UDK 577.1 : 61

ISSN 1452-8258

JOURNAL OF MEDICAL BIOCHEMISTRY

Published by
The Society of Medical Biochemists of Serbia
Belgrade

J Med Biochem

Vol. 41 · No 1 · 2022

JOURNAL OF MEDICAL BIOCHEMISTRY

available online at
http://www.dmbj.org.rs/jmb
http://scindeks.ceon.rs/journaldetails.aspx?issn=1452-8258

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JOURNAL OF MEDICAL BIOCHEMISTRY
Official Journal of the Society of Medical Biochemists of Serbia

Editorial Office
Society of Medical Biochemists of Serbia
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www.dmbj.org.rs

Publisher of electronic Journal issues

SCINDEKS
Serbian Citation Index

http://www.dmbj.org.rs/jmb
http://scindeks.ceon.rs/journaldetails.aspx?issn=1452-8258

Journal of Medical Biochemistry is published by financial support of Ministry of Education and Science of the Republic of Serbia

JOURNAL OF MEDICAL BIOCHEMISTRY

Publication dates: March, June, September, December

Editorial Office

Society of Medical Biochemists of Serbia,
Vojislava Ilića 94B/7, 11050 Belgrade, Serbia

JOURNAL OF MEDICAL BIOCHEMISTRY

Vol. 41 (2022)

Informacije o časopisu: Časopis izlazi tromesečno. Svako godište sadrži četiri broja. Rukopisi se dostavljaju na adresu uredništva. Autori se izveštavaju o prijemu rukopisa. Svi radovi se recenziraju.

Informacije o pretplati: Pretplata za organizacije i inostranstvo iznosi 400 € plus poštanski troškovi preračunato u dinare. Pretplata se šalje na tekući račun Društva medicinskih biohemičara Srbije:
160-375792-34, Banca Intesa, Beograd (sa naznakom »pretplata za JMB«).

Informacije o oglašavanju: Svi zahtevi koji se odnose na oglašavanje u Journal of Medical Biochemistry dostavljaju se na adresu Uredništva.

Informations for contributors: »Journal of Medical Biochemistry« publishes review articles, original research papers and preliminary communications dealing with clinical chemistry, medical biochemistry and related fields. Journal of Medical Biochemistry is published quarterly. Publication dates are: March, June, September, December. All manuscript should confirm to generally accepted usage in composition of scientific papers. For technical requirements, authors should consult a current issue. Contributions in two doublespaced typewritten copies should be addressed to The Editor, Journal of Medical Biochemistry, Society of Medical Biochemists of Serbia, Vojislava Ilića 94B/7, 11050 Belgrade, Serbia.

Information to subscribers: Subscribers rate per Volume (containing 4 issue) 400 € plus surface postage. Subscription orders and correspondance should be addressed to the Editorial Office, Journal of Medical Biochemistry, Society of Medical Biochemists of Serbia, Vojislava Ilića 94B/7, 11050 Belgrade, Serbia

Grafičko uređenje
Graphic design:

Danijela Mijailović

Štampa
Printed by:

SPRINT

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Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-31435

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 1–13, 2022

Review paper
Revijski rad

THE EFFECT OF RETICULOCYTE HEMOGLOBIN CONTENT ON THE DIAGNOSIS OF IRON DEFICIENCY ANEMIA: A META-ANALYSIS STUDY

EFEKAT SADRŽAJA RETIKULOCITNOG HEMOGLOBINA NA DIJAGNOSTIKOVANJE ANEMIJE USLED NEDOSTATKA GVOŽĐA: META-ANALIZA

Merve Kılıç[1,2], Aysel Özpınar[1], Mustafa Serteser[1], Meltem Kilercik[1], Muhittin Serdar[1]

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Summary

Background: Iron deficiency anemia (IDA) is the most common type of anemia worldwide and has many adverse effects on life quality. This meta-analysis study aims to show that reticulocyte hemoglobin content (CHr) is more effective than routinely used parameters in the diagnosis of IDA.
Methods: Comprehensive and systematic research was done using international databases including PubMed, Web of Science, Cochrane Library, Science Direct, and Google Scholar, which contain all articles published on IDA until December 29, 2020. Seventeen articles were included in the meta-analysis.
Results: The analyses found the Cohen's deffect size (Standardized Mean Difference) values of the parameters. Accordingly, CHr is 2.84 (95% CI 2.36 to 3.31), mean corpus volume (MCV) is 2.46 (95% CI 1.97 to 2.95), ferritin is 2.37 (95% CI 1.63 to 3.11), and transferrin saturation (TSAT) is 3.76 (95% CI 2.14 to 5.38). To diagnose IDA, the sensitivity value of the CHr concentration was found as 83.5% (95% CI 76.1 to 89.8), specificity value to be 91.8% (95% CI 85.5 to 96.4), and mean cut-off value as 28.2 pg.
Conclusions: The results of our study reveal the findings that CHr is a better biomarker than MCV and ferritin used in determining IDA, and its efficacy is lower than TSAT. It is very important to use it routinely for the pre-diagnosis of IDA, which is very important for public health. The groups in the study are heterogeneous but contain bias. Therefore, meta-analyses of studies with less heterogeneity of CHr are needed.

Kratak sadržaj

Uvod: Anemija usled nedostatka gvožđa (IDA) je najčešća vrsta anemije na svetu i ima mnogo negativnih efekata na kvalitet života. Cilj ove meta-analize je da pokaže da je koncentracija retikulocitnog hemoglobina (CHr) efikasnija od rutinski korišćenih parametara u dijagnozi IDA.
Metode: Sprovedeno je sveobuhvatno i sistematično istraživanje gde su upotrebljene međunarodne baze podataka, uključujući PubMed, Web of Science, Cochrane Library, Science Direct i Google Scholar, koje sadrže sve članke objavljene na temu IDA do 29. decembra 2020. Meta-analiza je obuhvatila sedamnaest članaka.
Rezultati: Analizom su utvrđene vrednosti parametara za veličinu efekta Koenovog d (standardizovana srednja razlika). Shodno tome, CHr je 2,84 (95% CI 2,36 do 3,31), srednja zapremina tela (MCV) je 2,46 (95% CI 1,97 do 2,95), feritin je 2,37 (95% CI 1,63 do 3,11), a zasićenje transferinom (TSAT) je 3,76 (95% CI 2,14 do 5,38). Da bi se dijagnostikovala IDA, utvrđena je vrednost osetljivosti koncentracije CHr na 83,5% (95% CI 76,1 do 89,8), vrednost specifičnosti 91,8% (95% CI 85,5 do 96,4), a srednja granična vrednost je 28,2 pg.
Zaključak: Rezultati naše studije ukazuju da je CHr bolji biomarker od MCV i feritina koji se koriste u određivanju IDA, a njegova efikasnost je niža od TSAT. Veoma je važno da se rutinski koristi za preddijagnozu IDA, što je veoma važno za javno zdravlje. Grupe u studiji su bile heterogene, ali imaju elemente pristrasnosti, pa je potrebno sprovesti meta-analize sa manjom heterogenosti CHr.

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Keywords: Reticulocyte Hemoglobin Content, Iron Deficiency Anemia, Transferrin saturation, Mean Corpus Volume, Ferritin, Meta-Analysis

Ključne reči: Sadržaj retikulocitnog hemoglobina, anemija usled nedostatka gvožđa, zasićenje transferina, srednja zapremina tela, feritin, meta-analiza

Introduction

Iron is an element that has essential functions for human life. While it is found in the structure of hemoglobin (Hb), which provides oxygen transport in the body, it also ensures the fulfilment of iron-related functions by joining the structure of enzyme systems in some tissues (1). Iron deficiency anemia (IDA) occurs when the iron intake in humans is less than its excretion, in other words, when a negative iron balance occurs in the body (increased need for iron, absorption disorders, chronic blood loss) as a result of insufficient iron for Hb synthesis in the stores (1, 2). IDA is the most common type of anemia, and it constitutes the most advanced stage of iron deficiency. The World Health Organization (WHO) describes a hemoglobin value of <130.0 g/L in men, <120.0 g/L in women, and <110.0 g/L in pregnant women as anemia (3, 4). IDA is more common in women than men due to conditions such as menstruation and pregnancy. Adolescence causes an increase in blood pressure due to rapid growth and development and insufficient iron stores. If this condition cannot be compensated, IDA may occur as a result of insufficient intake. The most important reasons for IDA in postmenopausal women and men are the formation and increase of gastrointestinal system (GIS) bleeding (5–7). IDA is characterized by hypochromia and microcytosis in erythrocytes, decreased serum ferritin and serum iron levels, TSAT, and increased total iron-binding capacity (4, 8). Low serum ferritin level in IDA is essential and should not always be associated with IDA. Again, because it is an acute phase reactant, its normal condition does not exclude IDA; the underlying etiology must be defined and regulated (9). In contrast to all these conditions, iron overload reduces the efficiency of iron utilization and induces oxidative stress formation (10). In addition to these, free erythrocyte zinc protoporphyrin (ER-ZPP), soluble transferrin receptor (sTfR), and reticulocyte hemoglobin content (CHr or Ret-He) are among the reliable laboratory test parameters used to describe IDA. Soluble transferrin receptor with increasing erythrocyte ER-ZPP value causes early deterioration of iron condition and emergence of IDA (11–14).

Bone marrow erythropoietic activity and intracellular iron requirement are important criteria in determining sTfR level. Therefore, in conditions associated with iron deficiency and induced erythropoiesis (sickle cell anemia, megaloblastic anemia, thalassemia, polycythemia, etc.), sTfR concentration increases, while aplastic anemia decreases (15, 16). Normal serum sTfR level is 3.5–8.5 mg/L. It is known that a high sTfR (>8.5 mg/L) level is an early and sensitive biomarker for the diagnosis of IDA (11).

The ratio of sTfR concentration to logarithmic ferritin level is also determinant in the differential diagnosis of IDA. A ratio of less than 1 is associated with chronic disease anemia, while the ratio higher than 2 is evaluated in favour of IDA (17).

The decrease in iron concentration increases zinc transport in the intestines, and therefore the increased concentration of ER-ZPP (80 μg/dL) in erythrocytes is associated with iron deficiency. However, routine use of ER-ZPP measurements is difficult and time-consuming due to automation difficulties (18).

CHr, also known as Ret-He, measures the amount of hemoglobin in reticulocytes and is an indicator of cell hemoglobination, reflecting the quality of newly produced reticulocytes. Microcytic, hypochromic red blood cell (RBC) is formed due to ongoing reticulocyte production when there is not enough iron. Thus, RET-He reflects an earlier measure of reduced hemoglobin status compared to hemoglobin and hematocrit (11, 19).

Reticulocytes are separated from the erythroblasts after Hb synthesis, pass into the peripheral blood and turn into mature erythrocytes within a few days. Therefore, CHr is the ideal parameter to be considered for real-time Hb synthesis. Reticulocyte hemoglobin content is affected only by the amount of iron unless there are hematopoietic disorders (10).

Determination of iron status is possible with RET-He measurement. RET-He is determined by automated fluorescence flow cytometry, which measures the mean values of the forward light scattering intensity of mature red blood cells and reticulocytes using a polymethine dye. The values obtained reflect the reticulocyte hemoglobin content (20). Reticulocyte hemoglobin content is more effective in diagnosing iron deficiency, determining early iron deficiency anemia, differentiation of beta-thalassemia feature, and more effective than the other parameters involved in iron metabolism (10, 12). CHr is a less variable parameter that performs better than ferritin in response to intravenous (IV) iron therapy, providing better diagnostic accuracy for iron (15, 16).

In the United States and Europe, CHr has been accepted as a marker in iron deficiency with a diagnostic threshold of 29 pg. However, there is no reference value agreed on the best value for its sensitivity and specificity (17).

This study examined the effectiveness of CHr in addition to routine parameters in determining IDA.

Methods

Literature Search Strategy

Comprehensive systematic research was carried out using international databases, including PubMed, Web of Science, Cochrane Library, Science Direct, and Google Scholar, to determine all studies on CHr as a biomarker of IDA.

Our study included all articles published until December 29, 2020. The words used as search criteria in this study are as follows: »Reticulocyte hemoglobin AND iron deficiency anemia OR iron deficiency«, »reticulocyte hemoglobin« OR »iron deficiency« OR »iron deficiency anemia«.

This systematic review and meta-analysis were carried out under the guidelines for Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Selection Criteria

The full texts of the articles related to the study were examined in terms of exclusion and inclusion criteria. Appropriate studies including clinical features of patients diagnosed with IDA and CHr used as a biomarker in the diagnosis of the disease were included in the meta-analysis.

Prospective/retrospective cohort, case-control, and cross-sectional studies were found suitable for inclusion in our study.

Repeated articles, reviews, case reports, expert opinions, letters, editorials, studies on experimental animals, studies not including control groups, studies where CHr is a biomarker but not related to iron deficiency, studies without available data, studies on results other than mean and standard deviation values, articles published in languages other than English, studies involving chronic patient groups with IDA, and studies only on iron deficiency were excluded (*Figure 1*).

Data Extraction and Quality Assessment

Data extraction, evaluation of literature quality, and evaluation of bias risk were carried out independently by two researchers (Serdar M. and Kılıç M.).

The following features were extracted: Article information (first author, year of publication), country, study designs, gender, device information, study example, patient/control information, the total number of samples, researched parameters. This information is shown in *Table I*. Microsoft Excel database was used to save the necessary information.

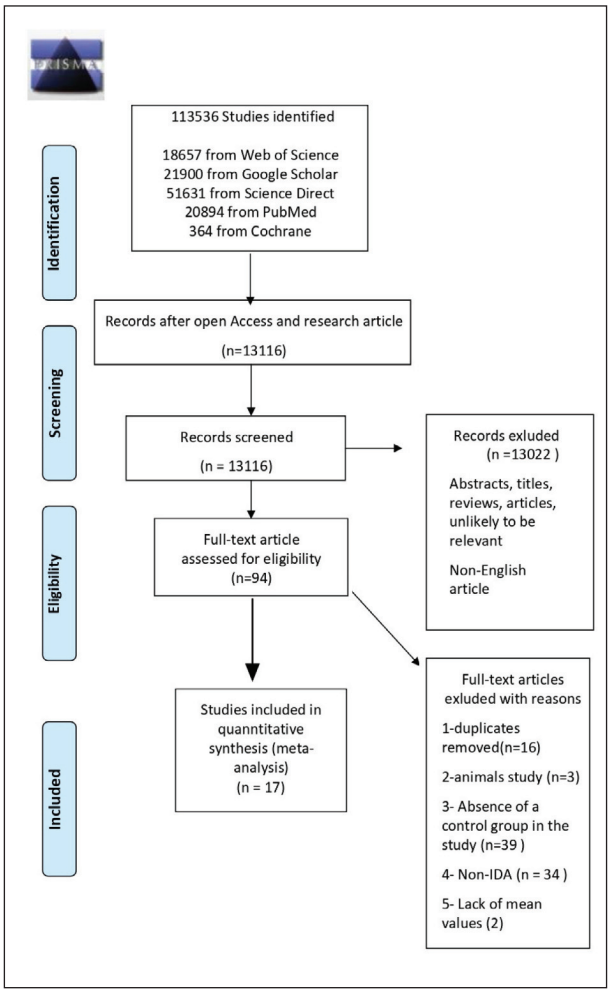

Figure 1 PRISMA flow diagram for inclusion of studies in this meta-analysis.

Statistical Analysis

The meta-analysis study was performed using MedCalc statistical software, version 19.0.7 (MedCalc Software, Ostend, Belgium). A meta-analysis was performed on Cohen's d effect size (Standardized Mean Difference) by taking the mean and standard deviation values. In the evaluation, the results obtained with the random-effects model of the studies with low bias risk were taken into consideration. The study aimed to perform group analysis of CHr, MCV, ferritin, and TSAT biomarkers according to the target range in IDA. Using Cochran's Q statistic and I^2 statistic respectively, statistical heterogeneity was evaluated. If the I^2 statistical value was above 50 per cent and the p-value was lower than 0.05, the heterogeneity was considered statistically significant.

Table I Features of the studies analyzed for iron deficiency anemia, Sysmex XN Aplpha/10/300/1000, XE 2100/5000 (Sysmex Corporation Kobe, Japan), Technicon H3, (Bayer, Germany), Advia 120, ADVIA 2120i (Siemens AG, Erlangen, Germany).

FIRST AUTHOR	YEAR	COUNTRY	DEVICE	STUDY DESIGN	PATIENT GROUP	PATIENT/ CONTROL	TOTAL NUMBER	FEMALE	MALE	RESEARCHED PARAMETERS
Chaipokam et al. (46)	2017	Thailand	Sysmex- XE Alpha	Prospective cohort	Adult-Anemia	Control, IDA, Talasemi, Talasemi trait, AI	267	190	77	MCV, CHr
Rehu et al. (35)	2011	Finland	ADVIA 120 and 2120	Retrospective	Adult-Anemia	Control, IDA, ACD	250	138	112	CHr, MCV
Brugnara et al. (8)	1999	Amerika	Technicon H3		Iron Deficiency Children	Control, IDA, ID	210	90	210	CHr, MCV, Ferritin
Balci et al. (47)	2016	Turkey	Sysmex ADVIA 2120i	Case-control	6–12 Years Old Children	Control, IDA, B_{12} vitamin deficiency, mixed anemia	112	52	60	CHr, MCV, Ferritin
Ceylan et al. (48)	2007	Turkey	ADVIA 120	Case-control	Adult-Anemia	Control, IDA, ID, Talasemi minor, B_{12} vitamin deficiency	131	91	40	CHr, MCV
Cai et al. (49)	2017	China	ADVIA 120	Case-control	Adults	Control, IDA, NIDA	140	100	40	CHr, MCV, Ferritin
LUO et al. (50)	2007	China	ADVIA 120	Case-control	Pre-menopausal Women-Anemia	Control, IDA, NIDA	142	142		CHr, MCV
Dinh et al. (51)	2020	Vietnam	Sysmex ADVIA 2120i	Retrospective	Adult-ESRD	Control, IDA, NIDA, ESRD, IDA-ESRD	312	188	124	CHr, MCV
Ageeli et al. (52)	2013	Saudi Arabia	ADVIA 2120i	Case-control	Adult-Anemia	Control, IDA, ACD, CRF	320	170	150	CHr, MCV, Ferritin, TSAT
Buttarello et al. (53)	2016	Italy	Sysmex XE-5000	Case-control	Adult-Anemia	Control, IDA, ID, NIDA, trait β talasemia	290			CHr, MCV, Ferritin
Uçar et al. (33)	2019	Turkey	Sysmex XN 1000	Case-control	Adult-Anemia	Control, IDA, ID, NIDA	217	171	46	CHr, MCV, Ferritin,TSAT
Toki et al. (54)	2017	Japan	Sysmex XN 300/XE	Case-control	Adult-Anemia	Control, IDA, ID, NIDA	211	148	63	CHr, MCV, Ferritin,TSAT
Vázquez-López et al. (55)	2019	Spain	ADVIA 120	Case-control	1–16 Years Old Children	Contol, IDA, ID	1239	620	619	CHr, MCV, Ferritin,TSAT
Malczewska-Lenczowska et al. (56)	2017	Poland	ADVIA 120	Case-control	Sports women	Control, ID stage I, ID stage II	219	219		CHr, MCV, Ferritin
Chinudomwong et al. (57)	2020	Thailand	Sysmex XN-10	Case-control	Adult-Anemia	Control, IDA, IDA-inflammation, NIDA-AI, NIDA-CKD, Talasemi	938	603	335	CHr, MCV, Ferritin
Urrechaga et al. (58)	2011	Spain	Sysmex XE 5000	Case-control	Anemia and Talasemia	Control, talasemia trait, mild IDA, severe IDA	473			CHr, MCV, Ferritin, TSAT
Rungngu et al. (59)	2016	Indonesia	Sysmex XE-2100	Cross-sectional	6–12 Years Old Children-Anemia	Control, IDA, NIDA	50	17	33	CHr, Ferritin

ACD: Anemia of chronic disease, AI: Inflammation anemia, CHr: Reticulocyte hemoglobin content, CKD:Chronic kidney disease, CRF: Chronic renal failure ESRD: End-stage renal disease, ID: Iron deficiency, IDA: Iron deficiency anemia, MCV:Mean corpuscular volume, NIDA: Non-iron deficiency anemia, TM: β-Talasemia minor, TSAT: Transferrin Saturation

Publication Bias

Two reviewers evaluated independently the risk of bias in each study using the Diagnostic Precision Study Quality Assessment Tool (QUADAS-2) recommended (18). Publication bias is demonstrated by analysis with a funnel plot.

Results

Literature Search and Study Characteristics

Based on the search strategy, 113,536 studies were evaluated among the databases specified. A total of 13,116 studies were included in the study after the records, except for studies with open access and research articles were excluded. Thirteen thou-

Table II Meta-analysis of reticulocyte hemoglobin content (CHr), Mean Corpuscular Volume (MCV), ferritin, Transferrin Saturation (TSAT), sensitivity and specificity of CHr to diagnose iron deficiency anemia articles.

Study	IDA (n)	IDA-CHr Main±SD	Control (n)	Control-CHr Main±SD	Effect Size	95% CI	MCV		FERRITIN		TSAT		SENSITIVITY			SPECIFICITY		
							EffectSize	95% CI	EffectSize	95% CI	Effect Size	95% CI	Sample size	Proportion (%)	95% CI	Sample size	Proportion (%)	95% CI
Chaipokam et al. 2017 (46)	53	21.2±5.5	99	33.1±2.4	-3.137	-3.62 to -2.65	-3.30	-3.80 to -2.80					53	83.0	70.2 to 91.9	99	80.8	71.6 to 88.0
Rehu et al. 2011 (35)	58	26.8±3.8	63	33.2±2	-2.12	-2.57 to -1.67	-1.16	-1.54 to -0.77					58	82.7	70.5 to 91.4	63	90.4	80.4 to 96.4
Brugnara et al. 1999 (8)	24	24.2±2.7	186	26.8±1.8	-1.349	-1.79 to -0.90	-1.08	-1.51 to -0.64	-0.09	-0.52 to 0.33			24	79.1	57.8 to 92.8	186	74.7	67.8 to 80.8
Balci et al. 2016 (47)	26	22.26±1	32	29.9±0.7	-8.692	-10.39 to -6.99	-3.20	-3.99 to -2.41	-4.26	-5.21 to -3.31								
Ceylan et al. 2007 (48)	41	21.8±3.3	34	28.2±1.7	-2.349	-2.94 to -1.75	-1.85	-2.40 to -1.30					41	85.3	70.8 to 94.43	34	1	84.6 to 99.9
Cai et al. 2007 (49)	56	23.3±4	46	31.8±2.5	-2.476	-2.99 to -1.95	-2.42	-2.93 to -1.90	-1.91	-2.38 to -1.44			56	87.5	75.9 to 94.82	46	91.3	79.2 to 97.5
Luo et al. 2007 (50)	30	23.5±3.1	71	32±1.1	-4.403	-5.15 to -3.65	-3.609	-4.27 to -2.94										
Dinh et al. 2020 (51)	59	23.4±3.2	145	31.2±1.2	-3.90	-4.38 to -3.41	-3.256	-3.69 to -2.81					59	98.3	90.9 to 99.9	145	97.9	94.0 to 99.5
Ageeli et al. 2013 (52)	100	22.9±2.9	60	30.9±1.3	-3.27	-3.76 to -2.79	-4.093	-4.64 to -3.53	-11.85	-13.2 to -10.5	-7.89	-8.82 to -6.96						
Buttarello et al. 2016 (53)	58	24.4±4.8	164	33±1.2	-3.23	-3.66 to -2.80	-2.141	-2.50 to -1.78	-1.31	-1.63 to -0.99			58	91.3	81.0 to 97.1	164	94.5	89.8 to 97.4
Uçar et al. 2019 (33)	52	21±4.1	54	36.6±7	-2.69	-3.22 to -2.15	-2.996	-3.55 to -2.43	-1.10	-1.51 to -0.69	-2.83	-3.37 to -2.29	52	90.3	78.9 to 96.8	54	100	93.3 to 100.0
Toki et al. 2017 (54)	72	23.4±4.9	67	33.8±2.5	-2.631	-3.08 to -2.17	-2.008	-2.41 to -1.59	-0.56	-0.90 to -0.22	-2.38	-2.82 to -1.94						
Vázquez-L et al. 2019 (55)	13	24.5±30	1153	31.1±1.7	-1.885	-2.43 to -1.33	-2.951	-3.51 to -2.39	-1.64	-2.19 to -1.09	-1.51	-2.06 to -0.96						
Malczewska-L et al. 2017 (56)	33	29.3±1.8	87	31.4±1.0	-1.567	-2.02 to -1.12	-0.457	-0.86 to -0.05	-2.44	-2.94 to -1.93								
Chinudomwong et al. 2020 (57)	133	20.6±9	155	33±1.4	-1.993	-2.27 to -1.71	-1.909	-2.18 to -1.63	-1.40	-1.66 to -1.14			133	73.6	65.3 to 80.9	155	96.7	92.6 to 98.9
Urrechaga et al. 2011 (58)	126	22.3±3.7	90	33.7±1.4	-3.827	-4.28 to -3.37	-3.235	-3.64 to -2.82	-2.44	-2.80 to -2.08	-4.39	-4.89 to -3.89						
Rungngu et al. 2016 (59)	16	25.8±4.8	34	29.8±1.3	-1.338	-1.99 to -0.68			-1.59	-2.27 to -0.91			16	43.7	19.7 to 70.1	34	85.2	68.9 to 95.0
Total (random effects)	950		2540		-2.846	-3.31 to -2.38	-2.463	-2.95 to -1.97	-2.38	-3.11 to -1.63	-3.77	-5.38 to -2.15	550	83.5	76.1 to 89.9	980	91.8	85.6 to 96.4

CI = Confidenceinterval, n = Sample size, SD = Standard Deviation

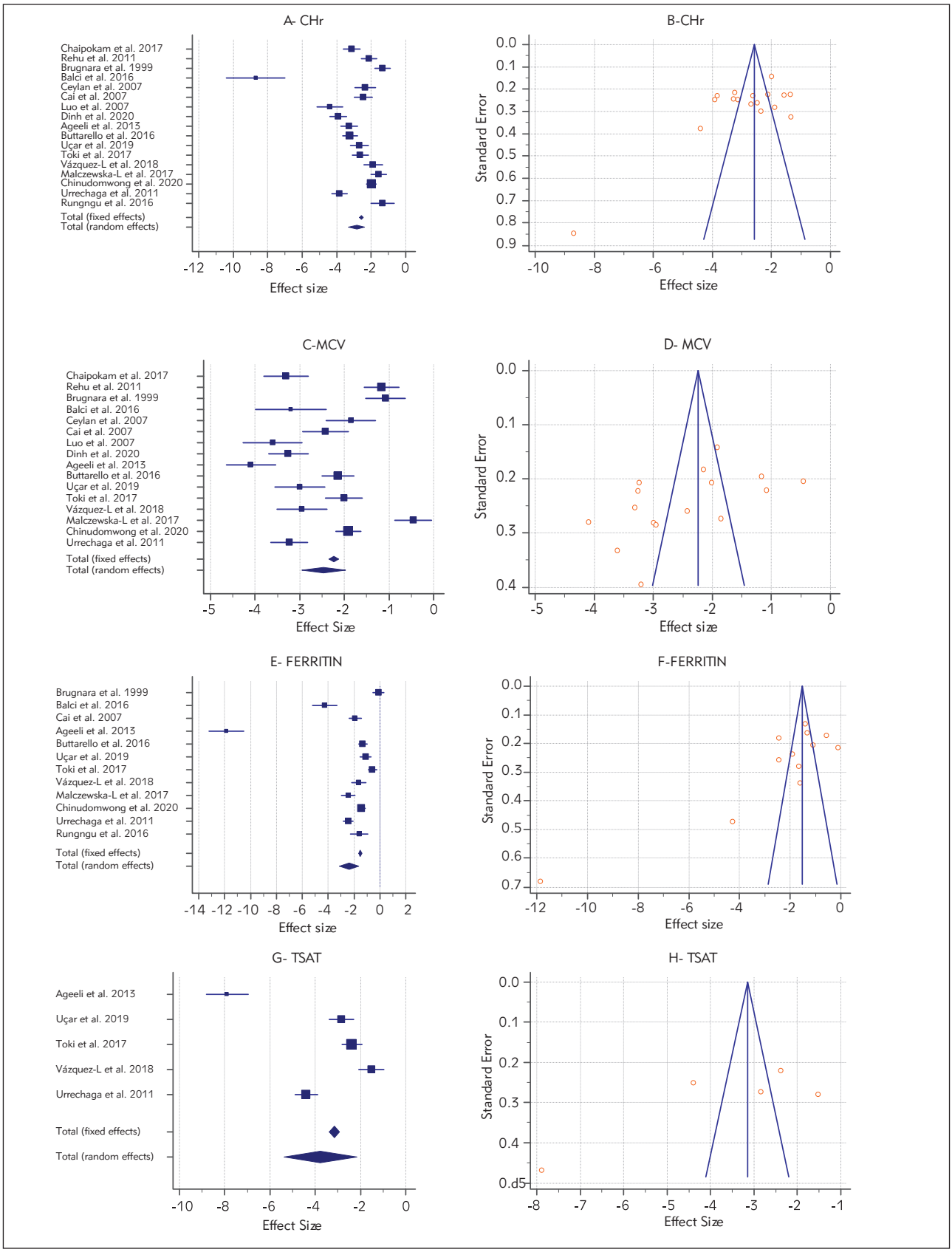

Figure 2A Forest plot of reticulocyte hemoglobin content (CHr), **B** Funnel plot of reticulocyte hemoglobin content, **C** Forest plot of mean corpuscular volume (MCV), **D** Funnel plot of mean corpuscular volume, **E** Forest plot of ferritin, **F** Funnel plot of ferritin, **G** Forest plot of transferrin saturation (TSAT), **H** Funnel plot of transferrin saturation.

sand twenty-two articles not suitable for the title and abstracts and published in languages other than English were excluded. From the remaining 94 pieces, 17 of them were included in the meta-analysis, excluding those with repeating records, animal studies, those that did not include a control group, and those related to chronic diseases other than iron deficiency. The flowchart and detailed literature search steps are shown in *Figure 1*.

Characteristics and Quality Studies

The articles on CHr, a biomarker used in patients with IDA, were included in our study, all in English until December 29, 2020. From the 17 articles, 2 of them were written in Thailand, 3 in Turkey, 2 in Spain, 1 in Finland, 1 in the United States, 2 in China, and one in Indonesia, Vietnam, Saudi Arabia, Italy, Poland, and Japan. The study contains 951 individuals with IDA and 3491 people, including 2540 control group members. The features of the included studies are shown in *Figure 1*.

Reticulocyte Hemoglobin Content

As a result of a detailed literature review, 17 articles including IDA data of CHr were included. In the meta-analysis performed with the low bias risk random-effects model in the included articles, the I^2 value of CHr was 93.52%, so intergroup heterogeneity was achieved (P <0.0001), and random effect size value 2.84 (95% CI 2.36 to 3.31) is shown in *Table II*. Publication bias is evaluated with a forest plot in *Figure 2A* and funnel plot in *Figure 2B*.

Mean Corpuscular Volume

After the literature review, 16 articles with MCV data were included. In the meta-analysis performed with the low bias risk random-effects model in the included articles, the MCV I^2 value was 94.71%, so intergroup heterogeneity was achieved (P <0.0001), and random effect size value 2.46 (95% CI 1.97 to 2.95) is shown in *Table II*. Publication bias is evaluated with a forest plot *Figure 2C* and funnel plot in *Figure 2D*.

Ferritin

12 articles with ferritin data were included. In the meta-analysis performed with the low bias risk random-effects model in the included articles, the ferritin I^2 value was 97.15%, so intergroup heterogeneity was achieved (P<0.0001), and random effect size value 2.37 (95% CI 1.63 to 3.11) is shown in *Table II*. Publication bias is evaluated with a forest plot *Figure 2E* and funnel plot in *Figure 2F*.

Transferrin Saturation

5 articles with TSAT data were included. In the meta-analysis performed with the low bias risk model of random effects in the included articles, the TSAT I^2 value was 97.70%. Therefore, intergroup heterogeneity was achieved (P<0.0001), and random effect size value 3.76 (95% CI 2.14 to 5.38) is shown in *Table II*. Publication bias is evaluated with a forest plot *Figure 2G* and funnel plot in *Figure 2H*.

Discussion

Anemia is a global health problem that is quite common worldwide and affects 43% of children under the age of five, 38% of pregnant women, and 29% of non-pregnant women (19). IDA brings along many complications such as growth retardation, neurocognitive deficiencies, impaired immune system, increased risk of premature, and impaired learning ability (20–24). It is therefore important to ensure accurate and timely diagnosis of the disease by preventing such adverse effects (25–27). Although there is no single and best test for determining iron deficiency, bone marrow aspiration, which is accepted as the gold standard, and the method of staining bone marrow macrophages and erythroid precursors with Prussian blue is used. However, the method is not suitable for routine use because it is expensive, subjective, and invasive (26, 28).

There are many biochemical parameters used in the diagnosis of IDA. However, as these parameters are affected by certain conditions, it is not easy to evaluate them. Serum ferritin concentration, serum iron level, TSAT, and total iron-binding capacity (TIBC) are the most widely used biochemical tests. Although serum ferritin level reveals the iron concentration accumulated in the body, factors such as acute and chronic inflation, malignancy, liver diseases, and excessive alcohol use increase independent of iron (29). Serum iron level decreases with infection, inflammation, and malignancy but increases with liver disease. Since the TSAT level is calculated on iron and TIBC, it is affected by changes in these values and does not always give an accurate result (29–32).

In recent years, CHr has become one of the parameters used to determine IDA (33, 34). reticulocytes, as the first erythrocytes produced in the bone marrow, transform into mature red blood cells a day or two after entering the bloodstream. Reticulocyte parameters have become one of the parameters used to reflect the iron status in a short time due to their shorter lifespan compared to erythrocytes and ability to provide information about bone marrow erythrocyte production (12, 35). CHr has a higher specificity and a lower coefficient of variation since it is not affected by inflammation like some parameters used in the diagnosis of IDA (36). Reticulocyte hemoglobin content data can be obtained from a few millimeters

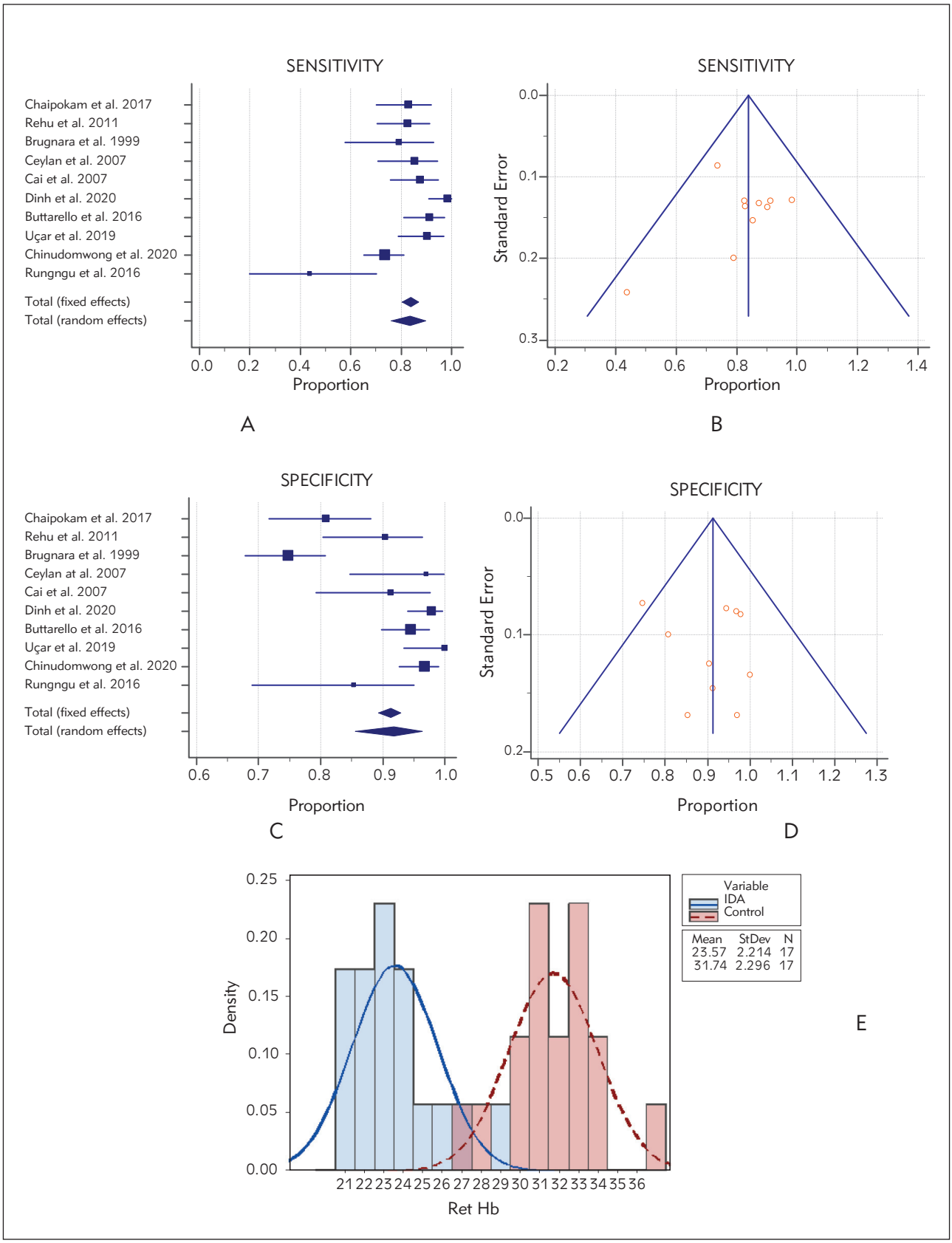

Figure 3A Forest plot meta-analysis of the sensitivity of reticulocyte hemoglobin content to diagnose iron-deficiency anemia articles, **B** Funnel plot meta-analysis of the sensitivity of reticulocyte hemoglobin content to diagnose iron-deficiency anemia articles, **C** Forest plot meta-analysis of the specificity of reticulocyte hemoglobin content to diagnose iron-deficiency anemia articles, **D** Funnel plot meta-analysis of the specificity of reticulocyte hemoglobin content to diagnose iron-deficiency anemia articles, the reticulocyte hemoglobin content mean cut-off value.

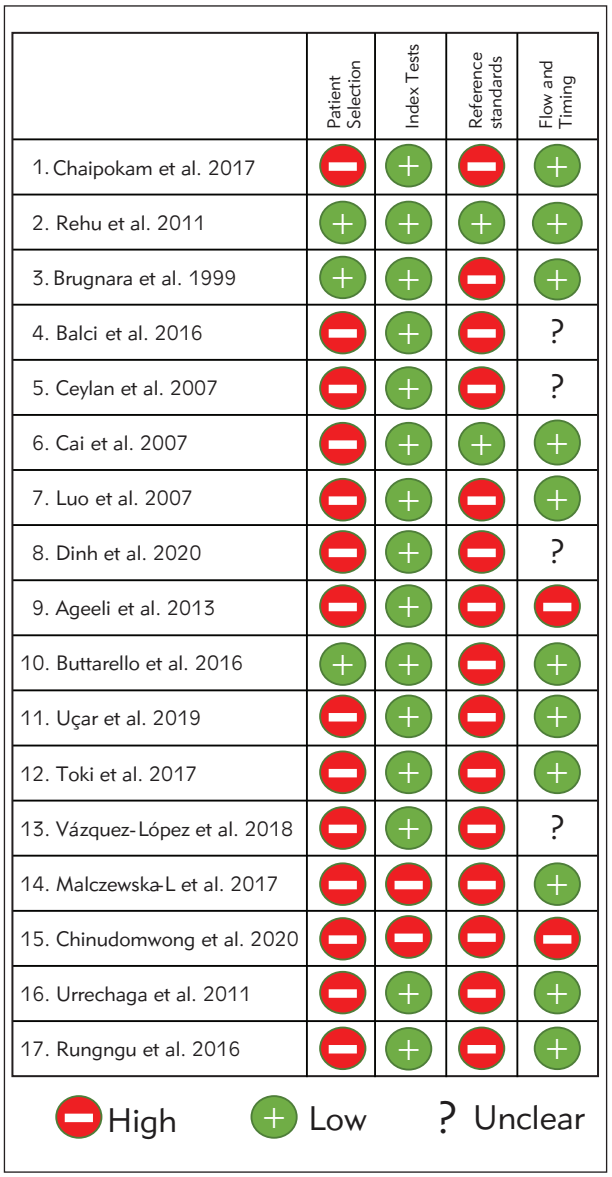

Figure 4 Methodological quality of the included studies (individual assessment).

of peripheral blood compared to bone marrow biopsy and is also advantageous because it is relatively inexpensive, convenient, and less invasive (37).

In this random-effect meta-analysis, studies on the efficiency of CHr in the diagnosis of IDA are summarized. For this meta-analysis, a total of 13,116 articles were examined, and 17 studies were included in the study according to the inclusion and exclusion criteria. The number of articles including parameters such as MCV, ferritin, and TSAT, which were used frequently in the past in the diagnosis of iron deficiency, varies (16 articles for MCV values, 12 articles for Ferritin values, and 5 articles for TSAT values were examined). The most effective examination is thought to be obtained by including each study that meets the criteria given in *Figure 1* for meta-analysis. The literature review exhibited the fact that this study is the first meta-analysis of CHr to determine the diagnosis of IDA.

A total of 3491 individuals, of which 2540 people were in the control group, and 951 people were with IDA, were included in this study. The inclusion criteria for the current study were determined as CHr, which is one of the important parameters in the diagnosis of IDA and containing at least one of the parameters of MCV, ferritin, or TSAT.

Ferritin is an indicator of the total amount of iron stored in the body. When the serum ferritin level shows values less than 15 mg/L, it is highly suggestive for the diagnosis of IDA. At values below 30 mg/L, the sensitivity is 92%, and the specificity is 98% (38, 39). Again, a ferritin level below 45 mg/L and the sensitivity of 85% and specificity of 92% are particular to IDA (40, 41).

A low MCV value alone is not sufficient for a diagnosis of IDA. Unless the MCV volume is greater than 95 μm] (95 fL), it should not be considered in IDA because this threshold has a sensitivity of 97.6% (40, 42).

TSAT is low in IDA, typically less than 10%, and in this case, the sensitivity is 48%, and the specificity is 88% (43, 44).

In the meta-analysis performed with the low bias risk random-effects model in the included articles, the sensitivity CHr I^2 value was 78.78% (95% CI 61.43 to 88.32), so intergroup heterogeneity was achieved ($P<0.0001$) (*Figure 3A–B*). The sensitivity of CHr to diagnose IDA was found as 83.5% (95% CI 76.1 to 89.8), and they are shown in *Table II*.

In the meta-analysis performed with the low bias risk random-effects model in the included articles, the specificity CHr I^2 value was 89.53% (95% CI 82.88 to 93.60), so intergroup heterogeneity was achieved ($P<0.0001$) (*Figure 3C–D*). The sensitivity of CHr to diagnose IDA was found as 91.8% (95% CI 85.5 to 96.4), and they are shown in *Table II*.

In conclusion, the meta-analysis study showed that CHr is a better marker than other more commonly used parameters in IDA. Many previous studies also support this conclusion. Also, this meta-analysis we conducted is important for being the first meta-analysis study regarding CHr and IDA.

The literature review revealed that different cut-off results related to CHr were obtained, and these values vary between 28–29 pg. The CHr mean cut-off value obtained in our study is 28.2, and this is shown in the histogram in *Figure 3E*.

There are important heterogeneity and bias problems in the studies. Patient selection and lack of reference methods are particularly important. There are important criteria differences for index tests. Also,

it is important to have significant group differences (pediatric patients, renal failure, etc.) in studies. It should be noted that there will be a significant change in methodologies over time (*Figure 4*).

All parameters of IDA, which are included in the research, have heterogeneity. CHr is a more effective marker in determining IDA compared to the routinely used MCV and ferritin levels. The effect size value of TSAT, one of the parameters examined, is above 0.80, and its selectivity is higher than CHr.

The study also has some limitations. These are as follows: like other meta-analysis studies in the literature, methodological differences are arising from combining studies conducted with different methods, and this may lead to bias. Since studies in which the diagnosis of IDA of CHr was evaluated in the meta-analysis were included, many parameters used routinely were excluded. IDA, erythropoiesis status, and chronic anemias were excluded from the study. In the study, no distinction was made according to gender and age.

Conclusion

This study is the first meta-analysis to evaluate the efficiency of CHr in the diagnosis of IDA. According to our results, CHr should be used additionally with the parameters used in the diagnosis of IDA. 'The results of our study reveal the findings that CHr is a better biomarker than MCV and ferritin used in determining IDA, and its efficacy is lower than TSAT. It is very important to routinely use it for the pre-diagnosis of IDA, which is very important for public health.' CHr alone provides important information about the current bioavailability of iron, but its use with other parameters removes uncertainty about the diagnosis and treatment of IDA. CHr is a very important parameter that can be used to evaluate a very common disease in the clinic, such as IDA' The heterogeneity index of the study results is quite high. Therefore, comprehensive studies with more homogeneous groups are needed to elucidate the relationship between IDA and CHr.

Acknowledgements. Not applicable.

Conflict of interest statement

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

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Received: March 21, 2021
Accepted: May 27, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-30659

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 14–20, 2022

Opinion paper
Mišljenje

SARS-COV-2 INFECTING ENDOTHELIAL CELLS, BIOCHEMICAL ALTERATIONS, AUTOPSY FINDINGS AND OUTCOMES IN COVID-19, SUGGEST ROLE OF HYPOXIA-INDUCIBLE FACTOR-1

SARS-COV-2 DOVODI DO INFEKCIJE ENDOTELIJELNIH ĆELIJA, MENJA BIOHEMIJSKE NALAZE I NALAZI AUTOPSIJE UKAZUJU NA ISHOD COVID-19 I ULOGU HIPOKSIJA-INDUCIBILNOG FAKTORA 1

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Summary

Researchers around the world have experienced the dual nature of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), 'tragically lethal in some people while surprisingly benign in others'. There have been congregating studies of the novel coronavirus disease (COVID-19), a disease that mainly attacks the lungs but also has mystifying effects on the heart, kidneys and brain. Researchers are also gathering information to ascertain why people are dying of COVID-19, whether it is solely a respiratory disorder, a coagulation disorder or multi-organ failure. Alterations in laboratory parameters like lactate, ferritin and albumin have been established as risk factors and are associated with outcomes, yet none have not been substantiated with a scientific biochemical rationale. SARS-CoV-2 affects the alveolar type II epithelial cells which significantly disturbs its surfactant homeostasis, deprives Na,K-ATPase of ATP, thereby disturbing the alveolar lining fluid which then gradually decreases the alveolar gaseous exchange initiating the intracellular hypoxic conditions. This activates AMP-activated kinase, which further inhibits Na,K-ATPase, which can progressively cause respiratory distress syndrome. The virus may infect endothelial cell (EC) which, being less energetic, cannot withstand the huge energy requirement towards viral replication. Therefore glycolysis, the prime energy generating pathway, must be mandatorily upregulated. This can be achieved by Hypoxia-inducible factor-1 (HIF-1). However, HIF-1 also

Kratak sadržaj

Istraživači širom sveta iskusili su dualnu prirodu teškog akutnog sindroma koronavirusa-2 (SARS-CoV-2), koji je »tragično letalan za neke ljude, a iznenađujuće benigan za druge«. Brojne su studije vezano za novo korona virus oboljenje (COVID-19), koji uglavnom oštećuje pluća, ali i ima i iznenađujuće efekte na srce, bubrege i mozak. Istraživači takođe sakupljaju podatke zašto ljudi umiru od COVID-19, bez obzira da li je to samo respiratorni poremećaj, poremećaj koagulacije ili je multi-organski poremećaj. Promene laboratorijskih parametara kako što su laktat, feritin i albumin su ustanovljeni kao faktori rizika, mada još uvek nisu naučno dovoljno potvrđeni. SARS-CoV-2 deluje na alveolarne tip II epitelijelne ćelije koje značajno oštećuju površinsku homeostazu, deluju na Na,K-ATPazu, odnosno vrše oštećenje alveolarne tečnosti koja zatim postepeno umanjuje izmenu alveolarnih gasova i dovodi do izmene intracelularnih hipoksičnih uslova. Ova aktivnosti AMP-aktivirane kinaze, koja zatim inhibira Na,K,ATPazu, što zatim može progresivno da prouzrokuje respiratorni distres sindrom. Virus može da inficira endotelijalne ćelije (EĆ) koje postaju manje energetske, i nisu sposobne da obezbede dovoljno energetskih potreba prema viralnoj replikaciji. Prema tome, glikoliza, primarni energetski put mora da se u prvom redu reguliše. Ovo može da se postigne sa Hipoksija inducibilnim faktorom-1 (HIF-1). Međutim, HIF-1 takođe aktivira transkripciju von Willebrandovog faktora plazminogen aktivator inhibitora-1, i deluje supresivno na oslobađanje trombomodulina. Ovo

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activates transcription of von Willebrand factor, plasminogen activator inhibitor-1, and suppresses the release of thrombomodulin. This in turn sets off the coagulation cascade that can lead to in-situ pulmonary thrombosis and micro clots. The proposed HIF-1 hypothesis justifies various features, biochemical alteration, laboratory as well as autopsy findings such as respiratory distress syndrome, increased blood ferritin and lactate levels, hypoalbuminemia, endothelial invasion, in-situ pulmonary thrombosis and micro clots, and multi-organ failure in COVID-19.

Keywords: novel coronavirus, COVID-19, SARS-CoV-2, severe acute respiratory syndrome coronavirus 2, hypoxia-inducible factor-1

nasuprot dovodi do koagulacione kaskade koja može da dovede in-situ pulmolarnu trombozu i mikro koagulaciju. Predložena HIF-1 hipoteza potvrđuje različite događaje, biohemijske promene, laboratorijske nalaze, kao i nalaze autopsije kao što su respiratorni distres sindrom, povećan nivo feritina i laktata u krvi, hipoalbuminemiju, endotelijalnu invaziju, in-situ pulmolarnu trombozu i mikro koagulaciju, kao i oštećenje brojnih organa u COVID-19.

Ključne reči: novi korona virus, COVID-19, SARS-CoV-2, težak akutni respiratorni sindrom koronavirusa 2, hipoinducibilni faktor-1

Introduction

The most crucial and curious question about the new coronavirus is "How deadly is it?" Majority of the new coronavirus disease-2019 (COVID-19) patients are asymptomatic (1). Of all the COVID-19 deaths, the share of deaths in 0–17 years old is only 0.06%, while in > 65 years is 73.6% (2). This clearly indicates that the virus is more lethal to older people. The causative agent of COVID-19, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), thus seems to be tragically lethal in some while being surprisingly benign in others. This disease mainly attacks the lungs but also has bewildering effects on the heart, kidneys and brain. Researchers are gathering information to determine what actually kills COVID-19 patients, whether it is pneumonia, blood clots or kidney failure (3). Presently, there is no discrete theory to explain all the features, symptoms, clinical and autopsy findings such as lethality of virus to older people, diffuse alveolar damage prevalent in younger patients, endothelial cell invasion, in-situ pulmonary thrombosis and micro clots, and multiple organ damage. Variation in laboratory parameters like lactate, ferritin and albumin have been established as risk factors and are associated with outcomes, but have not been substantiated with a scientific biochemical rationale. Here, I present the biochemical rationale and propose the Hypoxia-inducible factor-1 hypothesis to substantiate the various features, clinical laboratory as well as autopsy findings.

Material and Methods

Only PubMed/Scopus indexed articles published on the relevant topic along with the knowledge and understanding of biochemistry, acquired over three decades, have been used for proposing the hypothesis.

Results and Discussion

Entry of the virus in the body

SARS-CoV-2 enters a human host cell using the angiotensin-converting enzyme 2 (ACE2) as its receptor. Recent studies have indicated 10 to 20 times higher binding affinity of SARS-CoV-2 to ACE2 than the earlier SARS-CoV (4, 5) and higher expression of ACE2 on the epithelial cells of oral mucosa (6) and nasal epithelial cells (7). Thus, SARS-CoV-2 can replicate in these epithelial cells and can further penetrate inside the human body through these routes.

When SARS-CoV-2 enters through the mouth, it binds to and penetrates oral epithelial cells (OEC), hijacks its machinery, consumes its energy and nutrition for replication, and may even affect the gustatory system resulting in loss of taste function. Similarly, when it enters through the nose, the virus binds to and enters the nasal epithelial cells (NEC), possibly affecting the nasal olfactory epithelium and resulting in the loss of olfactory function. SARS-CoV-2 entering and damaging islets cells, resulting in acute diabetes, has been well documented (8). Taste reduction and smell reduction has been reported in 55.4% and 41.7% respectively of 204 COVID-19 patients (9). However, during the replication in OEC and NEC, the body gets sufficient time to tackle the virus through its own immune system. NEC also expresses the immune-associated genes (7). The nasal cavity is in continuation with the oral cavity, which allows the virus to slowly migrate to the oropharynx, nasopharynx, throat and the upper respiratory tract (URT) and can be detected in these regions. Since no major organ or critical function is lost, the COVID-19 individual may remain asymptomatic, or may have just mild symptoms. Anosmia, with or without dysgeusia has been manifested in patients with mild to no constitutional symptoms (10, 11). If the body's immune mechanism and lung defences are strong enough, it prevents the virus from invading the lower respiratory tract (LRT) and other organs and eventually the COVID-19 patient effectively fights off the viral invasion while being completely asymptomatic or having mild symptoms. This has been noticed in the majority of young COVID-19 individuals.

Why is the virus so lethal to older people?

Each and every inhalation can introduce new infectious agents and irritants into the respiratory system. To protect the lungs, the conducting airways are lined with ciliated airway epithelium. Whenever microorganisms try to invade the airway epithelium, they get trapped in the mucus layer, and the cilia beat in a coordinated manner, with considerable frequency, to remove the invading pathogens. However, aging slows the ciliary beat frequency, gradually decreases the number of cilia and ciliated cells resulting in the breakdown of the lungs first and foremost line of defense (12). In younger people, the airways are sensitive and the inhaled particles provoke vigorous coughing to expel the irritants, while in older people (>65 years), the coughing reflex either may not be triggered, due to less sensitive sensory receptors, or may be triggered ineffectively due to reduced respiratory muscle strength (13). In people > 65 years, the vital capacity is decreased, resulting in decreased exchange of gases, and the increased residual volume causes substantial air trapping in the lungs, making their alveolus a milieu for microbial growth. Alveoli in younger people (<65 years) have sufficient macrophages to clear the pathogens reaching alveoli, while alveoli in older people have fewer macrophages and more pro-inflammatory neutrophils that release free radicals and cytokines, which makes them more susceptible to cytokine storm that damages the alveolar structure (14). All of these result in high risk of SARS-CoV-2 migrating to the LRT and penetrating the deep lung tissue, causing serious lung complications in the already physiologically-challenged lungs of the elderly.

What if the SARS-CoV-2 reaches the deep lung tissue?

In lungs, the gaseous exchange is carried out in alveoli, which consists of alveolar type (AT) 1 and AT2 epithelial cells. AT1 cells, representing about 40% of the cell population but covering 90–95% of the alveolar surface area, contain scanty mitochondria and organelles. AT2 cells, representing 60% of the cell population while occupying only 5–10% of the area, are highly metabolically active with a large nucleus and their cytoplasm is rich in mitochondria, endoplasmic reticulum (ER) and prominent Golgi complex (GC) (15). A layer of alveolar lining fluid (ALF) covers the entire surface of the alveolar epithelium, and the regulation of its volume and composition is extremely important for optimal gaseous exchange (16). AT2 cells synthesize, assemble and regulate the secretion of functional surfactant (17). Surfactant when released produces a monolayer over alveolar epithelium surface, which reduces alveolar surface, opsonises pathogens and facilitates their clearance. Insufficient surfactant causes alveoli collapse, pulmonary edema & respiratory distress syndrome. Nearly 10% of the secreted surfactant pool is needed to be recycled per hour as the surfactant keeps getting inactivated. Primarily, AT2 cells cause this recycling, failing which, ineffective surfactant accumulates in the alveolus causing associated complications (18). Moreover, AT2 cells have Na,K-ATPase for the transepithelial ion transport which is crucial for the regulation of the ALF, to guarantee proper gaseous exchange (16).

In the alveolus, the AT2 cell has a high concentration of ACE2, all the infrastructure and energetics to support the replication and more importantly expression of > 20 other genes that are closely related to virus replication and transmission. This makes AT2 cells the most preferred target cells of COVID-19 in LRT (19).

Bioenergetics burden, hypoxia, ROS, down-regulation of Na,K-ATPase, breakdown of surfactant homeostasis in AT2 cells trigger lung damage

When SARS-CoV-2 enters an AT2 cell, it hijacks its entire cellular machinery and diverts its energy, essential amino acids (EAAs) and nutrition towards its own replication. If expenditure, exclusively towards the viral genome and structural proteins, are accounted for, then each virus costs a minimum of $1.7x10^7$ ATP along with a huge nutritional load of EAAs (20). However, this does not include the expenditure towards proteases, polyproteins 1ab, 5 and 3 sequences of subgenomic mRNA, lipids, carbohydrates, transport of molecules, assembly and disassembly process in ER-GC. The huge energy expenditure and hijacking of the entire cellular machinery of the host by the virus can significantly disturb the surfactant homeostasis and deprive Na,K-ATPase of sufficient ATP, as Na,K-ATPase itself requires around 40% of cellular energy for its normal functioning (21). Reduction in ATPase activity reduces the crucial transepithelial ion transport, which in turn disturbs the ALF resulting in decreased alveolar gaseous exchange, which is further worsened by the disturbed surfactant homeostasis. To cater for the additional energy required to support the fast viral replication, the AT2 mitochondrial electron transport chain (ETC) has to increase by consuming more glucose, fatty acids and oxygen. This increased ETC can result in more reactive oxygen species (ROS). Increased oxygen consumption with reduced oxygen delivery to the AT2 might gradually initiate intracellular hypoxic conditions in AT2. This hypoxia/ROS then activates AMP-activated kinase (AMPK) which further inhibits Na, K-ATPase. This greatly impairs lung fluid clearance (21) and can progressively increase the blood ferritin levels, as hypoxia causes more than four fold increase in ferritin content in alveolar cells. The increased blood ferritin content in COVID-19 has been abundantly reported.

