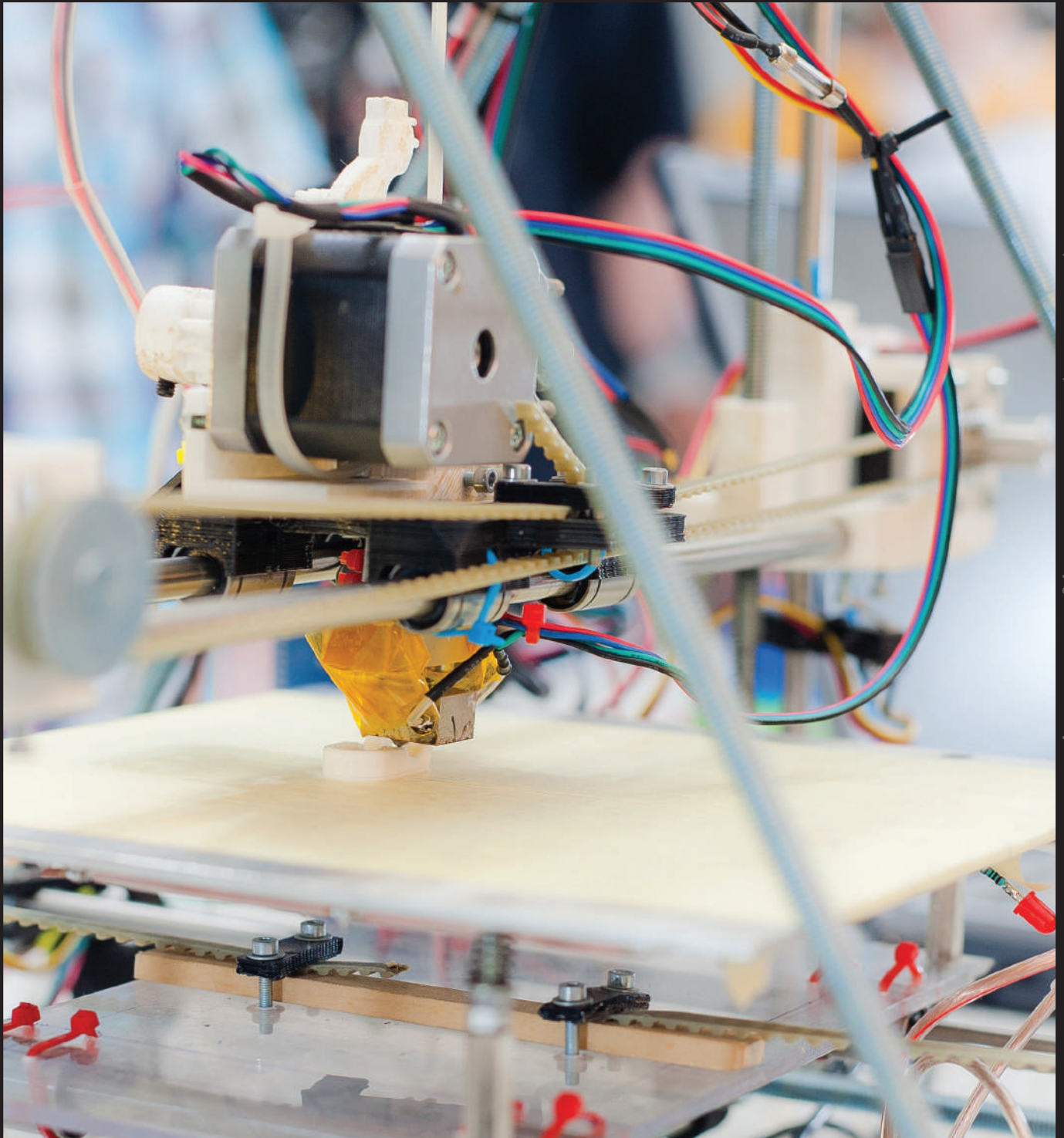


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SCIENCE

- 449** **Thyroid-Stimulating Hormone Levels within the Trimester-Specific Reference Intervals Are Correlated with Non-High-Density Lipoprotein Cholesterol and Remnant Cholesterol Concentrations in Pregnant Women**
Guocheng Wang, Feng Jin, Limin Xie, Xiaofen Zhang, Yawei Zhang, Xin Ni, Wei Li, Guojun Zhang
- 457** **Meteorin-like Protein and Asprosin Levels in Children and Adolescents with Obesity and Their Relationship with Insulin Resistance and Metabolic Syndrome**
Nariman Moradi, Reza Fadaei, Maryam Roozbehkia, Mitra Nourbakhsh, Mona Nourbakhsh, Maryam Razzaghy-Azar, Bagher Larijani
- 464** **Biclonal Gammopathies in South Tunisia: Clinical and Biological Characteristics**
Ameni Jerbi, Hend Hachicha, Aida Charfi, Faten Kallel, Sawsan Feki, Mourad Ben Ayed, Faten Ayadi, Rim Akrouf, Faten Frikha, Ali Amouri, Khaoula Kammoun, Moez Mdhafer, Mohamed Ben Hmida, Nabil Tahri, Zouheir Bahloul, Sofien Baklouti, Moez Elloumi, Hatem Masmoudi
- 469** **Association between Serum α -Klotho Levels and Behçet Disease**
Mohammad Reza Jafari Nakhjavani, Amir Ghorbanilaghaj, Aida Malek Mahdavi, Negin Nemati, Nadereh Rashtchizadeh, Sima Abedi Azar, Alireza Khabbazi
- 473** **Laboratory Strategies to Improve Allergy First-Line Screening in Primary Care**
Emilio Flores, Maite López-Garrigos, Álvaro Blasco, Javier Fernández, Carlos Leiva-Salinas, María Salinas
- 479** **Diagnostic Role of Circulating miRNAs in the Grading of Chronic Hepatitis B-Related Liver Fibrosis: A Systematic Review and Meta-Analysis**
Ting Jiang, Wenying Leng, Sen Zhong
- 489** **Evaluation of Antinuclear Antibody and Subserology Reflex Testing for the Diagnosis of Systemic Autoimmune Rheumatic Disorders in an Academic Teaching Hospital**
Delicia Duff, Niti Vyas, Janet Enderle, Rajkumar Rajendran
- 495** **Short-Term (6 Weeks) Experience of a Modular Workcell for Hemostasis Testing Including an Intelligent Data Manager at a Tertiary Care Hospital**
Hwan Tae Lee, So Young Lee, Ja Young Seo, Jeong-Yeal Ahn
- 502** **Comparison of IgG Against COVID-19 Between Postmenopausal and Nonmenopausal Women Vaccinated With Sinopharm Vaccine**
Fariba Nabatchian, Fatemeh Pakbaz, Mostafa Cheshrokh, Maryam Sajadi, Shadi Sharbati, Reza Afrisham
- 507** **Analysis of Specificity and Distribution Characteristics of Red Blood Cell Irregular Antibodies**
Hui Ni, Xiaoye Sun, Hui Cong
- 512** **3D Printing as an Effective Quality Assurance Implementation in Massive-Scale SARS-CoV-2 Testing at a SwabSeq Next-Generation Sequencing Laboratory**
Laila M. Sathe, Nishrat N. Khan, Jazmine M. Williams, Rosita Saul, Kane Jajieh, Maryam R. Sartippour, Rachel Young, Joanna Xie, Dawn M. Marquette, Tiffany Duncan, Eleazar Eskin, Valerie A. Arboleda
- 519** **An Assessment of Gender Equity and Parity Among “Nontraditional” Pathology Awards**
Jeremy W. Jacobs, Brian D. Adkins, Elizabeth S. Allen, Carlos Parra-Herran, Laura D. Stephens, Jennifer S. Woo, Garrett S. Booth
- 523** **Lipoprotein-Associated Phospholipase A₂ Correlates with Reduced Left Ventricle Ejection Fraction in Hemodialysis Patients**
Chen Jun-Feng, Jin Xiao-Ping, Zhang Juan, Yang Man-Li, Liu Fan, Fu Peng, Wu Qi-Shun, Shu Yang, Si Bo-Lin, Hu Yu-Wen, Chen Liang

- 527** **Urine Protein Immunofixation Electrophoresis: Free Light Chain Urine Immunofixation Electrophoresis Is More Sensitive than Conventional Assays for Detecting Monoclonal Light Chains and Could Serve as a Marker of Minimal Residual Disease**
Gurmukh Singh, Nkechi Arinze, David M. Manthei, Frederick V. Plapp, Roni J. Bollag
- 534** **Evaluation of Vitamin D Status and the Analysis of Risk Factors of Vitamin D Deficiency in Twin Pregnancies**
Juan Le, MS, Zhi-Hua Lv, PhD, Rui Peng, Yan Li, Shao-Ting Wang

CASE STUDY

- 543** **Autosomal Recessive Renal Hypouricemia Type 2 Presenting with Childhood Stroke in a Sri Lankan Child**
P. Mihika S. Fernando, R. A. Saraji R Gunasekara, Sabine Schröder, Christian Beet, Anura Jayawardena, Eresha A Jasinge
- 546** **Comprehensive Next-Generation Sequencing Testing in a Patient with TEMPI Syndrome**
Flavia Guimaraes Nunes Rosado, Danijela Lekovic, Jeffrey Gagan, James Malter, Weina Chen, David B. Sykes

The following are online-only papers that are available as part of Issue 54(5) online.

- e117** **Transfusion-Related Acute Lung Injury Caused by HLA-II Antibodies: A Case Report**
Xinxin Hao, Jue Wang, Tiegang Li, Qiushi Wang
- e121** **The Importance of Medical Laboratory Scientists and the Number of Doctoral Scientists that Began Their Career by Working on the Front Lines of Laboratory Medicine**
RL Sautter, DC Halstead
- e124** **Laboratory Diagnosis of Primary Amoebic Meningoencephalitis**
Juan U. Rojo, Rajkumar Rajendran, Jose H. Salazar
- e133** **Correction: The Importance of Medical Laboratory Scientists and the Number of Doctoral Scientists that Began Their Career by Working on the Front Lines of Laboratory Medicine**
- e134** **Clinical Educators' Teaching Approaches and Attributes in Laboratory Medicine**
Heather L. Phillips, Eleanor K. Jator, Shelley R. Latchem, Timothy A. Catalano
- e141** **Pathology – The Beginnings of Laboratory Medicine: Second in a Series**
Angela Tomei Robinson
- e152** **A rare occurrence of *Anaplasma*-associated peritonitis**
Valerie Bush, Jitendra Chaudhary, Devarajan Manu, Charles Hyman
- e157** **Heparin Concentration in Evacuated Tubes and Its Effect on pH, Ionized Calcium, Lactate, and Potassium in Venous Blood Gas Analysis**
Katherine Mahler, Rae Kerlin, JoAnna Jones, Jieli Li
- e161** **Effects of the Noise Reduction and Communication Management Headset System SLOS on Noise and Stress of Medical Laboratory Workers**
Jan Lehrke, Sören Lauff, Jan Mütcher, Martin G. Friedrich, Margarete Boos



ON THE COVER: Although references to “printing” in three dimensions (3D) can be found in the literature dating to the 1940s, the prototype for a 3D printing device was patented by Johannes Gottwald in 1971. Unlike most modern 3D printers, which typically use various photo-hardening polymers to form 3D printed devices, Gottwald’s “Liquid Metal Recorder” used molten metal that solidified on contact with the cooler printed device as it was being constructed. 3D printing is sometimes referred to as “additive manufacturing,” to distinguish it from machining devices that remove material to form an object. Early printers cost several hundred thousand dollars, but advances in computer technology drove down prices to less than \$200 for a basic model today. In this issue of *Laboratory Medicine*, Sathe and colleagues describe using a 3D printer to create plastic templates that overlaid specimen tube racks and identified the locations of controls in a SARS-CoV-2 testing laboratory.

Thyroid-Stimulating Hormone Levels within the Trimester-Specific Reference Intervals Are Correlated with Non-High-Density Lipoprotein Cholesterol and Remnant Cholesterol Concentrations in Pregnant Women

Guocheng Wang, MD,^{1,2} Feng Jin, PhD,² Limin Xie, MD,² Xiaofen Zhang, MD,² Yawei Zhang, PhD,³ Xin Ni, PhD,^{2,4} Wei Li, PhD,^{2,4} Guojun Zhang, PhD¹

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Keywords: thyroid stimulation hormone, non-high-density lipoprotein cholesterol, remnant cholesterol, pregnant women, reference interval, trimester

Abbreviations: TSH, thyroid-stimulating hormone; hCG, human chorionic gonadotropin; VD, verification database; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol. CLSI, Clinical and Laboratory Standard Institute; CHALLENGE, China Longitudinal Environmental, Genetic and Economic; ASCVD, atherosclerotic cardiovascular disease; TSHR, TSH receptor; ALL, entire pregnancy term; T3, third trimester

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ABSTRACT

Objective: Thyroid-stimulating hormone (TSH) levels are associated with serum lipid concentrations in the general nonpregnant population. Here, we aimed to establish trimester-specific reference intervals and to explore the associations of their variations within the specific reference intervals during pregnancy.

Methods: Trimester-specific reference intervals were established according to the Clinical and Laboratory Standard Institute EP28-A3c

guidelines using a direct sampling method based on a large prospective cohort. After making one-to-one matches, correlation analyses between TSH and lipid index levels, especially within the reference intervals, were conducted.

Result: A total of 1648 pregnant women for TSH and 2045 subjects for lipids were recruited to establish the trimester-specific reference intervals. The upper reference limit (90% confidence interval) of TSH for pregnant women in the first trimester is 3.95 (3.66–4.29) mIU/L, which is very close to the default value (4.0 mIU/L) recommended by the American Thyroid Association in 2017. Apart from triglyceride and high-density lipoprotein cholesterol, TSH levels were positively associated with the serum concentrations of total cholesterol, low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol (non-HDL-C), and remnant cholesterol (RC) either in the entire range or within the specific reference intervals. Of note, the positive correlations between TSH and non-HDL-C and RC were, albeit similarly weak ($r < 0.25$), relatively more robust ($P < .001$).

Conclusion: In this study, we showed positive correlations between TSH and lipid components within trimester-specific reference intervals, highlighting the need for the integrated management of pregnant women over age 35 and with nonoptimal lipid status in China.

Substantial alterations in maternal physiology take place during pregnancy as consequences of estrogen and progesterone changes and increasing metabolic demands. For instance, the serum thyroid-stimulating hormone (TSH) level reaches the lowest level in the first trimester of pregnancy partially due to an indirect inhibitory effect on human chorionic gonadotropin (hCG) level that sharply elevates at the same time.^{1,2} Circulating lipid levels rise by approximately 20% to 100% at the end of pregnancy.³

The relationships of serum concentrations between TSH and lipids in nonpregnant women have been well studied^{4–6} but are far

TABLE 1. Trimester-Specific Reference Intervals of Serum TSH and Lipid Levels in Pregnant Women^a

	T1			T2			T3		
	n	LRL	URL	n	LRL	URL	n	LRL	URL
TSH (mIU/L)	1150	0.08 (0.06–0.10)	3.95 (3.66–4.29)	372	0.05 (0.04–0.07)	4.23 (4.02–4.66)	126	0.20 (0.12–0.56)	5.92 (4.76–6.37)
TG (mmol/L)	894	0.45 (0.41–0.46)	2.17 (1.93–2.32)	129	0.54 (0.51–0.65)	3.05 (2.82–3.51)	1022	1.56 (1.43–1.62)	5.59 (5.28–6.06)
TC (mmol/L)	894	2.94 (2.84–3.00)	5.69 (5.60–5.89)	129	3.18 (2.93–3.41)	6.32 (5.84–6.78)	1022	4.29 (4.11–4.40)	8.93 (8.70–9.11)
HDL-C (mmol/L)	894	1.06 (1.03–1.09)	2.22 (2.16–2.26)	129	1.05 (0.95–1.24)	2.30 (2.26–2.52)	1022	1.24 (1.18–1.31)	2.82 (2.76–2.92)
LDL-C (mmol/L)	894	1.12 (1.06–1.16)	3.14 (3.04–3.30)	129	1.06 (0.75–1.29)	3.42 (3.31–3.63)	1022	1.89 (1.84–2.01)	5.33 (5.20–5.48)
Non-HDL-C (mmol/L)	894	1.55 (1.48–1.61)	4.04 (3.89–4.19)	129	1.59 (0.98–1.72)	4.42 (4.23–4.70)	1022	2.68 (2.61–2.75)	6.63 (6.43–6.71)
RC (mmol/L)	894	0.27 (0.25–0.29)	1.15 (1.10–1.18)	129	0.31 (0.23–0.32)	1.29 (1.13–1.56)	1022	0.35 (0.30–0.39)	1.79 (1.70–1.86)

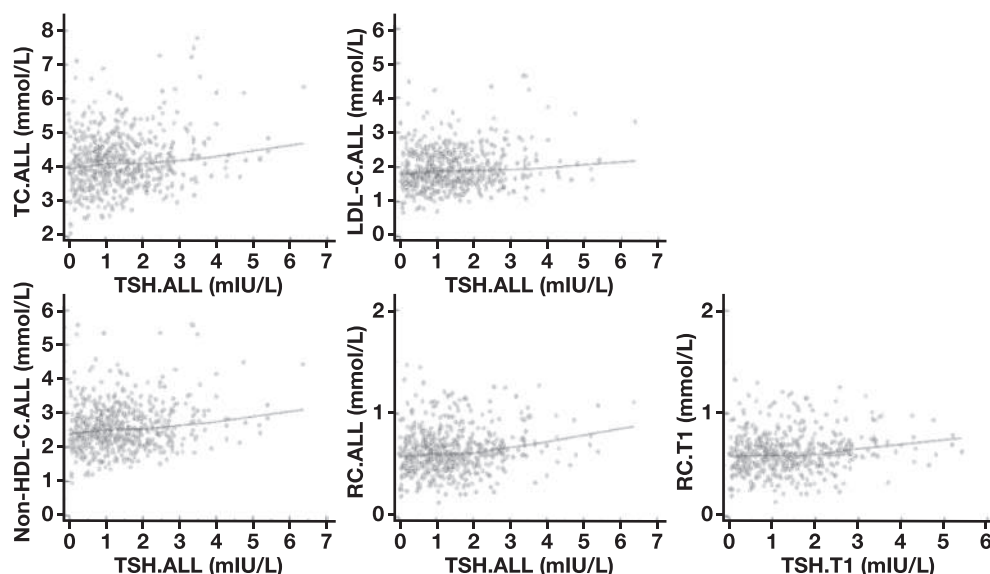
HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LRL, lower reference limit; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid-stimulating hormone; T1, first trimester; T2, second trimester; T3, third trimester; URL, upper reference limit.
^aNumbers in parentheses represent lower and upper bounds of the 90% confidence interval.

TABLE 2. Correlation Analysis between Thyrotropin and Lipids Using Data in the CHALLENGE Cohort^a

n	TSH-TG			TSH-HDL-C			TSH-TC			TSH-LDL-C			TSH-Non-HDL-C			TSH-RC		
	r	p		r	p		r	p		r	p		r	p		r	p	
ALL	596	–0.001 (–0.082 to 0.080)	.974	0.076 (–0.005 to 0.155)	.066	0.111 (0.031 to 0.189)	.007	0.091 (0.011 to 0.170)	.026	0.118 (0.038 to 0.196)	.004	0.124 (0.044 to 0.202)	.003					
T1	518	–0.073 (–0.158 to 0.014)	.100	0.053 (–0.033 to 0.139)	.225	0.081 (–0.005 to 0.166)	.066	0.059 (–0.028 to 0.144)	.182	0.083 (–0.003 to 0.168)	.059	0.111 (0.025 to 0.195)	.012					
T2	61	0.247 (–0.006 to 0.469)	.055	–0.085 (–0.330 to 0.170)	.515	0.023 (–0.230 to 0.273)	.862	0.048 (–0.206 to 0.296)	.714	0.094 (–0.162 to 0.338)	.471	0.095 (–0.161 to 0.339)	.467					
T3	17	–0.139 (–0.581 to 0.366)	.596	0.283 (–0.229 to 0.672)	.271	0.123 (–0.380 to 0.570)	.639	0.080 (–0.417 to 0.540)	.761	–0.026 (–0.500 to 0.461)	.922	–0.345 (–0.708 to 0.163)	.176					

ALL, entire pregnancy term; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid-stimulating hormone; T1, first trimester; T2, second trimester; T3, third trimester.
^aValues in bold indicate statistical significance ($P < .05$). Numbers in parentheses represent lower and upper bounds of the 95% confidence interval.

FIGURE 1. Scatter plots showing correlations between TSH and lipids of statistical significance ($P < .05$) in the CHALLENGE cohort. ALL, during the entire pregnancy; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid stimulating hormone; T1, first trimester. Dashed lines represent trend lines.



from clear in pregnant women due to vast physiological changes during pregnancy. In clinical practice, it is of utmost importance to keep the correlation between TSH and lipids in pregnancy in mind. Because serum TSH and lipids are rarely tested at the same time in routine prenatal examinations in China, awareness of the correlation can alert obstetricians to check the other if one is abnormal. Also, the correlation is an indicator that both thyroid function and lipid disorders should be corrected simultaneously because TSH affects lipid components directly by acting on TSH receptor (TSHR) expressed in hepatocyte membranes⁷ and indirectly by the deterioration of hepatic bile acid synthesis and insulin sensitivity.⁸ Moreover, considering particularly the physiological period of pregnancy (as described in the first paragraph) and large fluctuations of TSH and lipids levels, pregnancy and trimester-specific reference intervals are of extreme importance for precise diagnostics and targeted treatment. Meanwhile, considering diverse detection platforms and demographic gaps, population-specific reference intervals are also challenging.⁹ Until now, the correlations between TSH and lipids concentrations in reference intervals based on population-specific and trimester-specific specific reference intervals in pregnant women have been rarely reported.

In this study, we aimed to establish trimester and platform-specific reference intervals for TSH as well as circulating lipids and to explore the correlations between TSH and lipid concentrations in pregnant Chinese women of over 35 years of age and nonoptimal lipid status.

Materials and Methods

Subject Screening

A prospective cohort study entitled the China Longitudinal Environmental, Genetic and Economic (CHALLENGE) study was launched in March 2018 in the Shunyi district, Beijing, and more than 9000 pregnant women were consecutively enrolled. Based on this cohort, we

used the direct sampling method for establishing TSH and lipids reference intervals as well as correlation analysis. The inclusion criteria for candidate subjects were (1) a singleton pregnancy, (2) Chinese Han ethnicity, and (3) local residence time ≥ 1 year. The exclusion criteria were as follows: (1) personal or family history of thyroid or lipid dysfunction, (2) visible or palpable thyroid goiters, (3) thyroid peroxidase (>9 IU/mL) or thyroglobulin (>4 IU/mL) antibody positive, (4) overweight before pregnancy (body mass index ≥ 28 kg/mm²)¹⁰ or excessive weight gain during pregnancy (for prepregnancy normal-weight women >16 kg; for overweight >11.5 kg).¹¹

According to the guidelines of EP28-A3c, newly established reference intervals should be verified by the OOR (out of range) method by using another database of healthy pregnant women. The OOR is the ratio of data that is more than the upper reference limit and less than the lower reference limit. When OOR is equal to or less than 10%, the reference interval is validated and acceptable. Therefore, we collected circulating TSH and lipid data of 381 healthy pregnant women who attended regular prenatal screenings in our hospital (December 2020 to May 2021) as a verification database (VD). Establishing the VD had 2 aims: to verify the reference intervals from the CHALLENGE database by the OOR method and to conduct the same correlation analysis again after using CHALLENGE data to increase the reliability of our conclusions.

Laboratory Analysis

Three milliliters of venous blood was taken into a vacuum serum separator tube (Becton Dickinson) from each pregnant woman who had been required to fast for at least 8 h and had been sitting for at least 10 min before. Samples were centrifuged at 3000g of relative centrifugal force for 10 min after clot formation. At least 1 mL of serum was separated into 2 aliquots for biochemical analysis: 1 for TSH (reagent catalog No. 115448, Beckman Coulter), TPOAb (No. 213650, Beckman Coulter) and TgAb (No. 213833, Beckman Coulter) determination on an automated Beckman Coulter DXI800 analyzer; the other for triglyceride (TG, reagent catalog No. 200506, MC Bioengineering), total cholesterol (TC, No. 20200321,

MC Bioengineering), high-density lipoprotein cholesterol (HDL-C, No. 200629, MC Bioengineering), and low-density lipoprotein cholesterol (LDL-C, No. 200512, MC Bioengineering) testing on a Beckman Coulter AU2700 analyzer. The non-high-density lipoprotein cholesterol (non-HDL-C) level was calculated as TC minus HDL-C and the remnant cholesterol (RC) was calculated as non-HDL-C minus LDL-C.

Quality Assurance

When the laboratory data were extracted, inputted, and cleaned, a double check was applied. Inclusion and exclusion criteria were verified by a senior obstetrician for each pregnant woman included in the study analysis. The means (coefficient of variance) of 2 commercial internal quality controls were TSH, 9.2 mIU/L (5.28%) and 25.8 mIU/L (4.80%); triglyceride, 1.12 mmol/L (3.29%) and 2.98 mmol/L (2.24%); TC, 3.90 mmol/L (2.12%) and 7.08 mmol/L (1.74%); HDL-C, 1.23 mmol/L (4.50%) and 2.57 mmol/L (4.14%); LDL-C, 2.13 mmol/L (3.46%) and 3.71 mmol/L (3.12%). The relative biases of 5 National Center for Clinical Laboratories quality assessment materials for TSH, TG, TC, HDL-C, and LDL-C were qualified.

Statistical Analysis

Data were pooled, collated, and analyzed with Excel 365 (Microsoft) and MedCalc 20.0 (MedCalc Software). In adherence to the Clinical and Laboratory Standard Institute (CLSI) EP28-A3c guidelines, outliers were assessed using the one-third rule for D/R ratio proposed by Reed and Dixon,¹² where D is the difference between an extreme and its next value and R is the maximum difference of all the observations. Normality of data distribution was checked by the Kolmogorov-Smirnov test. In accordance with CLSI guidelines, all the reference intervals were defined as the ranges between the 2.5th and 97.5th percentiles of healthy pregnant women and obtained with the “Reference Intervals” submenu of the MedCalc statistical software. The correlation analysis was performed using Pearson’s correlation method. Differences were deemed as statistically significant when two-tailed *P* values equal to or less than .05.

Ethics Statement

This study was approved by the research ethics committee of Shunyi Maternal and Children’s Hospital of Beijing Children’s Hospital in China (ethical approval No. EASRN2021-01).

Results

Trimester-Specific Reference Intervals

During antenatal care visits in China, TSH levels were available in thyroid function tests and TG, TC, HDL-C, and LDL-C were part of routine lipid tests. A total of 1648 TSH and 8180 lipid (4 indexes) data matching the inclusion and exclusion criteria from March 2019 to May 2020 were validated for the establishment of trimester-specific reference intervals (TABLE 1). The median (range) weeks of data establishing reference intervals for the first, second, and third trimester were 8 (4–11), 14 (12–24), and 33 (25–41) for TSH, and 8 (6–11), 13 (12–24), and 36 (25–41) for lipids. Reference intervals were established according to CLSI EP28-A3c guidelines.

Relationship between TSH and Lipids in Pregnant Women Positive Correlations between TSH and Lipids

After making one-to-one matches with a unique number (national identification number), 596 pregnant women in the CHALLENGE co-

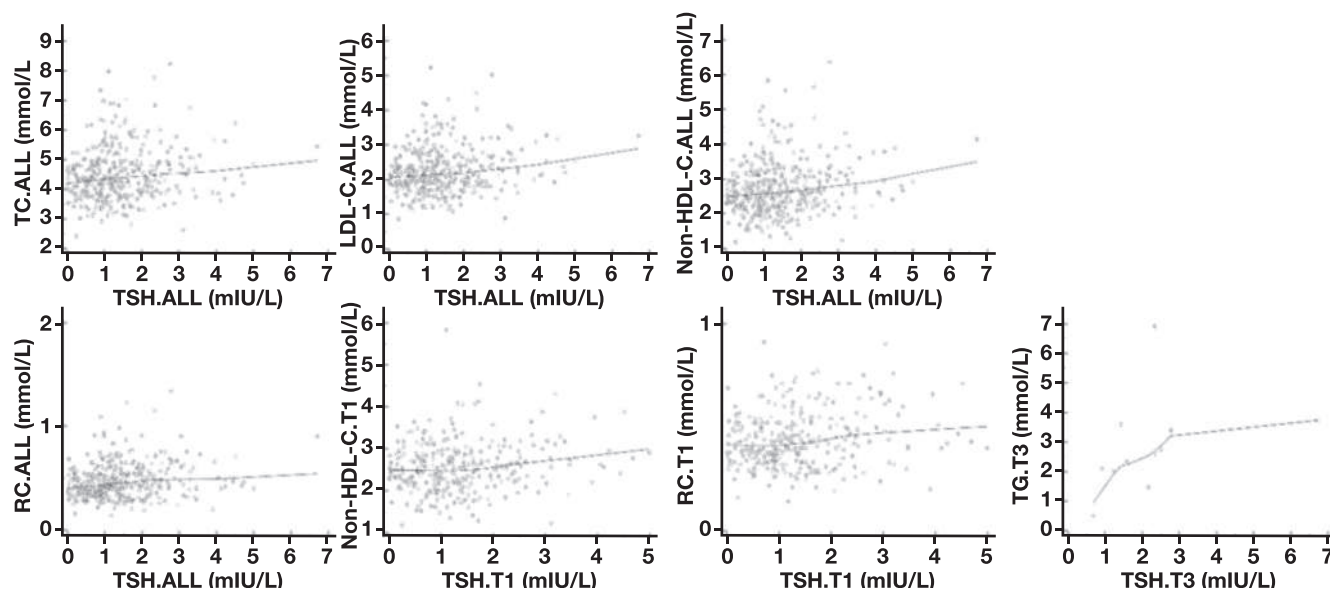
TABLE 3. Correlation Analysis between Thyrotropin and Lipids Using the Data in the Verification Database^a

	TSH-TG		TSH-HDL-C		TSH-TC		TSH-LDL-C		TSH-Non-HDL-C		TSH-RC	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
ALL	0.066 (−0.035 to 0.166)	.198	0.041 (−0.061 to 0.141)	.432	0.148 (0.048 to 0.245)	.004	0.145 (0.045 to 0.242)	.005	0.170 (0.071 to 0.266)	.001	0.192 (0.093 to 0.287)	.000
T1	−0.016 (−0.129 to 0.098)	.787	0.003 (−0.111 to 0.116)	.964	0.089 (−0.025 to 0.201)	.124	0.101 (−0.013 to 0.212)	.081	0.124 (0.011 to 0.235)	.032	0.134 (0.021 to 0.244)	.020
T2	0.025 (−0.212 to 0.258)	.839	0.044 (−0.193 to 0.276)	.716	0.030 (−0.207 to 0.263)	.806	0.006 (−0.229 to 0.241)	.960	0.016 (−0.220 to 0.250)	.894	0.033 (−0.204 to 0.266)	.789
T3	0.664 (0.106 to 0.904)	.026	0.091 (−0.538 to 0.655)	.790	0.291 (−0.374 to 0.758)	.386	0.336 (−0.330 to 0.779)	.312	0.391 (−0.273 to 0.803)	.235	0.610 (0.017 to 0.886)	.050

ALL, entire pregnancy term; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid-stimulating hormone; T1, first trimester; T2, second trimester; T3, third trimester.

^aValues in bold indicate statistical significance (*P* < .05). Numbers in parentheses represent lower and upper bounds of the 95% confidence interval.

