1.4^{A}	33.1 ± 2.0^{A}
T2	27.2 ± 1.5^{abA}	25.1 ± 1.2^{abA}	23.3 ± 2.2^{abA}
T3	21.6 ± 1.1^{abcA}	9.5 ± 1.7^{abcA}	10.5 ± 2.5^{abcA}
Group D2 at T0	58.7±1.9	41.2±1.7	47.8±1.4
T1	52.4 ± 1.5^{A}	37.2 ± 1.6^{A}	39.7 ± 2.5^{A}
T2	37.8 ± 1.6^{abAB}	29.4 ± 1.4^{abAB}	30.8 ± 2.6^{abAB}
T3	27.9 ± 1.7^{abcAB}	17.4 ± 1.8^{abcAB}	16.0 ± 2.7^{abcAB}

Note: The levels of serum IL-6, TNF-α and IL-8 display remarkable decreases in group D1 and D2 compared with those in group C ($P<0.05$) at T2 and T3, and the decreases are overtly larger in group D1 than those in group D2, and they are evidently greater at T3 than those at T2 ($P<0.05$). Intra-group comparison: $^{a}P<0.05$ *vs.* T0, $^{b}P<0.05$ *vs.* T1, and $^{c}p<0.05$ *vs.* T2. Inter-group comparison: $^{A}P<0.05$ *vs.* group C, and $^{B}P<0.05$ *vs.* group D1.

Table IV Content of serum ROS, MDA and SOD.

Group	ROS (U/L)	MDA (mmol/L)	SOD (U/mg)
Group C at T0	30.5±1.4	16.5±1.8	4.1±1.0
T1	32.5±1.7	17.6±1.4	3.4±1.4
T2	33.7±1.8	18.2±1.1	3.8±1.5
T3	34.8±1.9	17.0±1.3	4.3±1.2
Group D1 at T0	31.1±1.8	17.2±1.4	4.2±1.8
T1	28.1 ± 1.7^{A}	15.2 ± 1.0^{A}	6.8 ± 1.4^{A}
T2	18.4 ± 1.4^{abA}	10.5 ± 1.0^{abA}	12.6 ± 1.6^{abA}
T3	7.6 ± 1.9^{abcA}	4.6 ± 1.1^{abcA}	21.5 ± 1.5^{abcA}
Group D2 at T0	31.5±1.3	17.9±1.8	4.0±1.6
T1	27.8 ± 1.8^{A}	16.2 ± 1.4^{A}	5.2 ± 1.4^{A}
T2	22.1 ± 1.1^{abAB}	13.5 ± 1.3^{abAB}	8.6 ± 1.8^{abAB}
T3	14.6 ± 1.5^{abcAB}	8.4 ± 1.0^{abcAB}	15.8 ± 1.3^{abcAB}

Note: Compared with those in group C, the levels of serum ROS and MDA are prominently decreased ($P<0.05$) in group D1 and D2 at T2 and T3, while the SOD content is remarkably elevated ($P<0.05$), and the effects are markedly better in group D1 than those in group D2, and they are significantly superior at T3 to those at T2 ($P<0.05$).Intra-group comparison: $^{a}P<0.05$ *vs.* T0, $^{b}p<0.05$ *vs.* T1, and $^{c}P<0.05$ *vs.* T2. Inter-group comparison: $^{A}P<0.05$ *vs.* group C, and $^{B}P<0.05$ *vs.* group D1.

Table V $S_{ct}O_2$ in different groups.

Group	T0	T1	T2	T3
$S_{ct}O_2$ (%) Group C	81.5±1.5	70.5 ± 1.0^{a}	65.5 ± 1.2^{ab}	60.1 ± 1.7^{abc}
Group D1	82.1±1.1	80.7 ± 1.6^{A}	76.8 ± 1.6^{abA}	70.1 ± 1.9^{abcA}
Group D2	80.1±1.6	75.1 ± 1.9^{AB}	70.8 ± 1.7^{abAB}	65.1 ± 1.3^{abcAB}

Note: The $S_{ct}O_2$ is significantly lowered in group D1 and D2 at T2 and T3 compared with that at T0 and T1, and the decrease in group D1 is distinctly smaller than that in group D2 ($P<0.05$). Intra-group comparison: $^{a}P<0.05$ *vs.* T0, $^{b}P<0.05$ *vs.* T1, and $^{c}P<0.05$ *vs.* T2. Inter-group comparison: $^{A}P<0.05$ *vs.* group C, and $^{B}P<0.05$ *vs.* group D1.

Table VI PaO_2 and the intrapulmonary shunt Qs/Qt.

Group	PaO_2 (mmHg)	Qs/Qt (%)
Group C at T0	80.5±1.5	31.5±1.7
T1	285.1 ± 1.7^{a}	32.4±1.5
T2	186.1 ± 1.6^{ab}	33.9±1.6
T3	152.6 ± 1.9^{abc}	31.0±1.1
Group D1 at T0	81.5±1.0	32.1±1.5
T1	185.4 ± 1.9^{aA}	30.1 ± 1.2^{A}
T2	100.8 ± 1.4^{abA}	15.4 ± 1.1^{abA}
T3	$130.\ 9\pm1.5^{abcA}$	5.9 ± 1.3^{abcA}
Group D2 at T0	83.1±1.7	31.9±1.7
T1	180.4 ± 1.5^{aA}	29.1±1.5
T2	91.5 ± 1.7^{abAB}	20.1 ± 1.8^{abAB}
T3	110.6 ± 1.9^{abcAB}	10.4 ± 1.9^{abcAB}

Note: The Qs/Qt is significantly lower in group D1 and D2 than that in group C at T2 and T3 ($P<0.05$), while the PaO_2 content is notably raised ($P<0.05$), and such decrease and increase are significantly larger in group D1 than those in D2 group, and they are obviously greater at T3 than those at T2 ($P<0.05$). Intra-group comparison: $^{a}P<0.05$ *vs.* T0, $^{b}P<0.05$ *vs.* T1, and $^{c}P<0.05$ *vs.* T2. Inter-group comparison: $^{A}P<0.05$ *vs.* group C, and $^{B}P<0.05$ *vs.* group D1.