Fast viral replication continues damaging the other AT2 cells exponentially. The inflammatory cytokines of endothelial cells further exacerbates the deteriorating lung. Gaseous exchange is highly reduced and the alveolus becomes fully filled with fluid, or almost air-free, and ventilators also fail to sufficiently ventilate such COVID-19 patients, leading to death by respiratory failure. Older patients with more comorbid conditions tend to die early, while younger patients with no comorbidity continue to fight for longer. The accumulation of inactive surfactant along with the cell debris, free radical damage from oxygen, cytological pleomorphic AT2 cells can cause diffuse alveolar damage, which has been reported to be more prevalent in younger patients (3).

Respiratory failure cannot be the only cause – Endothelial cell invasion and HIF

During the clash between the immune system and SARS-CoV-2, the virus may escape into the circulatory system and spread easily because of the abundant expression of ACE2 on endothelial cells (EC) (22). With a higher binding affinity for ACE receptors (4, 5), SARS-CoV-2 can be more potent in spreading and infecting other organs via the bloodstream (3). The presence of viral elements within EC with evidence of EC death has also been reported (23).

The EC is not a major energy-requiring cell (24). It has low mitochondrial content and generates more than 80% of their energy requirement through glycolysis. The oxygen consumption, which contributes to just 15% of EC energy generation, has physiologically been kept low to facilitate ECs to transfer most of the oxygen to the perivascular tissues (25). SARS-CoV-2 infecting EC is bound to change its whole energetics, as the normally low energetic EC cannot withstand the huge energy requirement (20) towards viral replication. ECs are designed to generate energy mostly by anaerobic glycolysis, during which each glucose molecule is broken into 2 lactate molecules yielding only 2 molecules of ATP. Whereas, aerobically, each glucose molecule consuming oxygen and involving mitochondria, gets completely oxidized into 6 CO_2 molecules and generates 32 molecules of ATP. Thus, to sustain the viral replication on anaerobic glycolysis, the glycolysis must mandatorily be upregulated. This upregulation can be achieved by Hypoxia-inducible factor-1 (HIF-1), which is a transcriptional activator of genes involved in cell metabolism (27, 28).

HIF-1 is composed of a regulatory HIF-1α subunit and constitutively expressing HIF-1 subunit. HIF-1α subunits undergo oxygen-dependent hydroxylation by prolyl-hydroxylase domain containing protein (PHD). PHD-catalysed hydroxylation reactions require oxygen and alpha-ketoglutarate (2-oxoglutarate) as co-substrates, and iron and ascorbate as cofactors (29). The hydroxylated HIF-1α subunits are rapidly destroyed via the ubiquitin-proteasome system (UPS) pathway. In a cellular hypoxic environment, non-hydroxylated HIF-1α subunits escape UPS degradation, and combine with HIF-1 and co-activators forming a functional HIF-1 (26). Varieties of viral pathogens have been reported to activate the HIF-1 pathway. Hepatitis B virus, Vaccinia virus and Epstein-Barr virus can stabilize HIF-1α by interfering with prolyl hydroxylation or UPS degradation. Influenza A virus activates HIF-1 by inhibiting proteasome, that too under physiologically normal oxygen levels (normoxia), thereby mimicking a hypoxic response in normoxia (26, 30).

Thus, in COVID-19, it can be scientifically hypothesized that SARS-CoV-2 by some mechanism activates HIF-1 to manipulate the host cell environment for its own benefits. Accordingly, SARS-CoV-2 in EC activates HIF-1, which then induces the genes encoding all the glycolytic enzymes to upregulate glycolysis. Glucose transporters, phosphofructokinase-2 (generator of the most powerful glycolysis activator), phosphoglycerate kinase and pyruvate kinase, which are confirmed targets of HIF-1, are induced. HIF-1 inhibits pyruvate dehydrogenase via direct transactivation of pyruvate dehydrogenase kinase. Consequently, pyruvate entry into tricarboxylic acid (TCA) cycle is suppressed and instead generates lactate which effluxes from the tissues via monocarboxylate transporter MCT4, which is also upregulated by HIF-1 (31). Thus, based on this hypothesis, virus replication inside EC should cause very high consumption of glucose leading to malnutrition and high amounts of lactate in the blood. The same has been reported in 100 percent of the COVID-19 patients who died, thereby concluding that lactate levels can be used as indicators of disease progression (32). Additionally, HIF-1 activated in EC can upregulate pro-inflammatory cytokines (IL-1, 6 and 8) and platelet-activating factor to fuel neutrophils recruitment at the site of viral invasion (33). Increased serum IL-6 has been abundantly reported as a marker of disease progression and has been associated with fatal outcomes in COVID-19, thus favouring the proposed hypothesis.

Virally infected EC, as a damage control measure, can employ heme, as it greatly potentiates cell killing mediated by neutrophils and ROS. Consequently, EC upregulates heme oxygenase-1 (HO-1) to degrade and release Fe^{2+} from heme, and also upregulates ferritin (34). Ferritin heavy chain (FHC) ferroxidase inactivates Fe^{2+} to Fe^{3+} inside ferritin to preclude the generation of lethal hydroxyl radical from Fe^{2+} by Fenton reaction (35). HO-1 deficiency causing extensive EC damage amply demonstrates the significance of this defence system (34). HIF-1 upregulates the HO-1 gene (36) and consequently necessitates more ferritin. Thus, based on the proposed HIF-1 hypothesis, one can assume that high serum ferritin reflects alveolar (37) and intense endothelial invasion by SARS-CoV-2 leading to fatal outcomes, and the same has been reported abundantly.

HIF, coagulation abnormality and multi-organ failure

If SARS-CoV-2 indeed activates the HIF-1 pathway in EC, as hypothesized here, then it should also demonstrate its impact on procoagulant and anticoagulation behaviour of EC. Recently, it has been established that ECs produce surface regulatory proteins that prevent excessive coagulation. These include EC receptor thrombomodulin (TM), endothelial protein C receptor (EPCR), tissue factor pathway inhibitor (TFPI), and protein C (PC) (38). Under normal physiological conditions, TM-bound thrombin converts PC that is bound to EPCR, into activated protein C (APC). For APC to be effective, its complex with protein S (PS) synthesized by EC, must be formed (39). The resulting APC-PS complex, then inactivates the activated factor (F) VIII and FV and, therefore, limits the functions of FVIII-FIX (intrinsic tenase complex) and FX-FV (prothrombinase complexes) inhibiting coagulation under the normal physiological conditions (39). PS is also a cofactor of TFPI (40) which inhibits the tissue factor (TF)-FVII (extrinsic tenase complex) activation of FX (38). PS binds to activated FX and FV, inhibits activated FX independently, and downregulates thrombin generation (41). Thus, PS has a definite function in the inhibition of coagulation. HIF-1 downregulates PS expression, resulting in its inverse relationship with PS (42). The decreased PS might result in the inhibition of APC and TFPI. APC without its cofactor PS might fall short to prevent inactivation of activated FVIII and FV, the two cofactors essential for blood coagulation, thereby failing to prevent the FVIII-FIX intrinsic tenase and FX-FV prothrombinase complex. TFPI without the cofactor PS, may fail to inhibit TF–FVII extrinsic tenase complex activation of FX. Insufficient PS can result in an elevated amount of activated FIX, which increases the risk of venous thromboembolism. Hence, inhibition of activated FIX has been proposed as a treatment of venous thromboembolism (40). HIF-1 activates transcription of procoagulant molecule, von Willebrand factor (VWF) (43) and induces the exocytosis of EC Weibel-Palade (WP) body (store house of P-selectin and VWF) causing release of VWF and P-selectin at the EC surface (34) . HIF-1 induces transcription and translation of the plasminogen activator (PA) inhibitor-1 (44, 45) but does not induce tissue-PA (44) and suppresses the release of TM (46). Moreover, prostacyclin released by EC cannot exert its anti-aggregatory effect on platelets as adenylyl cyclase (downstream mediator) is reduced by HIF-1. As a result, cross-linked fibrin clots are formed on the surface of endothelium and this sets off the coagulation cascade (46). Based on the proposed hypothesis, small regions of the pulmonary capillary having SARS-CoV-2 infected ECs, can undergo coagulatory situation forming in-situ pulmonary thrombosis and micro clots. Thus in COVID-19, the pulmonary thrombotic events need not be embolic at all, instead, in-situ pulmonary thrombosis could be the culprit. The hypothesis also explains the findings in several studies that have reported a disproportionate high number of venous clotting events as pulmonary thrombi (47, 48) without an associated increase in deep vein thrombosis (DVT) (49). The hypothesis can also respond to the queries raised by some authors on whether the high number of pulmonary embolism (PE) are due to embolic events or are in-situ pulmonary thrombosis (49). Thrombosis reduces the blood flow, thereby restricting the delivery of nutrients and oxygen to downstream tissues and organs. This then, gradually induces necrosis and damage to the respective organs leading to death. Multiple organ damage has been reported in COVID-19 deaths (23, 32). Alternatively, large occlusive thrombi can detach and embolize and then occlude distal vessels resulting in thrombo-embolism. Similarly, venous thromboembolism, as a major cause of DVT and PE can be triggered (50) and the same has been reported as the cause of death (51).

Conclusions

The proposed HIF-1 hypothesis can rationalize various features, clinical laboratory and autopsy findings such as respiratory distress syndrome, increased blood ferritin and lactate levels, hypoalbuminemia, endothelial invasion, in-situ pulmonary thrombosis and micro clots, and multi-organ failure in COVID-19.

Future plans

A definite research plan can help to prove the mechanism by which the SARS-CoV-2 activates HIF-1. In COVID-19, HIF-1 can be activated by following mechanisms:

i) Intracellular hypoxic condition.

ii) Fe^{2+} may become a limiting factor due to increased ferritin

iii) Alpha-ketoglutarate may become limiting due to its conversion to glutamate, an AA consumed towards viral structural proteins.

iv) SARS-CoV-2 component or molecule interfering in PHD-catalysed hydroxylation reactions or UPS degradation.

Funding

This manuscript has not been previously published nor is not being considered for publication elsewhere.

Financial disclosure: Nothing to disclose »None declared.«

Dr. Ambade has nothing to disclose.

Conflict of interest statement

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

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Received: February 01, 2021
Accepted: June 13, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-31382

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 21–31, 2022

Original paper
Originalni naučni rad

ERROR EVALUATION IN THE LABORATORY TESTING PROCESS AND LABORATORY INFORMATION SYSTEMS

PROCENA GREŠKE U LABORATORIJSKOM PROCESU I U LABORATORIJSKOM INFORMACIONOM SISTEMU

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Summary

Background: The laboratory testing process consist of five analysis phases featuring the total testing process framework. Activities in laboratory process, including those of testing are error-prone and affect the use of laboratory information systems. This study seeks to identify error factors related to system use and the first and last phases of the laboratory testing process using a proposed framework known as total testing process-laboratory information systems.
Methods: We conducted a qualitative case study evaluation in two private hospitals and a medical laboratory. We collected data using interviews, observations, and document analysis methods involving physicians, nurses, an information technology officer, and the laboratory staff. We employed the proposed framework and Lean problem solving tools namely Value Stream Mapping and A3 for data analysis.
Results: Errors in laboratory information systems and the laboratory testing process were attributed to failure to fulfill user requirements, poor cooperation between the information technology unit and laboratory, inconsistency of software design in system integration, errors during inter-system data transmission, and lack of motivation in system use. The error factors are related to system development elements, namely, latent failures that considerably affected the information quality and system use. Errors in system development were also attributed to poor service quality.
Conclusions: Complex laboratory testing process and laboratory information systems require rigorous evaluation in minimizing errors and ensuring patient safety. The pro-

Kratak sadržaj

Uvod: Proces laboratorijskog ispitivanja sastoji se iz pet analitičkih faza u ukupnom procesu analiziranja. Aktivnosti u laboratorijskom procesu uključuju i one koje se odnose na utvrđivanje grašaka i utiču na laboratorijski informacioni sistem. Ovo izučavanje ima za cilj identifikaciju grešaka u odnosu na primenu sistema od prve do poslednje faze ispitivanja u laboratorijskom procesu primenom poznatog informacionog sistema za celokupni proces ispitivanja.
Metode: Primenili smo kvalitativno izučavanje procesa u dve privatne bolnice i medicinske laboratorije. Podatke smo sakupljali putem intervijua, na osnovu posmatranja i dokumentovanih metoda analiziranja uključuju i lekare, sestre, informacionog stručnjaka i laboratorijsko osoblje. Primenili smo poznati LEAN proces za rešavanje problema koji je poznat kao Value Stream Mapping i A3 za analizu podataka.
Rezultati: Greške u laboratorijskom informacionom sistemu i laboratorijskom procesu ispitivanja javljaju se uglavnom zbog loše saradnje između jedinice za informacione tehnologije i laboratorije, zbog lošeg informacionog sistema, prenosa podataka i motivacije za primenu sistema. Greške nastaju i zbog problema u razvoju samog kvaliteta informacionog sistema. Takođe greške su posledica lošeg servisiranja sistema.
Zaključak: Kompleksan laboratorijski proces ispitivanja i laboratorijski informacioni sistem iziskuju rigoroznu procenu i kontrolu grašaka i osgiranje sigurnosti pacijenata. Predloženi okvir i primena LEAN postupka su neohodni za procenu procesa laboratorijskog ispitivanja i laboratorijskog informacionog sistema koji moraju da budu rigorozni i sveobuvatni.

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List of abbreviations: total testing process (TTP); laboratory information systems (LIS); total testing process-laboratory information systems (TTP-LIS).

posed framework and Lean approach are applicable for evaluating the laboratory testing process and laboratory information systems in a rigorous, comprehensive, and structured manner.

Keywords: case study, error, evaluation, framework, laboratory information systems, Lean, patient safety, total testing process, socio-technical

Ključne reči: proučavanje slučaja, graška, procena, laboratorijski informacioni sistem, LEAN, sigurnost pacijenta, ukupni proces ispitivanja, socio-tehnološki proces

Introduction

A mistake or inefficiency in one of the stages of the laboratory testing chain can affect the overall process implementation and management, and subsequently physician diagnosis (1, 2). A laboratory information systems (LIS) expedites and facilitates interactions during the laboratory testing process (3). Involvement of multiple units in testing workflow requires effective use of LIS to monitor task performance, ensure a smooth process, and readily identify errors. Many errors identified in laboratory test results were caused by a complex, error prone, unreliable, and poorly designed LIS (4, 5). These outcomes are aggravated when the LIS linked patient and test data to other units and institutions and involved data exchange because of complex inter system interaction (6). Errors were also attributed to human factors, including patient misidentification and an erroneous test request (7).

Total testing process (TTP) (8) is a unique framework that guides the testing process as well as analyzing and minimizing testing error risk not only in the laboratory center, but also in other clinical units (7, 9). The TTP includes internal and external laboratory activities that involve one or more procedures requiring staff interaction. We proposed a TTP-LIS

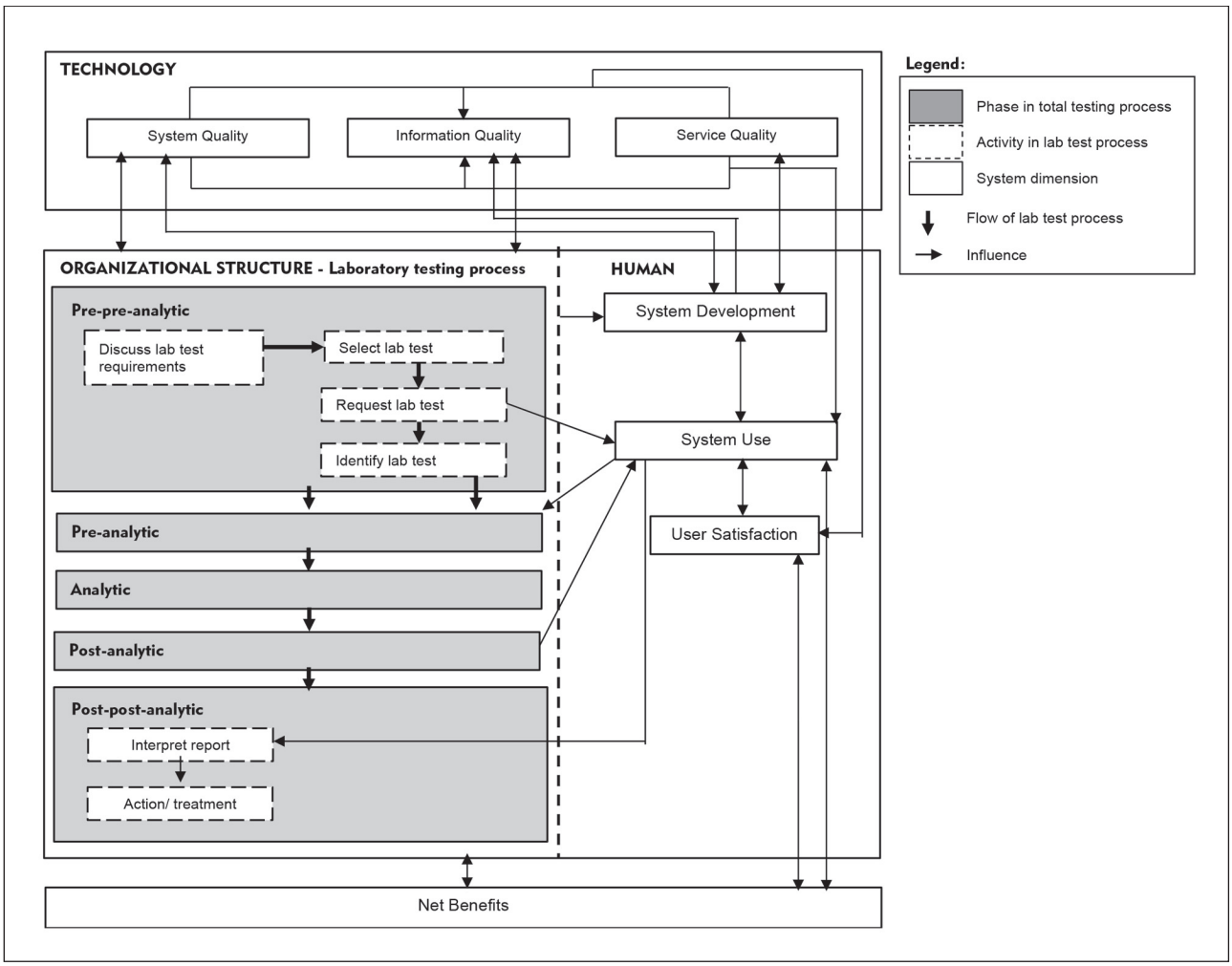

Figure 1 The proposed TTP-LIS framework.

framework on the basis of a combination of TTP and human, organization, technology and fit (HOT-fit) frameworks (10, 11). The HOT aspects are crucial elements that complement the evaluation of the LIS and lab testing process. The proposed framework aims to illustrate a systematic, coordinated, and optimized laboratory testing process and LIS flow to facilitate a rigorous error evaluation (12). The evaluation factors, dimensions, measures and their relationships are depicted in *Figure 1*.

Error evaluation can benefit from Lean, a quality improvement method that emphasizes on removing process waste, including error. Various Lean tools, such as value stream mapping (VSM), 5Why, and A3 problem-solving methods, have been widely used for process improvement (13). A3 is a structured approach to problem solving that uses a report tool to summarize the definition, scope, discovery process, findings, proposed action steps, and results from the problem analysis. A3 can be combined with other Lean tools, such as VSM and 5Why, to visualize and identify the root cause of problems. VSM is used to illustrate the overall process to identify waste/ problems and the appropriate solutions in the current and future state map, respectively. The problem can be scrutinized using the 5Why tool to identify its root cause and mitigation strategy by asking a series of question, either five times or any appropriate range. The study focused only on pre-pre-analytic and post-post-analytic phases of the TTP framework, given their high error rates (14, 15), compared to other phases.

Material and Methods

We conducted a subjectivist case study strategy employing qualitative methods in this summative evaluation to examine errors related to the LIS and the first and last phases of the lab testing process. A subjectivist approach enabled a comprehensive understanding of the healthcare context surrounding the management of LIS-induced error by generating detailed, insightful explanations (16, 17). We performed evaluation by applying the TTP-LIS framework at two premier private hospitals in Malaysia. These cutting-edge hospitals have been leading the national health care and are recognized by accreditation bodies such as the Malaysian Society for Quality in Health, Joint Commission International (XI), and Quality Management System (MS ISO 9001: 2015). The local Institutional Review Board deemed this study exempt from review. Author AA, a trained qualitative researcher, collected the data through interviews, non-participant observations, and document/artifact analysis methods.

Sampling

A purposeful snowball sampling method provided in-depth information from key informants. We identified participants from our initial contact with the lab director. We discussed the appropriateness of selected informants with the lab head based on their respective expertise, job scope and abilities in providing the required information. Finally, we recruited 15 participants, including clinicians and management, lab, and IT staff (*Table I*).

Table I Participant list and method description.

Method	Participant (N)	Description
Interview	Physician (2) Nurse (2) Lab head (1) Lab staff (2) IT staff (1)	• Semi structured interview questions were formulated according to the job description and role of participants
	Total = 8	
Document analysis	Physician (1) Lab head (1) Lab technician (1) IT staff (1)	• Lab test request form • Statistical report of the lab test request form • Statistical report of the lab test results (non/late access, location, and test type) • Monthly/annual report • Improvement in the lab testing process • LIS improvement report (based on modules/ functions/others)
Observation	Lab head (1) Lab staff (2)	• Process flow of the lab test request • Lab test report process
	Total = 15	

Data collection and analysis methods

The face-to-face, one-on-one interview lasted for one to two hours for each informant who we queried on lab testing process, LIS use, error and mistake incidents, their causes, and the strategies for mitigation and LIS improvement. We audio recorded and transcribed interviews. Observation took place in a medical lab for over a day on lab testing processes, from clinical requests to the production of lab results, to identify potential LIS-induced errors. We analyzed documents related to LIS' overall development, operation and management, process owner, backup system handling, and software and hardware management. We analyzed data thematically using the initial TTP-LIS evaluation framework (12). In addition, we employed three Lean tools, namely VSM, A3 *Problem Solving*, and 5Why to visualize the current process, its problems and root cause, and the desired (future) state of the first and last phases of lab testing (13). We validated and refined the TTP framework with an expert who reviewed and acknowledged the said framework as a comprehensive evaluation tool for the lab testing process and LIS.

Results

The hospitals PHA and PHB were established in the mid-1990s. They collaborated with a private laboratory, Lab C, which has managed most lab operations at all PH branches since 2000. The hospitals provide services to 3000 to 4000 patients at a time and provide educational services to medical and nursing students. Evaluation of the overall system used in the hospitals and laboratories involved the LIS, lab testing process and other health information systems (HISs). The LIS evolved from a stand-alone system that only supports internal laboratory operations to a system with extended functions that are connected to HISs. The LIS was also developed by the IT unit of Lab C whereas the HIS was outsourced and operated by the hospital IT unit. Both systems are integrated in a new platform. The IT staff in Lab C provide training to LIS users. *Figure* 2 illustrates the overall findings according to the proposed TTP-LIS framework.

Human factors

Overall, the LIS was optimized by the lab staff compared to the hospital staff. Many clinicians did not attend training because of time constraints and their heavy workload. Lack of training and exposure to LIS result in low system use. Users, particularly senior physicians and nurses, are reluctant to use the LIS to request lab tests and access its results for various reasons such as »wasting time, hassles to remember password, patient name or id« (Lab Head). According to a physician, »system use disrupted my task. Sometimes the LIS processes data slowly and requires time consuming access, while the network is disrupted during lab test request. The manual form saved more time.« A nurse stated that although »system use eased our task, our competency is low«.

LIS use is mandatory only in some PH branches, while others still operated manually. The LIS use started from the laboratory and expanded to clinical units. However, poor synergy and discrepancies between management and IT in planning and strategizing the LIS affect system development and the subsequent non-optimized LIS use in clinical units. Poor system development is also attributed to poor service quality in terms of responsiveness, assurance (service providers' skills, consideration and ability to provide trust and confidence (18)), and empathy from the service provider and hospital management. Decisions for system development were made according to individual or other interest including politics, such as conflict of interest and business profit, instead of system use. The integration of heterogeneous, outsourced, and in-house developed systems with different platforms, hardware, and software resulted in many system problems, such as unreadable information, unclear images (blurred, inappropriate pixel sizes, and display of system coding), and inaccessible information. These problems pose challenges to the clinical unit and the physicians' decision making pertinent to patient diagnosis or treatment because of inaccurate data. Subsequently, these issues affect system use, user satisfaction, and the lab test process. Physicians and nurses preferred the manual method in requesting lab tests and obtaining lab test results as they perceived as faster than those of LIS. Instead of increasing process efficiency, LIS use delayed tasks and disrupted the decision-making process. In short, system development outcomes significantly affect the system and information quality, and service quality determines the fulfillment of user requirements.

Technology factors

System quality influenced other factors including system development, system use, the lab testing process, and user satisfaction. We identified errors that stemmed from poor LIS functions, including the number of lab test results that are less than the actual number of applied tests. Moreover »[some] lab test results accessed from LIS showed unexpected analysis when the results are linked to diagnosis results from the CIS« (Dr. B).

Organizational factors

The whole lab testing process takes around 15–20 minutes, if there is no disruption, to paste bar code on specimen tubes and application form, entering request information in LIS, testing specimen and

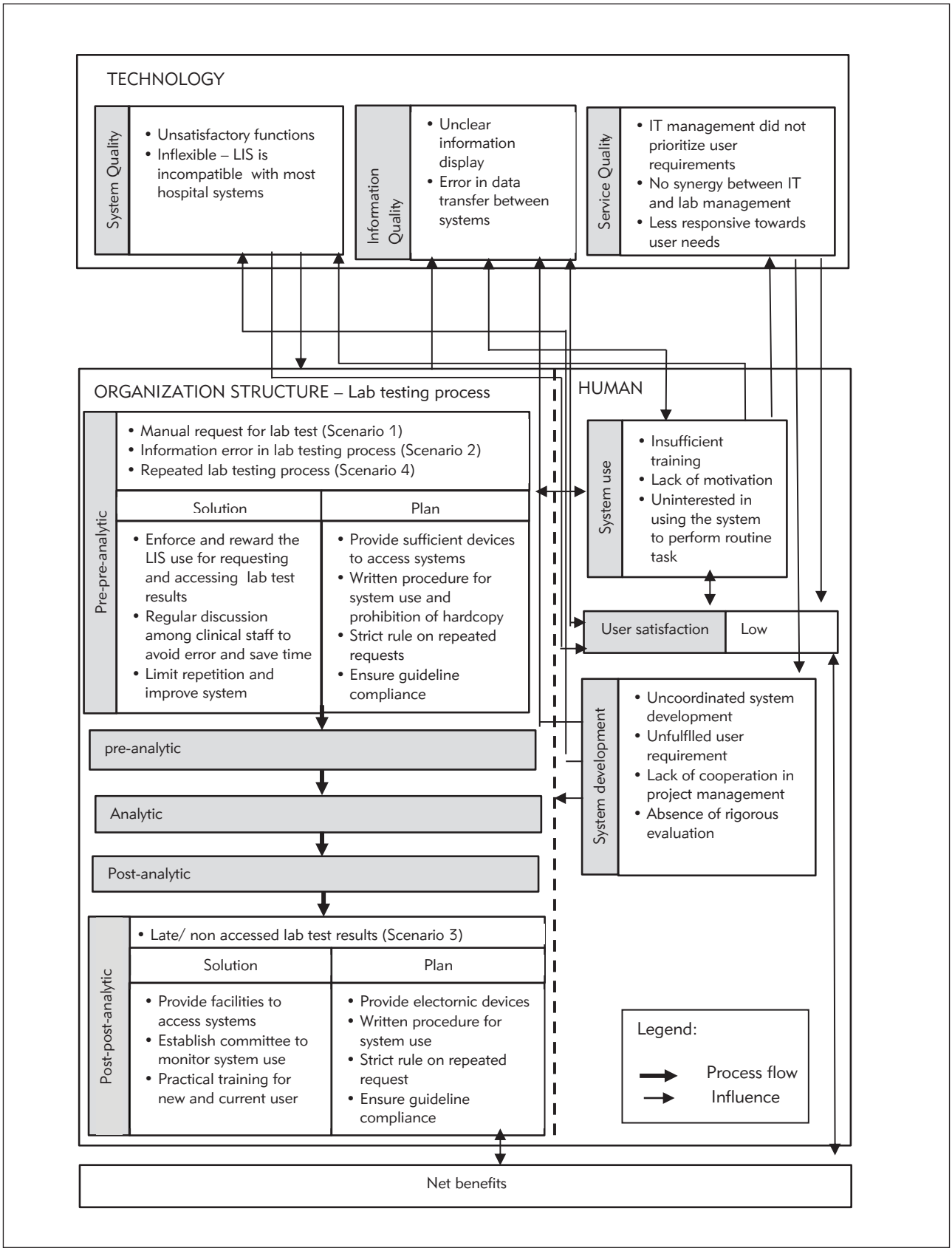

Figure 2 Error factors in the lab testing process and LIS.

verifying lab test results. We chose to analyze four process scenarios that were recommended by the informants according to their error impact on the overall workflow in terms of additional time, increased workload, material waste, and (most importantly) delay in patient treatment. Scenario 1 (manual request of the lab test process and printing lab test results) became problematic as it resulted in extra workload for lab staff to routinely check or request missing information on the manual form, file, print documents, and »...the patient code on manual forms need to be individually scanned and checked to ensure its consistency with the system« (lab head). Then, the lab test results must be printed and sent to physicians or nurses. Missing or lost results required another print out and the same goes for physicians who request patient lab test histories. Increased burden arises from the error chain, whereas a physician's error rippled to the lab unit and the prescribing process that involves lab test results.

Erroneous test request (Scenario 2) occurred due to several reasons, as claimed by the informants. »We must perform the test upon receiving the sample and request form. We would not able to identify the request as a mistake when the request information is consistent with those of the system« (lab staff). »Choosing the wrong test commonly happened in critical situations where [the] physician does not have time to check [the] test requested by the nurse« and the nurse »forgets to verify it with the physician.« A mistake is usually realized upon test completion. Non accessed/delayed lab test results (Scenario 3) recurred because of non-scrutinized processes or hasty decisions. According to the lab head, the situation affects staff efficiency, particularly when they must prioritize other urgent lab tests. Lab staff were puzzled when »a requested test results were not accessed upon its completion, [thereby] indicating that the test is not needed, [a situation] which wasted our time and resources to conduct the test.«

In Scenario 4, the repeated lab testing process is attributable to the inefficiency of the clinical unit and sample testing process. Lab testing is repeated when the laboratory or physician identified test results that are abnormal or fall outside the reference range lab test or unidentified errors were present in the test request. Upon realizing these abnormalities and erroneous request during results validation, the lab head ordered a second and correct test request, respectively. If the first and second test results are consistent, they are categorized as a critical case and the physician is contacted immediately. Result abnormalities are entailed for the second test, whereas erroneous request attributable to staff carelessness or inefficiency should be avoided. Similar to Scenario 2, the prescriber's verification is imperative before submitting the test request.

According to the four scenarios of the two lab testing processes for pre-pre-analysis and post-post-analysis (*Figure 1*), A3 diagrams are used to illustrate and elaborate upon the as-is and to-be processed elements as demonstrated in Scenario 2 (*Figure 2*). The process is related to lab test request by a nurse or clinical assistant using the LIS. A nurse was instructed by a physician to request for a lab test using a CIS. The nurse labelled sample tubes and stored them while waiting for a lab staff member to collect them. Then, the nurse directly entered the related information for requesting the lab test in the computer unit. However, the test type that she chose differed from that desired by the physician.

Normally, neither the nurse nor the lab head would realize the mistake until the physician checks the order before submitting it to the LIS. Therefore, the test was processed normally according to the requested test type. Upon the test completion, the results were generated, checked, and verified by lab head. Then, the results were submitted to the CIS via the LIS. A physician accessed the lab test results, only to realize that they are irrelevant. At this point, the charge was already forwarded to the finance unit for patient billing. This mistake required the physician to report the occurrence to the management and finance, and the charge must be paid by the hospital. Therefore, double checking and verifying test requests are critical to avoid a chain of problems. The physician is responsible for rechecking requests, and the nurse must remind the physician about it before submission. We illustrated the problems to aid in identifying the root cause and planning for mitigation as follows.

A3 *Problem Solving* report for Scenario 2

ISSUE

Mistake in selecting lab test type during the request through the LIS.

BACKGROUND

The nurse received instruction from the physician to request for a lab test via the LIS. The nurse did not realize that she had mistakenly chose the wrong test type during the request process.

FUTURE STATE

The to-be processed flow diagram is similar with that of the as-is process (*Figure 3*), except for the replacement of the two problems with the following two solutions.

SOLUTION STEPS

Detailed discussion among the medical team of a mitigation plan to avoid recurring mistakes and resources waste.

CURRENT STATE

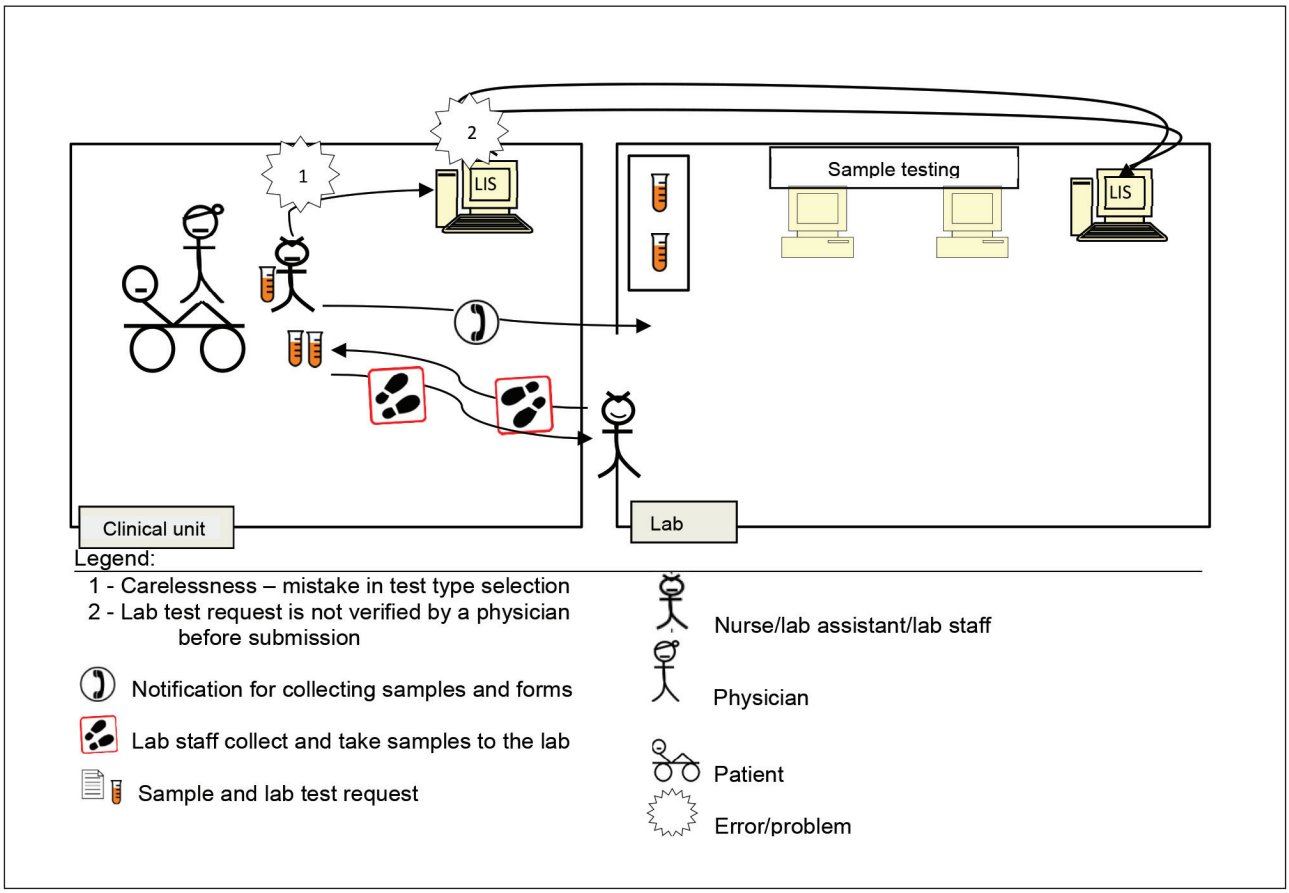

Figure 3 As-is Process of Scenario 2.

PROBLEM ANALYSIS

1. Carelessness – mistake in test type selection	
Why?	Lack of focus in receiving order
Why?	Irrelevant text/unclear test type selection/unfriendly user interface resulting in user misunderstanding
Why?	Poor system/ interface design
2. Lab test request is not verified by a physician before submission	
Why?	Slip due to forgetfulness
Why?	Urgency to submit the test request
Why?	Non-compliance with the standard operating procedure (SOP)

IMPLEMENTATION PLAN

Plan	Expected results
Lab test verification/auto verification is included as part of SOP in using LIS	The procedure for lab test verification is followed
Impose the procedure (e.g.: reminder on main web/mobile LIS interface, awareness campaign, training, and poster)	Physicians and staff are aware of and adhere to the procedure

COSTS/BENEFITS

Cost
System upgrade to include auto verification and alert functions
Awareness intervention programs
Benefits
Reduced mistakes in lab test requests
Increased efficiency and reduced turnaround time for lab test request and initializing patient treatment

Discussion

We went through a relatively challenging, iterative process of constructing structured and comprehensive socio-technical factors in the TTP-LIS framework (12). This study contributes to the existing knowledge by proposing a new framework based on the HOT-fit and TTP frameworks, as well as concepts in error management and process improvement, namely the Lean methods. The TTP-LIS framework features a comprehensive evaluation method of socio-technical factors that can be applied flexibly in other processes and systems in a similar or different clinical settings. The findings showed the practicality of the TTP-LIS framework as an evaluation tool in identifying errors and their causal factors. The use of Lean tools, namely, A3 report, VSM, and 5Why, enabled us to analyze and visualize the root cause of problems in an objective and structured manner (13, 32). The evaluation of LIS-induced error enabled the IT staff in both laboratory and hospital to collaborate in improving LIS quality by synchronizing system development to reduce system integration problems and considering system functions according to user requirements. Human, lab testing process, organization and technology factors are intertwined. Errors caused by human (4, 7) technology (5), and processes (3, 9) disrupted the lab testing process workflow. Human factors mainly contribute to errors in the lab testing process and LIS, as proven in other studies (7). Errors in system development and use that are attributed to human factors require continuous evaluation and monitoring to ensure quality. The LIS supports user needs (3, 19) and routine tasks and reduces problems (20). Mandatory use of the LIS among physicians and nurses is meant to increase the efficiency of routine tasks in the lab testing process. However, LIS use among clinicians is very low. In general, the findings can be categorized as follows: latent failure in system development, poor error management, and unsatisfactory lab testing process and LIS use.

Latent failure in system development

System development highly contributed to error occurrence in the LIS and HIS use in terms of introduction of new technology, heterogeneous software, human–computer interaction, and communication issues within the system developer team. These factors are consistent with other findings (3, 5, 6). These latent failures hinder the optimized potentials of the LIS. The case LIS developers really understand the requirements of the lab testing process and featured them as the main functions in LIS. In contrast, the HIS was outsourced; the hospital management team identified more general user requirements. This resulted in integration conflict and subsequent errors, including unclear data requirement and inappropriate graph generation that that affect physician decision making.

Latent failure is a major challenge for management and organizational decision makers. Strong collaboration between management with both hospital and laboratory units can aid in solving latent failure (21). During the system development, risk factors should also be considered apart from the cost. Heterogeneous system development methods increased error risk and cost. On the contrary, a unified system development method that considered

user requirement reduced error risk. The study can be extended to further understand latent failure factors and identify optimum strategies to address them.

Poor error management

In general, LIS-induced errors require tackling the problems at their root cause and employing a basic solution method from the socio-technical perspectives, before quality improvement and automation (3, 22–25), as proposed in our error management approach. Most identified errors can be mitigated through a joint, multi discipline collaboration from all staff. However, monitoring is imperative at the outset (26) to ensure guideline compliance. An error management method serves as a tool to mitigate errors identified by the system or through routine error checking at the end of a task completion. The absence of an error management system led to recurring errors (27, 28) that waste time, resources, and cost in terms of service or materials. Recurring errors also indicate ineffective and inefficient workflow and system use that negatively affects work motivation. Many error management strategies have been successfully proven in other industries and can be adopted in laboratory and clinical settings. These strategies include 1) reducing cognitive load through automated record, notes, and process (e.g., verification and checking); 2) enhanced information access; 3) imposing an error-proofing function for critical tasks such as preventing fatal drug instruction according to the dosage for certain patients; 4) checking error at its source (individual process step); 5) coordination of similar tasks; and 6) minimizing individual involvement in a single task (29–32).

Lab testing process and LIS use

User acceptance and sufficient training increase LIS use in lab testing workflow and subsequently ensure smooth flow and enhanced work quality (3, 7, 20, 33). However, a lean workflow is imperative prior to optimizing the process automation to improve the core issues in the workflow itself (3, 13). Various efforts have been made to reduce errors in routine monitoring, particularly in the early and final phases of the lab testing process, given that both phases involve clinical instead of lab staff who are more familiar with the related process. Therefore, inter departmental cooperation is crucial for avoiding recurring errors.

In short, although all scenarios involved simple errors and mistakes, they posed various possible implications, such as inefficiency, high workload, adverse events, and patient safety issues. Inappropriate testing is not only wasteful and costly, but also risky to patients (31). However, the processes can be streamlined and optimized through management and mitigation of process and error. Automated interventions such as an ordering system that alerts prescribers can educate them about requesting inappropriate or repeated testing (31). Moreover, auto verification is widely reported to have potential for facilitating safe, efficient, and reliable tools (30, 34). We proposed a comprehensive plan to avoid errors in the early and final lab testing process. The steps include

- analyzing and redesigning workflow according to Lean methods;
- establishing clear, written, and digital procedures;
- improving system training for users;
- outlining indicators for quality monitoring; and
- improving communication and synergy among healthcare and laboratory professionals.

The procedure for lab testing workflow must clarify patient identification; gathering, labelling, and transferring specimens; and analysis preparation. The responsible individual must understand and acknowledge the procedure and its importance, the potential risk, and effect on the sample and subsequently to the patient because of procedure noncompliance. All steps required ongoing training and efficient assessment.

Study limitations

The short duration of the observation limited the detail evaluation of possible error incident during the lab test process but this situation was offset with a briefing from the lab head. Moreover, documents related to LIS use and the lab testing process are restricted as they are regarded as private and confidential. Furthermore, manual requests for laboratory tests limit the evaluation of LIS use in clinical units, particularly in the pre-pre-analysis phase. Nevertheless, the rich interview data compensate for this constraint.

Author contributions: All authors accept responsibility for the entire content of this manuscript and have approved its submission.

Informed consent: We obtained informed consent from all individuals involved in this study.

Acknowledgements: This work was supported by the Ministry of Higher Learning Malaysia (Grant no.s FRGS/1/2018/ICT04/UKM/02/5 and ERGS/1/2011/STG/UKM/02/46).

Conflict of interest statement

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

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Received: March 19, 2021
Accepted: April 22, 2021

SCINDEKS
Serbian Citation Index
1J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-30420

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 32–39, 2022

Original paper
Originalni naučni rad

DIFFICULTIES IN THE DIAGNOSIS OF HbS/BETA THALASSEMIA: REALY A MILD DISEASE?

TEŠKOĆE U DIJAGNOZI HbS-BETA TALASEMIJE: ZAISTA BLAGA BOLEST?

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Summary

Background: HbS/β cases having clinical, hematologic and electrophoretic similarities cannot be sufficiently distinguished from sickle cell anemia cases and are misdiagnosed as sickle cell anemia. This study will investigate the congruence between the HPLC thalassemia scanning tests and the laboratory findings compared to the DNA sequence analysis results of the patients diagnosed with SCA between 2016 and 2020. This study also aims to indicate the current status to accurately diagnose sickle cell anemia and HbS/β in the light of hematologic, electrophoretic and molecular studies.

Methods: Fourteen patients who were diagnosed with SCA in hospitals at different cities in Turkey and followed by the Thalassemia Diagnosis, Treatment and Research Center, Muğla Sıtkı Koçman University were included in this retrospective study. The socio-demographic characteristics, hemogram, hemoglobin variant analysis results and DNA chain analysis results of the patients were taken from the database of the centre and then examined. The informed consents were taken from the patients. The patients were administered a survey containing questions about transfusion history and diagnostic awareness. The Beta-Thalassemia mutations were analysed using a DNA sequencer (Dade Behring, Germany) based on the Sanger method.

Results: According to the DNA sequence analysis, the results of these patients diagnosed with SCA in hospitals in different cities of Turkey were the following: of 14 patients, 8 had HbS/β^0, and HbS/β+ and one had HbS carrier, and one had Hb-O, and three had SCA. The patient with HbS

Kratak sadržaj

Uvod: Slučajevi HbS-β koji imaju kliničke, hematološke i elektroforetske sličnosti ne mogu se dovoljno razlikovati od slučajeva anemije srpastih ćelija i pogrešno se dijagnostikuju kao anemija srpastih ćelija. Ova studija će istražiti podudarnost između HPLC testova za skeniranje talasemije i laboratorijskih nalaza u poređenju sa rezultatima analize DNK sekvence pacijenata sa SCA dijagnozom između 2016. i 2020. Ova studija takođe ima za cilj da ukaže na trenutni status tačne dijagnoze anemije srpastih ćelija i HbS-β u svetlu hematoloških, elektroforetskih i molekularnih studija.

Metode: U ovu retrospektivnu studiju je bilo uključeno četrnaest pacijenata kojima je dijagnostikovan SCA u bolnicama u različitim gradovima Turske, a koje je pratio Centar za dijagnozu, lečenje i istraživanje talasemije, na Univerzitetu Muğla Sitki Kočman (Muğla Sıtkı Koçman). Socio-demografske karakteristike, hemogram, rezultati analize varijante hemoglobina i rezultati analize DNK lanca pacijenata su uzeti iz baze podataka centra i potom ispitani. Od pacijenata je obezbeđena informisana saglasnost. Pacijentima je data anketa koja je sadržala pitanja o istoriji transfuzije i svesti o dijagnozama. Mutacije beta-talasemije su analizirane pomoću DNK sekvencera (Dade Bering, Nemačka) na osnovu Sangerove metode.

Rezultati: Prema rezultatima analize DNK sekvence ovih pacijenata kojima je dijagnostikovan SCA u bolnicama u različitim gradovima Turske od 14 pacijenata je 8 imalo HbS-β^0 i HbS-β+, a jedan je bio HbS nosilac, jedan HbO, a tri osobe su imale SCA. Pacijent sa statusom nosioca HbS

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carrier status also contains three additional mutations, all of which are heterozygous. We discovered that although two of three mutations, which are c.315+16G>C and c.316-185C>T, are previously reported as benign, at least one of the two mentioned mutations, when combined with HbS, causes transfusion-dependent HbS/β.

Conclusions: Briefly, HbSS and HbS/β thalassemia genotypes cannot be definitely characterized by electrophoretic and hematologic data, resulting in misdiagnosis. c.315+16G>C and c.316-185C>T are previously reported as benign; at least one of the two mentioned mutations, when combined with HbS, causes transfusion-dependent HbS/β. In undeveloped or some developing countries, molecular diagnosis methods and genetic analyses cannot be used. If mutation analyses could be performed, then such differential diagnosis errors would reduce. However, if mutation analysis cannot be performed, other methods such as HPLC, capillary electrophoresis absolutely be sought to have insight into the parental carriage status.

Keywords: SCD, sickle cell anemia, HbS/β, HbSS, Sickle-β^0-thalassemia, genotype, fenotype

je takođe imao tri dodatne mutacije koje su sve heterozigotne. Otkrili smo da, iako su dve od tri mutacije, a to su c.315+16G>C i c.316-185C>T, prethodno prijavljene kao benigne, bar jedna od dve pomenute mutacije, u kombinaciji sa HbS, izaziva transfuziono zavisni HbS/β.

Zaključak: Ukratko, genotipovi HbSS i HbS/β talasemije ne mogu se definitivno okarakterisati elektroforetskim i hematološkim podacima, što rezultira pogrešnom dijagnozom. Utvrđeno je da bar jedna od dve pomenute mutacije, c.315+16G>C i c.316-185C>T, koje su prethodno prijavljene kao benigne, u kombinaciji sa HbS izaziva transfuziono zavisni HbS/β. U nerazvijenim zemljama, ili nekim zemljama u razvoju, metode molekularne dijagnoze i genetske analize se ne mogu koristiti. Ako bi se mogle izvršiti analize mutacija, onda bi se takve greške diferencijalne dijagnoze smanjile. Međutim, ako se analiza mutacija ne može izvršiti, apsolutno se traže druge metode poput HPLC-a, kapilarne elektroforeze kako bi se dobio uvid u status prenošenja sa roditelja na decu.

Ključne reči: SCD, anemija srpastih ćelija, HbS/β, HbSS, Srp-β^0-talasemija, genotip, fenotip

Introduction

Sickle cell diseases (SCD) affect millions of people worldwide. It is estimated that there are more than 300 million people who have (HbSS, SC, SD, SE, S/β, SO-Arab) or carry sickle cell disease, which is expected to be increased gradually (1, 2). 300,000–400,000 babies with SCA are born every year around the world, and tens of thousands of people have the most severe clinical phenotype of the disease – the homozygous HbSS form (3, 4). It can be seen everywhere in the world, but it has an epidemic course, especially in Sub-Saharan Africa, Saudi Arabia, India, Central and South America, Middle East countries and Mediterranean countries (5, 6). In Turkey, sickle cell disease and carriage are common, especially in the southern Mediterranean coasts, with a prevalence ranging from 0.3% to 44% (7).

Sickle cell disease is the most common one of the monogenic disorders caused by a point mutation in the 6th codon of the β-globin gene (HbS or HbB: c.20A> T) (6, 8). It originates from homozygous or compound heterozygous mutation of the abnormal hemoglobin S that forms as a result of the conversion of glutamic acid in the β-globin chain to valine (6, 9). SCD has a multi-systematic and complex physiopathology that can damage every organ and tissue in the body (8). The trigger of all complications is the deoxygenated HbS polymerization (6). The genotype is a key determinant of the clinical severity of SCD (4, 10).

More than 15 genotypes, which cause sickle cell disease, were determined (11). Homozygosis is the most common and severe genotype of the disease with the shortest survival and is called sickle cell anemia (SCA) (4, 11). Other main pair heterozygote SCD types are the hemoglobin S/DPunjab, hemoglobin S/E, hemoglobin SO-Arab, hemoglobin SC disease (HbS/C), sickle-β +-thalassemia (HbS/β+) and sickle-β^0-thalassaemia (HbS/β^0) forms. HbS/β cases are called HbS/β+ and HbS/β^0 according to the different hemoglobin A levels. Higher hemoglobin A and F levels are characterized by a milder phenotype. It is difficult to clinically distinguish S/β^0 thalassemia from sickle cell anemia (4, 8, 10). Therefore, parenteral carriage status and mutation should be analysed for an accurate diagnosis (4, 10).

In particular, HbS/β cases having clinical, hematologic and electrophoretic similarities cannot be sufficiently distinguished from sickle cell anemia cases, and are misdiagnosed as sickle cell anemia (10). Although they are clinically treated similarly, the specific profiles of the genetic and pathophysiological mechanisms of patients with HbS/β are not yet well known. On the other hand, there are limited data to describe the profile of clinical complications of HbS/β patients (8). The clinical phenotype severity varies even among individuals with the same genotype (12). The complication incidence varies over time in the same individuals and among different individuals (4, 11). Patients manifest a dramatic range of severity ranging from a milder clinical course to severe transfusion dependence and progressive organ damages (8).

One of the important points is that SCA manifests itself in the second 6-month postpartum period, while HbS/β may not manifest until puberty (7). However, it should be noted that even patients with mild SCD (HbSC and HbSβ+) forms may have vaso-occlusion attacks and hemolytic anemia as well as all serious and life-threatening complications, which are seen in SCA (13, 14). If a patient with HbS/β is misdiagnosed as SCA, this would have significant effects for the next generation; hence an accurate diagnosis

is crucial to prevent future diseases for the next generations.

This study will retrospectively investigate the congruence between the HPLC thalassemia screening tests and the laboratory findings in comparison with the DNA sequence analysis results to understand how accurately patients with SCA were diagnosed based on this information.

Materials and Methods

Ten (73.4%) female and four (26.6%) male patients, who admitted to the Thalassemia Diagnosis, Treatment and Research Centre, Muğla Sıtkı Koçman University Education and Research Hospital between January 1, 2016, and October 31, 2020, were included in this retrospective study. The ethics approval was taken from Muğla Sıtkı Koçman University Education and Research Hospital on July 3, 2020, and the Ethics Committee on September 26, 2020, with the document numbered 2020/10. This study was conducted following the Helsinki Declaration's principles.

Fourteen patients who were diagnosed with SCA in hospitals in different cities in Turkey and followed by the Thalassemia Diagnosis, Treatment and Research Centre, Muğla Sıtkı Koçman University were included in the study. The socio-demographic characteristics, hemogram, hemoglobin variant analysis results and DNA chain analysis results of the patients were taken from the database of the centre and then examined. The informed consents were taken from the patients. The patients were administered a survey containing questions about transfusion history and diagnostic awareness.

Their red blood cell index parameters were determined using Sysmex XN 1100 (Sysmex Diagnostic, Japan).

The hemoglobin variant analysis was performed using Primus Ultra II device (Trinity Biotech Diagnostic, Ireland) based on the high-pressure liquid chromatography (HPLC) byion exchange chromatography.

The Beta-Thalassemia mutations were analysed using a DNA sequencer (Dade Behring, Germany) based on the Sanger method.

Results

Our patient group consisted of 10 female (73.4%) and 4 male (26.6%) patients aged 6 months to 54. The main findings of the study; our patients had a wide range of clinical severity, ranging from mild joint pains to transfusion dependence.

According to the DNA sequence analysis, the results of these patients diagnosed with SCA in hospitals in different cities of Turkey were the following: of 14 patients, 8 had HbS/β^0 and HbS/$\beta+$ and one had HbS carrier, and one had Hb-O, and three had SCA.

The patient with HbS carrier status also contains three additional mutations, all of which are heterozygous. Although two of three mutations, which are c.315+16G>C and c.316-185C>T, are previously reported as benign, at least one of the two mentioned mutations, when combined with HbS, causes HbS/β status (15). This patient is transfusion-dependent and has pain crises. This patient's MCV value indicates microcytosis, which is compatible with HbS/β.

The patients in our study had the first manifestations and the initial diagnosis at 5, 6 or 12 years old.

Discussion

This study will investigate the congruence between the HPLC thalassemia scanning tests and the laboratory findings in comparison with the DNA sequence analysis results of the patients diagnosed with SCA between 2016 and 2020. This study also aims to indicate the current status to accurately diagnose sickle cell anemia and HbS/β in the light of hematologic, electrophoretic and molecular studies.

Table I Hematologic differences between SCA and S/Beta in literature.

Patients & Tests	HbSS	Hb S/β0	Hb S/β+
RBC Morphology	Normocytic	Microcytic	Microcytic
	Normochromic	Microchromic	Microchromic
Hemoglobin Electrophoresis			
A2 (%)	<3.5	>3.5	>3.5
F (%)	<10	<20	<20
A0 (%)	0	0	20–30
S (%)	>90	>80	>60
Phenotype	Usually Heavy	Medium-Heavy	Mild-Medium

Table II Hematologic and molecular diagnostic data of patients with SCA.

Pt.	Age	Sex	RBC (10^12cells/L)	HGB (g/L)	MCV (10^-14 L/cells	MCH (10^-11 g/cells)	B12 (pmol/L)	Folic Acid (nmol/L)	Hb S (%)	Hb A2 (%)	Hb A0 (%)	Hb F (%)	Genotype (Only pathogenic variants shown)	Phenotype
1	19	M	2.82	86	8.76	3.05	231	> 45	61.7	4.5	10.7	28	-	Hb S/β
2	38	F	3.69	84	7.05	2.28	312	24	46.4	5.8	46.2	1.6	heterozygous c.20A>T, heterozygous c.25_26delAA	Hb S/β0
3	40	F	3.46	91	7.80	2.63	188	> 45	44.9	5.4	44.2	5.5	heterozygous c.20A>T	Hb S Carrier
4	35	F	2.84	65	7.15	2.29	154	17	50.8	5.5	40.7	3.0	heterozygous c.20A>T, heterozygous c.316-106C>G	Hb S/β+
5	35	F	2.06	76	10.19	3.69	364	15	69.9	4.9	20.3	4.9	homozygous c.20A>T	Hb SS
6	54	M	5.48	120	6.55	2.19	-	-	-	5.3	14.9	1.8	heterozygous c.93-21 G>A, heterozygous c.364G>A	Hb O-Arab/ß+
7	38	F	3.37	74	6.71	2.20	-	-	72.5	6.1	13.2	8.2	herterozygousc.20A>T, heterozygous c.93-21G>A	Hb S/β+
8	24	F	2.55	59	7.37	2.31	-	17	76.7	6.9	9.0	7.4	heterozygous c.20A>T, heterozygous c.316-106C>G	Hb S/β+
9	42	M	2.84	86	8.59	3.03	441	39	82.2	4.9	2.9	10	homozygous c.20A>T	Hb SS
10	60	F	2.19	85	11.05	3.88	242	18	48.3	5.2	4.0	42.5	heterozygous c.315+1G>A, heterozygous c.20A>T	Hb S/β0
11	54	F	2.72	53	5.90	1.95	352	-	82.8	6.1	3.4	7.7	-	Hb S/β
12	26	M	3.45	96	7.68	2.78	245	12	77	3.7	4.6	14.7	homozygous c.20A>T	Hb SS
13	30	F	2.61	100	9.89	3.83	-	-	78.5	4.7	3.0	13.8	homozygous c.20A>T	Hb SS
14	18	F	3.79	92	7.28	2.43	-	-	69.5	6.5	11.4	12.6	heterozygous c.20A>T, heterozygous c.93-21G>A	Hb S/β+

Note:*RBC, red blood cell; Hb, hemoglobin concentration; MCV, mean cell volume; MCH, mean corpuscular hemoglobin

In the literature, HbS/β was characterized by fewer sickle cells and microcytic, hypochromic RBC, and a distinction was made between normocytic normochromic anemia and SCA to describe the explanatory variables between hemoglobin HbSS and HbS/β (*Table II*) (5, 6). However, splenomegaly is an important distinctive finding among the clinical symptoms. It was reported that SCA patients have hemolytic anemia with a progressive course in the second 6-month postpartum period and early childhood period as well as chronic splenomegaly, acute splenic sequestration crisis, splenic infarctions. However, HbS/β patients have no splenic infarction in the childhood period but have severe, palpable splenomegaly and associated sequestration crisis and rarely organ failure in the adulthood period (16, 17). In the multi-centre study by Belgemen Özer et al. (18), the hematologic, molecular and clinical data of 55 HbS/β patients were analyzed and compared with the literature data. It was reported that there were cases that were not consistent with the literature, with varying hemograms, hemoglobin electrophoresis and peripheral smear findings. In the study performed by Benites et al. (19) to compare the hematologic parameters of HbS/β^0 and HbS/β+ patients, a statistically significant difference was not found in any hemogram parameter except for leukocytes and platelets.