FIGURE 2. Scatter plots showing correlations between TSH and lipids of statistical significance ($P < .05$) in the verification database. ALL, during the entire pregnancy; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid stimulating hormone; T1, first trimester; T3, third trimester.



hort and 381 pregnant women in the VD were included for correlation analysis. The correlations that reached statistical significance are shown in bold font in **TABLE 2** and presented in the form of scatter plots in **FIGURE 1**. To validate the correlations, we conducted correlation analysis again in the VD and obtained similar results (**TABLE 3**, **FIGURE 2**).

Positive Correlations between TSH and Lipids within the Reference Intervals Based on Trimester-Specific Reference Intervals

The serum TSH and lipids concentrations of 526 pregnant women in the CHALLENGE cohort and 340 pregnant women in the VD fell within the trimester-specific reference intervals (**TABLE 1**) and constituted the normal range database. Similar correlation analysis results were obtained with these normal range data in the CHALLENGE cohort (**TABLE 4**) as well as VD (**TABLE 5**). Of note, the correlation results were almost completely consistent for RC. The correlations that reached statistical significance ($P < .05$) are in bold font (**TABLE 4** and **TABLE 5**) and shown in scatter plots (**FIGURE 3** and **FIGURE 4**).

Discussion

The formal term “reference interval” strongly advocated by the CLSI encompasses 95% values of relatively healthy reference subjects in most cases. Two sampling techniques, direct and indirect, are recommended in the well-accepted CLSI EP28-A3c guidelines¹² and are now widely used in clinical practice for establishing reference intervals. Despite the obvious advantages of time and cost saving, the indirect method using hospital data without the recruitment of healthy subjects has some technical limitations. For example, the value of acceptable deviation in the Hoffmann method, a widely-used indirect sampling technique, is to some extent subjective and determined by researchers.¹³ In line with our previous study,¹⁴ the applicable value of acceptable deviation for TSH in this study ($\alpha = 0.47$) was also greater than its default value ($\alpha = 0.05$)

and was 0.05 in lipid markers only for TG. Therefore, reference intervals established in a direct sampling method should be more precise and reliable. Based on a large prospective cohort with rigorous inclusion and exclusion criteria, we established trimester-specific reference intervals of TSH and lipids for pregnant Chinese Han women using a direct method.

Thyroid dysfunction has a strongly deleterious effect on pregnancy outcomes owing to the broad biological effects of thyroid hormone. The diagnosis of subclinical thyroid diseases (subclinical hypothyroidism and subclinical hyperthyroidism) and overt thyroid diseases in some cases relies on the first-line serum marker TSH. However, there was poor concordance among different TSH detection platforms and even the same platform.⁹ Moreover, hCG, which belongs to the same glycoprotein hormone family as TSH,¹ shows a strong thyroid-stimulating effect, especially when hCG increases exponentially in the early stage of pregnancy. As a consequence, the serum TSH concentration decreases sharply in the first trimester and then rises slowly to the original level at the end of pregnancy.² Therefore, establishing local trimester-specific reference intervals of TSH in the service population is critical for the precise management of thyroid dysfunction during pregnancy.

Concurrently, lipid metabolism is significantly affected by estradiol and progesterone fluctuations in pregnancy. Levels of all lipid parameters in the blood of pregnant women increase at different rates.³ With the rapid development of the economy in China, the population’s living style and dietary structure are experiencing dramatic changes.^{15,16} Additionally, there have been radical changes in China’s childbearing policy in recent years. Following the 1-child policy that had been in place for 30 years, the universal 2-child policy was implemented in 2016 and the 3-child policy came into effect in 2021. As a result, more and more women are becoming pregnant over the age of 35. These factors affect the fluctuations of blood lipid levels in pregnant women, underlining the urgency of trimester-specific and population-specific lipid reference intervals for the precise management of pregnancy. Besides the 4 traditional lipid indexes, non-HDL-C and RC were also explored for use in establishing reference intervals and correlation analysis in this study. We found that these 2 indexes had positive correlations with TSH, including data

TABLE 4. Correlation Analysis between Thyrotropin and Lipids within Trimester-Specific Reference Intervals in the CHALLENGE Cohort^a

n	TSH-TG			TSH-HDL-C			TSH-TC			TSH-LDL-C			TSH-Non-HDL-C			TSH-RC		
	r	p		r	p		r	p		r	p		r	p		r	p	
ALL	0.009	(-0.077 to 0.094)	.841	0.054	(-0.031 to 0.139)	.213	0.089	(0.003 to 0.173)	.042	0.066	(-0.020 to 0.150)	.132	0.099	(0.014 to 0.183)	.023	0.115	(0.030 to 0.198)	.008
T1	-0.055	(-0.147 to 0.037)	.240	0.038	(-0.054 to 0.130)	.415	0.061	(-0.031 to 0.152)	.194	0.035	(-0.058 to 0.127)	.460	0.066	(-0.027 to 0.157)	.163	0.108	(0.016 to 0.198)	.021
T2	0.211	(-0.051 to 0.445)	.113	-0.116	(-0.363 to 0.147)	.386	0.007	(-0.252 to 0.265)	.957	0.015	(-0.245 to 0.272)	.914	0.069	(-0.192 to 0.322)	.605	0.082	(-0.181 to 0.333)	.543
T3	0.198	(-0.349 to 0.645)	.478	0.202	(-0.346 to 0.647)	.470	0.077	(-0.453 to 0.567)	.785	0.011	(-0.504 to 0.520)	.970	-0.056	(-0.552 to 0.470)	.844	-0.281	(-0.693 to 0.271)	.311

ALL, entire pregnancy term; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid-stimulating hormone; T1, first trimester; T2, second trimester; T3, third trimester.

^aValues in bold indicate statistical significance (P < .05). Numbers in parentheses represent lower and upper bounds of the 95% confidence interval.

TABLE 5. Correlation Analysis between Thyrotropin and Lipids within the Trimester-Specific Reference Intervals in the Verification Database^a

n	TSH-TG			TSH-HDL-C			TSH-TC			TSH-LDL-C			TSH-Non-HDL-C			TSH-RC		
	r	p		r	p		r	p		r	p		r	p		r	p	
ALL	0.097	(-0.010 to 0.201)	.076	0.062	(-0.044 to 0.168)	.251	0.158	(0.053 to 0.260)	.004	0.142	(0.037 to 0.245)	.009	0.175	(0.070 to 0.276)	.001	0.220	(0.116 to 0.319)	.000
T1	0.048	(-0.074 to 0.168)	.438	0.021	(-0.101 to 0.141)	.740	0.120	(-0.001 to 0.238)	.053	0.119	(-0.002 to 0.237)	.054	0.153	(0.032 to 0.269)	.013	0.190	(0.071 to 0.305)	.002
T2	0.022	(-0.215 to 0.258)	.855	0.036	(-0.202 to 0.270)	.769	0.027	(-0.211 to 0.262)	.825	0.006	(-0.231 to 0.243)	.959	0.016	(-0.222 to 0.252)	.896	0.037	(-0.202 to 0.271)	.764
T3	0.600	(-0.107 to 0.904)	.088	0.517	(-0.224 to 0.879)	.154	0.467	(-0.286 to 0.863)	.205	0.433	(-0.324 to 0.852)	.244	0.517	(-0.224 to 0.879)	.154	0.628	(-0.063 to 0.912)	.070

ALL, entire pregnancy term; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid-stimulating hormone; T1, first trimester; T2, second trimester; T3, third trimester.

^aValues in bold indicate statistical significance (P < .05). Numbers in parentheses represent lower and upper bounds of the 95% confidence interval.

within the trimester-specific reference intervals. In clinical practice, values that fall outside of reference intervals must be marked with special labels on reports, as these values in most cases have been ignored. We consider the correlations when TSH and lipid levels are within the reference intervals for 2 purposes: to verify that correlations in fact exist between TSH and lipids within the reference intervals, although the correlations exist theoretically,

FIGURE 3. Scatter plots for the correlations between TSH and lipids within the specific reference intervals reaching statistical significance ($P < .05$) in the CHALLENGE cohort. nALL, during the entire pregnancy for data within the normal range; non-HDL-C, non-high-density lipoprotein cholesterol; nT1, first trimester for data within the normal range; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid stimulating hormone. Dashed lines represent trend lines.

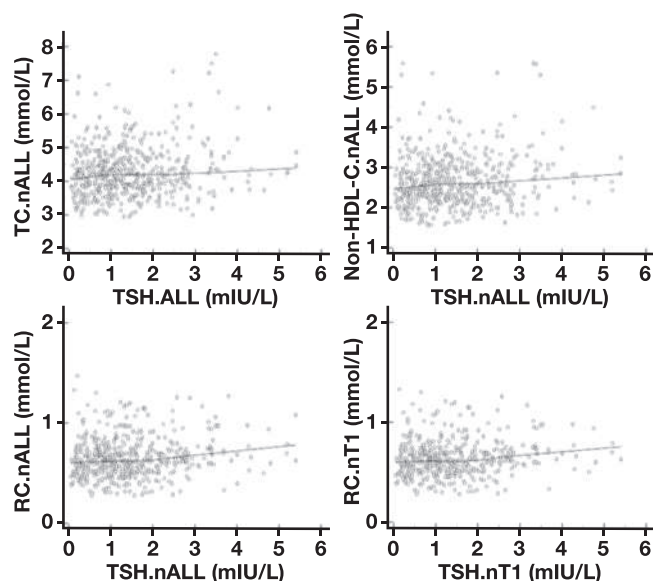
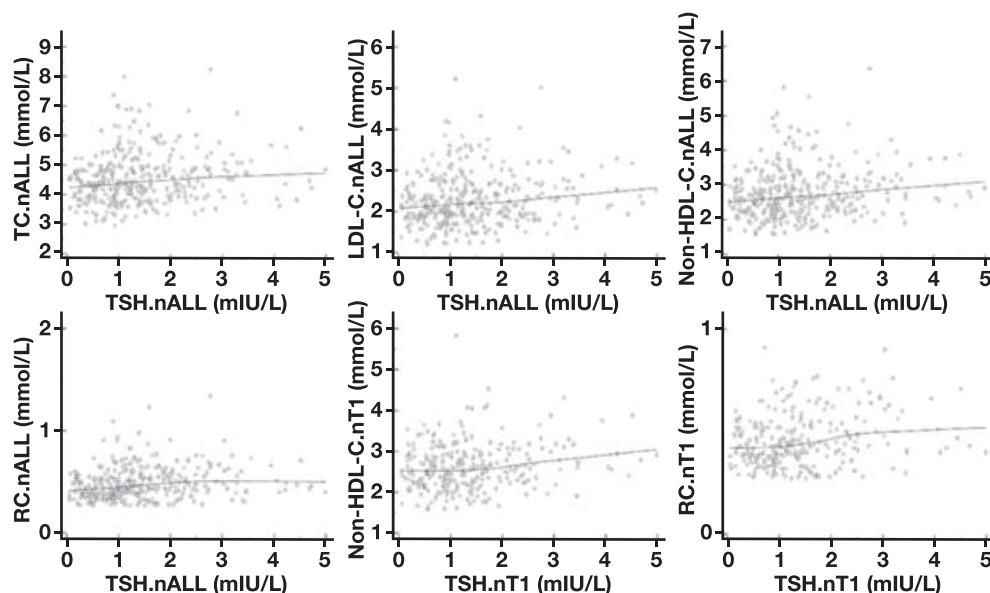


FIGURE 4. Scatter plots for the correlations between TSH and lipids within the specific reference intervals that reached statistical significance ($P < .05$) in the verification database. LDL-C, low-density lipoprotein cholesterol; nALL, during the entire pregnancy for data within the normal range; non-HDL-C, non-high-density lipoprotein cholesterol; nT1, first trimester for data within the normal range; RC, remnant cholesterol; TC, total cholesterol; TG, triglyceride; TSH, thyroid stimulating hormone. Dashed lines represent trend lines.



and to give more attention to the test results within the specific reference intervals for earlier identification and intervention. To the best of our knowledge, this is the first report of non-HDL-C and RC being used to establish trimester-specific reference intervals and explore correlations between them with TSH within the specific reference intervals during pregnancy.

Currently, LDL-C is one of the primary targets of lipid-lowering interventions.¹⁷ However, there are a number of limitations that cannot be ignored when we assess LDL-C in patients with hypertriglyceridemia and diabetes mellitus. Comparatively, non-HDL-C can be determined reliably in both fasting and nonfasting conditions and can more effectively guide the treatment of atherosclerotic cardiovascular disease (ASCVD) because TG measurement is not required.¹⁸ Moreover, non-HDL-C could represent all plasma atherogenic lipoprotein components and show a stronger predictive ability for ASCVD than LDL-C. It was observed in the Framingham cohort study that there was no association between LDL-C levels and ASCVD risk in patients with the same concentrations of non-HDL-C, and there was a strong correlation between non-HDL-C with future ASCVD events in subjects with the same LDL-C levels.¹⁹ As another advanced lipoprotein test, RC measurement can predict ASCVD events beyond LDL-C (elevated RC levels are associated with ASCVD independent of LDL-C),²⁰ and the circulating levels of RC but not LDL-C were associated with ASCVD outcomes in overweight or obese individuals.²¹ In our study, we found that there were positive correlations between RC and TSH in Chinese pregnant women.

It has been documented that TSH could affect lipid components independent of thyroid hormones.⁶ By acting on TSHR, which expresses in hepatocyte membranes, TSH could upregulate the expression of a rate-limiting enzyme (hepatic 3-hydroxy-3-methyl-glutaryl coenzyme A reductase) in cholesterol synthesis and enhance the activity of a key lipoprotein transcription factor (sterol regulatory element binding protein 1c). Additionally, TSH can also indirectly increase serum lipid concentrations by the deterioration of hepatic bile acid synthesis and insulin sensitivity. Given that the global epicenter of lipid-related risk is shifting from high-income countries in northwestern Europe and North

American to middle-income countries in East and Southeast Asia, TSH may therefore be another therapeutic target for the prevention and management of ASCVD in China.

A strength of this study is the use of normal data derived from our trimester-specific and service population-specific reference intervals. Therefore, the normal data that represent subtle variations of the corresponding indices were relatively precise. Our work also has a limitation that should be considered. The iodine status of each participant could not be determined. However, in China, a universal salt iodization program began early in 1994 and this program has been in place for almost 30 years. In recent years, there has been concern about more-than-adequate iodine intake in nonpregnant women.^{22,23} Considering the increased demand for iodine during pregnancy, the iodine status of pregnant women in our study may be regarded as appropriate. Additionally, the correlations between TSH and lipids in pregnant women were explored from different perspectives, as shown in **TABLE 2** to **TABLE 5**. There were some differences worth noting. The correlations between TSH and TG reached statistical significance only in the third trimester (T3) time period in VD (**TABLE 3**), and the correlations between TSH and LDL-C did not reach statistical significance in the CHALLENGE cohort (**TABLE 4**). The inconsistent results remind us that these correlations are still inconclusive and should be applied cautiously in clinical practice.

In conclusion, we established the trimester-specific and service population-specific reference intervals for TSH as well as circulating lipids in pregnant Chinese Han women; showed the positive correlations between TSH and several lipid components, including those within the specific reference intervals; and highlighted the need for the integrated management of TSH and lipid levels in pregnant women over age 35 years and of nonoptimal lipid status.

Funding

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

- Lapthorn AJ, Harris DC, Littlejohn A, et al. Crystal structure of human chorionic gonadotropin. *Nature*. 1994;369(6480):455–461. doi:10.1038/369455a0.
- Glinoe D. The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. *Endocr Rev*. 1997;18(3):404–433. doi:10.1210/edrv.18.3.0300.
- Mankuta D, Elami-Suzin M, Elhayani A, Vinker S. Lipid profile in consecutive pregnancies. *Lipids Health Dis*. 2010;9(1):58–62. doi:10.1186/1476-511X-9-58.
- Witte T, Ittermann T, Tham M, Riblet NB, Volzke H. Association between serum thyroid-stimulating hormone levels and serum lipids in children and adolescents: a population-based study of German youth. *J Clin Endocrinol Metab*. 2015;100(5):2090–2097. doi:10.1210/jc.2014-4466.
- Asvold BO, Bjoro T, Vatten LJ. Associations of TSH levels within the reference range with future blood pressure and lipid concentrations: 11-year follow-up of the HUNT study. *Eur J Endocrinol*. 2013;169(1):73–82.
- Wang F, Tan Y, Wang C, et al. Thyroid-stimulating hormone levels within the reference range are associated with serum lipid profiles independent of thyroid hormones. *J Clin Endocrinol Metab*. 2012;97(8):2724–2731. doi:10.1210/jc.2012-1133.
- Tian L, Song Y, Xing M, et al. A novel role for thyroid-stimulating hormone: up-regulation of hepatic 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase expression through the cyclic adenosine monophosphate/protein kinase A/cyclic adenosine monophosphate-responsive element binding protein pathway. *Hepatology*. 2010;52(4):1401–1409. doi:10.1002/hep.23800.
- Javed A, Balagopal PB, Vella A, et al. Association between thyrotropin levels and insulin sensitivity in euthyroid obese adolescents. *Thyroid*. 2015;25(5):478–484. doi:10.1089/thy.2015.0005.
- Wang G, Zhang G. The diverse upper reference limits of serum thyroid-stimulating hormone on the same platform for pregnant women in China. *Lab Med*. 2020;51(4):416–422. doi:10.1093/labmed/lmz089.
- American Society of Clinical Pathologists. Obesity: how the increase in incidence affects clinical values. *Lab Med* 2003;34(4):270–273.
- Rasmussen KM, Yaktine AL. *Weight Gain During Pregnancy: Reexamining the Guidelines*. Washington, DC: National Academies Press; 2009.
- CLSI. *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory*. Approved Guideline-Third Edition. Wayne, PA: Clinical and Laboratory Standards Institute, EP28-A3c. 2010.
- Feng G, Song W, Lü Y, et al. The application of Hoffmann method in the establishment of children's reference intervals of erythrocyte count. *Zhonghua Yi Xue Zazhi*. 2016;96(22):1742–1745.
- Zhang Y, Ma W, Wang G, Lv Y, Peng Y, Peng X. Limitations of the Hoffmann method for establishing reference intervals using clinical laboratory data. *Clin Biochem*. 2019;63(1):79–84. doi:10.1016/j.clinbiochem.2018.11.005.
- NCD Risk Factor Collaboration. Repositioning of the global epicentre of non-optimal cholesterol. *Nature*. 2020;582(7810):73–77.
- Pirillo A, Casula M, Olmastroni E, Norata GD, Catapano AL. Global epidemiology of dyslipidaemias. *Nat Rev Cardiol*. 2021;18(10):689–700. doi:10.1038/s41569-021-00541-4.
- Wilson PWF, Jacobson TA, Martin SS, et al. Lipid measurements in the management of cardiovascular diseases: practical recommendations a scientific statement from the national lipid association writing group. *J Clin Lipidol*. 2021;15(5):629–648. doi:10.1016/j.jacl.2021.09.046.
- Brunner FJ, Waldeyer C, Ojeda F, et al., Multinational Cardiovascular Risk Consortium. Application of non-HDL cholesterol for population-based cardiovascular risk stratification: results from the Multinational Cardiovascular Risk Consortium. *Lancet*. 2019;394(10215):2173–2183. doi:10.1016/S0140-6736(19)32519-X.
- Liu J, Sempos CT, Donahue RP, Dorn J, Trevisan M, Grundy SM. Non-high-density lipoprotein and very-low-density lipoprotein cholesterol and their risk predictive values in coronary heart disease. *Am J Cardiol*. 2006;98(10):1363–1368. doi:10.1016/j.amjcard.2006.06.032.
- Quispe R, Martin SS, Michos ED, et al. Remnant cholesterol predicts cardiovascular disease beyond LDL and ApoB: a primary prevention study. *Eur Heart J*. 2021;42(42):4324–4332. doi:10.1093/eurheartj/ehab432.
- Castaner O, Pinto X, Subirana I, et al. Remnant cholesterol, not LDL cholesterol, is associated with incident cardiovascular disease. *J Am Coll Cardiol*. 2020;76(23):2712–2724. doi:10.1016/j.jacc.2020.10.008.
- Shan Z, Chen L, Lian X, et al. Iodine status and prevalence of thyroid disorders after introduction of mandatory universal salt iodization for 16 years in China: a cross-sectional study in 10 cities. *Thyroid*. 2016;26(8):1125–1130. doi:10.1089/thy.2015.0613.
- Sun X, Shan Z, Teng W. Effects of increased iodine intake on thyroid disorders. *Endocrinol Metab (Seoul)*. 2014;29(3):240–247. doi:10.3803/EnM.2014.29.3.240.

Meteorin-like Protein and Asprosin Levels in Children and Adolescents with Obesity and Their Relationship with Insulin Resistance and Metabolic Syndrome

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Keywords: Metrnl, asprosin, obesity, metabolic syndrome, insulin resistance, children

Abbreviations: Metrnl, Meteorin-like protein; ELISA, enzyme-linked immunosorbent assay; BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; AMP, adenosine monophosphate; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; PPAR γ , peroxisome proliferator-activated receptor γ ; IL, interleukin; PKA, protein kinase A; SBP, systolic blood pressure; DBP, diastolic blood pressure; MetS, metabolic syndrome; IDF, International Diabetes Federation; FBS, fasting blood sugar; TG, triglyceride; TC, total cholesterol; SD, standard deviation; T2DM, type 2 diabetes mellitus.

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ABSTRACT

Objective: Two newly discovered adipokines, including Meteorin-like protein (Metrnl) and asprosin, have been implicated in glucose and insulin metabolism. This study aimed to investigate the associations of these adipokines with obesity in children and adolescents.

Methods: This study was performed on 35 normal-weight children and 35 children with obesity. Anthropometric and biochemical parameters

were determined. Serum concentrations of Metrnl, asprosin, and insulin were measured using enzyme-linked immunosorbent assay.

Results: Metrnl level was significantly lower in obese children than normal-weight children. Additionally, Metrnl was negatively correlated with body mass index (BMI), insulin, waist-to-hip ratio, and homeostatic model assessment of insulin resistance (HOMA-IR). Our results also revealed that circulating asprosin levels were significantly increased in obese children compared to the control subjects and were positively correlated with BMI, insulin, HOMA-IR, cholesterol, and LDL-C.

Conclusion: Obesity is accompanied by significant alterations in Metrnl and asprosin and therefore these adipokines, especially Metrnl, are suggested as new promising therapeutic targets for obesity and its associated metabolic imbalances.

Childhood obesity affects more than 30 million children aged under 5 years and over 340 million children and adolescents aged between 5 and 19 years; therefore, it is one of the most important public health problems worldwide.¹ The increasing prevalence of obesity is observed both in developing and developed countries and continues to spread even more in the former.² The issue of childhood and adult obesity remains an unresolved problem in developed countries.³ Several studies have focused on the link between childhood obesity, metabolism, and cardiovascular disorder risk factors. Evidence shows that obesity, dyslipidemia, and insulin resistance in childhood predispose children to metabolic syndrome, type 2 diabetes mellitus (T2DM), and cardiovascular disease, which progress with age.⁴ Therefore, prevention and treatment of obesity in children are necessary to prevent the progression of metabolic syndrome at an early age.⁵

Adipose tissue is a highly active endocrine organ, expressing and secreting various bioactive proteins and peptides called adipokines. These adipokines participate in the regulation of critical physiological functions, including glucose and lipid metabolism, insulin sensitivity, cardiovascular functions, immune response, and inflammation.⁶ The link between obesity and cardiovascular disease seems to be due to adipose tissue dysfunction, which may stem from an imbalance between

beneficial and harmful adipokines.⁷ Leptin has been shown to be associated with VEGF, one of the critical factors in the atherosclerotic process, in patients with obesity, underlining the connection between adipose tissue dysfunction and cardiovascular disease.^{8,9} The proper function of adipose tissue is an essential element in maintaining insulin sensitivity.¹⁰

Recently, a new adipokine, called Meteorin-like (Metrl) has been identified.¹¹ Previous studies have suggested that Metrl may function as an insulin-sensitizing factor.¹² Studies on Metrl-knockout and Metrl-overexpressing transgenic mice have shown that Metrl can regulate the expression of genes involved in adipocyte differentiation, lipid metabolism, and inflammation in adipose tissue and ultimately reduce insulin resistance.¹¹ It has been shown that Metrl regulates both lipogenesis and lipolysis by upregulation of their related genes in adipocytes.¹² The expression of peroxisome proliferator-activated receptor γ (PPAR γ), a key regulator of both adipocyte differentiation and function, is significantly increased by Metrl,^{12,13} suggesting that this adipokine may stimulate insulin sensitivity effects in adipose tissue via PPAR γ . Furthermore, Metrl induces the process of conversion of white to brown fat by increasing the cytokines interleukin (IL)4/IL13 and M2 macrophage activations.¹⁴ Given the fact that brown fat could protect mammals against obesity, insulin resistance, and T2DM, an increase in circulating Metrl consequently increases the body's total energy expenditure, which is associated with the browning of white fat sources, and improves glucose tolerance in obese diabetic rats.^{14,15}

Among the newly discovered adipokines, asprosin is a fasting-induced glucogenic protein hormone that activates the protein kinase A (PKA) pathway in the liver, which then causes the rapid release of glucose into the bloodstream.¹⁶ Pathological increase in plasma asprosin has been observed in both humans and mice with insulin resistance.¹⁷ Attenuation of this adipokine leads to a remarkable decrease in glucose and insulin levels via reducing hepatic glucose release.¹⁸ Therefore, targeting asprosin can be considered a useful therapeutic method in the treatment of metabolic syndrome, which is associated with hyperinsulinemia and T2DM.¹⁹

Considering obesity as a critical and prevalent health problem in children and its fundamental role in increasing long-term morbidity and mortality, unraveling early-age obesity-related alterations and introducing diagnostic and therapeutic targets at this age are essential elements in the management of obesity and its related metabolic aberrations. Metrl and asprosin, as newly discovered adipose tissue hormones, have not been previously studied in children and adolescents with obesity. Therefore, this study aimed to evaluate these 2 adipokines in children and adolescents and their association with obesity-related metabolic abnormalities.

Materials and Methods

Subjects

This study was conducted with children and adolescents aged between 8 and 18 years. The subjects were enrolled after careful clinical evaluation and obtaining written consent. After measuring their height and weight and calculating body mass index (BMI), a total of 70 people were selected and then divided into 2 groups, obese and control subjects. Participants with BMI higher than the 95th percentile related to their age and gender entered the obese group (35 people). The control group consisted of healthy children and adolescents with no history of obesity

who had normal weight, with BMI lower than the 85th percentile and higher than the 5th percentile related to their age and sex.

The presence of other diseases, such as hormonal, cardiovascular, and kidney diseases, was ruled out, and those with a history of disease or individuals whose BMI was lower than the 5th percentile for their age and sex were excluded from the study. The subjects were not taking any medications or supplements and had normal physical activity.

Complete histories were taken and all participants underwent clinical examinations. Height, weight, waist and hip circumference, and systolic (SBP) and diastolic (DBP) blood pressure were recorded. Metabolic syndrome (MetS) was diagnosed using the International Diabetes Federation (IDF) criteria, considering the presence of 3 of 5 components of the MetS criteria: waist circumference above the 90th percentile related to age and sex, fasting blood sugar (FBS) higher than 100 mg/dL, triglyceride (TG) level higher than 150 mg/dL, high-density lipoprotein cholesterol (HDL-C) lower than 40 mg/dL, DBP higher than 130 mmHg, or SBP higher than 85 mmHg.

This study was approved by the ethics committee of Iran University of Medical Sciences (IR.IUMS.FMD.REC.1398.290) before the experiment was started and was conducted in accordance with the principles set forth in the Helsinki Declaration. All the participants and/or their guardians gave informed consent prior to participation.

Sample Collection and Biochemical Measurement

Ten mL of fasting (12–16 h) venous blood was taken from each participant. After centrifugation and separation of serum, the levels of TG, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), HDL-C, and FBS were measured by colorimetric methods using laboratory kits (Pars Azmoon). Subsequently, plasma insulin, Metrl, and asprosin levels were measured by enzyme-linked immunosorbent assay (ELISA), using Aviscera-Bioscience kits. The homeostasis model assessment of insulin resistance (HOMA-IR), which is an indicator of insulin resistance, was calculated from the fasting concentrations of insulin and glucose using the following formula: $\text{HOMA-IR} = \text{fasting serum insulin } (\mu\text{U/mL}) \times \text{fasting plasma glucose (mmol/L)} / 22.5$.²⁰ Participants with HOMA-IR values greater than 3.16 were considered to have insulin resistance.²¹

Statistical Analysis

The obtained data were analyzed using the Statistical Package of Social Science Software program (SPSS), version 21. Continuous data were expressed as mean $\pm$ standard deviation (SD). Differences between the study groups were determined by unpaired *t* tests. Pearson correlation was used to examine the relationship between variables. *P* values less than .05 were considered statistically significant.

Results

Anthropometrics and Biochemical Measurements

The anthropometric and metabolic parameters of the study groups are shown in **TABLE 1**. There were no significant differences in age, FBS, SBP, and DBP, whereas BMI, waist circumference, hip circumference, and waist-to-hip ratio were found to be significantly higher in the obese group than the normal-weight group. Furthermore, the results showed significantly increased levels of insulin, TG, LDL-C, HDL-C, TC, HOMA-IR, and visfatin in the obese group compared with the control group.

TABLE 1. Anthropometric and Metabolic Characteristics of the Studied Groups^a

Variables	Control (n = 35)	Obese (n = 35)	P Value
Age (mo)	138.77 ± 25.65	132.57 ± 32.74	.381
BMI (kg/m ²)	18.41 ± 2.65	28.13 ± 5.75	<.001
MetS [yes (%)]	0 (0)	13 (37.1)	<.001
Insulin resistance [yes (%)]	3 (8.6)	26 (74.3)	<.001
Waist circumference (cm)	61.65 ± 6.57	84.80 ± 13.46	<.001
Hip circumference (cm)	78.37 ± 8.16	97.63 ± 13.63	<.001
Waist-to-hip ratio	0.79 ± 0.05	0.87 ± 0.10	<.001
SBP (mmHg)	9.54 ± 1.19	10.11 ± 1.45	.077
DBP (mmHg)	6.86 ± 0.88	7.26 ± 1.22	.120
FBS (mg/dL)	90.00 ± 4.92	92.06 ± 6.55	.142
Insulin (μU/mL)	7.75 ± 10.14	19.78 ± 9.65	<.001
HOMA-IR	1.76 ± 2.40	4.50 ± 2.23	<.001
Triglycerides (mg/dL)	73.94 ± 31.93	126.68 ± 69.72	<.001
Total cholesterol (mg/dL)	148.66 ± 19.06	171.54 ± 24.97	<.001
LDL-C (mg/dL)	75.80 ± 13.14	92.48 ± 16.82	<.001
HDL-C (mg/dL)	54.60 ± 7.50	47.66 ± 9.59	.001
visfatin (ng/mL)	1.49 ± 0.87	2.09 ± 1.03	.012

BMI, body mass index; DBP, diastolic blood pressure; FBS, fasting blood sugar; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

^aValues are expressed as mean ± SD except where otherwise indicated. Comparison of variables means in 2 groups was performed using Student t-test.

Serum Levels of Metrnl and Asprosin

Asprosin serum levels were significantly higher, whereas Metrnl levels were lower in the obese group than in the control group (FIGURES 1A and 1D). According to the IDF criteria, 13 cases were diagnosed with MetS. As shown in FIGURES 1B and 1E, the individuals with MetS showed increased asprosin level (7.36 ± 2.01 vs 8.93 ± 2.02 nmol/L) and decreased Metrnl serum level (91.69 ± 29.48 vs 69.35 ± 23.74 pg/mL), in comparison with those without MetS. Overall, 41 subjects had normal insulin sensitivity and 29 subjects were insulin resistant. The subjects with insulin resistance demonstrated a considerable increase in asprosin (6.69 ± 1.75 vs 9.00 ± 1.76 nmol/L) and a decrease in Metrnl circulating levels (100.4 ± 27.84 vs 69.38 ± 21.87 pg/mL), compared with the control group (FIGURES 1C and 1F). Taken together, these results indicate that serum levels of Metrnl and asprosin are altered in obesity, especially in those with MetS and insulin resistance.