Table VII Lung function indexes detected.

Group	Cdyn (mL/cm H_2O)	Pplat (cm H_2O)	Ppeak (cm H_2O)
Group C at T0	25.3±2.2	34.5±2.8	30.5±2.2
T1	26.3±2.7	33.4±2.4	29.4±2.8
T2	27.8±2.5	30.1±2.9	28.4±2.4
T3	28.1±2.9	29.8±2.0	27.6±2.5
Group D1 at T0	26.3±2.0	35.7±2.0	31.4±1.2
T1	28.4±2.8	30.4±2.7	30.1±1.8
T2	40.8 ± 2.9^{abA}	18.4 ± 2.9^{abA}	19.4 ± 1.3^{abA}
T3	52.7 ± 2.3^{abcA}	8.4 ± 2.3^{abcA}	9.1 ± 1.3^{abcA}
Group D2 at T0	25.9±2.1	35.1±2.4	31.8±2.0
T1	26.9±2.7	30.4±2.6	30.5±1.0
T2	34.8 ± 2.1^{abAB}	24.6 ± 2.8^{abAB}	25.8 ± 1.6^{abAB}
T3	44.8 ± 2.4^{abcAB}	16.7 ± 2.0^{abcAB}	17.6 ± 1.9^{abcAB}

Note: At T0 and T1, there are no significant differences in the Cdyn, Pplat and Ppeak among three groups. At T2 and T3, the Cdyn is significantly elevated, while the Pplat and Ppeak overtly decline ($P<0.05$), and group D1 has better effects in comparison with group D2, and the effects are obviously superior at T3 to those at T2 ($P<0.05$). Intra-group comparison: $^{a}P<0.05$ *vs.* T0, $^{b}P<0.05$ *vs.* T1, and $^{c}P<0.05$ *vs.* T2. Inter-group comparison: $^{A}P<0.05$ *vs.* group C, and $^{B}P<0.05$ *vs.* group D1.

Discussion

Hypoxemia is caused bright-to-left shunt and uneven distribution of alveolar ventilation and pulmonary perfusion in lungs, and the high ventilation/perfusion area interferes in the effective clearance of CO_2, which may result in hypercapnia (15). OLV will aggravate intrapulmonary shunt and dead space, while gravity and HPV confer protective effects (16). Besides, treatment strategies for OLV-induced hypoxemia, such as positive end-expiratory pressure ventilation and recruitment of alveoli, are not very effective in inhibiting the progression of the disease. In addition to the β-adrenergic receptors in bronchial smooth muscle, there are α1- and α2-adrenergic receptors expressed in the bronchial mucosa and ganglia (17). The bronchodilators currently used target the β-adrenergic receptors in the bronchial wall, and the effects of the bronchodilators targeting the α-adrenergic receptors have not been verified. Dexmedetomidine, a selective α-adrenergic receptor agonist, is reported to effectively repress histamine-induced bronchoconstriction and reduce the intrapulmonary shunt in healthy patients during OLV in an animal study (18). What's more, it is known that dexmedetomidine is capable of directly lowering pulmonary artery pressure, and will not increase pulmonary artery pressure in patients with pulmonary hypertension (19). Vickovic et al. (20) found that magnesium sulfate as an adjuvant to anesthesia in patients with arterial hypertension reduces hemodynamic changes during anesthesia. It was found in this study that there were no evident differences in the MAP, HR and SpO_2 among three groups at each observation time point, indicating that there is no influence on hemodynamics during OLV. ROS plays an important role in various tissue damage like liver damage. Reactive oxygen radicals have been associated with many diseases including autoimmune diseases like rheumatoid arthritis, diabetes mellitus, atherosclerosis, obesity, hypertension and cardiovascular diseases such as ischemia (21, 22). It can also trigger a cascade of cell damage and necrosis/apoptosis and subsequent pro-inflammatory response, further facilitating the progression of diseases (23). In this study, it was discovered that the serum IL-6, TNF-α and IL-8 levels were markedly down-regulated in group D1 and D2 compared with those in group C at T2 and T3, and the decreases were overtly larger in group D1 than those in group D2, and they were evidently greater at T3 than those at T2. Besides, the serum ROS and MDA levels were clearly reduced, while the SOD content obviously rose in group D1 and D2 at T2 and T3 compared with those in group C. Additionally, the effects were markedly better in group D1 than those in group D2, and they were significantly superior at T3 to those at T2.

In abdominal surgeries, anesthesia management based on brain saturation monitoring is able to shorten hospital stays and reduce cognitive dysfunc-

tion. In abdominal surgeries for the elderly, decreased cerebral blood oxygen level is almost always correlated with massive or continued hemorrhage and significantly down-regulated hemoglobin level (24). The results of this study manifested that the $S_{ct}O_2$ was evidently lowered in group D1 and D2 at T2 and T3 compared with that at T0, and the decrease in group D1 was distinctly smaller than that in group D2. PaO_2 triggers the contraction of capillaries by inhibiting the nitric oxide and cyclooxygenase pathways. Anesthetics and techniques may affect shunt by altering cardiac output, pulmonary vascular tone and modification of HPV. Furthermore, hemodynamic parameters and anesthesia needs are measured and evaluated as secondary outcomes, which may have effects on shunt (25, 26). Elhakim et al. (27) studied the effect of infusion of dexmedetomidine and found that dexmedetomidine reduces the shunt and improves oxygenation, and patients receiving epidural anesthesia with dexmedetomidine have lowered bispectral index values, intraoperative awareness and need for analgesia. It was found in this study that at T2 and T3, the Qs/Qt was overtly lowered, while the PaO_2 content was significantly elevated in group D1 and D2 compared with those in group C, and the effects were markedly better in group D1 than those in group D2, and they were significantly superior at T3 to those at T2. Moreover, an animal study revealed that dexmedetomidine increases pulmonary artery pressure and pulmonary vascular resistance *via* direct effects of its receptors on the vascular smooth muscle. Similar changes are also observed in healthy volunteers when the plasma concentration of dexmedetomidine infused reaches 1.9 ng/mL (27). In this study, it was revealed that no significant differences were detected in the Cdyn, Pplat and Ppeak among three groups at T0 and T1. At T2 and T3, the Cdyn was notably raised, while the Pplat and Ppeak were overtly reduced, and group D1 had overtly better effects in comparison with group D2, and the effects were obviously superior at T3 to those at T2. The results of this study are similar to the findings of above studies.