The results of our study on this controversial subject are mostly compatible with the literature. However, in analogy to the studies by Benites et al. (19) and Belgemen Özer et al. (18), our study had also patients who were incompatible with the literature. A HbSS patient had a low MCV; however, an elevated MCV was noted in some HbS/β patients, but it was found that this was caused by megaloblastic anemia. MCV/MCH is the basic distinctive hemogram finding between the two diseases; however, it is not sufficiently distinctive in cases of iron deficiency, B12-folic acid deficiency, nutritional deficiency, hypothyroidism, sideroblastic anemia, myelodysplastic conditions, HbSS accompanied by alpha-thalassemia mutation. Iron deficiency may cause the interpretation of HbSS as HbS/β, while megaloblastic anemia may cause the interpretation of HbS/β as Hb SS. Overlooking this may lead to erroneous diagnosis. However, it should be noted that it may be rarely detected at different values (20, 21).

In the study by Notarangelo et al. (22), HbS/β patients with confirmed molecular accuracy were classified according to the β mutation, and it was reported that some mutation differences with IVS-I-5 (G>C), IVS-I-5 (G>A) and IVS-I-110 had a more severe phenotype and clinical presentation, and some mutation differences were associated with a milder phenotype (8). The study by Belisário et al. (23) reported that the newly discovered 92 (C>T) and IVS-II-844 (C>A)/IVS-II-839 (T>C) mutation presented as a very mild HbS/β+ case.

The patient with HbS carrier status also contains three additional mutations, all of which are heterozy-

Table III Case histories.

Pt.	Diagnosis	Age at first diagnosis and first complaint	DNA sequencing at first diagnosis	Investigation of carrier status in parents at first diagnosis	Transfusion frequency	Splenectomy
1	BT Major At age 4: Hb S/β	Infancy. Unknown complaint	No	Yes. Unknown by the patient	Every 3 weeks	No
2	SCA and BTI At age 36: Hb S/β	5 years. Unknown complaint	No	No. Done at age 36	Every few months from five years of age	Yes
3	SCA and BTI At a later age: Hb S/β	Between 6–12 months. Jaundice	No	No. Done at a later age	Every 4–5 months	Yes
4	BT Major At age 17: Hb S/β	1.5 years. Unknown complaint	Yes	Yes. Carrier sibling	Every 2–4 months	Yes
5	At age 5: SCA At age 35: BTI At a later age: Hb S/β	5 years. Abdominal bloating and bone pain	Yes	No. Carrier sibling	Once a year	No
6	SCA	4–5 years. Fever and severe abdominal pain	No	No. Carrier sibling	Just once	No
7	Hb S/β	2 years. Crisis	No	Yes. Carrier sibling	Once or twice a year	No
8	At age 1.5: SCA At age 14: Hb S/β	1.5 years. Flu	No	Yes. Carrier sibling	Monthly	No
9	Hb S/β	5 years. Joint pain	No	Yes. Carrier sibling	Ten times a year	No
10	SCA	12 years. Never-ending pain crisis	No	No. Unknown by the patient	Twice a year	Yes
11	Hb S/β	6 years. Pains	No	Yes. Carrier sibling	Just once exchange transfusion	No
12	N/A	N/A	N/A	N/A	N/A	N/A
13	SCA	6 months. Unknown complaint	No	No. Affected sibling	3–4 times a year	No
14	SCA	2 years. Pain in joints, arms and feet	No	Yes. Three siblings with SCA	Once a year (First one this year)	Yes

gous. We discovered that although two of three mutations, which are c.315+16G>C and c.316-185C>T, are previously reported as benign, at least one of the two mentioned mutations, when combined with HbS, causes HbS/β status. This patient is transfusion-dependent and has pain crises (15). This patient's MCV value indicates microcytosis which is compatible with HbS/β. Especially in heterozygote individuals, the relationship between genotype and phenotype is important since it significantly affects the clinical severity. Some mutation differences are associated with milder phenotype, while some are associated with severe and progressive organ damages and predictable complications (24).

When examining the congruence between HPLC and DNA sequence analysis, patients in the study received transfusion regularly and used hydroxyurea. Therefore, hemoglobin A0, A2, F and S values may be misleading. However, the DNA sequence analysis results are the final diagnostic for HbS/β. On the other hand, a HbA2>3.5 is interpreted in favour of HbS/β, while a HbA2<3.5 is interpreted in favour

of SCA, in the literature. In the commonly used hemoglobin electrophoresis, since electrophoretic migrations and elution patterns of HbS and HbA2 are the same, they migrate together, and many abnormal hemoglobin values coincide at small values. Therefore, due to a methodological error in electrophoresis, the HbS band sometimes erroneously moves the HbA2 band over itself so that it seems to be high, or a part of the HbS band overlaps with the HbA2 band so that the HbA2 band seems to be higher than as is, eventually leading to diagnostic errors. Due to these errors, the normal HbA2 seems to be lowered so that accompanying alpha thalassemia can be assumed mistakenly, or the normal HbA2 seems to be elevated so that it can be classified as HbS/β. Similarly, the presence of paraprotein or a high concentration of polyclonal immunoglobulin may cause different hemoglobin band errors (5, 25, 26).

Although HPLC has more advantages, it should be noted that lowered HbA2 values may be measured due to errors caused by the co-elution of HbS and HbA2, analytic references, alpha thalassemia, iron deficiency or delta gene mutations (5). Electrophoresis has been commonly performed, but HPLC and DNA mutation analyses could not be performed in the past. However, today opportunities increase with advancing technology. Parenteral screening, molecular diagnosis methods and genetic consultation are recommended to make the final diagnosis, clarify the genotype-phenotype correlation and improve the predictability of complications.

The survey with patients in our study revealed that although the majority of patients are young, they do not know the nature of the disease and that parenteral screening was not performed for most patients in the diagnosis period. However, it is remarkable that the patients stated that they had known the two diseases. As is seen in the patients' statements in *Table III*, some patients were misdiagnosed and received the wrong treatment until very old age. It was found that the majority of the patients were diagnosed with SCA, but they had S/Beta. In our study, a patient had a major diagnosis of Beta-thalassemia since infancy, but it was found that she/he really had HbS/β when she/he was 36 years old. Similarly, the study by Eröz et al. (27) found that the patient aged 36 who was diagnosed with sickle cell anemia had beta-thalassemia based on the parenteral screening and mutation analysis results.

One of the important points is that SCA manifests itself in the second 6-month postpartum period, while HbS/β may not manifest until puberty (7). Given that SCD is a multi-systematic disease that may damage every organ and tissue in the body and has a complex pathophysiology, it may present with a wide range of clinical complications and organ damages. It may cause serious and life-threatening complications (13, 14). Patients who were admitted to a hospital with complaints of stroke and sequestration were reported (22, 28). Sequestration crisis is seen in HbSS or HbSβ^0 cases before the age of 5, while, in HbS/β cases, it may delay until puberty and early adulthood and progress rapidly, resulting in death as a result of hypovolemic shock (22).

Consequently, it was found that hematologic and electrophoretic studies cannot sufficiently distinguish between the two conditions, causing wrong diagnoses. Although epidemiological and laboratory data are well known, and molecular analyses are more accessible today, it should be noted that data may be overlooked and mistaken. For pediatric patients, it may be live-saving to identify children with HbSS and HbS/β thalassemia, to have information about the genotype, to estimate progressive organ damages and complications in patients with a more severe phenotype, to organize parent training to recognize the symptoms and to plan specific supervision, care and monitoring (29). The need for transfusion may be high due to severe splenomegaly, which is more commonly seen, especially in HbS/β patients. This transfusion need may be reduced after splenectomy (30, 31).

Also, if a patient with HbS/β is misdiagnosed as SCA, this would have significant effects for the next generation; hence an accurate diagnosis is a critical initial step to prevent future diseases for the next generations (25).

Conclusion

Briefly, HbSS and HbS/β thalassemia genotypes cannot be definitely characterized by electrophoretic and hematologic data, resulting in misdiagnosis. In undeveloped or some developing countries, molecular diagnosis methods and genetic analyses cannot be used. If mutation analyses could be performed, then such differential diagnosis errors would reduce. However, if mutation analysis cannot be performed, other methods such as HPLC, capillary electrophoresis should be sought to have insight into the parental carriage status.

Limitations of the study

The main limitation of this study was the small number of patients in our study population. The patients have received transfusion regularly, and we could not reach the DNA results of two patients. However, it provides an insight into HbSS – HbS/β in Turkey. To clarify this situation, the number of patients may be increased, and sex differences may be examined and analysed, which may fill a gap in the general population and affect the control of the disease.

Conflict of interest statement

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

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Received: January 18, 2021
Accepted: April 24, 2021

SCINDEKS Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-29914

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 40–46, 2022

Original paper
Originalni naučni rad

DO THE ETIOLOGY OF HYPONATREMIA AND SERUM SODIUM LEVELS AFFECT THE LENGTH OF HOSPITAL STAY IN GERIATRIC PATIENTS WITH HYPONATREMIA?

DA LI ETIOLOGIJA HIPONATREMIJE I NIVOI SERUMSKOG NATRIJUMA UTIČU NA DUŽINU BORAVKA U BOLNICI KOD GERIJATRIJSKIH BOLESNIKA SA HIPONATREMIJOM?

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Summary

Background: Hyponatremia can lead to a prolonged hospital stay and increased morbidity and mortality rates in geriatric patients. This study aimed to evaluate the effects of hyponatremia etiology and serum sodium (Na) levels on hospitalisation time in geriatric patients hospitalised due to hyponatremia.
Methods: The demographic characteristics, laboratory data, etiology of hyponatremia, and length of hospital stay were retrospectively recorded for 132 patients over 65 years of age who were hospitalised for hyponatremia.
Results: Of the 132 patients, 90 were female (68.2%), and 42 were male (31.8%). The serum Na levels of 66 (50%) patients were <120 mmol/L, those of 64 (48.5%) patients were 120–129 mmol/L, and those of two (1.5%) patients were >130 mmol/L. One hundred nine (82.6%) patients had hypoosmolar hyponatremia, 14 (10.6%) patients had isoosmolar hyponatremia, and nine (6.8%) patients had hyperosmolar hyponatremia. Also, 19.7% of the patients were hypovolemic, 37.9% were euvolemic, and 42.4% were hypervolemic. Hyponatremia etiology was congestive heart failure in 38 (28.8%) patients, syndrome of inappropriate antidiuretic hormone in 29 (22.0%) patients, gastrointestinal fluid loss in 24 (18.2%) patients, renal pathologies in 20 (15.2%) patients, the presence of drugs in 20 (15.2%) patients, and hypocortisolemia in one (0.8%) patient. The mean length of hospital stay for the patients was five (1–60) days. There was no statistically significant difference between the lengths of hospital stay

Kratak sadržaj

Uvod: Hiponatremija može dovesti do produženog boravka u bolnici i povećane stope morbiditeta i mortaliteta kod gerijatrijskih pacijenata. Cilj ove studije bio je da se procene efekti etiologije hiponatremije i nivoa natrijuma (Na) u serumu na vreme hospitalizacije kod gerijatrijskih pacijenata hospitalizovanih zbog hiponatremije.
Metode: Retrospektivno su zabeležene demografske karakteristike, laboratorijski podaci, etiologija hiponatremije i dužina boravka u bolnici za 132 pacijenta starijih od 65 godina koji su hospitalizovani zbog hiponatremije.
Rezultati: Od 132 pacijenta, 90 su bile žene (68,2%), a 42 muškarci (31,8%). Nivoi Na u serumu 66 (50%) pacijenata su bili < 120 mmol/L, kod 64 (48,5%) pacijenata su bili 120–129 mmol/L, a kod dvoje (1,5%) pacijenta > 130 mmol/L. Sto devet (82,6%) pacijenata je imalo hipoosmolarnu hiponatremiju, 14 (10,6%) bolesnika je imalo izoosmolarnu hiponatremiju, a devetoro (6,8%) pacijenata je imalo hiperosmolarnu hiponatremiju. Takođe, 19,7% pacijenata bilo je hipovolemično, 37,9% euvolemično, a 42,4% hipervolemično. Kod 38 (28,8%) pacijenata etiologija hiponatremije je bila kongestivna srčana insuficijencija, kod 29 (22,0%) pacijenata sindrom neodgovarajućeg antidiuretskog hormona, gubitak gastrointestinalne tečnosti kod 24 (18,2%) pacijenata, bubrežne patologije kod 20 (15,2%) pacijenata, prisustvo lekova kod 20 (15,2%) pacijenata, a kod jednog (0,8%) pacijenta hipokortizolemija. Prosečna dužina boravka u bolnici za pacijente bila je pet (1–60) dana. Nije bilo statistički značajne razlike između dužine boravka u bolnici na osnovu etiologije hiponatremije

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based on hyponatremia etiology and serum Na levels (p=0.861 and p=0.076). It was observed that the lengths of stay for patients who developed hyponatremia during their hospitalisation in various clinics were longer than those for patients who presented to the emergency department (p<0.001).

Conclusions: In this study, it was determined that the length of hospital stay did not change with the etiology of hyponatremia and serum Na level at the time of admission, but patients who developed hyponatremia during their hospitalisation had longer hospitalisation times.

Keywords: hyponatremia, geriatrics, length of stay

i nivoa Na u serumu (p = 0,861 i p = 0,076). Uočeno je da su dužine boravka kod pacijenata koji su razvili hiponatremiju tokom hospitalizacije u raznim klinikama bile duže nego kod pacijenata koji su došli na odeljenje hitne pomoći (p < 0,001).

Zaključak: U ovoj studiji je utvrđeno da se dužina boravka u bolnici nije menjala sa etiologijom hiponatremije i nivoa Na u serumu u trenutku prijema, ali su pacijenti koji su razvili hiponatremiju tokom hospitalizacije imali duže vreme hospitalizacije.

Ključne reči: hiponatremija, gerijatrija, dužina boravka

Introduction

Hyponatremia is the most common electrolyte abnormality observed in clinical practice. It can be seen in about 30% of hospitalised patients and can lead to a wide range of clinical symptoms, from asymptomatic to severe and even life-threatening (1, 2).

In order to determine the diagnosis and treatment in patients presenting with hyponatremia, grouping is performed according to patients' serum osmolality and volume status. Serum osmolality is grouped as hypoosmolar at <280 mmol/kg, isoosmolar at 280–295 mmol/kg, and hyperosmolar at >295 mmol/kg, with a further categorisation of hypovolemic, euvolemic, or hypervolemic hyponatremia according to volume status. Symptoms depend on the severity and duration of the hyponatremia. Acute hyponatremia is defined by the onset of symptoms within 48 h. Patients with acute hyponatremia develop neurologic symptoms caused by cerebral edema due to water movement into the brain. These may include seizures, impaired mental status, or coma and death. Hyponatremia developing over longer than 48 h is considered chronic hyponatremia. Treatment depends on the acute or chronic onset, the patient's volume status, and the severity and nature of the symptoms.

Age is a strong independent risk factor for hyponatremia, and older patients constitute a high-risk group for its occurrence (2–5). Symptoms such as nausea, vomiting, headache, stupor, coma, and seizures are associated with acute hyponatremia and fatigue, cognitive impairment, and gait defects are associated with chronic hyponatremia; also, falls, poor bone quality (e.g., osteoporosis), and negative effects of fractures are more frequent and severe in geriatric patients (6–10). Hyponatremia also prolongs the hospitalisation time remarkably and increases the cost of medical care substantially (11). It is unknown whether there is a relationship between the etiology of hyponatremia or initial serum sodium (Na) levels and the length of hospital stay. This study intended to evaluate the clinical features, hyponatremia etiologies, and hospitalisation durations of hyponatremic patients over 65 years of age and determine whether there is a relationship between hyponatremia etiology and the length of hospital stay.

Materials and Methods

Study Population

In this study, 132 patients aged 65 years who were hospitalised in our clinic due to hyponatremia were evaluated retrospectively. Patients with complete data were included in the study. Patients aged <65 years and those with hyperglycemia, hyperlipidemia, or paraproteinemia that could cause pseudohyponatremia were excluded from the study. The approval of the local ethics committee was obtained (18.09.2019/94).

The demographic characteristics of the patients (age, gender) and their places of presentation (emergency room, other clinics), presenting complaints (nausea, vomiting, confusion, seizure, fever, dyspnea, edema, general condition disorder, fatigue, anorexia), physical examination findings, volume statuses (hypovolemic, euvolemic, hypervolemic), hyponatremia etiologies, treatments (hypertonic saline, isotonic saline, water restriction, diuretics, ultrafiltration), and laboratory data (glucose, urea, creatinine, serum osmolality, Na, potassium (K), urine Na, urine osmolality, hemoglobin, thyrotropin (TSH), and serum cortisol levels) were recorded. The normal serum Na range is 135–145 mmol/L, serum osmolality is 275–293 mmol/kg serum water, and urine osmolality is 500–850 mmol/kg water. Hyponatremia is defined as an Na of <135 mmol/L, and severe hyponatremia is defined as serum Na of <120 mmol/L.

Statistical Analysis

The IBM SPSS 21.0 statistical software package for Windows was used for the statistical analysis of the data. For all data, the normality assumption was evaluated via the Shapiro-Wilk test. Numerical data are indicated by median (minimum-maximum), and categorical data are indicated by numbers (percentage). The Mann-Whitney U test was used to compare numerical data between two groups, and the Kruskal-

Wallis test was used to compare more than two groups. Values of $p<0.05$ were considered statistically significant.

Results

Of the 132 patients, 90 were female (68.2%), 42 were male (31.8%), and the mean age was 74.97±7.14 years. Severe hyponatremia (Na of <120 mmol/L) was detected in 66 (50%) patients.

While 97 (73.5%) patients presented to the emergency department with complaints related to hyponatremia, 35 (26.5%) patients were found to have developed hyponatremia during their hospitalisation in various clinics. Dyspnea and edema were observed in 28 (21.2%) patients, nausea/vomiting in 26 (19.7%) patients, confusion in 26 (19.7%) patients, fatigue and anorexia in 15 (11.4%) patients, seizures in eight (6.1%) patients, fever in eight (6.1%) patients, and general condition disorder in five (3.8%) patients, whereas 16 (12.1%) patients were asymptomatic. The median systolic blood pressure of the patients was 120 (68–250) mmHg, and the mean diastolic blood pressure was 70 (39–130) mmHg. The physical examination findings of the patients at the time of admission are presented in *Table I*. Hypovolemic hyponatremia was detected in 26 (19.7%) patients, euvolemic hyponatremia in 50 (37.9%) patients, and hypervolemic hyponatremia in 56 (42.4%) patients.

Serum Na level was 119.50 (99–131) mmol/L at the time of admission, 125 (105–139) mmol/L at the 24th hour of treatment, and 128.50 (108–144) mmol/L at the 48th hour of treatment ($p<0.001$). The serum Na levels of 66 (50%) patients were <120 mmol/L, those of 64 (48.5%) patients were 120–129 mmol/L, and those of two (1.5%) patients were >130 mmol/L. One hundred nine (82.6%) patients had hypoosmolar hyponatremia, 14 (10.6%) patients had isoosmolar hyponatremia, and nine (6.8%) patients had hyperosmolar hyponatremia (*Table II*).

The etiology of hyponatremia was congestive heart failure in 38 (28.8%) patients, syndrome of inappropriate antidiuretic hormone (ADH) secretion (SIADH) in 29 (22.0%) patients, gastrointestinal fluid loss in 24 (18.2%) patients, renal pathologies in 20 (15.2%) patients, the presence of drugs in 20 (15.2%) patients, and hypocortisolemia in one (0.8%) patient. In 51.7% of patients with SIADH, the cause was an infection, with the most common (66.7%) reason being pneumonia.

Sixty-eight (51.5%) patients were treated with hypertonic saline, 20 (15.2%) with isotonic saline, 24 (18.2%) with water restriction and diuretics, and 20 (15.2%) with only water restriction. It was observed that seven (5.3%) patients required ultrafiltration.

The mean length of hospital stay for the patients was 5 (1–60) days. There was no statistically significant difference between the lengths of hospital stay in terms of hyponatremia etiologies ($p=0.861$). In addition, serum Na levels at the time of presentation did not show a statistically significant difference in terms of hyponatremia etiologies ($p=0.065$). It was observed that the lengths of hospital stay and serum Na levels at the time of presentation were similar in female and male patients ($p=0.440$ and $p=0.230$).

Table I Demographic features, complaints, and physical examination findings of the patients.

	n (%)
Gender	
Female	90 (68.2)
Male	42 (31.8)
Place of application	
Emergency department	97 (73.5)
Different clinics	35 (26.5)
Complaints	
Dyspnea/edema	28 (21.2)
Nausea/vomiting	26 (19.7)
Confusion	26 (19.7)
Asymptomatic	16 (12.1)
Fatigue/anorexia	15 (11.4)
Seizures	8 (6.1)
Fever	8 (6.1)
General condition disorder	5 (3.8)
Physical examination	
Rales in the lung	
Present	42 (31.8)
Absent	90 (68.2)
Ascites	
Present	5 (3.8)
Absent	127 (96.2)
Pretibial edema	
Present	36 (28.3)
Absent	91 (71.7)
Volume	
Hypovolemic	26 (19.7)
Euvolemic	50 (37.9)
Hypervolemic	56 (42.4)

Table II Laboratory values of the patients.

	Median (min–max)
Glucose (mmol/L)	5.44 (3.77–6.72)
Urea nitrogen (mmol/L)	7.32 (0.51–36.03)
Creatinine (μmol/L)	79.56 (13.26–739.9)
Potassium (mmol/L)	4.40 (2.30–6.70)
TSH (mIU/L)	1.1 (0.92–5.2)
Serum cortisol (nmol/L)	317.2 (110.3–717.2)
Hemoglobin (g/L)	112 (62–179)
Serum Na level at the time of admission (mmol/L)	119.50 (99–131)
Serum Na level at the 24th hour of treatment (mmol/L)	125 (105–139)
Serum Na level at the 48th hour of treatment (mmol/L)	128.50 (108–144)
Serum osmolality (mmol/kg)	259 (212–309)
Urine osmolality (mmol/kg)	224 (36–782)
Urine Na (mmol/L)	45.5 (4–321)
Na groups	n (%)
<120 mmol/L 120–129 mmol/L 130–135 mmol/L	66 (50) 64 (48.5) 2 (1.5)
Osmolality groups	n (%)
Hypoosmolar Isoosmolar Hyperosmolar	109 (82.6) 14 (10.6) 9 (6.8)

TSH: Thyrotropin, Na: sodium

In addition, there was no statistically significant difference between the duration of hospital stay in patients with serum Na levels of <120 mmol/L and 120–129 mmol/L at the time of admission (p=0.076). It was observed that the lengths of hospital stay for patients who developed hyponatremia during their hospitalisation in various clinics was longer than those of patients who presented to the emergency department (p<0.001), but serum Na levels were higher in patients who developed hyponatremia during hospitalisation (p<0.001) (*Table III*).

Table III Duration of hospitalisation and sodium levels at the time of patients' admission according to gender, etiology, place of application, and patients' duration of hospitalisation according to sodium groups.

	Duration of hospitalisation	p	Na levels at the time of admission	p
Gender				
Female	4 (1–60)	0.440	119 (101–131)	0.230
Male	7 (1–43)		121 (99–129)	
Etiology				
Congestive heart failure	4 (1–30)	0.861	119 (99–128)	0.065
SIADH	6 (1–59)		121 (109–129)	
Gastrointestinal fluid loss	4 (1–36)		119 (101–126)	
Renal pathologies	7 (1–60)		122.5 (105–129)	
Drugs	4 (1–43)		120.5 (108–131)	
Place of application				
Emergency department	3 (1–59)	<0.001	118 (99–130)	<0.001
Different clinics	10 (2–60)		124 (111–131)	
Na groups				
<120 mmol/L	4 (1–59)	0.076	-	-
120–129	6 (1–60)			

Na: Sodium, SIADH: syndrome of inappropriate antidiuretic hormone secretion

Discussion

Hyponatremia is the most common electrolyte disorder in hospitalised patients and society. Hyponatremia prevalence in society is ~8%, and this prevalence increases significantly with age (3, 4). Hyponatremia is reported to be associated with an increased risk of mortality and poor prognosis in older individuals (3, 12).

The higher rate of hyponatremia in the elderly is related to the deterioration of the water excretion capacity associated with aging and the more frequent exposure of this group to drugs and diseases associated with hyponatremia (2, 13). The decrease in the glomerular filtration rate due to aging causes impaired water excretion capacity. In addition, the decrease in intrarenal prostaglandin production seen at older ages may cause impaired water excretion

capacity (14). Another factor contributing to hyponatremia in this group is the fact that the age-related decrease in total body water percentage causes further fluctuations in serum Na concentration. Higher sensitivity to osmotic stimuli can be seen in the geriatric population (15, 16). Elderly individuals frequently use drugs known to cause hyponatremia (such as thiazide diuretics, selective serotonin reuptake inhibitors, and nonsteroidal anti-inflammatory drugs), and they often suffer from diseases that may be associated with hyponatremia (for example, diabetes mellitus, infections, heart failure, liver diseases, malignancies, and endocrinopathies) (17, 18). Many elderly patients with hypertension or heart failure maintain a low-salt diet, which can cause a low serum Na concentration. In this population, a decrease in protein intake due to overlapping diseases may play a role in the development of hyponatremia by impairing water excretion (19, 20).

Diuretics and SIADH are among the most common causes of hyponatremia in the elderly (20, 21). In one prospective study that included only elderly hospitalised patients, the most common causes of hyponatremia were SIADH and diuretics. In the same study, the two most common causes of SIADH were lower respiratory tract infection and stroke (22). In the study of Chatterjee et al. (23), gastrointestinal fluid loss, cerebrovascular accident, and pulmonary sepsis were found to be the most frequent causes of hyponatremia. In the work of Babaliche et al. (24), SIADH was also the most common cause of hyponatremia in 46% of patients, followed by renal pathologies in 13%, gastrointestinal compromise in 11%, cardiac causes in 10%, cirrhosis in 10%, and drugs in 10%. In addition, Ishikawa et al. (25) reported that 40% of patients presenting with hyponatremia aged 65 and above had hypothalamic-pituitary-adrenal dysfunction. Although congestive heart failure was reported in other studies as a less common cause of hyponatremia than diuretics and SIADH, the most common cause of hyponatremia in our study was congestive heart failure, the second most common cause was SIADH (23, 24). Contrary to the study of Ishikawa et al. (25), hyponatremia due to hypopituitarism was very rare in our study group. This may be because patients with hypopituitarism are asymptomatic for long periods, and their need for hospitalisation is less than those of other patients. Because only hospitalised patients were included in our study, the rate of hypopituitarism may be lower than expected.

The importance of early recognition of hyponatremia and prompt intervention is critical (26). In a large multicenter trial with 151,486 patients, it was shown that all types and grades of dysnatremias were related to increased risk-adjusted and raw hospital mortality rates. The odds ratios for mild, moderate, and severe hyponatremia were 1.32, 1.89, and 1.81, respectively (27). Moreover, in addition to mortality, hyponatremia prolongs the hospitalisation time remarkably and increases medical care costs (11). In our study, the length of hospital stay due to hyponatremia was observed to be 5 (1–60) days, and this duration did not change according to the etiology of hyponatremia or the patient's gender or initial serum Na levels. It was observed that patients who applied to the emergency department had lower Na levels but shorter hospital stays than patients who developed hyponatremia during their hospitalisation in other clinics.

In their study, including 100 patients with moderate to severe hyponatremia who were monitored in the intensive care unit, Babaliche et al. (24) reported that 59% of the patients were male and 41% were female, with a slight dominance of the male gender. In the work of Sood et al. (28), the male-to-female ratio was 1.25:1. In other studies in the literature, male gender dominance is observed in patients with hyponatremia (23, 29). Contrary to these studies, in our study, 68.2% of patients with hyponatremia were female. Since our study consists of randomly recruited patients for a certain period of time, the gender result may be due to this.

In the study of Sood et al. (28), including 106 hyponatremic patients, 90% were hypoosmolar, 9% hyperosmolar, and 1% were isoosmolar, while 40% were euvolemic, 31% were hypervolemic, and 29% were hypovolemic. In the study of Chatterjee et al. (23), 50.74% of the patients were euvolemic, 26.86% were hypervolemic, and 22.4% were hypovolemic, while in the study of Babaliche et al. (24), 50% were euvolemic, 33% were hypervolemic, and 17% were hypovolemic. In our study, 42.4% of the patients were hypervolemic, 37.9% were euvolemic, and 19.7% were hypovolemic while 82.6% had hypoosmolar hyponatremia, 10.6% had isoosmolar hyponatremia, and 6.8% had hyperosmolar hyponatremia.

In their study, Sood et al. (28) reported that 42% of patients had severe hyponatremia, 48% had moderate hyponatremia, and 10% mild hyponatremia. Similarly, in our study, severe hyponatremia was detected in 50% of the hospitalised geriatric patients. It was observed that Na levels were 120–129 mmol/L in 48.5% and 130–135 mmol/L in 1.5% of the patients.

Pillai et al. (30) observed that among intensive care unit admissions, the symptoms attributed to hyponatremia included nausea (69.3%), malaise (80%), drowsiness (61.3%), confusion (41.3%), lethargy (24%), frequent falls (1.3%), convulsions (2.7%), altered sensorium (41.3%), and delirium (9.3%). Krishnamurthy and Srinivas (31) reported that the symptoms found in hyponatremia patients were vomiting (29.6%), giddiness (2.4%), altered sensorium (8.5%), headache (9.2%), chest pain (6.4%), generalized weakness (8.4%), fever (12.3%), cough (15.2%),

loss of consciousness (0.7%), nausea (22.5%), loose stools (5%), increased fatigability (10.4%), breathlessness (17.8%), abdominal pain (8.8%), difficulty in micturition (0.9%), lower limb swelling (3.6%), and seizures (6.4%) (31). In our study, dyspnea and edema were observed in 28 (21.2%) patients, nausea/vomiting in 26 (19.7%) patients, confusion in 26 (19.7%) patients, fatigue and anorexia in 15 (11.4%) patients, seizures in eight (6.1%) patients, fever in eight (6.1%) patients, and general condition disorder in five (3.8%) patients, whereas 16 (12.1%) patients were asymptomatic.

In acute symptomatic hyponatremia, hypertonic saline solution is commonly used to acutely increase serum Na levels and prevent serious neurological symptoms. Hypovolemic hyponatremia is treated with adequate fluid resuscitation to reduce ADH secretion stimulation. Normal saline is often used to suppress the hypovolemic stimulus that causes ADH release (32, 33). In patients with SIADH, careful administration of hypertonic fluids may be required, along with discontinuation of suspicious drugs and reduced water consumption. In these cases, furosemide can also be administered to prevent circulatory overload, especially if elderly patients have concomitant cardiac dysfunction. Furosemide increases free water excretion and leads to higher serum Na. Our study observed that 51.5% of the patients were treated with hypertonic saline, 15.2% with isotonic saline, 18.2% with water restriction and diuretics, and 15.2% with only water restriction. It was also observed that 5.3% of the patients required ultrafiltration.

In conclusion, there is an increasing tendency for hyponatremia to occur with increased age, comorbidities, and the use of drugs. In our study, congestive heart failure and SIADH were determined to be the most common causes of hyponatremia in geriatric patients. Nausea, vomiting, and dyspnea were the most common symptoms. It was determined that the length of hospital stay did not change with the etiology of hyponatremia, gender, or serum Na level at the time of admission. However, patients who developed hyponatremia during their hospitalisation had longer hospitalisation times.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of interest statement

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

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Received: January 19, 2021
Accepted: May 28, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-30927

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 47–52, 2022

Original paper
Originalni naučni rad

ROLE OF ARTERIAL BLOOD GAS (ABG) AS A VALUABLE ASSESSMENT TOOL OF DISEASE SEVERITY IN SARS-COV-2 PATIENTS

ULOGA ARTERIJSKIH GASOVA KRVI (AGK) KAO VREDNOG PARAMETRA ZA PROCENU TEŽINE OBOLJENJA U SARS-COV-2 PACIJENATA

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Summary

Background: COVID-19 is caused by a novel coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The foremost predominant complication of SARS-CoV-2 is arterial hypoxemia thereby disturbing lung compliance, requiring mechanical ventilation.
The aim of the current research study is to analyze role of ABG as a valuable assessment tool of disease severity in SARS-CoV-2 patients.
Methods: 170 arterial blood samples were collected from patients admitted in Intensive Care Unit (ICU) of Sri Guru Ram Das Charitable Hospital, Amritsar. They were analyzed for arterial blood gas using ABG analyzer. Parameters of ABG such as pH, pCO_2, HCO_3, O_2 saturation, ionized calcium (iCa) and calculated ionized calcium (at pH 7.4) was calculated for all the samples.
Results: Continuous variables were described as medians with interquartile ranges (IQRs) and categorical variables as percentages and frequencies. Spearman correlation test was done for calculation of correlation between pH and other ABG parameters. Analysis of arterial blood gas revealed significant negative correlation ($p<0.05$) between pH and pCO_2 and significant positive correlation ($p<0.05$) between pH and HCO_3 and between pH and delta ionized calcium. Low levels (98.2%) of ionized calcium were observed while monitoring the ABG findings though weak negative correlation ($p<0.05$) was observed between pH and iCa.
Conclusions: Our study suggests that ABG analysis acts as a momentous indicator for critically ill patients admitted in Intensive Care Unit (ICU). Estimation of iCa in this critical

Kratak sadržaj

Uvod: COVID-19 je izazvan novim koronavirusom koji izaziva težak respiratorni sindrom koronavirus 2 (SARS-CoV-2). Predominantna komplikacija SARS-CoV-2 je arterijska hipoksemija koja dovodi do oštećenja pluća i zahteva mehaničku ventilaciju. Svrha ovog izučavanja je da analizira ulogu AGK kao vrednog parametra za procenu težine oboljenja kod SARS-CoV-2 pacijenata.
Metode: Uzeto je 170 arterijskih uzoraka krvi od pacijenata koji su bili primljeni u Intenzivnu jedinicu (ICU) u Sri Guru Ram Das Charitable Hospital, Amristar. Analizirani su arterijski gasovi krvi primenom ABG analizatora. Parametri ABG (AGK) bili su pH, pCO_2, HCO_3, O_2 saturacija, jonizovani kalcijum (iCA) i izračunati jonizovani kalcijum (na pH 7,4) za sve analizirane uzorke.
Rezultati: Za sve promenljive parametre izračunata je medijana i interkvartilne oblasti (IQRs) i varijable kako što su procenti i frekvencije. Korišćen je Spearman korelacioni test za izračunavanje korelacije između pH i i drigih ABG parametara. Analizom arterijskih gasova krvi dobijena je značajna negativna korelacija ($p<0,05$) između pH i pCO_2 i značajna pozitivna korelacija ($p<0,05$) između pH i HCO_3 i između pH i delta jonizovanog kalcijuma. Niski nivoi (98,2%) jonizovanog kalcijuma su nađeni pri praćenju ABG nalaza u toku nedeljne negativne korelacije ($p<0,05$) kad je praćena između pH i iCa.
Zaključak: Naše izučavanje ukazuje da ABG analiza ima ulogu trenutnog indikatora za kritično bolesne pacijente primljene u Intenzivnu Jedinicu (ICU). Određivanje iCa u ovom kritičnom momentui ima veoma značajnu ulogu u

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care setting acts as a distinctive biochemical feature of SARS-CoV-2 disease, as an initial assessment tool, for hypocalcemia.

Keywords: arterial blood gas, SARS-CoV-2, ionized calcium, hypocalcemia

praćenju SARS CoV-2 oboljenja kao inicijalno sredstvo ispitivanja hipokalcijemije.

Ključne reči: arterijski gasovi krvi, SARS-CoV-2, jonizovani kalcijum, hipokalcijemija

Introduction

The outbreak of Novel coronavirus disease (COVID-19) was initially noticed in a seafood market in Wuhan city in Hubei Province in Mid-December, 2019 and subsequently speeded to 214 countries worldwide (1). World Health Organization (WHO) declared this outbreak as a »Public Health Emergency of International Concern (PHEIC) on 30th January, 2020 and later declared COVID-19 as pandemic on 11th March, 2020 (2).

The novel coronavirus belongs to lineage B of the genus beta – coronavirus of the coronavirus family which includes SARS-CoV (Severe Acute Respiratory syndrome) and MERS – CoV (Middle East Respiratory Syndrome) (3).The seventh member of the corona virus family to infect humans is 2019 novel coronavirus (2019-nCoV) (4). Coronaviruses (CoVs) are positive sense single stranded RNA viruses of the family coronaviridae that infect a wide host range to produce disease ranging from common cold to severe illness (5).

SARS-CoV2 affects different people in various ways. Most infected people develop mild to moderate illness and may recover without hospitalization. Primarily transmitted through the respiratory tract, the most common clinical presentations of symptomatic individuals infected with SARS-CoV-2 include fever, dyspnea, cough, fatigue, and sore throat. In advanced cases, patients may rapidly develop respiratory failure with acute respiratory distress syndrome, and even progress to death (6).

The foremost predominant complication of SARS-CoV-2 is arterial hypoxemia thereby disturbing lung compliance requiring mechanical ventilation. Arterial blood gas (ABG) analysis provides information regarding patient oxygenation, ventilation adequacy and acid base levels.

Or in other words we can say, it tells the activity in both respiratory system and metabolic system. Both the systems work together in order to maintain the pH in normal range. If one system is disturbed the other will try to compensate. Thus, the current research was aimed to analyze the role of ABG as a valuable assessment tool of disease severity in SARS-CoV2 patients. We also hypothesized that which parameter among ABG analysis can play a significant role and to what extent as regards to disease severity.

Material and Methods

Participants

In the present observational study 170 samples of ABG were collected from 17 critically ill patients severely affected with the disease and admitted in Intensive Care Unit (ICU) of Sri Guru Ram Das Charitable Hospital, Amritsar, Punjab (India) during the period from October 2020 to December 2020. They were laboratory confirmed tested positive cases for corona virus. The diagnosis was established by reverse-transcriptase polymerase chain reaction (RT-PCR) method by testing nasal and pharyngeal swab specimens according to World Health Organization (WHO) interim guidance criteria. Hematology testing was conducted on H560 Hematology analyzer (Erba Mannheim). Analysis of ABG was done on ABG analyzer (Siemens make) in clinical laboratory of the hospital for arterial samples of all admitted patients whose stay was more than 5 days. The clinical outcome was monitored until the discharge of the patient. No written informed consent of patient was required as the study included only undisclosed information. The study was approved by the institutional ethics committee.

Before proceeding with the analysis, the pre-analytical errors were taken into consideration to avoid false results for pH and ionized calcium (iCa). To achieve correct heparin and blood concentration correct blood volume of sample was collected with immediate mixing after sampling to avoid false low levels of iCa. After collection of the sample the blood was immediately analyzed in the clinical laboratory for ABG (within 20 minutes) to avoid any discrepancy in the results because loss of pCO_2 from the collected sample may increase pH as alkaline pH increases protein bound calcium and decreases iCa levels. On the other hand acidic pH decreases protein bound calcium and increases iCa levels. Even haemolysis results in false levels of low ionized calcium.

Among the ABG parameters pH, pCO_2, HCO_3 and ionized calcium (iCa) were mainly taken into consideration. Ionized calcium levels were calculated both as actually measured levels and corrected mathematically at pH 7.4 to avoid influence of sample handling. Delta ionized calcium was also calculated to move towards more accuracy.

Calculation of corrected ionized calcium (at pH 7.4) and delta ionized calcium

Following formula was used to calculate corrected ionized calcium (7).

Corrected ionized calcium = Measured ionized calcium × [1 – 0.53 × (7.4 – actual pH)]

Delta ionized calcium is the difference between ionized calcium and calculated ionized calcium at pH 7.4

Statistical analysis

The statistical analysis was performed using Statistical Package for Social Science program (version 16.0; SPSS Inc., Chicago, IL). In the present observational study 6 to 15 (Average 10) successive readings of ABG were noted for each patient. For the descriptive analysis, continuous variables were described as medians with interquartile ranges (IQRs) and categorical variables as percentages and frequencies. Spearman correlation test was done for calculation of correlation between pH and other ABG parameters. The test with p value < 0.05 was considered statistically significant.

Results

Demographic and clinical characteristics of SARS-CoV-2 patients are summarized in *Table I*. On initial hospital evaluation, median levels of total calcium, total proteins, albumin and NLR ratio was 1.98 mmol/L (1.64–2.34), 67 g/L (58–74), 26 g/L (15.50–35.50) and 8.7 (6.5–29.5) respectively. With median value of 1.98 mmol/L all the patients were found to be hypocalcemic. Normal levels of serum proteins though on lower side were observed with median value 67 g/L.

All the patients also showed Hypoalbuminemia with median value of 26 g/L. Whereas, NLR the ratio of absolute count of neutrophil to lymphocyte with median level 8.7 was also calculated as a stress factor in clinical ICU practice.

Among the total critically ill patients 65% were males. Fourteen (82%) out of seventeen patients were having comorbidities i.e. 41% were diabetic, 6% were hypertensive whereas 35% were found to be both diabetic and hypertensive. 18% were found to be having no comorbidity. Out of seventeen patients, seven (41%) could not survive. In non-survivors T2DM (86%) was the most common comorbidity followed by HTN (14%). Initially, for the observations of acid-base imbalance 53% were suffering with mixed disorder of acid-base balance (respiratory alkalosis and metabolic acidosis), 41% with respiratory alkalosis and 6% with respiratory acidosis. After monitoring 6 to 15 successive readings of the ABG samples for each patient we analyzed a total of 170 samples for which median (IQR) was calculated. For pH it was 7.43 (7.29–7.56), for pCO_2 33.6 mm/Hg (14.05–53.97), for HCO_3 and iCa 23.1 mmol/L (13–32.79) and 0.86 mmol/L (0.05–1.57) respectively and for O_2 saturation 95.3% (85.2–105.2).

Table II revealed percentage occurrence of abnormal levels of ABG parameters of SARS-CoV-2 patients in which low levels of ionized calcium was 98% as compared to abnormal levels of pH being 40%, pCO_2 being 52%, HCO_3 being 58% and low saturation of oxygen being 46%.

Table I Demographic and clinical characteristics of SARS-CoV-2 patients.

Parameter	Median (IQR) or N (%)
Age, years (n=17)	62 (31.5–83.5)
Male (n=17)	11 (65%)
Female (n=17)	6 (35%)
With Comorbidities (HTN, T2DM) (T2DM) (HTN) No comorbidity	14 (82%) 6 (35%) 7 (41%) 1 (6%) 3 (18%)
Acid Base Imbalance Respiratory Alkalosis Respiratory Acidosis Mixed disorder	 7 (41%) 1 (6%) 9 (53%)
Non survivors (T2DM) (HTN)	7 (41%) 6 (86%) 1 (14%)
Total Calcium, mmol/L (n=17)	1.98 (1.64–2.34)
Total Protein, g/L (n=17)	67 (58–74)
Serum Albumin, g/L (n=17)	26 (15.50–35.50)
NLR (n=17)	8.7 (6.5–29.5)
pH (n=170)	7.43 (7.29–7.56)
pCO_2, mmHg (n=170)	33.6 (14.05–53.97)
HCO_3, mmol/L (n=170)	23.1(13–32.79)
O_2, sat % (n=170)	95.3 (85.2–105.2)
Ionized Calcium, mmol/L (n=170)	0.86 (0.05–1.57)

HTN – hypertension; DMT2 – diabetes mellitus type 2; NLR – neutrophil-to-lymphocyte ratio; pCO_2 – partial pressure of carbon dioxide; HCO_3 – bicarbonate ion; O_2sat – oxygen saturation

Table II Percentage occurrence of abnormal levels of ABG parameters in SARS-CoV-2 patients.

Parameter	Readings n=170 (%)
pH	68 (40)
pCO_2	88 (51.8)
HCO_3	99 (58.2)
O_2 saturation	78 (45.9)
Ionized Calcium	167 (98.2)

Table III depicts Spearman correlation to see the association of pH with other parameters of ABG. A strong negative correlation was observed between pH and pCO_2 (*Figure 1*) and a marginal negative correlation were observed between pH and ionized calcium (*Figure 2*). No association was found between pH and O_2 saturation (*Figure 3*). Whereas, a strong positive correlation was observed between pH and HCO_3 (*Figure 4*) and between pH and delta ionized calcium

Table III Spearman correlation of pH with pCO_2, HCO_3, ionized calcium and O_2 sat in SARS-CoV-2 patients.

Parameter	Patients with SARS-CoV-2 pH	
	r_s	p value
pCO_2	-0.335	< 0.001*
HCO_3	0.399	<0.001*
Ionized Calcium	-0.151	0.048*
O_2 saturation	0.015	0.847
Delta ionized calcium	0.213	0.005*

*p<0.05 considered statistically significant. rs – Spearman correlation

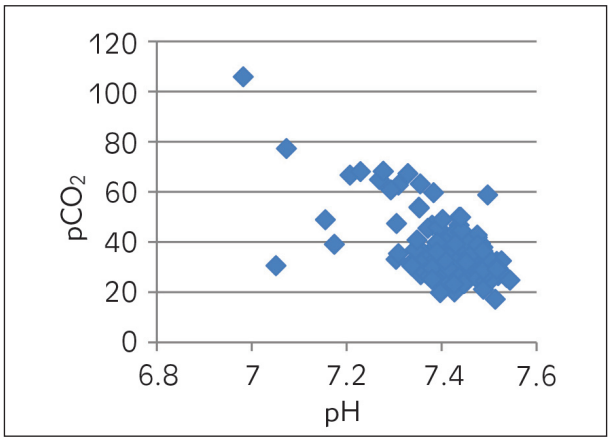

Figure 1 pH vs pCO_2.

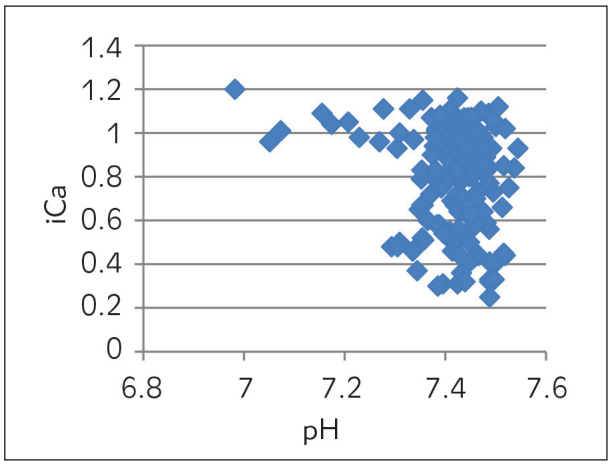

Figure 2 pH vs ionized calcium (iCa).

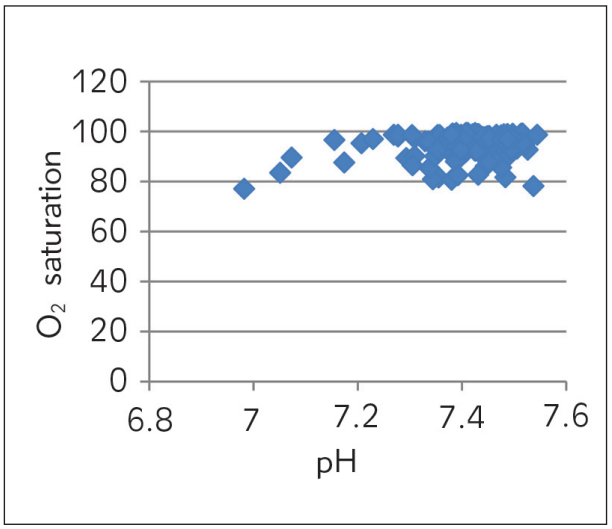

Figure 3 pH vs O_2 saturation.

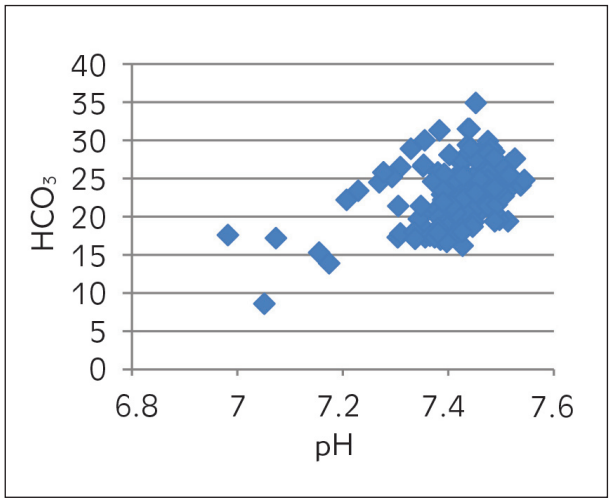

Figure 4 pH vs HCO_3.

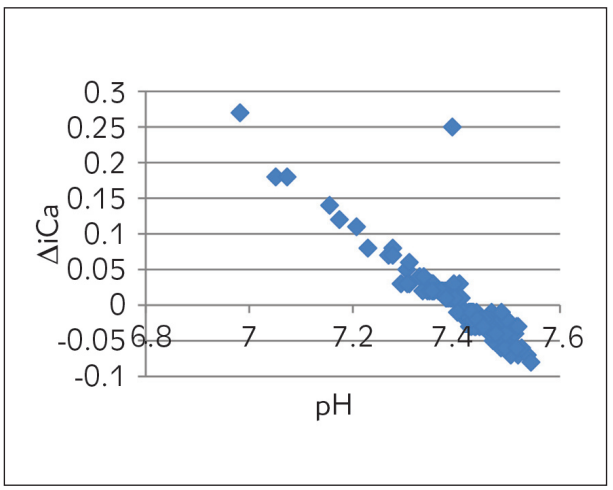

Figure 5 pH vs delta ionized calcium (Δ iCa).

(*Figure 5*). Delta ionized calcium is the difference between ionized calcium and calculated ionized calcium at pH 7.4. *Figure 5* shows both positive (for acidic) and negative (for alkalosis) correlation for pH and delta ionized calcium. Since, our number of cases was mostly of alkalosis (41%) and mixed disorder (53%) as compared to acidosis (6%) the graph depicts more of negative correlation.

Discussion

A low level of calcium is a common laboratory abnormality in viral infection and pneumonia (8). Previous studies reported that calcium played a vital role in viral infections and replicative mechanisms of SARS-CoV, MERS-CoV and Ebola virus. Calcium ions directly interacted with fusion peptides of these viruses promoting their replication (9, 10). Hypocalcemia is a common phenomenon among critically ill patients, its prevalence ranges from 15% to 88% in adults (11). Similar to recent study by Zhou et al. (12) our study revealed hypocalcemia in all cases regardless of severity of their condition in the early stage of viral infection. Recently, Filippo et al. (13) observed 80% hypocalcemic patients in their study.

Considering age factor 59% of our patients were older than 60 years. Median age of the studied patients was 62 years old which suggested that aged people were more susceptible to severe COVID-19. In another study by Liu et al. (14) 63.6% of their COVID-19 patients were also of older age (>65 years). Age factor does affect the recovery capacity of a person at the later stages of infection because the liver and kidney function of an individual declines as age advances leading to decrease in intestinal calcium absorption due to low levels of 25- hydroxy cholecalciferol. Similarly, we observed in our study, that the severe critically ill patients who could not survive were older (>60 years) in age.

In our present study among the non-survivors T2DM (86%) was the most common comorbidity. In Diabetes Mellitus secretion of Parathyroid Hormone (PTH) declines, resulting in decrease disrupted calcium homeostasis that is cellular calcium depletion occurs. PTH stimulates calcitriol (1,25 dihydroxy cholecalciferol), which regulates calcium homeostasis in the body. Calcitriol induced Ca^{2+} signals (oscillations) regulate insulin secretion from the pancreatic cells (15, 16). Thus, the rapid increase in intracellular Ca^{2+} triggers insulin release.

The perception that acid base imbalance in diabetes is confined to metabolic acidosis is also challenged by our results. The most common disturbance observed was mixed disorder of acid-base balance present in 9 patients. Respiratory disturbance predominantly respiratory alkalosis was present in 7 patients whereas, only 1 patient suffered with respiratory acidosis.

Calcium is predominantly bound to albumin. Almost 30–55% in the plasma and a decrease in serum albumin will also cause hypocalcemia. Ionized calcium binds to negatively charged sites on protein molecules. Therefore, Hypoalbuminemia is associated with Pseudohypocalcemia which is reduction in total calcium concentration even though there are normal iCa levels (17).

To sustain normal organ function calcium homeostasis has to be maintained. Changes in intracellular calcium homeostasis can also promote the activation of inflammatory pathways leading to increase in tumor necrosis factor (TNF) and IL-6 (18, 19). In addition, hypoxia of tissue and organ induces cell membrane damage resulting in calcium influx (14).

Chernow et al. (20) in their study demonstrated that hypocalcemia was associated with prolonged stay in ICU and increased mortality, similar to our study, in which 41% were non-survivors, with stay in ICU more than 5 days. Moreover, poor prognosis observed in hypocalcemic patients with severe SARS-CoV-2 was similar to many recent studies (14). Similar to our observations, some authors also identified hypocalcemia as a relevant and independent risk factor for worse clinical outcome, associated with higher mortality in hospitalized and critically ill patients admitted in ICU (14, 21–23). Though our results showed marginal significant correlation of pH and iCa as such but strong correlation between pH and delta iCa cannot be ignored too. Moreover, 98% prevalence of low levels of iCa as compared to other parameters of ABG is also worth mentioning.

Limitation

The main limitation of our study is that the sample size was relatively small. We could only proceed with the samples from patients admitted from October to December. Larger studies are needed to confirm our findings. Possibility of bacterial-infection also could not be ruled out because of high NLR ratio in these studied SARS-CoV-2 patients. We consider that additional studies are required to support these findings.

Conclusion

Our study suggests that ABG analysis acts as a momentous indicator for critically ill patients admitted in Intensive Care Unit (ICU). Estimation of iCa in this critical care setting acts as a distinctive biochemical feature of SARS-CoV-2 disease, as an initial assessment tool, for hypocalcemia, has a potential impact on its severity. It is also suggested that ionized calcium- the physiologically active component of total calcium should be preferred as routine method for determining the level of calcium in all patients. Moreover, iCa is also easy to measure (within 15 to 20 minutes) making clinicians in identifying severe patients at initial hospital valuation. Hypocalcemia represents a novel prospective treatment goal worth to be treated at the earliest for improvements in patient care.

Acknowledgments. The authors are grateful to Professor Malkinder Singh, Department of English of Khalsa College Amritsar for vetting the paper with respect to grammatical mistakes

Conflict of interest statement

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

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Received: February 18, 2021
Accepted: June 10, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-31250

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 53–61, 2022

Original paper
Originalni naučni rad

INTERFEROGRAMS PLOTTED WITH REFERENCE CHANGE VALUE (RCV) MAY FACILITATE THE MANAGEMENT OF HEMOLYZED SAMPLES

INTERFEROGRAMI PREDSTAVLJENI SA PROMENOM REFERENTNE VREDNOSTI (RCV) MOGU POTPOMOĆI ORGANIZACIJI HEMOLIZOVANIH UZORAKA

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Summary

Background: The European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group for Preanalytical Phase (WG-PRE) have recommended an algorithm based on the reference change value (RCV) to evaluate hemolysis. We utilized this algorithm to analyze hemolysis-sensitive parameters.
Methods: Two tubes of blood were collected from each of the 10 participants, one of which was subjected to mechanical trauma while the other was centrifuged directly. Subsequently, the samples were diluted with the participant's hemolyzed sample to obtain the desired hemoglobin concentrations (0, 1, 2, 4, 6, 8, and 10 g/L). ALT, AST, K, LDH, T. Bil tests were performed using Beckman Coulter AU680 analyzer. The analytical and clinical cut-offs were based on the biological variation for the allowable imprecision and RCV. The algorithms could report the values directly below the analytical cut-off or those between the analytical and clinical cut-offs with comments. If the change was above the clinical cut-off, the test was rejected. The linear regression was used for interferograms, and the hemoglobin concentrations corresponding to cut-offs were calculated via the interferograms.
Results: The RCV was calculated as 29.6% for ALT. Therefore, ALT should be rejected in samples containing >5.9 g/L hemoglobin. The RCVs for AST, K, LDH, and T. Bil were calculated as 27.9%, 12.1%, 19.2%, and 61.2%, while the samples' hemoglobin concentrations for test rejection were 0.8, 1.6, 0.5, and 2.2 g/L, respectively.