Correlation Analysis of Metrnl and Asprosin

Correlation analysis was performed in the entire study population and, separately, obese and control groups, and the results are presented in TABLE 2 and TABLE 3. Metrnl was found to be negatively correlated with BMI, waist circumference, hip circumference, waist-to-hip ratio, insulin, and HOMA-IR in the whole population. Additionally, Metrnl showed significant inverse correlations with BMI and waist circumference in the obese group. On the other hand, the results revealed that asprosin was positively correlated with BMI, waist circumference, hip circumference, insulin, HOMA-IR, TG, cholesterol, and LDL-C. In addition, asprosin showed significant positive correlations with both insulin and HOMA-IR in the obese group. Moreover, asprosin was observed to

be inversely correlated with visfatin, SBP, and DBP in the control group. There was a considerable negative correlation between asprosin and Metrnl serum levels in the entire studied population.

Discussion

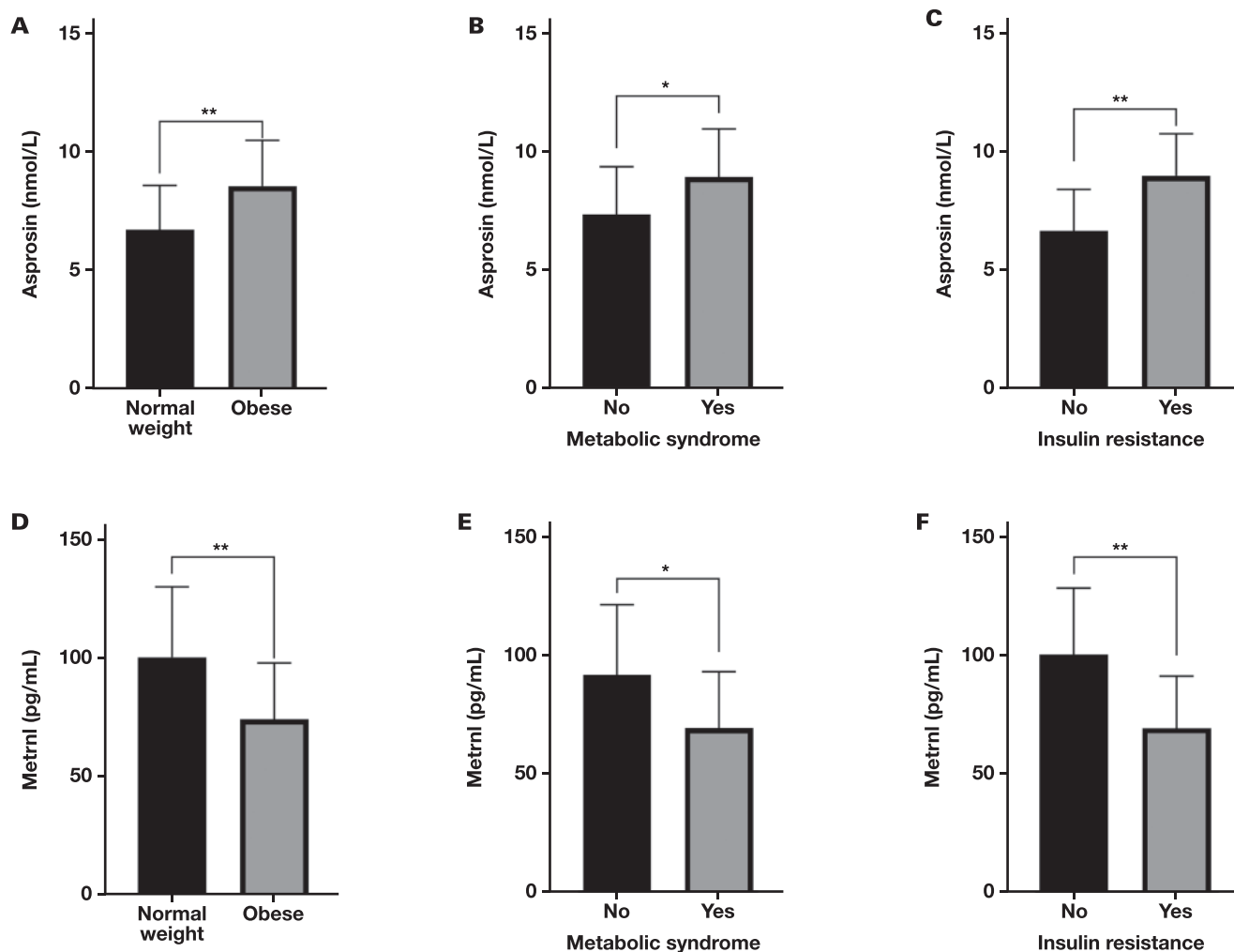
In this study, Metrnl was found to be significantly lower in children and adolescents with obesity than in controls, with a negative correlation with obesity-related indicators, including BMI and waist circumference. These results are in line with those presented by Fouani et al,²² who showed a significant decrease in serum Metrnl levels in overweight/obese women with polycystic ovary syndrome. Similarly, Dadmanesh et al²³ demonstrated lower serum levels of Metrnl in patients with coronary artery disease and T2DM and its negative association with insulin resistance and inflammatory cytokines.

Our results also showed that Metrnl serum concentrations were significantly decreased in obese children and adolescents with metabolic syndrome as well as those with insulin resistance. A significant negative correlation between Metrnl and indices of insulin resistance, including insulin levels and HOMA-IR, was also observed. A similar relationship has been reported in previous studies.²² Shreds of evidences from animal models have shown that Metrnl can act as a local insulin-sensitizer in white adipose tissue through the PPARγ pathway.¹² Metrnl is also able to enhance insulin action in adipose tissue by stimulating the insulin-induced phosphorylation of AKT.¹¹ However, the results of some studies are in contrast to the proposed role of Metrnl in obesity. Wang et al²⁴ showed that serum levels of Metrnl were elevated in patients with newly diagnosed T2DM and significantly correlated with lipid and glycemic profile and insulin resistance. Al-Khairi et al²⁵ reported elevated plasma levels of Metrnl in individuals with T2DM and obesity as well as a significant correlation between Metrnl and BMI in obese subjects. However, the results of some other studies are indicative of no association between serum Metrnl and BMI.^{26,27} These controversies might arise from the heterogeneous nature of the participants and confounding factors that are primarily found in adult subjects, methodological differences, or the obesity cutoffs. In our study, Metrnl was observed to be negatively correlated with BMI, insulin, waist-to-hip ratio, and HOMA-IR in children and adolescents with obesity, which is consistent with the biological effects that had been previously attributed to Metrnl through in vivo and in vitro studies.¹¹ Regarding the insulin-sensitizing action of Metrnl, lower levels of this adipokine in children and adolescents with obesity might contribute to exacerbating insulin resistance in these subjects.

Many favorable functions have been described for Metrnl through various in vitro and in vivo studies. Metrnl has been found to be capable of repairing the ischemic heart²⁸ and protecting the heart against cardiac dysfunction, upregulating thyroid hormone secretion, inducing uncoupler proteins,²⁹ increasing osteoblast differentiation and bone mineralization,³⁰ suppressing proinflammatory responses,³¹ regenerating skeletal muscle,³² and ameliorating insulin resistance in skeletal muscle cells.³³ Therefore, this molecule can be considered valuable in human biology with beneficial effects that are lost in children with obesity early in life.

Animal studies have shown that intravenous administration of Metrnl improves glucose tolerance, attenuates pancreatic inflammation, and reduces the inflammatory cytokines.³⁴ On the other hand, interventions to reduce body weight, such as bariatric surgery, have been shown to augment Metrnl levels,³⁵ indicating that there is a potential for this molecule to be manipulated in obesity.

FIGURE 1. Comparison of asprosin and Meteorin-like protein (Metrl) in various studied groups. A, Asprosin levels in normal-weight and obese subjects. B, Serum asprosin levels according to the presence of metabolic syndrome. C, Asprosin serum levels according to the presence of insulin resistance. D, Serum levels of Metrl in normal-weight and obese participants. E, Serum Metrl levels according to the presence of metabolic syndrome. F, Metrl serum levels according to the presence of insulin resistance. * $P < .05$; ** $P < .01$.



Asprosin, a novel adipokine, is pathologically increased in individuals with insulin resistance.¹⁷ The association between asprosin and metabolic syndrome has been investigated in some previous studies.¹⁹ Naemian et al³⁶ found elevated serum concentrations of asprosin in patients with T2DM as well as positive and significant correlations among asprosin and BMI, FBS, HOMA-IR, and TC/HDL-C ratio in T2DM patients. In addition, they showed that subjects with higher BMI had higher concentrations of asprosin than individuals with lower BMI. Our results reveal that circulating asprosin levels were significantly increased in children and adolescents with obesity compared with the controls, which is consistent with the findings of a study by Silister et al,³⁷ who showed that serum asprosin levels were significantly different among children with normal weight, overweight, and obesity. Similarly, a prospective cohort study found higher asprosin concentrations in obese participants than in controls. Nevertheless, some contradictory results exist; for instance, a cross-sectional study showed that serum asprosin concentrations were significantly decreased in children with obesity aged between 6 and 14 years compared with healthy normal-weight children, and were negatively correlated with BMI,³⁸ suggesting

the conflicting role of asprosin in obesity. The data also showed a remarkable relationship between asprosin and insulin resistance as well as MetS. Consistently, higher asprosin levels in children with IR than non-IR children and its correlation with the parameters of MetS have been reported.^{39,40}

The strength of this study was to consider subjects at younger ages, including children and adolescents, because the causes of childhood obesity are complicated and heterogeneous,⁴¹ and the management of obesity in childhood differs from that in adulthood.⁴² Prevention and treatment of childhood obesity should be prioritized because children with obesity are more likely to develop serious complications and health problems.⁴² A limitation of this study was its small sample size; however, we attempted to compensate by carefully selecting the cases and matching the control group to limit the intervening factors.

Conclusions

The results of this study show the associations of Metrl and asprosin, as newly identified adipokines, with obesity, MetS, and insulin resistance

TABLE 2. Correlation Analysis of Meteorin-like Protein with Anthropometric and Biochemical Data

	Entire Study Population (n = 70)		Obese (n = 35)		Control (n = 35)	
	<i>r</i>	<i>P</i>	<i>R</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age	−0.086	.446	−0.251	.145	−0.053	.752
BMI	−0.514	<.001	−0.383	.023	−0.277	.107
Waist circumference	−0.458	<.001	−0.425	.011	0.071	.685
Hip circumference	−0.417	<.001	−0.265	.124	−0.108	.535
Waist-to-hip ratio	−0.264	.027	−0.313	.067	0.324	.057
SBP	−0.162	.181	−0.030	.862	−0.125	.473
DBP	−0.065	.591	0.119	.496	−0.088	.614
FBS	−0.108	.372	0.028	.873	−0.102	.560
Insulin	−0.388	.001	−0.300	.080	−0.131	.453
HOMA-IR	−0.387	.001	−0.305	.075	−0.133	.445
Triglycerides	−0.104	.391	0.034	.847	0.293	.087
Cholesterol	−0.107	.378	0.149	.394	0.105	.546
LDL-C	−0.117	.334	0.186	.284	0.074	.672
HDL-C	0.105	.385	−0.043	.806	−0.112	.520
Visfatin	0.026	.832	0.081	.643	0.291	.089

BMI, body mass index; DBP, diastolic blood pressure; FBS, fasting blood sugar; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

TABLE 3. Correlation Analysis of Asprosin with Anthropometric and Biochemical Data

	Entire Study Population (n = 70)		Obese (n = 35)		Control (n = 35)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age	−0.040	.745	0.139	.424	−0.165	.343
BMI	0.430	<.001	0.298	.082	−0.084	.633
Waist circumference	0.383	.001	0.243	.159	−0.213	.218
Hip circumference	0.364	.002	0.294	.086	−0.202	.244
Waist-to-hip ratio	0.215	.075	−0.003	.986	−0.023	.895
SBP	−0.058	.636	−0.042	.809	−0.338	.047
DBP	−0.101	.404	−0.061	.726	−0.426	.011
FBS	−0.050	.682	−0.182	.294	−0.096	.583
Insulin	.470	<.001	0.400	.017	0.226	.191
HOMA-IR	.453	<.001	0.367	.030	0.224	.195
Triglycerides	.222	.065	0.132	.449	−0.184	.289
Cholesterol	.268	.025	0.106	.546	0.046	.795
LDL-C	.256	.032	0.108	.537	−0.022	.899
HDL-C	−0.210	.081	−0.177	.309	0.114	.515
Visfatin	.030	.804	0.056	.748	−0.335	.049

BMI, body mass index; DBP, diastolic blood pressure; FBS, fasting blood sugar; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

in children and adolescents, which suggests a possible role for these adipokines in the pathogenic mechanisms of obesity. The alteration of these 2 markers in childhood obesity indicates their potential as therapeutic targets and diagnostic markers in managing obesity and its associated metabolic aberrations. However, the mechanisms through which asprosin and Metrnl affect obesity have not been fully elucidated yet. Further observational studies are needed to demonstrate the roles of these adipokines in the etiology of childhood obesity and its associated disorders.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Di Cesare M, Sorić M, Bovet P, et al. The epidemiological burden of obesity in childhood: a worldwide epidemic requiring urgent action. *BMC Med.* 2019;17(1):212. doi:10.1186/s12916-019-1449-8.
2. Wang Y, Pan L, Wan S, et al. Increasing prevalence of overweight and obesity in Yi farmers and migrants from 2007 to 2015 in China: the Yi migrant study. *BMC Public Health.* 2018;18(1):659. doi:10.1186/s12889-018-5577-4.
3. Lean ME, Astrup A, Roberts SB. Making progress on the global crisis of obesity and weight management. *BMJ.* 2018;361:k2538.
4. Mottaghi A, Mirmiran P, Pourvali K, Tahmasbpour Z, Azizi F. Incidence and prevalence of childhood obesity in Tehran, Iran in 2011. *Iran J Public Health.* 2017;46(10):1395.
5. Fornari E, Maffei C. Treatment of metabolic syndrome in children. *Front Endocrinol.* 2019;10:702.
6. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab.* 2004;89(6):2548–2556. doi:10.1210/jc.2004-0395.
7. Nakamura K, Fuster JJ, Walsh K. Adipokines: a link between obesity and cardiovascular disease. *J Cardiol.* 2014;63(4):250–259. doi:10.1016/j.jcc.2013.11.006.
8. Skrypnik D, Mostowska A, Jagodziński PP, Bogdański P. Association of rs699947 (–2578 C/A) and rs2010963 (–634 G/C) single nucleotide polymorphisms of the VEGF Gene, VEGF-A and leptin serum level, and cardiovascular risk in patients with excess body mass: a case-control study. *J Clin Med.* 2020;9(2):469. doi:10.3390/jcm9020469.
9. Skrypnik D, Skrypnik K, Pelczyńska M, et al. The effect of Plantago major supplementation on leptin and VEGF-A serum levels, endothelial dysfunction and angiogenesis in obese women—a randomised trial. *Food Funct.* 2021;12(4):1708–1718. doi:10.1039/d0fo01878c.
10. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol.* 2008;9(5):367–377. doi:10.1038/nrm2391.
11. Zheng S-l, Li Z-y, Song J, Liu J-m, Miao C-y. Metrnl: a secreted protein with new emerging functions. *Acta Pharmacol Sin.* 2016;37(5):571–579. doi:10.1038/aps.2016.9.
12. Li Z-Y, Song J, Zheng S-L, et al. Adipocyte Metrnl antagonizes insulin resistance through PPAR γ signaling. *Diabetes.* 2015;64(12):4011–4022. doi:10.2337/db15-0274.
13. Semple RK, Chatterjee VKK, O'Rahilly S. PPAR γ and human metabolic disease. *J Clin Invest.* 2006;116(3):581–589. doi:10.1172/JCI28003.
14. Rao RR, Long JZ, White JP, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell.* 2014;157(6):1279–1291. doi:10.1016/j.cell.2014.03.065.
15. Kim SH, Plutzky J. Brown fat and browning for the treatment of obesity and related metabolic disorders. *Diabetes Metab J.* 2016;40(1):12–21. doi:10.4093/dmj.2016.40.1.12.
16. Romere C, Duerschmid C, Bournat J, et al. Asprosin, a fasting-induced glucogenic protein hormone. *Cell.* 2016;165(3):566–579. doi:10.1016/j.cell.2016.02.063.
17. Wang Y, Qu H, Xiong X, et al. Plasma asprosin concentrations are increased in individuals with glucose dysregulation and correlated with insulin resistance and first-phase insulin secretion. *Mediators Inflamm.* 2018;2018:1–7. doi:10.1155/2018/9471583.
18. Zhang X, Jiang H, Ma X, Wu H. Increased serum level and impaired response to glucose fluctuation of asprosin is associated with type 2 diabetes mellitus. *J Diabetes Investig.* 2020;11(2):349–355. doi:10.1111/jdi.13148.
19. Yuan M, Li W, Zhu Y, Yu B, Wu J. Asprosin: a novel player in metabolic diseases. *Front Endocrinol.* 2020;11:64. doi:10.3389/fendo.2020.00064.
20. Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment: insulin resistance and β -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* 1985;28(7):412–419.
21. Keskin M, Kurtoglu S, Kendirci M, Atabek ME, Yazici C. Homeostasis model assessment is more reliable than the fasting glucose/insulin ratio and quantitative insulin sensitivity check index for assessing insulin resistance among obese children and adolescents. *Pediatrics.* 2005;115(4):e500–e503. doi:10.1542/peds.2004-1921.
22. Fouani FZ, Fadaei R, Moradi N, et al. Circulating levels of Meteorin-like protein in polycystic ovary syndrome: a case-control study. *PLoS One.* 2020;15(4):e0231943. doi:10.1371/journal.pone.0231943.
23. Dadmanesh M, Aghajani H, Fadaei R, Ghorban K. Lower serum levels of Meteorin-like/subfatin in patients with coronary artery disease and type 2 diabetes mellitus are negatively associated with insulin resistance and inflammatory cytokines. *PLoS One.* 2018;13(9):e0204180. doi:10.1371/journal.pone.0204180.
24. Wang K, Li F, Wang C, et al. Serum levels of Meteorin-like (Metrnl) are increased in patients with newly diagnosed type 2 diabetes mellitus and are associated with insulin resistance. *Med Sci Monit.* 2019;25:2337.
25. AlKhairi I, Cherian P, Abu-Farha M, et al. Increased expression of Meteorin-like hormone in type 2 diabetes and obesity and its association with irisin. *Cells* 2019;8(10):1283.
26. El-Ashmawy HM, Selim FO, Hosny TA, Almassry HN. Association of low serum Meteorin like (Metrnl) concentrations with worsening of glucose tolerance, impaired endothelial function and atherosclerosis. *Diabetes Res Clin Pract.* 2019;150:57–63. doi:10.1016/j.diabres.2019.02.026.
27. Chung HS, Hwang SY, Choi JH, et al. Implications of circulating Meteorin-like (Metrnl) level in human subjects with type 2 diabetes. *Diabetes Res Clin Pract.* 2018;136:100–107. doi:10.1016/j.diabres.2017.11.031.
28. Huynh K. Meteorin-like protein repairs the ischaemic heart via receptor KIT in endothelial cells. *Nat Rev Cardiol.* 2022;19(9):575. doi:10.1038/s41569-022-00752-3.
29. Şekerci G, Erden Y, Tekin S. Effects of Meteorin-like hormone on endocrine function of hypothalamo-hypophyseal system and peripheral uncoupling proteins in rats. *Mol Biol Rep.* 2022;49(7):5919–5925. doi:10.1007/s11033-022-07374-5.
30. Huang R, Balu AR, Molitoris KH, et al. The role of Meteorin-like in skeletal development and bone fracture healing. *J Orthop Res.* 2022;40(11):2510–2521.
31. Jung TW, Pyun DH, Kim TJ, et al. Meteorin-like protein (METRNL)/IL-41 improves LPS-induced inflammatory responses via AMPK or PPAR δ -mediated signaling pathways. *Adv Med Sci.* 2021;66(1):155–161. doi:10.1016/j.advms.2021.01.007.
32. Baht GS, Bareja A, Lee DE, et al. Meteorin-like facilitates skeletal muscle repair through a Stat3/IGF-1 mechanism. *Nat Metab.* 2020;2(3):278–289. doi:10.1038/s42255-020-0184-y.
33. Lee JO, Byun WS, Kang MJ, et al. The myokine Meteorin-like (metrnl) improves glucose tolerance in both skeletal muscle cells and mice by targeting AMPK α 2. *FEBS J.* 2020;287(10):2087–2104. doi:10.1111/febs.15301.
34. Yao Z, Lin P, Wang C, Wang K, Sun Y. Administration of metrnl delays the onset of diabetes in non-obese diabetic mice. *Endocr J.* 2021;68(2):179–188. doi:10.1507/endocrj.EJ20-0351.
35. Jamal MH, AlOtaibi F, Dsouza C, et al. Changes in the expression of Meteorin-like (METRNL), irisin (FNDC5), and uncoupling proteins (UCPs) after bariatric surgery. *Obesity (Silver Spring)* 2022;30(8):1629–1638. doi:10.1002/oby.23473.
36. Naiemian S, Naeemipour M, Zarei M, et al. Serum concentration of asprosin in new-onset type 2 diabetes. *Diabetol Metab Syndr.* 2020;12(1):1–8.
37. Sünnetçi Silistre E, Hatipoğlu HU. Increased serum circulating asprosin levels in children with obesity. *Pediatr Int.* 2020;62(4):467–476. doi:10.1111/ped.14176.
38. Long W, Xie X, Du C, et al. Decreased circulating levels of asprosin in obese children. *Hormone Res Paediatr.* 2019;91(4):271–277. doi:10.1159/000500523.
39. Li X, Liao M, Shen R, et al. Plasma asprosin levels are associated with glucose metabolism, lipid, and sex hormone profiles in females

- with metabolic-related diseases. *Mediators Inflamm.* 2018;2018:1–12. doi:[10.1155/2018/7375294](https://doi.org/10.1155/2018/7375294).
40. Wang M, Yin C, Wang L, et al. Serum asprosin concentrations are increased and associated with insulin resistance in children with obesity. *Ann Nutr Metab.* 2019;75(4):205–212. doi:[10.1159/000503808](https://doi.org/10.1159/000503808).
41. Lytle LA. Examining the etiology of childhood obesity: the IDEA study. *Am J Community Psychol.* 2009;44(3-4):338–349. doi:[10.1007/s10464-009-9269-1](https://doi.org/10.1007/s10464-009-9269-1).
42. Hoey H. Management of obesity in children differs from that of adults. *Proc Nutr Soc.* 2014;73(4):519–525. doi:[10.1017/s0029665114000652](https://doi.org/10.1017/s0029665114000652).

Biclonal Gammopathies in South Tunisia: Clinical and Biological Characteristics

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Keywords: paraproteinemias, immunofixation, electrophoresis, immunoglobulin, Tunisia, epidemiology

Abbreviations: BGs biclonal gammopathies; MM, multiple myeloma; Ig, immunoglobulin; BGUS, BG of undetermined significance; MGs, monoclonal gammopathies; HCs, heavy chains, LC, light chain; IF, immunofixation; IEP, immunoelectrophoresis; SPE, serum protein electrophoresis; DMGMs, double monoclonal gammopathy manifestations

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ABSTRACT

Objective: Biclonal gammopathies (BGs) are rare situations characterized by the production of 2 monoclonal proteins. There are no available data on BGs in North Africa. We aimed to estimate the prevalence of BGs in our population and describe their clinical and laboratory features.

Methods: We conducted a 31-year retrospective study including patients with persistent double monoclonal bands based on the results of immunofixation/immunoelectrophoresis.

Results: A total of 35 patients with available clinical data (sex ratio, M/F = 1.53; mean age, 70 ± 10.87 years [range, 45–90 years]) were included. The main associated conditions were multiple myeloma (MM) (40%), BG of undetermined significance (BGUS) (34%), and lymphoproliferative diseases (23%). Only one-third of the patients had

2 monoclonal spikes on serum protein electrophoresis. The most common paraprotein combinations were immunoglobulin (Ig)G-IgG (25%) and IgG-IgA (23%) with different light chains in one-half of the cases. The mean follow-up was 25.6 months (median, 12 months). No BGUS evolved into a malignant disease.

Conclusion: BGs are rare in clinical laboratory routine but must be accurately identified by the pathologist. Our cohort is characterized by a high prevalence of BGUS compared with MM.

Biclonal gammopathies (BGs) are defined as a clonal proliferation of plasma cells or B-lymphoid progenitors that produces 2 different monoclonal proteins known as M-proteins or paraproteins. These 2 paraproteins may result from production from 2 different plasma cell clones or a single plasma cell clone.^{1,2} Unlike monoclonal gammopathies (MGs), BGs are not common. Their prevalence is reported to be 1.5% to 4% among patients with MG.¹⁻³

Patients with BG can express M-proteins with different isotypes of heavy chains (HCs) that are light chain (LC) isotype-matched, or same HC isotypes with different LC isotypes, or different HC isotypes with different LC isotypes. The latter situation would signify true BG, most likely arising from 2 or more distinct cell populations.²

Although clinical characteristics and therapeutic responses of BGs have been reported to be similar to those of MG,^{4,5} recognizing BGs is important for the appropriate interpretation and follow-up. For multiple myeloma (MM) patients, evolution and treatment response can vary between clones. Further treatment decisions may be therefore indicated.⁵

On the other hand, BGs are known to be associated mainly with biclonal gammopathy of undetermined significance (BGUS), B lymphoproliferative disorders, and less commonly with MM.⁴ However, the distribution of these diseases can vary worldwide.^{2,5,6}

There are limited studies of BG in the literature, mainly reported as isolated case reports. In Tunisia, there are no known studies that have analyzed BG. We aimed to estimate the prevalence of BGs in our laboratory in a long-term study, to describe their clinical and laboratory features as well as the underlying diseases with follow-up for available cases.

Materials and Methods

Study Population

We conducted a 31-year retrospective study from January 1992 to June 2022. Among all cases with a confirmed MG, we included those with BG based on the results of immunofixation (IF)/immunoelectrophoresis (IEP) and whose clinical data were available. Medical records of patients with BG were retrieved. We collected data on patients' underlying diseases and initial clinical symptoms including laboratory data. We excluded patients with a pattern of complete immunoglobulin (Ig) molecule, along with excess free LCs with the same isotype.

Immunofixation

IF was performed with Hydragel IF (HYDRAGEL 1, Bence-Jones; Sebia) manually until 2010; then Automate (Sebia) was used according to the manufacturer's instructions to identify the isotype of Ig and its LC.

To avoid the possibility of false-positive double monoclonal gammopathy because of Ig polymerization, serum was pretreated with beta-mercapthanol (1%), which is a strong reducing agent breaking disulfide bonds in proteins. Then IF was performed again. Only sera with persistent double monoclonal bands were included.

Quantitative Ig assays for IgG, IgA, IgM, and were determined by nephelometry on a BN-Prospect System instrument (Siemens).

Statistical analyses were performed using SPSS version 22.0.

Results

Prevalence of BG

Among all serum samples received in our immunology laboratory with requests for IF between January 1992 and June 2022 (n = 15,717), we had a total of 2005 cases (13%) of MGs. Among them, there were 58 cases of BG accounting for 3% of all MG cases and 0.4% of all serum

samples received during the study period. Only patients with clinical records available were included in this study (n = 35).

Patient Characteristics

Epidemiology

Medical records were available for 35 patients (60%). Twenty-two patients (63%) were men, and 13 (37%) were women (sex ratio, M/F = 1.53). The mean age at diagnosis was 70 ± 10.87 years (range, 45–90 years). Twenty-five patients (71%) were aged 65 years or over and only 2 patients (6%) were aged less than 50 years.

Patient's Clinical Characteristics and Associated Diseases

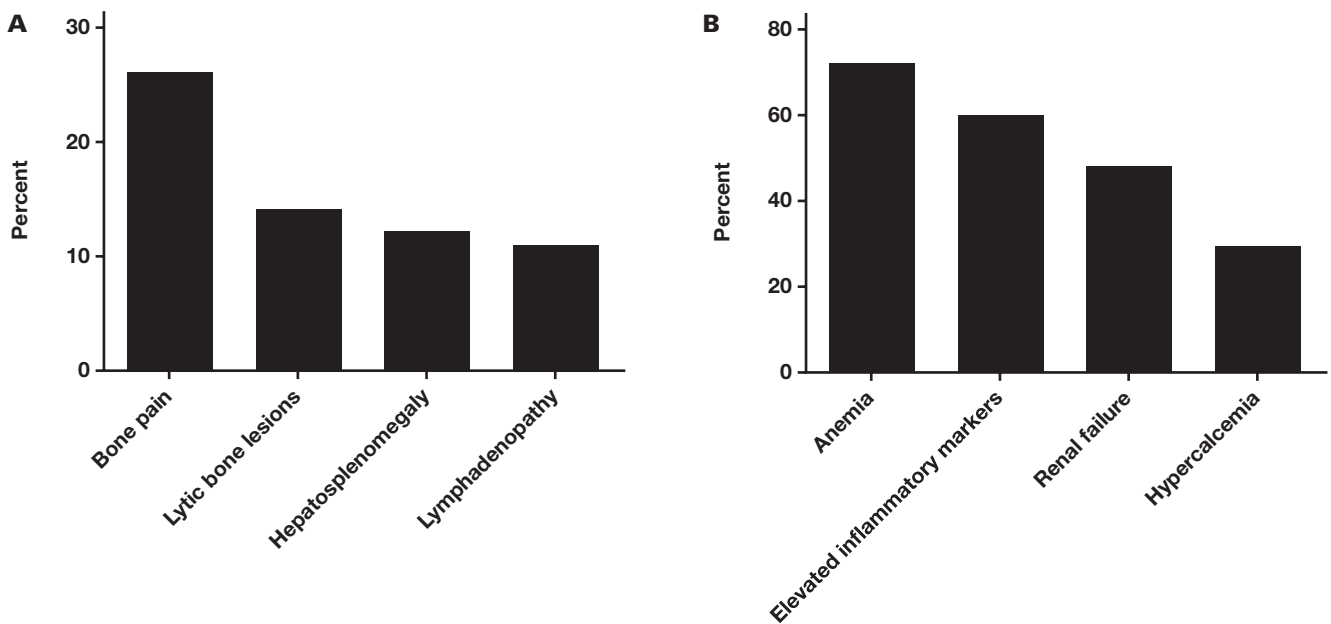
The most frequent clinical signs at diagnosis are shown in **FIGURE 1A**. The most frequent diagnoses were MM (40%), BGUS (34%), and lymphoproliferative diseases (23%) (**TABLE 1** and **supplementary material**). BG was identified in 1 patient already known to have MM associated with amyloidosis following chemotherapy.

MM patients (mean age, 72 ± 8 years) were older than those with BGUS (mean age, 69 ± 15 years; *P* = .5) or lymphoproliferative diseases (mean age, 68 ± 8 years; *P* = .3) with more frequent bone pain (*P* = .2) and bone osteolytic lesions (*P* = .6). Hepatosplenomegaly and lymphadenopathy were associated with lymphoproliferative diseases (*P* = .01 and .02, respectively). Five patients (14%) (3 BGUS, 1 MM, and 1 B-cell lymphoma) had associated autoimmune diseases (**supplementary material**; patients 15, 16, 21, 33, and 35)

Follow-up

The mean follow-up was 25.6 months (median, 12 months) for included patients. Of the 12 patients with BGUS, 7 patients (58%) had no malignant transformation of their disease within a mean follow-up period of 5 ± 3.55 years, 4 patients were lost to follow-up, and 1 patient died. Of the 14 patients with MM, 4 patients died because of disease progression or complications related to disease, 2 are still alive, and the remaining 8 patients were lost to follow-up.