Conclusions

According to our results, the present study demonstrated that dexmedetomidine is able to effectively mitigate inflammatory response and oxidative stress, lower oxygenation and Qs/Qt and improve SctO2 and lung function during OLV with good effects. Our study found that patients undergoing open pulmonary lobectomy and observing hemodynamic parameters, inflammatory factors, oxidative stress indicators, and changes in $S_{ct}O_2$, PaO_2 and Qs/Qt as well as lung function at different time points, provides theoretical and experimental bases for the popularization and application of dexmedetomidine. Although our study provided a good experimental basis for the research and development of adrenergic receptor drugs, further studies in dexmedetomidine patients are still required for indications of antioxidative therapy during anaesthesia.

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Conflict of interest statement

The authors reported no conflict of interest regarding the publication of this article.

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INDIRECT ESTIMATION OF REFERENCE INTERVALS FOR THYROID PARAMETERS USING ADVIA CENTAUR XP ANALYZER

INDIREKTNA PROCJENA REFERENTNIH INTERVALA ZA PARAMETRE ŠTITNE ŽLIJEZDE UPOTREBOM ADVIA CENTAUR XP ANALIZATORA

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Summary

Background: The aim of this study was to determine the reference intervals (RIs) for thyroid stimulating hormone (TSH), free thyroxine (FT4), free triiodothyronine (FT3) and FT3/FT4 ratio using indirect methods.
Methods: We analyzed 1256 results TSH, FT4 and FT3 collected from a laboratory information system between 2017 and 2021. All measurements were performed on a Siemens ADVIA Centaur XP analyzer using the chemiluminescent immunoassay. We calculated the values of the 2.5th and 97.5th percentiles as recommended by the IFCC (CLSI C28-A3).
Results: The RIs derived for TSH, FT4, FT3 and FT3/FT4 ratio were 0.34–4.10 mIU/L, 11.3–20.6 pmol/L, 3.5–6.32 pmol/L and 0.21–0.47, respectively. We found a significant difference between calculated RIs for the TSH and FT4 and those recommended by the manufacturer. Also, FT3 values were significantly higher in the group younger than 30 years relative to the fourth decade (5.26 *vs.* 5.02, p=0.005), the fifth decade (5.26 *vs.* 4.94, p=0.001), the sixth decade (5.26 *vs.* 4.87, p<0.001), the seventh decade (5.26 *vs.* 4.79, p<0.001) and the group older than 70 years old (5.26 *vs.* 4.55, p<0.001). Likewise, we found for TSH values and FT3/FT4 ratio a significant difference (p <0.001) between different age groups.
Conclusions: The establishing RIs for the population of the Republic of Srpska were significantly differed from the rec-

Kratak sadržaj

Uvod: Cilj ove studije bio je da se odrede referentni intervali (RI) tireotropnog hormona (TSH), slobodnog tiroksina (FT4), slobodnog trijodotironina (FT3) i odnosa FT3/FT4 indirektnom metodom procene referentnih intervala.
Metode: Analizirali smo 1256 dobijenih vrednosti TSH, FT4 i FT3 u periodu između 2017. i 2021. godine. Rezultate smo uzeli iz laboratorijskog informacionog sistema. Sva merenja su izvedena na Siemens ADVIA Centaur XP analizatoru pomoću hemiluminiscentnih imunohemijskih testova. Izračunali smo vrednosti 2,5-og i 97,5-og percentila prema preporuci IFCC-a (CLSI C28-A3).
Rezultati: Procenjeni RI za TSH, FT4, FT3 i odnos FT3/FT4 bili su 0,34–4,10 mIU/L; 11,3–20,6 pmol/L; 3,5–6,32 pmol/L i 0,21–0,47. Utvrdili smo značajnu razliku između izračunatih RI za TSH i FT4 i onih koje preporučuje proizvođač. Takođe, vrednosti FT3 bile su značajno veće u grupi mlađoj od 30 godina u odnosu na četvrtu deceniju (5,26 *vs.* 5,02; p = 0,005), petu deceniju (5,26 *vs.* 4,94; p = 0,001), šestu deceniju (5,26 *vs.* 4,87; p<0,001), sedmu deceniju (5,26 *vs.* 4,79; p<0,001) i grupu stariju od 70 godina (5,26 *vs.* 4,55; p<0,001). Isto tako, za vrednosti TSH i odnos FT3/FT4 pronašli smo značajnu razliku (p <0,001) između različitih dobnih grupa.

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ommended RIs by the manufacturer for TSH and FT4. Our results encourage other laboratories to develop their own RIs for thyroid parameters by applying CLSI recommendations.

Keywords: reference intervals, indirect methods, thyroid parameters

Zaključak: Procenjene vrednosti referentnih intervala za TSH i FT4 za stanovništvo Republike Srpske značajno su se razlikovale od preporučenih RI od strane proizvođača. Naši rezultati podstiču druge laboratorije da izrade sopstvene RI za parametre štitne žlezde primenom CLSI preporuka.