Kratak sadržaj

Uvod: Radna grupa za preanalitičku fazu (WG-PRE) Evropske Federacije za kliničku hemiju i laboratorijsku medicinu (EFLM) preporučila je algoritam koji se zasniva na promeni referentne vrednosti (RCV) za procenu hemolize. Mi smo koristili ovaj algoritam za analiziranje osetljivih hemolizovanih parametara.
Metode: Uzete su dve epruvete krvi od svakog od 10 učesnika, jedna koja je bila predmet mehaničke traume a druga je direktno centrifugirana. Istovremeno uzorci su bili razblaženi sa hemolizovanim uzorcima učesnika da bi se dobile željene koncentracije hemoglobina (0, 1, 2, 4, 6, 8 i 190 g/L). Analiziranje ALT, AST, K, LDH, T. Bil je izvedeno primenom Beckman Coulter AU680 analizatora. Analitičke i kliničke cut-off vrednosti bile su zasnovane na biološkoj varijaciji za dozvoljivu nepreciznost i RCV. Algoritmi su odgovarali direktno vrednostima ispod kliničke cut-off ili one između analitičke i kliničke cut-off. Ako je promena bila iznad kliničke cut-off test je odbačen. Linearna regresija je korišćena na interferograme i koncentracije hemoglobina koje su odgovarale cut-off vednostima izračunatih preko interferograma.
Rezultati: RCV je izračunat kao 29,6% za ALT. Prema tome potrebno je odbaciti uzorke za ALT koji su imali >5,9 g/L hemoglobina. RCV za AST, K, LDH i T. Bil izračunati su kao 27,9%, 12,1%, 19,2% i 61,2%, dok su uzorci sa koncentracijom hemoglobina za odbacivanje testa bili 0,8, 1,6, 0,5 i 2,2 g/L.

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Conclusions: Algorithms prepared with RCV could provide evidence-based results and objectively manage hemolyzed samples.

Keywords: hemolysis, interference, preanalytical phase, laboratory errors, reference change value

Zaključak: Algoritimi pripremljeni sa RCV mogu da obezbe rezultate zasnovane na dokazima i objektivno procene hemolizovane uzorke.

Ključne reči: hemoliza, interferencija, preanalitička faza, laboratorijske greške, promene referntnih vrednosti

Introduction

The International Organization for Standardization (ISO) has established that laboratory errors may arise at every point related to the laboratory in the process, from the test request to diagnosis and treatments interrelated to test results (1). This comprehensive definition showed that the laboratory's responsibilities are not only restricted to analyze the test and report its results. Nowadays, when laboratory results are considered crucial in clinical decisions, laboratories have a significant role, directly or indirectly, in the diagnosis and treatment of patients in many aspects (2). Thus, laboratory errors should be handled with a holistic approach, evaluated, and fixed (3).

Most of the errors evident in laboratories arise from the preanalytical process (4). Notably, the most common problem in this process is that of inappropriate samples (5). Among inappropriate samples, hemolyzed samples are the most common. Evaluation of all samples sent to the laboratory in a previous study, revealed that 2.2% of them were hemolyzed samples (6). Among samples accepted from emergency departments, up to 30% have been reported to be hemolyzed (7). Hemolysis can be in vivo and in vitro. In vivo hemolysis occurs during various diseases or treatments and constitutes only 2% of all hemolyzed samples accepted to the laboratory (8). In vitro hemolysis is almost the sole cause of hemolyzed samples encountered in laboratories and is caused by the following: blood collection process, sample handling process, sample processing process, and individual differences (9).

Hemolysis can be evaluated using two methods. The first is the classical method based on the technician's visual assessment. The visual evaluation method is discouraged because of its subjectivity and low reproducibility. Even if the color indicator charts with varying degrees of hemolysis, sample photographs are used for evaluation; this method's reliability and sensitivity are relatively low (10, 11). The second method uses hemolysis index (H-index), a tool that enables estimating the sample free hemoglobin (Hb) concentration using automated systems. The H-index can report the degree of hemolysis to both the laboratory specialist and physician. Therefore, it can enable the evaluation of the test results affected by hemolysis in the analyzed sample. Automated systems are reportedly more reliable than visual evaluation in determining hemolysis, and therefore, it is recommended for use instead of the visual method (12). Currently, the management of hemolyzed samples involves applying four options according to the hemolysis level, as follows: analysis of the sample followed by reporting the result; reporting the result and its interpretation; rejection based on the test; thorough sample rejection (13). For correct implementation, the serum index should be determined based on the test for the management of hemolysis.

The C56-A guideline of the Clinical and Laboratory Standards Institute (CLSI) recommends that interference studies should first be carried out by in vitro diagnostic (IVD) manufacturers and that the laboratories should design, as well as use, their algorithms. It also states that the reference change value (RCV) can be used as the allowable total error criterion for interference studies (14). Fraser stated that when the interfering effect of the substance on the test result exceeds the RCV, it could change the actual level of the measured analytes, with a clinically significant difference (15). The entire procedure was presented in an opinion letter that was recommended for clinical biochemistry tests published by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Working Group for Preanalytical Phase (WG-PRE) in 2018 (16). The EFLM WG-PRE has proposed an algorithm in line with this data to evaluate hemolysis interference for clinical chemistry tests. Interferograms were created by adding the allowable analytical coefficient of variation (CV) values and RCV as evaluation criteria after graphs were drawn by calculating the percentage change according to the Hb concentration for each test. Recent studies recommend a new algorithm that allows the interpretation of the test result through interferograms (12).

In this study, we aimed to develop algorithms to be used in the alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), potassium (K), and total bilirubin (T. Bil) tests for our laboratory, with respect to these suggestions, and to compare the data obtained with the manufacturer's statements.

Materials and Methods

This study was performed at the Medical Biochemistry Laboratory of the Gaziosmanpasa Training and Research Hospital, with the approval of the local ethics committee (decision date & number: April 10, 2019-56), in accordance with the Declaration of Helsinki. The study was explained to all volunteers and then informed consent was obtained.

Study design and sample preparation procedure

The sample size was determined to be 10 people, as per the CLSI EP 07A2 guideline. Acute or chronic disease, regular medication use, bleeding disorder, and pregnancy were selected as the exclusion criteria. Two tubes of blood were collected from 10 apparently healthy volunteers aged between 18 and 50 years. A single experienced phlebotomist was appointed to perform blood collection, to avoid hemolysis caused by phlebotomy. The samples were collected in the collection tubes (BD Barricor 5.0 mL, 13x100 mm, Becton, Dickenson and Company, NJ, USA) using the blood collection device (BD Vacutainer Holder, Becton, Dickenson and Company, NJ, USA) equipped with a needle (BD Vacutainer Eclipse 21G, BD-Belliver Industrial Estate, Plymouth PL6 7BP, UK).

One of the paired tubes was centrifuged without any intervention to obtain the hemolysis-free samples. The blood from the other tube was passed through the needle 10 times using an injector to achieve mechanical hemolysis (12). The mechanical trauma method was preferred to obtain samples similar to hemolyzed samples sent to the laboratory. This method ensured that the hemolyzed sample contains leukocytes and thrombocytes (13). Subsequently, the tubes were centrifuged at 2000 × *g* for 10 min following the manufacturer's recommendation.

Free Hb concentrations were measured in the separated plasma samples by the spectrophotometric method using the auto hematology analyzer (Mindray BC 6800, Shenzhen Mindray Bio-Medical Electronics Co., China; imprecision of Hb: 1.0%). It was confirmed that one of the samples had an Hb concentration of 0 g/L, while the other had a Hb concentration >10 g/L. To obtain samples with the desired free Hb concentrations (1, 2, 4, 6, 8, and 10 g/L), the hemolyzed samples were diluted with the hemolysis-free sample of the same volunteer. The samples were prepared individually for each volunteer, thus preventing dilution bias and inter-individual differences from affecting the values obtained. After obtaining the diluted samples, each sample was analyzed duplicate on the Mindray BC 6800 analyzer to confirm the desired Hb concentration. The process for sample preparation is shown in *Figure 1*.

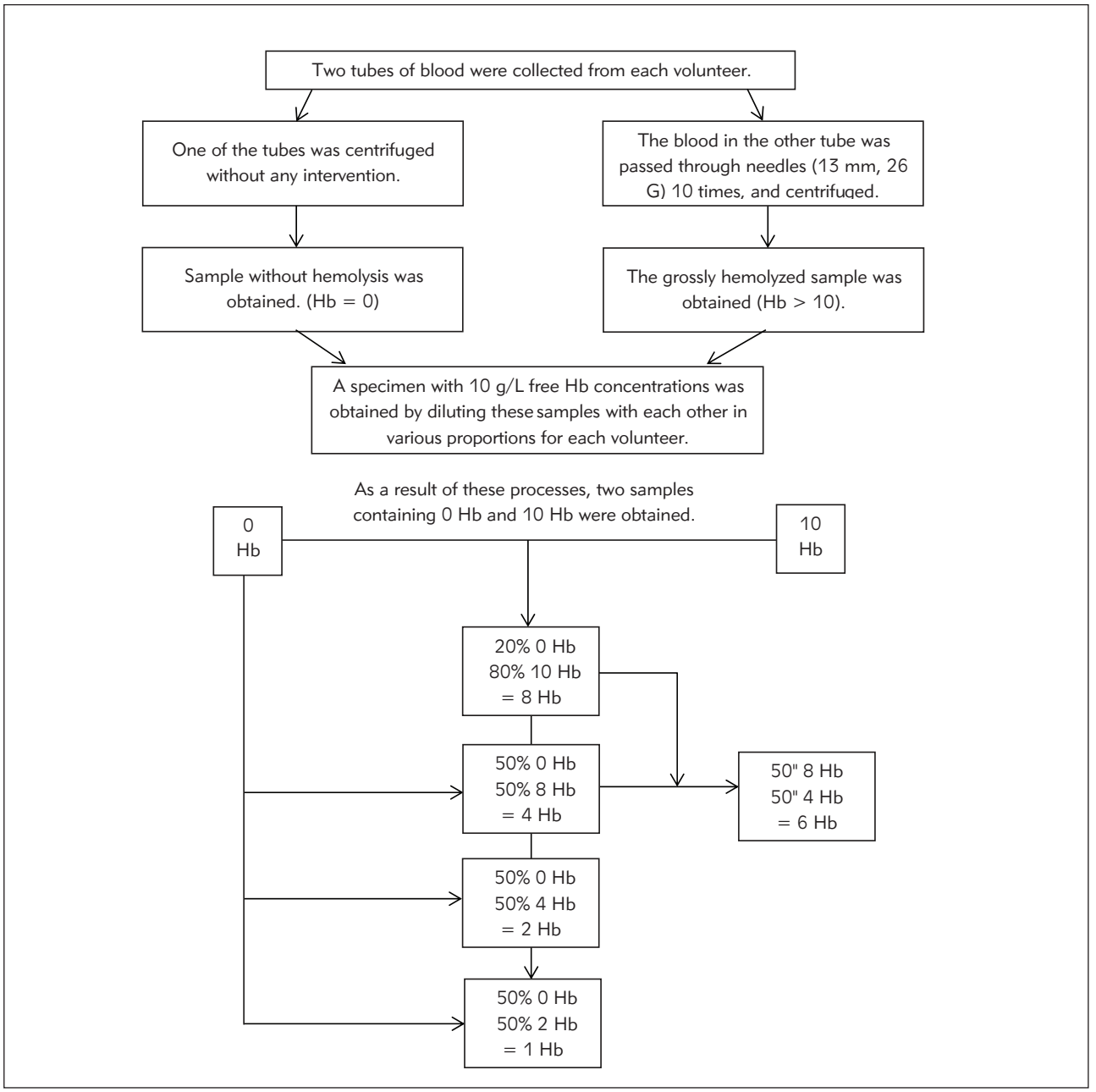


Figure 1 Preparation of hemolyzed samples. The samples were prepared individually for each volunteer (n=10). As a result, samples with the desired Hb concentrations (0, 1, 2, 4, 6, 8, and 10 g/L) were procured. Hb was expressed as g/L.

Evaluated tests and reagents

ALT, AST, LDH, K, and T. Bil tests were performed on a Beckman Coulter clinical chemistry analyzer AU680 (Beckman Coulter, Brea, CA). Tests were analyzed in duplicate for each sample. The manufacturer's original reagents were used in this study. The principles of the tests have been elaborated in the subsequent text.

- ALT: IFCC (without pyridoxal phosphate activation)
- AST: IFCC (without pyridoxal phosphate activation)
- LDH: Lactate to pyruvate, IFCC
- K: Ion-selective electrodes, indirect
- T. Bil: 3,5-dichlorophenyl-diazonium tetrafluoroborate (DPD) with caffeine, and a surfactant

In the technical sheets of reagents, it has been reported that hemolyzed samples should not be used for AST, K, and LDH tests. No Hb concentration, H-index limit, or bias of interference has been specified. It has been reported that the interference could be up to 10% in samples with 5 g/L free Hb for ALT and 0.45 g/L free Hb for T. Bil. H-index values are expressed qualitatively on the Beckmann AU680 analyzer. The cut-off degrees and the corresponding approximate Hb concentration ranges are presented in *Table I*.

Statistical analysis

The percentage difference was calculated for the samples from each patient. Next, the mean percentage differences were calculated for each Hb concentration using the percentage differences for 10 different volunteers. The Shapiro–Wilk test was used to evaluate whether the values were normally distributed. All values in our study were distributed normally, and the results are presented as the mean ± SD. Subsequently, interferograms were plotted for the evaluated tests. The scatter plots and linear regression method were used when creating interferograms, and the Hb concentrations corresponding to analytical and clinical cut-offs were calculated using the regression equation of the graphs. Analytical cut-offs were determined according to the desirable allowable CV (analytically acceptable imprecision (I %)) values of the biological variation (BV) databases. Clinical cut-offs were determined according to the RCV. Intra-individual CV (CV_I) for tests was obtained from the EFLM BV database and Westgard Desirable BV database (17, 18). The Hb concentration for sample rejection was determined to be 10 g/L following the recommendation of EFLM (16). The formulas for the calculation are as follows:

Percent difference (%)

$$= \left(\frac{Result\ of\ hemolyzed\ sample - Result\ of\ nonhemolyzed\ sample}{Result\ of\ nonhemolyzed\ sample}\right) x\ 100$$

$$I\ (\%) = 0.5 \times CV_I$$

$$RCV\ (\%) = \sqrt{2} \times 1.96 \times \sqrt{(CV_A^2 + CV_I^2)}$$

I: Analytically acceptable imprecision

CV_A: Laboratory analytical CV

CV_I: Within-subject BV

For laboratory analytical CV (CV_A) calculation, two levels of internal quality controls (Beckman Coulter Control Serum 1–2, Inc., USA), which were run on 20 different days, were used. The following formula was used for calculation (16):

$$CV_A = \frac{QC\ Level\ 1\ CV_A + QC\ Level\ 2\ CV_A}{2}$$

The calculations were performed, and interferograms were plotted, using MedCalc® Statistical Software version 19.6.4 (MedCalc Software Ltd, Ostend, Belgium) and Microsoft Office 365 (Microsoft Excel Software, Microsoft Corporation, US).

Table I The cut-off degrees and the corresponding approximate hemoglobin (Hb) concentration ranges in Beckmann Coulter AU680 analyzer.

Cut-offs specified by the manufacturer	Approximate free Hb concentration ranges (g/L)
0	Hb≤0.5
+	0.5<Hb≤1
++	1<Hb≤2
+++	2<Hb≤3
++++	3<Hb≤5
+++++	5<Hb

Results

According to the specified tests using samples with determined Hb concentrations, mean percentage differ-

Table II Mean percentage differences (%) between samples from each subject (n=10) for determined hemoglobin (Hb) values, according to the specified tests.

Tests	Hb=1 g/L	Hb=2 g/L	Hb=4 g/L	Hb=6 g/L	Hb=8 g/L	Hb=10 g/L
Alanine aminotransferase, U/L	6.7 ± 3.5	11.4 ± 4.2	19.6 ± 7.6	31.0 ± 12.3	39.6 ± 15.6	48.2 ± 15.3
Aspartate aminotransferase, U/L	46.1 ± 11.3	83.2 ± 17.9	162.9 ± 37.2	253.8 ± 58.3	354.8 ± 86.4	458.3 ± 92.6
Lactate dehydrogenase, U/L	50.7 ± 24.9	126.9 ± 45.3	287.7 ± 83.3	517.1 ± 102.0	659.6 ± 152.7	794.3 ± 163.1
Potassium, mmol/L	8.0 ± 4.7	15.3 ± 5.1	29.3 ± 7.8	47.3 ± 7.5	61.0 ± 10.8	74.3 ± 12.5
Total Bilirubin, mmol/L	-20.3 ± 12.6	-51.9 ± 22.5	-115.4 ± 45.5	-181.3 ± 78.6	-247.1 ± 101.2	-295.2 ± 110.5

All values are presented as mean ± SD (%). Normal distribution of values was proved by Shapiro-Wilk test (p> 0.05).

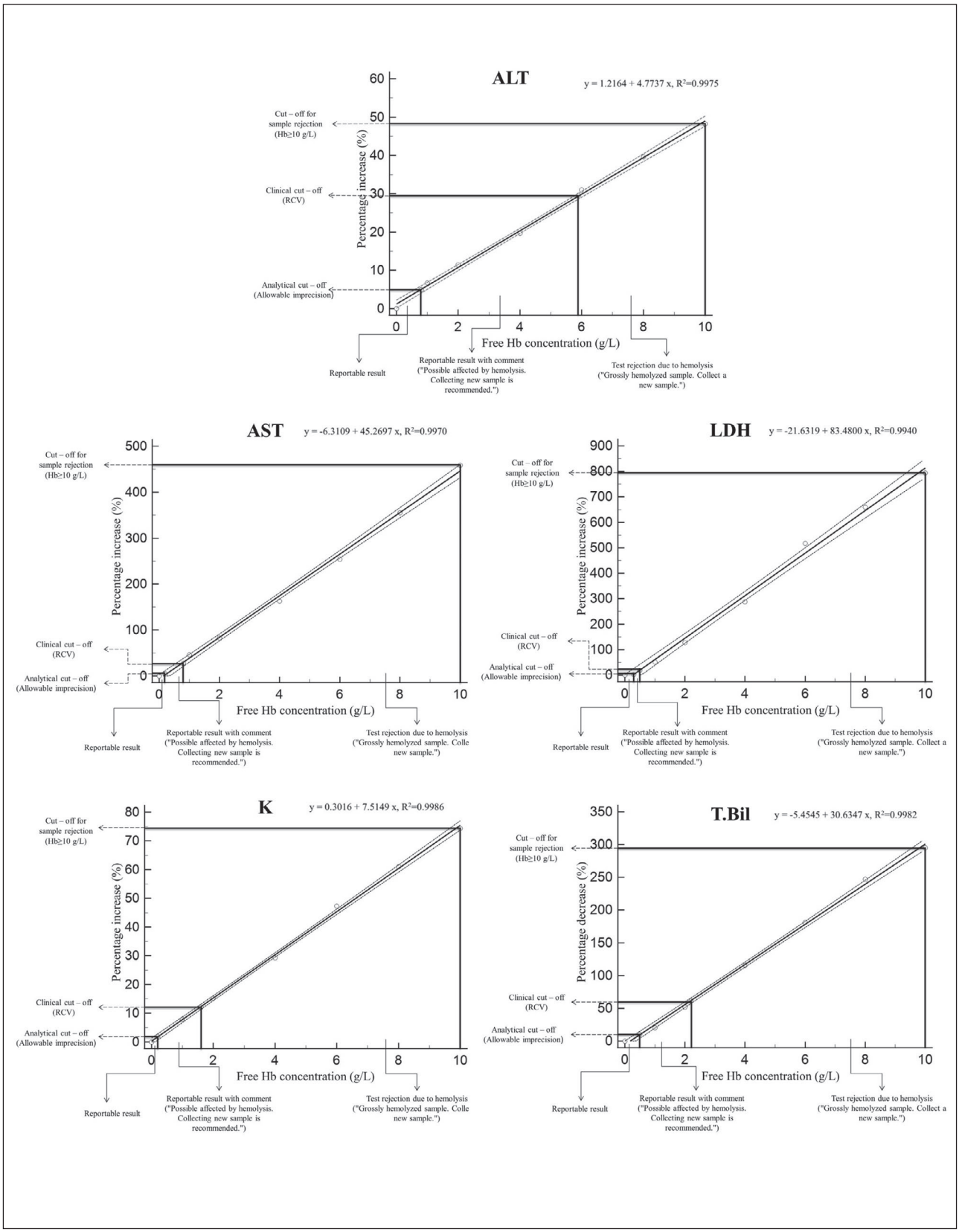
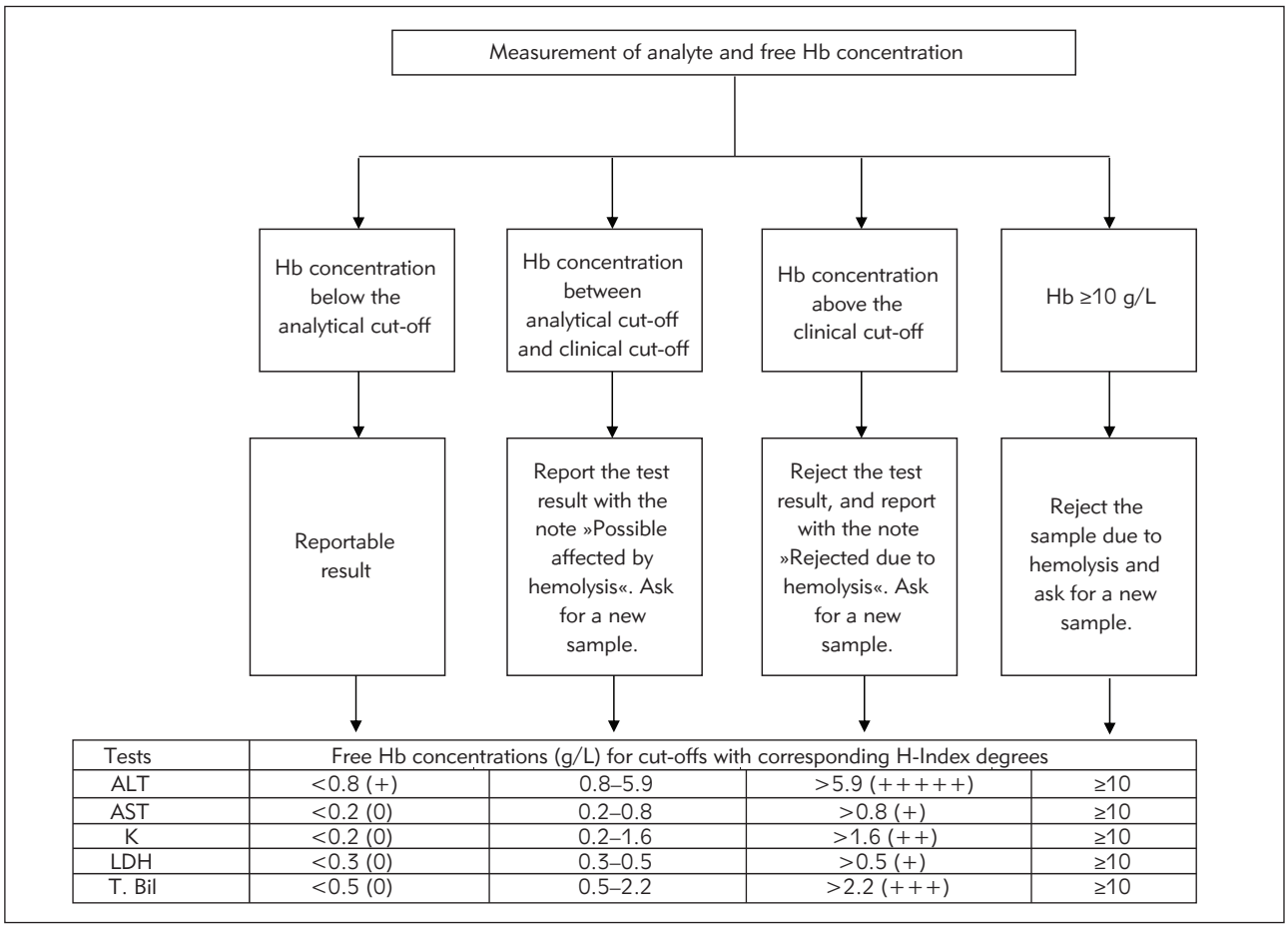


Figure 2 Interferograms of the evaluated tests. Analytical cut-off: desirable allowable imprecision from the biological variation databases, clinical cut-off: reference change value (RCV). Regression equations and coefficient of determinations (R^2) are presented in the corners of the figures. The regression line represents the 95% CI. Hb: Hemoglobin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, K: Potassium, LDH: Lactate dehydrogenase, T. Bil: Total bilirubin.

Table III Regression equations (95% CI), R^2, CV_A and CV_I values (%) of the evaluated tests. Analytical and clinical cut-off values (%) with corresponding free hemoglobin (Hb) concentrations calculated via regression equations.

Tests	Regression equation (95% CI)	R^2	CV_A (%)	CV_I (%)	Desirable imprecision (Analytical cut-off - %)	Hb[a] (g/L)	RCV (Clinical cut-off - %)	Hb[b] (g/L)
ALT	y = 1.22 (-0.33 – 2.76) + 4.77 (4.50 – 5.05) x	0.99	3.5	10.1	5.0	0.8	29.6	5.9
AST	y = -6.31 (-22.25 – 9.63) + 45.27 (42.43 – 48.11) x	0.99	3.0	9.6	4.8	0.2	27.9	0.8
K	y = 0.30 (-1.51 – 2.11) + 7.51 (7.19 – 7.84) x	0.99	1.5	4.1	2.0	0.2	12.1	1.6
LDH	y = -21.63 (-63.39 – 20.13) + 83.48 (76.05 – 90.91) x	0.99	4.6	5.2	2.6	0.3	19.2	0.5
T. BIL	y = -5.63 (-14.83 – 3.57) + 30.66 (29.03 – 32.30) x	0.99	3.4	21.8	10.9	0.5	61.2	2.2

[a] Corresponding to analytical cut-off [b]Corresponding to clinical cut-off. CI: Confidence interval, R^2: Coefficient of determination, CV_A: Analytical CV of the laboratory, CV_I: Within – subject Biological Variation, Analytical cut-off: Desirable allowable imprecision values from Biological Variation databases, Clinical cut-off: RCV (Reference change value). ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, K: Potassium, LDH: Lactate dehydrogenase, T.Bil: Total bilirubin.



Tests	Free Hb concentrations (g/L) for cut-offs with corresponding H-Index degrees			
ALT	<0.8 (+)	0.8–5.9	>5.9 (+++++)	≥10
AST	<0.2 (0)	0.2–0.8	>0.8 (+)	≥10
K	<0.2 (0)	0.2–1.6	>1.6 (++)	≥10
LDH	<0.3 (0)	0.3–0.5	>0.5 (+)	≥10
T. Bil	<0.5 (0)	0.5–2.2	>2.2 (+++)	≥10

Figure 3 Basic presentation of the composed algorithms. Hb concentrations corresponding to cut-offs were calculated via the regression equation. H-index degrees were adapted to the results in line with the cut-offs of the manufacturer. Hb: Hemoglobin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, K: Potassium, LDH: Lactate dehydrogenase, T. Bil: Total bilirubin.

ences (%) between the samples are presented in *Table II*. The interferograms of the tests are shown in *Figure 2*. The desirable allowable impression (I %) – analytical cut-off for ALT was obtained as 5.0%. The RCV – clinical cut-off was calculated as 29.6% (*Table III*). Hb concentrations corresponding to these values were calculated as 0.8 and 5.9 g/L based on the regression equation (*Table III*). Accordingly, for samples with Hb concentration <0.8 g/L, ALT results could be reported directly; with 0.8–5.9 g/L, ALT results could be reported with interpretation (*Table III*). Since the percentage change values of ALT in samples with Hb >5.9 g/L were greater than the RCV, it was deemed appropriate for rejection (*Table III*). For AST, LDH, K, and T.Bil, I % values and RCVs were found to be 4.8%, 27.9%; 2%, 12.1%; 2.6%, 19.2%; and 10.9%, 61.2%, respectively (*Table III*). The Hb concentration cut-offs obtained from the regression equation for the four tests were found to be 0.2, 0.8 g/L; 0.2, 1.6 g/L; 0.3, 0.5 g/L, and 0.5, 2.2 g/L; respectively (*Table III*). The H-index measurement results in each hemolyzed sample were observed in agreement with the manufacturer's H-index cut-offs. However, it was observed that the ratio of change in analyte results due to hemolysis did not fully comply with the manufacturer's statements.

The CV_A, CV_I, analytical cut-off (I) and clinical cut-off values (RCV), regression equations (95% CI), coefficient of determinations (R^2), and the corresponding Hb levels to cut-off values are presented in *Table III*. A simple presentation of the algorithms is shown in *Figure 3*.

Discussion

In our study, the hemolysis interference was evaluated for five clinical biochemistry parameters, based on the BV desirable I% (analytical cut-off) and RCV% (clinical cut-off). Accordingly, to determine the extent to which the tests were affected by hemolysis, LDH, AST, K, T. Bil, and ALT tests were performed, with respect to Hb concentration ranging from low to high.

For LDH, if the Hb concentration of the sample was <0.3 g/L, the test result could be reported directly; moreover, it could be reported with comment for 0.3–0.5 g/L Hb, and the test had to be rejected for >0.5 g/L Hb. For AST and K, the corresponding Hb values (in g/L) were found to be <0.2, 0.2–0.8, and >0.8, and <0.2, 0.2–1.6, and >1.6, respectively (*Figure 3*). As per the manufacturer's recommendation, these three tests should be rejected on the hemolyzed samples. As the initial cut-off value determined by the manufacturer for the H-index is 0.5 g/L, it can be assumed that the hemolyzed sample was meant for samples containing 0.5 g/L Hb. In another study, the upper limit of Hb reference value for hemolysis-free plasma samples was found to be 0.13 and 0.10 g/L in two different biochemistry analyzers (19). Although no information is available in the literature for our device, these values may be considered valid for our study, since it involved working with plasma samples containing lithium heparin. In fact, the Hb concentrations corresponding to the analytical cut-offs we calculated in our study for LDH, AST, and K seem close to these values. Besides, the tests most affected by hemolysis were those of LDH, AST, and K. However, our findings – particularly those associated with K – do not seem entirely consistent with the manufacturer's statement. It has been observed that K can be analyzed on samples containing up to 1.6 g/L Hb.

When the Hb concentration of the sample for T. Bil was <0.5 g/L, the result could be reported directly. It was observed that for 0.5–2.2. g/L Hb, the result could be reported with comment and that it should be rejected for >2.2 g/L Hb. For ALT, these Hb values were found to be <0.8, 0.9–5.9, and >5.9, respectively (*Figure 3*). The manufacturer stated that the hemolysis interference could be less than 10% in samples containing Hb up to 0.45 g/L for T. Bil and 5 g/L for ALT. Although this information is consistent with our findings, it is insufficient to manage samples containing 0.45 and 5 g/L in the T. Bil and ALT, respectively. According to our findings, samples containing up to 2.2 g/L and 5.9 g/L could be analyzed for T. Bil and ALT, respectively, and the result could be reported with the interpretation.

Perovic and Dolvic (20) evaluated the hemolysis levels of 25 clinical biochemistry parameters using RCV and compared the results with the manufacturer's statements. Similar to our study, the Beckman Coulter AU480 clinical chemistry analyzer and reagents from the same manufacturer were used in this study. It was observed that the findings within the reference range obtained in this study were consistent with our findings, and in the same study, it was stated that the manufacturer's statements were insufficient for ALT tests, among the tests evaluated. Studies on other devices and reagents have also obtained results that do not comply with the manufacturer's declarations (21–24). On the other hand, the general approach assumes the significant change affecting the result as ± 10% and presents the cut-off values accordingly. However, this ratio is far from being a proper criterion for every test (24). Using RCV or other criteria instead of ± 10% change value as a standard for each test can also support their flexibility (24, 25). Additionally, rejection rates may decrease by accepting RCV as a cut-off for test rejection (23). Based on these data, the use of RCV and these algorithms by manufacturers while performing interference studies will make the H-Index more beneficial.

Moreover, several problems have been observed with the routine use of the H-index. EFLM WG-PRE had a call for IVD manufacturers to provide more clarity, with respect to serum indexes, in 2018 (26). One problem is that the H-index is reported as degrees in some devices and free Hb concentrations in other devices. EFLM WG-PRE recommended a harmonization in reporting results via the use of a common unit, free Hb (g/L). Another problem is that manufacturers do not adequately report the interference specifications of the kits according to their H-index. For laboratories, inadequate information could be a significant problem in the management of hemolyzed samples. This situation was also evaluated in a large survey study conducted by EFLM WG-PRE, with the participation of 1405 laboratories in 37 European countries. It has been reported that many laboratories indicate heterogeneity of data on interference as the reason for avoiding serum indexes, and 67% of laboratories using serum indexes use the cut-off values recommended by the manufacturer without verification (27, 28). On the other hand, EFLM WG-PRE assumes that IVD producers did not fully comply with CLSI guidelines while performing interference studies; therefore, verification studies should be carried out by the laboratories (28). Furthermore, internal, and external quality control evaluations for H-index are recommended, given the critical importance of H-index results in the evaluation of other test results (26). It is thought that the approaches in which pre-

analytical variables are considered, especially in external quality control assessments, can provide efficient use of the H-index (12).

Our findings support this point of view. As seen in our study, it is thought that presenting the hemolysis interference with the qualitative cut-offs given based on approximate concentrations recommended by the manufacturer instead of the free Hb concentrations may be unsatisfactory for the evaluation and interpretation of hemolysis. Moreover, it would be more beneficial to use interferograms, instead of a single cut-off value, for managing hemolyzed samples. Interferograms provide more precise information for testing or sample rejection; hence, they allow laboratories the flexibility to accept the sample, and analyze or reject the tests. It is necessary to be careful when deciding to reject the samples that have been submitted to the laboratory. The decision on which sample is inappropriate, decision on tests that cannot be carried out in these samples, or decision to reject the sample should be based on evidence-based information. The blood sample is submitted to laboratories with the intent to elucidate crucial information about the patient being examined. The importance of correct interpretation of the test results, and their subsequent reporting, by a laboratory professional is analogous to that of accurate interpretation of a physical examination by a physician (29). The laboratory specialist's process for extensive assessment of the sample also includes the determination of which sample is suitable for analyzing or the determination of which test will be carried out. Analyzing the samples and reporting the correct result should be one of the priorities of laboratories due to the prolonged diagnostic process, additional cost, and other problems related to patient/doctor safety that may arise due to incorrect results and rejection (16). When we collectively evaluate these findings and opinions, the use of H-index and other serum indexes with interferograms appears to be beneficial for laboratories.

There are some limitations to this study. First, only five parameters affected by hemolysis were selected. A study including all clinical biochemistry tests would yield a more comprehensive result. Second, the reference method for Hb measurement is the hemoglobin cyanide method measured spectrophotometrically (30). However, the Mindray BC 6800 auto hematology analyzer used in our routine laboratory was used in our study to measure the Hb value. It is reported in the literature that the device we use is satisfactory in terms of analytical performance for Hb measurement (31). Nevertheless, using the reference method could provide more accurate results. Finally, the test results we evaluated in our study are within the reference ranges valid for the relevant test. Conducting evaluations in concentrations exceeding the reference range may help use the H-index more effectively.

Therefore, using the H-index with RCV and adapting the information to interferograms could be advantageous for laboratories to identify and manage hemolyzed samples. Single cut-off values are not suitable for use in interferograms alone. As observed in our study, it seems more appropriate to perform local studies to verify these values and determine the analytical and clinical cut-offs.

Research funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

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

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Received: March 11, 2021
Accepted: June 11, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-33096

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 62–70, 2022

Original paper
Originalni naučni rad

CUL4A ATTENUATES LPS-INDUCED ACUTE KIDNEY INJURY VIA BLOCKING NF-κB SIGNALING PATHWAY IN SEPSIS

CUL4A UBLAŽAVA LPS-INDUKOVANU AKUTNU INSUFICIJENCIJU BUBREGA BLOKIRANJEM NF-κB SIGNALNOG PUTA U SLUČAJU SEPSE

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Summary

Background: Acute kidney injury (AKI) is a common disease that can develop into end-stage kidney disease. Sepsis is one of the main causes of AKI. Currently, there is no satisfactory way to treat septic AKI. Therefore, we have shown the protective function of Cul4a in septic AKI and its molecular mechanism.
Methods: The cellular and animal models of septic AKI were established by using lipopolysaccharide (LPS). Western blot (WB) was employed to analyze Cul4a expression. RT-qPCR was employed to test the expression of Cul4a, SOD1, SOD2, GPX1, CAT, IL-6, TNF-α, Bcl-2, IL-1β, Bax and KIM-1 mRNA. ELISA was performed to detect the contents of inflammatory factors and LDH. CCK-8 was utilized to detect cell viability. Flow cytometry was utilized to analyze the apoptosis. DHE-ROS kit was used to detect the content of ROS.
Results: Cul4a was down-regulated in cellular and animal models of septic AKI. Oxidative stress is obviously induced by LPS, as well as apoptosis and inflammation. However, these can be significantly inhibited by up-regulating Cul4a. Moreover, LPS induced the activation of the NF-κB pathway, which could also be inhibited by overexpression of Cul4a.
Conclusions: Cul4awas found to be a protective factor in septic AKI, which could inhibit LPS-induced oxidative stress, apoptosis and inflammation of HK-2 cells by inhibiting the NF-κB pathway.

Keywords: sepsis, AKI, Cul4a, oxidative stress, inflammation, apoptosis

Kratak sadržaj

Uvod: Akutna insuficijencija bubrega (AKI) je česta bolest koja može prerasti u kritičnu fazu oštećenja bubrega. Sepsa je jedan od glavnih uzroka AKI. Trenutno ne postoji zadovoljavajući način lečenja septičke akutne insficijencije bubrega. Stoga smo pokazali zaštitnu funkciju Cul4a kod septičke insuficijencije bubrega i njegov molekularni mehanizam.
Metode: Ćelijski i životinjski modeli septičke akutne insuficijencije bubrega su izazvani upotrebom lipopolisaharida (LPS). Za analizu ekspresije Cul4a korišćen je Vestern blot (WB). RT-qPCR je korišćen za ispitivanje ekspresije mRNA Cul4a, SOD1, SOD2, GPKS1, CAT, IL-6, TNF-α, Bcl-2, IL-1β, Bax-a i KIM-1 mRNA. Izvršena je ELISA analiza za otkrivanje sadržaja upalnih faktora i LDH. Za utvrđivanje održivosti ćelija korišćen je CCK-8. Za analizu apoptoze korišćena je protočna citometrija. Za otkrivanje sadržaja ROS-a korišćen je DHE-ROS komplet.
Rezultati: Cul4a je snižen u ćelijskim i životinjskim modelima septičkog AKI. Oksidativni stres je očigledno izazivan LPS-om, kao i apoptoza i upala. Međutim, oni se mogu značajno inhibirati regulacijom Cul4a. Štaviše, LPS je indukovao aktivaciju NF-kB signalnog puta, što bi takođe moglo biti inhibirano prekomernom ekspresijom Cul4a.
Zaključak: Utvrđeno je da je Cul4a zaštitni faktor u slučajevima akutne septičke insuficijencije bubrega, što, inhibirajući NF-κB signalni put, može sprečiti oksidativni stres izazvan LPS-om, apoptozu i upalu ćelija HK-2.

Ključne reči: sepsa, AKI, Cul4a, oksidativni stres, zapaljenje, apoptoza

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Introduction

AKI refers to a syndrome in which a patient's renal function is significantly declined rapidly due to various causes, and a series of clinical symptoms occur, including increased serum creatinine (Cr), decreased urine output, electrolyte disturbance, acid-base imbalance, etc. In severe cases, acute brain edema, acute heart failure, or even life-threatening may occur. Suffering from AKI can lead to increased demand for renal replacement therapy, increased risk of death, and more treatment costs, which will bring a heavy burden to patients and society (1–3). AKI could be induced by many factors, including sepsis, ischemia-reperfusion injury, and nephrotoxic drugs. A large clinical study showed that in critically ill patients, septic shock is the main factor in the onset of AKI, accounting for 47.5% of the total population (4)

Sepsis is a continuous and excessive inflammatory response and immunosuppression caused by pathogen invasion. It is an important factor for organ failure of the body and can lead to shock and even death of patients (5). Sepsis is a common clinical systemic critical illness. In critically ill patients, the fatality rate reaches 35% (6), which seriously threatens the life and health of patients (7). Studies found that sepsis patients had a 50% risk of developing AKI, and such patients have a poor prognosis and high mortality (8, 9). In clinical practice, the treatment of septic AKI is often implemented through strategies such as fluid replacement, diuretics and antibiotics. However, the fatality rate of septic AKI has not been significantly reduced (10). Therefore, exploring new sepsis AKI treatment drugs is of great significance for alleviating kidney damage and saving patients' lives and health.

Cullin4A (Cul4a) belongs to the E3 ligase ubiquitin family in the ubiquitin-proteasome system (UPS) and determines the substrate specificity of ubiquitination modification (11–13). Cul4a ubiquitin ligase has a wide range of substrates and acts a pivotal part in a series of biological processes such as signal transduction, transcription regulation, cell cycle regulation, maintenance of genome stability, and embryo development (14, 15). At present, Cul4a has been extensively studied in various cancers (16, 17), but its function in kidney disease such as septic AKI is still unclear.

Here, we describe a new role of Cul4a, which is to inhibit inflammation and apoptosis mediated by oxidative stress in septic AKI. This will provide a potential new treatment for septic AKI.

Materials and Methods

Rat septic AKI model

Ten male Sprague-Dawley (SD) rats (Shanghai Experimental Animal Center of Chinese Academy of Sciences) were raised in an SPF environment. The breeding room has a temperature of 22–25 °C and a humidity of about 50%. LPS (5 mg/kg) was injected intraperitoneally to establish a septic AKI model.

Cell treatment

HK-2 cells, human renal cortex proximal convoluted tubule epithelial cell line, were purchased from Yaji Biotechnology Co., Ltd (Shanghai, China). The cells were cultured in a culture medium composed of DMEM/F-12 (Gibco, Rockville, MD, USA) and 10% FBS (Gibco, Rockville, USA) at 37 °C with 5% CO_2. The medium needs to be changed every 24 hours. 500 ng/mL LPS was used to induce cell damage.

The Cul4a overexpression plasmids (Sangon Biotech, Shanghai, China) was transfected into HK-2 cells using Lipofectamine™ 3000 in accordance with the instructions.

Western blot

Radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) was used to extract the total protein in HK-2 cells. The concentration was examined by the BCA method. After incubation with the loading buffer, the same amount of protein (30 μg) from each group was added into SDS-PAGE. The voltage was set to 120 volts. When the protein is sufficiently separated, it is transferred to the PVDF membrane. The current was set to 300 mA. After the membranes were blocked by QuickBlock™ Blocking Buffer (Beyotime, Shanghai, China), primary antibodies (Cul4a, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IκKα, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IκBα, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, Abcam, Cambridge, MA, USA, Rabbit, 1:1000) were added and incubated at 4 °C. The next day, the secondary antibody was used to incubate the membranes. The electrochemiluminescence (ECL) developer was added dropwise to develop imaging, and the grey value was semi-quantitatively analyzed according to Image J software.

RT-qPCR analysis

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA in HK-2 cells following the protocols. The complementary deoxyribose nucleic acid (cDNA)was synthesized using Real Master Mix (Bio-Rad, Hercules, CA, USA). RT-qPCR was performed using the Prism 7900 System. GAPDH was utilized to normalize the expression of mRNAs. All the primers were listed in *Table I*.

Table I Real-time PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
Cul4a	CAAGACAGGGAGGTTCCA	TCTCCACACAGGCAATCA
TNF-α	AGGCACTCCCCCAAAAGATG	CCACTTGGTGGTTTGTGAGTG
IL-1β	ATGCCACCTTTTGACAGTGATG	GAAGGTCCACGGGAAAGACA
IL-6	GCCTTCTTGGGACTGATGCT	CTGCAAGTGCATCATCGTTGT
SOD1	CAATGTGGCTGCTGGAA	TGATGGAATGCTCTCCTGA
SOD2	GCCGTGTTCTGAGGAGAG	GTCGTAAGGCAGGTCAGG
GPX1	TTGAGAAGTGCGAGGTGAA	TCCGCAGGAAGGTAAAGAG
CAT	TGGTTTTCACCGACGAG	TTTGCCTTGGAGTATCTGG
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
IκKα	AAACCAGAAAATTGTTGTGGACT	ATCGAATCCCAGACCCTATATCAC
IκBα	TAAGCAAAATCCTGACCTGGTGT	GCTCGTCCTCTGTGAACTCC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction

Determination of malondialdehyde (MDA)

The level of MDA in the supernatant of HK-2 cells was examined by Lipid Peroxidation (MDA) Assay Kit (Abcam, Cambridge, MA, USA) according to the instructions.

Determination of ROS production

The contents of ROS in HK-2 cells was tested using DCFH-DA (MCE, Nanjing, China). The cells were incubated with DCFH-DA (5 μmol/L) for 30 min in the dark. Then the cells were collected by 0.05% trypsin-EDTA solution. After that, the cells were suspended in a fresh medium. Finally, the level of ROS was tested by a flow cytometer.

Enzyme-linked immunosorbent assay (ELISA)

The supernatant of HK-2 cells was collected. The contents of inflammatory cytokines (IL-6, IL-1β, TNF-α) and LDH were detected by commercial ELISA kits (Elabscience, Wuhan, China) following the protocols.

Flow Cytometry

The HK-2 cells were collected by trypsin and centrifugation. Then the cells were resuspended in a 200 μL binding buffer. After that, 5 μL Annexin V-FITc and 5 μL PI were added into the binding buffer. Finally, 10 min later, the apoptosis rate was measured by a flow cytometer.

TUNEL staining

The apoptosis was examined by TUNEL staining with a TUNEL kit (Roche, USA) following the manufacturers' instructions. The nucleus was stained by DAPI. The images were observed by the inverted fluorescence microscope.

Statistical analysis

The measurement data were described as the mean±standard deviation (SD). All statistical analyses were performed by GraphPad Prism 8.0. One-way analysis of variance (ANOVA) or Student's t-test was used for comparison. Significance was accepted at $P<0.05$.

Results

Cul4a was down-regulated in LPS-treated HK-2 cells

First, the expression of Cul4a in the LPS-treated HK-2 cells was detected through WB. Compared with the control group, Cul4a expression in HK-2 cells of the LPS group was significantly reduced (*Figure 1A*). At the same time, the level of Cul4a mRNA was also examined, and the result was consistent with the protein level (*Figure 1B*). In addition, we constructed the rat model of septic AKI and also detected Cul4a expression in the kidney. Cul4a expression in the LPS group was less than that in the sham group (*Figure 1C* and *D*). To further study the function of Cul4a, we transfected the Cul4a overexpression plasmid into cells and verified the transfection efficiency of the plasmid from the protein and mRNA levels (*Figure 1E* and *F*).

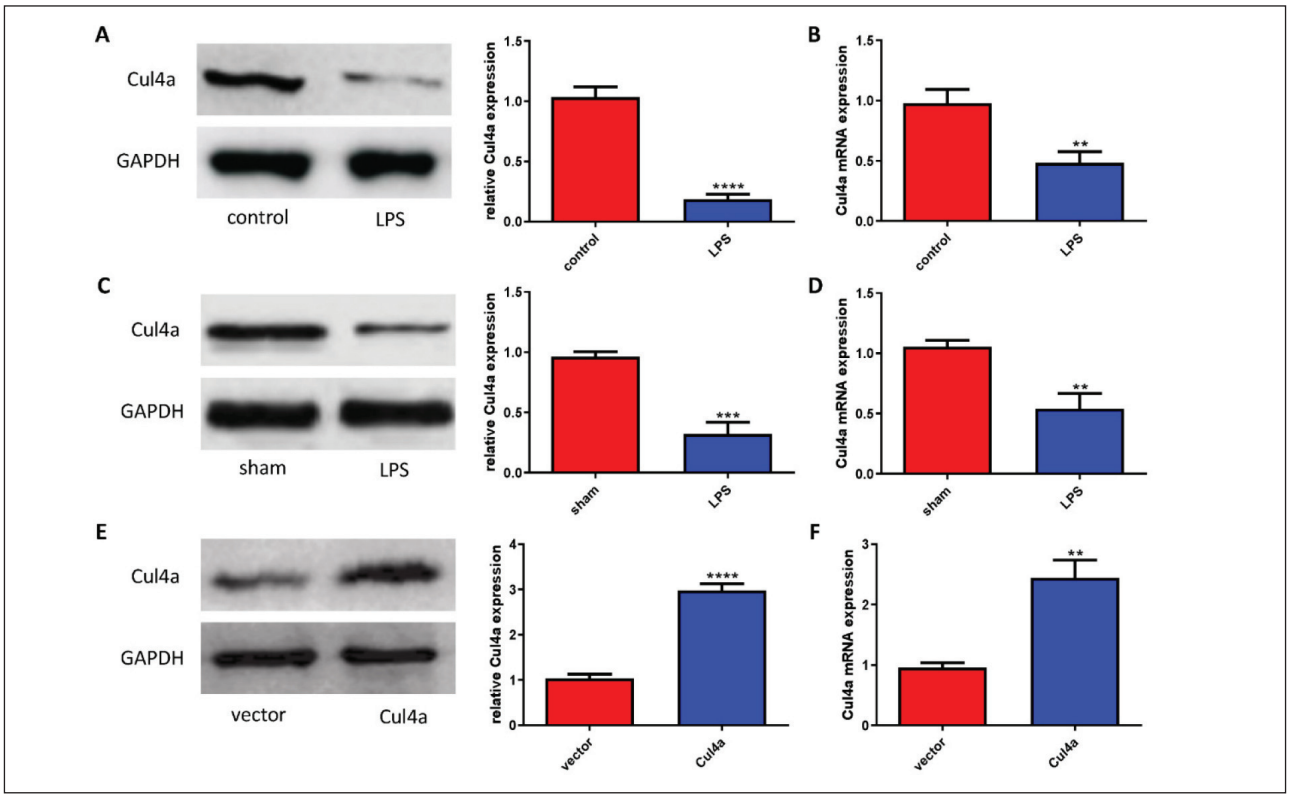

Figure 1 Cul4a was down-regulated in LPS-treated HK-2 cells. (A) Western blot showed the expression of Cul4a in HK-2 cells ("****" $p<0.0001$ vs. control, n=3). (B) Cul4a mRNA expression in HK-2 cells was detected by RT-PCR ("**" $p<0.01$ vs. control, n=3). (C) Western blot showed the expression of Cul4a in kidney tissues of rats ("***" $p<0.001$ vs. sham, n=3). (D) Cul4a mRNA expression in kidney tissues of rats was detected by RT-PCR ("**" $p<0.01$ vs. sham, n=3). (E) The expression of Cul4a in HK-2 cells transfected with plasmids ("***" $p<0.001$ vs. control, n=3). (F) Cul4a mRNA expression in HK-2 cells transfected with plasmids was detected ("**" $p<0.01$ vs. control, n=3).

Overexpression of Cul4a inhibited LPS-induced oxidative stress

Through RT-qPCR, we detected the levels of SOD1, SOD2, GPX1, and CAT mRNA. Compared with the control group, the levels of those mRNA in the LPS group were remarkably reduced, suggesting that oxidative stress exists in septic AKI. However, compared with the LPS+vector group, the levels of those mRNA in the LPS+Cul4a group were significantly increased (*Figure 2A~2D*). The content of MDA in the cell supernatant was also detected. Overexpression of Cu14a notably inhibited the content of MDA induced by LPS (*Figure 2E*). In addition, Cul4a also markedly decreased the production of ROS induced by LPS (*Figure 2F*).

Overexpression of Cul4a inhibited LPS-induced inflammation

We also tested the inflammatory response in HK-2 cells. LPS obviously induced the production of inflammatory cytokines (IL-6, IL-1β, TNF-α) mRNA in HK-2 cells. While up-regulating Cul4a suppressed their expression *(Figure 3A~3C*). We also tested the contents of inflammatory cytokines in the cell supernatant. The content of inflammatory cytokines in the LPS+Cul4a group was markedly lower than those in the LPS+vector group (*Figure 3D~3F*).

Overexpression of Cul4a inhibited LPS-induced apoptosis

Through the CCK-8 assay, the cell viability was detected. The treatment of LPS significantly reduced the viability of HK-2 cells, but overexpression of Cul4a could reverse this (*Figure 4A*). Furthermore, overexpression of Cu14a can also reduce the release of LDH (*Figure 4B*). We also tested the expression of KIM-1 mRNA and found that LPS can significantly induce the expression of KIM-1, while Cul4a can reduce this (*Figure 4C*). The expression of apoptosis-related genes was also detected. The level of Bcl-2 mRNA in the LPS treatment group was significantly reduced, while the level of Bax was significantly increased. Overexpression of Cu4a can reverse the above results (*Figure 4D* and *4E*). In addition, the rate of apoptosis was tested through flow cytometry and TUNEL staining. The results suggested that overexpression of Cul4a can inhibit LPS-induced apoptosis of HK-2 cells (*Figure 4F* and *4G*).

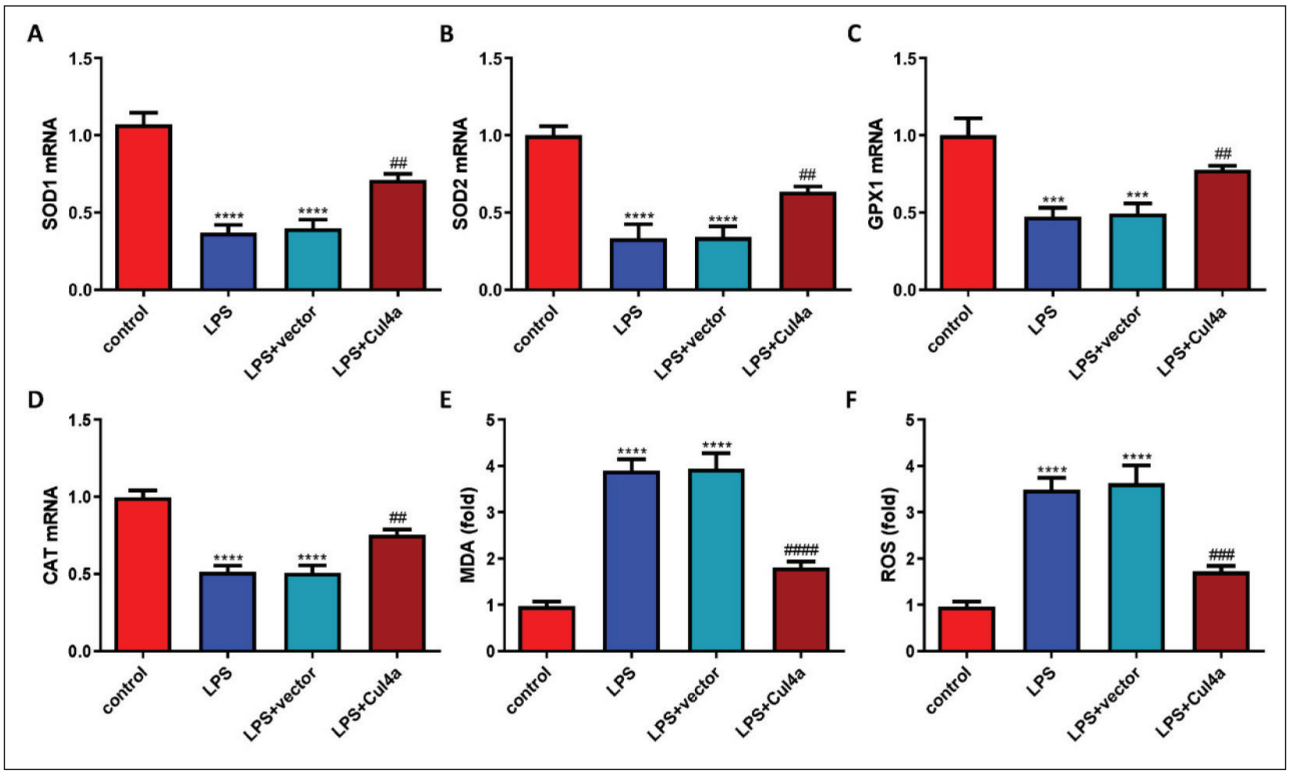

Figure 2 Overexpression of Cul4a inhibited LPS-induced oxidative stress of HK-2 cells. (A~D) The levels of SOD1, SOD2, GPX1, CAT mRNA were detected through RT-qPCR("***" $p<0.001$ vs. control, "****" $p<0.0001$ vs. control, "##" $p<0.01$ vs. LPS+vector, n=3). (E) The contents of MDA were detected ("****" $p<0.0001$ vs. control, "####" $p<0.0001$ vs. LPS+vector, n=3). (F) The production of ROS in HK-2 cells was detected ("****" $p<0.0001$ vs. control, "###" $p<0.001$ vs. LPS+vector, n=3).