FIGURE 1. Most frequent clinical (A) and biological (B) features in patients with bicalonal gammopathy.



Of the 14 patients with MM, 4 patients died because of disease progression or complications related to disease, 2 are still alive, and the remaining 8 patients were lost to follow-up.

Laboratory Test Results

Serum Protein Electrophoresis

Serum protein electrophoresis (SPE) was performed for all patients. Only 11 patients among 35 (31%) had 2 monoclonal spikes. Twenty-two patients (65%) had a monoclonal spike mainly in the gamma region. For the 2 remaining patients, 1 had a normal electrophoretic pattern and the other had a polyclonal increase of gamma globulins at the SPE.

Immunofixation

Among the 35 patients with BG included, the most common M-protein combinations were IgG-IgG (25%) and IgG-IgA (23%). We had 1 case of triclonal gammopathy (IgG-IgG-IgA) (TABLE 2).

The LCs of the monoclonal Ig were different (kappa K and lambda λ) in approximately one-half of the BGs (18 cases; 51%). The LCs were of the same isotype (κκ or λλ) in 10 cases (28%) and 7 cases (20%), respectively.

TABLE 1. Spectrum of Hematologic Diagnoses Identified in Patients with Biclonal Gammopathy (n = 35)

Hematologic Diagnosis	No. (%) of Patients
MM	14 (40)
BGUS	12 (34)
Lymphoproliferative diseases	8 (23)
Non-Hodgkin lymphoma	5 (14)
Chronic lymphocytic leukemia	2 (6)
Waldenström's macroglobulinemia	1 (3)
Extramedullary plasmocytoma	1 (3)
Total	35 (100)

BGUS, biclonal gammopathy of undetermined significance; MM, multiple myeloma.

Other Laboratory Tests

The most observed biological features were anemia and elevated inflammatory markers such as elevated erythrocyte sedimentation rate and increased α globulins fraction in electrophoresis (FIGURE 1B).

Discussion

Unlike MGs, there are limited data on BGs^{1,2,5-8} and there is no study on this rare pathology in Tunisian or African populations. We performed a retrospective study in which we described the clinical and laboratory features of BGs in the southern Tunisian population.

It is noteworthy that the terms BGs and double monoclonal gammopathy manifestations (DMGMs) are often used interchangeably. However, they are pathogenically different. Patients with DMGMs can express 2 different M-proteins that differ in HC isotypes with the same LCs or LC isotypes with the same HC isotype. However, patients with true BG express M-proteins that differ in HC and LC isotypes.^{2,3}

We chose to exclude patients with a complete monoclonal Ig along with an additional band constituted by excess free LCs with different mobility but similar isotype. This particular pattern could not be considered as BG as it lacks 2 distinct isotypes of HCs and did not possess different LC subtypes. This pattern has been associated with a worse prognosis because of renal dysfunction, extramedullary plasmacytomas, and shorter survival.^{9,10}

Prevalence of BG

In this study, the prevalence of BG is 3% among all MGs, which is in line with other studies that reported BGs prevalence to be between 1.5% to 4%^{1-3,6,8} (TABLE 3). Comparing available data in literature must take into account environmental factors, population epidemiological characteristics, and the sensitivity of the techniques used to identify and characterize M-proteins. We used the IF technique, which is reported to be more sensitive than agarose gel or capillary zone electrophoresis in detecting and identifying MGs.¹¹ Studies that use electrophoresis and immunosubstraction rather

TABLE 2. Heavy and Light Chain Combinations in the 35 Biclonal Gammopathy Patients Included

Heavy Chain Combinations	No. (%)	Light Chain Combinations (No.)		
		λλ	Κλ	κκ
IgG-IgG	9 (25)	2	4	3
IgG-IgA	8 (23)	2	2	4
IgG-IgM	5 (14)	1	1	3
IgA-IgM	3 (8)	0	3	0
IgG-IgD	2 (6)	0	2	0
IgG-LC	2 (6)	0	2	0
IgA-IgA	1 (3)	1	0	0
IgA-IgD	1 (3)	0	1	0
IgA-LC	2 (6)	0	2	0
IgM-LC	1 (3)	0	1	0
IgG-IgG-IgA	1 (3)	1	0	0
Total	35 (100)	7 (20%)	18 (51%)	10 (29%)

Ig, immunoglobulin; LC, light chain.

TABLE 3. Comparison of Our Data with Those Reported in the Literature

Study (Country)	No. of Patients Included	Prevalence of BG, %	Isotype Combinations No. (%)					Light Chain Combinations No. (%)			Underlying Disease No. (%)			
			IgG-IgG	IgG-IgA	IgG-IgM	IgA-IgA	Others	λλ	κλ	κκ	MM	BGUS	Lymphoproliferative Diseases	Extramedullary Plasmacytoma
Kyle et al ¹ (USA)	57	1.5	6 (11)	29 (51)	8 (14)	1 (2)	13 (22)	7 (12)	16 (28)	21 (37)	9 (16)	37 (65)	11 (19)	0
Riddell et al ¹³ (Canada)	28	2.5	0	10 (36)	10 (36)	0	8 (28)	2 (7)	12 (43)	9 (32)	11 (39)	6 (22)	11 (39)	0
Nilsson et al ⁷ (Sweden)	20	1.9	9 (45)	7 (35)	2 (10)	0	2 (10)	2 (10)	12 (60)	6 (30)	7 (35)	8 (40)	4 (20)	1 (5)
Guastafierro et al ¹⁴ (Italy)	34	2.8	9 (26)	4 (12)	6 (18)	2 (6)	13 (38)	4 (12)	19 (56)	10 (29)	6 (18)	16 (47)	12 (35)	0
Garcia et al ⁶ (Spain)	47	2.9	17 (36)	9 (19)	10 (21)	2 (5)	9 (19)	9 (19)	18 (38)	20 (43)	9 (19)	20 (43)	18 (38)	0
Sharma et al ² (India)	15	1.6	2 (13)	6 (40)	1 (7)	3 (20)	3 (20)	1 (6)	7 (47)	7 (47)	12 (82)	1 (6)	1 (6)	1 (6)
Our study (Tunisia)	35	3	9 (25)	8 (23)	5 (14)	1 (3)	2 (6)	7 (20)	18 (51)	10 (29)	14 (40)	12 (34)	8 (23)	1 (3)

BG, biclonal gammopathy; BGUS, BG of undetermined significance; Ig, immunoglobulin; MM, multiple myeloma.

than immunofixation may underestimate the prevalence of BGs. Also, some authors included only patients with 2 M-proteins at the initial diagnosis because of the retrospective design of their studies. Nevertheless, additional M-proteins may emerge later following observation or treatment.^{5,12} In our study, we tried to include both cases. Indeed, we had 1 patient with MM associated with amyloid light chain amyloidosis that developed an additional monoclonal peak after treatment. Previous studies have demonstrated that patients with MM in whom a second and unrelated clone emerged during therapy had improved overall survival compared with patients without a secondary clone.¹²

Epidemiological Characteristics and Underlying Diseases

In our study, the mean age at diagnosis was 70 years and the sex was predominantly male, which is consistent with that described in other monoclonal^{15–17} and biclonal series.^{1,2,6–8,13,18}

The largest patient group of BGs in our cohort was the MM group (40%) followed by the BGUS group (34%) and lymphoproliferative diseases (23%). However, in most of the published data, BGUS is the most common diagnosis. This is in accordance with previous MG cohorts. The most frequent diagnosis of MGs has been MGUS in Western studies and MM in previous North African studies.^{8,15,17} It is worthwhile to note that the prevalence of MM seems to have decreased compared with the previous Tunisian cohort study published 20 years ago.¹⁵ This may suggest a change in disease distribution due to better awareness about the diagnosis of MGUS in our country. A large-scale updated study about MGs would be more informative.

Immunochemical Profile

The most common compositions of Ig in our series were the IgG-IgG (25%) and IgG-IgA (23%) combinations, which is similar to the findings in the series of Garcia et al⁶ and Nilsson et al⁷ but different from those observed in the series of Kyle et al,¹ Sharma et al,² Guastafierro et al,⁸ and Riddell et al¹⁶ (TABLE 3).

The differences in the composition of the gammopathies between the different series could be explained by the discrepancy either in

epidemiological data or in underlying diseases. This could be also due to the different levels of sensitivity of the paraprotein detection technique employed, the small number of patients, or erroneous inclusion criteria related to the polymerization of monoclonal components.

The results of the LC combinations showed a predominance of K-λ chains association (51%), which is consistent with most published data.^{7,13,14} Twenty-nine percent of BGs had the K-K LC combination. K LCs are reported to be the most frequent in the literature.⁶ An LC combination of the same type could be explained by a common clonal origin for the 2 paraproteins in many patients with BG, as demonstrated by previous molecular studies.^{3,19}

Evolution

In our study, as with the series of Riddell et al¹⁶ and Garcia et al⁶, no BGUS evolved into malignant hematological disease. However, the follow-up of these patients was heterogeneous and limited to only some of the patients in the study. In the literature, the rate of progression for BGUS is reported to be 1%/year, which is similar to MGUS.⁵

Our study includes the first and only Tunisian and African cohort. The main limitation of this study is its retrospective design. However, considering the low incidence of BGs, the retrospective study allowed including this number of patients.

In conclusion, BGs are rare in clinical laboratory routines but must be accurately identified by the pathologist. As far as is known, BGs share epidemiological characteristics with MGs. Compared to previous local MG studies, our cohort is characterized by an increased prevalence of BGUS compared with MM. This distribution seems to be closer to the European and American studies. None of our patients with BGUS developed MM.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Kyle RA, Robinson RA, Katzmann JA. The clinical aspects of biclonal gammopathies review of 57 cases. *Am J Med.* 1981;71(6):999–1008. doi:10.1016/0002-9343(81)90326-0.
2. Sharma S, Gupta P, Aggarwal R, Malhotra P, Minz RW, Bansal F. Demystifying biclonal gammopathy: a pathologist's perspective. *Lab Med.* 2019;50(4):357–363. doi:10.1093/labmed/lmz006.
3. Tschumper RC, Dispenzieri A, Abraham RS, Henderson KJ, Jellinek DF. Molecular analysis of immunoglobulin genes reveals frequent clonal relatedness in double monoclonal gammopathies. *Blood Cancer J.* 2013;3(4):e112–e117. doi:10.1038/bcj.2013.12.
4. Decaux O, Leroy H, Ianotto J, et al. A369 clinical and biological features of biclonal gammopathies: a review of 203 cases. *Clin Lymphoma Myeloma.* 2009;9(February):S60–S61. doi:10.1016/s1557-9190(11)70561-3.
5. Mullikin TC, Rajkumar SV, Dispenzieri A, et al. Clinical characteristics and outcomes in biclonal gammopathies. *Am J Hematol.* 2016;91(5):473–475. doi:10.1002/ajh.24319.
6. García-García P, Enciso-Alvarez K, Diaz-Espada F, Vargas-Núñez JA, Moraru M, Yebra-Bango M. Gammopatías biclonales: estudio retrospectivo de 47 pacientes. *Rev Clin Esp.* 2015;215(1):18–24. doi:10.1016/j.rce.2014.07.003.
7. Nilsson T, Norberg B, Rudolph O, Jacobsson L. Double gammopathies: incidence and clinical course of 20 patients. *Scand J Haematol* 1986;36(1):103–106.
8. Guastafierro S, Ferrara MG, Sica A, Parascandola RR, Santangelo S, Falcone U. Serum double monoclonal components and hematological malignancies: only a casual association? Review of 34 cases. *Leuk Res.* 2012;36(10):1274–1277. doi:10.1016/j.leukres.2012.05.008.
9. Vani B, Ritu A, Praveen S, Pankaj M, Minz R. Excess free light chains in serum immunofixation electrophoresis: attributes of a distinctive pattern. *Indian J Hematol Blood Transfus.* 2018;34(4):632–635. doi:10.1007/s12288-018-0926-0.
10. Gurmukh S, Xu, H. Light chain predominant intact immunoglobulin monoclonal gammopathy disorders: shorter survival in light chain predominant multiple myelomas. *Lab Med.* 2021;52(4):390–398.
11. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol.* 2014;15(12):e538–e548. doi:10.1016/S1470-2045(14)70442-5.
12. Zou D, An G, Zhu G, et al. Secondary monoclonal gammopathy of undetermined significance is frequently associated with high response rate and superior survival in patients with plasma cell dyscrasias. *Biol Blood Marrow Transplant.* 2014;20(3):319–325. doi:10.1016/j.bbmt.2013.11.022.
13. Mseddi-hdiji S, Haddouk S, Ben AM, Tahri N, Elloumi M, Baklouti S. Gammopathies monoclonales en Tunisie: analyse épidémiologique, immunochimique et étiologique d'une série de 288 cas [Monoclonal gammopathies in Tunisia: epidemiological, immunochemical and etiological analysis of 288 cases]. *Pathol Biol.* 2005;53(1):19–25.
14. Adhane A, Mohammed B, Ali A, et al. Monoclonal plasma cell dyscrasia: immunochemical and etiological parameters of 151 cases living in the Eastern area of Morocco. *PAMJ Clin Med.* 2021;5(7):1–10.
15. Belouni R, Allam I, Cherguelaine K, et al. Epidemiological and immunochemical parameters of monoclonal plasma cell dyscrasias of 2121 cases in Algeria. *Curr Res Transl Med.* 2020;68(2):67–70. doi:10.1016/j.retram.2019.11.003.
16. Riddell S, Traczyk Z, Paraskevas F, Isreal LG. The double gammopathies: clinical and immunological studies. *Medicine.* 1986;65(3):135–142.
17. Gastélum-Cano JM, Fragoso-Flores J, Noffal-Nuño VM, Deffis-Court M. An unusual pattern in serum protein electrophoresis to take in mind: a case report. *Pract Lab Med.* 2021;24(December 2020):0–4.
18. Ouzzif Z, Doghmi K, Bouhsain S, et al. Monoclonal gammopathies in a Moroccan military hospital. *Rheumatol Int.* 2012;32(10):3303–3307. doi:10.1007/s00296-011-2093-6.
19. Kriangkum J, Motz SN, Debes Marun CS, et al. Frequent occurrence of highly expanded but unrelated b-cell clones in patients with multiple myeloma. *PLoS One.* 2013;8(5):e64927. doi:10.1371/journal.pone.0064927.

Association between Serum α -Klotho Levels and Behçet Disease

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Keywords: Behçet disease, α -klotho, klotho proteins, endothelial dysfunction, vasculitis, superoxide dismutase 2

Abbreviations: BD, Behçet disease; ED, endothelial dysfunction; FGF, fibroblast growth factor; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; ICBD, International Criteria for Behçet's Disease; SOD2, superoxide dismutase 2; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; RF, rheumatoid factor

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ABSTRACT

Background: Endothelial dysfunction (ED) has a well-known role in promoting vascular inflammation in Behçet disease (BD). α -klotho is involved in regulation of endothelial function, and its reduction has been reported to be associated with ED.

Objective: To assess serum α -klotho in patients with BD, compared with healthy control individuals.

Methods: In a cross-sectional study, 55 patients with BD and 30 age- and sex-matched healthy controls were enrolled, and their serum levels of α -klotho were measured.

Results: Common clinical symptoms in patients with BD were oral aphthous ulcers, uveitis, and genital ulcers. Median (IQR) serum α -klotho levels in the BD and control groups were 0.30 (0.20–0.70) and 1.00 (0.70–2.52) ng/mL, respectively. The difference was statistically significant ($P = .005$). No significant correlation was observed between serum α -klotho and age ($r = 0.194$; $P = .14$). Serum α -klotho levels in patients with uveitis were significantly lower.

Conclusion: α -klotho may have a role in the pathogenesis of ED and is a potential biomarker for uveitis in BD.

Behçet disease (BD) is a type of systemic vasculitis that mainly affects young adults. Recurrent oral aphthous and genital ulcers, skin lesions, and uveitis—as well as involvement of the central nervous system, musculoskeletal and vascular systems, and gastrointestinal tract—are common manifestations.¹ The etiology and pathogenesis of BD are unknown. However, the main pathological manifestation is infiltration of inflammatory cells in the perivascular area and the walls of the capillaries, veins, and arteries of various sizes.² BD is an inflammatory disease with autoimmune and autoinflammatory features that is caused by the activation of the immune system after exposure to environmental factors in genetically predisposed individuals.^{3–5} Endothelial dysfunction (ED) is a hallmark of BD.^{6,7} Endothelial activation has a well-known role in promotion of vascular inflammation and thrombosis in BD.^{6,7}

The α -klotho protein is an essential component of endocrine fibroblast growth factor (FGF) receptor complexes and exists in transmembrane and soluble forms.⁸ α -klotho primarily is known as an antiaging agent.⁸ Also, α -klotho is involved in the regulation of endothelial function; its reduction has been reported to be associated with ED⁹ and may have a role in the regulation of immune response.⁸ Several study reports^{10–14} have presented the association between α -klotho and various rheumatic diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc).

The growing evidence regarding α -klotho dysregulation in patients with autoimmune diseases and ED has led to the hypothesis that these peptides can be used as a biomarker in BD. To our knowledge, there has been no investigation regarding the role of α -klotho in BD and its correlation with clinical manifestations and activity of disease. So, this work of research aimed to assess the serum α -klotho levels in patients with BD, compared with those levels in healthy subjects.

Methods

Subjects

In a cross-sectional study, 55 patients diagnosed with BD and 30 age- and sex-matched healthy controls were enrolled. Inclusion criteria were diagnosis of BD according to the International Criteria for Behçet's Disease (ICBD)¹⁵ and age ≥ 16 years. Patients with BD were selected consecutively from the outpatient BD clinic of the Connective Tissue Diseases Research Center of Tabriz University of Medical Sciences, Iran, between February 2021 and December 2021. The exclusion criteria were impaired renal function, liver disease, diabetes mellitus, thyroid and/or

parathyroid disorders, any other autoimmune diseases or overlap syndromes, malignant neoplasms, pregnancy and breastfeeding, ever smoking, and/or currently taking antioxidant supplements. The research protocol was approved by the ethics committee of Tabriz University of Medical Sciences, Tabriz, Iran; informed written consent was gained from all subjects before inclusion in the study. During the study, all personal information was kept confidential, and accordingly, we performed other steps to act according to ethical and humanitarian considerations.

Clinical and Biochemical Measurements

All participants were examined in a multidisciplinary clinic by a rheumatologist and, if necessary, by an ophthalmologist and other specialists. Data related to the demographic characteristics of participants and clinical manifestations of patients with BD were recorded. We collected 5 mL of venous blood specimens after 12-hour overnight fasting. The serum specimens were isolated from whole blood and were kept at -70°C until biochemical measurements were performed. Serum levels of α -klotho were measured using a human α -klotho ELISA kit in an ELISA plate reader (Model Stat Fax 2100, Awareness Technologies). The specimens collected from the studied groups were tested by A. Ghorbanihaghjo simultaneously, using the same reagent lot number.

Statistical Analysis

Statistical analysis was performed using SPSS software, version 16.0 (IBM). Normality of variables distribution was evaluated using the Kolmogorov-Smirnov test. Categorical and normally distributed quantitative variables were displayed as No. (%) and mean (SD), respectively. Variables not normally distributed were presented as median (IQR). Between-groups comparisons were made via χ^2 testing, independent sample t -testing, and Mann-Whitney U testing, as appropriate. Correlations between variables were analyzed using the Pearson correlation coefficient; $P < .05$ was considered statistically significant.

Results

The study included 55 patients with BD and 30 healthy controls. Demographic and clinical characteristics of participants are presented in **TABLE 1**. There was no significant difference in age and sex between patients with BD and controls. The most common clinical symptoms in patients with BD were oral aphthous ulcers, uveitis, and genital ulcers.

We compared serum α -klotho levels in the BD and control groups (**FIGURE 1**). Median (IQR) serum α -klotho levels in BD and control groups were 0.30 (0.20–0.70) and 1.00 (0.70–2.52) ng/mL, respectively. The difference was significant ($P = .005$).

We assessed the correlation between serum α -klotho levels and age: no statistically significant correlation was observed ($r = 0.194$, $P = .14$) (**FIGURE 2**). Also, we assessed the association between serum α -klotho levels and categorical variables, including sex and the involvement of various organs, in patients with BD (**TABLE 2**). Serum α -klotho in patients with uveitis was significantly lower (**TABLE 2**). There was no significant correlation between serum α -klotho level and involvement of other organs (**TABLE 2**).

Discussion

ED is characterized by decreased vasodilation and predisposes patients to prothrombotic and proinflammatory states.¹⁶ Several study reports

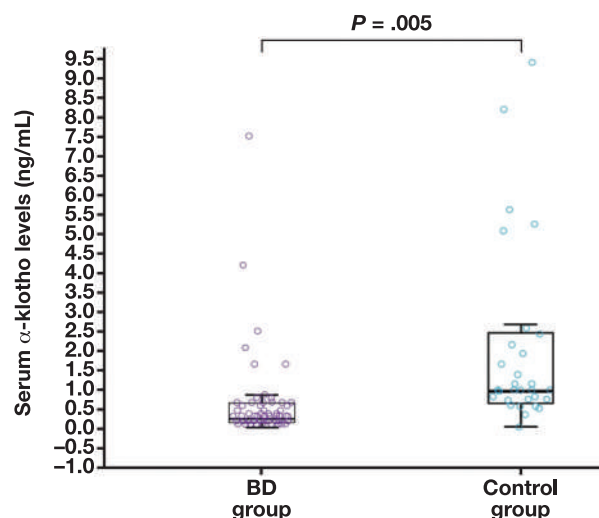
TABLE 1. General Characteristics and Biochemical Factors of Study Subjects

Characteristic	Patients with BD (n = 55)	Healthy Controls (n = 30)	P Value ^a
Age, y, mean (SD)	40.6 (9.7)	43.1 (7.8)	.30
Male, No. (%)	36 (65.5)	20 (66.7)	.55
Oral aphthous ulcer, No. (%)	51 (92.7)	—	—
Uveitis, No. (%)	43 (78.2)	—	—
Genital ulcer, No. (%)	28 (50.9)	—	—
Skin lesions, No. (%)	21 (38.2)	—	—
Vasculitis, No. (%)	12 (21.8)	—	—
Arthritis, No. (%)	11 (20.0)	—	—
CNS involvement, No. (%)	2 (3.6)	—	—

BD, Behçet disease; CNS, central nervous system.

^a $P < .05$ was considered statistically significant. P values indicate comparison between groups (χ^2 testing).

FIGURE 1. Serum α -klotho levels in the studied groups. BD, Behçet disease.



mentioned ED in various types of vasculitis, including BD.^{14–16} ED is a well-known feature of BD.^{17–21} Various factors, including oxidative stress and hyperhomocysteinemia, are reported to play a role in ED when it manifests in the context of BD.^{17,22,23}

The klotho proteins originally were known as antiaging proteins. However, it has been characterized that these proteins have an important role in protecting vasculature. Possible mechanisms include increased expression of superoxide dismutase 2 (SOD2) that facilitates the catalysis of superoxide generated by mitochondrial respiration into hydrogen peroxide²⁴; upregulating endothelial nitric oxide synthase, the enzyme that controls nitric oxide production in endothelial cells⁵; and activation of the PI3K/Akt pathway, which leads to increased expression of Nrf2 (a transcription factor that enhances the expression of genes encoding antioxidant defense proteins under oxidative conditions)²⁵; and inhibiting apoptosis of endothelial cells.²⁶ In addition to its protective role against oxidative stress, klotho reduces the expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells.²⁷ Klotho also decreases the

FIGURE 2. Correlation between serum α -klotho levels and age in patients with Behçet disease ($r = 0.194$; $P = .14$).

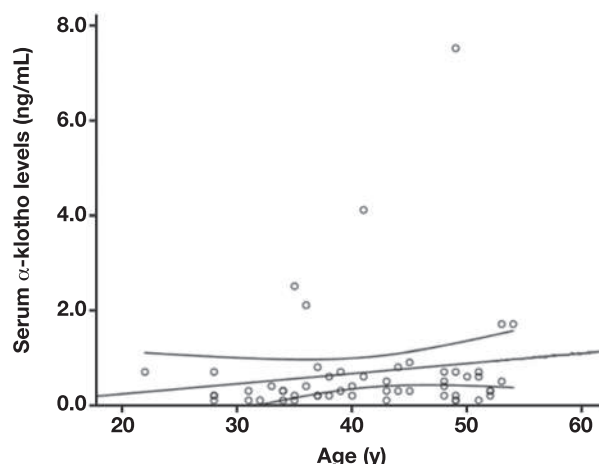


TABLE 2. Serum α -klotho Levels in Patients with BD (n = 55)

Characteristic	Serum α -klotho Level, Median (IQR)		P Value
	With BD	No BD	
Male	0.35 (0.20–0.70)	0.40 (0.20–0.70)	.55
Uveitis	0.30 (0.20–0.60)	1.20 (0.15–2.40)	.02
Genital ulcer	0.30 (0.10–0.70)	0.40 (0.20–0.70)	.36
Skin lesions	0.30 (0.20–0.60)	0.30 (0.20–0.70)	.83
Phlebitis	0.35 (0.20–0.60)	0.30 (0.20–0.70)	>.99
Arthritis	0.30 (0.20–0.70)	0.35 (0.20–0.70)	.76
CNS involvement	2.10 (0.10–4.10)	0.30 (0.20–0.70)	— ^a

BD, Behçet disease; CNS, central nervous system; IQR, interquartile range.

^an = 2 were considered to be too few patients for analysis.

activation of NF- κ B and suppresses the expression of TNF- α , IL-6, and IL-8.^{27,28} Ushigusa et al¹² reported an association between reduction of α -klotho protein with ED and neuronal damage in patients with SLE.

In addition to the role of klotho proteins in the regulation of endothelial-cell function, these proteins may also play a role in modulating immune-system function.⁸ Data on the relationship between klotho proteins and the immune system are limited to animal models, in the literature.⁸ Odaka et al²⁹ reported underdevelopment of the immune system and impairment of B-cell differentiation in klotho-knockout mice. Liu et al²⁸ reported an anti-inflammatory property of α -klotho. It has been shown³⁰ that α -klotho suppresses TNF- α -induced proinflammatory cytokine production, such as monocyte chemoattractant protein-1, IL-6, and IL-8, in the kidneys in a mouse model of diabetes.³⁰

The present study results show downregulation of α -klotho in patients with BD. Also, we found lower serum α -klotho levels in patients with BD who have uveitis. Downregulation of α -klotho was reported in RA, SLE, and SSc.⁸ According to Witkowski et al,¹⁰ α -klotho was downregulated at the mRNA and protein levels in CD4⁺ lymphocytes from subjects with RA. In a cohort of patients with RA, higher plasma concentrations of α -klotho were associated with higher levels of autoantibodies: rheumatoid factor (RF), anticitrullinated peptide

antibodies, and disease activity score of -28 .¹¹ In another study report, Ushigusa et al¹² demonstrated that a lower level of soluble α -klotho in cerebrospinal fluid was an important factor for predicting neuropsychiatric SLE. In studies carried out by Talotta et al¹³ and Hajjalilo et al,¹⁴ serum concentration of α -klotho was found to be lower in patients with SSc than in healthy controls. However, it was neither related to the severity of the disease nor to organ involvement.^{13,14}

To our knowledge, this is the first study in the literature on the serum level of α -klotho in BD. The limitations of the present study were the relatively small sample size and the cross-sectional design of the study. In conclusion, our findings showed that α -klotho could have a role in the pathogenesis of ED in BD—namely, that α -klotho may be a biomarker for uveitis in BD.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

- Mendes D, Correia M, Barbedo M, et al. Behçet's disease—a contemporary review. *J Autoimmun*. 2009;32(3-4):178–188. doi:10.1016/j.jaut.2009.02.011
- Demirelli S, Degirmenci H, Inci S, Arisoy A. Cardiac manifestations in Behçet's disease. *Intractable Rare Dis Res*. 2015;4(2):70–75. doi:10.5582/irdr.2015.01007
- Sadeghi A, Davatchi F, Shahram F, et al. Serum profiles of cytokines in Behçet's disease. *J Clin Med*. 2017;6(5):49.
- Zeidan MJ, Saadoun D, Garrido M, Klatzmann D, Six A, Cacoub P. Affiliations expand Behçet's disease physiopathology: a contemporary review. *Auto Immun Highlights*. 2016;7(1):4. doi:10.1007/s13317-016-0074-1
- Lucherini OM, Lopalco G, Cantarini L, et al. Critical regulation of Th17 cell differentiation by serum amyloid-A signalling in Behçet's disease. *Immunol Lett*. 2018;201:38–44. doi:10.1016/j.imlet.2018.10.013
- Kayıkcioglu M, Aksu K, Hasdemir C, et al. Endothelial functions in Behçet's disease. *Rheumatol Int*. 2006;26(4):304–308. doi:10.1007/s00296-005-0590-1
- Toledo-Samaniego N, Oblitas CM, Peñaloza-Martínez E, et al. Arterial and venous involvement in Behçet's syndrome: a narrative review. *J Thromb Thrombolysis*. 2022;54:162–171. doi:10.1007/s12399-022-02637-1
- Russell DL, Oates JC, Markiewicz M. Association between the anti-aging gene Klotho and selected rheumatologic autoimmune diseases. *Am J Med Sci*. 2021;361(2):169–175. doi:10.1016/j.amjms.2020.10.021
- Martín-Núñez E, Donate-Correa J, Muros-de-Fuentes M, Mora-Fernández C, Navarro-González JF. Implications of klotho in vascular health and disease. *World J Cardiol*. 2014;6(12):1262–1269. doi:10.4330/wjcv.v6.i12.1262
- Witkowski JM, Soroczyńska-Cybula M, Bryl E, Smoleńska Z, Jóźwik A. Klotho—a common link in physiological and rheumatoid

arthritis-related aging of human CD4+ lymphocytes. *J Immunol.* 2007;178(2):771–777. doi:[10.4049/jimmunol.178.2.771](https://doi.org/10.4049/jimmunol.178.2.771)

11. Alvarez-Cienfuegos A, Cantero-Nieto L, Garcia-Gomez JA, Robledo G, González-Gay MA, Ortego-Centeno N. FGF23-Klotho axis in patients with rheumatoid arthritis. *Clin Exp Rheumatol.* 2020;38(1):50–57.
12. Ushigusa T, Ichinose K, Sato S, et al. Soluble α -klotho is a potential biomarker associated with neuropsychiatric systemic lupus erythematosus. *Clin Immunol.* 2016;165:29–34. doi:[10.1016/j.clim.2016.03.001](https://doi.org/10.1016/j.clim.2016.03.001).
13. Talotta R, Atzeni F, Bongiovanni S, et al. Measurement of serum alpha-klotho in systemic sclerosis patients: results from a pivotal study. *Ann Rheum Dis.* 2016;75(2):[10.1136/annrheumdis-2016-eular.1683](https://doi.org/10.1136/annrheumdis-2016-eular.1683).
14. Hajjalilo M, Noorabadi P, Tahsini Tekantapeh S, Malek Mahdavi A. Endothelin-1, α -Klotho, 25(OH) Vit D levels and severity of disease in scleroderma patients. *Rheumatol Int.* 2017;37(10):1651–1657. doi:[10.1007/s00296-017-3797-z](https://doi.org/10.1007/s00296-017-3797-z)
15. International Team for the Revision of the International Criteria for Behçet's Disease. Evaluation of the international criteria for Behçet's disease (ICBD). *Clinic Exp Rheumatol.* 2006;24(suppl 42):S13.
16. Herrmann J, Lerman A. The endothelium: dysfunction and beyond. *J Nucl Cardiol.* 2001;8(2):197–206. doi:[10.1067/mnc.2001.114148](https://doi.org/10.1067/mnc.2001.114148)
17. Chambers JC, Haskard DO, Kooner JS. Vascular endothelial function and oxidative stress mechanisms in patients with Behçet's syndrome. *J Am Coll Cardiol.* 2001;37(2):517–520. doi:[10.1016/s0735-1097\(00\)01137-2](https://doi.org/10.1016/s0735-1097(00)01137-2)
18. Protogerou A, Lekakis J, Stamatelopoulos K, et al. Arterial wall characteristics in patients with Adamantiades-Behçet's disease. *Adv Exp Med Biol.* 2003;528:399–404. doi:[10.1007/0-306-48382-3_82](https://doi.org/10.1007/0-306-48382-3_82)
19. Ozdemir R, Barutcu I, Sezgin AT, et al. Vascular endothelial function and plasma homocysteine levels in Behçet's disease. *Am J Cardiol.* 2004;94(4):522–525. doi:[10.1016/j.amjcard.2004.04.073](https://doi.org/10.1016/j.amjcard.2004.04.073)
20. Oflaz H, Mercanoglu F, Karaman O, et al. Impaired endothelium-dependent flow-mediated dilation in Behçet's disease: more prominent endothelial dysfunction in patients with vascular involvement. *Int J Clin Pract.* 2005;59(7):777–781. doi:[10.1111/j.1742-1241.2005.00477.x](https://doi.org/10.1111/j.1742-1241.2005.00477.x)
21. Caliskan M, Yilmaz S, Yildirim E, et al. Endothelial functions are more severely impaired during active disease period in patients with Behçet's disease. *Clin Rheumatol.* 2007;26(7):1074–1078. doi:[10.1007/s10067-006-0449-1](https://doi.org/10.1007/s10067-006-0449-1)
22. Freitas JP, Filipe P, Yousefi A, Emerit I, Guerra Rodrigo F. Oxidative stress in Adamantiades-Behçet's disease. *Dermatology.* 1998;197(4):343–348. doi:[10.1159/000018029](https://doi.org/10.1159/000018029)
23. Aksu K, Turgan N, Oksel F, et al. Hyperhomocysteinaemia in Behçet's disease. *Rheumatology.* 2001;40(6):687–690.
24. Yamamoto M, Clark JD, Pastor JV, et al. Regulation of oxidative stress by the anti-aging hormone klotho. *J Biol Chem.* 2005;280(45):38029–38034. doi:[10.1074/jbc.M509039200](https://doi.org/10.1074/jbc.M509039200)
25. Cui W, Leng B, Wang G. Klotho protein inhibits H₂O₂-induced oxidative injury in endothelial cells via regulation of PI3K/AKT/Nrf2/HO-1 pathways. *Can J Physiol Pharmacol.* 2019;97(5):370–376. doi:[10.1139/cjpp-2018-0277](https://doi.org/10.1139/cjpp-2018-0277)
26. Tyurenkov IV, Perfilova VN, Nesterova AA, Glinka Y. Klotho protein and cardio-vascular system. *Biochemistry (Mosc).* 2021;86(2):132–145.
27. Maekawa Y, Ishikawa K, Yasuda O, et al. Klotho suppresses TNF- α -induced expression of adhesion molecules in the endothelium and attenuates NF- κ B activation. *Endocrine.* 2009;35(3):341–346. doi:[10.1007/s12020-009-9181-3](https://doi.org/10.1007/s12020-009-9181-3)
28. Liu F, Wu S, Ren H, Gu J. Klotho suppresses RIG-I-mediated senescence-associated inflammation. *Nat Cell Biol.* 2011;13(3):254–262. doi:[10.1038/ncb2167](https://doi.org/10.1038/ncb2167)
29. Okada S, Yoshida T, Hong Z, et al. Impairment of B lymphopoiesis in precocious aging (klotho) mice. *Int Immunol.* 2000;12(6):861–871. doi:[10.1093/intimm/12.6.861](https://doi.org/10.1093/intimm/12.6.861)
30. Zhao Y, Banerjee S, Dey N, et al. Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine)⁵³⁶ phosphorylation. *Diabetes.* 2011;60(7):1907–1916. doi:[10.2337/db10-1262](https://doi.org/10.2337/db10-1262)

Laboratory Strategies to Improve Allergy First-Line Screening in Primary Care

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Keywords: allergy testing, primary care, screening, laboratory interventions, patient outcome improvement, cost reduction

Abbreviations: sIgE, serum IgE; PC, primary care; GPs, general practitioners; SC, specialized care; EMRs, electronic medical records; LIS, laboratory information system

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ABSTRACT

Background: There are nonestablished protocols in use for first-line allergy screening based on IgE testing. These protocols attempt to address an unmet need for sustainability of clinical laboratories, at a time when demand is increasing.