Ključne reči: referentni intervali, indirektne metode, parametri štitne žlezde

Introduction

To make an appropriate diagnosis of thyroid disease and for more cost-effective monitoring of patients with altered thyroid function, it is necessary to ensure a quality and accurate laboratory analysis of thyroid function parameters: thyroid stimulating hormone (TSH), free thyroxine (FT4) and free triiodothyronine (FT3). TSH is the most sensitive marker for diagnosing subclinical functional thyroid disease. It is determined by the third generation methods with a sensitivity of 0.01 mIU/L (1). However, standardization and harmonization of methods are still problematic and can lead to significant practical problems and have clinical consequences in the interpretation of laboratory findings (2–5). Furthermore, it has been shown that TSH reference intervals (RIs) should be redefined in different countries due to variability in regional iodine intake as well as used analytical methods (2).

For these reasons, it is necessary to make reference values for one's own population and not to use external sources, *i.e.*, values proposed by the manufacturer. Using accurate RIs (median with 2.5th or 97.5th percentile) is imperative for laboratory professionals because comparing individual results with RIs is crucial for medical decisions. The validity of RI for serum TSH primarily affects hypothyroidism's diagnostic accuracy.

The direct method for a RI calculation is a chiefly recommended technique (6). An alternative approach is the indirect method based on routinely collected patient samples used for diagnostic or monitoring purposes (7, 8).

Understanding the effects of within and between individual variability, analytical and preanalytical variability (3, 9), disease pathophysiology, and diagnosing the disease is crucial for both methods (10). However, using the indirect approach in establishing RIs from patients' results is the simplest way to collect data and is significantly cheaper. Numerous studies explain the benefit of establishing indirect RLs for TSH, FT4 and FT3 from large databases stored in laboratory information systems (11–14). Also, RIs should be obtained in subjects whose thyroid dysfunction was ruled out based on biochemical filtration. For the establishment RI for TSH, TSH results should be excluded if FT3 and FT4 are outside the RI proposed by the manufacturer. This way of collecting data for RIs makes the reference population more similar to patients, including identical preanalytical conditions (15). Laboratories are encouraged to use indirect methods to estimate RIs according to well-defined and recommended criteria by the International Clinical Federation Commission on Chemistry (IFCC) (CLSI C28-A3) (6, 8). In *Figure 1* we have presented the proposed criteria used in the indirect determination of RIs for thyroid parameters.

The goal of our study was to use indirect methods to estimate RIs for TSH, FT4, FT3 and FT3/FT4 ratio from results of the patients obtained during routine laboratory work. The investigation is conducted on the Republic of Srpska population.

Materials and Methods

In this study, we analyzed the results of thyroid parameters (TSH, FT4, FT3) which have been collected from the laboratory information system (LIS) of the University Clinical Centre of the Republic of Srpska, Banja Luka. The measurements were performed on an ADVIA Centaur XP analyzer (Siemens Healthineers USA, United States) using the chemiluminescent immunoassay (CLIA). The collection period for the analyzed thyroid parameters was from October 1, 2017, to April 1, 2021.

The 1328 participants were enrolled in this study, older than 18 years, with predominantly female subjects (84%). The blood samples from outpatients were taken during the morning, between 7:00 and 11:00 a.m., at fasting. We excluded patients with positive antithyroid-peroxidase antibodies (>60 IU/mL) and antithyroglobulin antibodies (>4,1 IU/mL). Only the first result of each patient was included.

We evaluated patients' values within the RIs recommended by the manufacturer. Thus, when we estimate RIs for TSH, the FT4 and FT3 values should be within RIs but TSH values can be within, above or below RIs recommended by the manufacturer.

Quality control was performed using corresponding commercial control samples with low, medium, and high concentrations. The limit of quantitation (LoQ, functional sensitivity) of the ADVIA Centaur TSH3-Ultra assay was 0.008 mIU/L. Intra- and inter-assay coefficients of variation on the three levels of controls were for TSH 1.97%, 1.95%, 2.26% and 4.13%, 4.28%, 3.99%; for FT4 3.33%, 2.23%,

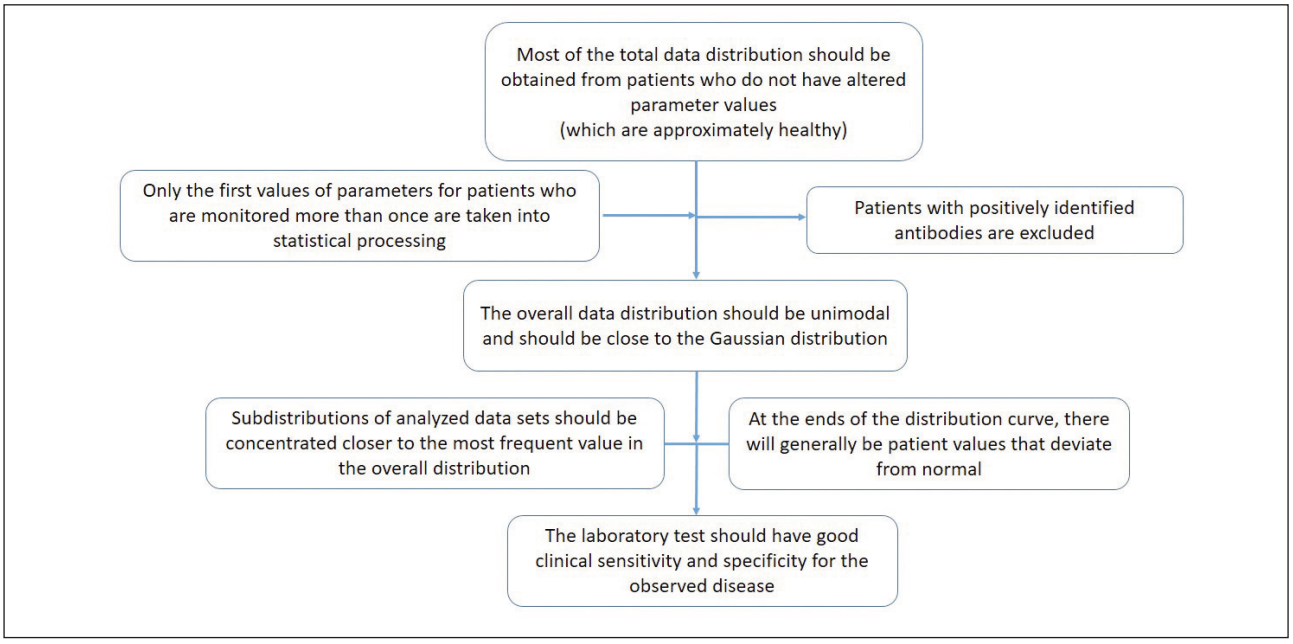

Figure 1 Proposed criteria for indirect method determination of RIs.