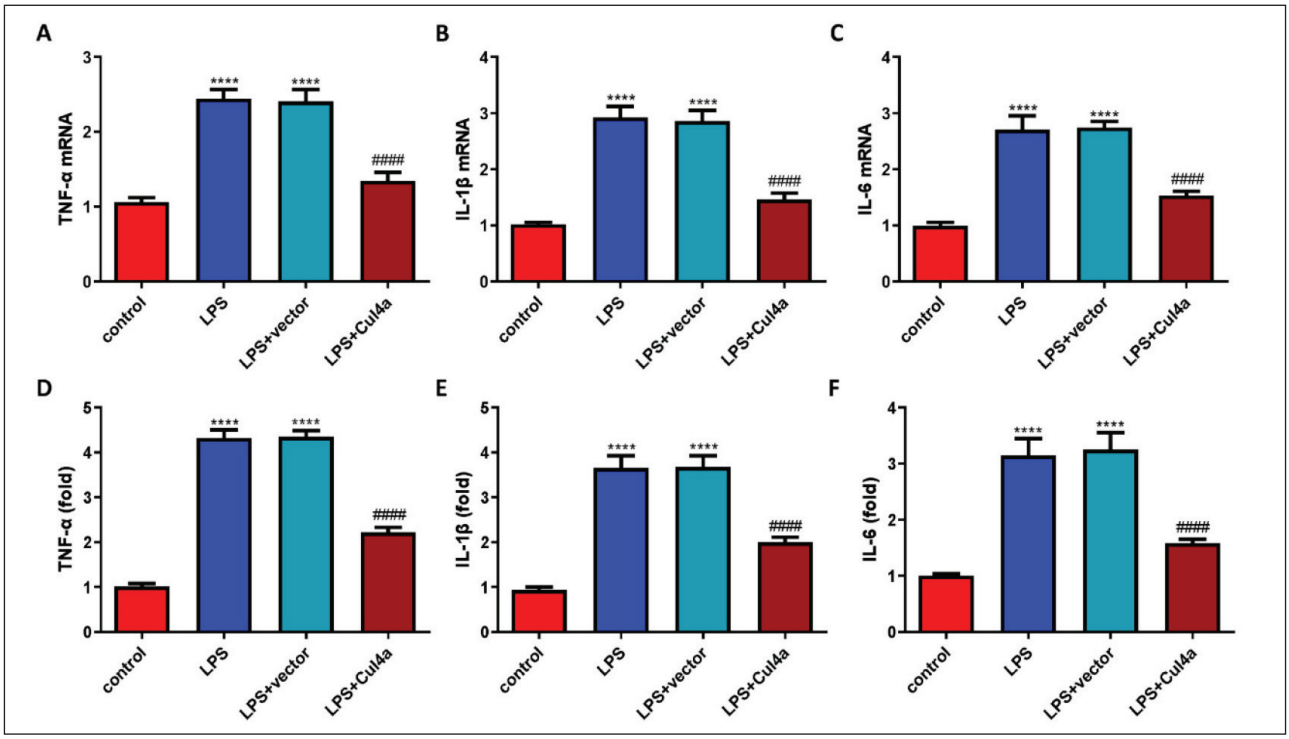

Figure 3 Overexpression of Cul4a inhibited LPS-induced inflammation of HK-2 cells. (A~C) The levels of TNF-α, IL-1β, IL-6 mRNA were detected through RT-qPCR ("****" $p<0.0001$ vs. control, "####" $p<0.0001$ vs. LPS+vector, n=3). (D~F) The contents of TNF-α, IL-1β, IL-6 in the supernatant were detected ("****" $p<0.0001$ vs. control, "####" $p<0.0001$ vs. LPS+vector, n=3).

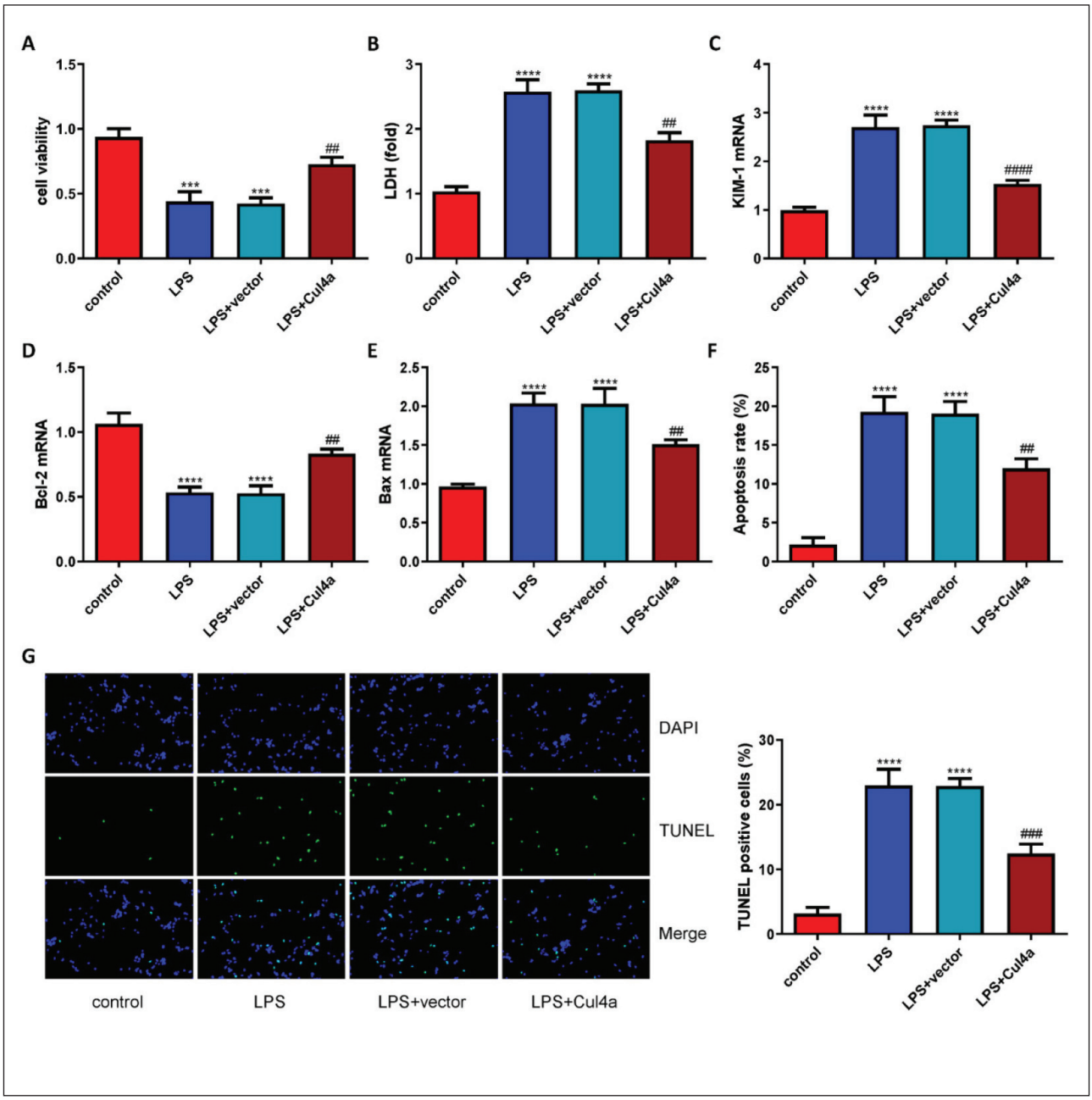

Figure 4 Overexpression of Cul4a inhibited LPS-induced apoptosis of HK-2 cells. (A) The viability of HK-2 cells was detected ("***" $p<0.001$ vs. control, "##" $p<0.01$ vs. LPS+vector, n=3). (B) The contents of LDH in the supernatant were detected ("****" $p<0.0001$ vs. control, "##" $p<0.01$ vs. LPS+vector, n=3). (CDE) The levels of KIM-1, Bcl-2, Bax mRNA were detected ("****" $p<0.0001$ vs. control, "##" $p<0.01$ vs. LPS+vector, "####" $p<0.0001$ vs. LPS+vector, n=3). (F) The rate of apoptosis was detected by flow cytometry ("****" $p<0.0001$ vs. control, "##" $p<0.01$ vs. LPS+vector, n=3). (G) Results of TUNEL staining in each group (200×) ("****" $p<0.0001$ vs. control, "###" $p<0.001$ vs. LPS+vector, n=3).

Cul4a inhibited the NF-κB pathway

Since the NF-κB pathway plays an important role in oxidative stress, inflammation, and apoptosis, we tested the marker proteins of this signalling pathway. The treatment of LPS significantly increased the expression of IκKα but decreased the expression of IκBα. However, overexpression of Cul4a significantly reversed their expression (*Figure 5A*). The expression of IκKα mRNA and IκBα mRNA was also detected, and the results were consistent with the previous results (*Figure 5B* and *5C*).

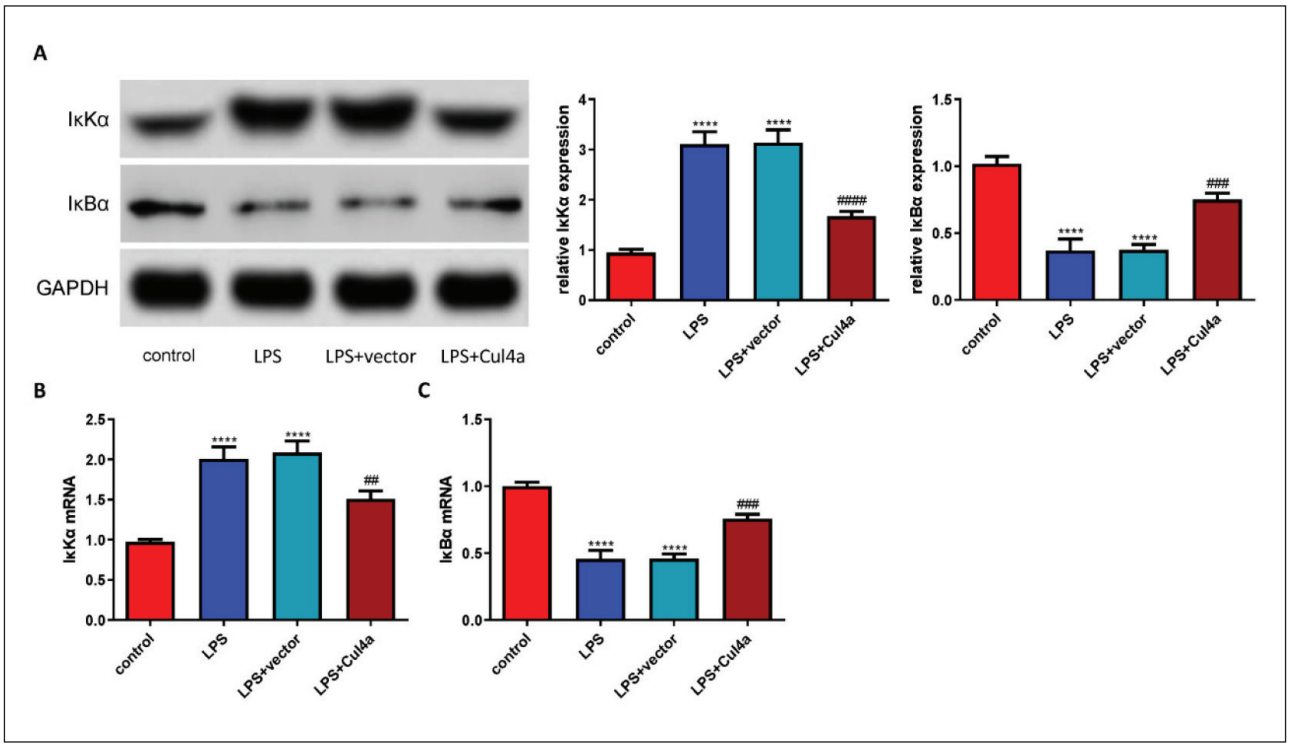

Figure 5 Cul4a inhibited the NF-κB signalling pathway. (A) The expression of IκKα and IκBα was detected ("****" $p<0.0001$ vs. control, "###" $p<0.001$ vs. LPS+vector, "####" $p<0.0001$ vs. LPS+vector, n=3). (B and C) The levels of IκKα and IκBα mRNA were detected ("****" $p<0.0001$ vs. control, "##" $p<0.01$ vs. LPS+vector, "###" $p<0.001$ vs. LPS+vector, n=3).

Discussion

In this present study, we revealed the protective role of Cul4a in septic AKI. We have revealed for the first time that Cul4a is down-regulated in septic AKI. Overexpression of Cul4a can significantly inhibit oxidative stress, inflammation and apoptosis, thereby reducing septic AKI. This protective effect was achieved at least in part by inhibiting the NF-κB signalling pathway.

The pathogenesis of AKI caused by sepsis is very complicated, which may be related to the increase of inflammatory factors, oxidative stress, and apoptosis (18, 19). Some scholars have suggested that inhibiting apoptosis, improving immune inflammation, and oxidative stress damage can help prevent AKI caused by sepsis (20). Apoptosis, especially renal tubular epithelial cell apoptosis, plays a key role in septic AKI. Sepsis may cause kidney cell apoptosis through endoplasmic reticulum stress, death receptor pathway, and mitochondrial pathway (21). In addition, sepsis can cause the body's systemic immune-inflammatory response and oxidative stress damage. Inflammation and oxidative stress can damage the glomeruli and renal tubules, leading to kidney damage, and, ultimately, renal insufficiency (22). Inflammatory factors such as IL-6 and TNF-α and oxidative stress indicators such as SOD can be used as early diagnostic markers for AKI caused by sepsis, and these markers have important values for judging the prognosis of the disease. Many studies have shown that inhibiting inflammatory factors and oxidative stress can help improve septic AKI. Chen et al. (23) proved that hydrogen sulfide could reduce septic AKI by inhibiting inflammation and oxidative stress. Rutin has also been shown to alleviate septic AKI in mice by inhibiting oxidative stress, inflammation, and apoptosis in the kidney (24).

NF-κB is a nuclear transcription factor involved in inflammation, oxidative stress and apoptosis (25, 26). Studies have found that the expression of NF-κB in the kidney tissue of septic AKI rats increases. When NF-κB translocates to the nucleus, it can activate the downstream inflammatory factors and oxidative stress indicators such as SOD, etc., thereby promoting the aggravation of inflammatory response and oxidative stress damage and aggravating kidney damage. And inhibition of the NF-κB signalling pathway could reduce renal inflammation, oxidative stress, and apoptosis, thereby reducing renal injury.

Our study demonstrates the regulatory role of Cul4a in septic AKI. Overexpression of Cul4a can remarkably inhibit LPS-induced oxidative stress and inhibit the production of inflammatory factors and apoptosis of HK-2 cells. The protective effect of Cul4a is at least partially achieved by inhibiting the NF-κB pathway.

Conclusion

To sum up, Cul4a was found to be a protective factor in septic AKI, and overexpression of Cul4a could inhibit LPS-induced oxidative stress, inflammation, and apoptosis of HK-2 cells by inhibiting the NF-κB pathway.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Received: July 13, 2021
Accepted: July 29, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-26452

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 71–78, 2022

Original paper
Originalni naučni rad

EFFECTS OF ADIPONECTIN, PLASMA D-DIMER, INFLAMMATION AND TUMOR MARKERS ON CLINICAL CHARACTERISTICS AND PROGNOSIS OF PATIENTS WITH OVARIAN CANCER

EFEKAT ADIPONEKTINA, D-DIMERA U PLAZMI, ZAPALJENJSKIH PROCESA I TUMORSKIH MARKERA NA KLINIČKE KARAKTERISTIKE I PROGNOZU KOD PACIJENTKINJA SA RAKOM JAJNIKA

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Summary

Background: To investigate the effects of adiponectin (ADPN), plasma D-dimer (D-D), inflammation, and tumour markers on clinical characteristics and prognosis of patients with ovarian cancer.

Methods: A total of 80 patients with ovarian cancer treated in our hospital from April 2017 to November 2019 were enrolled as study subjects and evenly divided into an observation group (patients with ovarian cancer) and a control group (patients with the benign ovarian tumour) based on the results of the postoperative pathological biopsy. The levels of ADPN, plasma D-D, inflammatory factors, and serum tumour markers [carbohydrate antigen 125 (CA125), human epididymis protein 4 (HE4), and risk of ovarian malignancy algorithm (ROMA)] were compared between the two groups. The diagnostic value of serum tumour markers CA125, HE4, and ROMA in ovarian cancer was explored. The correlations of ROMA changes with the changes in the levels of ADPN, plasma D-D, high-sensitivity C-reactive protein (hs-CRP), CA125, and HE4 were analysed. Additionally, the related risk factors affecting the development of ovarian cancer were subjected to univariate and multivariate logistic regression analyses.

Results: In comparison with the control group, the observation group exhibited a lowered ADPN level ($p<0.05$), notably raised levels of plasma D-D, inflammatory factors hs-CRP and interleukin-6 (IL-6) and serum tumour markers

Kratak sadržaj

Uvod: Cilj ove studije bio je da ispita efekte adiponektina (ADPN), D-dimera u plazmi (D-D), upala i tumorskih markera na kliničke karakteristike i prognozu kod pacijentkinja sa rakom jajnika.

Metode: U studiju je uključeno ukupno 80 pacijentkinja sa rakom jajnika lečenih u našoj bolnici od aprila 2017. do novembra 2019. godine. Pacijentkinje su ravnomerno podeljene u grupu za posmatranje (pacijentkinje sa rakom jajnika) i kontrolnu grupu (pacijentkinje sa benignim tumorom jajnika) na osnovu rezultata postoperativne biopsije. Između dve grupe upoređivani su nivoi ADPN, D-D u plazmi, inflamatorni faktori i serumski tumorski markeri [ugljenohidratni antigen 125 (CA125), humani protein epididimisa 4 (HE4) i algoritam rizika za malignitet jajnika (ROMA)]. Istražena je dijagnostička vrednost tumorskih markera u serumu CA125, HE4 i ROMA kod karcinoma jajnika. Analizirane su korelacije promena ROMA sa promenama nivoa ADPN, D-D u plazmi, C-reaktivnog proteina visoke osetljivosti (hs-CRP), CA125 i HE4. Pored toga, povezani faktori rizika koji utiču na razvoj raka jajnika podvrgnuti su univarijantnim i multivarijantnim logističkim regresionim analizama.

Rezultati: U poređenju sa kontrolnom grupom, posmatrana grupa je imala sniženi nivo ADPN ($p < 0,05$), značajno povišene nivoe D-D u plazmi, inflamatornog faktora hs-CRP i interleukina-6 (IL-6) i serumskih tumorskih markera CA125

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CA125 and HE4 and an evidently increased ROMA ($p<0.05$). Besides, the detection of serum ROMA showed the highest specificity and sensitivity and low false-positive rate and false-negative rate. The changes of ROMA were positively correlated with the changes in the levels of plasma D-D, hs-CRP, CA125, and HE4 ($p<0.05$) and negatively associated with the changes in ADPN level ($p<0.05$). The results of the univariate analysis showed that abnormal ADPN, D-D, hs-CRP, IL-6, CA125, and HE4 levels were related to risk factors affecting the development of ovarian cancer. It was found through multivariate logistic regression analysis that decreased ADPN level and increased D-D, hs-CRP, IL-6, CA125, and HE4 levels were independent risk factors affecting the development of ovarian cancer.

Conclusions: In the case of ovarian cancer, the ADPN level declines, while the levels of plasma D-D, inflammatory factors, and serum tumour markers CA125, HE4, and ROMA rise obviously. Besides, the ROMA level displays a positive relation to the content of CA125, HE4, plasma D-D, and inflammatory factors and a negative association with the ADPN level.

Keywords: adiponectin, plasma D-dimer, inflammatory factors, tumour markers, ovarian cancer

i HE4 i jasno uvećan ROMA ($p < 0,05$). Pored toga, otkrivanje serumskog ROMA pokazalo je najveću specifičnost i osetljivost i nisku stopu lažno pozitivnih i lažno negativnih rezultata. Promene ROMA su u pozitivnoj korelaciji sa promenama nivoa D-D u plazmi, hs-CRP, CA125 i HE4 ($p < 0,05$), a negativno su povezane sa promenama u nivou ADPN ($p < 0,05$). Rezultati univarijantne analize pokazali su da su abnormalni nivoi ADPN, D-D, hs-CRP, IL-6, CA125 i HE4 povezani sa faktorima rizika koji utiču na razvoj karcinoma jajnika. Kroz multivarijantnu logističku regresionu analizu utvrđeno je da su smanjeni nivo ADPN-a i povećani nivoi D-D, hs-CRP, IL-6, CA125 i HE4 bili nezavisni faktori rizika koji utiču na razvoj karcinoma jajnika.

Zaključak: U slučaju karcinoma jajnika, nivo ADPN opada, dok nivoi D-D u plazmi, inflamatorni faktori i tumorski markeri u serumu CA125, HE4 i ROMA očigledno rastu. Pored toga, nivo ROMA pokazuje pozitivnu vezu sa sadržajem CA125, HE4, D-D u plazmi i inflamatornim faktorima, a negativnu povezanost sa nivoom ADPN.

Ključne reči: adiponektin, D-dimer u plazmi, inflamatorni faktori, tumorski markeri, rak jajnika

Introduction

Patients with ovarian cancer tend to be at the middle or advanced stage when definitely diagnosed, and some of them even lost the opportunities for surgery (1). Ovarian cancer, the most common gynaecological malignancy, is characterized by an insidious onset, inconspicuous early symptoms, challenging diagnosis, and a poor prognosis, so it has a high fatality rate (2). The knowledge, as well as diagnosis and treatment methods for ovarian tumours, have been significantly improved as the living standards of people and economic level elevate, and medical technology has developed rapidly in China. However, the fatality and disability rates of ovarian cancer remain high (3).

Studies have manifested that the 5-year survival rate of patients with early ovarian cancer is more than 90% after regular treatment (4), and that of those with middle or advanced ovarian cancer, especially those who have lost opportunities for surgical treatment, is less than 20% (5). For this reason, improving the survival rate and quality of life of patients with ovarian cancer is the focus and hotspot in current studies. Early diagnosis and early treatment of ovarian cancer are particularly important. However, most patients seek medical advice only after complications occur since they have no specific symptoms and signs in the early stage (6). In addition, the specificity and sensitivity of tumour markers applied in the diagnosis of ovarian cancer in clinical practice need to be improved (7). A study denoted that patients with malignant tumours, including those with ovarian cancer, have significant changes in the levels of adiponectin (ADPN), plasma D-dimer (D-D), and related inflammatory factors (8). In this study, therefore, the effects of ADPN, plasma D-D, inflammation, and tumour markers on the prognosis of ovarian cancer were explored. It is now reported as follows.

Materials and Methods

General data

A total of 80 patients with ovarian cancer treated with surgery in our hospital from April 2017 to November 2019 were enrolled as study subjects. After surgery, these patients were definitely diagnosed based on the results of the pathological biopsy. All subjects signed enrolment consent. This study was approved by the Ethics Committee of our hospital. The diagnosis and treatment were conducted as per the fourth edition of the *Guidelines for the Diagnosis and Treatment of Ovarian Malignancies* (2015). Before surgery, a tentative diagnosis was made combined with the patient's clinical symptoms, imaging examination findings, and relevant biochemical auxiliary examinations. Then, surgical treatment was carried out. After the surgery, patients underwent regular chemoradiotherapy and biologic therapy. All patients enrolled had complete clinical data and were followed up regularly. Exclusion criteria were: patients with mental illness, those undergoing neoadjuvant chemotherapy before enrolment, those with unclear diagnosis, those lost to follow-up, those with respiratory failure, long-term administration of glucocorticoids, those with systemic immune system diseases or severe liver and kidney dysfunction, those who had not signed the enrolment consent, those with malignant tumours in other parts, those with a KPS score less than 60, or those with tumour cachexia. The enrolled patients were evenly divided into an observa-

tion group (patients with ovarian cancer) and a control group (patients with the benign ovarian tumour) based on the results of the postoperative pathological biopsy. In the observation group, patients were aged 19–60 years old with (49.9±2.7) on average, the duration of gynaecological symptoms was 1–6 month with a mean of (2.8±0.5) months, the tumour diameter was 1–15 cm with an average of (8.1±2.1) cm, and 21 had ascites. In the control group, the age was 18–60 years old with (50.0±2.6) on average, the duration of gynaecological symptoms was 1–6 month with a mean of (2.9±0.6) months, the tumour diameter was 1–15 cm with an average of (8.0±2.0) cm, and 20 had ascites. There were no statistically significant differences in the age, duration of gynaecological symptoms, tumour diameter, and the ratio of ascites, as well as the levels of ADPN, plasma D-D, inflammation, and tumour markers at the time of enrolment, between the two groups ($p>0.05$).

Methods

All patients enrolled were treated with surgery and diagnosed by postoperative pathological biopsy. Before surgery, fasting elbow venous blood was collected from patients in the morning for examinations of ADPN, plasma D-D, inflammatory factors [high-sensitivity C-reactive protein (hs-CRP) and interleukin-6 (IL-6)] and tumor markers [carbohydrate antigen 125 (CA125), human epididymis protein 4 (HE4) and risk of ovarian malignancy algorithm (ROMA)]. Besides, the above-related factors affecting the incidence of ovarian cancer were analysed in all patients enrolled to determine the correlations of serum biochemical indicators with the changes in the ROMA level.

Observation indexes

The levels of ADPN, plasma D-D, inflammatory factors, and serum tumour markers CA125, HE4, and ROMA were compared between the two groups. The diagnostic value of serum tumour markers CA125, HE4, and ROMA in ovarian cancer was explored. The correlations of the changes of ROMA with the changes in the levels of ADPN, plasma D-D, hs-CRP, CA125, and HE4 were analysed. Additionally, the related risk factors affecting the development of ovarian cancer were subjected to univariate and multivariate logistic regression analyses.

Assessment criteria

Hs-CRP (normal value: 10 mg/L) was detected via immunoturbidimetry, and IL-6 (normal value: 0.37–0.46 ng/L), D-D (normal value: <200 μg/L), ADPN (normal value: 3.62–13.06 μg/mL), CA125 (normal value: 40 μg/L), HE4 (normal value: 70 pmol/L) and ROMA (normal value: 0–11.4%) were examined through enzyme-linked immunosorbent assay (ELISA).

Statistical processing

SPSS 20.0 was used for statistical processing. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used to compare the mean between two groups, and the χ^2 test was adopted for comparison of the ratio between two groups. The correlations of ROMA level with the levels of ADPN, plasma D-D, hs-CRP, CA125, and HE4 were analysed through the Pearson method, and univariate and multivariate logistic regression analyses were performed on the related risk factors affecting the development of ovarian cancer. $P<0.05$ suggested that the difference was statistically significant.

Results

Comparisons of the levels of ADPN, plasma D-D, and inflammatory factors between the two groups

Compared with those in the control group, the level of ADPN declined ($p<0.05$), while the levels of plasma D-D and inflammatory factors his-CRP and IL-6 rose significantly in the observation group ($p<0.05$) (*Table I*).

Comparisons of serum tumour marker levels between the two groups

The levels of CA125, HE4, and ROMA were remarkably higher in the observation group than those in the control group ($p<0.05$) (*Table II*).

Table I Comparisons of ADPN, plasma D-D and inflammatory factor levels between the two groups ($\bar{x}\pm s$).

	ADPN (μg/mL)	D-D (μg/L)	Hs-CRP (mL/L)	IL-6 (ng/L)
Observation	1.1±0.2	362.3±21.4	16.3±2.8	0.68±0.05
Control	8.6±1.1	158.6±8.3	8.3±0.6	0.41±0.02
t	42.426	56.128	17.669	31.710
p	0.000	0.000	0.000	0.000

Table II Comparisons of CA125, HE4 and ROMA levels between the two groups ($\bar{x}\pm s$).

	CA125 (40 μg/L)	HE4 (pmol/L)	ROMA (%)
Observation	85.6±10.5	106.8±2.5	23.6±1.5
Normal group	18.9±2.3	45.6±1.2	8.9±0.6
t	39.245	139.578	57.547
p	0.000	0.000	0.000

Diagnostic value of serum CA125, HE4, and ROMA in ovarian cancer

The detection of serum ROMA showed the highest specificity and sensitivity and low false-positive rate and false-negative rate.

Correlation analysis of ROMA level with ADPN, plasma D-D, hs-CRP and serum CA125 and HE4 levels

The ROMA level was positively associated with plasma D-D, hs-CRP, and serum CA125 and HE4 levels ($p<0.05$) and negatively related to the ADPN level ($p<0.05$) (*Figure 1–5*).

Table III Diagnostic value of serum CA125, HE4, and ROMA in ovarian cancer.

	Specificity	Sensitivity	False-positive rate	False-negative rate
CA125	69.8	67.3	15.6	30.8
HE4	67.8	57.8	32.1	42.1
ROMA	81.2	83.3	18.9	32.8

Table IV Correlation analysis of ROMA level with ADPN, plasma D-D, hs-CRP and serum CA125 and HE4 levels.

	r	*p*
ADPN	-0.8921	0.000
Plasma D-D	0.9781	0.000
Hs-CRP level	0.9196	0.000
CA125	0.9792	0.000
HE4	0.9570	0.000

Univariate analysis of related risk factors affecting the incidence of ovarian cancer

It was revealed through univariate analysis that abnormal ADPN, D-D, hs-CRP, IL-6, CA125, and HE4 levels were related risk factors affecting the incidence of ovarian cancer (*Table V*).

Multivariate logistic regression analysis of related risk factors affecting the incidence of ovarian cancer

The multivariate logistic regression analysis results showed that lowered ADPN level and raised

Table V Univariate analysis of related risk factors affecting the incidence of ovarian cancer.

Item		Ovarian cancer	Benign ovarian	χ^2	*p*
ADPN level	Normal	9	27	19.090	0.000
	Abnormal	31	13		
D-D level	Normal	7	31	26.125	0.000
	Abnormal	33	11		
Hs-CRP level	Normal	7	26	18.620	0.000
	Abnormal	33	14		
IL-6 level	Normal	13	29	12.832	0.000
	Abnormal	27	11		
CA125 level	Normal	8	25	6.994	0.008
	Abnormal	32	15		
HE4 level	Normal	11	25	9.899	0.002
	Abnormal	29	15		

Table VI Multivariate logistic regression analysis of related risk factors affecting the incidence of ovarian cancer.

Item	β	SE	W	OR	p	95% CI
Decreased ADPN level	1.145	0.390	11.139	5.211	0.009	0.539–2.478
Increased D-D level	1.923	0.542	4.464	2.361	0.021	1.593–9.242
Increased hs-CRP level	1.714	0.603	17.11	5.526	0.008	3.106–9.834
Increased IL-6 level	1.966	0.454	18.781	7.135	0.000	2.933–17.358
Increased CA125 level	1.605	0.382	17.717	4.972	0.000	2.354–10.490
Increased HE4 level	1.049	0.440	5.646	2.853	0.017	1.201–6.773

Table VI Multivariate logistic regression analysis of related risk factors affecting the incidence of ovarian cancer.

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Increased IL-6 level	1.966	0.454	18.781	7.135	0.000	2.933–17.358
Increased CA125 level	1.605	0.382	17.717	4.972	0.000	2.354–10.490
Increased HE4 level	1.049	0.440	5.646	2.853	0.017	1.201–6.773

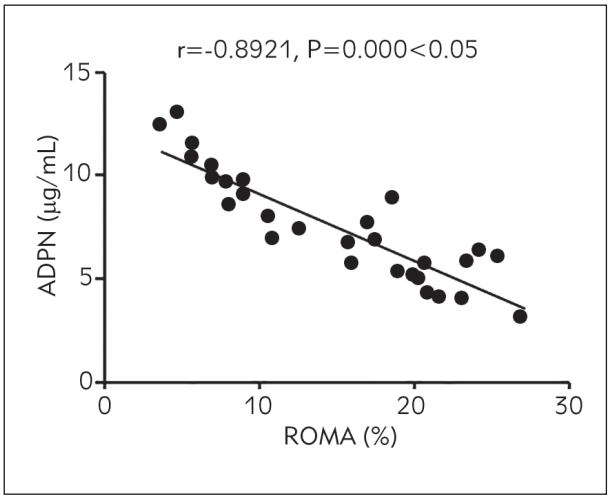

Figure 1 Correlation between ROMA level and ADPN level.

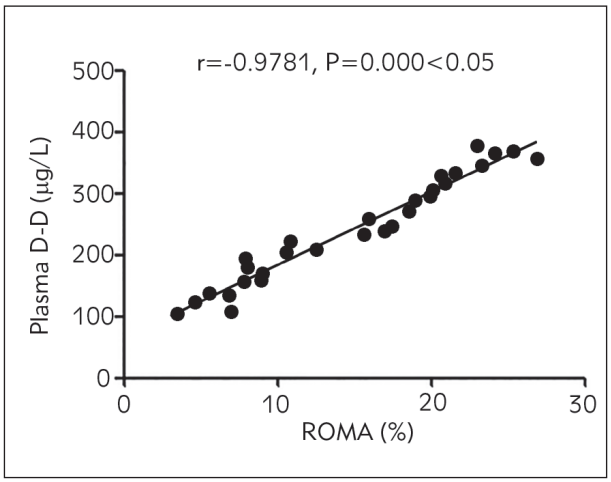

Figure 2 Association between ROMA level and plasma D-D level.

D-D, hs-CRP, IL-6, CA125, and HE4 levels were independent risk factors affecting the incidence of ovarian cancer (*Table VI*).

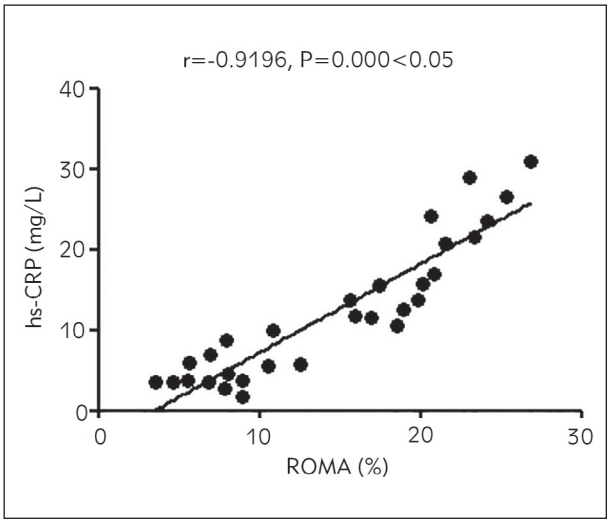

Figure 3 Relationship between ROMA level and hs-CRP level.

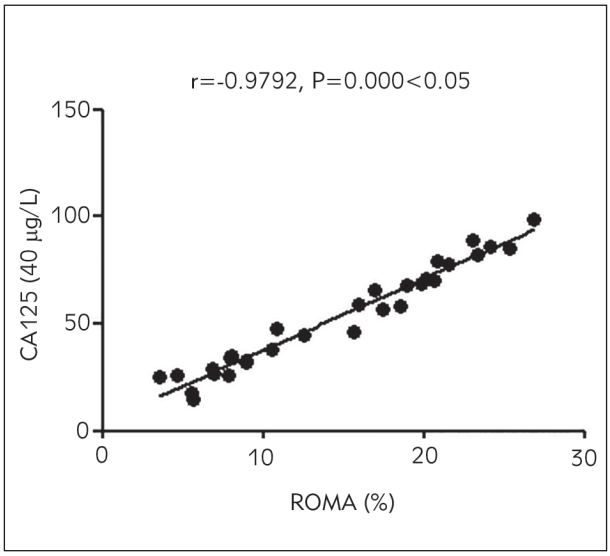

Figure 4 Correlation between ROMA level and serum CA125 level.

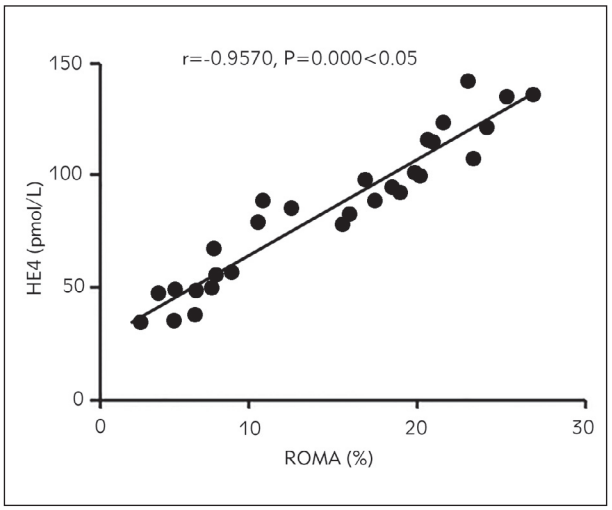

Figure 5 Relation between ROMA level and serum HE4 level.

Discussion

As the most common gynaecological malignant tumour in the clinic, ovarian cancer ranks first in terms of mortality rate among various gynaecological tumours (9). As to its treatment, surgical treatment combined with postoperative adjuvant chemoradiotherapy and biological therapy is the main approach. For patients at the early stage receiving early intervention, the 5-year survival rate is over 90%, while with the progression of the disease, the 5-year survival rate is less than 20% in patients at the middle or advance stage loss the opportunities for surgery. Besides, it is prone to relapse after treatment, leading to a poor prognosis. Therefore, the early diagnosis of ovarian cancer is very important (10). Currently, most scholars believe that many factors cause ovarian cancer, and the major ones are environmental factors, genetic factors, and lifestyle habits (11). Besides, the prognosis of patients is affected by such factors as the clinical stage after onset, tumour histopathological type, and differentiation degree, surgical treatment, and postoperative psychological status (12). Hence, strengthening the monitoring of patients with ovarian cancer and making early diagnosis and early treatment is of great significance for improving patients' prognosis and quality of life.

As medical technology continuously advances, the prognosis of patients with ovarian cancer has been improved significantly, especially for those with early diagnosis and treatment. However, the prognosis of patients in the middle or advanced stage remains poor. As a result, effective early diagnosis is very important for ameliorating the prognosis and quality of life and prolonging patients' survival time with ovarian cancer. The detection of tumour markers has been widely adopted in the prediction of various tumour-related diseases. The malignant tumour cells in patients with ovarian cancer will secrete and release related protein antigens during proliferation, increasing the level of responsive tumour markers. However, tumour markers with high specificity and sensitivity have not been detected yet.

In this study, the correlations of ADPN, plasma D-D, inflammatory factors, and tumour markers with the prognosis of patients were analysed. The results uncovered that compared with those in the control group, the level of ADPN declined, while the levels of plasma D-D and inflammatory factors hs-CRP and IL-6 rose significantly in the observation group, suggesting that patients with malignant ovarian tumour have a lowered ADPN level and increased levels of D-D and inflammatory factor levels. Also, the levels of serum tumour markers CA125, HE4, and ROMA were compared between the two groups. It was found that the levels of CA125, HE4, and ROMA were remarkably higher in the observation group than those in the control group, implying that patients with ovarian cancer exhibit obviously raised levels of serum CA125 and HE4 levels and an abnormal ROMA level. Moreover, the diagnostic value of serum CA125, HE4, and ROMA in ovarian cancer was explored, and it was discovered that the detection of serum ROMA showed the highest specificity and sensitivity and low false-positive rate and false-negative rate. This indicates that ROMA detection is an effective way for early comprehensive diagnosis of ovarian cancer. Furthermore, the ROMA level correlations with ADPN, plasma D-D, his-CRP, and serum CA125 and HE4 levels were analysed. The results revealed that the ROMA level was positively associated with plasma D-D, hs-CRP, and serum CA125 and HE4 levels and negatively related to ADPN level, suggesting that with the increase in ROMA, ADPN, plasma D-D, hs-CRP and serum CA125 and HE4 levels are raised, whereas ADPN level is lowered. Lastly, the results of univariate and multivariate logistic regression analyses showed that abnormal ADPN, D-D, hs-CRP, IL-6, CA125, and HE4 levels were related risk factors affecting the development of ovarian cancer, and lowered ADPN level and raised D-D, hs-CRP, IL-6, CA125, and HE4 levels were independent risk factors affecting the development of ovarian cancer.

A study demonstrated that ROMA is an ideal predictor for the risk of ovarian cancer in patients with ovarian cancer (13). Compared with single detection and combined detection of CA125, HE4, CA724, and CEA levels, ROMA detection is more ideal and more valuable in the identification of benign and malignant gynaecological mass-related diseases in the pelvic cavity. It may be related to a larger AUC of ROMA prediction, which effectively avoids the subjective interpretation of different detection methods and different testers on the results to observably improve the diagnostic specificity and sensitivity to ovarian cancer (14). Another study reported that the inflammatory response *in vivo* is negatively associated with anti-tumour immune function in patients with malignant tumours (15). In the case of malignant tumours, the inflammatory response *in vivo* is significantly enhanced, leading to disordered

proliferation of tumour cells and evasion from immune surveillance, and the self-renewal, migration, metastasis, and normal apoptosis of normal cells are suppressed in the body, changing the microenvironment of tissue blood vessels and cell matrix, thereby inducing the growth of tumour cells (16).

Fat metabolism disorders and adipocyte dysfunction in tumour tissues will lead to overtly decreased ADPN level (17), and at the same time, activate the AMP-activated protein kinase (AMPK) pathway, further resulting in abnormal transduction of ADPN production signals (18). Moreover, in malignant tumours, significant insulin resistance may occur in the body, leading to abnormal transduction of plasma insulin signals related to the occurrence of malignant tumours and abnormal energy metabolism of central sensors and cells, thus inducing malignant tumours. Finally, the research on the tumour marker CA125 revealed that CA125, which is regarded as a tumour marker related to ovarian cancer by some scholars, is generally expressed on the surface of mesothelial tissues such as the endometrium and the epithelium of the reproductive tract (19). In the case of carcinomatosis of ovarian cells, serum CA125 is significantly increased, which can be detected in patients with malignant tumours like pancreatic cancer and breast cancer, so it only acts as a non-specific tumour marker for ovarian cancer in clinical application. HE4 is mainly secreted by ovarian cancer cells and serve as an indicator for the diagnosis of early ovarian cancer in some studies. Besides, HE4 is only secreted in early ovarian cancer cells, so the specificity and sensitivity of HE4 detection to ovarian cancer in various stages need to be improved (20).

In conclusion, patients with ovarian cancer have a reduced ADPN level and distinctly elevated levels of plasma D-D, inflammatory factors, and serum tumour markers CA125, HE4 and ROMA. Besides, the ROMA level has a positive relation to the content of CA125, HE4, plasma D-D and inflammatory factors, and a negative association with ADPN level.

Conflict of interest statement

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

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Received: May 09, 2020
Accepted: November 05, 2020

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-30135

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 79–90, 2022

Original paper
Originalni naučni rad

ANEMIA OF INFLAMMATION IN PATIENTS WITH COLORECTAL CANCER: CORRELATION WITH INTERLEUKIN-1, INTERLEUKIN-33 AND GALECTIN-1

ANEMIJA U INFLAMACIJI PACIJENATA SA KOLOREKTALNIM KARCINOMOM: KORELACIJA SA INTERLEUKINOM-1, INTERLEUKINOM-33 I GALEKTINOM-1

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Summary

Background: Patients with colorectal cancer (CRC) have anemia often present as a consequence of chronic bleeding from tumor. The exact role of IL-33, Galectin-l and IL-l in the pathological genesis of anemia in colorectal cancer patients has not been elucidated yet. The main goal of this research was to analyze Gal-l, IL-l and IL-33 systemic values in anemic and non-anemic CRC patients.
Methods: Concentrations of IL-33, Galectin-1 and IL-1 have been studied in blood samples of 55 CRC patients (27 without anemia and 28 with anemia).
Results: CRC patients with anemia had more severe and local advanced disease compared to CRC non-anemic patients. Anemia positively correlated with higher nuclear grade, lymph and blood vessel invasion, as well as with higher TNM stage, detectable metastatic lesions in lung and liver and peritoneal carcinomatosis. Significantly higher IL-33, Gal-1 and IL-1 concentration have been found in sera of patients with CRC and detected anemia. CRC patients mostly had microcytic anemia, while ferritin values were in normal range. Analysis revealed positive mutual correlation between serum values of galectin-1, IL-1 and IL-33 in CRC patients. Level of hemoglobin negatively cor-

Kratak sadržaj

Uvod: Pacijenti sa kolorektalnim karcinomom (CRC) imaju anemiju koja je često prisutna kao posledica hroničnog krvarenja iz tumora. Tačna uloga IL-33, galektin-1 i IL-1 u patološkoj genezi anemije kod pacijenata sa kolorektalnim karcinomom još nije razjašnjena. Glavni cilj ovog istraživanja bio je analiza sistemskih vrednosti Gal-1, IL-1 i IL-33 kod anemičnih i neanemičnih CRC pacijenata.
Metode: Koncentracije IL-33, galektin-1 i IL-1 ispitivane su u uzorcima krvi 55 pacijenata sa CRC (27 bez anemije i 28 sa anemijom).
Rezultati: CRC pacijenti sa anemijom imali su težu i lokalno uznapredovalu bolest u poređenju sa CRC neanemičnim pacijentima. Anemija je pozitivno korelirala sa višom nuklearnom invazijom, invazijom limfe i krvnih sudova, kao i sa višim TNM stadijumom, uočljivim metastatskim lezijama u plućima i jetri i peritonealnoj karcinomatozi. Značajno veće koncentracije IL-33, Gal-1 i IL-1 pronađene su u serumima pacijenata sa CRC i otkrivenom anemijom. CRC pacijenti su uglavnom imali mikrocitnu anemiju, dok su vrednosti feritina bile u normalnom opsegu. Analiza je otkrila pozitivnu međusobnu korelaciju između serumskih vrednosti galektina-1, IL-1 i IL-33 kod pacijenata sa CRC. Nivo hemoglobina u nega-

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related with serum IL-33, Gal-1 and IL-1. We have analyzed the Receiver Operating Characteristic (ROC) curves of serum IL-33, Gal-1 and IL-1 showed that these cytokines can be treated as additional markers for anemia of inflammation in CRC patients.
Conclusions: Predomination of Galectin-1, IL-1 and IL-33 in anemic CRC patients implicates on their potential role in anemia genesis and further development.

Keywords: Anemia, Colorectal carcinoma, Gal-1, IL-1, IL-33

tivnoj korelaciji sa serumskim IL-33, Gal-1 i IL-1. Analizirali smo ROC krive seruma IL-33, Gal-1 i IL-1 što je ukazalo da se ovi citokini mogu tretirati kao dodatni markeri za anemiju upale kod pacijenata sa CRC.
Zaključak: Predominacija galektin-1, IL-1 i IL-33 kod anemičnih CRC pacijenata implicira na njihovu potencijalnu ulogu u nastanku anemije i daljem razvoju.

Ključne reči: anemija, koloreklatni karcinom, GAL-1, IL-1, IL-33

Introduction

CRC is the second most often cause of death from malignancy in the world. Regarding women population, CRC is the second most frequent cancer and the third regarding men. There is a significant increase of the colorectal cancer incidence due to unhealthy lifestyles and ageing of world population (1). The revised World Cancer Research Fund and American Institute for Cancer Research report that the high consumption of red meat and meat products, obesity and alcohol raise the possibility of CRC development, while on the other hand activity and exercising are protective factors (2).

Anemia is common in patients with CRC and can be one of the most often extraintestinal signs of CRC, which is present in 30%–75% of patients (3, 4). Several studies have reported an association between anemia, both microcytic or normocytic, and poorer cancer specific survival of patients due to systemic inflammation and nutritional status (5, 6). Anemia in CRC patients is often the consequence of chronic bleeding from the tumor; which can be occult from a right colon tumor or visible presence of blood in feces from left colon or rectal cancers (4). However, anemia can also be caused during systemic inflammatory response to the tumor with higher concentrations of inflammatory cytokines such as interferon gamma (IFN-γ), different sorts of interleukins (IL-1, IL-6, IL-8 and IL-10) and tumor necrosis factor alpha (TNF-α) that directly or indirectly stimulate progression of anemia (3, 7).

IL-33, by biding to its receptor ST2, stimulates *in vivo* growth of CRC cells both in human and murine and their sphere formation and inhibits tumor apoptosis induced by chemotherapy (8). Galectin-1 (Gal-1) is Galactoside-binding lectin with many functions, which is involved in different phases of tumorigenesis: stimulation of cell growth and migration, cells interactions, angiogenesis, tumor–immune escape (9). IL-1 is produced by various cells, such as myeloid cells types, mostly after inflammatory or stress conditions (10). Previous studies confirmed importance of IL-1 in different processes, for example genesis of tumor cells, their development and invasiveness, stimulation or inhibition of immune response against tumor (10). The exact contribution of these mediators in pathological genesis of anemia in CRC patients has not been elucidated yet.

The main goal of this research has been to analyze Gal-1, IL-1 and IL-33 values both in anemic and non-anemic CRC patients.

Methods

Ethical statement

This study was performed in Clinical Center of Kragujevac (Gastroenterology Center) and Faculty of Medical Sciences (Center for Molecular Medicine and Stem Cell Research), University of Kragujevac, Serbia. All examined subjects gave informed consent. Ethics Committees of the Clinical Center of Kragujevac, Serbia (Approval Number 01/1627) and Faculty of Medical Sciences, University of Kragujevac, Serbia (Approval Number 01-311/6) have given ethical approvals. All research procedures were carried out in accordance with the Declaration of Helsinki and the Principle of Good Clinical Practice.

Patients

For the purpose of this research, 55 CRC patients were involved and examined. CRC was confirmed after endoscopic and histopathological examination. Patients with previous treatments (chemotherapy, radiation, antibiotics, immunosuppressive agents, salicylates, corticosteroids and biological therapy) or without adequate clinical documentation available were not included. Excluding criteria also were comorbidity with other gastroenterological diseases (esophageal reflux disease, ulcer disease, helicobacter pylori infection, malabsorptive syndrome, diverticulous bowel disease), autoimmune, inflammatory, malignant and infectious diseases or active bleeding. We collected and investigated clinical data about age, sex (determined with physical examination), cancer size, localization, presence of metastasis, invasion of vascular and lymphatic vessels, TNM clinical stage, histological differentiation rate and nuclear grade (using American Joint Committee on Cancer – AJCC classification from 7th edition in 2010).

Measurement of serum Galectin-1, IL-33 and IL-1

Serum levels of cytokines of interest were measured, as previously described (11). Shortly, 10 mL of blood were taken from each examined subject at 8am before any therapy. Collected samples have been stored at -80 °C, until ELISA testing, which was performed according to the instructions of manufacturer (Systems R&D, Minneapolis, USA).

Tumor markers measurement

Levels of tumor markers, such as carcinoembryonic antigen-CEA, alpha-fetoprotein-AFP and cancer antigen CA 19-9, in sera of each patient were evaluated in the laboratory of Clinical Center Kragujevac by CLIA methods (chemiluminescence enzyme immunoassay).

Statistical analysis

With SPSS software (20.0) we have performed all data analyses. Our results were presented as (mean ± standard error). To determine statistical significance between the means of two groups we used Mann-Whitney U-test or Student's t-test, where adequate and for the correlation between the markers of interest with anemia in CRC patients, we used Pearson's or Spearman's correlation, also where adequate. We used the Chi Square Test to evaluate the statistical significance of data presented in tables. Statistically significant p-values were for $p \leq 0.05$.

Results

All 55 adult patients diagnosed with CRC have been included in this research (27 without anemia, 28 with anemia). Clinico-pathological features of all patients are shown in *Table I*. The analysis of the data also did not show any significant difference regarding the distribution of age and gender.

CRC patients with anemia have severe and local advanced disease

We have formed two groups according to the hemoglobin concentration (with and without anemia). This classification was based on the presence of anemia, which is diagnosed when hemoglobin concentration is less than 130 g/L in male patients and less than 120 g/L in female patients. Evaluation of nuclear grade of colorectal cancer was made in patients with the presence or absence of anemia, using AJCC classification (12). According to nuclear grade all CRC patients have been split into groups I, II and III. Results have shown that anemic patients had significantly higher nuclear grade of colorectal cancer in comparison to non-anemic patients ($p = 0.003$; *Figure 1A*). Moreover, according to the evidence of detectable invasion of blood or lymph vessels we have split all CRC patients into groups (+ or -). Significantly higher percentage of CRC patients with diagnosed anemia had detectable invasion of blood and lymph vessels in comparison to CRC patients without anemia, respectively ($p = 0.009$; $p = 0.008$; *Figure 1B*, *1C*). Further, anemia positively correlated with higher nuclear grade ($r = 0.489$; $p = 0.002$), lymph ($r = 0.444$; $p = 0.006$) and blood vessel invasion ($r = 0.439$; $p = 0.007$).

Table I Clinico-pathological characteristics of examined patients.

Characteristics	Colorectal carcinoma (CRC) patients	
	Without anemia	With anemia
Sex (male/female)	17/10	16/12
Age (mean (range))	63.4 (50–75)	66.5 (53–82)
Hemoglobin, g/L	138.04±9.33	96.77±4.34
MCV	89.36±11.09	70.77±6.63
Platelets ($x10^9$/L)	314.23±12.46	408.63±29.55
Histological differentiation rate (well/moderate)	6/21	7/21
AFP	3.26±1.64	238.84±128.30
CEA	33.79±22.25	223.14±109.11
CA 19-9	81.57±70.51	931.07±190.05

Anemia correlates with more progressive form of CRC

According to TNM clinical stage, we have split all CRC patients into two groups (I+II or III+IV). CRC patients with anemia had significantly advanced TNM stage (or had TNM score III or IV), while CRC patients without anemia (or with normal level of hemoglobin) mostly had TNM stage I or II ($p = 0.011$; *Figure 2A*). According to the evidence of liver metastasis, lung metastasis or peritoneal carcinomatosis, we have split all CRC patients into groups (+ or -). Higher percentage of CRC patients with anemia had detectable metastatic lesions in liver and lung as well as peritoneal carcinomatosis, respectively, compared to CRC patients without anemia ($p = 0.003$; $p = 0.031$; $p = 0.004$; *Figure 2B*, *2C*, *2D*). Additionally, anemia positively correlated with higher TNM stage ($r = 0.431$; $p = 0.009$), detectable metastatic lesions in lung ($r = 0.422$; $p = 0.019$) and liver ($r = 0.428$; $p = 0.002$) and peritoneal carcinomatosis ($r = 0.417$; $p = 0.003$), respectively. Further, tumor markers, such as CEA, AFP and CA 19-9 have been signifi-

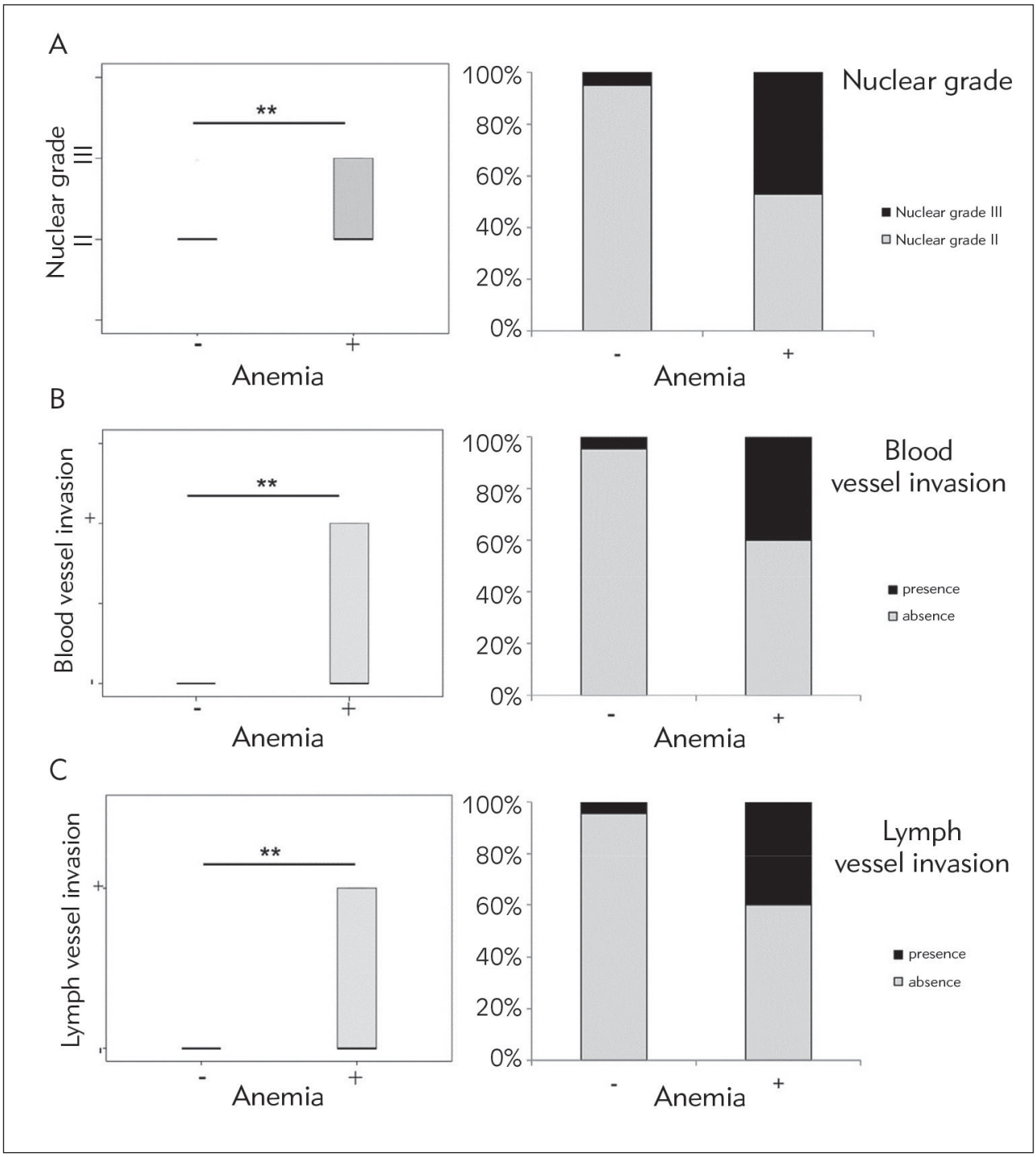


Figure 1 CRC patients with anemia have severe and local advanced disease: **A.** Patients with CRC and anemia had higher nuclear grade of colorectal cancer. **B.** Higher percentage of blood vessels invasion or **C.** lymph vessels invasion in comparison to CRC patients without anemia. Chi-Square test was used (* $p < 0.05$; ** $p < 0.01$).

cantly higher in patients with CRC and anemia in comparison to patients with CRC without anemia (*Table I*). Moreover, results have shown that hemoglobin negatively correlates with tumor markers CEA ($r = -0.475$; $p = 0.001$), AFP ($r = -0.383$; $p = 0.007$) and CA 19-9 ($r = -0.610$; $p = 0.001$), respectively. Negative correlations were also detected between MCV and CEA ($r = -0.343$; $p = 0.020$), AFP ($r = -0.277$; $p = 0.049$) and CA 19-9 ($r = -0.369$; $p = 0.014$), respectively.