Objective: To present a novel protocol for first-line allergy screening and to evaluate the implementation benefits for patients, the health care system, and payers.

Methods: We carried out an observational retrospective study analyzing 4359 interventions on primary care testing requests. Interventions included overriding redundant serum IgE (sIgE) testing for allergen mixes, extracts included in mixes, low-prevalence extracts, and milk and egg molecular components without previous positive results when exposed to extracts. We also added prevalent allergen testing.

Results: The strategy saved 683 tests from being performed unnecessarily. Test volume decline was primarily driven by the cancelation of 2186 egg and milk components tests; 561 tests were added for mixes, together with 942 allergen extracts tests.

Discussion: The results of this study show how the allergy laboratory plays a key role in actively managing demand for sIgE testing, leading to optimized diagnosis.

The prevalence of allergies in the past 20 to 30 years has notably increased, especially in Western countries.^{1,2} Allergies are becoming a major public health concern.² Patients present with a broad variety of symptoms, which may be respiratory, skin-related, digestive, or even systemic. Current diagnosis of allergies is based on the correlation of symptoms together with proof of sensitization (presence of allergen-specific IgE) using different in vivo and in vitro tests in which serum IgE (sIgE) testing plays a crucial role.^{3,4}

The primary care (PC) department is the frontline for detection of patients with allergy in Spain and the rest of Europe.⁵ The findings of a study by Brożek et al⁶ have shown that general practitioners (GPs) do not receive specific training on the management of allergic diseases. For this reason, it is crucial to ensure interaction between PC offices (where patients are identified and receive primary prophylaxis and symptomatic treatment) and specialized care (SC) offices, where patients receive personalized diagnosis and secondary prophylaxis. This interaction should be based on clear clinical guidelines and the optimization of available resources. However, skin testing is not practical in PC and therefore is not recommended.

The accessibility of sIgE tests and the short response times from clinical laboratories make them extremely valuable in PC to confirm sensitization.⁶ Also, the use of sIgE blood testing in PC has been proven to significantly increase the ability to identify patients with atopic dermatitis and has 3 main effects: reducing the number of poorly classified cases, reducing the number of cases of unknown type, and providing recommendations for allergen avoidance strategies.⁷

The decision to allow GPs to request sIgE blood testing for screening has a significant impact on health department budgets because this type of request significantly increases the number of patients who may have access to these tests. One should also evaluate the savings derived from accurate diagnosis in PC, which avoids further visits, unnecessary treatments, and unnecessary referrals to specialist care. The emergence of sIgE tests for allergen mixes allows for reduction of the number of requests for sIgE tests for allergen extracts. The findings of 2 studies^{8,9} have proven that this is a useful and cost-effective strategy as first-line

screening because its positive results allow for diagnosing allergies with a PPV of 90.2%, although it does not allow identifying the disease-causing allergen. We stress that having clear protocols regarding the use of these tests is the key to making them truly cost-effective. In this regard, clinical laboratories can play a crucial role in improving the appropriateness of requests and obtaining the maximum benefit from each test performed.

Allergies are local in nature due to the variability of the most frequent allergens in each region. As a result, it is necessary for each region/laboratory/hospital to conduct studies on the cost-effectiveness of the diagnostic protocols used in PC so that the detection, referral, or treatment of the allergic pathology is the most optimal for the patient and the health care system.

The demand for serological tests for allergies continues to grow over time. Active management of the demand for sIgE from PC, regulated through the experience of the clinical laboratory, can lead to improved selection and diagnosis of patients with allergies while reducing the cost of testing in our area of influence, as has already been demonstrated for other conditions such as anemia.¹⁰

The aim of this work of research was to quantify the demand for sIgE tests from PC; to explain the strategies applied in our area of influence, in hopes of improving the appropriateness of requests for sIgE testing in PC; and to evaluate the benefits that such strategies have brought to patients, clinicians, and payers. We hope that other laboratories may be able to adapt these strategies to their needs.

Materials and Methods

This work was an observational retrospective study. It was carried out between January 1, 2014, and December 31, 2019. Our laboratory is located at Hospital San Juan de Alicante, Spain. It has 370 beds, serves a population of 234,551 inhabitants in an urban-rural setting, and has 9 PC centers assigned to it, with their respective auxiliary offices. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Hospital San Juan de Alicante, Spain.

Specimen Collection

For this study we analyzed all serum specimens from patients referred for first-line in vitro allergy testing from PC centers that serve children and adults, from the population served by the hospital. We included 3912 specimens from patients aged 10 to 99 years, all of whom are White.

Protocol between PC and SC Centers for Managing Patients with Allergies

In January 2014, a new protocol was established at the PC centers in our area of influence. All GPs working in these centers were specifically trained by allergy specialists on the new protocol, and the rationale behind each intervention was explained.

In general terms, it was established that for patients with clinical symptoms of respiratory allergy (rhinoconjunctivitis and/or asthma), general practitioners should request sIgE testing for aeroallergen mixes (ImmunoCAP Phadiatop, Thermo Fisher Scientific) and sIgE for allergen extracts according to clinical history. Likewise, for patients with suspected allergies, allergen-specific sIgE testing should be requested according to clinical history (in the case of clearly

suggestive symptoms) or food-mix sIgE (ImmunoCAP Allergen fx5, Thermo Fisher Scientific).

Symptomatic treatment and/or allergen avoidance (if a clear culprit was pinpointed by clinical records) should be prescribed for patients showing positive results in a screening test if these results correlated with symptoms. If these tactics were insufficient to achieve symptom control, referral to the specialist allergy service was advised. Cases of anaphylaxis were referred directly to the allergy service.

According to our internal evaluation, the requests for sIgE tests for allergen extracts that can be replaced by Phadiatop testing in our area of influence are the following: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat dander, dog dander, *Olea europaea*, *Lolium perenne*, *Cynodon dactylon*, *Parietaria judaica*, and *Aspergillus fumigatus* (TABLE 1). In the case of fx5, the composition is known and is as follows: egg white, milk, fish, wheat, peanuts, and soy.

This protocol for allergy management in PC was agreed on by PC and SC allergy-treating clinicians and implemented for automation in our laboratory information system (LIS), manufactured by GestLabs. Electronic medical records (EMRs) were captured using Orion Clinic (Orion Health) and ABUCASIS (Indra).

Additional interventions by the laboratory to improve the appropriateness of requests were as follows:

- No redundant sIgE testing for allergen mixes (Phadiatop and fx5): when receiving requests for 1 or both tests with a prior negative result within the previous 2 months or with a previous positive result (considering positive results to be $>1 \text{ kU}_A/\text{L}$, which is 3 times the upper reference interval limit) within the previous 3 years, the test was reported as canceled, and the previous result was displayed, specifying the testing date. The specimens were kept for 1 month in case there was a clinical reason for them to be retested.
- No sIgE testing for allergen extracts included in Phadiatop or fx5: when receiving a request for sIgE for allergen extract only (except for highly specific histories), sIgE was replaced by Phadiatop or fx5 if the requested allergen was included in the mixes. Likewise, when receiving requests for sIgE for allergen extracts and mix testing, only Phadiatop testing was performed. When the test results were positive, an explanatory note was included, indicating that this result was sufficient to refer the patient to SC according to their history, if

TABLE 1. Allergen Extract Tests with Positive Results that Showed a 100% Correlation in Positivity with Phadiatop Results in Concurrent Determinations

Allergen Extract Test	No. of Concurrent Tests
<i>Dermatophagoides pteronyssinus</i>	97
<i>Dermatophagoides farinae</i>	97
Cat dander	87
Dog dander	85
<i>Olea europaea</i>	83
<i>Lolium perenne</i>	83
<i>Cynodon dactylon</i>	83
<i>Parietaria judaica</i>	72
<i>Aspergillus fumigatus</i>	52

considered appropriate by the general practitioner, due to symptom severity.

- No sIgE testing for low prevalent aeroallergen and food allergen extracts in our region: the test was not performed, and clinical justification was requested.
- No sIgE testing for milk and egg molecular components without prior positivity to allergen extracts: when milk (α and β -lactalbumin and casein) and egg (ovalbumin and ovomucoid) component sIgE tests were requested, the specimens were first tested for allergen extracts, and if the results were positive, then the specimens were tested for molecular components.
- Automatic registration of additional tests.
- In patients with respiratory symptoms (rhinitis and/or asthma), the laboratory information system (LIS) automatically registered sIgE tests (information on patients suspected of having rhinitis and/or asthma was automatically transferred to the LIS by the EMR systems) for allergen extracts of *Salsola kali* and *Cupressus arizonica*, if not requested, due to their high prevalence in our region and their absence of representation in Phadiatop testing.
- In patients with urticaria and dermatological symptoms, LIS also added sIgE testing for allergen extracts of *Anisakis* (information on patients suspected of having food allergies was automatically transferred to the LIS by the EMR systems) due to the high prevalence of this allergen (which is not represented in fx5) in our coastal region.

Blood Tests

All sIgE testing for allergen extracts or allergen mixes was performed by ImmunoCAP assay using the Phadia 250 instrument (Thermo Fisher Scientific) according to the supplier instructions. ImmunoCAP Phadiatop and/or fx5 was (were) used for mixes. Both tests are described as in vitro qualitative and semiquantitative assays based on fluoroenzyme immunoassay technology for graded determination of IgE antibodies specific to inhalant/food allergens in human serum or plasma. ImmunoCAP whole allergens testing uses the same technology but allows quantitative determination of specific IgE against a specific allergen.

The ImmunoCAP test displayed 4% to 9% CV in the reporting range, from 0.1 to 100 kU_A/L. Results were considered positive when greater than 0.35 kU_A/L.

Study of Aeroallergens Represented and Not Represented in Phadiatop

A retrospective analysis was carried out on test results for the most frequently requested allergens in our area of influence between January 2008 and December 2014. For these allergens, the results of sIgE for allergen extract were compared with the results from the Phadiatop test. Allergens with positive results that were 100% related to positive or negative Phadiatop results were selected as long as both tests were performed concurrently.

Study of Allergens That Are Nonprevalent in the Health Care Area

We carried out a retrospective analysis on test results for allergens considered to be not prevalent in our area of influence between January 2008 and December 2014. We decided not to proceed with requests cor-

responding to allergen extracts with positivity of less than 5%, except for highly suggestive clinical histories.

Data Analysis

We obtained data from the LIS and performed descriptive analysis on the data obtained. Categorical variables were presented as No. (%). To monitor the evolution of demand, requests for sIgE were normalized to the requests for serum creatinine in percentages during the same period of time.

Results

Evolution over Time of the Number of Requests for sIgE from Primary Care

GP requests for allergen extracts and allergen mix sIgE tests during the study period resulted in 17,557 tests being performed. There was a significant increase in the demand for sIgE testing over time (**FIGURE 1A**). We also observed a clear upward trend in the number of requests during the months of April and October (the peaks of allergic respiratory pathologic manifestations). In April, especially, 45% more tests were requested in 2019 than in 2014. This upward trend also occurred in January, with a 22% increase. In July, however, the trend was more stable, with a decrease in the number of requests in the last 2 years studied and an overall negative trend of -1.5% compared to 2014 (**FIGURES 1A-1E**). All cases showed significant variability over the years, due to the intrinsic variability of allergic pathologies, which are influenced by multiple, continuously changing factors.

Strategies for Improving Requests and Their Impact

TABLE 2 shows the results of the different strategies for improving requests. There were interventions on 4355 requests for allergen-specific sIgE, representing 25% of all tests. The balance between the number of tests saved and additional tests requested was negative, with 683 tests not performed.

No Redundant Testing for Allergen Mixes (Phadiatop and fx5)

This strategy resulted in 312 fewer tests, with directly associated cost reductions. Therefore, 312 serum specimens were stored, for which no further testing was requested.

Replacement of sIgE Tests for Allergen Extracts Included in Phadiatop or fx5 When Included in These Tests

There were interventions in 1673 such requests. At least 2 allergen extract tests were replaced by 1 Phadiatop/fx5 testing, resulting in a minimum savings of 837 tests.

No sIgE Testing for Allergen Extracts That Are Not Prevalent in the Influence Area

Requests for the following allergen extracts declined due to positivity of less than 5%: *Betula verrucosa*, *Acacia longifolia*, *Picea excelsa*, *Acer negundo*, *Fagus grandifolia*, *Pinus strobus*, *Quercus alba*, *Alnus incana*, *Castanea sativa*, *Corylus avellana*, *Juniperus sabinoides*, and *Rumex acetosella* (**TABLE 3**).

This strategy resulted in 312 allergen extract tests that were not analyzed. Therefore, 312 serum specimens were stored, for which

FIGURE 1. The evolution of the demand for specific sIgE tests from primary care (PC) providers. A, Annual total requests. B, Requests during January ($\Delta = 22\%$), during April ($\Delta = 45\%$) (C), during July ($\Delta = -1.5\%$) (D), and during October ($\Delta = 14\%$) (E). The number of sIgE requests was normalized to creatinine. The y-axis displays the number of sIgE tests per each 100 creatinine tests.

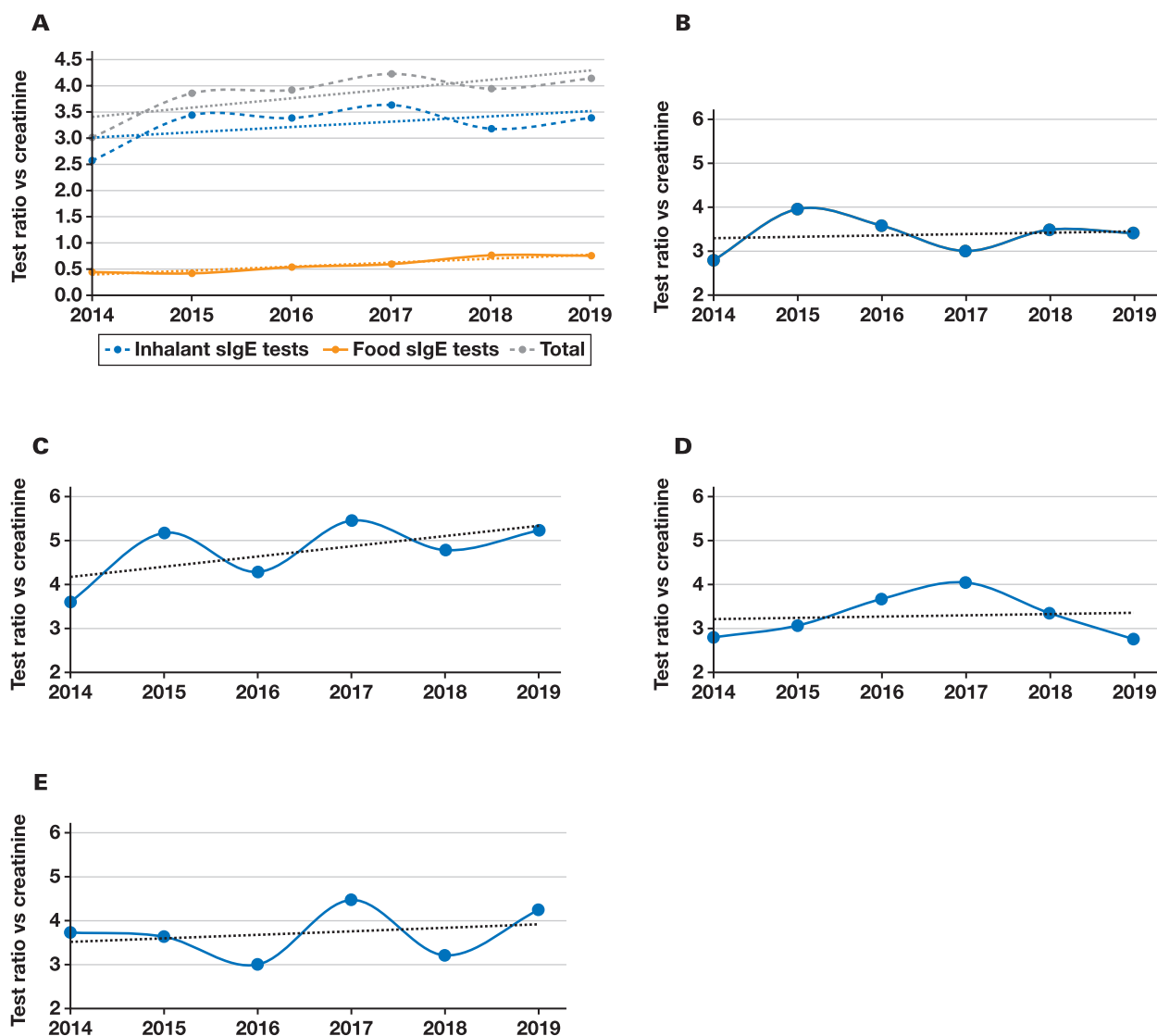


TABLE 2. Strategies for Improving Testing Requests and Their Impact in Number of Tests Performed and Costs of Testing

Strategy	Interventions	No. and Type(s) of Test(s) Saved/Added	Financial Balance ^a
Redundant tests not performed (Phadiatop/fx9)	312	–312 Phadiatop/fx5	–€2184 to –€4368
Replacement of sIgE tests for allergen extracts included in Phadiatop or fx5	1673	–1673 allergen extracts; +873 Phadiatop/fx5 ^b	–€3258 to –€6515
No sIgE tests for allergen extracts not present in the influence area	312	–312 allergen extracts	–€1747 to –€3494
No sIgE testing for milk and egg components without prior positive results for allergen extracts	803	+722 allergen extracts, –2186 components	–€8198 to –€16,396
Extension of extract testing for the high-prevalence allergens <i>Salsola kali</i> and <i>Cupressus arizonica</i>	950	+1900 allergen extracts	+€4200 to +€8400
Extension of high-prevalence allergens: allergen extract tests for <i>Anisakis</i> spp.	305	+305 allergen extracts	+€1708 to +€3416
Total	4355	+561 Phadiatop/fx5, +942 allergen extracts, –2186 components Total balance: –683 tests	–€9480 to –€18,960

^aDisplayed as a range because test prices vary.

^bObtained value considering that each Phadiatop/fx5 was replaced by only the minimum number of allergen extract tests ($n = 2$).

TABLE 3. Positive Results for Nonprevalent Allergens in the Area

Allergen (Pollen)	Tests, No. (Positive Results, %)
Eastern white pine (<i>Pinus strobus</i>)	101 (1)
Birch (<i>Betula verrucosa</i>)	41 (2)
Acacia (<i>Acacia longifolia</i>)	37 (0)
Fir (<i>Picea excels</i>)	22 (0)
Maple (<i>Acer negundo</i>)	9 (0)
Beech (<i>Fagus grandifolia</i>)	8 (0)
Oak (<i>Quercus alba</i>)	6 (0)
Gray alder (<i>Alnus incana</i>)	5 (0)
Spanish chestnut (<i>Castanea sativa</i>)	5 (0)
Savin juniper (<i>Juniperus sabinoidea</i>)	3 (0)
Hazel (<i>Corylus avellana</i>)	2 (0)
Red sorrel (<i>Rumex acetosella</i>)	2 (0)

studies of the previously indicated low prevalence allergen were never again requested.

No sIgE Testing for Milk and Egg Components without Prior Positivity for Allergen Extracts

Requests for milk and egg component tests were replaced with their respective allergen extracts if these had not been ordered, or they were deleted pending the outcome of the allergen extracts testing. This process involved the addition of 312 allergen extracts tests for egg and 410 tests for cow's milk. The savings due to negative results of the allergen extract tests were 1160 tests for milk components and 1026 in egg components. The balance was 2186 fewer component tests performed.

Additional Registration of High Prevalence Allergens

According to our internal evaluation, *Salsola kali* and *Cupressus arizonica* are not included in Phadiatop, but in our experience, there is high sensitization to these allergens in our region. We intervened in the testing requests for 950 patients with respiratory symptoms, adding sIgE tests to both extracts, resulting in 1900 additional tests performed.

Similarly, we know that *Anisakis* spp are not included in fx5; however, in our influence area, which is a Mediterranean coastal area, the prevalence of *Anisakis* spp is high, and allergy to this parasite is underdiagnosed.¹¹ In this instance, we intervened in 305 requests with symptoms of urticaria, to which the sIgE testing for *Anisakis* spp was added, which meant performing 305 additional tests. The return on investment was that 27.9% of the interventions yielded positive results for *Salsola kali* and/or *Cupressus arizonica*, and 11.8% yielded positive results for *Anisakis* spp (TABLE 4).

Discussion

Requests for sIgE tests from PC centers increased at a higher rate than requests for other basic biochemistry tests, such as creatinine, which is included in most laboratory test requests. This net increase in demand for sIgE tests in recent years coincides with the widely documented increase in the prevalence of allergic diseases in Western countries.^{1,2} The overall results indicate a clear trend of increasing requests between

TABLE 4. Results of Extending sIgE Tests to Prevalent Allergen Extracts in the Influence Area

Variable	<i>Salsola kali</i> and <i>Cupressus arizonica</i>	<i>Anisakis</i> spp
Cases, No.	950	305
Age, y, median IQR	27–30	29–40
Female sex, No. (%)	516 (54.3%)	197 (64.6%)
Cases testing positive, No. (%)	265 (27.9%)	36 (11.8%)

the years 2014 and 2019. However, the monthly study shows variability in months and years due to the seasonal nature of allergies, which also vary from year to year due to different pollination rates. The most significant increase in testing occurs in April, which coincides with typical pollen patterns and is an indicator of the relevance of inhalant allergies for PC professionals in our region.

The strategies we implemented for decreasing excess testing demand decreased the number of unnecessary specific sIgE tests by 2186. These strategies allowed for greater test reduction than that yielded by strategies aimed at correction of underrequesting (which resulted in 1503 additional tests). Our strategies generated direct savings (number of tests) and indirect savings (reductions in unnecessary workload for laboratory staff, reductions in PC visits, and reductions in unnecessary medication) while also improving diagnosis and patient care.

Phadiatop/fx5 tests were performed for 1673 requests for sIgE for the allergen extracts that are included in those tests. Although performing these tests required prior review by the physician and increased the number of Phadiatop/fx5 tests performed, it reduced the number of tests per patient to less than half of the previous amounts. The high impact of this strategy is probably due to a lack of knowledge on the part of PC prescribing physicians, for whom allergen mixes are a useful tool.⁷

The elimination of sIgE tests for mixes requested at the wrong time was another strategy that was simple to implement in the LIS. This strategy allowed savings in costs and tests used, while making the most use of the information provided by sIgE testing.

The elimination of sIgE tests for extracts of allergens not prevalent in this area requires adequate knowledge of the most prevalent and allergenic local allergens but also allowed us to avoid unnecessary testing without losing information on the patient sensitization profiles. In this sense, each laboratory, in agreement with SC centers, must carry out a study to determine which allergen extracts are not appropriate for study in a certain area.

The strategy with the greatest impact was not testing for milk and egg components without prior results for allergen extracts: this strategy saved 2186 unnecessary sIgE tests for components. This action had no impact on the characterization of patient sensitization profiles because patients who tested positive for allergen extracts were also tested for allergen components. The results probably reflect the need to train our PC professionals in detection of food allergies.

Finally, the addition of sIgE tests to the most important sources of allergies in our region, which was fully automated in the LIS, resulted in a net increase in testing and costs, which was clearly offset by savings realized by the use of subsequent strategies. In fact, more than 300 patients were classified as being allergic to *Salsola kali* and *Cupressus arizonica* or *Anisakis* spp—patients who would not have been diagnosed if merely the clinician requests had been followed. Also, indirect savings

were generated by preventing new requests/PC visits resulting from not finding the disease-causing allergen in the first analysis or because the patient could not be classified as having allergies, causing confusion for the physician and patient.

The balance of all the aforementioned information shows that our strategy in the rationalization of sIgE tests is functional and adequate in our environment—it has already been shown in similar strategies related to other biochemical biomarkers.^{10,11} The net financial balance associated with our strategy falls within the savings range of €9480 to €18,960, depending on the sIgE test price, which varies across the different laboratories due to public tenders, volume, taxes, etc.

The study has certain limitations. First, due to the regional variability in allergic pathologies and the importance of factors such as race in the patient sensitization profile, the data in this manuscript may not be extrapolated to all other laboratories, regions, countries, or hospitals. Also, we were unable to compare the percentage of patients diagnosed with allergies before and after implementation because that information is not automatically recorded by the medical records system and it necessarily implies the detailed review of a vast amount of clinical records. However, to our knowledge, this is the first study in the literature to describe an accurate strategy, agreed on between all stakeholders involved in allergy diagnosis, to improve allergy screening while reducing costs to the health care system. Given our data, we encourage allergists, PC physicians and pediatricians, and laboratory professionals to work together to evaluate how to adapt the strategy described in this manuscript in their daily routines.

Second, allergy screening tests are not available in all regions. However, there is a huge amount of evidence suggesting the usefulness of these tests worldwide,^{9,12–15} even when not used as part of a well-defined screening strategy. As a result, allergy screening could be a useful option for clinical laboratories to incorporate into their testing portfolio.

Conclusions

The results of this study show how the allergy laboratory, when working closely with PC and SC centers, plays a key role in managing the active demand for sIgE testing. The laboratory leads to better selection of testing and diagnosis of patients with allergies, which improves patient outcome while reducing the direct and indirect costs of allergy testing, providing benefits to payers as well.