2.54% and 2.50%, 4.00% , 2.33 %; for FT3 3.08 %, 2.35 %, 2.47 % and 4.05%, 2.87%, 2.76 %, respectively.

The estimated parameters were included in the external quality assessment scheme (Riqas, Randox). The RIs for TSH, FT4 and FT3 provided by the manufacturer were 0.55–4.78 mIU/L, 11.5–22.7 pmol/L and 3.5–6.5 pmol/L, respectively.

Statistical analysis

Reference limits (RLs) were determined using statistical programmes MedCalc, version 12.1.4.0 (MedCalc Software, Belgium) and SPSS version 24.0 (SPSS Inc, USA). D'Agostino-Pearson test for normal distribution was used to test the distribution of the analyzed parameters. Suspected outliers were identified and omitted using the Tukey method (16, 17). To estimate the indirect reference limits (RLs) for all the analyzed thyroid parameters non-parametric percentile method was used. Lower and upper limits, as 2.5^{th} and 97.5^{th} percentiles, were presented with 90% confidence interval (CI) for each limit. Considering Fraser's theory of »allowable bias« in laboratory tests, we tested whether the indirectly estimated RI significantly differs from the RI recommended by the manufacturer (18, 19). We used a procedure proposed by Ozarda et al. (19) to normalize the RL differences. Firstly, we calculated the critical value for the upper RL differences (UL) and lower RL differences (LL). The numerator was equivalent to the upper limit (UL) ratio or lower limit (LL) ratio computed as a ratio of absolute differences in average UL (or LL) between indirectly estimated RLs and recommended RLs. The denominator corresponds to the standard deviation in calculating the RI, estimated as the average difference between UL and LL recommended by the manufacturer. To assess whether the calculated RIs differ from the recommended ones, we used the criterion of optimal analytical specification or desirable bias limit in laboratory tests as one-eighth (0.125) of the denominator. The RLs were considered divergent when the ratio exceeded the »optimal limits« for analytic bias (>0.125).

Additionally, thyroid parameters were analyzed according to decades of life and presented as box plots. To reveal the significance of differences between the subgroups relative to decades of life, the ANOVA test with a post-hoc Tukey test was performed.

Results

In the *Figure 2* we present the distribution of the analyzed thyroid parameters. All thyroid parameters show a skewed distribution with a long tail toward higher values. For further analyses, all values were log-transformed and we used Tukey's method for detecting outliers. After removing the outliers, the indirect reference values were determined in 1256 from 1328 data.

Calculated RLs for FT3/FT4 ratio were: 0.21 (0.20–0.22) for 2.5th percentile (90% CI) and 0.47 (0.46–0.48) for 97.5th percentile (90% CI), with median value (90% CI) of 0.33 (0.325–0.335). Reference interval width for indirectly calculated *vs.* recommended reference limits was 3.76 *vs.* 4.23,

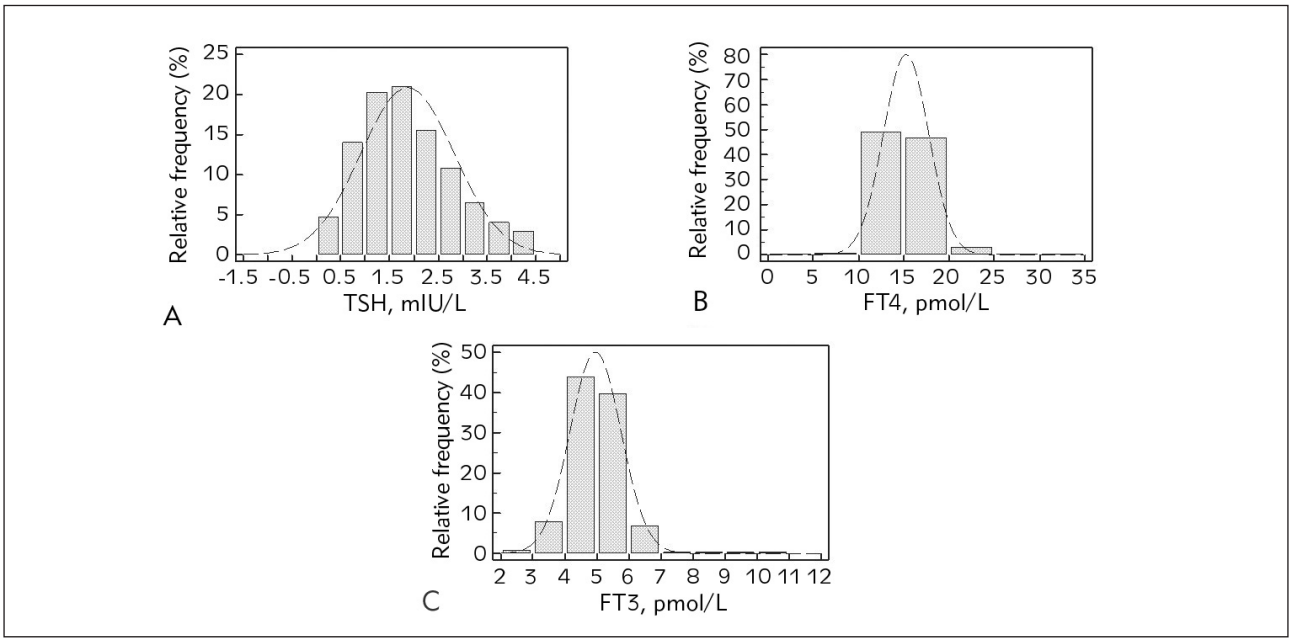

Figure 2 Distribution of the analyzed thyroid parameters: thyroid stimulating hormone, TSH; free thyroxine, FT4; free triiodothyronine FT3.