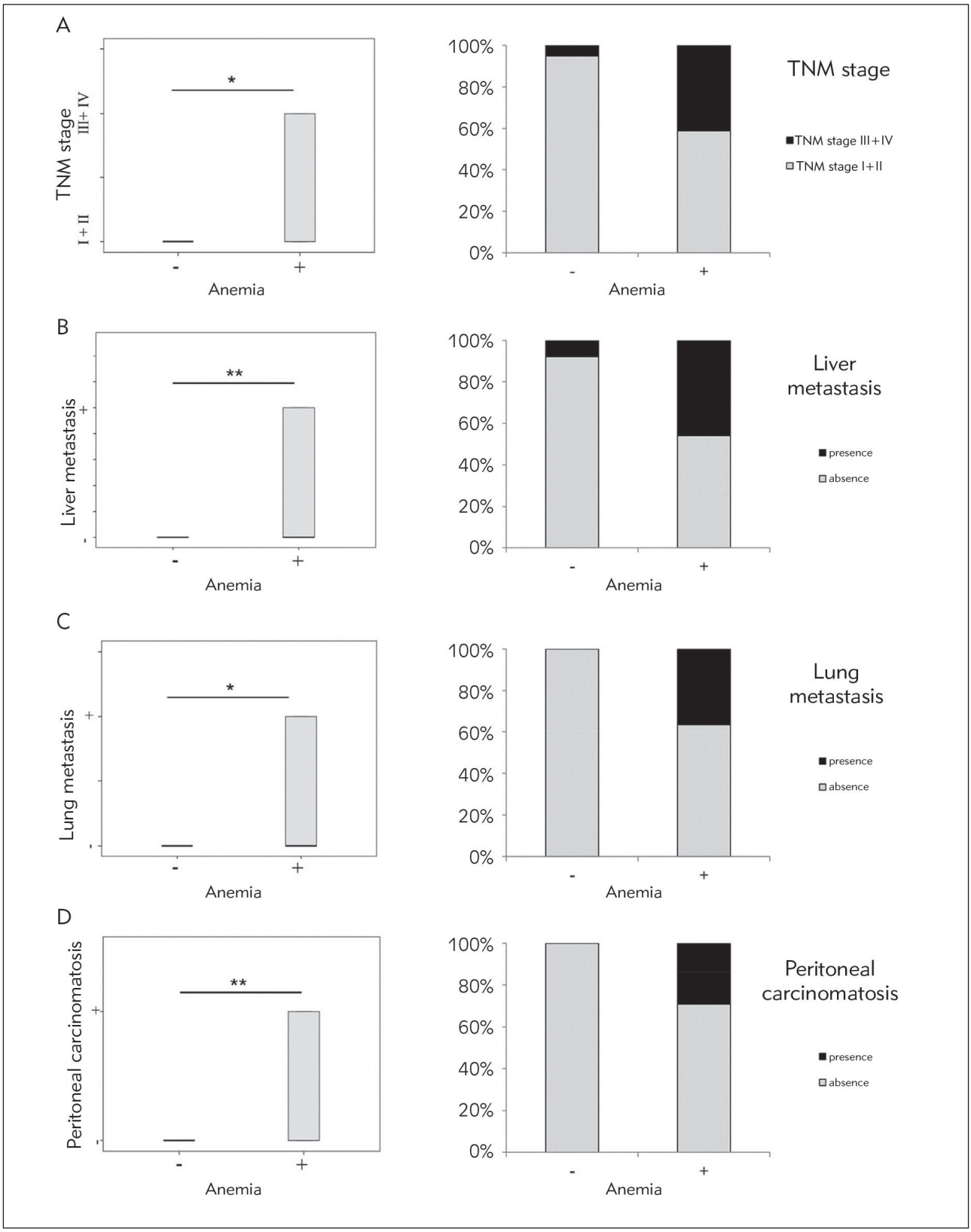


Figure 2 Anemia correlates with more progressive form of CRC: **A.** Patients with CRC and anemia had significantly advanced TNM stage, **B.** higher percentage of detectable metastatic lesions in liver, **C.** lung or **D.** peritoneal carcinomatosis compared to CRC patients without anemia. Chi-Square test was used (* $p < 0.05$; ** $p < 0.01$).

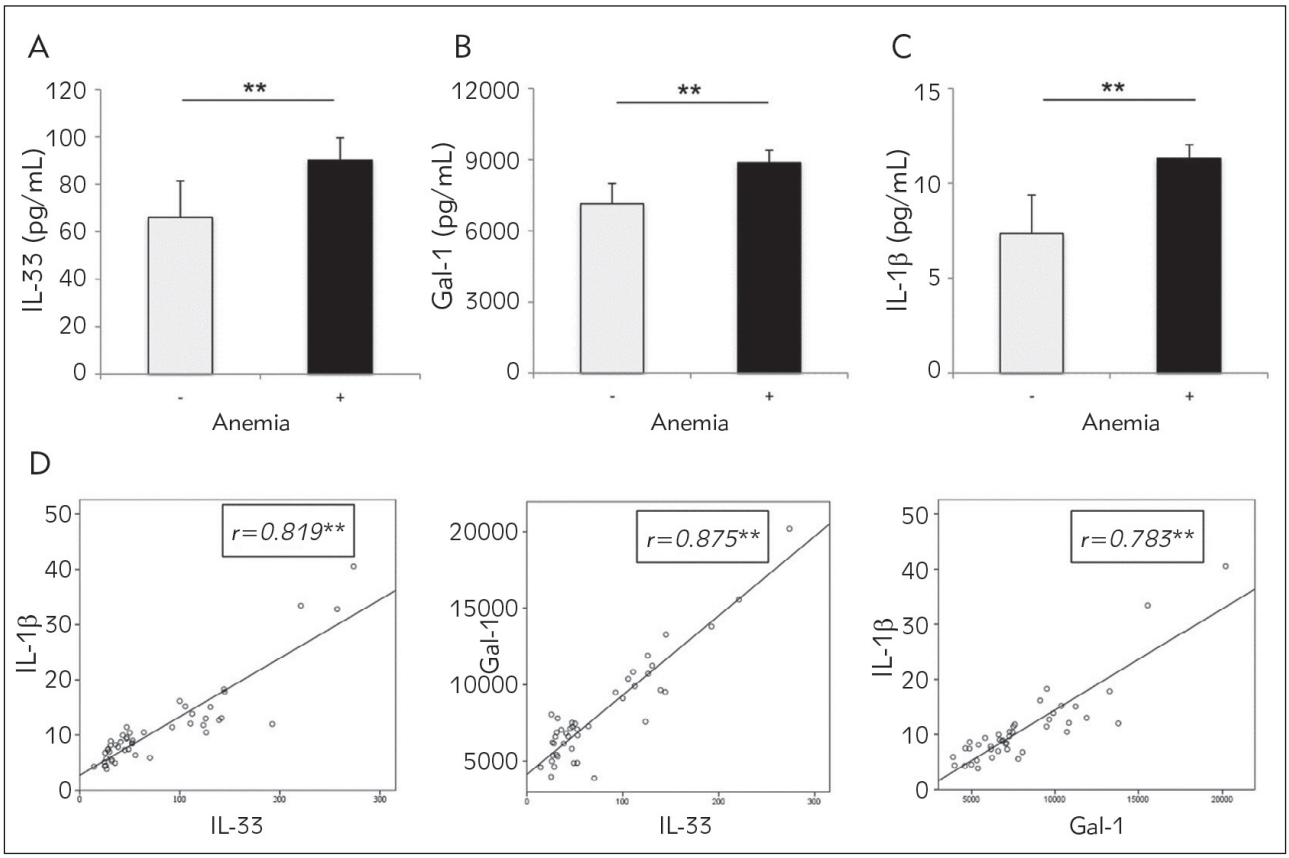


Figure 3 A, B, C Anemic CRC patients had higher concentration of IL-33, Gal-1 and IL-1 β in serum compared to non-anemic CRC patients. **D.** Positive correlation between galectin-1 and IL-33, galectin-1 and IL-1 and IL-33 and IL-1 values in serum of CRC patients. Correlation was determined using Spearman's test (* $p < 0.05$; ** $p < 0.01$).

Patients with CRC and anemia had increased IL-33, Gal-1 and IL-1 values in serum

Serum values of cytokines of interest were analyzed in serum of CRC patients with/without diagnosed anemia. Significantly higher concentration of IL-33 ($p = 0.003$), Gal-1 ($p = 0.009$) and IL-1 ($p = 0.005$) have been found in serum of CRC patients with detected anemia (*Figure 3A*, *3B*, *3C*). Positive correlation was detected between CA 19-9 and IL-1 ($r = 0.321$; $p = 0.029$) and IL-33 ($r = 0.438$; $p = 0.002$), respectively. Moreover, analysis revealed positive relation between serum values of galectin-1 and IL-33 ($r = 0.772$; $p = 0.001$), galectin-1 and IL-1 ($r = 0.828$; $p = 0.001$) and IL-33 and IL-1 ($r = 0.879$; $p = 0.001$) in CRC patients (*Figure 3D*).

CRC patients mostly had microcytic anemia

Further, analysis of the volume of red blood cells (mean corpuscular volume – MCV) was made in CRC patients with and without anemia. MCV has been significantly lower in patients with CRC and detected anemia in comparison to patients CRC with normal hemoglobin level ($p = 0.001$; *Figure 4A*). In order to confirm previous results, we have formed two groups of CRC patients according to the MCV values and analyzed IL-33, Gal-1 and IL-1 values in serum. CRC patients with microcytic anemia (MCV<83fL) had significantly higher IL-33 ($p = 0.009$), Gal-1 ($p = 0.002$) and IL-1 ($p = 0.013$) in sera in comparison to CRC patients with normocytic anemia or without anemia (*Figure 4B*, *4C*, *4D*). Additionally, strong positive relation has been detected in comparison of galectin-1 and IL-33 ($r = 0.870$; $p = 0.001$), galectin-1 and IL-1 ($r = 0.795$; $p = 0.001$) and IL-33 and IL-1 ($r = 0.826$; $p = 0.001$) values in serum of CRC patients with diagnosed microcytic anemia (*Figure 4E*, *4F*, *4G*). Further, we measured ferritin in all patients. Ferritin values were in normal range and without any significant difference in ferritin levels when comparing anemic CRC patients to non-anemic CRC patients (data not shown).

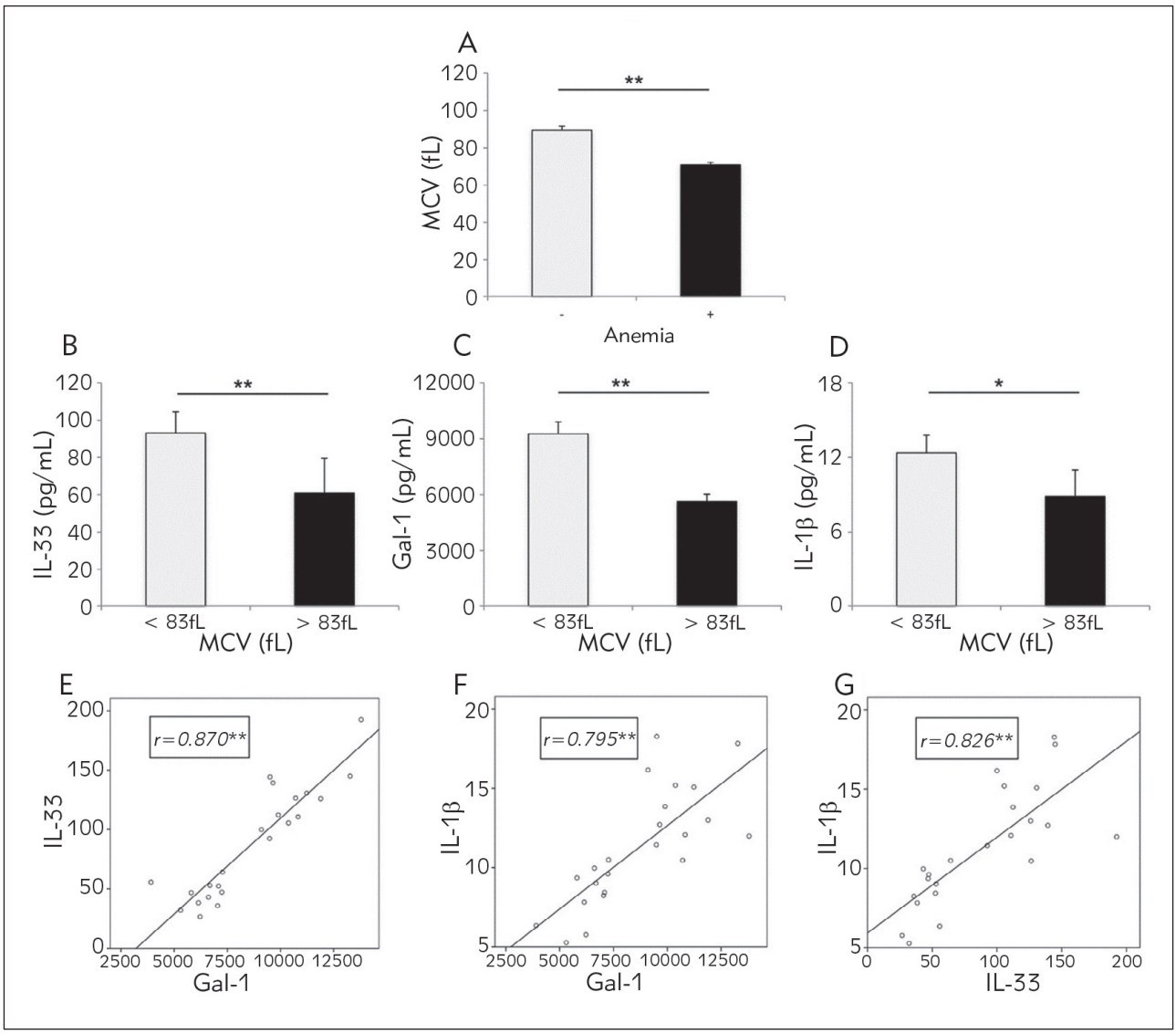


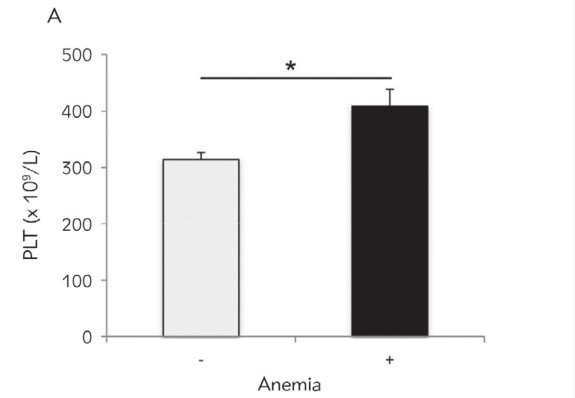
Figure 4 A Significantly lower MCV of red blood cells in anemic CRC patient. **B, C, D.** Significantly higher IL-1, Gal-1 and IL-1 β in serum of CRC patients with microcytic anemia in comparison to CRC patients with normocytic anemia or without anemia. **E, F, G.** Strong positive correlation was detected between galectin-1 and IL-33, galectin-1 and IL-1 β and IL-33 and IL-1 β, values in serum of CRC patients with diagnosed microcytic anemia. Correlation was determined using Spearman's test (* p< 0.05; ** p< 0.01).

Thrombocytosis correlates with anemia

Additional blood analysis revealed significantly higher number of platelets in CRC patients with detected anemia compared with non-anemic CRC patients (p = 0.03; *Figure 5*). Thrombocytosis has positive correlation with IL-33 (r = 0.404; p = 0.004), Gal-1 (r = 0.333; p = 0.024), IL-1 (r = 0.335; p = 0.020) values in serum, respectively (*Figure 5*).

IL-33, Gal-1 and IL-1 concentrations negatively correlate with hemoglobin

Finally, we have tested correlation of serum level of hemoglobin with IL-33, Gal-1 and IL-1 values in serum. The results have shown moderate negative correlation between hemoglobin and serum IL-33 (r = -0.595; p = 0.001), Gal-1 (r = -0.629; p = 0.001) and IL-1 (r = -0.572; p = 0.001), respectively (*Figure 6*). Examination of ROC curves of IL-33, Gal-1 and IL-1 values in serum showed that these cytokines could predict anemia in CRC patients (*Figure 6*). Our data demonstrate that IL-33 (sensitivity 95.8%, specificity 45.8%) Gal-1 (sensitivity



B

Variables	Thrombocytosis Spearman's rho
IL-33	0.404**
Gal-1	0.333*
IL-1β	0.335*

Figure 5 A Patients with CRC and anemia had significantly higher number of platelets compared to non-anemic patients with CRC. **B.** Positive correlation between thrombocytosis and IL-33, Gal-1 and IL-1 β, respectively. Correlation was determined using Spearman's test (* $p < 0.05$; ** $p < 0.01$).

59.1%, specificity 66.7%) and IL-1 (sensitivity 75%, specificity 75%) can be potential markers for paraneoplastic anemia in CRC patients. The optimal cut off levels of cytokines estimated for confirmation of anemia in CRC patients was 31.58 pg in mL (IL-33), 7257.07 pg in mL (Gal-1), 8.9624 pg in mL (IL-1).

Discussion

In this present research, we have shown that CRC patients with anemia had significantly higher nuclear grade in comparison to CRC non-anemic patients (*Figure 1*). CRC patients with diagnosed anemia had significantly higher TNM stage as well as invasion of lymph and blood vessels and detectable metastatic lesions in lung and liver and peritoneal carcinomatosis compared to CRC non-anemic patients (*Figure 2*). Higher concentrations of CEA, AFP, CA 19-9 were detected in patients with CRC and detectable anemia (*Table I*). Tumor markers negatively correlated with hemoglobin and MCV. As previous study confirmed positive correlation between tumor markers and CRC progression, our data implicate on more severe form of disease in anemic patients (13). Our finding of severe and more progressive disease in

A

Variables	Hemoglobin Spearman's rho
IL-33	-0.595**
Gal-1	-0.629**
IL-1β	-0.572**

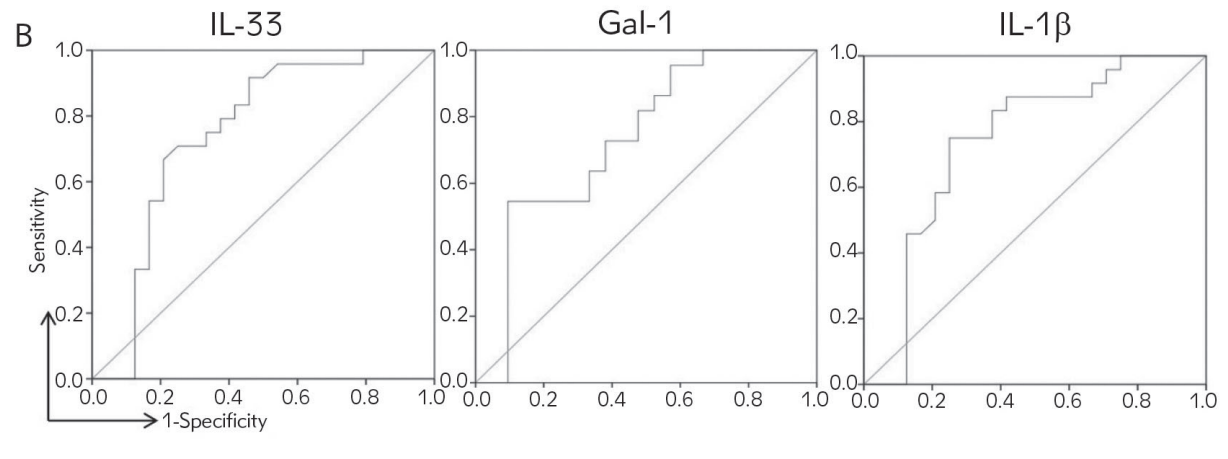


Figure 6 A Moderate negative correlation between serum hemoglobin level and IL-33, Gal-1 and IL-1 β, respectively. **B.** Specificity and sensitivity of IL-33, Gal-1 and IL-1 in serum. Correlation was determined using Spearman's test (* $p < 0.05$; ** $p < 0.01$).

anemic CRC patients are in line with many previous studies (14). As anemia is common in patients with CRC and can be one of the most often extraintestinal signs of CRC, we have measured MCV in these patients to confirm the type of anemia (3). Analyses have confirmed significantly lower levels of MCV in anemic patients with CRC in comparison to non-anemic patients with CRC. According to these results, it is possible that predomination of microcytic anemia in CRC patients suggests on possible gastrointestinal bleeding. However, normal range of ferritin values in CRC patients with anemia implicate on other causes of detected anemia, but bleeding. According to Moreno et al. (15) decreased MCV with normal ferritin values implicate on chronic disease anemia. Anemia can also be a consequence of systemic inflammatory response in presence of tumor (3). Cancer related anemia occurs without bleeding and results from chronic inflammation and synthesis of pro-inflammatory cytokines simultaneously by different kinds of cells, both immune and cancer cells (16). The synthesized pro-inflammatory cytokines can cause anemia by different pathological mechanisms (7). They stimulate hepatic production of hepcidin. Hepcidin is known as an inhibitor of iron absorption in the duodenum. Further, cytokines can facilitate iron storage in macrophages. This retention of iron in macrophages causes limited availability of iron for erythroid cells. Moreover, previous studies showed that cytokines with proinflammatory activities can have a direct inhibitory influence on erythroid progenitors' proliferation and differentiation or they can activate macrophages and thus increasing the process of erythrophagocytosis (3, 7).

As previous studies clearly confirmed the importance of proinflammatory cytokines in biology of colorectal cancer (3, 7), our research was further based on the investigation of the systemic concentrations of proinflammatory cytokines. IL-1 is a pleiotropic cytokine involved in the few processes in tumorigenesis such as tumor growth, metastasis, and angiogenesis (10). IL-1 can also act indirectly via stimulation of production of matrix metalloproteinases and different kinds of cytokines, for example IL-6, IL-8, TNFα, TGF and VEGF (17). Our study showed significantly higher IL-1 concentration in patients with CRC and detected anemia compared to non-anemic CRC patients as well as positive association between CA 19-9 and IL-1 and negative association between hemoglobin and IL-1 (*Figure 3, Figure 6*). Previous studies are in line with our results suggesting a significant role of IL-1 in the development and pathological genesis of anemia. IL-1 affects iron metabolism, thus disabling utilization of iron for synthesis of hemoglobin (17). IL-1 decreases level of erythropoietin *in vitro*, modulates erythropoietin receptors and also inhibits production of erythroid progenitors (18).

Interleukin-33 (IL-33) is a member of IL-1 cytokine family. Its role in immune response regulation after cellular stress or damage is crucial (19) and also is involved in pathogenesis of CRC development. IL-33 has many functions, for example in host immunity against tumor as an inhibitor, in angiogenesis as a promoter and can also induce tumor stroma remodeling. It can also affect the activation of NF-kB transcriptional factor; stimulate production of proinflammatory cytokines thereby promoting inflammation (20). Our result revealed significantly higher systemic IL-33 concentration in anemic CRC patients, positive association between CA 19-9 and IL-33 and negative association between hemoglobin and IL-33 (*Figure 3*, *Figure 6*), suggesting on possible role of this proinflammatory cytokine in pathogenesis of anemia in CRC patients. Other study showed that IL-33 is highly expressed in endothelial cells thus promoting the expansion of different kind of hematopoietic precursors and regulating myelopoiesis *in vitro* and *in vivo*, implicating its role in hematopoiesis (21, 22). Stankovic et al. (23) revealed that during acute inflammation IL-33/ST2 axis plays an essential role in metabolism of Fe, mainly by increasing concentration of iron at the place of inflammation and thus decreasing blood mean corpuscular hemoglobin. The most often cause for development of microcytic anemia is iron deficiency, while on the other hand iron sequestration facilitated by IL-1 and IL-33 might be an alternative cause of anemia in CRC patients.

Galectin-1 in CRC exhibits different aspects of tumor progression, such as cell adhesion, tumor cell transformation and growth. Also, it enhances migration of cancer cells, their proliferation and metastasis, inhibition of apoptosis and stimulation of angiogenesis (24, 25). During the progression of CRC, galectin-1 is often significantly changed, meaning that overexpression of galectin-1 (24) correlates with poorly differentiated, invasive form of CRC with lymph node metastasis and shorter patient survival (25). Our previous study has also shown importance of Galectin-1 in the pathology of CRC. We noticed fecal Gal-1 in feces samples of patients with more advanced form of CRC (11). Moreover, Gal-1 positively correlates tumor markers, such as AFP and CA 19-9 and with tumor histological differentiation stage (11). According to available information, this is the first research, which investigates connection between systemic Gal-1 and anemia in CRC patients. In our study, we showed that patients with CRC and detected anemia had significantly higher level of systemic Galectin-1 compared to the group of CRC patients without anemia (*Figure 3*). Moreover, Galectin-1 negatively correlated with hemoglobin (*Figure 6*). IL-1 and IL-33 are members of the same IL-1 family of cytokines and thus share many effector functions (10, 19). Galectin-1 and IL-33 share the same tumor promoting effect in CRC, by helping tumor cells in bypassing of apoptosis (8, 26, 27). It is possible that Gal-1 through NK- B signaling pathway promotes proinflammatory cytokines production, such as IL-1

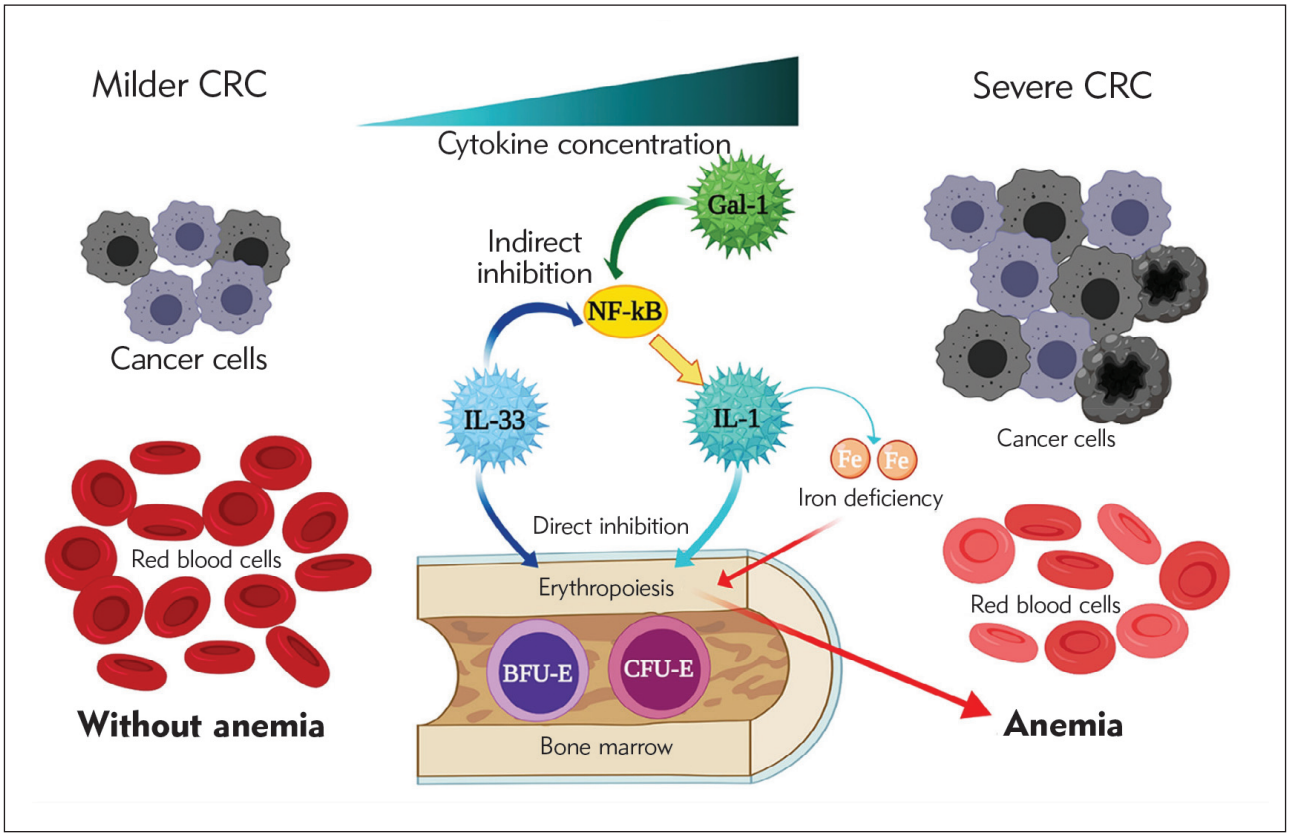


Figure 7 Potential synergistic effect of IL-1 and IL-33 in pathogenesis of anemia of inflammation in CRC patients: 1. IL-1 and IL-33 directly inhibit erythropoiesis and disable synthesis of hemoglobin via suppression of iron utilization; 2. Gal-1 and IL-33 activate NF-kB transcriptional factor and indirectly stimulate proinflammatory cytokines production, such as IL-1 production, which then inhibits erythropoiesis. In turn subsequent anemia correlates with severe and more progressive CRC. (*Created by the authors with BioRender.com)

production and subsequently attenuates hematopoiesis.

Revelation of positive correlation between systemic IL-1, IL-33 and galectin-1 levels in serum of anemic CRC patients suggested on synergistic effect of these cytokines in pathogenesis of anemia in CRC patients. Based on previous data, it is believed that IL-1 and IL-33 directly disable synthesis of hemoglobin via suppression of iron utilization, while indirectly Gal-1 and IL-33 activate NF-kB transcriptional factor and stimulate proinflammatory cytokines production, such as IL-1 production that in turn inhibits erythropoiesis, as described in *Figure 7*. Further, examination of ROC curves of Gal-1, IL-33 and IL-1 revealed that these cytokines could predict anemia of inflammation in CRC patients (*Figure 6*).

Reactive thrombocytosis, an elevated platelet, can be caused due to deficiency of iron, infection (acute or chronic) and different inflammatory diseases. Nowadays, cancers are frequently connected with paraneoplastic thrombocytosis. Thrombocytosis often accompanies cancer growth and metastatic dissemination (28). Recent studies revealed possible association between thrombocytosis and poor prognosis for patients with CRC (29). Paraneoplastic elevation of platelet count is associated with worse overall survival, as well as cancer-specific and disease-free survival in CRC patients. Moreover, there are strong evidence regarding significant correlation between tumor location, higher TNM clinical stage (T3-4), presence of metastasis or lymph node and vessels invasion, and also poor histological differentiation with thrombocytosis in CRC patients (30, 31). In our study, we noticed significantly higher number of platelets in the group of CRC patients with anemia in comparison to the group of non-anemic CRC patients (*Figure 5*). Systemic values of cytokines Gal-1, IL-1 and IL-33 positively correlated with higher platelets number (*Figure 5*). Previous study claimed that IL-1 stimulates megakaryopoesis thus increasing the number of platelets (32). The other study showed that mice with over expression of IL-33 had detectable anemia and thrombocytosis (22). Possible explanation for thrombocytosis and positive correlation between higher number of platelets and mediators of interest is that enhanced levels of IL-1 and IL-33 could directly induce megakaryopoiesis and subsequently thrombocytosis.

Conclusion

Presented data revealed higher Galectin-1, IL-1 and IL-33 values in serum of CRC patients with anemia, establishing them as new players in genesis and progression of anemia of inflammation (*Figure 7*). These findings point on mechanism of anemia genesis in CRC patients, besides occult bleeding, that deserves further clarification.

Acknowledgements: The Serbian Ministry of Education, Science and Technological Development (175069 and 175071), project with PR China (06/2018) and the Faculty of Medical Sciences Kragujevac (projects JP04/15, JP15/19 and JP11/18), Serbia, have supported this study.

Conflict of interest statement

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

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Received: January 01, 2021
Accepted: July 11, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-31711

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 91–99, 2022

Original paper
Originalni naučni rad

LX-8000R AND URISED 2 FULLY AUTOMATED URINE ANALYZERS COMPARISON TO MANUAL MICROSCOPIC EXAMINATION

POREĐENJE DVA POTPUNO AUTOMATIZOVANA ANALIZATORA LX-8000R I URISED 2 SA MIKROSKOPSKIM MANUELNIM ISPITIVANJEM URINA

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Summary

Background: Urinalysis has an important place in evaluating kidney and urinary tract infections. Automated urine analyzers enhance productivity and turnover in laboratories and economize time and labor required for analysis. In the present study, we evaluated and compared analytic and diagnostic performance of UriSed2 with LX-8000R, which is a novel image-based automated urine sediment analyzer.
Methods: A total of 178 urine samples sent to our laboratory were evaluated by the two urine analyzers and standard manual microscopy. Precision and comparison studies were done in accordance with CLSI guidelines.
Results: Sensitivity assessment revealed similar outcomes with both UriSed2 and LX-8000R devices for erythrocyte count (RBC), whereas UriSed2 device yielded higher outcomes for leukocyte count (WBC) and epithelial cells (EPI) than LX-8000R analyzer. Specificity of UriSed2 for WBC and RBC was higher than that of LX-8000R device. According to Gamma statistics, both urine analyzers showed perfect consistency for WBC, RBC and EPI cell counts. Manuel microscopy revealed statistically significant correlation between LX-8000R and UriSed2 in terms of WBC and RBC. Manual evaluation by Bland-Altman analysis demonstrated lower WBC and RBC values and higher EPI as compared to both UriSed2 and LX-8000R devices. As the result of Passing-Bablok regression analysis, both devices were found to be inconsistent with manual microscopy.
Conclusions: We think that evaluation of automated urine analyzers will be more meaningful when they are evaluated

Kratak sadržaj

Uvod: Analiza urina ima važno mesto u proceni infekcija bubrega i urinarnog trakta. Automatski analizatori urina povećavaju produktivnost i promet u laboratorijama i štede vreme i rad potreban za analizu. U ovoj studiji smo procenili i uporedili analitičke i dijagnostičke performanse UriSed2 sa LX-8000R, koji je novi automatizovani analizator za analizu sedimenta urina.
Metode: Ukupno 178 uzoraka urina poslanih u našu laboratoriju procenjeno je pomoću dva analizatora urina i standardne ručne mikroskopije. Studije preciznosti i poređenja rađene su u skladu sa smernicama CLSI.
Rezultati: Procena osetljivosti pokazala je slične ishode sa uređajima UriSed2 i LX-8000R za broj eritrocita (RBC), dok je uređaj UriSed2 dao veće rezultate za broj leukocita (WBC) i epitelnih ćelija (EPI) od analizatora LX-8000R. Specifičnost UriSed2 za WBC i RBC bila je veća nego kod LX-8000R uređaja. Prema Gamma statistici, oba analizatora urina pokazala su savršenu konzistenciju za broj ćelija WBC, RBC i EPI. Manuelova mikroskopija otkrila je statistički značajnu korelaciju između LX-8000R i UriSed2 u odnosu na WBC i RBC. Ručna procena Bland-Altmanovom analizom pokazala je niže vrednosti WBC i RBC i veći EPI u poređenju sa UriSed2 i LX-8000R uređajima. Kao rezultat Passing-Bablokove regresijske analize, utvrđeno je da oba uređaja nisu u skladu s ručnom mikroskopijom.
Zaključak: Smatramo da će evaluacija automatizovanih analizatora urina biti značajnija kada se vrednuju zajedno sa uzorcima urina i kliničkim nalazima pacijenata, poredeći ih sa ručnom mikroskopijom.

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together with urine samples and patient clinical findings in addition to comparing with manual microscopy.

Keywords: Automated urine analyzer, LX-8000R, Urised 2, manual microscopy

Ključne reči: automatizovani urinski analizator, LX-8000R, Urised 2, manuelna mikroskopija

Introduction

Urinalysis, which is an important test in clinical medicine, is used for screening, diagnosis and monitoring of urinary system diseases as well as the diseases detected through urinary system (1). Urinalysis in daily clinical settings is important in terms of detecting hematuria and proteinuria, which are the initial signs of kidney diseases. In addition, it is important also in assessing urinary erythrocyte morphology and in distinguishing a glomerular disease from non-glomerular disease (2). Urinalysis consists of physical appearance of urine, chemical analysis and microscopic examination of urine sediment. Manual microscopic method for urinalysis is a time-consuming and labor-intensive technique requiring well-trained and experienced technicians (1). Microscopic examination of urine is affected by a number of factors including speed and duration of centrifugation, amount of urine left in the tube, dye usage, and experience and training of the analyst. Despite all these disadvantages, manual microscopy is the reference method for examination of urine sediment (3). However, fully automated urine analyzers are recommended by many international institutions for minimizing these effects and for standardization of microscopic analysis (4).

UriSed2 device captures images from the urine centrifuged in a disposable tube by a digital camera attached to a bright-field microscope of 400× magnification. Then the sediment is identified and classified by image-processing software. The automated process used in the UriSed2 analyzer is similar to that used at manual microscopic examination. The images thereafter can be controlled again by a technician, when necessary. UriSed2 software is able to distinguish red blood cells (RBC), white blood cells (WBC), squamous epithelial cells (EPI) and other sediment particles (5, 6).

LX-8000R device works by taking real-time picture of the cells and field image from the urine sample by Flowcell Digital Imaging. It performs the analysis of visible cells in the urine sample and strip analysis simultaneously. It does not require pretreatment or include centrifugation. Because of single-probe distribution system, a small amount of urine is subjected to both microscopic and chemical analyses, which are then reported together. Hence, test time is shortened and working productivity is enhanced providing advantage for pediatric patients as well as patients that have difficulty in giving urine sample (7).

In the literature, no study is encountered about the performance of LX-8000R device. The present study aimed to evaluate analytic performance of the UriSed2 (77 Elektronika Kft, Hungary) and LX-8000R (Hangzhou Longx Technology, Zhejiang, China) automated analyzers by comparing with manual microscopic analysis.

Materials and Methods

The study was performed using 178 urine samples collected into the clean tubes without preservatives, which have been obtained from the patients applied to the Training and Research Hospital for routine policlinic visit. The urine samples were obtained from the specimens at an amount of 30 mL at the least. The samples were analyzed in small groups on different days. The urine samples, which were divided into three different tubes at an amount of 10 mL for each, were analyzed in an hour at the latest. The study was approved by the Health Sciences Institute Diyarbakır Gazi Yaşargil Training and Research Hospital Ethics Committee (No: 20.12.2019 – 385).

RBC, WBC, EPI, lipid cylinder and crystals (calcium oxalate dihydrate, uric acid, triple phosphate) were studied in both devices and compared with manual microcopy.

UriSed 2

UriSed2 analyzer pipes the sample from 2 mL urine into a 0.2 mL disposable tube. The tube filled is then centrifuged at 2000 rpm for 10 seconds. Fifteen different fields from various points of the sample can be displayed on the screen by an automated CMOS (Complementary Metal Oxide Semiconductor) fixed cam. The images are recorded in three types as bright-field, phase contrast and composite, and then evaluated by Auto Image Evaluation Module (AIEM). Urinary particles are classified as RBC, WBC, leukocyte clusters, hyaline cylinders, pathologic cylinders, EPI, non-squamous epithelial cells, bacteria, yeast, mucosa, sperm, and sub-crystals (calcium-oxalate monohydrate, calcium-oxalate dehydrate, uric acid or triple phosphate) (8). Misclassified images can be checked and corrected by the technician, when necessary.

LX-8000R

It performs the analysis of visible cells in the urine sample and strip analysis at the same time. The samples are put into the device without need for pretreatment. Minimum 3 mL of urine sample is adequate for analysis. The device as well, which does not include centrifugation, is subjected to tube mixing procedure before analysis to provide homogenous mixture of urine. The urine, which is obtained by a single probe at a single step, is subjected to both microscopic and chemical analyses and the results are reported together. For chemical analysis, it is studied by the method of dripping on a strip. Specific Gravity, Color and Turbidity parameters of the urine sample are measured by Refractometry technique. There is a high resolution phase contrast microscope with 10X and 40X lenses on it. Real-time cell picture and images of 20 different fields are taken from the urine by flowcell digital imaging for microscopic analysis. Based on famous light-microscopic morphology detection method, urine tangible ingredient detection combines digital medical image processing technology, computer multimedia technology and artificial intellectualizing technology, which enables detection operators to clearly observe the urine tangible ingredients in the quantitative flow counting chamber under the digital microscope system inserted in Analysis System via large screen display, and then get the detection report of clinical significance according to cells per volume or numbers of tubes. Camera focus settings are automatically made by the software for each sample. Automatic probe wash is performed after each urine sampling (internal and external wash) to prevent contamination of samples. During the procedure, real images of the sample are displayed on the screen without adding any chemical substance into the urine sample. Urinary particles are classified as dysmorphic RBC, RBC, WBC, leukocyte clusters, cylinders, EPI, bacteria, yeast, mucus, sperm and crystals. Other subgroup particles can be defined manually on the screen. In sediment analysis, the parameters are shown on the result screen as XX/μL or p/Hpf. (7). Misclassified images can be checked and corrected by a technician, when necessary.

Technical properties of microscopic and chemical units of the UriSed2 and LX-8000R automated urine analyzers are given in *Table I*.

Table I Technical specifications of the microscopic and the chemical stripe analyzers.

	Urised 2	LX-8000R
Microscopic Analyzers		
Throughput	Up to 120 tests/hour	Up to 100 tests/hour
Methodology	Whole view field microscopic image	Flowcell digital imaging
Batch size	100 test tubes	60 test tubes
Min. sample volume	2 mL	3 mL
Detected particle classes	RBC (red blood cells); WBC (white bloodcells and WBC clumps); HYA (hyaline casts); PAT (pathological casts); EPI (squamous epithelial cells); NEC (nonsquamous epithelial cells); BACc (bacteria cocci); BACr (bacteria rods) YEA (yeast) CRY (crystals): [CaOxm (calcium-oxalate monohydrate),CaOxd (calcium-oxalatedihydrate), URI (uric acid), TRI (triple phosphate)]; MUC (mucus); SPRM (sperm); Further classes for manual subclassification are also available!	RBC (red blood cells); Dysmorphic RBC; WBC (white blood cells and WBC clumps); EPI (squamous epithelial cells); BACc (bacteria cocci); YEA (yeast) CRY (crystals); MUC (mucus); SPRM (sperm); Other subgroups particulates can be manually defined on the screen.
Chemical Stripe Analyzers		
Max. throughput	Up to 250 tests/hour	Up to 200 tests/hour
Strips capacity	150 strips	200 strips
Methodology	Reflectance photometer	Reflectance photometer
Min. sample volume	2 mL	3 mL
Memory	Max 10.000 results	Max 100.000 results.
Test wavelengths	4 discrete wavelengths	3 discrete wavelengths
Evaluated parameters	Bilirubin, Urobilinogen, Ketones, Protein, Glucose, Ascorbic acid, Nitrite, Leucocytes, Blood, pH, Specific gravity	Bilirubin, Urobilinogen, Ketones, Protein, Glucose, Ascorbic acid, Nitrite, Leucocytes, Blood, pH, Specific gravity, UMA

Manual Microscopy

For microscopic examination, 10 mL of urine sample is centrifuged at 2000 rpm (400 g) for 5 min and the sediment obtained was examined within 30 minutes. After eliminating 9 mL of urine sample, the remaining 1 mL urine sample was re-suspended, 20 UL sediment was piped onto a microscope slide and covered with a coverslip (18 × 18 mm). Microscopic examination was done with a light microscope (Olympus, CX21) at 100× and 400× magnifications. The particles were counted per field, and the results were classified semi-quantitatively within intervals or as negative and positive. All urine samples were evaluated by three persons (2 technicians and a medical biochemistry specialist doctor) that have more than 10 years of experience. In order to minimize the inter-observer variability, examinations were performed using the same microscope.

Manual microscopy was used as the reference method for all computations.

Precision Study

Within-run precision was assessed by measuring RBC and WBC for a total of 20 times in a day using high-level and low-level quality control materials. LX series urine analyzers Urine Dipstick / Microscopics Control Level 1-Level 2 (Hangzhou Longxin Technology. Zhejiang, China) was used for LX-8000R device, and KOVA Liqua-Trol with Microscopics Level I-II urinalysis Control (KOVA International. California, United States) was used for UriSed2 device.

Between-run precision was assessed by measuring RBC and WBC for a total of 20 times (5 times in a day for four days) using both quality control materials. Precision of each method was assessed by calculating % variation coefficient (CV %).

Statistical Analysis

SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) for Windows package program, which is a windows-based software, and MedCalc statistics software (MedCalc Software, Mariakerke, Belgium) were used for the statistical analyses of study data. The data are presented as percentage (%), mean ± standard deviation (SD), correlation coefficient (r), variation coefficient (CV%), 95% confidence interval (95% CI), consistence rate and weighted kappa (κ).

As Urised2 and LX-8000R analyzers use different methods of microscopic analysis, relationship and differences between the results of WBC, RBC and EPI were compared using Passing-Bablok and Bland-Altman graphics. Spearman correlation test was used to investigate the consistency of the microscopic analysis of each device. Correlation coefficient (r) was interpreted as following; <0.3 negligible correlation, 0.3–0.5 low correlation, 0.5–0.7 moderate correlation, 0.7–0.9 high correlation, and >0.9 very high correlation (9). For gamma statistics and weighted kappa values, it was considered that 0.5–0.75 represents good agreement and >0.75 represents excellent agreement (10). A p value <0.05 was considered statistically significant.

Table II Semi-quantitative range classification of urine particles.

Parameters	Negative	Few	Moderate	High	Many
Erythrocyte (cells/HPF)	0–4	5–10	11–20	21–50	≥51
Leukocyte (cells/HPF)	0–4	5–10	11–20	21–50	≥51
Epithelial cell (cells/HPF)	0–4	5–10	11–20	21–50	≥51

Results

The present study compared the data obtained from the analyses of 178 urine samples in two different fully automated urine analyzers.

The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of LX-8000R and UriSed 2 as compared to manual urinalysis is demonstrated in *Table III*. The sensitivity of UriSed2 device for WBC, RBC and EPI was 90.36%, 63.79% and 70.59%, respectively; and the specificity was 87.23%, 94.12%, and 87.16%, respectively. The sensitivity of LX-8000R device for WBC, RBC and EPI was 84.34%, 65.52% and 51.47%, respectively; and the specificity was 78.72%, 84.87% and 95.41%, respectively.

Within-run and between-run coefficients of RBC and RBC variations for each method are demonstrated in *Table IV*.

Gamma statistics comparisons for WBC, RBC and EPI counts between manual urinalysis and LX-8000R and UriSed2 urine analyzers are demonstrated in *Table V* and *Table VI*. Gamma values for WBC, RBC and EPI were 0.886, 0.782 and 0.770, respec-

Table III Diagnostic accuracy of automated urine analysers compared to manual microscopy.

Parameter	Analyzer	Sensitivity	Specificity	PPV (%)	NPV (%)
WBC	LX-8000R	84.34	78.72	77.77	85.05
	UriSed 2	90.36	87.23	86.20	91.11
RBC	LX-8000R	65.52	84.87	67.85	83.47
	UriSed 2	63.79	94.12	84.09	84.21
EPI	LX-8000R	51.47	95.41	87.50	75.91
	UriSed 2	70.59	87.16	77.41	82.60

Table IV Within-run and between-run precision of microscopic analysis by LX-8000R and UriSed 2.

		Within-run precision				Between-run precision			
		Level 1 (Low)		Level 2 (High)		Level 1 (Low)		Level 2 (High)	
Analyzer	Parameter (cells/HPF)	Mean±SD	%CV	Mean±SD	%CV	Mean±SD	%CV	Mean±SD	%CV
LX-8000R	RBC	*	*	33.85±6.20	18.32	*	*	38.45±6.52	16.97
	WBC	*	*	36.10±8.60	23.83	*	*	33.75±9.97	29.55
UriSed 2	RBC	*	*	47.60±7.42	15.59	*	*	38.22±10.18	26.63
	WBC	1.32±0.51	39.29	32.79±4.13	12.60	1.03±0.73	70.58	28.81±4.96	17.22

*SD and CV did not get calculated because the mean value of erythrocyte and leukocyte results was 0.

Table V Comparison of the numbers of WBC, RBC and EPI cell counted by the manual method and the LX-8000R.

		Number of WBC (cells / HPF)						Number of RBC (cells / HPF)						Number of EPI (cells / HPF)					
Manual Microscopy (cells / HPF)		0–4	5–10	11–20	21–50	≥51	Total	0–4	5–10	11–20	21–50	≥51	Total	0–4	5–10	11–20	21–50	≥51	Total
	0–4	74	18	3	0	0	95	74	18	3	0	0	95	105	3	1	1	0	110
	5–10	6	8	6	0	0	20	6	8	6	0	0	20	11	7	2	0	0	20
	11–20	4	1	5	3	1	14	4	1	5	3	1	14	12	3	2	0	0	17
	21–50	2	1	4	13	3	23	2	1	4	13	3	23	8	9	3	4	0	24
	51	1	0	0	2	23	27	1	0	0	2	23	27	2	3	1	1	0	7
	Total	87	28	18	18	27	178	87	28	18	18	27	178	138	25	9	6	0	178
	Gamma	0.886						0.782						0.770					
	p	< 0.001						< 0.001						< 0.001					

Table VI Comparison of the numbers of WBC, RBC and EPI cell counted by the manual method and the UriSed 2.

		Number of WBC (cells / HPF)						Number of RBC (cells / HPF)						Number of EP (cells / HPF)					
Manual Microscopy (cells / HPF)		0–4	5–10	11–20	21–50	≥51	Total	0–4	5–10	11–20	21–50	≥51	Total	0–4	5–10	11–20	21–50	≥51	Total
	0–4	102	11	3	3	1	120	113	4	2	1	0	120	96	6	6	1	1	110
	5–10	9	8	0	2	3	22	11	7	2	2	0	22	11	8	1	0	0	20
	11–20	9	1	2	2	0	14	7	2	2	2	1	14	6	5	5	1	0	17
	21–50	0	0	0	1	1	2	0	0	0	1	1	2	3	5	11	5	0	24
	51	2	0	0	1	17	20	3	0	0	1	16	20	0	0	2	2	3	7
	Total	122	20	5	9	22	178	134	13	6	7	18	178	116	24	25	9	4	178
	Gamma	0.938						0.880						0.805					
	p	< 0.001						< 0.001						< 0.001					

Table VII The correlation coefficients of microscopic analysis results between the manual microscopy and automated analyzers.

Parameters		LX-8000R r*	UriSed 2 r*
Manual Microscopy	RBC	0.554	0.639
	WBC	0.731	0.793
	EPI	0.601	0.615

*r; Spearman correlation of coefficient, p< 0.001.

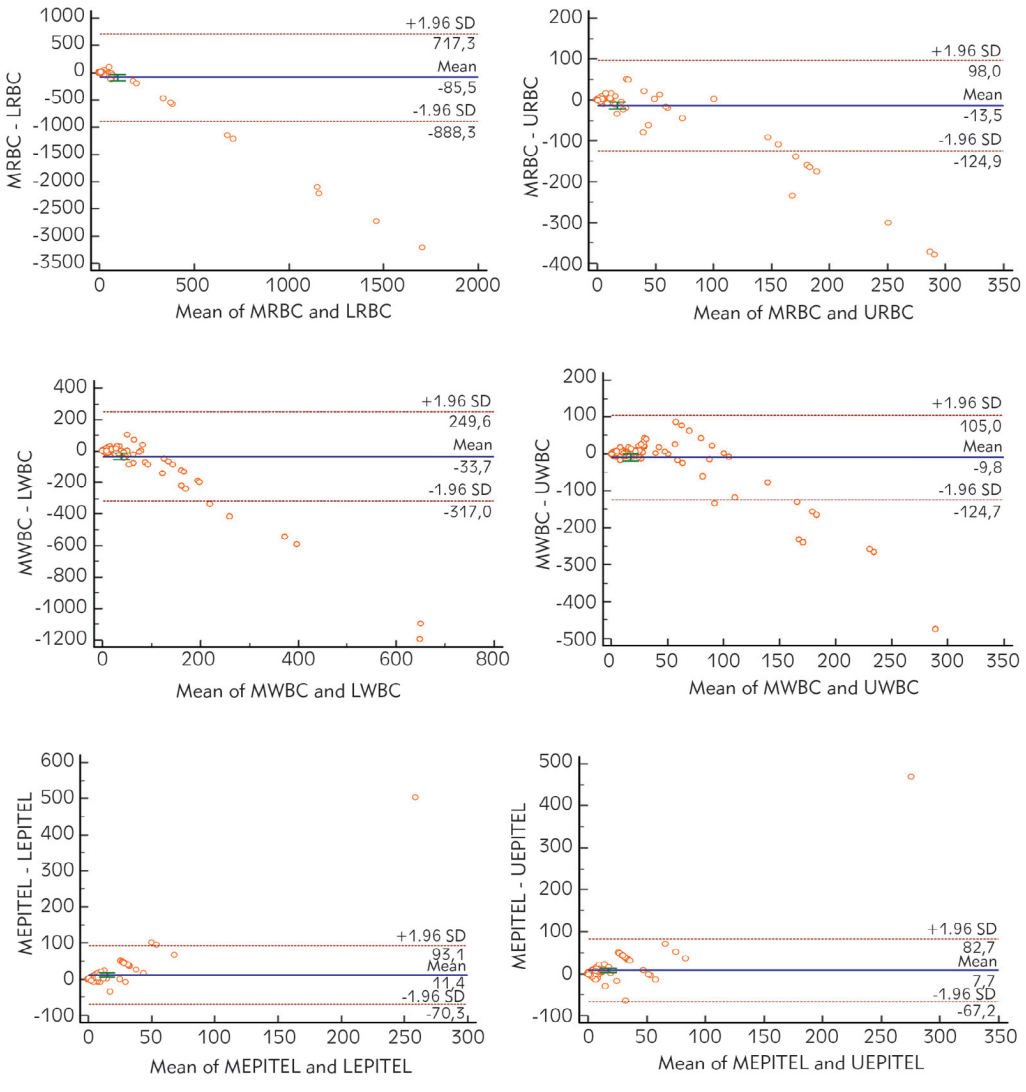
tively (p <0.001) for LX-8000R, and 0.938, 0.880 and 0.805, respectively (p <0.001) for UriSed2.

The correlation between manual urinalysis and LX-8000R and UriSed2 devices was r = 0.731 and 0.793, respectively for WBC (p<0.001); r = 0.554 and 0.639, respectively for RBC (p<0.001), and r = 0.601 and 0.615, respectively for EPI (p<0.001), and these correlations were statistically significant (*Table VII*).

Table VIII The results of the Passing-Bablok regression analysis plots for WBC, RBC and EPI counts.

Analytes	Comp	Equation	Intercept (95% CI)	Slope (95% CI)	Cusum p
WBC	1 vs 2	y = 0.00 + 0.60 x	0.00 –(-0.25 to 0.00)	0.60 (0.50 to 0.75)	0.07
	1 vs 3	y = 0.00 + 1.04 x	0.00 –(0.00 to 0.00)	1.04 (-84.92 to 84.92)	0.01
RBC	1 vs 2	y = 0.00 + 0.45 x	0.00 –(0.00 to 0.00)	0.45 (0.23 to 0.58)	0.01
	1 vs 3	y = 0.00 + 0.75 x	0.00 –(0.00 to 0.00)	0.75 (0.52 to 1.00)	0.01
EPI	1 vs 2	y = 0.00 + 5.00 x	0.00 –(0.00 to 0.00)	5.00 (3.40 to 8.50)	0.01
	1 vs 3	y = 0.00 + 2.00 x	0.00 –(0.00 to 0.00)	2.00 (1.45 to 2.58)	0.01

Comp - comparisons: 1) Manual Microscopy, 2) LX-8000R, 3) UriSed 2



MRBC: Manuel RBC, MWBC: Manuel WBC, MEPITEL: Manuel EPI
LRBC: LX-8000R RBC, LWBC: LX-8000R WBC, LEPITEL: LX-8000R EPI
URBC: UriSed 2 RBC, UWBC: UriSed 2 WBC, UEPITEL: UriSed 2 EPI

Figure 1 Bland-Altman plots.

With Bland-Altman analysis, the mean of differences between manual microscopy and LX-8000R was as following: WBC -33.7, RBC -85.5, and EPI 11.4, whereas the mean of differences between manual microscopy and UriSed2 was as following: WBC -9.8, RBC -13.5, and EPI 7.7. Accordingly, while lower results were obtained for WBC and RBC in both UriSed2 and LX-8000R devices as compared to manual urinalysis, higher results were obtained for EPI (*Figure 1*).

In the present study, Passing-Bablok regression analysis was used to compare the microscopic analyzers of each device. Regression analysis revealed a linear correlation between manual microscopy and LX-8000R device for WBC (p=0.07). However, it was determined that there is significant deviation from linearity for RBC and EPI (p=0.01 and p=0.01, respectively) with LX-8000R vs. manual microscopy and that manual microscopy and LX-8000R device are not concordance for RBC and EPI count. Results of the regression analysis for the outcomes with manual microscopy vs. UriSed2 device demonstrated significant deviation from linearity for WBC, RBC and EPI (p=0.01, 0.01 and 0.01, respectively), and it was observed that manual microscopy and UriSed2 device are not concordance for WBC, RBC and EPI count (*Table VIII*).

In the present study, uric acid crystal was detected in 4 samples by manual microscopy, whereas it was detected in one sample with LX-8000R device and in none of the samples with UriSed2 device. While manual microscopy detected Ca oxalate crystals in 10 samples, LX-8000R detected Ca oxalate crystals in 9 samples, and UriSed2 device detected in 6 samples. Manuel microscopy detected 1 lipid cylinder, whereas any of the two urine analyzer detected no lipid cylinder.

Discussion

In urine sediment analysis, automated urine analyzers contribute to the reduction of workforce and potential assay variations and shortening of turnaround time. Manual microscopy remains to be the »gold standard« despite the methodological problems and disadvantages such as that many factors reduce the precision and accuracy of results (11, 12). Manual microscopy is not only labor-intensive and time consuming, but also has between-technicians differences and low reproducibility (10). Currently available automated urine analyzers enhance productivity and turnover in the laboratories by increasing the reproducibility and productivity/throughput and are considered to reduce the time and labor required to process urine samples (13, 14).