Acknowledgments

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Platts-Mills TAE. The allergy epidemics: 1870–2010. *J Allergy Clin Immunol*. 2015;136(1):3–13. doi:10.1016/j.jaci.2015.03.048
2. Kupryś I, Kuna P. Epidemics of allergic diseases: a new health problem in the modern world [in Polish]. *Pol Merkur Lekarski*. 2003;14(83):453–455.
3. Casas ML, Esteban A, González-Muñoz M, et al. Proyecto VALIDA: Validation of Allergy *In vitro* Diagnostics Assays (Herramientas y recomendaciones para la valoración de las pruebas in vitro en el diagnóstico de la alergia). *Adv Lab Med/Av en Med Lab*. 2020;1(4):20200022. doi:10.1515/almed-2020-0022
4. Kwong KYC, Eghrari-Sabet JS, Mendoza GR, Platts-Mills T, Horn R. The benefits of specific immunoglobulin E testing in the primary care setting. *Am J Manag Care*. 2011;17(17):S447–S459.
5. Jutel M, Papadopoulos NG, Gronlund H, et al. Recommendations for the allergy management in the primary care. *Allergy*. 2014;69(6):708–718. doi:10.1111/all.12382
6. Brożek JL, Bousquet J, Agache I, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines—2016 revision. *J Allergy Clin Immunol*. 2017;140(4):950–958. doi:10.1016/j.jaci.2017.03.050
7. Duran-Tauleria E, Vignati G, Guedan MJ, Petersson CJ. The utility of specific immunoglobulin E measurements in primary care. *Allergy*. 2004;59(Suppl 7):35–41. doi:10.1111/j.1398-9995.2004.00566.x
8. Dekker FW, Dzn JDM, Kramps JA, Kaptein AA, Vandenbroucke JP, Dijkman JH. The Phadiatop *in vitro* test for allergy in general practice: is it useful? *Fam Pract*. 1990;7(2):144–148. doi:10.1093/fampra/7.2.144
9. Zeng G, Hu H, Zheng P, et al. The practical benefit of Phadiatop test as the first-line *in vitro* allergen-specific immunoglobulin E (sIgE) screening of aeroallergens among Chinese asthmatics: a validation study. *Ann Transl Med*. 2018;6(8):151. doi:10.21037/atm.2018.04.06
10. Del Amo Del Arco N, Márquez Liétor E, Ramos Corral R, Guillén Santos R, Bernabeu Andreu FA, Cava Valenciano F. Effectiveness of an intervention to improve demand management of laboratory tests related to anaemia in primary care [in Spanish]. *J Healthc Qual Res*. 2020;35(5):291–296. doi:10.1016/j.jhqr.2019.12.002
11. Furundarena JR, Uranga A, González C, et al. Initial study of anaemia profile for primary care centres with automated laboratory algorithms reduces the demand for ferritin, iron, transferrin, vitamin B₁₂ and folate tests. *J Clin Pathol*. 2022;75(2):94–98. doi:10.1136/jclinpath-2020-207130
12. Hwang H, Kwon J, Kim JY, Lee HH, Oh CE, Choi G-S. The RIDA allergy screen versus the Phadiatop test in 430 consecutive patient specimens. *Lab Med*. 2016;47(1):20–29. doi:10.1093/labmed/lmv002
13. Mikkelsen S, Dinh KM, Boldsen JK, et al. Combinations of self-reported rhinitis, conjunctivitis, and asthma predicts IgE sensitization in more than 25,000 Danes. *Clin Transl Allergy*. 2021;11(1):e12013. doi:10.1002/ctt2.12013
14. Vidal C, Gude F, Boquete O, et al. Evaluation of the phadiatop test in the diagnosis of allergic sensitization in a general adult population. *J Investig Allergol Clin Immunol*. 2005;15(2):124–130.
15. Chang YC, Lee TJ, Huang CC, Chang P-H, Chen Y-W, Fu C-H. The role of Phadiatop tests and total immunoglobulin E levels in screening aeroallergens: a hospital-based cohort study. *J Asthma Allergy*. 2021;14:135–140. doi:10.2147/JAA.S292710.

Diagnostic Role of Circulating miRNAs in the Grading of Chronic Hepatitis B–Related Liver Fibrosis: A Systematic Review and Meta-Analysis

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Keywords: miRNA, liver fibrosis, chronic hepatitis B, systematic review, meta-analysis, biomarker

Abbreviations: CHB, chronic hepatitis B; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio; SROC, summary receiver operating characteristic curve; CIs, confidence intervals; HBV, hepatitis B virus; TP, true-positive; FN, false-negative; TN, true-negative; FP, false-positive; QUADAS-2, Quality Assessment of Diagnostic Accuracy Studies-2; SROC, summary receiver operating characteristic; I^2 , inconsistency index

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ABSTRACT

Objective: miRNAs are considered potential biomarkers that can be used for the grading of chronic hepatitis B (CHB)–related liver fibrosis. This meta-analysis aims to elucidate the diagnostic performance of miRNAs.

Methods: Databases were used to search for meta-analyses. A bivariate model was used to calculate pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR). In addition, the area under the summary receiver operating characteristic curve (AUC) and 95% confidence intervals (CIs) were calculated.

Results: A total of 9 studies with 1159 patients with CHB-related liver fibrosis were assessed. For diagnosis of significant liver fibrosis, the pooled sensitivity, specificity, PLR, NLR, DOR, and AUC were 0.73 (95% CI, 0.68–0.78), 0.78 (95% CI, 0.70–0.84), 3.32 (95% CI, 2.52–4.37), 0.34 (95% CI, 0.30–0.39), 9.70 (95% CI, 7.10–13.24), and 0.81 (95% CI, 0.77–0.84), respectively.

Conclusion: miRNAs are potential biomarkers of CHB-related liver fibrosis.

Liver fibrosis is characterized by abnormal connective tissue hyperplasia resulting from liver injury caused by various pathogenic factors. It is an intermediate stage of several chronic liver diseases and can eventually develop into cirrhosis and liver cancer if not treated promptly and effectively.¹ Chronic hepatitis B (CHB) is caused by hepatitis B virus (HBV) infection and is characterized by chronic inflammation, hepatocyte necrosis, or liver inflammatory lesions. Continuous progression of CHB may lead to liver fibrosis. According to the World Health Organization, there are more than 292 million patients with CHB worldwide, accounting for approximately 3.9% of the global population, and there are approximately 90 million persons with CHB in China.² In addition, approximately 1% to 2% of patients with CHB worldwide have liver fibrosis.² According to previous basic or clinical studies, liver fibrosis may be prevented or reversed.^{3,4} Therefore, early detection and accurate diagnosis of liver fibrosis are important for subsequent treatment.

Liver biopsy is the gold standard for diagnosing liver fibrosis and assessing its severity and is the most accurate diagnostic method available at present. However, it is easily influenced by sampling differences such as size, number, and location of liver samples or diagnostic level of case sample observers, thus imposing a certain risk. At present, some noninvasive methods are used for evaluating the stage of liver fibrosis in clinical settings. Transient elastography is the preferred noninvasive method for measuring liver stiffness, and its accuracy in the diagnosis of liver fibrosis in viral hepatitis is high.⁵ Yasaka et al⁶ used deep learning and deep convolutional neural networks to analyze the enhanced magnetic resonance imaging findings of the liver of 534 patients, which showed high diagnostic accuracy in the classification of liver fibrosis.

In addition to traditional imaging modalities, noninvasive diagnostic methods based on the detection of serum biomarkers can be used for the classification of persons with liver fibrosis. For example, a meta-analysis of 11 articles involving 1897 patients with liver fibrosis aged >18 years reported that serum chitinase-3-like protein 1 is a promising marker for the staging of liver fibrosis.⁷ The miRNAs are short, noncoding RNAs containing 18 to 25 nucleotides that regulate gene expression at the posttranscriptional level.⁸ Previous studies

have shown that the expression of some miRNAs is stage-dependent, and the expression profiles of miRNAs among patients with different degrees of liver fibrosis are different.^{9,10} In recent years, scholars have attempted to examine the diagnostic efficacy of circulating miRNAs in CHB-related liver fibrosis; however, the results have been inconsistent. Therefore, this meta-analysis aims to comprehensively evaluate the diagnostic efficacy of circulating miRNAs in CHB-related liver fibrosis.

Patients and Methods

Search Strategy

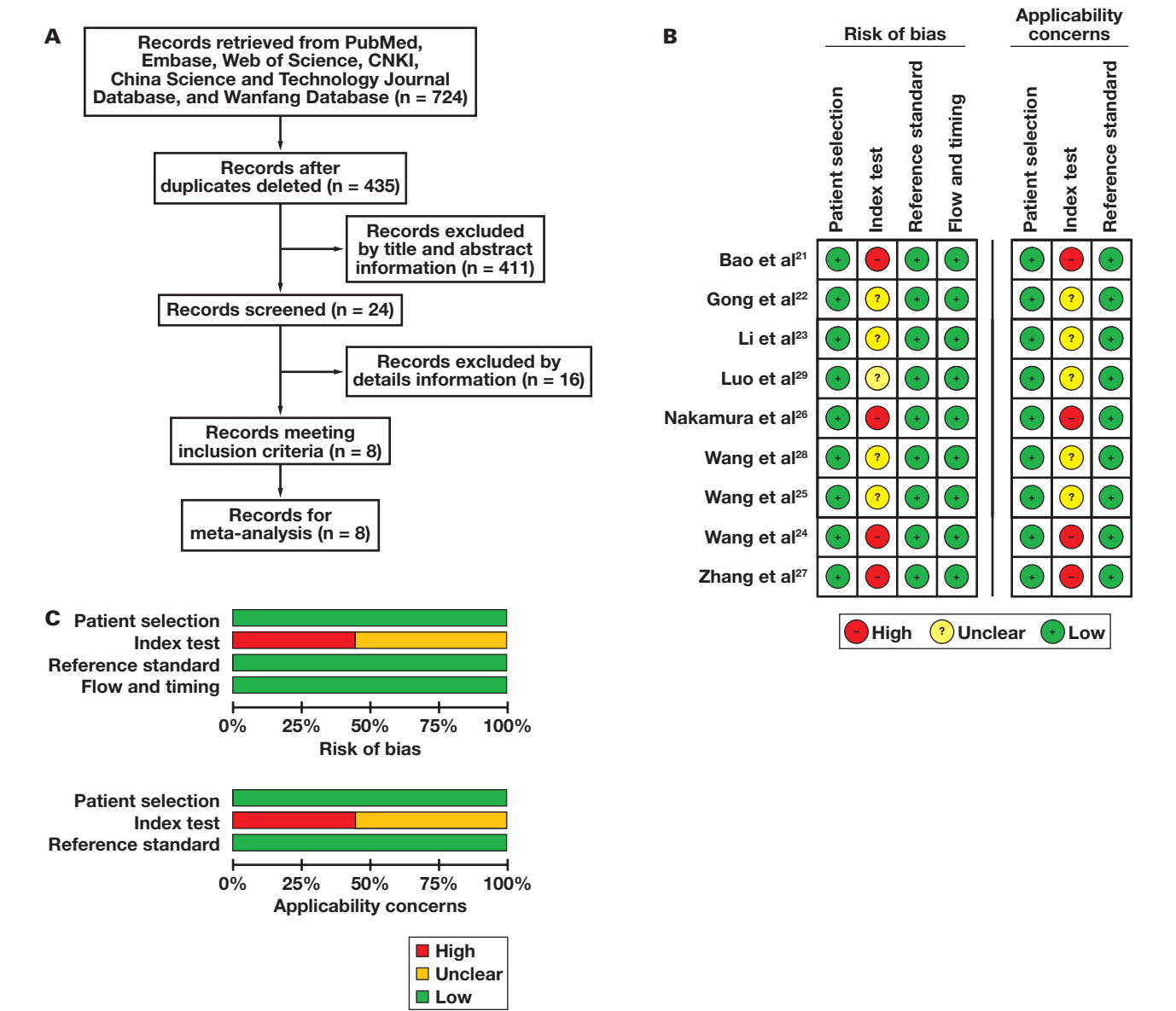
The PubMed, Embase, Web of Science, China National Knowledge Infrastructure, China Science and Technology Journal Database, and

Wanfang databases were used to search for published articles reporting on the correlation between miRNAs and CHB-related liver fibrosis using Boolean queries. The retrieval time was defined as the time from the establishment of each database to September 2022.

Inclusion and Exclusion Criteria

The inclusion criteria were as follows: (1) studies evaluating the diagnostic performance of miRNAs for the classification of CHB-related liver fibrosis; (2) studies using histopathology as a means of diagnosis of CHB-related liver fibrosis; (3) studies in which the degree of liver fibrosis was graded according to the METAVIR,¹¹ Scheuer,¹² or other relevant staging systems; (4) studies including sufficient data (including true-positive [TP], false-negative [FN], true-negative [TN] and false-positive [FP] results) for constructing a 4-grid table to evaluate diagnostic performance; (5) similar articles published

FIGURE 1. A, Flowchart of the meta-analysis. B, Methodological quality summary chart created using the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) tool. C, Methodological quality graph plotted using the QUADAS-2 tool.



by the same authors, of which the most recently published articles or those with the largest sample size were selected. The exclusion criteria were as follows: (1) studies on animal experiments; (2) case reports, meeting proceedings, and reviews with unavailable or missing primary data; (3) studies on patients with co-infection with other hepatitis viruses.

Data Extraction and Quality Evaluation

Two authors independently screened the literature according to the abovementioned inclusion and exclusion criteria, merged the literature obtained from the 6 databases, and removed the duplicate literature. The authors read titles and abstracts for preliminary screening. After the preliminary screening, they read the full text and refined the selected articles according to the inclusion and exclusion criteria. Finally, relevant data were extracted from the included articles, including the name of the first author; publication year; language; nationality, number, sex, and age of patients included in the articles; type of sample; detection method; type of miRNA; and TP, FN, TN, and FP results. Thereafter, the quality of the included articles was evaluated based on items in the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2).¹³ According to the actual content of each included article, 14 items were evaluated as “yes,” “no,” and “unclear,” and the conclusion that the risk of bias and applicability were “high,” “low,” or “unclear” was reached. The Review Manager 5.3 software was used to plot a quality evaluation chart of the included articles. Literature inclusion, data extraction, and quality evaluation were independently conducted by 2 researchers. When there were inconsistencies, a third researcher was appointed to discuss and make subsequent decisions.

Statistical Analysis

The Stata software (version 15.0) (Stata Corporation) was used for statistical analysis. A bivariate model was used to calculate the pooled effect size indicators including sensitivity, specificity, diagnostic odds ratio (DOR), positive likelihood ratio (PLR), negative likelihood ratio (NLR), area under the summary receiver operating characteristic (SROC) curve and their 95% confidence intervals (CIs). The basic idea of bivariate model fitting is as follows: owing to differences in the diagnostic boundary points and diagnostic test operation specifications among various studies, large differences were observed among various studies evaluating diagnostic performance, and the heterogeneity among these studies was strong. The conventional fixed effects model could not be used; however, a random effects model could be used for analysis.^{14–16} The heterogeneity of studies was mainly assessed using Cochran's Q test and by evaluating the inconsistency index (I^2).^{17,18} P values of $<.05$ were considered statistically significant, and I^2 values of $>50\%$ suggested high heterogeneity among studies. If the heterogeneity among studies was high, meta-regression and subgroup analyses were conducted to explore the source of heterogeneity. Robustness tests were used to determine whether the results of the meta-analysis were significantly affected by some studies. The Fagan nomogram was used to demonstrate the relationship among the pretest probability, likelihood ratio, and posttest probability.¹⁹ In addition, the publication bias of articles was mainly evaluated via Deek's funnel plot analysis,²⁰ and a P -value of $<.05$ indicated significant publication bias.

TABLE 1. Basic Characteristics of Included Studies

First Author	Year	Language	Country	Type of Sample	Assay	Criterion	miRNA	Internal/Control	No. of Patients	Age, y	Sex (Male/Female)
Wang Q	2021	English	China	Serum	qRT-PCR	Scheuer	miR-92a-3p, miR-146a-5p	Cel-miR-39	282	36 (30–46)	185/97
Zhang Q	2021	English	China	Plasma	qRT-PCR	Scheuer	miR-3162, miR-1225, miR-1238, miR-4721, miR-H7	Cel-miR-39	58	S0: 40.7 $\pm$ 14.9, S1: 33.8 $\pm$ 9.4, S2: 38.0 $\pm$ 8.4, S3: 39.4 $\pm$ 13.2, S4: 50.1 $\pm$ 12.6	S0: 6/4, S1: 10/5, S2: 8/5, S3: 6/4, S4: 7/3
Gong X	2021	Chinese	China	Serum	qRT-PCR	Scheuer	miR-223	U6	168	S0-1: 38.9 $\pm$ 9.5, S2: 37.7 $\pm$ 9.7, S3: 36.2 $\pm$ 9.3, S4: 37.5 $\pm$ 8.6	S0-1: 36/22, S2: 25/17, S3: 22/11, S4: 20/15
Wang TZ	2019	English	China	Plasma	RT-qPCR	Fibrosis-4 scores	miR-122-5p, miR-223, miR-29c-3p	Unknown	92	F0: 34.7 $\pm$ 12.8, F1: 34.8 $\pm$ 13.0, F2: 40.2 $\pm$ 10.1, F3: 38.0 $\pm$ 10.2, F4: 45.5 $\pm$ 9.3	F0-9/2, F1: 13/3, F2: 8/4, F3: 7/6, F4: 29/11
Li W	2019	Chinese	China	Serum	RT-qPCR	Fibrosis-4 scores	miR-21	Unknown	73	41.8 $\pm$ 12.0	48/25
Wang Y	2018	Chinese	China	Serum	RT-qPCR	Scheuer	miR-122	U6	138	37.0 $\pm$ 10.6	72/66
Bao S	2017	English	China	Serum	RT-qPCR	Scheuer	miR-29a, miR-29b, miR-29c, miR-143, miR-223, miR-21, miR-374	Cel-miR-39	123	S0-2: 28 (26–36), S3-4: 39 (31–49)	S0-2: 45/24, S3-4: 39/15
Nakamura M	2017	English	Japan	Serum	RT-qPCR	METAVIR	miR-122	Cel-miR-39	91	41.0 $\pm$ 10.4	66/25
Luo K	2022	Chinese	China	Serum	RT-qPCR	Scheuer	miR-21, miR-148b	U6	134	32.4 $\pm$ 8.3	75/59

NR, not reported; RT-qPCR, real-time reverse transcriptase-polymerase chain reaction.

Results

Literature Search

After the search strategy was defined, 724 Chinese and English articles were selected from 6 databases of which 289 duplicate articles were excluded and 435 articles were included. After reading the titles and abstracts for preliminary screening and excluding animal studies, reviews, conference abstracts, case reports, studies on nonhepatic fibrosis, and articles with undetected circulating miRNA expression levels, 24 articles were preliminarily selected. The remaining articles were further screened according to the inclusion and exclusion criteria by reading the full text. Eventually, 9 articles^{21–29} were included in the meta-analysis, with 4 Chinese and 5 English articles, involving 1159 patients with CHB-related liver fibrosis. The process of literature inclusion is shown in **FIGURE 1A**. The 9 included articles contained data on 19 miRNAs and were published between 2017 and 2022, and the study participants were mainly from Japan and China. The histological scoring systems used were not identical among studies, with 1 study using the METAVIR scoring system,¹¹ 6 studies using the Scheuer scoring system,¹² and 2 studies using the Fibrosis-4 Index for Liver Fibrosis.³⁰ Nonsignificant fibrosis was defined as a METAVIR score of $\leq$ F2, Scheuer score of $\leq$ S1 or S2, or Fibrosis-4 index of $\leq$ F1. Significant fibrosis was defined as a METAVIR score of $\geq$ F3, Scheuer score of $\geq$ S2 or S3, or Fibrosis-4 index of $\geq$ F2. The basic characteristics of included studies are shown in **TABLES 1** and **2**.

TABLE 2. Diagnostic Accuracy and Cut-Off Values

Author, Year	TP	FP	FN	TN	Cut-off Value	Regulation Mode
Wang Q, 2021	111	10	47	114	2.1	Upregulated
Wang Q, 2021	107	25	51	99	1.5	Upregulated
Nakamura M, 2017	23	26	7	35	Unknown	Downregulated
Zhang Q, 2021	28	0	5	25	Unknown	Upregulated
Zhang Q, 2021	26	0	7	25	Unknown	Upregulated
Zhang Q, 2021	20	5	13	20	Unknown	Upregulated
Zhang Q, 2021	13	0	20	25	Unknown	Upregulated
Zhang Q, 2021	17	2	16	23	Unknown	Upregulated
Wang TZ, 2019	47	7	18	20	0.066	Downregulated
Wang TZ, 2019	44	10	21	17	0.983	Downregulated
Wang TZ, 2019	60	16	5	11	0.283	Downregulated
Bao S, 2017	39	16	15	53	Unknown	Downregulated
Bao S, 2017	31	19	23	50	Unknown	Downregulated
Bao S, 2017	33	8	21	61	Unknown	Downregulated
Bao S, 2017	42	20	12	49	Unknown	Downregulated
Bao S, 2017	46	28	8	41	Unknown	Downregulated
Bao S, 2017	46	30	8	39	Unknown	Downregulated
Bao S, 2017	43	30	11	36	Unknown	Downregulated
Wang Y, 2018	53	8	25	52	Unknown	Downregulated
Li W, 2019	32	7	6	28	0.9835	Upregulated
Gong X, 2021	50	33	18	67	1.62	Upregulated
Luo K, 2022	77	8	26	23	1.5	Upregulated
Luo K, 2022	85	11	18	20	2	Downregulated

FN, false negative; FP, false positive; TN, true negative; TP, true positive.

Literature Quality Assessment

The QUADAS-2 scale was used to evaluate the quality of the included articles. The scale is divided into 4 aspects as follows: (1) patient selection, (2) index test, (3) reference standard, and (4) flow and timing. The results showed that the quality of the included articles was high, and a low risk was observed in patient selection, reference standard, and flow and timing. However, a high or unknown risk was observed in the index test. These results suggested that the threshold was not prespecified, and some studies did not clearly define the threshold. The selection of a test threshold to optimize sensitivity and specificity may improve diagnostic efficiency. Overall, the studies included were of high quality. The results are shown in **FIGURES 1B** and **1C**.

Meta-Analysis

The data of the 9 included articles were extracted and integrated. The pooled sensitivity was 0.73 (95% CI, 0.68–0.78) with an I^2 value of 71.27% (**FIGURE 2A**). The pooled specificity was 0.78 (95% CI, 0.70–0.84) with an I^2 value of 83.40% (**FIGURE 2B**). The pooled PLR was 3.32 (95% CI, 2.52–4.37) with an I^2 value of 62.87% (**FIGURE 2C**). The pooled NLR was 0.34 (95% CI, 0.30–0.39) with an I^2 value of 52.35% (**FIGURE 2D**). The pooled DOR was 9.70 (95% CI, 7.10–13.24) with an I^2 value of 99.95% (**FIGURE 2E**). Significant heterogeneity was observed in some studies. The area under the SROC curve was 0.81 (95% CI, 0.77–0.84) (**FIGURE 2F**). As shown in **FIGURE 3A**, when miRNAs were tested for all individuals with a 50% pretest probability of having significant CHB-related liver fibrosis, a positive result increased the posttest probability of

FIGURE 2. Forest plots of pooled sensitivity (A), pooled specificity (B), pooled PLR (C), pooled NLR (D), pooled DOR (E), and the pooled area under the SROC curve (F). DOR, diagnostic odds ratio; NLR, negative likelihood ratio; PLR, positive likelihood ratio; SROC, summary receiver operating characteristic.

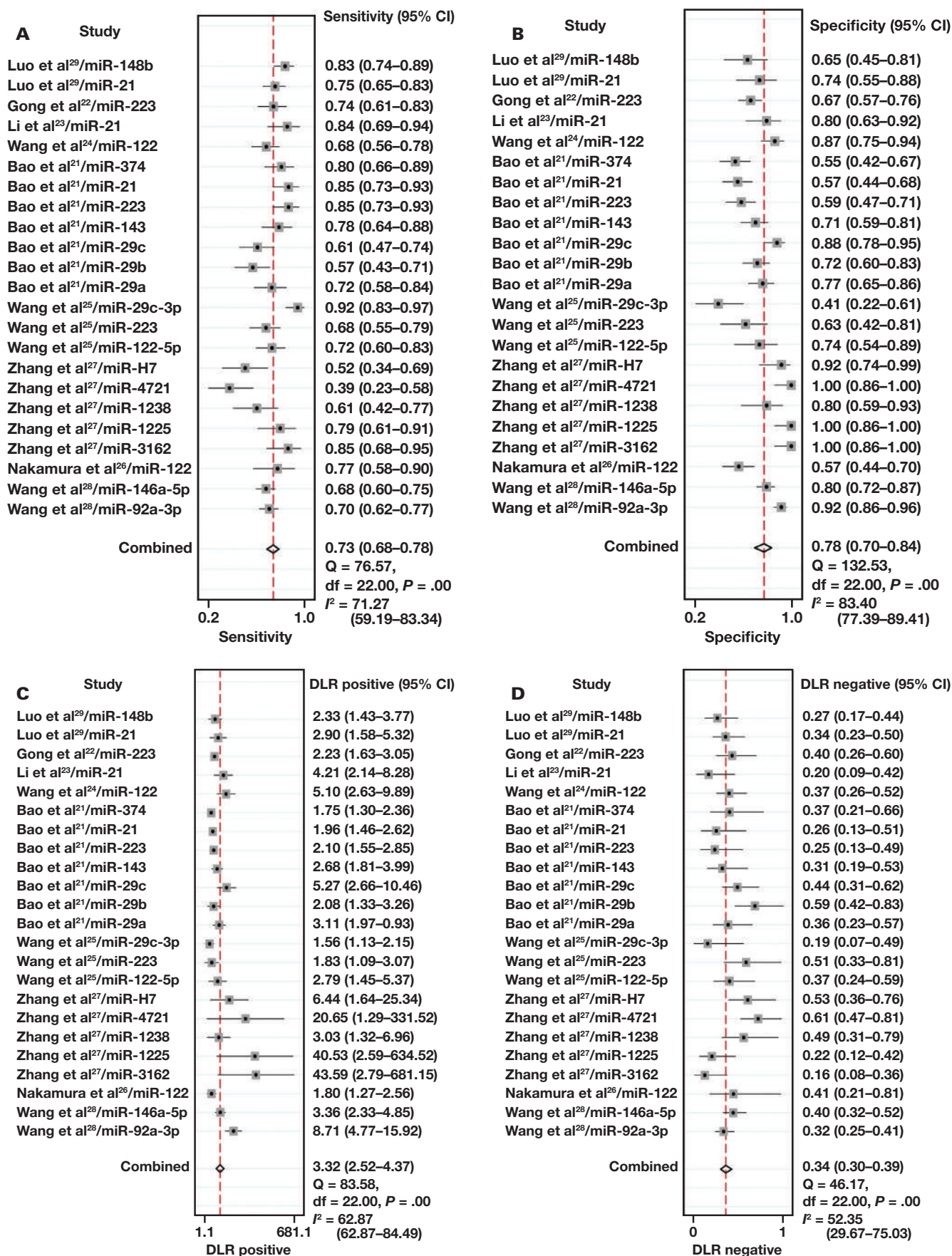
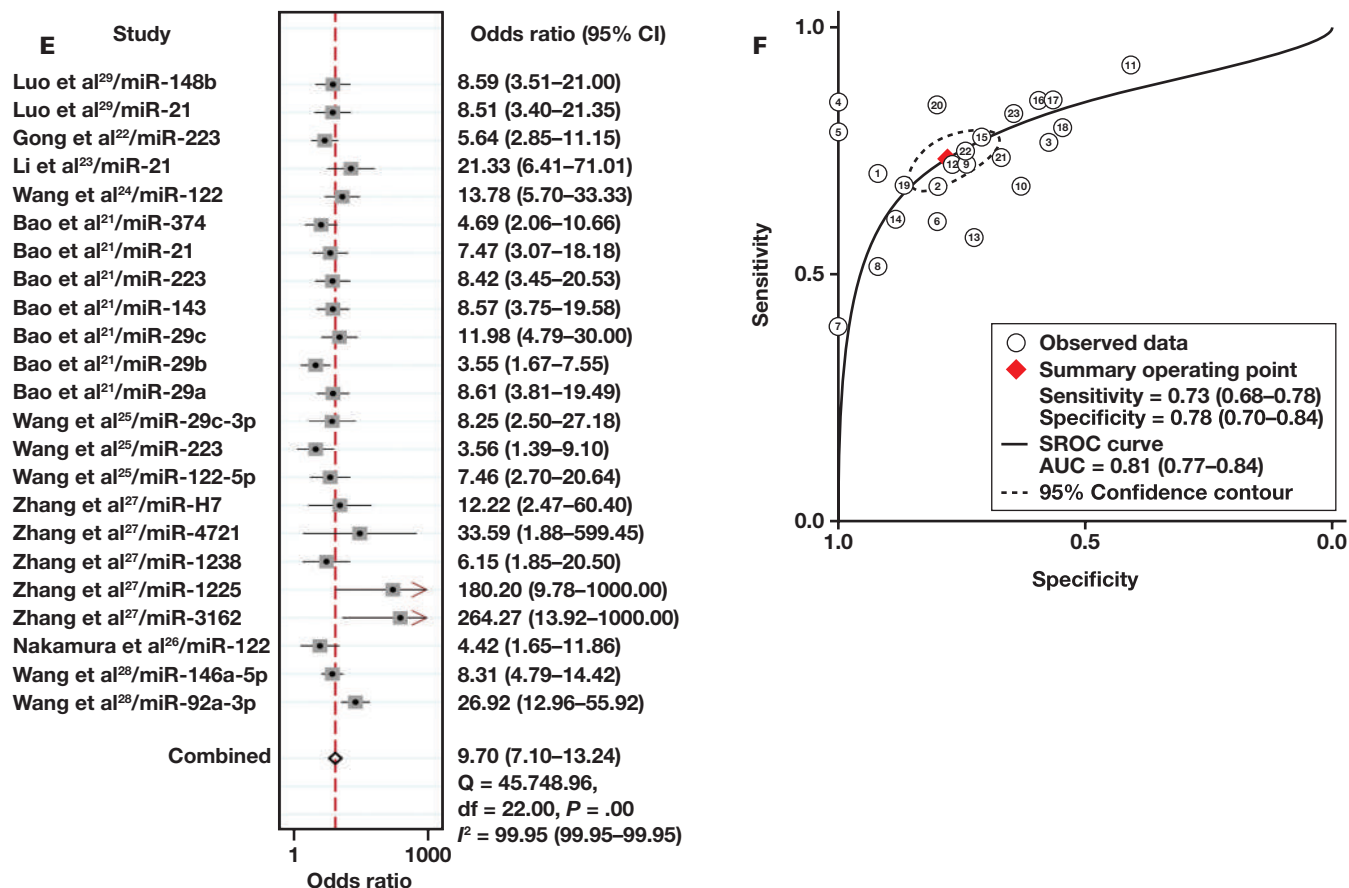


FIGURE 2. (cont)



having significant CHB-related liver fibrosis to 77%, whereas a negative result decreased the posttest probability to 25%.

Meta-Regression and Subgroup Analyses

The following factors were considered the possible sources of heterogeneity: whether miRNAs were highly expressed in patients with severe CHB-related liver fibrosis, whether the study was published in Chinese or English, whether the sample size was >100, whether serum samples were used, whether the internal miRNA reference was cel-mir-39 or U6, and whether the grading system used for liver fibrosis was the Scheuer scoring system or Fibrosis-4 Index for Liver Fibrosis. Based on meta-regression analysis, high miRNA expression, a sample size of >100, English language, serum samples, the use of cel-mir-39 or U6 as internal mRNA references, and the Scheuer scoring system were identified as the sources of significant heterogeneity in pooled sensitivity, and serum samples and the Fibrosis-4 Index for Liver Fibrosis were identified as the sources of significant heterogeneity in pooled specificity (FIGURE 3B). In addition, subgroup analysis revealed that studies reporting low miRNA expression in patients with significant CHB-related liver fibrosis, Chinese studies, studies with small sample size (<100), studies in which serum samples were used, and studies using internal miRNA references other than cel-mir-39 and U6 and a scoring system other than the Scheuer scoring system had higher pooled sensitivity. Studies in which serum samples and the Fibrosis-4 Index for Liver Fibrosis were not used had higher pooled specificity (TABLE 3).

Robustness Tests

Goodness-of-fit and bivariate normality analyses (FIGURES 4A and 4B) showed that the bivariate model was moderately robust. Influence analysis revealed 5 outliers of which 2 were found through outlier detection (FIGURES 4C and 4D). After removing these 5 studies, pooled sensitivity was 0.74 (95% CI, 0.70–0.77) with an I² value of 52.95%, pooled specificity was 0.75 (95% CI, 0.68–0.81) with an I² value of 80.38%, pooled PLR was 2.97 (95% CI, 2.38–3.69) with an I² value of 51.98%, pooled NLR was 0.35 (95% CI, 0.31–0.39) with an I² value of 0, and pooled DOR was 8.52 (95% CI, 6.60–10.99) with an I² value of 99.99%. The area under the SROC curve was 0.80 (95% CI, 0.76–0.83). After deleting these studies, the results were not significantly different from the previous results, indicating that the included studies were relatively robust.

Publication Bias

The publication bias was examined via Deek's analysis and the result showed that there was no publication bias (P = .51) (FIGURE 5).