Table I Indirect estimation of RLs for the overall analyzed thyroid parameters determined on ADVIA Centaur XP Siemens immunochemistry analyzer.

Analyzed thyroid parameters	2.5th percentile (90% CI)	50th percentile (90% CI)	97.5th percentile (90% CI)	Siemens manufacturer's reference limits
TSH, mIU/L	0.34 (0.27–0.39)	1.73 (1.18–2.48)	4.10 (3.96–4.19)	0.55–4.78
FT4, pmol/L	11.3 (11.0–11.5)	15.01 (13.6–16.5)	20.6 (20.1–21.0)	11.5–22.7
FT3, pmol/L	3.5 (3.3–3.6)	4.9 (4.5–5.4)	6.4 (6.3–6.6)	3.5–6.5

Presents median, lower, and upper limits for all three analyzed thyroid parameters with corresponding 90 % CI.

Table II Comparison of the RLs calculated by indirect method with manufacturer recommended RLs.

		TSH, mIU/L	FT4, pmol/L	FT3, pmol/L
Nominator	| LLi – LLr |	0.21	0.2	0
	| ULi – ULr |	0.68	2.1	0.1
Denominator	ULr – LLr	4.23	11.2	3
RL differences	LL	0.049	0.018	0
	UL	0.161	0.188	0.033

RL, reference limit; LLi, lower reference limit calculated using the indirect method; LLr, lower reference limit recommended by the manufacturer; ULi, upper reference limit calculated using the indirect method; ULr, upper reference limit recommended by the manufacturer; ΔLL, critical lower limit ratio; ΔUL, critical upper limit ratio.

9.3 *vs*. 11.2, and 2.9 *vs*. 3.0, for TSH, FT4 and FT3, respectively. Further, we calculated critical values for UL and LL. Results were presented in *Table II*. We found that there was a difference between the calculated and recommended ULs for TSH and FT4.

In the next step, we analyzed parameters according to age groups (*Figure 3*). We have stratified groups as follows: younger than 30 years old (N=222), the fourth decade of life from 31 to 40 years old (N=320), the fifth decade of life from 41 to

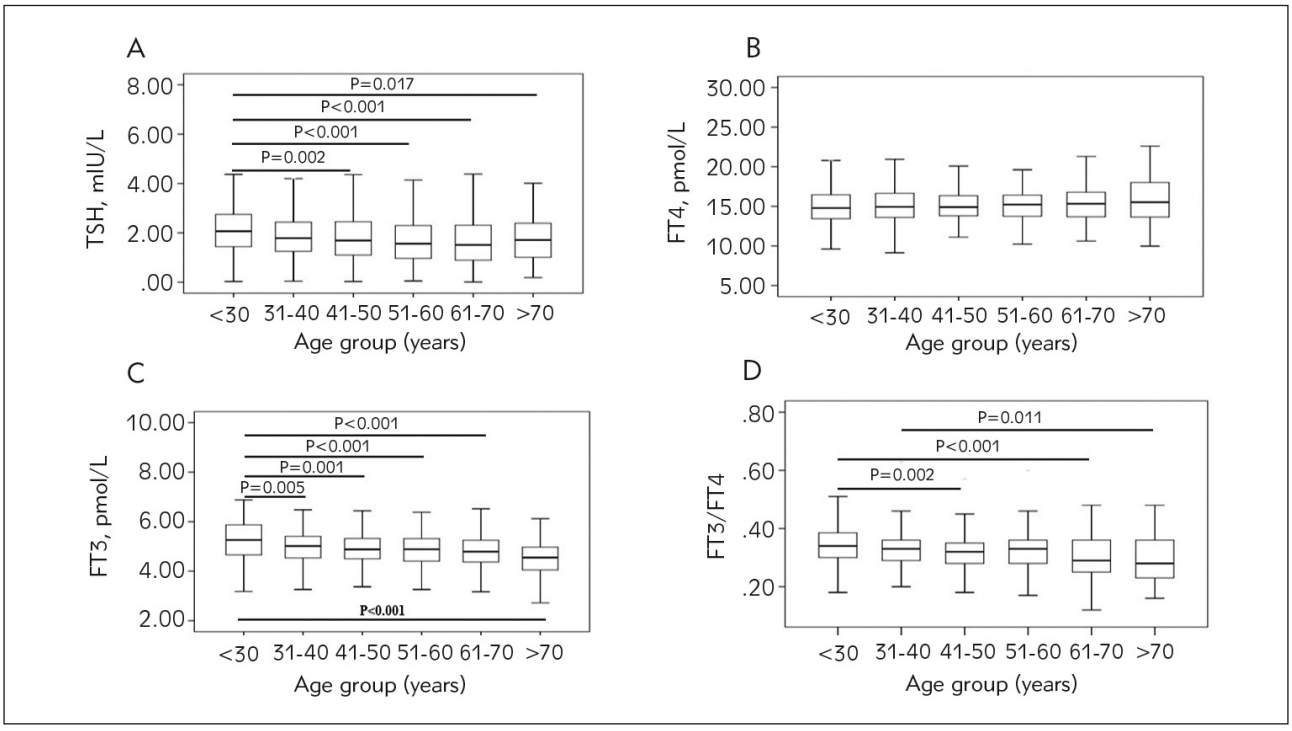

Figure 3 Median and interquartile range for the overall analyzed TSH, FT4, FT3 and FT3/FT4 values relative to decades of life.

50 years old (N=301), the sixth decade of life from 51 to 60 (N=164), the seventh decade of life from 61 to 70 (N=167) and older than 70 years old (N=82).