Bottini et al. (15) stated that UriSed is a precise method with inter- and intra-assay precision ranging from 8%–15%. In addition, they stated that UriSed, depending on the particle count, shows much lower variation than that observed in manual urinalysis. Budak et al. (16) reported higher within-run and between-run CVs with UriSed device as compared to iQ200 and UF-1000 devices. Moreover, they detected slightly higher false-negative RBC and WBC reports with UriSed than the other devices. They suggested sampling and centrifugation steps used in the analytic method of the UriSed device as the origins of this situation. Ma et al. (17) detected that UriSed has acceptable levels of within-run and between-run precision (CV < 20%) for RBC, WBC, Cylinder, EPI and bacteria (BAC). Zaman et al. (6) found within-run precision at low and high control levels to be 17.8% and 6.7%, respectively for RBC and 17% and 4.4%, respectively for WBC. In the present study, the mean value of the LX-8000R and UriSed2 devices' within-run precision and between-run precision for RBC at low control level was found to be zero; and CV% could not be calculated. Within-run precision at high control level yielded better CV% values for WBC and RBC with UriSed2 device (CV% 12.60 and 15.59, respectively) than LX-8000R device (CV% 23.83 and 18.32, respectively). Between-run precision at high control level yielded better CV% values for WBC with UriSed2 device (CV% 17.22) than LX-8000R device (CV% 29.55), whereas better CV% values with LX-8000R device (CV% 16.97) than UriSed2 device (CV% 26.63) for RBC.

Akin et al. (19) compared the analytic performance of UriSed with that of iQ200 automated analyzer and detected significant correlation between two methods. Laiwejpithaya et al. (1) compared RBC, WBC ve EPI cell counts obtained by UriSed 3 and UX-2000 automated urine analyzers with those obtained by manual microscopy. As a conclusion, they reported that UriSed 3 and UX-2000 devices have almost similar performance for RBC and WBC counts but UriSed 3 is more reliable for EPI cell count (1). Budak et al. (16) determined consistency by 91.5% between UF-1000i and UriSed, 92.2% between iQ200 and UriSed, and 89.5% between UF-1000i and iQ200 for RBC, whereas they found consistency by 82.2% between UF-1000i and UriSed and 83.7% between iQ200 and UriSed for WBC. In the same study, epithelial cell count demonstrated consistency by 86.6% between UF-1000i and iQ200, 85.1% between UF-1000i and UriSed, 87.6% between iQ200 and UriSed, and 90.2% between UF-1000i and iQ200 (16). In a study, Ercin (18) compared Urised2 and FUS200 microscopic analyzers and found WBC, RBC and EPI cell counts to be within the same range and reported excellent consistency for these three parameters (Gamma value is 0.916, 0.770 and 0.961, respectively; p <0.001). Jintasuthanont et al. (10) compared the results of UriSed analyzer with that of manual microscopy; they found the gamma value to be 0.837 for WBC, 0.918 for RBC, and 0.939 for squamous epithelial cell count, and they detected high correlation between the results obtained by full-automated analyzer and the

results obtained by manual microscopy. In the present study, WBC, RBC and EPI gamma values were 0.886, 0.782 and 0.770, respectively for LX-8000R ($p < 0.001$) and 0.938, 0.880 and 0.805, respectively for UriSed2 ($p < 0.001$). Based on these outcomes, it was determined that both urine analyzers show excellent consistency with manual microscopy for WBC, RBC and EPI cell counts.

Ercin (18) reported high correlation between Urised2-LabUmat2 and FUS200-H800 devices for WBC count, but moderate correlation for RBC count. Moreover, according to the Passing-Bablok regression analysis in this study, they reported that there is no consistency between these two methods because of the presence of remarkable deviation from linearity for WBC and RBC counts ($p < 0.05$ and $p = 0.01$, respectively) but that they are concordance for EPI cell count ($p = 0.65$). In addition, Bland-Altman agreement plot graphics demonstrated that automated microscopy units of the two devices showed acceptable performance for WBC (-29.3 ± 1.96 SD), RBC (43.3 ± 1.96 SD) and EPI (-4.0 ± 1.96 SD) cell counts (18). Yalçınkaya et al. (3) compared FUS200 and Urised3 urine analyzers where Deming regression analysis yielded a correlation coefficient of 0.961 and 0.961 for WBC and RBC counts, respectively. When they investigated the consistency between two urine analyzers for negative-positive test results, they found the kappa value to be 0.79 (good consistency) for WBC and 0.42 (moderate consistency) for RBC (3). In the present study, Bland-Altman analysis revealed lower mean of differences between manual microscopy and UriSed2 device for WBC, RBC, EPI (-9.8, -13.5 and 7.7, respectively) suggesting that manual microscopy and UriSed2 device are concordant. The mean of differences between manual microscopy and LX-8000R device for WBC, RBC and EPI was -33.7, -85.5 and 11.4, respectively suggesting that manual microscopy and LX-8000R device are not concordant for WBC and RBC counts but more concordant only for EPI. As a method similar to manual microscopy, centrifuging the urine in the UriSed2 device and screening 15 fields equivalent to 400x magnification and then having the images checked by a technician suggests that the outcomes might be more concordant.

Passing-Bablok regression analysis demonstrated a linear correlation between manual urinalysis and LX-8000R device for WBC ($p = 0.07$) but remarkable deviation from linearity for RBC and EPI ($p = 0.01$ and $p = 0.01$, respectively). Regarding manual microscopy vs. UriSed2, it was determined that there is a remarkable deviation from linearity for WBC, RBC and EPI cell counts ($p = 0.01$, 0.01 and 0.01, respectively) and that manual microscopy and UriSed2 device are not concordant for WBC, RBC and EPI cell counts. Considering these outcomes, we can say that both devices, in general, are not concordant with manual microscopy; however, this might result from semi-quantitative evaluation by manual microscopy.

According to Yalçınkaya et al. (3), urine samples can be analyzed in UriSed 3 and FUS-200 and give reproducible outcomes, UriSed 3 have higher specificity for RBC and higher sensitivity for WBC than FUS-200. They determined that FUS-200 analyzer have higher PPV for WBC but lower PPV for RBC than UriSed 3. They reported almost perfect and similar NPV with FUS-200 and UriSed 3 analyzers for both cell types (3). Ma et al. (17) detected >80% sensitivity and specificity for RBC, WBC and EPI cells between UriSed device and manual microscopy. Mittal et al. (2) determined the sensitivity, specificity, PPV and NPV of UriSed2 urine analyzer to be 89.2%, 93.3%, 96.8% and 78.8%, respectively for RBC and to be 86%, 91%, 96% and 74.3%, respectively for WBC. In the present study, while UriSed2 device was more sensitive for WBC and EPI (90.36% and 70.59%) than LX-8000R device (84.34% and 51.47%), both devices have nearly similar sensitivity for RBC (63.79% and 65.52%). UriSed2 device had higher specificity, PPV and NPV for WBC and RBC (87.23% and 94.12%; 86.20% and 84.09%; and 91.11% and 84.21%, respectively) than LX-8000R device (78.72% and 84.87%; 77.77% and 67.85%; and 85.05% and 83.47%, respectively).

Bottini et al. (15) stated that UriSed urine analyzer is a precise and accurate alternative to microscopy. They stated also that routine use of these automated urine analyzers would enable better workflow and reduce the turnaround time and, in addition, examination of the images displayed on the screen of UriSed device would potentially eliminate the need for microscopic examination for most of the urine samples (15).

The fact that the LX-8000R device, which is evaluated in the present study, obtains images from 20 different fields in the urine sample by real-time cell picture and field image method without centrifugation can be considered as a limitation. We think that better analysis can be achieved with higher number of images. We also think that evaluation of images from 15 different fields in a centrifuged urine using UriSed2 is a better approach for urinalysis. Despite overall outcomes and statistics, we suggest that evaluation of automated urinalysis devices together with samples and patient clinical findings in addition to comparing with manual microscopy will be more meaningful.

Today, although automated urine analyzers reduce workload in the laboratories, they need to be developed further for they can accurately recognize the pathological elements occurring in the urine due to methods and software.

Conflict of interest statement

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

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Received: April 06, 2021
Accepted: August 16, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-33374

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 100–107, 2022

Original paper
Originalni naučni rad

SSRP1 AFFECTS THE GROWTH AND APOPTOSIS OF GASTRIC CANCER CELLS THROUGH AKT PATHWAY

SSRP1 UTIČE NA RAST I APOPTOZU ĆELIJA RAKA ŽELUCA KROZ AKT SIGNALNI PUT

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Summary

Background: We aimed to determine the SSRP1's potential influence on the apoptosis and proliferation of gastric cancer (GC) cells and its regulatory mechanism.
Methods: SSRP1 expression in GC cells and tissues was detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The interrelation between clinicopathological characteristics of GC patients and SSRP1 expression was analysed via χ^2 test, and the correlation between SSRP1 expression and overall survival rate was analysed using Kaplan-Meier survival analysis. After the knockdown of SSRP1 in AGS cells, the SSRP1 expression, colony formation ability, cell viability, cell cycle changes, apoptosis rate, and migration and invasion ability were detected through qRT-PCR, colony formation assay, CCK8 assay, flow cytometry and transwell test, respectively. Finally, the effects of down-regulation of SSRP1 on the expressions of phosphorylated-protein kinase B (p-AKT), B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax) were explored using Western blotting.
Results: SSRP1 displayed a high expression in GC cells and tissues. SSRP1 expression was closely interrelated to the TNM stage, lymph node metastasis and tumour size. The survival rate of patients was markedly shorter in the high expression group than in the lower expression group. After the knockdown of SSRP1 in cells, the viability and colony formation ability of AGS cells were inhibited. In addition, the cell ratio in the G1 phase was increased, while that in the S phase declined, and the cell invasion and migration were obviously weakened. It was found from Western blotting that the knockdown of SSRP1 could evidently suppress

Kratak sadržaj

Uvod: Naš cilj je bio da otkrijemo potencijalni uticaj SSRP1 na apoptozu i proliferaciju ćelija raka želuca (GC) i njegov regulatorni mehanizam.
Metode: Ekspresija SSRP1 u GC ćelijama i tkivima je detektovana kvantitativnom lančanom reakcijom polimeraze reverzne transkripcije (qRT-PCR). Odnos između kliničko-patoloških karakteristika pacijenata sa GC i ekspresije SSRP1 je analiziran pomoću Hi-kvadratnog testa (χ^2), a korelacija između ekspresije SSRP1 i ukupne stope preživljavanja pomoću Kaplan-Meier analize preživljavanja. Nakon obaranja SSRP1 u ćelijama AGS, ekspresija SSRP1, sposobnost formiranja kolonije, vitalnost ćelija, promene ćelijskog ciklusa, stopa apoptoze i sposobnost migracije i invazije su otkriveni pomoću qRT-PCR, testa formiranja kolonije, CCK8 testa, protočne citometrije i transvel testa, redom. Konačno, efekti smanjenja SSRP1 na ekspresije fosforilisane protein kinaze B (p-AKT), B-ćelijskog limfoma-2 (Bcl-2) i Bcl-2 pridruženog Ks proteina (Bax) su ispitani pomoću Vestern blota.
Rezultati: SSRP1 je pokazao visoku ekspresiju u GC ćelijama i tkivima. Ekspresija SSRP1 je bila usko povezana sa stadijumom TNM, metastazama u limfnim čvorovima i veličinom tumora. Stopa preživljavanja pacijenata je bila znatno kraća u grupi sa visokom ekspresijom nego u grupi sa nižom ekspresijom. Nakon obaranja vrednosti SSRP1 u ćelijama, došlo je do inhibicije vitalnosti i sposobnosti formiranja kolonija AGS ćelija. Pored toga, povećana je elijska stopa u G1 fazi, dok je u S fazi opala, dok su invazija i migracija ćelija očigledno oslabljene. Vestern blot metodom je utvđeno da bi obarenje SSRP1 moglo očigled-

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the protein levels of Bcl-2 and p-AKT but promote the protein expression of Bax, indicating that silencing SSRP1 can inhibit the proliferative capacity and increase the number of GC cells through inactivating the AKT signalling pathway.
Conclusions: SSRP1 rose up in GC tissues and cells. Reduction of SSRP1 can inhibit the proliferative capacity and increase the number of GC cells through inactivating the AKT signalling pathway.

Keywords: SSRP1, gastric cancer, proliferation, apoptosis

no potisnuti nivo proteina Bcl-2 i p-AKT, ali bi promovisalo ekspresiju proteina Bak, ukazujući da smanjenje SSRP1 može inhibirati proliferativni kapacitet i povećati broj GC ćelija kroz inaktivaciju AKT signalnog puta.
Zaključak: SSRP1 je porastao u tkivima i ćelijama GC. Smanjenje SSRP1 može inhibirati proliferativni kapacitet i povećati broj GC ćelija kroz inaktivaciju AKT signalnog puta.

Ključne reči: SSRP1, rak želuca, proliferacija, apoptoza

Introduction

Gastric cancer (GC) is a frequently seen malignancy seriously threatening human health. According to the latest statistics, the morbidity rate of GC ranks 4th among all malignant tumours in the world, and its mortality rate has jumped to 3rd place (1, 2). The 5-year survival rate of GC patients in China has dramatically risen with the early gastroscopy and prompt surgical intervention in recent years (3). However, the 5-year survival rate of advanced GC remains low (4). Therefore, further uncovering the pathogenesis of GC for preventing the occurrence of GC and improving the prognosis is of great significance.

Structure-specific recognition protein 1 (SSRP1) was originally identified as a high-mobility group 1 (HMG1)-related DNA-binding protein in 1991, and its biological functions can be attributed to its HMG domain (5). SSRP1, a subgroup of histone chaperones, boosts chromatin transcription (FACT) complex and regulates most processes associated with chromatins, such as replication, DNA repair, and transcription (6–9). It is believed that SSRP1 dramatically influences the development and occurrence of tumours, and its expression is upregulated in such tumours as hepatocellular cancer (10), colorectal cancer (11), nasopharyngeal cancer (12) and glioma (13). Knockdown of SSRP1 *in vitro* can suppress the proliferative capacity of glioma U251 and U87 cells by inactivating the MAPK pathway (13) and reducing the growth of NSCLC cells (14). Nevertheless, the biological role and mechanism of SSRP1 in gastric cancers remain unclear.

To clarify SSRP1's function in GC and its mechanism of action, therefore, SSRP1 expression in GC cells and tissues was explored through a series of functional assays, and its effects on the proliferative capacity, apoptosis, invasion and migration of GC cells via the protein kinase B (AKT) pathway were determined, thereby providing new ideas for the targeted clinical treatment of GC.

Materials and Methods

Patients and tissue specimens

A total of 40 paired GC specimens were surgically resected in our hospital. The patients had complete clinicopathological data and follow-up data. Specimens receiving 5-min fast freezing in liquid nitrogen were reserved at -80 °C. The present research was reviewed and obtained approval of the Hospital Medical Ethics Committee, and the written informed consent was obtained from all participants.

Cell culture and transfection

AGS, MGC-803, FU97, BGC-823, GES-1 and SGC-7901 cells were cultured in 1640 medium from Invitrogen (Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) provided by GIBCO/BRL (Rockville, MD, USA), 100 U/mL penicillin and 100 mg/mL streptomycin from Beyotime (Shanghai, China) under 5% CO_2 at 37 °C. AGS cells undergone siRNA (GenePharma, Suzhou, China) or NC transfection using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) *as per* the guidance of the manufacturer si-SSRP1 sense: 5′-GCCAUGUCUACAAGUAUGATT-3′, and antisense: 5′-UCAUACUUGUAGACAUGGCTT-3′.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNAs underwent RT into cDNAs on GoScript RT System (Promega, Madison, WI, USA) by an All-in-One miRNA RT kit (GeneCopoeia, Rockville, MD, USA). Then SYBR Green human miRNA kit (GeneCopoeia, Rockville, MD, USA) and GoTaq qPCR Master Mix (Promega, Madison, WI, USA) were utilised for qRT-PCR. The procedures of thermal cycler were set below: 95 °C for 10 min, (95 °C for 30 s) × 40 cycles, primer annealing at 55 °C for 30 s and 72 °C for 30 s. Each assay was repeated for 3 times.

Western blotting

The cells were harvested and lysed with 1× cell lysate from Cell Signaling Technology (Danvers, MA, USA) with phenylmethylsulfonyl fluoride (PMSF) (1 mmol/L) at 4 °C for 30–60 min. Subsequent to 10-min centrifugation at 12,000 g, the cytoplasmic protein samples were obtained, and bicinchoninic acid (BCA) protein test kit from Thermo Fisher Scientific (Waltham, MA, USA) was utilised for measurement of

protein concentration. Then, total protein samples (20–50 μg) were isolated via 10–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane from Millipore (Billerica, MA, USA). The membrane was blocked by 5% skim milk at room temperature for 2 h, and undergone primary antibody (1:1,000) incubation at 4 °C nightlong. On the following day, TBS with 0.05% (v/v) Tween-20 (TBST) was utilised for rinsing the membrane, and it received 2-h incubation with secondary antibodies jugated by horseradish peroxidase (1:5,000, Lianke Biotech Co., Ltd., Hangzhou, China) at indoor temperature. Following rinsing, the membrane was observed using an electrochemiluminescence (ECL) assay kit (PerkinElmer, Inc., Rockford, IL, USA) and Tanon 5500 gel imaging system from Tanon Science & Technology (Shanghai, China).

Detection of proliferative activity via cell counting kit-8 (CCK8) test

Cells undergoing transfection were paved onto a ninety-six-well plate (1000 cells/well), and cell proliferation was measured once a day *as per* the manufacturer's instructions. In a word, each well was added with 10 μL of CCK8 solution (KeyGen BioTECH, Nanjing, China) for 2-h cell incubation at 37 °C. Finally, the solution was assessed at 450 nm via spectrophotometry. The assay was repeated for 3 times in each group.

Colony formation experiment

Cells receiving transfection were paved onto a 6-well plate (200 cells/well), cultivated for 2 weeks, fixed with paraformaldehyde and dyed by Giemsa dye. Then formed colonies were counted, and the colony formation rate in each plate was calculated. The assay was repeated for 3 times in each group.

Examination of cell cycle and apoptosis rate via flow cytometry

Following at least two hours of immobilisation in cold methanol, cells received RNase A/propidium iodide (PI) solution incubation. After that, the FACSCalibur system from Becton-Dickinson (Franklin Lakes, NJ, USA) was utilised for distribution assessment of cell cycle, and the proportion of cells in varying phases was detected via Modfit 2.0 software. Flow cytometry and Annexin V/PI dying (Life Technologies, CA, USA) were implemented for apoptotic cell determination. This test was implemented thrice, and findings were presented as mean ± SD. The proportion of apoptotic cells was analysed using FlowJo7.6.1 software.

Transwell assay

The motility of tumour cells *in vitro* was assessed using chambers for Transwell migration from Corning (Corning, NY, USA) and those for invasion from BD Biosciences (Franklin Lakes, NJ, USA). Briefly, 200,000 cells containing siRNA or not were added into medium supplemented with 2% FBS, and the lower chamber was added with the medium with 10% FBS for 48 h. Then cells migrating/invading the base of the upper membrane were dyed with 0.1% crystal violet dye. Next, the cells were counted in five fields stochastically chosen using a microscope.

Statistical analysis

Data were processed via Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA). Metrical data were displayed by mean ± SD ($\bar{x}\pm$SD). Intergroup differences were processed through t-test and one-way analysis of variance (ANOVA). Comparison of enumeration data was implemented using χ^2 test. Survival curves were plotted using the Log-rank test (Kaplan-Meier method). $P<0.05$ reflected statistically significant differences.

Results

SSRP1 displayed a high expression in GC tissues and cells

It was found from qRT-PCR that SSRP1 expression rose up in 40 GC tissues compared with that in para-carcinoma normal ones (*Figure 1A*). SSRP1 expression was further detected in GES-1 as well as AGS, FU97, MGC-803, BGC-823 and SGC-7901. It was discovered that SSRP1 expression rose in GC cell lines, and it showed the highest value in AGS cell lines, so AGS cell lines were selected for subsequent knockdown assay (*Figure 1B*). According to the median expression of SSRP1, the patients were allocated into high- and low-expression groups, and the interrelations ofSSRP1 expression with clinical and pathological features of patients was further analysed. The results revealed that the patients in the high-expression group displayed a larger tumour size and advanced TNM stage, and are often accompanied by lymph node metastasis, showing statistically significant differences ($P<0.05$) (*Table I*). Besides, the Kaplan-Meier survival curves were analysed. It was confirmed that patients in the high-expression group had a lower survival rate relative to that in the low-expression group (HR=2.3980, P=0.0369), indicating that SSRP1 predicts the unfavourable prognosis of GC patients. The above findings demonstrate that SSRP1 is highly expressed in GC patients, and it can affect the TNM stage, lymph node metastasis and tumour size of GC patients, indicating a poor prognosis.

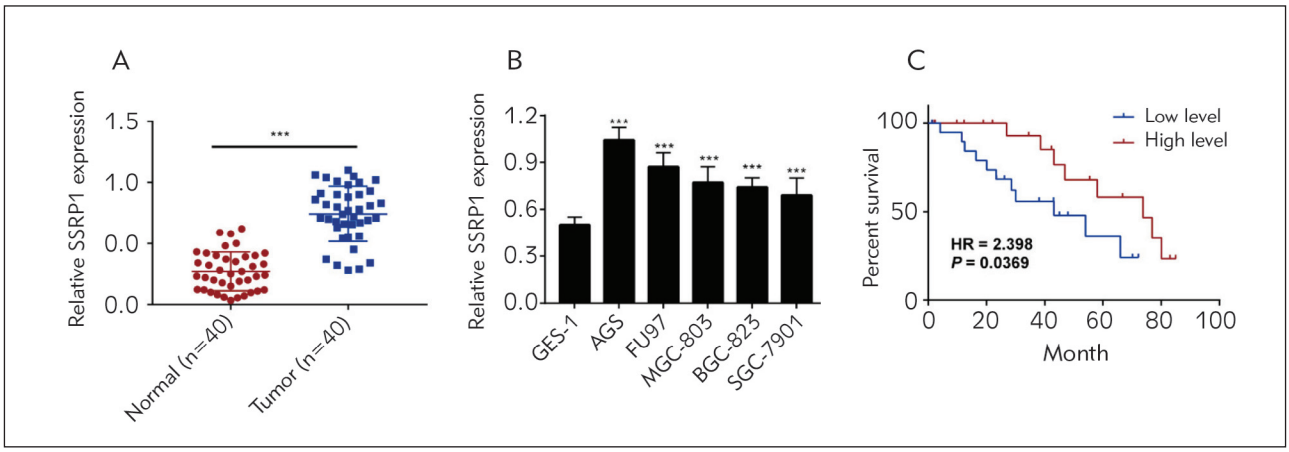

Figure 1 SSRP1 displays high expressions in GC cells and tissues. (A) It is found from qRT-PCR that SSRP1 expression obviously rose in 40 cases of GC tissues relative to that in para-carcinoma normal tissues. (B) The expression of SSRP1 is detected in GES-1 as well as AGS, FU97, MGC-803, BGC-823 and SGC-7901. (C) Kaplan-Meier survival analysis. Patients had a lower survival rate in the high-expression group relative to that in the low-expression group.

Table I Relations of SSRP1 expression with clinical and pathological features in GC patients (n=40).

Clinicopathologic features	Number of cases	SSRP1 expression		P-value
		Low (n=20)	High (n=20)	
Age (years)				0.752
≤60	19	10	9	
>60	21	10	11	
Gender				0.749
Male	23	11	12	
Female	17	9	8	
Tumour size				0.022*
≤5CM	15	11	4	
>5CM	25	9	16	
TNM stage				0.027*
I+II	19	13	6	
III+IV	21	7	14	
Tumour differentiation				0.113
Well/Moderate	21	13	8	
Poor	29	17	12	
Lymph node metastasis				0.004*
Absent	19	14	5	
Present	21	6	15	
Distant metastasis				
Positive		11	8	0.342
Negative			9	12

Knockdown of SSRP1 inhibited GC cell proliferation

To explore SSRP1's role in GC development, AGS cell lines were further selected for knockdown assay and whether changes in SSRP1 affect GC cells was explored. QRT-PCR findings ascertained that SSRP1 mRNA expression dropped obviously after AGS cells were transfected with si-SSRP1 (*Figure 2A*). Then SSRP1's impact on AGS cell activity was assessed. It was unveiled from the CCK8 assay showed that cell activity was obviously weakened after knockdown of SSRP1 in contrast to that in the control group (*Figure 2B*). According to the colony formation assay, the capacity of AGS cells treated with siRNA to form colonies also became weaker (*Figure 2C*), implying that knocking down SSRP1 impedes GC cells to proliferate.

SSRP1 influenced GC cells to proliferate and their apoptosis by impeding the AKT signaling pathway

SSRP1's impacts on the cycle and apoptosis of AGS cells were explored using flow cytometry. As shown in *Figure 3A*, at 48 h after treatment of AGS cells with siRNA, cell proportion became larger in the G1 phase but smaller in the S phase, confirming that knockdown of SSRP1 can inhibit the transition of the G1/S phase. In addition, it was found from flow cytometry that the apoptosis rate rose markedly after the knockdown of SSRP1 (*Figure 3B*). As a classical signal transduction pathway, the AKT pathway is implicated in apoptosis and proliferation. Furthermore, the results of Western blotting manifested that knockdown of SSRP1 could suppress the protein expressions of phosphorylated AKT (p-AKT) and B-cell lymphoma-2 (Bcl-2), but evidently promote Bax protein expression (*Figure 3C* & *3D*), indicating that silencing SSRP1 may inhibit GC cells from proliferating and promoting their apoptosis through the AKT signalling pathway.

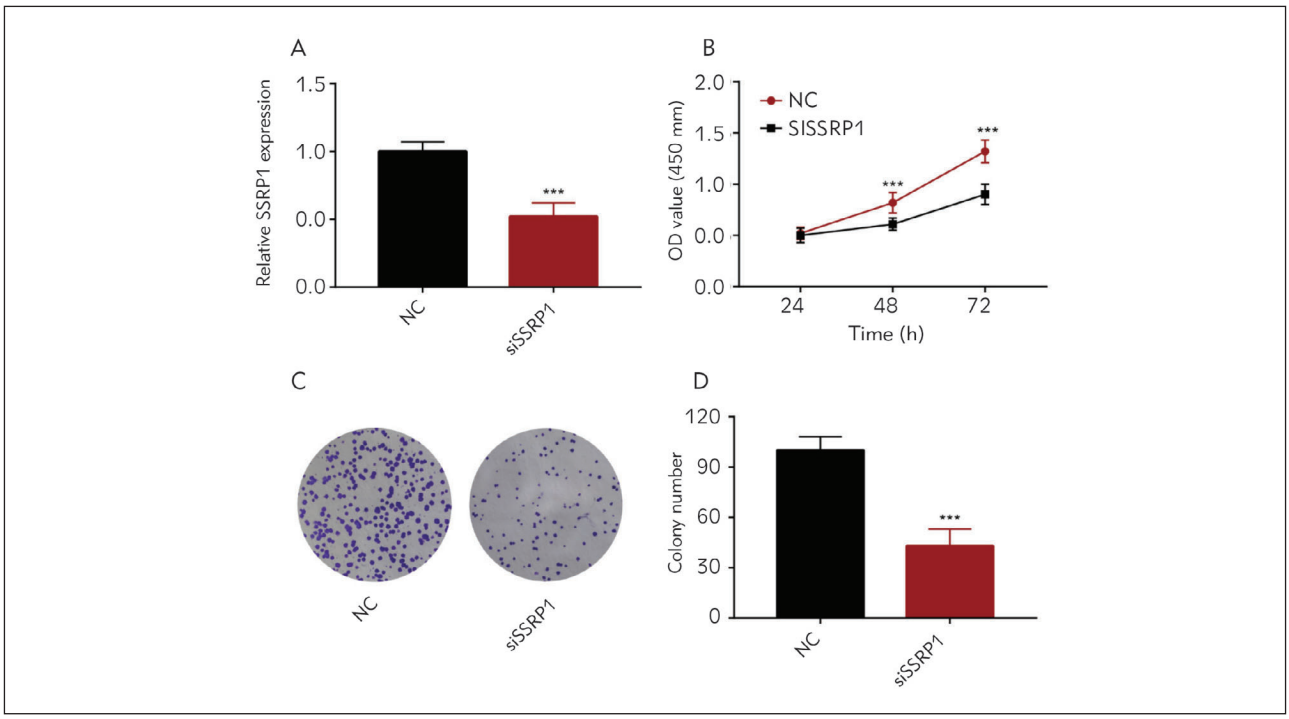

Figure 2 Decreasing SSRP1 impedes GC cells to proliferate. (A) QRT-PCR findings reveal that the mRNA expression of SSRP1 is obviously suppressed by knockdown of SSRP1 at 48 h after AGS cells are transfected with siRNA. (B) The results of the CCK8 assay show that the AGS cell viability declines after the knockdown of SSRP1. (C) Colony formation assay findings show that the colony formation ability of AGS cells becomes weaker after the knockdown of SSRP1.

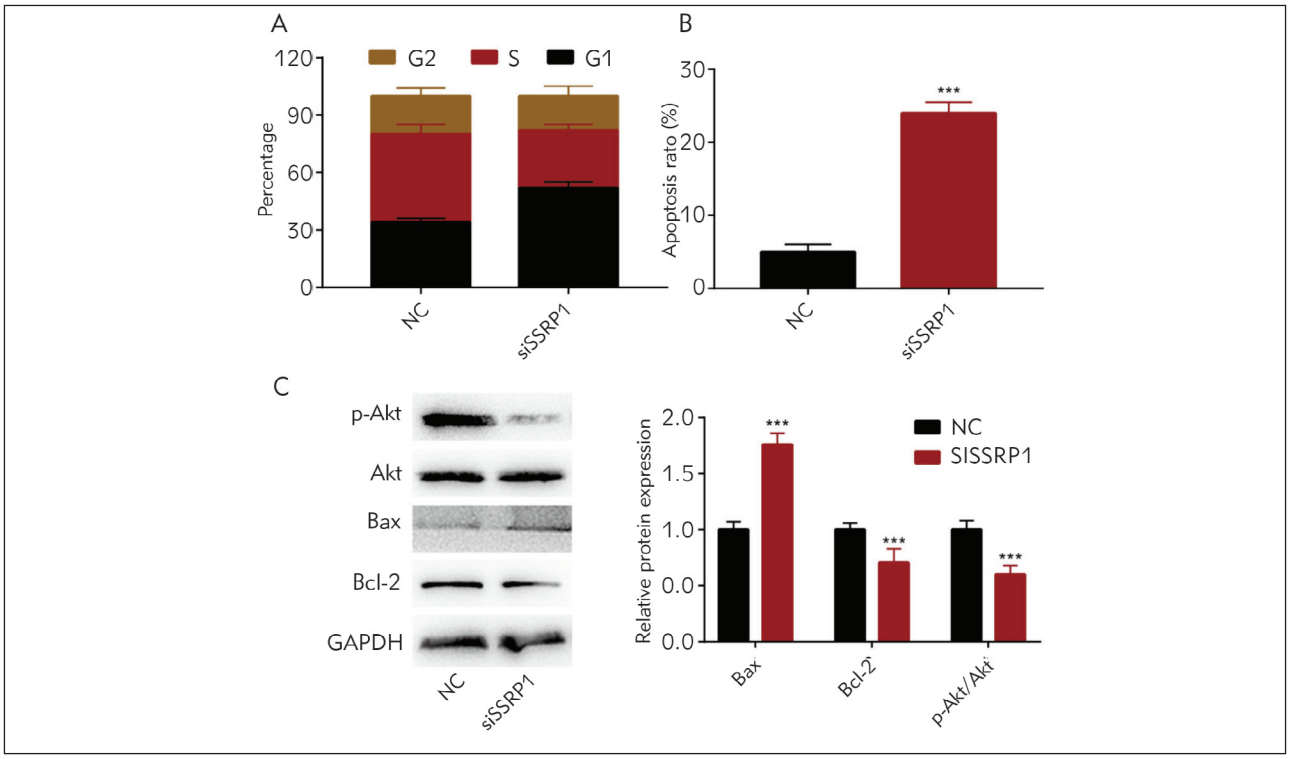

Figure 3 SSRP1 affects GC cells to proliferate and their apoptosis via impeding the AKT signalling pathway. (A) Flow cytometry findings manifest that cell proportion rises in the G1 phase and declines in the S phase, and no significant changes are found in cell proportion in the G2 phase. (B) Flow cytometry findings manifest that the apoptosis rate rises markedly after the knockdown of SSRP1. (C & D) Western blotting results show that knockdown of SSRP1 can reduce the expressions of p-AKT and Bcl-2 proteins but increase the protein expression of Bax.

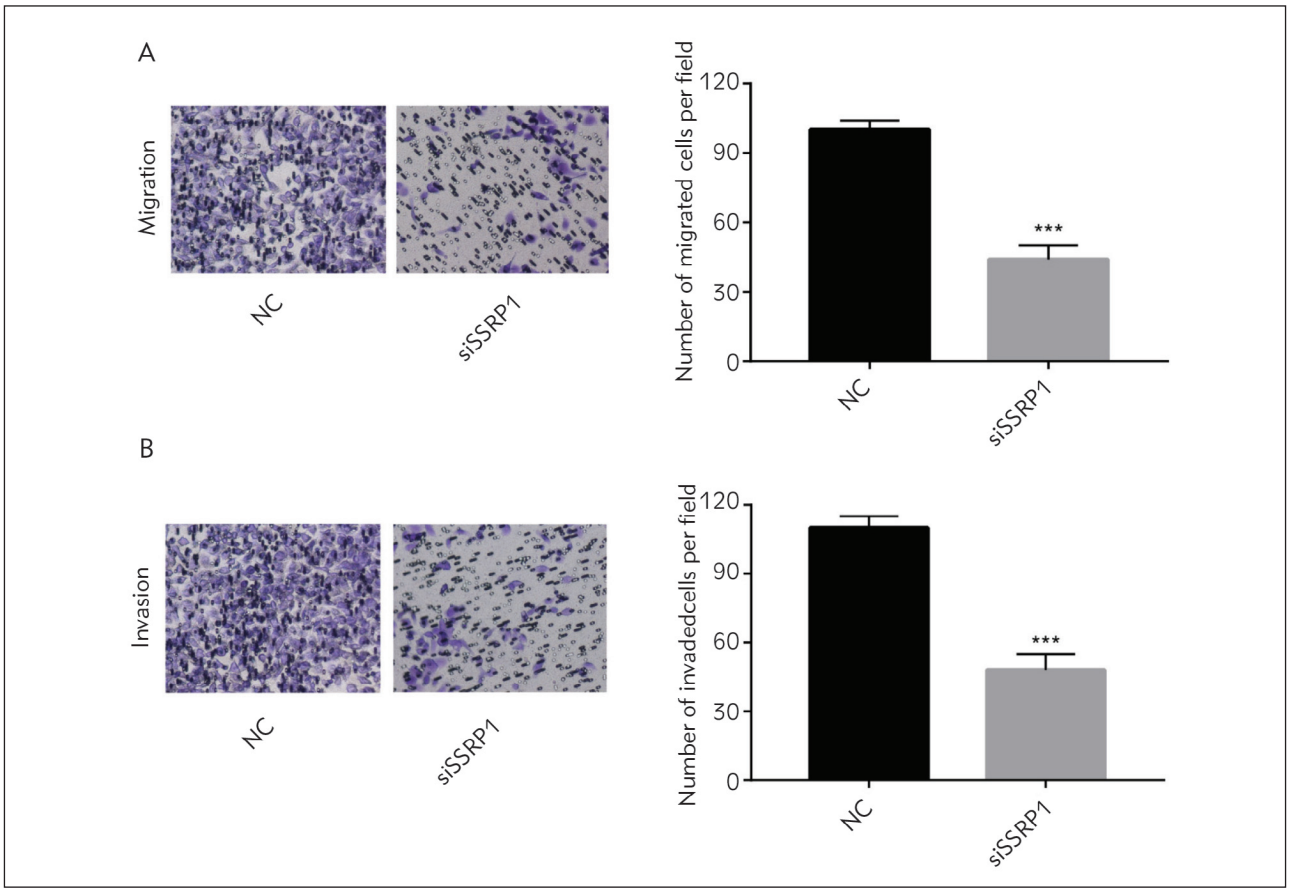

Figure 4 Down-regulation of SSRP1 suppresses GC cells to migrate and invade. (A & B) Transwell assay findings show that after reducing SSRP1, the AGS cell migration and invasion capacities remarkably decline.

Down-regulation of SSRP1 suppressed GC cells to migrate and invade

As confirmed above, down-regulation of SSRP1 can impede GC cells to proliferate and promote their apoptosis. Then, SSRP1's influences on the GC cells' abilities to migrate and invade were further explored. Transwell assay findings showed that after SSRP1 knockdown, the cell migration and invasion ability remarkably declined (*Figure 4A* & *4B*). Thus, it can be inferred that decreasing SSRP1 can impair the migration and invasion capacities of GC cells.

Discussion

It is reported that SSRP1, a subunit of histone chaperone protein FACT, can destroy the nucleosomes and histone substitutes (15, 16). Moreover, it is implicated in DNA damage repair and replication as well as cell proliferation and apoptosis (14, 17). In the current research, it was disclosed that SSRP1 rose in GC tissues and cells, consistent with previous studies on cancers, including NSCLC (14), breast cancer (18, 19) and liver cancer (10). The survival rate of patients was lower in the high-expression group than in the low-expression group, indicating that highly expressed SSRP1 predicts the unfavourable prognosis of GC patients. Additionally, the results of a series of *in vitro* experiments confirmed that knockdown of SSRP1 could inhibit the vitality, colony formation ability and cell cycle transition of GC cells and induce apoptosis, and may also suppress cell migration and invasion.

SSRP1 is a key regulatory factor for keeping normal DNA replication because it is related to MCM helicase and can promote the unwinding activity of MCM helicase DNA on the nucleosome template (20, 21). Breaking the FACT-MCM complex can hinder the beginning of DNA replication. Inhibiting SSRP1 can suppress cells to grow and hamper the S-phase cell cycle progression owing to suppression on the replication fork advancement (20). Proliferation signals accompanied by enhanced DNA replication are the markers for cancer cells (22). In this study, it was found that decreasing SSRP1 repressed the viability and colony formation ability of GC cells, which is a characteristic feature of GC cells.

In this study, it was confirmed that SSRP1 played a role in regulating GC cells to proliferate and regu-

lated their cycle, apoptosis, invasion, and migration capacities. It was unveiled that knockdown of SSRP1 could lead to cell cycle arrest and induce apoptosis. Moreover, after the knockdown of SSRP1, the protein expression of Bcl-2 could be inhibited, whereas that of Bax could be promoted. During the progression of GC, metastasis is the fatal event, so early diagnosis of highly metastatic primary tumours is of important significance for the treatment and prognosis of patients. In this study, the findings revealed that SSRP1 knockdown impeded GC cells to migrate and invade. In addition, it is reported that SSRP1 is implicated in the classical PI3K/AKT signalling pathway and affects the apoptosis and proliferation of colon cancer cells via the AKT pathway (23). As the main signalling pathway in the downstream of many growth factor receptors, a classical signalling pathway related to cell apoptosis and survival, the PI3K/AKT pathway, is the most active signalling pathway in human tumours. Moreover, the PI3K/AKT pathway facilitates the proliferation and malignant transformation and impedes tumour cell apoptosis by phosphorylating PI3K and AKT proteins (24, 25). Serine/threonine kinase AKT, classified into AKT1-3, is a key PI3K signal transduction factor in mammals. Numerous studies have unravelled that the PI3K/AKT pathway can promote GC cells to proliferate and invade (26, 27). In this study, therefore, the involvement of the AKT pathway in alterations of GC cell proliferation and apoptosis following interference with SSRP1 was explored. The findings manifested that knockdown of SSRP1 reduced the expression of p-AKT, indicating that SSRP1 regulates GC cell proliferation, cycle, apoptosis, invasion and migration via activating the AKT pathway.

Conclusion

Knockdown of SSRP1 affects GC cells to proliferate and their apoptosis through the AKT pathway, which provides a new possible therapeutic strategy and diagnostic target for GC.

Conflict of interest statement

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

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Received: August 7, 2021
Accepted: August 28, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-32903

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 108–114, 2022

Original paper
Originalni naučni rad

SIPA1 BOOSTS MIGRATION AND PROLIFERATION, AND BLOCKS APOPTOSIS OF GLIOMA BY ACTIVATING THE PHOSPHORYLATION OF THE FAK SIGNALING PATHWAY

SIPA1 POSPEŠUJE MIGRACIJU I PROFILACIJU, I BLOKIRA APOPTOZU GLIOME AKTIVIRANJEM FOSFORILACIJE SIGNALINOG PUTA FOKALNE ADHEZIONE KINAZE

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Summary

Background: We aimed to analyze the regulatory effects of SIPA1 (signal-induced proliferation-associated protein 1) on glioma progression and the dominant signaling pathway.
Methods: Differential level of SIPA1 in glioma and normal tissues and cells was determined. Migratory, proliferative, apoptotic and cell cycle progression changes in A172 cells with overexpression or knockdown of SIPA1 were examined. Finally, protein levels of phosphorylated FAKs in A172 cells intervened by SIPA1, and the FAK inhibitor PF-562271 were detected.
Results: SIPA1 was upregulated in glioma cases. Knockdown of SIPA1 reduced migratory and proliferative rates of glioma cells, increased apoptotic cell rate, and declined cell ratio in the S phase. The knockdown of SIPA1 also downregulated cell cycle proteins. In addition, SIPA1 upregulated phosphorylated FAKs in A172 cells and thus boosted malignant phenotypes of glioma.
Conclusions: SIPA1 is upregulated in glioma that boosts migratory and proliferative potentials of glioma cells by activating the phosphorylation of the FAK signaling pathway.

Keywords: SIPA1, FAK, phosphorylation, glioma

Kratak sadržaj

Uvod: Cilj nam je bio da analiziramo regulatorne efekte SIPA1 na progresiju glioma i dominantni signalni put.
Metode: Određen je diferencijalni nivo SIPA1 u gliomu i normalnim tkivima i ćelijama. Ispitivane su migratorne, proliferativne, apoptotičke i promene u progresiji ćelijskog ciklusa u ćelijama A172 sa prekomernom ekspresijom ili obaranjem SIPA1. Konačno, otkriveni su nivoi proteina fosforilisanih FAK u ćelijama A172 sa intervencijom SIPA1 i inhibitorom FAK PF-562271.
Rezultati: SIPA1 je uvećan u slučajevima glioma. Pad SIPA1 je smanjio migracijsku i proliferativnu stopu ćelija glioma, povećao apoptotičku ćelijsku stopu i smanjio ćelijski odnos u S fazi. Snižavanjem SIPA1 takođe su sniženi i proteini ćelijskog ciklusa. SIPA1 je povećao fosforilisani FAK u ćelijama A172 i tako pojačao maligne fenotipe glioma.
Zaključak: SIPA1 je povećan kod glioma i pojačava migratorne i proliferativne potencijale ćelija glioma aktiviranjem fosforilacije signalnog puta FAK.

Ključne reči: SIPA1, FAK, fosforilacija, gliom

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Introduction

Gliomas are the leading malignant tumors of the central nervous system that originate from glial cells of the neuroectoderm. They represent 80% of primary intracranial malignancies (1). The average survival time of glioma is only 12–14 months (2). Currently, surgery combined radiotherapy, chemotherapy and biotherapy is preferred to glioma patients, although they can only prolong the survival for months. The prognosis of glioma is extremely poor (3). Clarification of the pathogenesis of glioma and the involvement of differentially expressed genes in glioma contributes to the improvement of clinical outcomes (4).

SIPA1 (signal-induced proliferation-associated protein 1) is a protein relevant to tumor invasiveness and metastasis. It is located on human chromosome 11q13.3, containing a zinc finger at the C terminal and a GTPase activator that is highly homologic with Rap1GAP at the N terminal (5, 6). RapGAP protein constitutes Rap1GAP and SIPA1 (7). As a specific RapGAP protein, SIPA1 negatively regulates Rap1 by converting it to the inactivate GDP-bound state, thus translocating signals into nuclei that mediates gene transcription (8). Rap1 is highly homologic with Ras, sharing similar functions in regulating cell-cell connection, secretion, and adhesion (9). In addition, SIPA1 also participates in the mediation of cell clonality, adhesion, and migration (10). This study aims to explore the regulatory effects of SIPA1 on glioma and the dominant signaling pathway.

Materials and Methods

Collection of glioma samples

Glioma samples (n=32) were surgically resected and collected. Glioma cases were pathologically confirmed, and they did not have preoperative glioma treatments. Normal brain tissue samples (n=24) resected during craniocerebral decompression in patients with brain traumas were collected as controls. The Ethic Committee of The Central Hospital of Jamusi City approved this study, and written informed consent was obtained from each patient.

Cell culture

The GBM-derived T98G and A172 cell lines, the grade III astrocytoma-derived U87 cell line and astrocyte cell line NHA (American Type Culture Collection (ATCC) (Manassas, VA, USA)) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in an incubator containing 5% CO_2 at 37 °C. Cell passage was conducted at 80% of confluence.

Cell transfection

Cells seeded in a 6-well plate were grown to 80% of confluence, followed by the transfection of vectors constructed by GenePharma (Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was examined by quantitative real-time polymerase chain reaction (qRT-PCR) at 24 h.

qRT-PCR

Cells were lysed in TRIzol (Invitrogen, Carlsbad, CA, USA) for 5 min, followed by incubation in 200 μL of chloroform. After 12,000×g centrifugation at 4 °C for 5 min, the upper layer was collected and incubated with 500 μL of isopropanol. After 12,000×g centrifugation at 4 °C for 10 min, the precipitant was washed in 1 mL of 75% ethanol and diluted in 20 μL of diethylpyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA concentration was measured using NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Using the PrimeScript™ RT Master Mix, reversely transcribed complementary deoxyribose nucleic acids (cDNAs) were further subjected to qPCR.

SIPA1-Forward: 5′-TGCAAGATGGTGGCAGTCCTC-3′; SIPA1-Reverse: 5′-CTGCCCGCCTCCGACATGATC-3′; GAPDH-Forward: 5′-ACACCATGGGGAAGGTGAAG-3′; GAPDH-Reverse: 5′-GTGACCAGGCGCCCAATA-3′; Cyclin A2-Forward: 5′-CGCTGGCGGTACTGAAGTC-3′; Cyclin A2-Reverse: 5′-GAGGAACGGTGACATGCTCAT-3′; Cyclin D1-Forward: 5′-GCTGCGAAGTGGAAACCATC-3′; Cyclin D1-Reverse: 5′-CCTCCTTCTGCACACATTTGAA-3′; Cyclin E1-Forward: 5′-AAGGAGCGGGACACCATGA-3′; Cyclin E1-Reverse: 5′-ACGGTCACGTTTGCCTTCC-3′.

Transwell

Cell suspension (5×10^4/L) in serum-free medium and medium containing 10% FBS were respectively applied at the top and bottom of a Transwell insert pre-coated with 200 mg/mL Matrigel. After 24-h cell culture, cells migrated from the top to the bottom were fixed in 70% ethanol for 30 min and dyed in 0.2% crystal violet for 10 min, which were then observed and counted.

5-Ethynyl-2′- deoxyuridine (EdU)

Cell suspension (2×10^5/L) was seeded in a 96-well plate and stained with EdU as recommended by the commercial kit (Beyotime, Shanghai, China). EdU-positive cells in 3 random fields per well were captured for calculating using Image J software (NIH, Bethesda, MD, USA).

Flow cytometry

After 5-min centrifugation at 1,000 r/min and phosphate-buffered saline (PBS) washing twice, the precipitant was induced with 5 μL of Annexin V/FITC and 10 μL of Propidium Iodide (PI) in the dark for 15 min. Cell apoptosis was analyzed by detecting FL1 (488 nm wavelength) and FL2 gate (633 nm wavelength). In addition, cell cycle distribution was analyzed using the CellQuestTMD Analysis Software (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot

After 30-min lysis of cells, and 15-min centrifugation at 4 °C, 12,000 rpm, protein samples were prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (30 μg per lane) and transfer on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking non-specific antigens on membranes, they were induced with primary and secondary antibodies under indicated conditions. Protein signals were detected using Luminol substrate solution.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical processing. Data were expressed as $\bar{x} \pm s$, and differences between groups were compared using the independent t-test. A significant difference was set at $P<0.05$.

Results

Upregulation of SIPA1 in glioma

Compared with normal brain tissues, mRNA and protein levels of SIPA1 were remarkably upregulated in glioma (*Figure 1A, 1B*). Meanwhile, SIPA1 was more highly expressed in glioma cell lines than astrocytes (*Figure 1C, 1D*). A172 cells were used for the following experiments since they expressed a relatively high abundance of SIPA1 in the three tested glioma cell lines.

Knockdown of SIPA1 suppressed migratory and proliferative potentials of glioma

SIPA1 level was effectively suppressed by transfection of si-SIPA1 in A172 cells (*Figure 2A*). After the knockdown of SIPA1, the migratory cell number (*Figure 2B*) and EdU-positive ratio (*Figure 2C*) were markedly reduced. In addition, cell apoptosis was stimulated by transfection of si-SIPA1 (*Figure 2D*). Flow cytometry data showed that the knockdown of SIPA1 in A172 cells arrested cell cycle progression in the G1 phase, which was further supported by the downregulation of cell cycle proteins Cyclin A2, Cyclin D1 and Cyclin E1 in glioma cells with SIPA1 knockdown (*Figure 2E, 2F*).

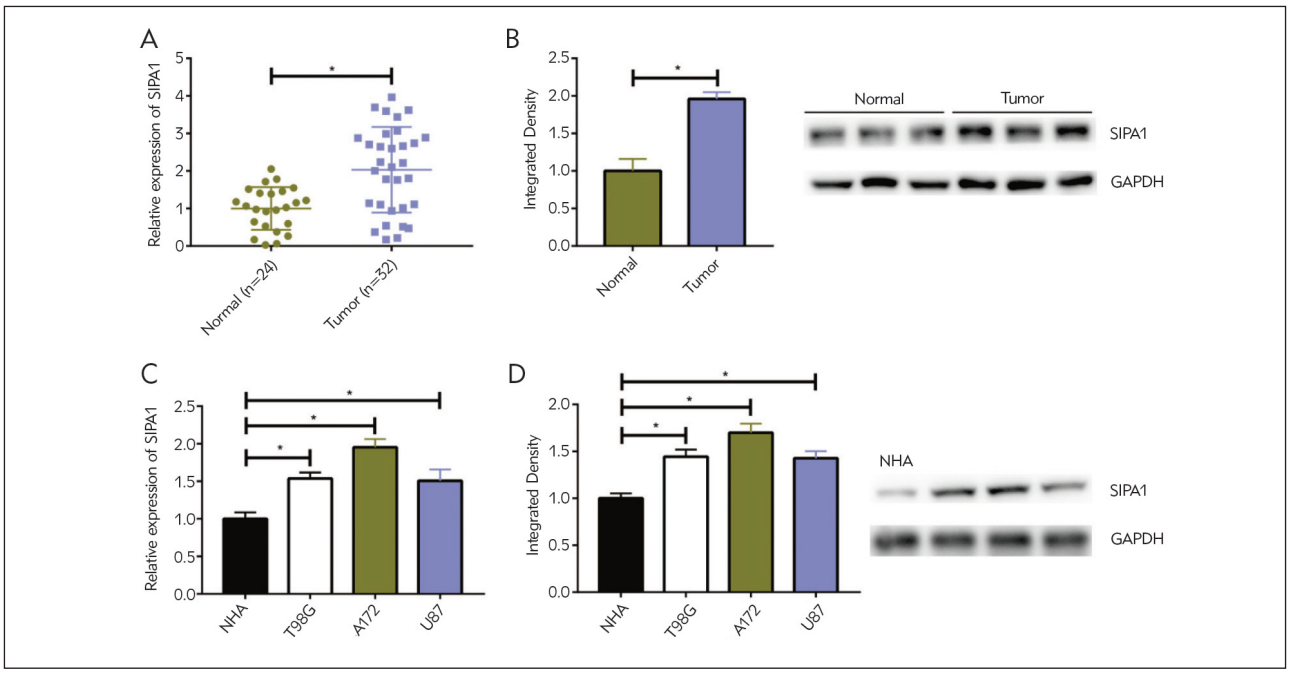

Figure 1 Upregulation of SIPA1 in glioma. (A) The mRNA level of SIPA1 in glioma and normal brain tissues; (B) The protein level of SIPA1 in glioma and normal brain tissues; (C) The mRNA level of SIPA1 in glioma cell lines; (D) The protein level of SIPA1 in glioma cell lines; *$P<0.05$.

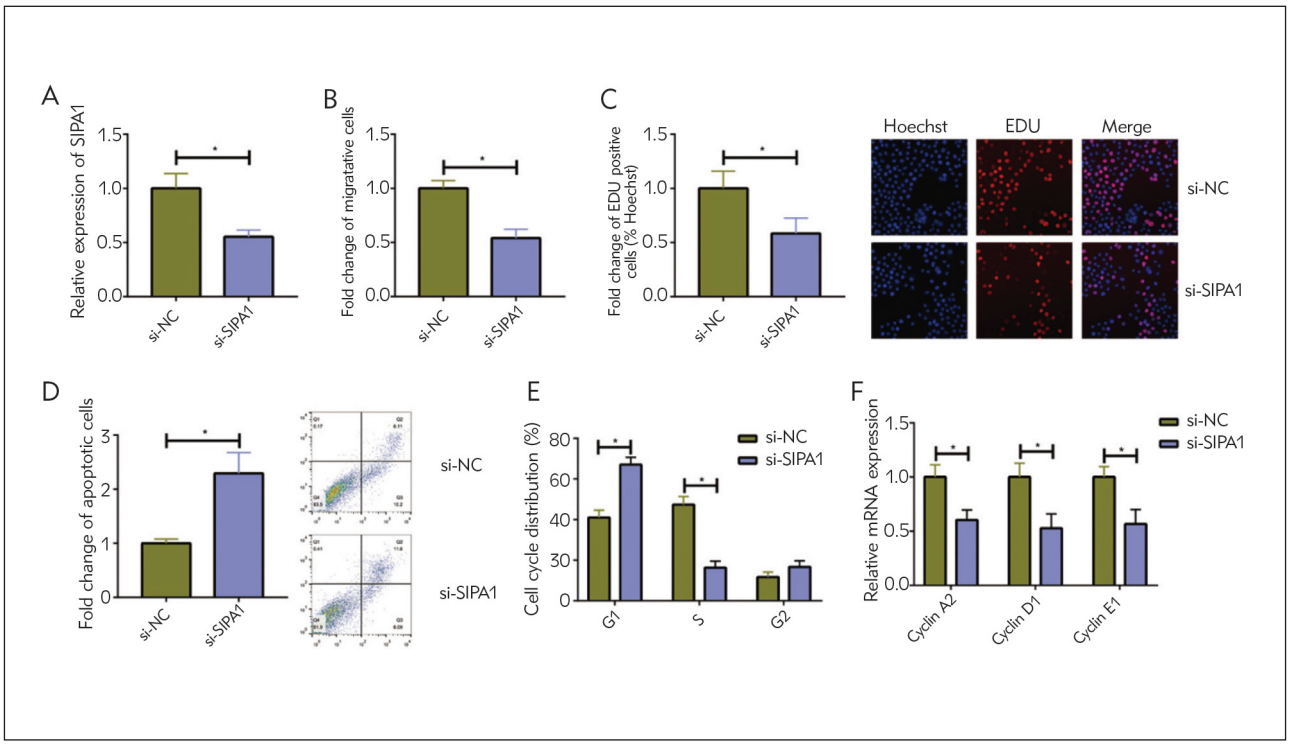

Figure 2 Knockdown of SIPA1 suppressed migratory and proliferative potentials of glioma. (A) Transfection efficacy of si-SIPA1 in A172 cells; (B) Migration in A172 cells with SIPA1 knockdown; (C) EdU-positive ratio in A172 cells with SIPA1 knockdown (magnification = 40×); (D) Apoptosis in A172 cells with SIPA1 knockdown; (E) Cell cycle distribution in A172 cells with SIPA1 knockdown; (F) Relative levels of Cyclin A2, Cyclin D1 and Cyclin E1 in A172 cells with SIPA1 knockdown; *P<0.05.

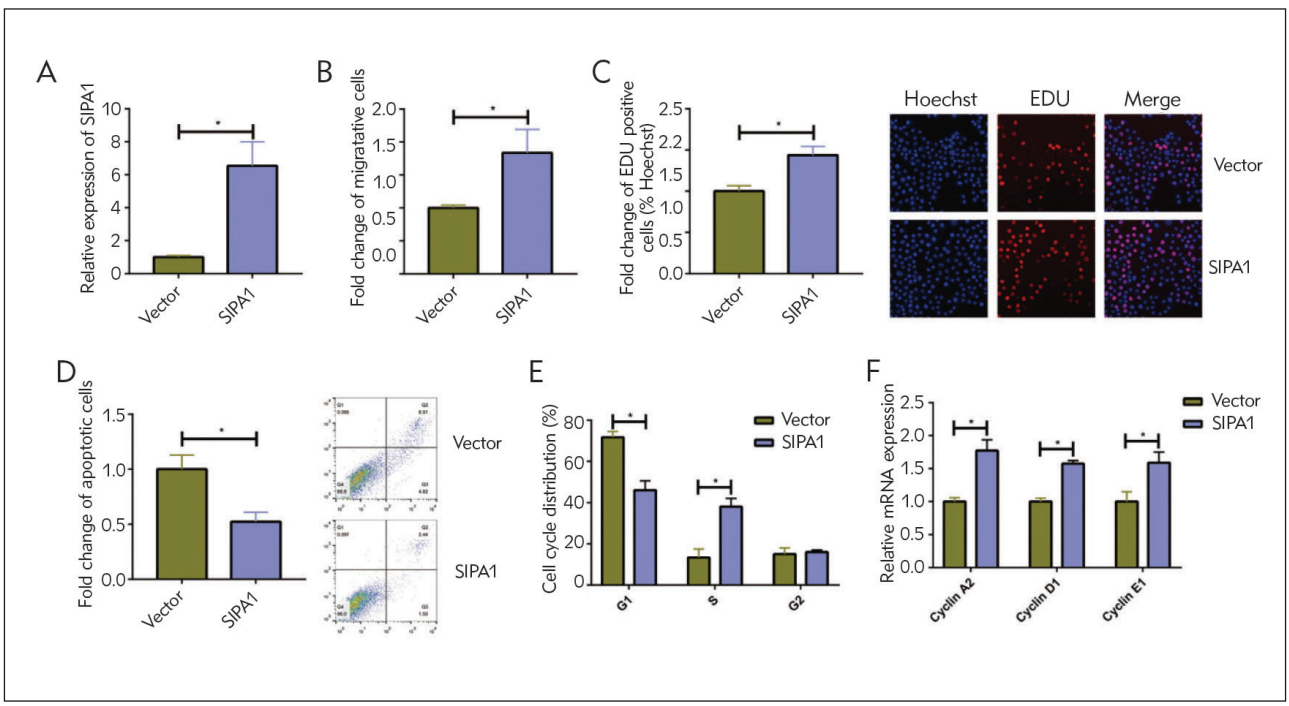

Figure 3 Overexpression of SIPA1 suppressed migratory and proliferative potentials of glioma. (A) Transfection efficacy of SIPA1 overexpression vector in A172 cells; (B) Migration in A172 cells with SIPA1 overexpression; (C) EdU-positive ratio in A172 cells with SIPA1 overexpression (magnification = 40×); (D) Apoptosis in A172 cells with SIPA1 overexpression; (E) Cell cycle distribution in A172 cells with SIPA1 overexpression; (F) Relative levels of Cyclin A2, Cyclin D1 and Cyclin E1 in A172 cells with SIPA1 overexpression; *P<0.05.