Discussion

Liver fibrosis is a repair response caused by various pathogenic factors acting on the liver. It can lead to abnormal activation of hepatic stellate cells, massive proliferation, and secretion of the extracellular matrix, resulting in excessive deposition of collagen fibers in the liver.¹ Early and prompt diagnosis of liver fibrosis is very important to assess

FIGURE 3. A, Fagan nomogram for assessing the posttest probabilities of miRNAs for predicting significant liver fibrosis. B, Univariable meta-regression analysis for the sensitivity and specificity of miRNAs for predicting significant liver fibrosis. * $P < .05$; ** $P < .01$; *** $P < .001$.

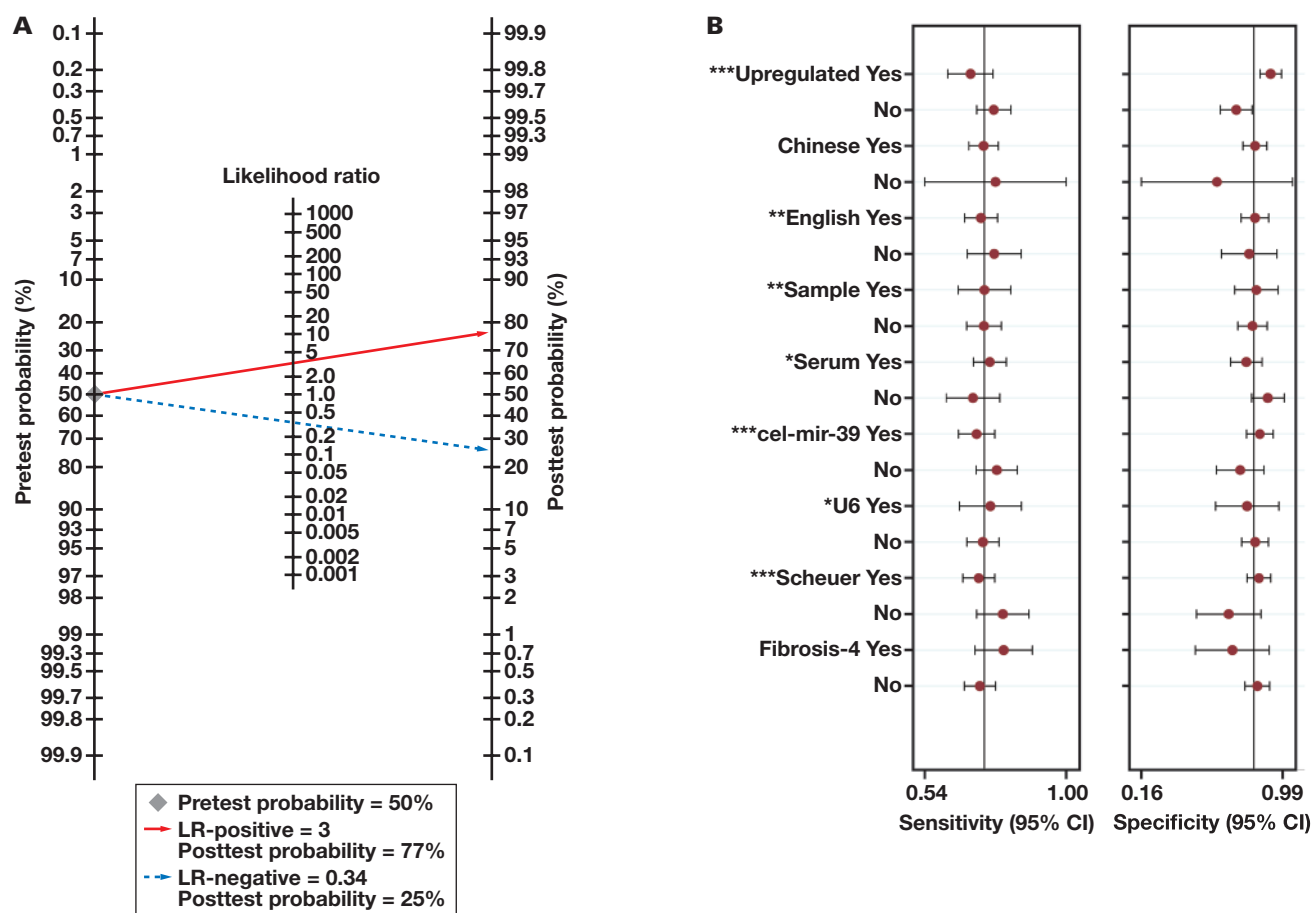
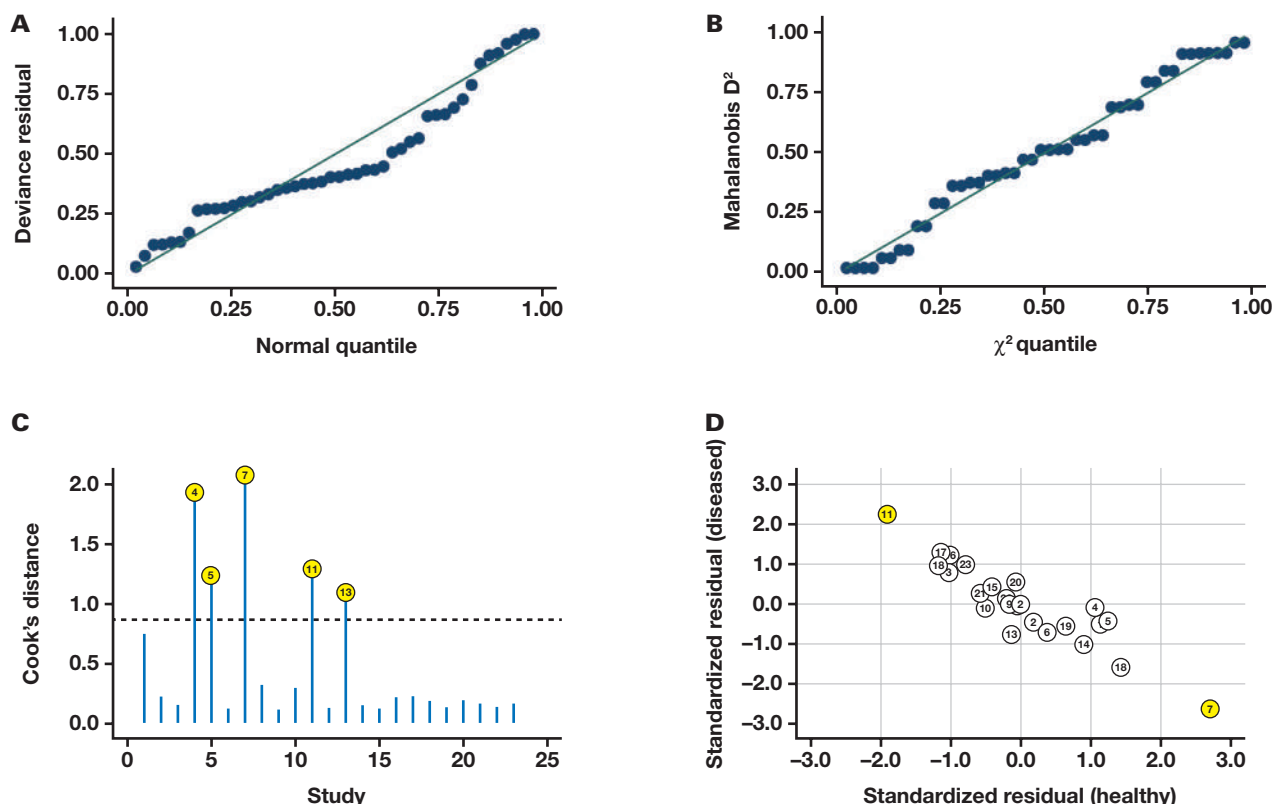


TABLE 3. Results of Univariable Meta-Regression and Subgroup Analyses

Parameter	Category	No. of Studies	Sensitivity	P1	Specificity	P2
Upregulated	Yes	10	0.69 (0.62–0.76)	<.01	0.87 (0.81–0.93)	.75
	No	13	0.76 (0.71–0.82)		0.68 (0.59–0.77)	
China	Yes	22	0.73 (0.68–0.78)	.41	0.79 (0.72–0.85)	.34
	No	1	0.77 (0.54–1.00)		0.57 (0.16–0.99)	
English	Yes	18	0.72 (0.67–0.78)	<.01	0.79 (0.71–0.86)	.3
	No	5	0.77 (0.68–0.85)		0.75 (0.60–0.91)	
Sample >100	Yes	6	0.73 (0.65–0.82)	<.01	0.79 (0.67–0.91)	.16
	No	17	0.73 (0.68–0.79)		0.77 (0.69–0.85)	
Serum	Yes	15	0.75 (0.70–0.80)	.03	0.74 (0.65–0.82)	<.01
	No	8	0.70 (0.61–0.78)		0.86 (0.77–0.95)	
Cel-miR-39	Yes	15	0.71 (0.65–0.77)	<.01	0.81 (0.74–0.89)	.51
	No	8	0.77 (0.71–0.84)		0.70 (0.57–0.84)	
U6	Yes	4	0.75 (0.65–0.85)	.03	0.74 (0.57–0.92)	.15
	No	19	0.73 (0.68–0.78)		0.79 (0.71–0.86)	
Scheuer	Yes	18	0.72 (0.66–0.77)	<.01	0.81 (0.74–0.87)	.88
	No	5	0.79 (0.71–0.88)		0.64 (0.46–0.82)	
Fibrosis-4	Yes	4	0.80 (0.70–0.89)	.1	0.66 (0.46–0.86)	.04
	No	19	0.72 (0.67–0.77)		0.80 (0.73–0.87)	

FIGURE 4. Influence analysis and outlier detection. A, Goodness-of-fit. B, Bivariate normality. C, Influence analysis. D, Outlier detection.



the progression of chronic liver disease, the clinical outcome of patients and the efficacy of antifibrotic drugs. Liver biopsy is considered the gold standard for the diagnosis and grading of liver fibrosis. However, several disadvantages of liver biopsy, such as large sample error and high bleeding risk, limit its widespread application in clinical practice. As noninvasive biomarkers, circulating miRNAs have great potential in the diagnosis of liver fibrosis. Therefore, this meta-analysis aimed to comprehensively evaluate the diagnostic efficacy of circulating miRNAs in CHB-related liver fibrosis.

Serological markers of liver fibrosis mainly include direct and indirect markers. The indirect markers include transaminase, bilirubin, coagulation function test, and platelet count. Most factors affect the formation of liver fibrosis during the progression of liver disease, which can represent the change in liver function but cannot accurately reflect the degree of liver fibrosis.³¹ miRNAs are widely present in tissues, plasma, serum, or other body fluids with stable expression and are not degraded by endogenous ribonucleases. They participate in various physiological and pathological processes in the body and serve as biomarkers in various diseases.^{32–34} Therefore, miRNAs can be used as noninvasive biological markers for the grading of liver fibrosis. In addition to the use of individual miRNAs, multiple miRNAs can be combined for the grading of CHB-related liver fibrosis.^{29,35} Long et al³⁶ constructed an 88-miRNA signature, which can distinguish between patients with cirrhosis and CHB based on serum samples.

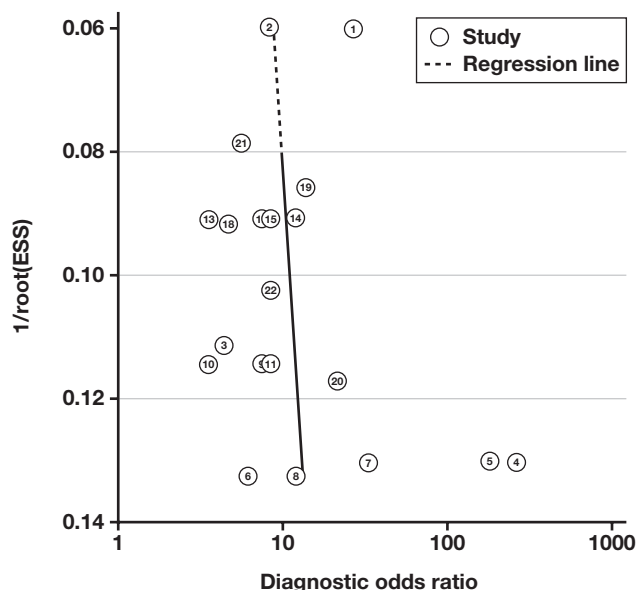
In this meta-analysis, relatively more studies were found to use miR-223, miR-21, and miR-122. The miR-223 is involved in the process of fibrosis in cardiomyocytes³⁷ and the lungs.³⁸ The serum level of miR-223 in patients with CHB is increased, which is a risk factor for liver fibrosis

in patients with CHB.³⁹ CHB-related liver fibrosis has unique pathogenesis. A recent study showed that the X gene of HBV can upregulate the expression of miR-21 in hepatocytes,⁴⁰ indicating that simple antagonism of TGF- β 1 cannot effectively inhibit CHB-related liver fibrosis.⁴¹ The miR-122 plays an important role in lipid metabolism, hepatitis virus replication, liver fibrosis, and liver cancer.⁴² It inhibits the proliferation of hepatic stellate cells, reduces collagen production, and plays an antifibrotic role.⁴³ In this meta-analysis, miR-122 expression was found to be low in patients with significant liver fibrosis in the included studies. Therefore, the 3 types of miRNAs have been used as biomarkers for the grading of liver fibrosis; however, additional studies are required for verification.

In this meta-analysis, significant heterogeneity was observed among studies, and some heterogeneous factors were identified via meta-regression analysis. In addition to common factors such as sample size and publication language, different levels of miRNA expression, sample sources, staging methods, and internal references of miRNA contributed to the heterogeneity. A study reported that miRNA expression was different in the plasma and serum of the same patients.⁴⁴ In addition, another study reported that miRNA expression was significantly higher in the serum samples of patients with hepatitis and liver cirrhosis than in their plasma samples, and miRNA expression evaluated using different internal references was also different.⁴⁵ Therefore, caution is required when comparing the miRNA data of different extraction kits and sample types, which may explain the differences in pooled sensitivity and specificity among studies.

Furthermore, 2 classification systems of liver fibrosis were identified as a source of significant heterogeneity. The Scheuer scoring system was used more commonly than the Fibrosis-4 Index for Liver

FIGURE 5. Deeks' funnel plot asymmetry test for assessing publication bias. $P = .51$.



Fibrosis in the included studies. The Scheuer scoring system relies on pathological tissue biopsy for the grading of liver fibrosis, whereas the Fibrosis-4 Index for Liver Fibrosis only relies on the age, platelet count, alanine aminotransferase levels, and aspartate aminotransferase levels of patients for the grading of liver fibrosis. Therefore, the classification of the Fibrosis-4 Index for Liver Fibrosis may not be accurate but can be easily applied. However, we found that both miR-122 and miR-223 had good diagnostic efficacy for liver fibrosis as determined by both grading systems. Therefore, miRNAs are promising biomarkers for the graded diagnosis of CHB-related liver fibrosis.

This meta-analysis has some limitations. First, significant heterogeneity was observed. However, some possible sources of heterogeneity were identified. Second, miRNA expression in the included studies was evaluated according to clinical criteria after the grading of CHB-related liver fibrosis, which may increase the population selection bias. Third, the included studies are Asian, and the applicability of results to other populations may be open to discussion. In addition, the statistical methods used in the included studies could not be controlled, which may have affected the results.

Conclusions

Circulating miRNAs are potential markers that can be used to grade CHB-related liver fibrosis, especially in Asian patients. Large and well-designed studies should be performed to determine the role of promising miRNAs in the grading of CHB-related liver fibrosis.

Acknowledgments

We thank Bullet Edits for linguistic editing and proofreading of the manuscript. All analyses in the present meta-analysis are aligned with the established medical ethics guidelines. This meta-analysis is based on published articles. Therefore, informed consent was not required.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

- Aydın MM, Akçali KC. Liver fibrosis. *Turk J Gastroenterol*. 2018;29:14–21. doi:10.5152/tjg.2018.17330
- Polaris Observatory Collaborators. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. *Lancet Gastroenterol Hepatol*. 2018;3:383–403.
- van der Meer AJ, Sonneveld M, Schouten JN, Janssen HL. [Reversibility of hepatic fibrosis]. *Ned Tijdschr Geneesk*. 2014;158:A6790.
- Roehlen N, Crouchet E, Baumert TF. Liver fibrosis: mechanistic concepts and therapeutic perspectives. *Cells*. 2020;9:875. doi:10.3390/cells9040875
- Agbim U, Asrani SK. Non-invasive assessment of liver fibrosis and prognosis: an update on serum and elastography markers. *Expert Rev Gastroenterol Hepatol*. 2019;13:361–374. doi:10.1080/17474124.2019.1579641
- Yasaka K, Akai H, Kunitatsu A, Abe O, Kiryu S. Liver fibrosis: deep convolutional neural network for staging by using gadoxetic acid-enhanced hepatobiliary phase MR images. *Radiology*. 2018;287:146–155. doi:10.1148/radiol.2017171928
- Huang X, Zhuang J, Yang Y, et al. Diagnostic value of serum chitinase-3-like protein 1 for liver fibrosis: a meta-analysis. *Biomed Res Int*. 2022;2022:3227957. doi:10.1155/2022/3227957
- Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol*. 2007;23:175–205. doi:10.1146/annurev.cellbio.23.090506.123406
- Li BB, Li DL, Chen C, et al. Potentials of the elevated circulating miR-185 level as a biomarker for early diagnosis of HBV-related liver fibrosis. *Sci Rep*. 2016;6:34157. doi:10.1038/srep34157
- Halász T, Horváth G, Pár G, et al. miR-122 negatively correlates with liver fibrosis as detected by histology and FibroScan. *World J Gastroenterol*. 2015;21:7814–7823. doi:10.3748/wjg.v21.i25.7814
- Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology*. 1996;24:289–293. doi:10.1002/hep.510240201
- Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology*. 1994;19:1513–1520.
- Whiting PF, Rutjes AW, Westwood ME, et al., QUADAS-2 Group. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. *Ann Intern Med*. 2011;155:529–536. doi:10.7326/0003-4819-155-8-201110180-00009
- Chu H, Cole SR. Bivariate meta-analysis of sensitivity and specificity with sparse data: a generalized linear mixed model approach. *J Clin Epidemiol*. 2006;59:1331–1332.
- Reitsma JB, Glas AS, Rutjes AW, Scholten RJ, Bossuyt PM, Zwinderman AH. Bivariate analysis of sensitivity and specificity produces informative summary measures in diagnostic reviews. *J Clin Epidemiol*. 2005;58:982–990. doi:10.1016/j.jclinepi.2005.02.022
- Harbord RM, Whiting P, Sterne JA, et al. An empirical comparison of methods for meta-analysis of diagnostic accuracy showed hierarchical models are necessary. *J Clin Epidemiol*. 2008;61:1095–1103. doi:10.1016/j.jclinepi.2007.09.013
- Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med*. 2002;21:1539–1558. doi:10.1002/sim.1186
- Ioannidis JP. Interpretation of tests of heterogeneity and bias in meta-analysis. *J Eval Clin Pract*. 2008;14:951–957. doi:10.1111/j.1365-2753.2008.00986.x
- Caraguel CG, Vanderstichel R. The two-step Fagan's nomogram: ad hoc interpretation of a diagnostic test result without calculation. *Evid Based Med*. 2013;18:125–128. doi:10.1136/eb-2013-101243

20. Deeks JJ, Macaskill P, Irwig L. The performance of tests of publication bias and other sample size effects in systematic reviews of diagnostic test accuracy was assessed. *J Clin Epidemiol*. 2005;58:882–893. doi:10.1016/j.jclinepi.2005.01.016
21. Bao S, Zheng J, Li N, et al. Serum MicroRNA levels as a noninvasive diagnostic biomarker for the early diagnosis of hepatitis B virus-related liver fibrosis. *Gut Liver*. 2017;11:860–869. doi:10.5009/gnl16560
22. Gong X, Wan S, Fu C. The expression and clinical significance of miR-223 in serum from patients with hepatitis B and advanced liver fibrosis. *J Pathog Biol*. 2021;16(10):1202–1206.
23. Li W, Zhao Z, Li Y. Value of serum miR-21 level in evaluating the degree of liver fibrosis in patients with chronic hepatitis B. *Chin Hepatol*. 2019;24(1):48–51.
24. Wang Y, Chen X, Li Y, et al. Expression and clinical significance of serum microRNA-122 in liver fibrosis of patients with chronic hepatitis B. *Chin J Infect Dis*. 2018;36(9):533–536.
25. Wang TZ, Lin DD, Jin BX, Sun XY, Li N. Plasma microRNA: a novel non-invasive biomarker for HBV-associated liver fibrosis staging. *Exp Ther Med*. 2019;17:1919–1929. doi:10.3892/etm.2018.7117
26. Nakamura M, Kanda T, Jiang X, et al. Serum microRNA-122 and Wisteria floribunda agglutinin-positive Mac-2 binding protein are useful tools for liquid biopsy of the patients with hepatitis B virus and advanced liver fibrosis. *PLoS One*. 2017;12:e0177302. doi:10.1371/journal.pone.0177302
27. Zhang Q, Zhang Q, Li B, et al. The diagnosis value of a novel model with 5 circulating miRNAs for liver fibrosis in patients with chronic hepatitis B. *Mediators Inflamm*. 2021;2021:6636947. doi:10.1155/2021/6636947
28. Wang Q, Hu Q, Ying Y, et al. Using next-generation sequencing to identify novel exosomal miRNAs as biomarkers for significant hepatic fibrosis. *Discov Med*. 2021;31:147–159.
29. Luo K, Ding L, Liu M. Serum miR-21 and miR-148b levels in patients with chronic hepatitis B and their predictive value for liver tissue inflammation grading and fibrosis staging. *J Prac Hepatol*. 2022;25(3):335–338.
30. Sterling RK, Lissen E, Clumeck N, et al., APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology*. 2006;43:1317–1325. doi:10.1002/hep.21178
31. Ebrahimi H, Naderian M, Sohrabpour AA. New concepts on pathogenesis and diagnosis of liver fibrosis; a review article. *Middle East J Dig Dis*. 2016;8:166–178. doi:10.15171/mejdd.2016.29
32. Durante G, Broseghini E, Comito F, et al. Circulating microRNA biomarkers in melanoma and non-melanoma skin cancer. *Expert Rev Mol Diagn*. 2022;22:305–318. doi:10.1080/14737159.2022.2049243
33. Zafari N, Bahramy A, Majidi Zolbin M, et al. microRNAs as novel diagnostic biomarkers in endometriosis patients: a systematic review and meta-analysis. *Expert Rev Mol Diagn*. 2022;22:479–495. doi:10.1080/14737159.2021.1960508
34. Gu Y, Yuan J. Diagnostic significance of miRNAs as potential biomarkers for human renal cell carcinoma: a systematic review and meta-analysis. *Expert Rev Anticancer Ther*. 2022;22:437–444. doi:10.1080/14737140.2022.2051483
35. Chen YJ, Zhu JM, Wu H, et al. Circulating microRNAs as a fingerprint for liver cirrhosis. *PLoS One*. 2013;8:e66577. doi:10.1371/journal.pone.0066577
36. Long XR, Zhang YJ, Zhang MY, Chen K, Zheng X, Wang HY. Identification of an 88-microRNA signature in whole blood for diagnosis of hepatocellular carcinoma and other chronic liver diseases. *Aging*. 2017;9:1565–1584.
37. Liu X, Xu Y, Deng Y, Li H. MicroRNA-223 regulates cardiac fibrosis after myocardial infarction by targeting RASA1. *Cell Physiol Biochem*. 2018;46:1439–1454. doi:10.1159/000489185
38. Qu SJ, Zhao L, Song ZZ, Shen WP, Ju P, Li YM. Expression and significance of miR-223 in rats with pulmonary fibrosis. *Eur Rev Med Pharmacol Sci*. 2020;24:3951–3958. doi:10.26355/eurev_202004_20864
39. Loureiro D, Tout I, Narguet S, Benazzouz SM, Mansouri A, Asselah T. miRNAs as potential biomarkers for viral hepatitis B and C. *Viruses*. 2020;12:1440. doi:10.3390/v12121440
40. Ye L, Kan F, Yan T, et al. Enhanced antiviral and antifibrotic effects of short hairpin RNAs targeting HBV and TGF- β in HBV-persistent mice. *Sci Rep*. 2017;7:3860. doi:10.1038/s41598-017-04170-1
41. Hou Z, Quan J. Hepatitis B virus X protein increases microRNA-21 expression and accelerates the development of hepatoma via the phosphatase and tensin homolog/phosphoinositide 3-kinase/protein kinase B signaling pathway. *Mol Med Rep*. 2017;15:3285–3291. doi:10.3892/mmr.2017.6363
42. Miyaaki H, Ichikawa T, Kamo Y, et al. Significance of serum and hepatic microRNA-122 levels in patients with non-alcoholic fatty liver disease. *Liver Int*. 2014;34:e302–e307. doi:10.1111/liv.12429
43. Li J, Ghazwani M, Zhang Y, et al. miR-122 regulates collagen production via targeting hepatic stellate cells and suppressing P4HA1 expression. *J Hepatol*. 2013;58:522–528. doi:10.1016/j.jhep.2012.11.011
44. Foye C, Yan IK, David W, et al. Comparison of miRNA quantitation by Nanostring in serum and plasma samples. *PLoS One*. 2017;12:e0189165. doi:10.1371/journal.pone.0189165
45. Wang Y, Liang Z, Gao Y, et al. Factors influencing circulating MicroRNA level in the studies of hepatocellular carcinoma biomarker. *Neoplasma*. 2015;62:798–804. doi:10.4149/neo_2015_096

Evaluation of Antinuclear Antibody and Subserology Reflex Testing for the Diagnosis of Systemic Autoimmune Rheumatic Disorders in an Academic Teaching Hospital

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Keywords: antinuclear antibody, subserology, systemic autoimmune rheumatic disease, SARD, safety net, panel testing, reflex testing

Abbreviations: ANA, antinuclear antibody; SARD, systemic autoimmune rheumatic disorder; ACR, American College of Rheumatology; dsDNA, anti-double-stranded DNA; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; SS, Sjögren's syndrome; anti-Sm, anti-Smith; anti-SSA, anti-SS-related antigen A; anti-SSB, anti-SS-related antigen B; anti-Scl-70; anti-topoisomerase I; anti-CCP, anti-cyclic citrullinated peptide; IRB, institutional review board; PLR, positive likelihood ratio

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ABSTRACT

Objective: The aim of this study was to examine appropriate utilization of antinuclear antibody (ANA) screening tests with follow-up subserology tests (reflex testing) for diagnosing systemic autoimmune rheumatic disorder (SARD).

Methods: We conducted a retrospective chart review of 3003 SARD-test orders at an academic teaching hospital from January to December 2019. Testing patterns were categorized as American College of Rheumatology (ACR)-recommended reflex testing, panel testing, or single subserology testing. We described testing patterns, assessed their diagnostic accuracy, and explored factors associated with reflex testing.

Results: Reflex testing accounted for 79.7% of SARD test-ordering, whereas improper testing (panel or single subserology) accounted for the other 20.3%. Reflex testing was associated with significantly more SARD diagnoses than improper testing ($P = .004$). Testing patterns were significantly associated with race/ethnicity ($P = .008$), with reflex

testing being less frequent than improper testing in Hispanics and Whites.

Conclusion: In summary, one-fifth (20.3%) of testing patterns for suspected SARD did not follow the ACR-recommended guidelines for using reflex testing. Use of reflex testing was associated with an increased frequency of SARD diagnosis.

Clinical laboratory diagnostic tests are continually being evaluated to ensure efficient utilization. Antinuclear antibody (ANA) and subserology tests for evaluating suspected systemic autoimmune rheumatic disorders (SARDs) are examples of such tests. In 2013, the American College of Rheumatology (ACR) listed ANA and subserology tests among the top 5 most overutilized laboratory tests and indicated that there is an overall lack of consistency of ANA and subserology test ordering by clinicians.¹ This lack of structure may lead to patient harm, unnecessary follow-up testing, inaccurate diagnoses, incorrect treatment, and reduced quality of life.² Thus, the ACR made a Choosing Wisely recommendation advising against the ordering of subserology tests, such as anti-double-stranded DNA (dsDNA), without a positive ANA screening test or clinical suspicion of autoimmune disease.¹ Furthermore, the ACR recommendations emphasized the need to order ANA testing for patients with clinical symptoms and to perform subserologies after obtaining a positive ANA, a strategy known as “reflex testing.” Although this guidance clarified the framework for proper utilization of ANA and subserology analyses, overutilization of these tests for assessing SARD continues.

Currently, the American Autoimmune Related Diseases Association estimates that 50 million Americans have an autoimmune disease, the majority of whom are women.³ Globally, approximately 3%–5% of the population have some form of an autoimmune disease, and the prevalence of these disorders is expected to increase.⁴ There are at least 80 recognized SARDs, which are a subgroup of autoimmune diseases. Systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), and scleroderma are among the most common SARDs.⁵ SARDs are collectively characterized by the presence of

widespread systemic inflammation, which can lead to multiorgan failure and death.^{6,7} Nonspecific symptoms of these disorders often complicate clinician's attempts to establish a definitive SARD diagnosis. This emphasizes the importance of accurate and appropriate use of ANA and subserology testing for assessing these disorders.

FIGURE 1 depicts the reflex testing algorithm recommended by the ACR to prevent overuse of ANA and subserology testing.¹ One of the main goals of formulating this algorithm was to simplify the complexity of ANA and subserology testing for providers who are not rheumatic disease specialists.⁸ A factor contributing to the accuracy of the algorithm is that it encourages providers to carefully consider the patient's symptoms, in addition to ANA screening and titer results. The algorithm also emphasizes the ACR's view that negative ANA screening tests are typically followed by negative subserology results; therefore, subserology testing after a negative ANA result is generally wasteful and unnecessary. However, in rare cases, it may be necessary to perform subserology tests without a prior positive ANA (ie, "single serology testing") or at the same time as testing for ANA (ie, "panel testing"). These alternatives may be appropriate for individuals undergoing follow-up testing for a previously diagnosed autoimmune disease.⁹

One hospital that serves as a prime model of the ACR's suggested ANA and subserology test utilization is the Denver Health Hospital. The general policy of this safety net hospital is to only perform subserology testing after a positive ANA is obtained, which reflects reflex testing recommended by the ACR guidance.⁹ Like many hospitals in the country, our institution lacks a standard framework for SARD testing. As a first step in reducing overuse of ANA and subserology testing, we conducted a retrospective review to identify and analyze test ordering patterns at our institution that do not follow ACR's recommendations for the use of reflex testing. Analysis of these patterns allowed determination of the prevalence and diagnostic accuracy of the observed patterns for the diagnosis of SARD.

The main hypothesis of this study was that providers are not ordering ANA and subserology testing for suspected SARD as recommended by the ACR. To test this hypothesis, we described ANA and subserology ordering patterns at our institution over a 12-month period and compared these patterns with those reported at Denver Health Hospital. For more in-depth analysis, we also randomly selected 2 subgroups

of patients from our study population—1 group that underwent reflex testing and another group that underwent improper testing (single serology or panel testing)—to identify possible patient characteristics associated with the test-ordering pattern, determine the frequency of specific SARD diagnoses and the association between test patterns and diagnosis of SARD, and evaluate the clinical utility of ANA and subserology tests for diagnosing specific SARDs.