Differences in the reference values for the analyzed thyroid parameters relative to the decades of life were estimated using the Tukey HSD post hoc test, as set in one way analysis of variance (ANOVA). First, we found an overall significance value for the difference between groups for TSH (F (5.1251) = 6.147, $p<0.001$), FT3 (F (5.1251) =12.015, $p<0.001$) and FT3/FT4 ratio (F (5,1251) = 5.276, $p<0.001$). A Tukey post hoc test revealed that the TSH values were statistically significantly higher in the group younger than 30 years relative to the fifth decade (2.06 *vs.* 1.69, $p=0.002$), the sixth decade (2.06 *vs.* 1.56, $p<0.001$), the seventh decade (2.06 *vs.* 1.51, $p<0.001$) and the group older than 70 years old (2.06 *vs.* 1.71, $p=0.017$). Additionally, FT3 values were statistically significantly higher in the group younger than 30 years relative to the fourth decade (5.26 *vs.* 5.02, $p=0.005$), the fifth decade (5.26 *vs.* 4.94, $p=0.001$), the sixth decade (5.26 *vs.* 4.87, $p<0.001$), the seventh decade (5.26 *vs.* 4.79, $p<0.001$) and the group older than 70 years old (5.26 *vs.* 4.55, $p<0.001$). FT3/FT4 index was statistically significantly higher in the group younger than 30 relative to the fifth decade (0.34 *vs* 0.32, $p=0.002$), to the seventh decade (0.34 *vs* 0.29, $p<0.001$) and relative to the group older than the 70 years old (0.34 *vs.* 0.30, $p=0.011$).

Discussion

In this study, we established reference values for TSH, FT4 and FT3 in the population of the Republic of Srpska by indirect method *i.e.* using data stored in our information system. There was a statistically significant difference between calculated RIs for the TSH and FT4 and those recommended by the manufacturer. This indicates that it is necessary to define laboratory and method specific RLs for these thyroid parameters. The RIs for TSH in this study was apparently lower (0.34–4.1 mIU/L) than by the manufacturer (0.55–4.78 mIU/L). The RIs for TSH obtained on different populations, but the same analyzer (Siemens analyzer) show differences in the lower and upper limit of RIs in the ranges from 0.32 to 1.01 mIU/L and 3.00 to 5.51 mIU/L, respectively (20-24). Therefore, our results are between these values but do not match them, which also favors establishing ours RIs. Also, this study's results agree with the general opinion that the upper TSH reference limits for outpatients should be below 4.5 mIU/L (25). Nevertheless, laboratory guidelines show that more than 95% of healthy people have TSH below 2.5 mIU/L (26) which has not been confirmed in our study (*Figure 2*). Today, it seems to have the most published data on RIs on the Roche platform although it is necessary to publish RIs as many as possible for other platforms as well. The published data provide security to laboratory professionals in their daily, routine work. Our previous study showed that the TSH values obtained on Roche and Siemens ana-

lyzers well agree (the slope for the correlation of Roche and Siemens was 1.11 using the Passing–Bablok regression method) (2). Also, in a similar study, we determined the TSH, FT4 and FT3 RIs for our population on a Roche analyzer (27). We have noticed significant differences for TSH in the lower and upper limits (0.34 *vs.* 0.65 mIU/L and 4.1 *vs.* 5.39 mIU/L). This can be explained by the possible influence of environmental factors over the years, primarily the effect of iodine status. Research showed that in 2006 in the Republic of Srpska (28), there was not enough iodine in the diet, what could lead to such a high upper limit of TSH. The last study revealed a significantly lower value, indicating a significant improvement of the iodine status (unfortunately, there is no recent data on iodine in the diet in the Republic of Srpska). Also, the reason for this could be different methods for assessing RIs, the number of samples in the studies and the smaller number of men in the indirect method. The absence of a decline in serum FT4 values in our study further contributes to the evidence that there is adequate iodine intake in our population.

In addition, our results are more in line with the RIs population of the Republic of Serbia for TSH (0.35–4.10 *vs.* 0.42–3.67 mIU/L), if indirect method was used for determination of reference values (12).

According to ages, the shown changes for TSH are not clinically useful, which is in line with the results of other studies (29, 30). Reasons for these changes may be due to physiological variables (e.g., menstrual cycle phase), individual variables, variables present in some non-thyroid diseases, iatrogenic factors such as thyroid and non-thyroid drugs, phlebotomy time, etc.

Surprisingly, both of our studies reported almost the same upper limit for FT4 and FT3 RIs (20.6 *vs.* 20.18 and 6.4 *vs.* 6.33 pmol/L), which to encourage us the future use of RIs obtained by indirect methods.

The best compliance of our RIs with the proposed values by the manufacturer was for FT3 which is, ultimately, crucial for a complete assessment of the success of the therapy. In addition, this cross-sectional study indicates that FT3 values change with ageing. Therefore, the existence of an age-related decrease in the circulating FT3 levels might represent a physiological mechanism already shown in some studies (31, 32).

The IFCC has so far made great efforts to standardize measurement for thyroid function tests, particularly for TSH, taking into account the different platforms used to measure these parameters. However uniform reference values for thyroid parameters have not yet been achieved. Therefore, routine clinical laboratories are advised to determine their own RIs following accepted consensus standards, such as those of the IFCC, National Academy of Clinical Biochemistry and CLSI (33, 34).

Additionally, we have examined RLs for FT3/FT4 ratio as useful parameter to detect thyroid disfunction (35–38). Some authors have pointed out that this ratio is positively correlated with TSH within the reference range of thyroid function in adults (36). Our result of median value of FT3/FT4 ratio was in agreement with the parameter values examined by Chen and associates (35). To our knowledge our study is the first that examined changes in the FT3/FT4 ratio by decades of age. Our results were also in agreement with Strich et al. (39) investigation. The authors have confirmed that TSH enhancement of FT4 to FT3 conversion is age dependent. These results indicate the importance of determining and monitoring free hormones ratio as an additional parameter that can help clinicians in assessing thyroid function. Also, more studies indicate a significant relationship between FT3/FT4 ratio and other diseases (39, 40). The FT3 / FT4 ratio would be useful in everyday practice.