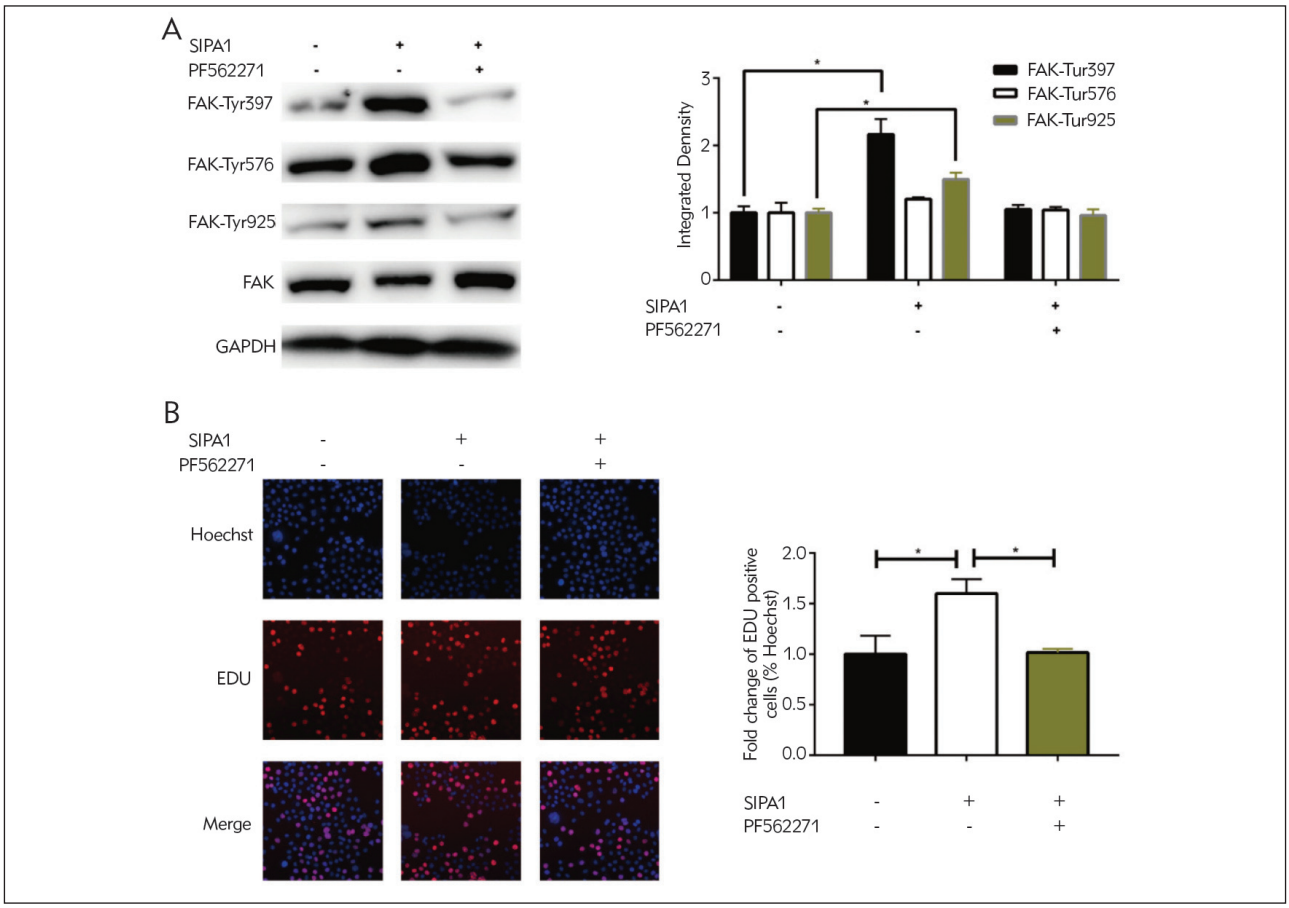

Figure 4 Overexpression of SIPA1 activated phosphorylation of FAK. A172 cells were transfected with negative control or SIPA1 overexpression vector, followed by either treatment of PF-562271 or not; (A) Protein levels of Phospho-FAK (Try397), Phospho-FAK (Try576), Phospho-FAK (Try925) and FAK; (B) EdU-positive ratio in A172 cells (magnification = 40×); *P<0.05.

Overexpression of SIPA1 boosted migratory and proliferative potentials of glioma

We analyzed phenotype changes of A172 cells overexpressing SIPA1 further (*Figure 3A*). Overexpression of SIPA1 markedly enhanced migratory and proliferative potentials of glioma cells (*Figure 3B*, *3C*). In addition, the apoptosis rate was reduced in A172 cells overexpressing SIPA1 (*Figure 3D*). Besides, overexpression of SIPA1 remarkably prolonged the S phase and upregulated Cyclin A2, Cyclin D1 and Cyclin E1 (*Figure 3E*, *3F*).

Overexpression of SIPA1 activated phosphorylation of FAK

Interestingly, overexpression of SIPA1 in A172 cells upregulated Phospho-FAK (Try397), Phospho-FAK (Try576) and Phospho-FAK (Try925), which were reversed by treatment of the FAK inhibitor PF-562271 (*Figure 4A*). To further explore the involvement of the phosphorylated FAK in SIPA1-induced glioma progression, proliferative ability in glioma cells overexpressing SIPA1 intervened by PF-562271 was examined. As expected, the intervention of PF-562271 reduced the EdU-positive rate, indicating that the phosphorylation of FAK did participate in glioma progression boosted by SIPA1 (*Figure 4B*).

Discussion

Glioma is a complicated malignant tumor. Its pathogenesis remains largely unclear, and brain traumas, nitrite food, occupational hazard and radiation exposure may be potential risk factors of glioma. Besides, immune factors are closely associated with the development of glioma, involving Treg, $CD3^+T$, $CD4^+T$ and $CD8^+T$ cells (11). Therefore, differentially expressed genes in gliomas have been well concerned. They can be utilized as specific biomarkers for guiding the screening, diagnosis and treatment, and predicting the prognosis of glioma (12). The development of targeted therapy based on these biomarkers is a promising approach to improving glioma patients' poor prognosis (13, 14).

The cancer-associated role of SIPA1 differs in human malignant tumors. Hunter et al. (15) first

identified that SIPA1 is located on the metastasis efficiency modifier locus mtes1. SIPA1 SNP remarkably influences the function of Rap GTPase. They demonstrated that intervention of SIPA1 in nude mice markedly alters the metastatic ability of cancer cells. Minato et al. (16) suggested that SIPA1 prevents cell adhesion to fibronectin by inhibiting Rap1GTP in Hela cells. Through mediating the interaction between SIPA1 and Rap1GTP, AF-6 inhibits integrin-induced cell adhesion (17). The diasporin pathway can regulate transcription of extracellular matrix (ECM) genes, pTEN and Trp53, which is a tumor progression-associated pathway regulated by SIPA1 (18, 19). In hematological malignancies, SIPA1 acts as a tumor-suppressor gene. SIPA1 knockout mice showed T cell non-responsiveness before bone marrow dysfunction, which eventually leads to the development of delayed myeloid leukemia (20). SIPA1 is distributed in nuclei of breast cancer cell line MDA-MB-231. By promoting the expression of integrin 1 by acting on its promoter, SIPA1 further activates the phosphorylation of FAK, and thus regulates invasiveness and morphology of breast cancer cells through the MMP9 signaling and F-actin, respectively (21). *In vivo* overexpression of SIPA1 enhances tumorigenesis of prostate cancer in SCID mice by inhibiting the binding between collagen and fibronectin, thus inactivating ECM-induced activation of Rap1. Meanwhile, overexpression of SIPA1 downregulates Brd4, which further attenuates the binding between prostate cancer cells and ECM (22). The regulatory effect of SIPA1 on the migratory capacity of colorectal carcinoma (CRC) is quite the opposite of that in breast cancer and prostate cancer. A clinical trial involving 94 CRC patients revealed that the relative level of SIPA1 is negatively correlated to metastatic lymphatic rate. Knockdown of SIPA1 markedly triggers the migratory ability of CRC cells (23). In the present study, SIPA1 was highly expressed in glioma cases, which boosted migratory and proliferative capacities of glioma cells and inhibited cell apoptosis.

FAK is overactivated or over-phosphorylated in multiple types of cancer cells, leading to malignant migration, proliferation, adhesion and EMT (24). In addition to the kinase function, FAK also serves as a scaffold for protein complexes that regulates cancer development (25). Here, overexpression of SIPA1 upregulated Phospho-FAK (Try397), Phospho-FAK (Try576) and Phospho-FAK (Try925), which were reversed by treatment of the FAK inhibitor PF-562271. The treatment of PF-56271 abolished the boosted proliferative ability of glioma by overexpression of SIPA1. It is indicated that the phosphorylation of FAK was involved in the glioma progression boosted by SIPA1.

There were limitations in the present study. First of all, the sample size of glioma and normal brain tissues was limited. Therefore, the clinical significance of SIPA1 in glioma needs to be further validated in more samples. Secondly, *in vivo* experiments are lneeded to verify the tumorigenic role of SIPA1 in glioma.

Conclusion

SIPA1 is upregulated in glioma, which boosts malignant progression of glioma by activating the phosphorylation of the FAK signaling pathway.

Financial Disclosure

The authors declared that this study had received no financial support.

Conflict of interest statement

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

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Received: August 07, 2021
Accepted: August 26, 2021

SCINDEKS
Serbian Citation Index
J Med Biochem 2022; 41 (1) DOI: 10.5937/jomb0-31941

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 115–121, 2022

Original paper
Originalni naučni rad

ESTABLISHING REFERENCE INTERVALS FOR VON WILLEBRAND FACTOR MULTIMERS

USPOSTAVLJANJE REFERENTNIH INTERVALA ZA MULTIMERE FAKTORA VON WILLEBRAND

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Summary

Background: von Willebrand factor (VWF) multimers (VWF:MM) methodologies are technically difficult, laborious, time consuming, non-standardized and results vary between laboratories. A new semi automated VWF:MM assay is available for routine use (Sebia). Due to lack of reference values for VWF:MM fractions, results interpretation can be challenging in some cases. The aim of this study was to determine reference intervals for low molecular weight (LMWM), intermediate molecular weight (IMWM) and high molecular weight (HMWM) multimers.
Methods: By the international cooperation initiated between 4 countries (Estonia, Latvia, France, and USA) 131 samples of relatively healthy individuals were analyzed for VWF:MM (in total 51 males and 80 non-pregnant females aged 17–69 years). Reference intervals were calculated according to CLSI C28-A3 standard.
Results: The proposed reference intervals for VWF:MM were calculated for LMWM 10.4–22.5%, IMWM 22.6–37.6%, HMWM 45.6–66.6%. Age related differences were

Kratak sadržaj

Uvod: Metodologija multimera (VWF:MM) von Willebrand faktora (VWF) tehnički je teška, naporna, digotrajna, nestandardizovana i rezultati se razlikuju u različitim laboratorijama. Novi poluautomatski VWF:MM test (Sebia) dostupan je za rutinsku upotrebu. Zbog nedostatka referentnih vrednosti za VWF:MM frakcije, tumačenje rezultata može u nekim slučajevima biti izazovno. Cilj ove studije bio je da se odrede referentni intervali za multimere niske molekularne mase (LMWM), srednje molekularne mase (IMWM) i visoke molekularne težine (HMWM).
Metode: Međunarodnom saradnjom započetom između 4 zemlje (Estonija, Letonija, Francuska i SAD) 131 uzorak relativno zdravih pojedinaca analiziran je na VWF:MM (ukupno 51 muškarac i 80 žena koje nisu bile trudne u dobi od 17–69 godina). Referentni intervali su izračunati prema CLSI C28-A3 standardu.
Rezultati: Predloženi referentni intervali za VWF:MM izračunati su za LMWM 10,4–22,5%, IMWM 22,6–37,6% i HMWM 45,6–66,6%. Starosne razlike su primećene u

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List of abbreviations: VWF, von Willebrand factor; VWF:MM, von Willebrand factor multimers; LMWM, low molecular weight multimers; IMWM, intermediate molecular weight; HMWM, high molecular weight multimers; VWD, von Willebrand disease

seen in IMWM and HMWM ($p<0.001$ and 0.038). There was no gender related difference observed. Geographically LMWM results of France were different from the other regions ($p<0.05$).

Conclusions: Quantification of VWF:MM fractions, in addition to qualitative assessment of VWF:MM patterns, has the potential to aid in differential diagnosis of von Willebrand disease (VWD) subtypes. The reference values calculated in this study can be used in future research to establish clinical decision limits.

Keywords: von Willebrand factor, von Willebrand factor multimers, quantitative analysis, reference intervals

IMWM i HMWM ($p < 0,001$ i 0,038). Nije uočena razlika vezana za pol. Geografski rezultati LMWM iz Francuske bili su različiti od ostalih regiona ($p < 0,05$).

Zaključak: Kvantifikovanje frakcija VWF:MM, pored kvalitativne procene VWF:MM uzoraka, može da pomogne i u diferencijalnoj dijagnozi podtipova von Willebrandove (VWD) bolesti. Referentne vrednosti izračunate u ovoj studiji mogu se koristiti u budućim istraživanjima za utvrđivanje granica kliničkih odluka.

Ključne reči: von Willeberandov faktor, multimeri von Willebrandovog faktora, kvantitativna analiza, referentni intervali

Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder with an approximate prevalence of about 1–2 % in the general population (1–3), although the true incidence is unknown (4). VWF plays an important role in regulation of normal hemostasis and facilitates progression of bleeding or thrombotic disorders with platelet and endothelial dysfunction (5–6). VWD arises due to structural and/or quantitative abnormalities of von Willebrand factor (VWF), a large multimeric glycoprotein with adhesive functions through binding to FVIII, to platelet surface glycoproteins, and to constituents of subendothelial connective tissue (5–7).

VWD is classified into 3 main types: type 1, a partial quantitative deficiency; type 2, a qualitative defect that is further subdivided into 4 categories, 2A, 2B, 2N, and 2M; and type 3, a complete absence of VWF (1). Correct classification of the type/subtype of the VWD is important in patients' management and the therapeutic approach (1).

As VWF has diverse functions, laboratory testing for VWD and other VWF-related disorders (i.e., thrombotic thrombocytopenic purpura (8) or a variety of cardiac lesions that result in clearance of larger multimers, such as aortic regurgitation, mitral insufficiency, and hypertrophic cardiomyopathy (9)) require complex laboratory assessment (3, 10). The first-line tests typically include evaluation of VWF antigen (VWF:Ag), different VWF activity (VWF:Ac) assays (e.g. ristocetin cofactor assay (VWF:RCo), VWF activity measured as VWF binding to the glycoprotein Ib (GPIb) receptor on the platelet surface (VWF:GPIbM), collagen binding (VWF:CB) etc.) and factor VIII activity (FVIII:C) (4).

VWF multimeric assay is a second-line analysis used in the diagnosis and classification of different VWD subtypes (11). VWF circulates in plasma as low, intermediate, and high molecular weight (LMWM, IMWM, and HMWM, respectively) multimers (12, 13). The absence of HMWM is the cardinal feature that distinguishes type 1 from type 2A and 2B VWD, whereas the different subtypes of type 2 VWD can be differentiated by more subtle alterations of the inner structure of smaller multimers (4, 10, 11).

Historically, VWF multimers are analyzed by in-house developed electrophoresis techniques and densitometric analysis of Western blots (7, 14). These methodologies are technically difficult, laborious, time consuming and non-standardized (2, 12). The development of a relatively rapid semi-automated commercial VWF multimer kit assay (Hydragel 5/Hydragel 11 von Willebrand multimers, Sebia, France) may represent a first step toward standardization. This method was already shown to provide adequate information for characterization and classification of congenital VWD subtypes (12, 14, 15). Moreover, results correlate with the clinical status, diagnosis of inherited or acquired VWD, if used and interpreted by experienced professionals (12, 14).

In addition to qualitative interpretation of multimer patterns, the Sebia PHORESIS software allows quantification of VWF:MM band patterns, and calculation of the percentage values of each molecular weight multimer fraction. Quantitative multimer analysis might be needed for the detection of subtle abnormalities and changes following therapeutic interventions (7, 16). Due to lack of reference values for VWF:MM fractions, result interpretation can be challenging in some cases.

Thus, in the present study we used densitometry to determine reference intervals for LMWM, IMWM and HMWM fractions.

Materials and Methods

Study subjects

To collect a larger sample size an international cooperation was initiated between 4 countries (Estonia, Latvia, France, and USA). The list of participating institutions were as follows: L1 (two institutions from Baltic countries: L1A – Laboratory of North Estonia Medical Centre, Tallinn Estonia; L1B – Riga East University Hospital, Riga, Latvia), L2 (Department of Biology, Foch Hospital, Suresnes, France), and L3 (University of Utah / ARUP Laboratories, Salt

Lake City, Utah, United States). Both Estonian and Latvian samples were analyzed in the Laboratory of North Estonia Medical Centre, thus accounted as one group L1.

In total 134 healthy volunteers were recruited for this study, but after outlier exclusion 131 samples were analyzed: 51 males and 80 non-pregnant females aged 17–69 years.

Acceptance criteria: no history of hemorrhagic episodes; no usage of any interfering medication for at least 10 days before blood collection; normal VWF results (VWF:Ag; VWF:Ac – VWF:GPIbM (L1), VWF:GPIbR (L2) and VWF:RCo (L3); VWF:Ac/VWF:Ag ratio); written consent provided. Blood donor plasmas were not used because the questionnaire for blood donors do not include information regarding family bleeding history, individual mild bleeding episodes and are not screened for VWD routinely. The study was performed according to the Declaration of Helsinki and was approved by appropriate local or national ethical committees or local Institutional Review Board at each institution.

Sample collection and specimen processing

Samples for the reference interval studies were collected from apparently healthy individuals according to the participating institutions' locally approved venous blood sampling procedures and in concordance with ethical laws of each participating country. Briefly, peripheral venous blood specimens were collected into light blue-top vacuum tubes [3.2% sodium citrate tubes (BD Vacutainer, L1A, L3 or Sarstedt, L2) or 3.8% NC Buffered Citrate (Vacutest KIMA srl, L1B)], centrifuged (within 2 hours after sampling) at a speed and time required to consistently produce platelet-poor plasma (residual platelet count less than 10 $x10^9$L):

L1A – 1500 g for 15 minutes at room temperature

L2B – 1500 g for 15 minutes at room temperature, aliquoted, stored frozen at -70 °C and transported on dry ice to L1A

L2 – 2000 g for 15 minutes at 15 °C (twice)

L3 – 1700 g for 15 minutes at room temperature

Samples were aliquoted and stored frozen (at least –20 °C) until testing (within 30 days). Aliquots were thawed in a water bath (+37 °C) for 5 minutes and mixed well before testing.

VWF multimers method and densitometry

The VWF multimers method, developed by Sebia (France), is described in detail elsewhere (3, 4). It was used by the participating laboratories without deviation from the original Sebia assay protocol. In brief, citrated plasma samples were analyzed on the Hydrasys 2 instrument (Sebia, France) with ready to use SDS agarose gels (Hydragel 5 von Willebrand multimers, Sebia). Densitometry of VWF multimer patterns was carried out with a transmission scanner (Sebia Gelscan Instrument) which allows scanning and data storage of the results. Data acquisition is performed by a bidimensional calibrated CCD sensor. The instrument, when connected to a PC with the Sebia PHORESIS software, allowed the operator to display the gel images, curves, curves overlapping, and quantification of multimer band patterns according to the manufacturer recommendation (LMWM 1-3 bands; IMWM 4–7 bands; and HMWM 8^{th} band and above).

The percentage values of each molecular weight multimer fraction was provided by the software. The calculation was made by applying the ratio of the area of each fraction and the total area under the curve. The multimer patterns of the plasma samples studied were, if necessary, compared with the reference pool pattern analyzed on the same gel. The total area under the curve of each sample was directly proportional to the amount of antigen (VWF:Ag).

Statistical analysis

All statistical analysis was performed with MedCalc® software (MedCalc Software, Belgium) version 18.11.6. and IBM SPSS statistics version 23. Descriptive statistics was used to analyze demographic data and laboratory characteristics. The data was analyzed according to age, gender and geographic location. The results were expressed as median (interquartile range [IQR]). The difference between variables was tested using the Mann-Whitney test. P values of <0.05 were considered statistically significant.

Reference intervals were established using a robust method following CLSI C28-A3 standard to calculate the 2.5^{th} and 97.5^{th} percentiles and associated 90% confidence intervals (CI) for each VWF multimeric fraction. Data distributions were tested for normality by Shapiro-Wilk test. Outlier detection was performed by Grubs double sided and Tukey methods.

Results

Study subjects

Data and samples were collected from 131 healthy volunteers (51 males and 80 non-pregnant females), from Baltic Region (L1), France (L2) and United States (L3). The demographic characteristics and laboratory findings are summarized in *Table I*.

Table I Characteristics of study groups and corresponding results of VWF:MM fractions.

	L1 (n=31)	L2 (n=64)	L3 (n=36)
Age range (years)	18–69	17-62	19–61
Age, median (IQR)	34 (23–46)	40.5 (30.3–51.8)	30 (24.3–36.0)
males/females	7/24	27/37	17/19
LMWM, % median (IQR)	15 (12.7–17.2)	16.1 (14.5–19.1)	14 (12.4–16.0)
LMWM lowest / highest value	9.8–23.0	10.7–23.3	9.7–19.9
IMWM, % median (IQR)	29.2 (26.7–31.2)	29 (27.2–30.6)	30.7 (26.3–34.2)
IMWM lowest / highest value	22.8–36.4	21.4–35.8	21.3–38.6
HMWM, % median (IQR)	55.4 (51.1–60.2)	54.5 (52.2–58.1)	55.9 (51.3–59.6)
HMWM lowest / highest value	43.2–66.2	45.1–65.2	44.4–68.2

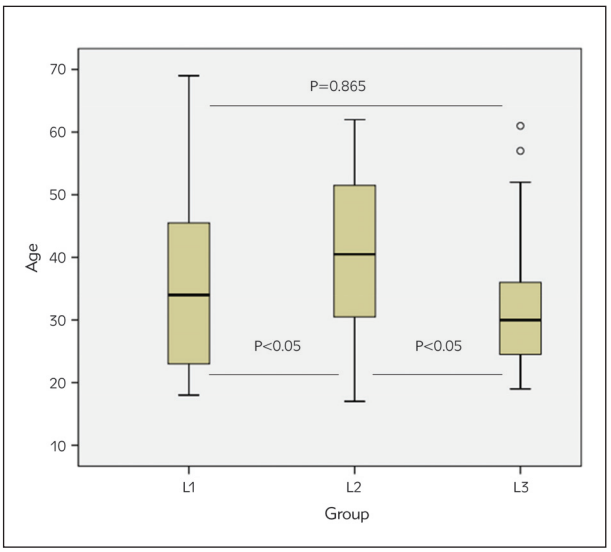

Figure 1 Age differences between subjects of different geographical locations. L1, samples from Baltic region; L2, samples from France; L3, samples from United States.

Participants' age was between 17 and 69 years. Subjects from L3 were younger than from L1 and L2: medians (IQR) were 30 (24.3–36.0), 34 (23–46) and 40.5 (30.3–51.8), respectively. As presented in *Figure 1*, there was no significant difference in age between L1 and L3 (P=0.865), but the differences between L2 vs L1 and L2 vs L3 were statistically significant (p<0.05).

To assess possible differences in VWF multimers fractions data from the 3 participating regions was compared.

Age related difference in VWF multimers fractions

VWF multimers patterns were analyzed for age-related differences and are shown in *Figure 2*. Visually LMWM tend to increase with increasing age, although changes are not statistically significant. IMWM variations were found to be statistically significant (P<0.001), but values fluctuate with two intervals with increasing values, and one shift of decreasing values. HMWM tend to decrease with increasing age, and this finding is statistically significant (P=0.038).

Gender related difference in VWF multimers fractions

As shown in *Figure 3A*, there was no significant difference between males and females in VWF multimers structure: LMWM (P=0.067), IMWM (P=0.507), HMWM (P=0.060).

Geographical locations related difference in VWF multimers fractions

Table I and *Figure 3B* summarize the results of the VWF structure related parameters.

The LMWM were higher in group L2 (16.1 [14.5–19.1]) than in group L1 (15 [12.7–7.2]) and group L3 (14 [12.4–16.0]). The differences between L2 vs L1 and L2 vs L3 were statistically significant (p<0.05) but clinically irrelevant, difference between L1 vs L3 was insignificant (P=0.260). There was no significant difference in IMWM and HMWM between geographical locations.

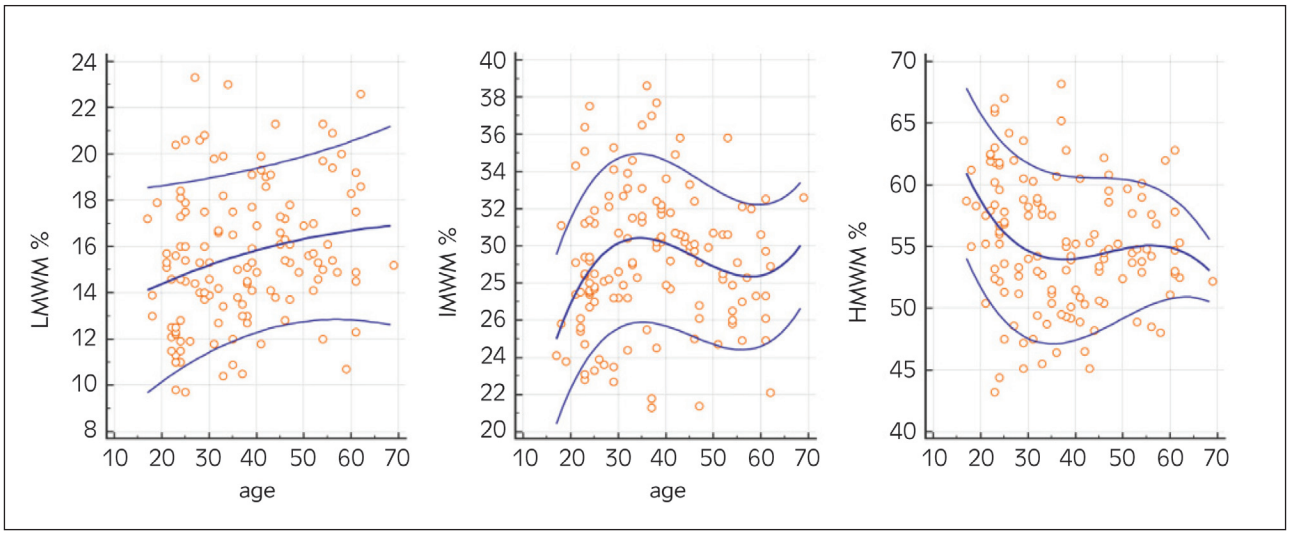

Figure 2 Age-related differences of LMWM, IMWM and HMWM in study population. LMWM, low-molecular-weight multimers; IMWM, intermediate-molecular-weight multimers HMWM, high-molecular-weight multimers. Blue lines represent 0.1, 0.5 and 0.9 centiles.

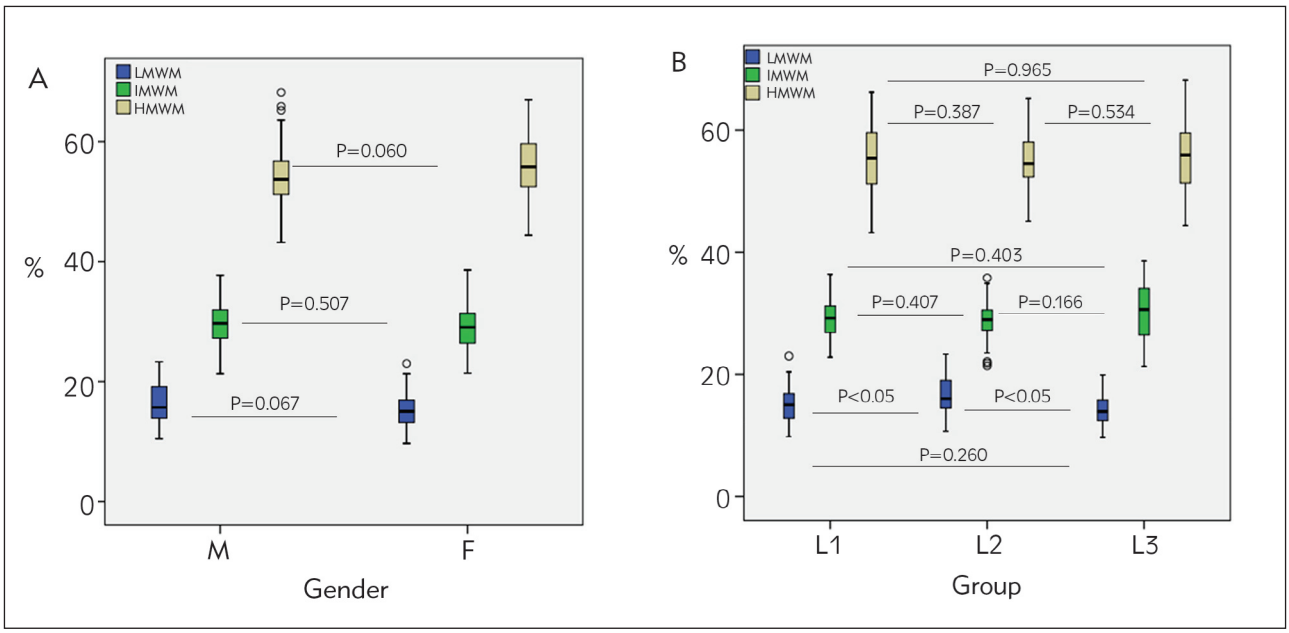

Figure 3 Differences of LMWM, IMWM and HMWM percentage values by gender (A) and between geographical locations (B). L1, samples from Baltic region; L2, samples from France; L3, samples from United States; LMWM, low-molecular-weight multimers; IMWM, intermediate-molecular-weight multimers HMWM, high-molecular-weight multimers.

Calculation of reference intervals

Values of the three testing locations for the LMWM, IMWM and HMWM were distributed normally, thus reference values were calculated based on a normal distribution.

The proposed reference intervals for VWF:MM are presented in *Table II*.

Table II Proposed reference intervals for VWF:MM.

	Low Molecular Weight	Intermediate Molecular Weight	High Molecular Weight
Lower limit, % [90% CI]	10.4 [9.9–11.0]	22.6 [21.8–23.3]	45.6 [44.5–46.7]
Upper limit, % [90% CI]	22.5 [21.5–23.5]	37.6 [36.4–38.7]	66.6 [65.1–68.0]

Discussion

VWF multimeric analysis is essential for diagnosis and subtyping of VWD and acquired von Willebrand syndrome (AVWS) (5, 9, 17–19). There is still a need for interlaboratory standardization of this method. Indeed, interlaboratory comparability and reproducibility of this analysis are insufficient due to the predominant use of locally developed VWF multimer methods by laboratories worldwide (22, 25). The new semi-automated VWF multimer technique can help in standardization (26): it helps to reduce the interlaboratory variability and the variability between different measurement runs. Densitometry could contribute to its standardization by offering a reproducible quantification and additional visualization of VWF multimer patterns and permitting a precise quantitative comparison of sample patterns with those of a reference plasma curve (20).

Several independent investigators have previously reported on the analytical performance evaluation of the new Sebia technique with either 5-gel and 11-gel formats (3, 12, 14, 15, 18, 20, 21, 23). Details of analytical performance of the Sebia method are beyond the scope of our current study. In brief, this new assay provides a clear pattern of VWF multimer distribution on the gels and densitometry scans. It demonstrates acceptable performance results and has the major advantage of being performed within one working day.

In published data for evaluation of the accuracy of the new Sebia assay researchers have used different approaches. They have compared plasma samples from patients presenting with different types of VWD with samples from healthy volunteers (21), commercial Standard Human Plasma (23), donors and commercial frozen normal donor plasmas (14). Reference intervals were not originally defined by the manufacturer. Due to lack of reference values for VWF:MM fractions, results interpretation can be challenging in some cases. HMWM have the greatest role in VWF functional activity (13), therefore reference intervals for HMWM are most important in clinical decision making.

In 2018, Bowyer et al. (14) investigated multimeric patterns in 51 samples collected from healthy volunteers and using commercial frozen normal donor plasma (Cryocheck; Precision Biologic, Halifax, NS, Canada). In this study ranges for HMWM varied 35–58.5%, but authors noted that Gaussian distribution was not observed for HMWM. Importantly, the storage condition for the commercial Cryocheck Normal Donor Set is at -40 to -80 °C. Storage and transport issues that allowed plasmas to reach temperatures outside of this range potentially could have affected the establishment of HMWM lower intervals using this donor set.

A group of researchers from Belgium (21) has calculated normal reference intervals for VWF multimers fractions using samples from 40 healthy volunteers. They have reported intervals for HMWM as 40.8–63.2%.

The intervals determined in these previous studies were similar to our results, but they were calculated using a relatively low powered sample size. According to the CLSI guidelines C28–A3 (24), the sample size can be considered to be representative when it is larger than 120, therefore in the current study we established the reference intervals of LMWM, IMWM and HMWM fractions in 131 relatively healthy adults, in order to obtain a more acccurate result.

An interesting finding was the relationship of certain multimer fractions with the age of study individuals. The tendency of LMWM to increase and HMWM to decrease with increased age is seen in our data. Meanwhile, IMWM values are variable during adult life. Nevertheless, definitive conclusions cannot be made due to the small sample size of the study. Discovered tendencies, especially the tendency of HMWM to decrease with increasing age, could potentially be analyzed in detail in future larger studies.

It should be noted that multimer fraction separation and their percentage values calculation is based on the scanned gel and are not directly measured quantitatively, thus an interpretation of »gray zone« should be considered in future studies evaluating clinical decision making possibilities.

To conclude, the quantification of VWF:MM fractions is an additional valuable tool to supplement the qualitative visual assessment of VWF:MM patterns. It potentially has the value to aid in differential diagnosis of VWD and AVWS subtypes. The reference values calculated in this study can be used in future research to establish clinical decision limits.

Acknowledgments. We are grateful to Sebia (France) for the funding of this project. We would like to thank all the medical/laboratory staff and the volunteers who contributed to this study. The preliminary results of this study were discussed at IFCC C-RIDL (Scientific Committee of IFCC on Reference Intervals and Decision Limits) closed meeting during Euromedlab2019 Barcelona congress and presented at the ISTH 2020 Virtual Congress (abstract/e-poster).

Conflict of interest statement

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

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Received: April 23, 2021
Accepted: July 30, 2021

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 122–128, 2022

News from the Society of Medical Biochemists of Serbia

NEWS FROM THE SOCIETY OF MEDICAL BIOCHEMISTS OF SERBIA SCIENTIFIC FOUNDATION »PROFESSOR IVAN BERKEŠ«

Prepared by Professor Dr Nada Majkić-Singh,
Society of Medical Biochemists of Serbia Director

Appreciating the work of their teacher and a renowned expert Professor Dr Ivan Berkeš, the Society of Medical Biochemists of Yugoslavia (now the Society of Medical Biochemists of Serbia) and the Faculty of Pharmacy, University of Belgrade, created the **Scientific Foundation »Professor Ivan Berkeš«** in 1997 and established the **»Annual Scientific Conference«** dedicated to the life and work of Professor Ivan Berkeš. The scientific foundation was established on the proposal of the Professor Dr Nada Majkić-Singh, also a long-term organizer of the Annual Scientific Conferences. Beginning in 1998, Scientific Conferences have been presenting masters and doctoral theses of members of the Society and related disciplines during the previous year.

On November 16th, 2010, the Thirteenth Annual Scientific Conference dedicated to the 100th anniversary of the birth of Professor Ivan Berkeš was held in Belgrade, during which experts from Belgrade (N. Majkić-Singh), Zagreb (S. Margetić) and Skopje (S. Efremova-Aaron), the cities where Professor Ivan Berkeš lived and worked, gave their lectures. Also, diplomas and money awards were traditionally presented by the Scientific Fund »Dr Ivan Berkeš« to the best students of the Faculty of Pharmacy in Belgrade: Zora Ćetković and Jelena Joksić.

The purpose of the **Scientific Foundation** is to award monetary awards and diplomas to the best graduates of the Faculty of Pharmacy in the previous year, of both study profiles (graduate pharmacist-medical biochemist and graduate pharmacist; now: master of pharmacy-medical biochemist and master of pharmacy), who were the quickest to graduate and with the highest average grade. So far, twenty-two Scientific Annual Conferences have been held, with fifty laureates of the Fund who were awarded diplomas and monetary awards, with their biographies presented in the Monograph.

In 2020, the Society of Medical Biochemists of Serbia marked the 110th anniversary of the birth of Professor Ivan Berkeš, on which occasion the Monograph-LIFE AND WORK OF PROFESSOR IVAN BERKEŠ and FOUNDATION LAUREATES were prepared: WHERE ARE THEY AND WHAT ARE THEY DOING TODAY?

Laureats of the Scientific Foundation Professor Ivan Berkeš in 2021

Awards from the Foundation »Professor Ivan Berkeš« in 2021 were awarded to the best students of Faculty of Pharmacy, University of Belgrade graduated in the school year 2020/2021. This year recipients were:

SUZANA SAVIĆ

(Master of Pharmacy-Medical Biochemists)

and

ANDREA ATANASOV

(Master of Pharmacy)

The awards delivered Professor Nada Majkić-Singh and Professor Nataša Bogavac-Stanojević on behalf of Society of Medical Biochemists of Serbia and Department of Medical Biochemistry, Faculty of Pharmacy, Univeristy of Beograde during III KONGRES TIMA MEDICINSKIH BIOHEMIČARA (TMB-BPSA) on November 7th at the Pharmaceutical Faculty, University of Belgrade.

On this occasion, the Society of Medical Biochemists congratulates them once again on their achievements in the field of medical biochemistry and pharmacy.

Suzana Savić je rođena 13.03.1997. godine u Loznici. Osnovnu školu završila je u Malom Zvorniku kao đak generacije. U istom mestu, 2016. godine završila je gimnaziju – opšti smer. Upisala je Farmaceutski fakultet u Beogradu, smer farmaceut – medicinski biohemičar 2016. godine, a diplomirala u junu 2021. godine. Dobitnica je nagrade Profesor dr Ivan Berkeš kao najbolji student diplomirani farmaceut – medicinski biohemičar u školskoj 2020/2021 godini. Tokom studija nagrađena je tri puta za redom godišnjom nagradom Farmaceutskog fakulteta za najbolje studente. Suzana Savić se za vreme studija bavila naučno-istraživačkim radom na Katedri za Medicinsku biohemiju Farmaceutskog fakulteta Univerziteta u Beogradu pod mentorstvom Prof. dr Aleksandre Zeljković. Učestvovala je na XIII Mini – kongresu studenata Farmaceutskog fakulteta u Beogradu 2021. godine na kom je prezentovala naučno-istraživački rad na temu »Uticaj primene monakolina K na efikasnost sinteze i apsorpcije holesterola«, koji je proglašen za najbolji rad iz oblasti medicinske biohemije.

Suzana Savić, born on 13.03.1997. in Loznica. She went to the elementary school in her hometown Mali Zvornik and finished it as a student of the generation. In the same town, in 2016, she finished high school. Same year she started her studies in the Faculty of Pharmacy in Belgrade (Master of pharmacy – medical biochemist), and graduated in June of 2021. She received »Professor Dr Ivan Berkeš« award as the best student of the class 2020/2021. During her studies, she got annual award of the Faculty of Pharmacy for the best students, three times in a row. Also Suzana Savić, during her studies, was engaged in student scientific research at Department of Medical Biochemistry, Faculty of Pharmacy, University of Belgrade under the mentorship of Prof. Dr. Aleksandra Zeljković. She participated in XIII Mini-Congress of Students at the Faculty of Pharmacy in Belgrade in 2021, where she presented a student scientific research paper on the topic »The effect of monacolin K administration on efficiency of the cholesterol synthesis and absorption«, which was named the best work in the field of medical biochemistry.

Andrea Atanasov rođena je 15.12.1997. godine u Beogradu. Osnovnu školu i Devetu gimnaziju »Mihailo Petrović Alas«, prirodno-matematički smer, završila je u Beogradu. Farmaceutski fakultet Univerziteta u Beogradu upisala je 2016. godine, a diplomirala u septembru 2021. godine sa prosečnom ocenom 9,75. Diplomski rad pod nazivom »Fenotip CYP2C19 homozigotnih i hemizigotnih transgenih miševa u osamnaestom danu embrionalnog razvića« odbranila je na Katedri za fiziologiju. Tokom srednje škole bila je polaznik Istraživačke stanice »Petnica« na programu hemije. Dobitnica je nagrade Naučnog fonda »Profesor Ivan Berkeš« kao najbolja studentkinja na smeru Farmacija koja je diplomirala u školskoj 2020/2021 godini. Za vreme studija bila je nagrađivana nekoliko godina od strane Farmaceutskog fakulteta za postignut uspeh. Stipendista je Fonda za mlade talente Republike Srbije – Dositeja. Tokom studija bila je stipendista grada Beograda i Ministarstva prosvete, nauke i tehnološkog razvoja Republike Srbije. Učestvovala je na XIV Mini-kongresu studenata Farmaceutskog fakulteta kao autor studentskog naučno-istraživačkog rada. Doktorske akademske studije na Farmaceutskom fakultetu Univerziteta u Beogradu upisala je školske 2021/2022 godine pod mentorstvom Doc. Dr Marina Jukića. Od oktobra 2021. godine radi kao istraživač-pripravnik na Farmaceutskom fakultetu.

Andrea Atanasov was born on 15th December 1997 in Belgrade. She finished elementary school and The IX Gymnasium »Mihailo Petrović Alas« – natural sciences and mathematics, in Belgrade. She enrolled at the University of Belgrade – Faculty of Pharmacy in 2016, and graduated in September 2021 with an average grade of 9.75. She defended her graduate thesis entitled »CYP2C19 phenotype of homozygous and hemizygous transgenic mice on the eighteenth day of embryonic development« at the Department of Physiology. During high school, she attended Chemistry program at the »Petnica« Research Station. She received award for the best graduated student (Pharmacy programme) during 2020/2021 at the University of Belgrade-Faculty of Pharmacy from the Scientific Foundation »Professor Ivan Berkeš«. During her studies, she was awarded for her success by the Faculty of Pharmacy for several years. She is a scholarship holder of the Fund for Young Talents of the Republic of Serbia – Dositeja. During her studies, she was a scholar of the City of Belgrade and the Ministry of Education, Science and Technological Development, Republic of Serbia. She participated in the XIV Mini-Congress of Students of the Faculty of Pharmacy as the author of student research work. She enrolled in doctoral academic studies at the Faculty of Pharmacy of the University of Belgrade in the 2021/2022 school year under the supervisor of Assist. Prof. Marin Jukić. Since October 2021, she has been working as a Graduate Researcher at the Faculty of Pharmacy.

Professor Nada Majkić-Singh and Professor Nataša Bogavac-Stanojević address to the participants of the III TMBP-BPSA kongres, Novembar 7, 2021, Pharmaceutical Faculty, Belgrade.

Professor Nada Majkić-Singh and Professor Nataša Bogavac-Stanojević address to the participants of the III KONGRES TIMA MEDICINSKIH BIOHEMIČARA (TMB-BPSA) on behalf of the Scientific Fund »Professor Ivan Berkeš«.

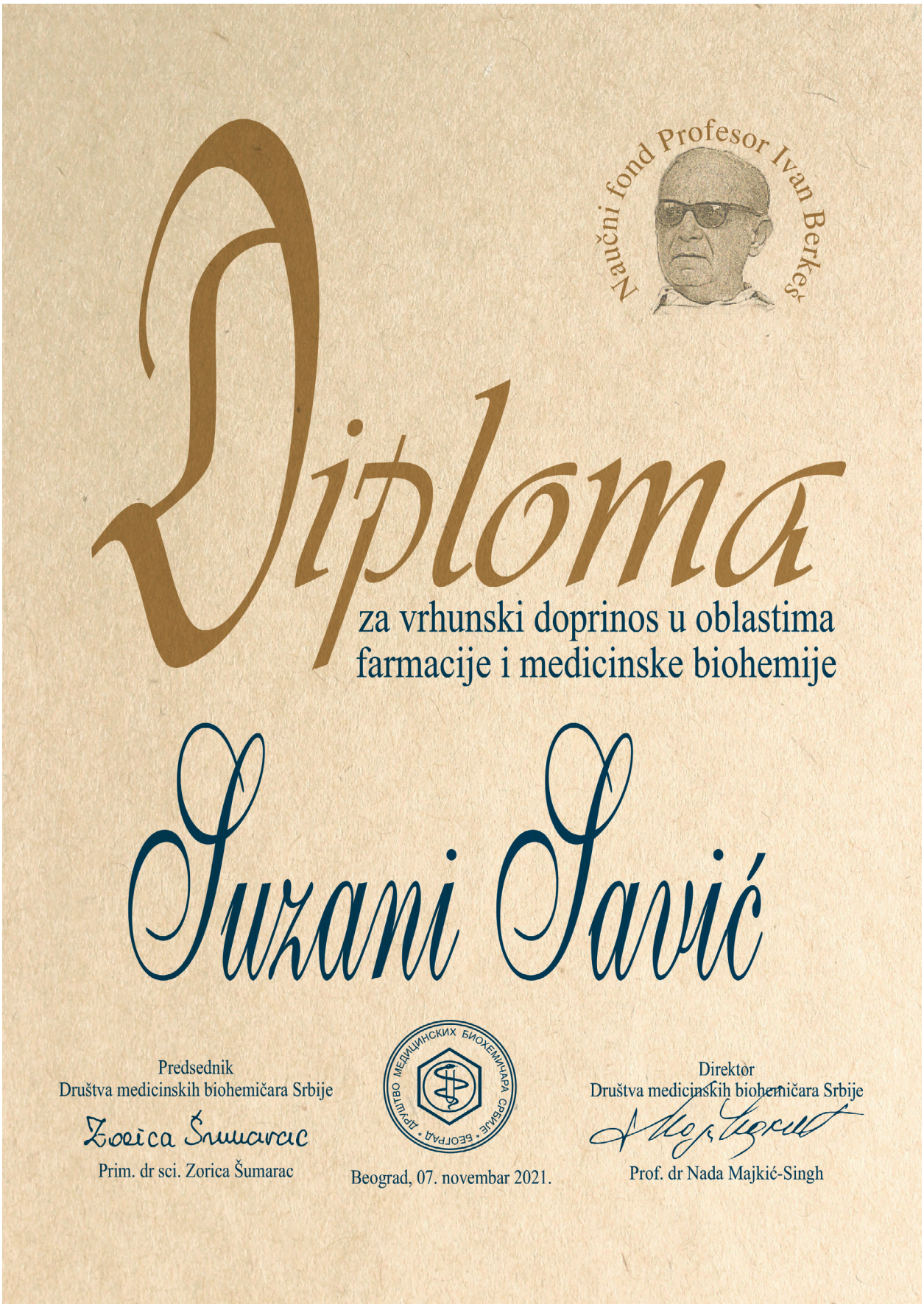
Professor Nada Majkić-Singh deliver Award of the Professor Ivan Berkeš Foundation to Suzana Savić, the winner for 2021.

Suzana Savić, the winner of the Scientific Foundation »Professor Ivan Berkeš« with Nada Majkić-Singh and Nataša Bogavac-Stanojević.

Andrea Atanasov, the winner of the Scientific Foundation »Professor Ivan Berkeš« with Nada Majkić-Singh and Nataša Bogavac-Stanojević.

Andrea Atanasov and Suzana Savić after delivering the Awards with Nada Majkić-Singh and Nataša Boghavac-Stanojević.

From left to right: Suzana Savić, Nada Majkić-Singh, Teodora Tumbas (TMB-BPSA Coordinator), Nataša Bogavac-Stanojević and Andrea Atanasov, Novembar 7, 2021, Pharmaceutical Faculty, Belgrade.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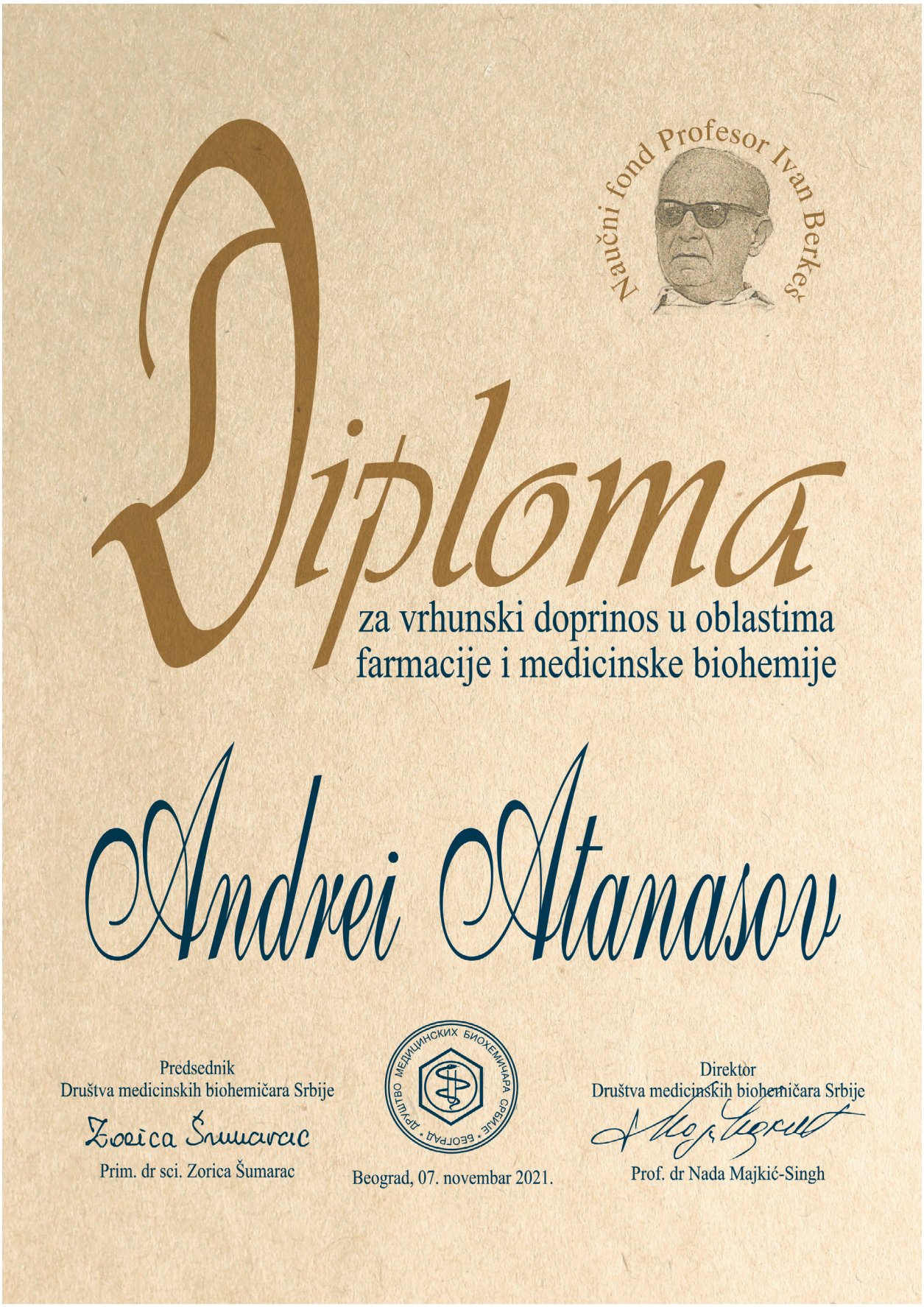
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UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 129–132, 2022

News from the Society of Medical Biochemists of Serbia

III CONGRESS OF THE TEAM OF MEDICAL BIOCHEMISTS (TMB-BPSA) UNDER THE TOPIC: FEMALE ENDOCRINOLOGICAL DISORDERS OF THE 21ST CENTURY (PCOS, INSULIN RESISTANCE, HASHIMOTO'S THYROIDITIS)

Teodora Tumbas, TMB-BPSA Cordinator

The TMB Congress was held for the first time in 2017 as the first such congress among students of pharmacy-medical biochemistry at the national level. The congress is held every two years.

The aim of the congress is to provide students and graduates of all biomedical sciences with the opportunity to inform about a very current topic among women and health professionals around the world, and to apply new knowledge in future practice and in everyday life as health professionals with the right and secure information.

Team of Medical Biochemists – Belgrade Pharmacy Students' Association -BPSA (TMB-BPSA) organized a congress named »Women endocrine disorders of XXI century« on 6th and 7th of November, Faculty of Pharmacy University of Belgrade. It contained a theoretical and practical part held at the Faculty of Pharmacy, respecting all epidemiological measures. We thought that additional attention had to be given to this topic because it is widespread nowadays, as with our students, but also wider female population. The theoretical part of the congress, which contained four sessions, through lectures of spec. med. biochem. Violeta Stanojević (Preanalytical variations), then of Prim. Dr Snežana Polovina (Preparation of a patient for blood and urine sampling for hormone analysis), spec. med. biochem. Ana Petrović Mucok (Laboratory diagnosis of Hashimoto thyroiditis), Prof. Dr Aleksandra Zeljković (Laboratory diagnosis of Insulin resistance and syndrome of polycystic ovaries), ass. spec. med. biochem. Biljana Glišić (Panel of laboratory tests for women in reproductive period) on the first day of the congress, then on the second day following with lectures of prof. Aleksandra Stefanović (Examination of ovarian reserve), prof. dr Miloš Žarković (Hashimoto tyrioditis), Prof. Dr Đuro Macut (Meaning of healthy reproductive system in reproductive period of women), mr. ph. med. biochem. Marija Milanović (A balanced diet in the treatment of PCOS) and Prof. Dr Aleksandra Buha Đorđević (Decoding the role of exposome in endocrine health) we have successfully realized the theoretical part of the congress. Then, the following was the practical part where the students of pharmacy – medical biochemistry had the opportunity to solve clinical case connected to listened lectures. After the first two sessions on the first day of the project, the panel discussion »When I grow up, I will be a medical biochemist« where we explained more profoundly the journey from diploma to the job and all the experiences and skills they can acquire during studies. The closing ceremony was shown out by Awards ceremony for the best students of generation which the *Society of Medical Biochemists Serbia* gave with Professor Nada Majkić-Singh being the representative on behalf of the »Professor Ivan Berkeš Foundation«.

We have to give special thanks to Faculty of Pharmacy Belgrade, and all sponsors and partners on this project.

For more project from our student organization, you can find us on social networks @bpsa_beograd or website www.napser.org.

Figure 1 Day 1. Grand opening with Teodora Tumbas, coordinator of the project giving a welcome speech.

Figure 2 Day 1. Panel discussion »When I grow up, I want to be a medical biochemist«. From left to right, medical biochemists: Branko Subošić, Lidija Kostadinov, Sanja Erceg, Nebojša Korićanac, Sandra Cvitak, Tamara Gojković

Figure 3 Organizing committee of Third Congress of Team of medical biochemists (TMB-BPSA). From left to right: Tamara Lukić, Anđela Marković, Jelena Pavić, Katarina Krsmanović, Aleksandra Karić, Maša Veljković, Zorica Lučanović, Isidora Crnčević, Teodora Tumbas, Anđela Aćimović, Tamara Stojković, Stanislava Glišović, Sajma Bačevac.

Figure 4 Day 2. Auditorium of congress and Professor Dr Miloš Žarković with his lecture.

Figure 5 Closing ceremony, with Society of Medical Biochemists of Serbia giving an annual award for best students in front of the Scientific Foundation »Professor Ivan Berkeš«. From left to right: mr. ph. med. biochem. Suzana Savić (Awarded), Professor Dr Nada Majkić Singh, Teodora Tumbas, Professor Dr Nataša Bogavac Stanojević, mr. ph. Andrea Atanasov (Awarded)

UDK 577.1 : 61 **ISSN 1452-8258**

J Med Biochem 41: 133, 2022

Technical reports
Obaveštenja

PROGRAM NAUČNIH I STRUČNIH SKUPOVA I EDUKATIVNIH SEMINARA

• 2022, April 10–14, Munich, Germany
EUROMEDLAB

• 2022, June 26–30, Seoul, Korea
XXIV IFCC WorldLab Seoul 2022

• 2022, September, Belgrade, Serbia
XXII Srpski kongres medicinske biohemije i laboratorijske medicine sa međunarodnim učešćem
16th Belgrade Symposium for Balkan Region

• 2022, November Belgrade, Serbia
XXIII Scientific Annual Ivan Berkeš Conference

Informacije o održavanju skupova mogu se dobiti od Sekretarijata Društva medicinskih biohemičara Srbije
e-mail: dmbj@eunet.rs; dmbs@eunet.rs; www.dmbj.org.rs

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INSTRUCTIONS FOR AUTHORS

1. Scope and Policy of the Journal

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

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