This study has some limitations, primarily the small number of male respondents in the research and no recent data on sufficient iodine.

Conclusion

The establishing and using your own thyroid hormone RIs provides a much better basis for diagnosing or considering treatment for thyroid dysfunction than using a manufacturer interval. The our study indicates the need for greater use of the FT3 / FT4 ratio in routine work. In addition, these results should encourage more laboratories to apply CLSI recommendations in determining RIs for thyroid parameters, for their specific populations.

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Conflict of interest statement

The authors reported no conflict of interest regarding the publication of this article.

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News from the Society of Medical Biochemists of Serbia

16th NTK – ACCREDITED LABORATORIES CONFERENCE-SOCIETY OF MEDICAL BIOCHEMISTS OF SERBIA

INTERNATIONAL CONVENTIONS ON QUALITY IN THE ORGANIZATION OF THE UNITED ASSOCIATION OF SERBIA FOR QUALITY (UASQ), BELGRADE, SERBIA

Prepared by the Assistant Professor Dr Neda Milinković,

Department of Medical Biochemistry, Pharmaceutical Faculty, Belgrade University Belgrade, Serbia

Under the auspices of Society of Medical Biochemists of Serbia (SMBS), the 16th NTK – Accredited Laboratories Conference was held on September 08, 2021. This Conference was traditionally held within International Conventions on Quality in the organization of the United Association of Serbia for Quality (UASQ), Belgrade, Serbia. Lectures within the Conference were accredited by the National Accreditation Body of Serbia, held via the online platform Cisco webex, and lasted 6-hours. The Conference was chaired by Prof Nada Majkić-Singh (SMBS), Belgrade, Prof Zorica umarac, Center for Medical Biochemistry, Clinical Center of Serbia, Belgrade and Assistant Professor, Neda Milinković, Department of Medical Biochemistry, University of Belgrade - Faculty of Pharmacy, Belgrade, Serbia.

The 16th NTK-Accredited Laboratory Conference was dedicated to innovations and trends in the implementation of overall quality management in routine medical biochemical laboratories, which contributes to the development of safe laboratory diagnostics. The first lecture was given by Tamara Gojković, Teaching Assistant at the Department of Medical Biochemistry, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia. Topic of this presentation was »Validation of chromatographic methods in biochemical laboratories«. This lecture was dedicated to the process of validation of specific chromatographic techniques in the bio analytical laboratory. The challenges in quality control of individual phases of chromatographic techniques were discussed. The complexity of the criteria and guidelines that are an integral part of the validation protocol of pre-analytical and analytical procedures of chromatographic technique was one of the topic. The second lecture was given by Miron Sopić, Assistant Professor at the Department of Medical Biochemistry, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia. The topic of this lecture was »Pre-analytical and analytical quality control qPCR«. It was dedicated to the explanation of quality control management in the preanalytical and analytical phase of specific molecular diagnostic tests, with reference to the qPCR technique. It was discussed of the importance of the qPCR method for a wide range of testing and diagnostic areas. The review was also on the current guidelines and requirements of the ISO 20395:2019 standard, which must be followed in order to obtain reliable results. The third lecture was given by Dr Vera Lukić, from the Institute for Health Protection of Serbian Railway Workers, Belgrade, Serbia with the topic »Hemolysis index: current recommendations and concerns«. It was dedicated to the explanation of the most common causes of laboratory errors that occur in the pre-analytical phase of laboratory testing. Participants were acquainted with the requirements of ISO 15189:2014 standards related to quality control, especially in the pre-analytical phase of laboratory testing, as well as how to objectively and reliably could detect possible interferences from the quality of the sample composition being analyzed. Automatic measurement of hemolysis index in routine conditions was presented, what were advantages and possible challenges. The fourth lecture was presented by Neda Milinković, Assistant Professor at the Department of Medical Biochemistry, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia. The topic of this lecturer was »Bias in medical biochemistry – what laboratory staff should know?«. This lecture was dedicated to explaining the correct assessment of bias, what are the possible principles of determination and practical application in routine medical laboratories. It was explained what available data the laboratory staff can use from the test manufacturer. The practical application of bias in relation

to the interpretation of laboratory results was discussed. The importance of knowing bias regarding the challenges in standardization and harmonization of routine measurement methods was also topic of this Conference.

The participants of this conference are extremely satisfied with the content of the lectures and presentations, which was confirmed by the average grade of 5.0, which was given to the all lecturers and presentations.

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STATISTICAL GUIDELINES

These guidelines are designed to help authors prepare statistical data for publication and are not a substitute for the detailed guidance required to design a study or perform a statistical analysis. Each section of a scientific paper is addressed separately.

Summary

The number and source of data must be stated and conclusions which have a statistical basis must be substantiated by inclusion of pertinent descriptive statistics [mean or median, standard deviation (SD) or interquartile range, percentage coefficient of variation (%CV), 95% confidence limits, regression equations, etc.].

Methods

Experimental design, subject selection and randomization procedures should be described and analytical precision quoted when appropriate. The hypotheses to be tested by a statistical procedure must be stated and where appropriate power calculations for the sample size used should be given (it is recommended that the power is <80%). In case-control studies, clearly define how cases and controls were selected and what matching has taken place.

Statistical tests should be described but need not be referenced unless they are unusual or are applied in a non-standard way. Computer software used should be referenced.

If the paper is reporting the results of a diagnostic trial read the STARD statement (1) and for a clinical trial read the CONSORT statement (2) to improve the quality of your report.

Results

Unnecessary precision, particularly in tables, should be avoided. Rounded figures are easier to compare and extra decimal places are rarely important. Descriptive statistics require an additional digit to those used for the raw data. Percentages should not be expressed to more than one decimal place and not be used at all for small samples.

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.

References

1. Bossuyt PM, Reitsma JB, Bruns DE, et al., for the STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Ann Clin Biochem 2003; 40: 357–63.
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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