Methods

We performed a retrospective chart review of patients with suspected SARD who underwent ANA and/or subserology testing at our institution from January 2019 through December 2019. Our institution is an approximately 800 bed academic teaching hospital. At this institution, for ANA and/or subserology testing, the Immuno Concepts Hep-2000 Fluorescent ANA-Ro Slides on the Autoimmune FastTrack (AFT) 3100 automated analyzer with Immuno Concepts Image Navigator and BioRad Bioplex 2100 were the primary testing method used for ANA screens and subserology testing. All positive/negative slides were interpreted daily by a trained medical laboratory scientist (MLS) with a follow-up review by a pathologist prior to reporting to the patient's chart. The cut-off titer for an ANA screen to be considered as a positive test is 1:80 dilution for adults and 1:40 for pediatric patients. Following a positive ANA screen, additional subserology tests were performed based on individual test orders placed by the physician. The subserology tests included anti-dsDNA, anti-Smith (anti-Sm), anti-ribonucleoprotein, anti-Sm/RNP, anti-SS-related antigen A (anti-SSA), anti-SS-related antigen B (anti-SSB), anti-histidyl-tRNA synthetase, anti-ribosomal P, anti-chromatin, anti-topoisomerase I (anti-Scl-70), anti-centromere, and anti-cyclic citrullinated peptide (anti-CCP). We used the International Classification of Diseases, 10th version code M35.9 to identify patients suspected of SARD. We collected data regarding the following: patient demographics (age, sex, ethnicity), provider department, provider type (medical resident, medical doctor, physician assistant, nurse practitioner), laboratory tests (ANA screening and titers, subserology tests), and diagnoses. For our study, we only included patients with initial ANA and reflex tests and/or initial single subserology tests and excluded patients who were previously diagnosed with a SARD.

FIGURE 1. American College of Rheumatology recommendations for systemic autoimmune rheumatic disorder (SARD) testing.

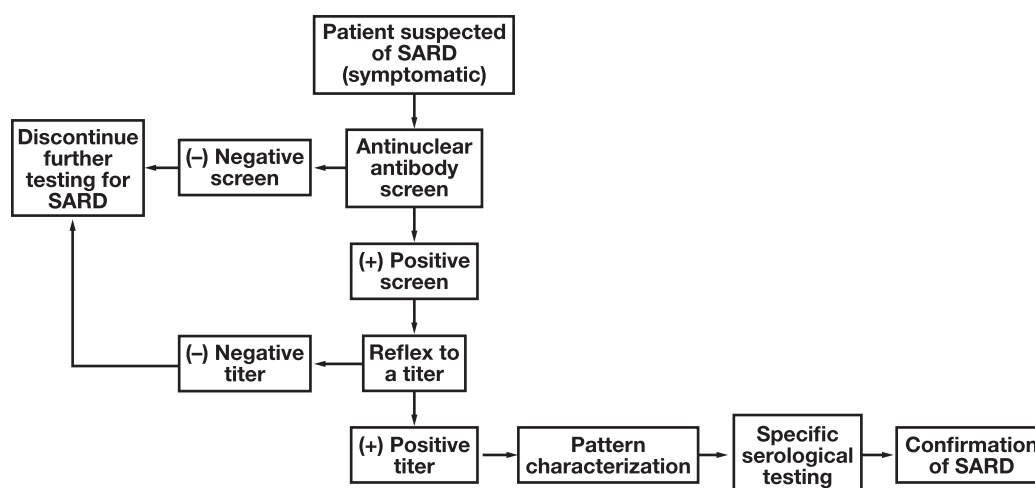


TABLE 1. Demographics of the Systemic Autoimmune Rheumatic Disorder Testing Population at Our Institution (January 2019–December 2019)

Characteristic	Improper Testing (n = 563)	Reflex Testing (n = 2374)	Total (n = 2937)	P Value
Age, mean $\pm$ SD (range), y	48.0 $\pm$ 18.4 (1–89)	46.3 $\pm$ 20.1 (1–96)	46.6 $\pm$ 19.8 (1–96)	.067
Sex, No. (%)				
Male	117 (20.8)	818 (34.5)	935 (31.8)	<.001
Female	446 (79.2)	1556 (65.5)	2002 (68.2)	
Race/ethnicity, No. (%)				
Caucasian	329 (58.4)	1403 (59.1)	1732 (59.0)	.081
Hispanic	112 (19.9)	562 (23.6)	674 (22.9)	
Black	96 (17.1)	329 (13.9)	425 (14.5)	
Asian	2 (3.7)	61 (2.6)	82 (2.8)	
Other ^a	5 (0.9)	19 (0.8)	24 (0.8)	

SD, standard deviation.

^aIncludes American Indian and Pacific Islander.

Statistical Analysis

Data are presented as mean $\pm$ standard deviation except where otherwise indicated. Unpaired *t*-test was used to compare means between groups. The χ^2 analysis was used to compare categorical data. Data analysis was performed using Excel and SPSS, with *P* values < .05 indicating statistical significance. Our institutional review board (IRB) considered this study to be a quality assessment/quality improvement study that did not require IRB approval or oversight.

Results

A total of 2937 patients were included in the study. **TABLE 1** summarizes their characteristics. The mean patient age was 46.6 $\pm$ 19.8 y (range, 1–96 y), and the majority of patients were White (59.0%) and female (68.2%). Most patients underwent ACR-recommended testing (n = 2374; 80.8%), whereas the remainder underwent improper (panel or single serology) testing (n = 563; 18.1%).

The 2937 patients underwent a total of 3003 ANA and subserology tests for suspected SARD. Of these 3003 SARD test-ordering patterns, 2401 (79.7%) followed the ACR recommendation, as shown in **TABLE 2**. Panel testing accounted for 497 tests (17.0%) and singular serology testing accounted for the remaining 105 tests (3.3%), for a combined improper testing rate of 20.3%. By comparison, of the 3221 ANA and subserology tests performed at Denver Health Hospital, 3010 (93.4%) followed the ACR recommendations, 211 (6.6%) were panel testing, and 0 (0%) were single serology testing.⁹

The 3003 SARD tests were ordered by 21 departments. The percentage of improper testing was equal to or less than the total 6.6% rate of improper testing found at Denver Health Hospital in only 4 departments: Gastroenterology (5%), Orthopedics (2%), Psychiatry (0%), and Radiology (0%). It should be noted that both Psychiatry and Radiology departments ordered only a single ANA reflex test each in the study period, which is statistically not valid (**TABLE 3**). The Family Medicine department ordered the highest number of all tests (n = 778) and the highest number of ACR-recommended tests (n = 679). Emergency Medicine ordered the highest number of panel tests (n = 119), and Internal Medicine ordered the highest number of single serology tests (n = 28). The departments with the lowest frequency of ACR-recommended test

TABLE 2. Ordering Patterns for Antinuclear Antibodies and Subserology Tests at Our Institution and Denver Health Hospital

	Tests, No. (%)	Improper Testing Patterns (%)
Our Institution		
Reflex testing	2401 (79.7)	0.0
Panel testing	497 (17.0)	17.0
Single subserology testing	105 (3.3)	3.3
Total	3003 (100)	20.3
Denver Health Hospital ^a		
Reflex testing	3010 (93.4)	0.0
Panel testing	211 (6.6)	6.6
Single subserology testing	0 (0)	0.0
Total	3221 (100)	6.6

^aData obtained from reference 9.

ordering were the subspecialties of internal medicine including Neurology (62.0%), Dermatology (57.4%), and Rheumatology (46.0%), as well as Otolaryngology (45.5%).

TABLE 4 shows the characteristics of the 364 patients randomly selected for the subgroup analysis: 182 in the improper testing group and 182 in the reflex testing group. The mean age of all patients was 46.2 y; 76.6% were women, 54.7% were White, and 54.5% had commercial health care insurance. The χ^2 analysis revealed that race/ethnicity was significantly different between groups (*P* = .008), with improper testing being more frequent than reflex testing in White and Hispanic patients. Other patient characteristics (age, sex, and insurance payor type) were similar between groups.

TABLE 5 summarizes the SARD diagnoses in the 2 subgroups. Of the 364 subjects, 29 (8.0%) were diagnosed with SARD. The most commonly diagnosed SARDs were SLE (n = 9; 2.5%), RA (n = 8; 2.2%), SS (n = 6; 1.6%), antiphospholipid syndrome (n = 2; 0.5%), scleroderma (n = 1; 0.3%), and unspecified SARD (n = 3; 0.8%). The percentage of patients diagnosed with SARD was significantly higher in the reflex testing group (n = 22; 11.5%) than in the improper testing group (n = 7; 3.6%) (*P* = .004) (**TABLE 6**).

TABLE 3. Systemic Autoimmune Rheumatic Disorder Testing Patterns According to Department

Department	Reflex Testing, No. (%)	Panel Testing, No. (%)	Single Subserology Testing, No. (%)	Total, No. (%)
Allergy and Immunology	12 (80.0)	1 (16.7)	2 (13.3)	15 (0.5)
Cardiology	12 (75.0)	4 (25.0)	0 (0)	16 (0.5)
Dermatology	66 (57.4)	43 (37.4)	6 (5.2)	115 (3.8)
Emergency Medicine	417 (76.0)	119 (21.7)	13 (2.4)	549 (18.3)
Family Medicine	679 (87.3)	82 (10.5)	17 (2.2)	778 (25.9)
Gastroenterology	338 (98.5)	5 (1.5)	0 (0)	343 (11.4)
Gerontology	18 (66.7)	7 (25.9)	2 (7.4)	27 (0.9)
Hematology/Oncology	29 (82.9)	4 (11.4)	2 (5.7)	35 (1.2)
Internal Medicine	299 (76.5)	64 (16.4)	28 (7.2)	391 (13.0)
Infectious Disease	13 (86.7)	2 (13.3)	0 (0)	15 (0.5)
Nephrology	70 (86.4)	10 (12.3)	1 (1.2)	81 (2.7)
Neurology	49 (62.0)	24 (30.4)	6 (7.6)	79 (2.6)
Obstetrics and Gynecology	14 (73.7)	5 (26.3)	0 (0)	19 (0.6)
Ophthalmology	23 (88.5)	2 (7.7)	1 (3.8)	26 (0.9)
Orthopedics	83 (97.6)	2 (2.4)	0 (0)	85 (2.8)
Otolaryngology	10 (45.5)	11 (50.0)	1 (4.5)	22 (0.7)
Pediatrics	163 (92.1)	12 (6.8)	2 (1.1)	177 (5.9)
Psychiatry	1 (100)	0 (0)	0 (0)	1 (0.03)
Radiology	1 (100)	0 (0)	0 (0)	1 (0.03)
Rheumatology	104 (46.0)	98 (43.4)	24 (10.6)	226 (7.5)
Urology	1 (33.3)	2 (66.7)	0 (0)	3 (0.1)
Total	2401	497	105	3003

TABLE 4. Demographics and Health Care Insurance Payor Types of the Improper Testing and Reflex Testing Groups

Variables	Improper Testing (n = 182)	Reflex Testing (n = 182)	Total (n = 364)	P Value
Age, mean $\pm$ SD, y	47.8 $\pm$ 19.6	45.2 $\pm$ 19.7	46.2 $\pm$ 19.4	.131
Sex, No. (%)				
Male	50 (27.5)	35 (19.2)	85 (23.4)	.063
Female	132 (72.5)	147 (80.8)	279 (76.6)	
Race/ethnicity, No. (%)				
Caucasian	107 (58.8)	92 (50.5)	199 (54.7)	.008
Hispanic	27 (14.8)	54 (29.7)	81 (22.3)	
Black	42 (23.1)	31 (17.0)	73 (20.1)	
Asian	6 (3.3)	5 (2.7)	11 (3.0)	
Payor type, No. (%)				
Commercial	100 (54.9)	99 (54.4)	199 (54.5)	.269
Medicare	35 (19.2)	44 (24.2)	79 (21.7)	
Medicaid/CHIP	35 (19.2)	34 (18.7)	69 (19.0)	
Self-pay/county	12 (6.6)	5 (2.7)	17 (4.7)	

CHIP, Children's Health Insurance Program; SD, standard deviation.

TABLE 7 shows the clinical utility of ANA and subserology tests for the 4 most common SARDs (SLE, RA, SS, and scleroderma) in our study population, including the pretest probability, positive likelihood ratio (PLR), pretest odds, posttest odds, and posttest probability.

Discussion

In this study, we analyzed 3003 SARD test-ordering patterns in 2937 patients and found that testing was more commonly ordered for females than males (68.2% vs 31.8%), and the mean age of all patients

was 46.6 years. These results are comparable to those of other studies, showing a similar predominance of women and a similar average age (~46.9 years) for SARD testing.^{10,11}

When we examined whether ANA and subserology testing of patients with suspected SARD was in alignment with ACR's recommendations, we found that the majority (79.7%) of ordered tests were in accordance

TABLE 5. Systemic Autoimmune Rheumatic Disorder Diagnoses in the Improper Testing and Reflex Testing Groups (n = 364)

SARD Diagnosis	Diagnosis (n)	Frequency (%)
Systemic lupus erythematosus	9	2.5
Rheumatoid arthritis	8	2.2
Sjögren's syndrome	6	1.6
Antiphospholipid syndrome	2	0.5
Scleroderma	1	0.3
Unspecified ^a	3	0.8
Total	29	8.0

SARD, systemic autoimmune rheumatic disorder.

^aIndividuals diagnosed with SARD, but for whom the specific disorder was not determined during the study period.

TABLE 6. Frequency of Systemic Autoimmune Rheumatic Disorder Diagnosis in the Reflex Testing vs Improper Testing Groups (n = 364)

Group	Diagnosis (n)	No Diagnosis (n)	χ^2 Value	P Value
Improper testing	7	175	8.4303	.004
Reflex testing	22	160		
Total	29	335		

TABLE 7. Clinical Utility of Antinuclear Antibody and Subserology Tests for Common Systemic Autoimmune Rheumatic Disorder Diagnoses at Our Institution^a

Value	Disorder				
	Systemic Lupus Erythematosus	Rheumatoid Arthritis	Sjögren's Syndrome		Scleroderma
Antinuclear antibody					
Pretest probability (%)	2.5	2.2	1.6		0.3
Positive likelihood ratio	2.2	0.9	1.0		1.8
Pretest odds	0.03	0.02	0.02		0.004
Posttest odds	0.06	0.02	0.08		0.008
Posttest probability (%)	4.0	2.1	1.6		0.8
Subserology tests					
Type of antibody	Anti-dsDNA	Anti-CCP	Anti-SSA	Anti-SSB	Anti-Scl-70
Pretest probability (%)	2.5	2.2	1.6	1.6	0.3
Positive likelihood ratio	14.0	13.4	5.4	6.7	4.3
Pretest odds	0.03	0.02	0.02	0.02	0.003
Posttest odds	0.4	0.3	0.09	0.11	0.01
Posttest probability (%)	26.4	21.5	8.1	9.8	1.3

Anti-CCP, anti-cyclic citrullinated peptide; anti-dsDNA, anti-double-stranded DNA; anti-Scl-70, anti-topoisomerase I; anti-SSA, anti-SS-related antigen A; anti-SSB, anti-SS-related antigen B.

^aThe calculations were based on published sensitivity and specificity values for ANA and subserology tests, respectively: antinuclear antibody for systemic lupus erythematosus, 95% and 57%; antinuclear antibody for RA, 41% and 56%; antinuclear antibody for Sjögren's syndrome, 48% and 52%; antinuclear antibody for scleroderma, 85% and 54%; anti-dsDNA, 70% and 95%; anti-CCP, 67% and 95%; anti-SSA, 70% and 87%; anti-SSB, 40% and 94%; and anti-Scl-70, 43% and 90%.^{15–18}

with these recommendations. However, 20.3% of test ordering did not align with these recommendations (ie, was improper testing): panel testing accounted for 17.0% of the test-ordering patterns, whereas single subserology testing without a prior positive ANA accounted for 3.3%. Our improper ordering rate was higher than the 6.6% reported in the Denver Health Hospital study.⁹ We also found that the majority of improper testing was conducted by subspecialties of internal medicine. The relative overutilization of ANA and subserology testing at our institution likely reflects a lack of standardization of SARD testing, unlike the framework established at the safety net hospital that the results of ANA screening tests be obtained before ordering specific SARD confirmatory subserology. Our lack of a similar framework likely also explains the low number of departments with an improper testing rate ≤6.6%.

When we evaluated factors potentially associated with improper testing compared with ACR-recommended reflex testing, we found an association between test-ordering patterns and race/ethnicity. Of note, reflex testing was more frequent than improper testing in Hispanic patients. Previous studies have noted disparities between different racial/ethnic groups regarding diagnosis and outcomes of SARDs.¹² A recent study of patients with RA noted that the median time to referral to a rheumatologist after onset of symptoms was 6 to 8 months for most groups but 22.7 months for Hispanics.¹³ Several factors may contribute to delayed specialist referral and initiation of appropriate treatment, including language barriers, socioeconomic status, cultural differences, and health literacy.¹³ As a result of the known poorer outcomes of SARD in Hispanic patients, clinicians may be attempting to improve diagnoses in this population by overusing ANA and subserology tests.

Our results also showed that reflex testing was associated with an increased frequency of SARD diagnoses compared with improper testing. Individuals undergoing assessment for suspected SARD, if examined by a specialist (eg, rheumatologist, dermatologist, neurologist) who understands the use of each specific serology test and is knowledgeable in the various

rheumatic diseases is able to obtain a definitive SARD diagnosis, as it simplifies the workflow and aims to increase the diagnostic accuracy of the testing process.⁸ In our study, we noted that positive ANA screening tests, titers, and patterns were generally investigated by a specialist. Overall, reflex testing and appropriate investigation by a specialist shows potential for improving the ability to obtain a definitive SARD diagnosis if implemented as a permanent part of an institution's SARD-testing workflow.

In terms of the specific test ordering performed for assessing potential SARD, we performed a comprehensive assessment of the testing utility based on our 2 subgroups. This analysis allowed us to evaluate the significance of positive test results in symptomatic SARD patients. The pretest and posttest calculations provided useful insights into the diagnostic utility of these tests in patients presenting with suspected SARD. Symptomatic individuals with a positive ANA and accompanying positive subserology for a specific SARD would have an increased PLR. The best use of ANA testing alone was for evaluating SLE (PLR of 2.2); for the other common SARDs values (RA, SS, and scleroderma), ANA alone was less clinically useful, as indicated by their PLRs ranging from 0.9 to 1.8. These findings are consistent with previously published low sensitivity and specificity of ANA testing for the non-SLE disorders.¹⁴ Specific subserology testing was more beneficial for assessing all SARDs, with PLRs ranging from 4.3 for anti-Scl-70 (for scleroderma) to anti-dsDNA (for SLE). These findings were consistent with previous literature showing higher sensitivity and specificity of subserology testing for these disorders.¹⁴ Overall, the clinical usefulness of ANA and subserology testing depends on the sensitivity, specificity, and prevalence of SARD in the population. Given that SARDs are relatively uncommon disorders, our results support the need to carefully use ANA and subserology testing in the appropriate patient population, that is, in patients with clinical findings suggestive of SARD.

Our study has some limitations, including the relatively short timeframe for data collection (12 months) and the possibility of selection bias, which is inherent in all cross-sectional retrospective chart review studies. Furthermore, the published sensitivities and specificities used for analyzing the utility of the tests were based on only the indirect immunofluorescent method and do not account for other methods used for detecting ANAs.

Conclusion

When comparing test-ordering patterns against a safety-net hospital that can be a model for ACR-recommended ANA and subserology reflex testing, we found an elevated rate of improper SARD test ordering. Our study provided an opportunity to identify the prevalence of ordering patterns, identify the departments' ordering specific test patterns, evaluate possible associations to test ordering, determine the diagnostic benefit of testing, and examine the clinical utility of ANA and subserology testing for the diagnosis of SARD. For future analyses, an additional area of review would be the actual implementation of the ACR framework and a comparison of pre- and postimplementation outcomes, which will allow for a more comprehensive overview and understanding of the overall benefit of using reflex testing for the evaluation of SARD.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Yazdany J, Schmajuk G, Robbins M, et al., American College of Rheumatology Core Membership Group. Choosing wisely: the American College of Rheumatology's Top 5 list of things physicians and patients should question. *Arthritis Care Res (Hoboken)*. 2013;65(3):329–339. doi:10.1002/acr.21930
2. Abeles AM, Abeles M. The clinical utility of a positive antinuclear antibody test result. *Am J Med*. 2013;126(4):342–348. doi:10.1016/j.amjmed.2012.09.014
3. Autoimmune Disease – National Stem Cell Foundation. <https://nationalstemcellfoundation.org/glossary/autoimmune-disease/>
4. Wang L, Wang FS, Gershwin ME. Human autoimmune diseases: a comprehensive update. *J Intern Med*. 2015;278(4):369–395. doi:10.1111/joim.12395
5. Theofilopoulos AN, Kono DH, Baccala R. The multiple pathways to autoimmunity. *Nat Immunol*. 2017;18(7):716–724. doi:10.1038/ni.3731
6. Brandt JE, Priori R, Valesini G, Fairweather D. Sex differences in Sjögren's syndrome: a comprehensive review of immune mechanisms. *Biol Sex Differ*. 2015;6:19. doi: 10.1186/s13293-015-0037-7
7. Haag H, Liang T, Avina-Zubieta JA, De Vera MA. How do patients with systemic autoimmune rheumatic disease perceive the use of their medications: a systematic review and thematic synthesis of qualitative research. *BMC Rheumatology*. 2018;2(1):9. doi:10.1186/s41927-018-0017-8
8. Tonutti E, Bizzaro N, Morozzi G, et al; Study Group on Autoimmune Diseases of the Italian Society of Clinical Pathology and Laboratory Medicine. The ANA-reflex test as a model for improving clinical appropriateness in autoimmune diagnostics. *Autoimmun Highlights*. 2016;7(1):9. doi:10.1007/s13317-016-0080-3
9. Davis L, Goldstein BL, Tran V, et al Applying choosing wisely: antinuclear antibody (ANA) and subserology testing in a safety net hospital system. *Open Rheumatol J*. 2015;9:82–87.
10. Angum F, Khan T, Kaler J, Siddiqui L, Hussain A. The prevalence of autoimmune disorders in women: a narrative review. *Cureus*. 2020;12. doi:10.7759/cureus.8094
11. Rajendran R, Salazar JH, Seymour RL, Laposata M, Zahner CJ. Overutilization and underutilization of autoantibody tests in patients with suspected autoimmune disorders. *Diagnosis (Berlin)*. 2021;8(4):497–503. doi:10.1515/dx-2020-0139
12. Barton JL, Trupin L, Schillinger D, et al. Racial and ethnic disparities in disease activity and function among persons with rheumatoid arthritis from university-affiliated clinics. *Arthritis Care Res (Hoboken)*. 2011;63(9):1238–1246. doi:10.1002/acr.20525
13. Riad M, Dunham DP, Chua JR, et al. Health disparities among Hispanics with rheumatoid arthritis: delay in presentation to rheumatologists contributes to later diagnosis and treatment. *J Clin Rheumatol*. 2020;26(7):279–284. doi:10.1097/RHU.0000000000001085
14. American Academy of Family Physicians. Likelihood ratios https://www.aafp.org/dam/AAFP/documents/journals/afp/Likelihood_Ratios.pdf.
15. Ali Y. Rheumatologic tests: a primer for family physicians. *Am Fam Physician*. 2022;98(3):164–170.
16. Habash-Bseiso DE, Yale SH, Glurich I, Goldberg JW. Serologic testing in connective tissue diseases. *Clin Med Res*. 2005;3(3):190–193. doi:10.3121/cmcr.3.3.190
17. Stankovic A. *Sjogrens Syndrome (SS)*. 2019.
18. Didier K, Bolko L, Giusti D, et al. Autoantibodies associated with connective tissue diseases: what meaning for clinicians? *Front Immunol*. 2018;9:541. doi:10.3389/fimmu.2018.00541

Short-Term (6 Weeks) Experience of a Modular Workcell for Hemostasis Testing Including an Intelligent Data Manager at a Tertiary Care Hospital

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Abbreviations: TLA, total laboratory automation; TATs, turnaround times; PT, prothrombin time; aPTT, activated partial thromboplastin time; SD, standard deviation; CLSI, Clinical Laboratory Standards Institute; LIS, laboratory information system; FDP, fibrinogen degradation products

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ABSTRACT

Objective: Modular workcells could be a better solution than total laboratory automation (TLA) in hemostasis laboratories. Here, we evaluated the impact of implementing a modular workcell (HemoCell) with an intelligent data management facility (HemoHub).

Methods: We compared the turnaround times (TATs), numbers of rerun samples, and rerun times pre- and postimplementation of the HemoCell at Gil Medical Center. Prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer, and fibrinogen were evaluated.

Results: The TAT standard deviations (SDs) and maximum TAT values decreased after HemoCell implementation, although the mean TATs for PT, aPTT, and D-dimer were increased. Numbers of rerun samples were increased (18.1/day vs 44.7/day). However, rerun times were reduced, and SDs were decreased during the post-HemoCell period compared with pre-HemoCell. Additionally, technologists needed smaller working space and less labor.

Conclusion: The modular workcell could improve quality and efficiency by providing more consistent TATs and shorter rerun times in the hemostasis laboratory.

Many tests are performed in modern laboratories, and improvements in quality, efficiency, standardization, organization, and safety are ongoing. Clinical laboratory performance has substantially improved with the automation of preanalytical, analytical, and postanalytical test phases.^{1,2} Total laboratory automation (TLA) is representative of laboratory automation. However, there are some limitations to TLA, such as high short-term costs, space requirements, and differential requirements for sample management, and proneness to bottlenecks.³ Therefore, laboratories should choose automation systems that are suited to the individual laboratory conditions.

The automation of hemostasis testing needs to be carefully considered. Centrifuges are required to run at different speeds for coagulation and clinical chemistry tests.^{4,5} The Clinical Laboratory Standards Institute (CLSI) guideline H21-A5 currently recommends the centrifuge method (eg, 1500g for no less than 15 min at room temperature) for platelet-poor plasma.⁶ Shorter centrifugation times, higher centrifugal forces, or the use of a centrifuge brake can affect hemostasis test results.^{7,8} Furthermore, although some studies reported that short-term storage at 4°C or storage for >4 h at room temperature did not impair test quality, CLSI H21-A5 recommends that samples for testing of hemostasis analytes be transported at 18°C to 24°C, centrifuged to achieve platelet-poor plasma, and tested within 1 h of collection or within a maximum of 4 h.^{6,9,10} In a TLA system, the performance of sample reruns involves the manual retrieval of the sample from refrigerated storage. Da Rin and Lippi¹¹ reported that a rerun of plasma retrieved after 30 min of refrigerated storage did not substantially impair the quality of coagulation testing. However, sample rerun on a TLA system still requires manual work, including sample tracking, retrieval, and manual reloading. A potential alternative that addresses the limitations of TLA in hemostasis laboratories is the use of modular workcells, which require less floor space than TLA systems.^{5,12}

The HemoCell Specialized Lab Automation modular workcell (Werfen), hereafter called HemoCell, was implemented at Gil Medical Center, Republic of Korea, to improve the quality and efficiency of

tests. Therefore, we investigated whether a modular workcell system does in fact improve quality and efficiency by comparing turnaround times (TATs), rerun times, and the number of rerun samples before and after HemoCell implementation. The TATs and rerun times were measured and expressed as the mean $\pm$ standard deviation (SD). We also described HemoCell efficiency in terms of working area and labor requirements. The expectations of HemoCell implementation were an improvement in the quality and standardization of results by means of decreased TATs and rerun times with a low SD and an improvement in workflow efficiency due to reduced manual sample handling and working radius. Here, we present our short-term experience of the HemoCell modular workcell system in a hemostasis laboratory.

Materials and Methods

Setting

This study was approved by the Institutional Review Board of Gil Medical Center, Republic of Korea (approval No. GDIRB2021-026) and was performed in the hemostasis laboratory of Gil Medical Center (1450 beds). This laboratory processes approximately 377,000 tests annually. The HemoCell system was installed in the laboratory in March 2021 and is the first modular workcell for hemostasis tests to be used in Korea. It comprises an ACL TOP 750 LAS system, a HemoHub Intelligent Data Manager (HemoHub), HemosIL reagents, and a Thermo Scientific TCAutomation track.^{13,14} HemoHub connects all Werfen hemostasis systems and manages multiple networked sites using a single workstation with a centralized interface. It provides an overall view of critical results and analyzer status in real-time, automatic sample reruns and access to past results. Samples are aliquoted into cuvettes in the HemoCell system and can be maintained at 36.5°C for >1 h in the ACL TOP 750 LAS and directly used for reruns without sample retrieval. Before HemoCell installation, the laboratory used 2 ACL TOP 750 CTS

stand-alone analyzers with sample racks, and most reruns required the manual steps of sample tracking, retrieval, barcode reading, and manual reloading.

Study Design

We compared the TATs, number of rerun samples, and rerun times before and after HemoCell implementation. We defined TAT in this study as the time between sample registration and the availability of the last result in the laboratory information system (LIS). During the pre-HemoCell period, samples were registered at the reception desk and manually loaded into an ACL TOP 750 CTS analyzer using sample racks. In contrast, samples are automatically registered using a robotic arm and carrier when loaded through the HemoCell input module. We extracted laboratory data from the LIS and ACL TOP 750 instruments to evaluate the impact of the HemoCell system. We evaluated 6 weeks of operation (30 working days, 8:00 AM–5:00 PM on weekdays) before and after HemoCell implementation. To minimize the effects of the run-in period, we allowed 6 months of observation before our evaluation so that adjustments and changes could be made. Only routine analyses, including prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer, and fibrinogen, were evaluated as both immediately needed (stat) and routine tests. Since antithrombin, fibrinogen degradation products, and dilute Russell viper venom time screening and confirmatory tests were not performed daily, they were excluded because of low test volumes and rerun rates. The rerun rules applied during this study are listed in **TABLE 1**. The target TATs were 60 and 120 min for stat and routine tests, respectively. Tests that took ≥ 120 min were excluded because most involved inadequate sample volumes and required resampling before analysis.

Statistical Analysis

Data were analyzed using IBM SPSS v23.0 statistics software (IBM). The Mann-Whitney *U* test was used for independent sample analysis. *P* values < .05 were considered statistically significant. The TATs and rerun times were reported as medians to account for skewed

TABLE 1. List of Rerun Rules for Hemostasis Analytes

Test	Pre-HemoCell	Post-HemoCell ^c
PT	≤ 13 sec and delta percent change $(\text{[current} - \text{past result]}/\text{past result}) \geq 63.8\%$ ^a	≤ 13 sec and delta percent change $(\text{[current} - \text{past result]}/\text{past result}) \geq 63.8\%$
	> 13 sec and delta percent change $(\text{[current} - \text{past result]}/\text{past result}) \geq \pm 20.0\%$ ^a	> 13 sec and delta percent change $(\text{[current} - \text{past result]}/\text{past result}) \geq \pm 20.0\%$
	Extended mode when > 60 sec ^b	Extended mode when > 60 sec
		Out of reference range when first visit
aPTT	Delta percent change $(\text{[current} - \text{past result]}/\text{past result}) \geq \pm 20.0\%$ ^a	Delta percent change $(\text{[current} - \text{past result]}/\text{past result}) \geq \pm 20.0\%$
	< 25 sec or > 120 sec ^b	< 25 sec
		Out of reference range when first visit
		Autovalidation only when $(\text{original result} - \text{rerun result})/\text{rerun result} < 20.0\%$
D-Dimer	15 $\times$ dilution when > 7268 ng/mL FEU ^b	15 $\times$ dilution when > 7268 ng/mL FEU
	100 $\times$ dilution when $> 128,000$ ng/mL FEU ^b	100 $\times$ dilution when $> 128,000$ ng/mL FEU
Fibrinogen	5 $\times$ dilution > 930 mg/dL ^a	5 $\times$ dilution > 930 mg/dL

aPTT, activated partial thromboplastin time; FEU, fibrinogen equivalent unit; PT, prothrombin time.

^aThese reruns were manually performed from retrieval.

^bThese reruns were automatically performed from samples on the racks.

^cThese reruns were automatically performed from aliquots in the cuvettes.

distributions, which were performed using Microsoft Power BI Desktop (Microsoft).

Results

Total Test and Rerun Test Volumes

Our hemostasis laboratory processed 12,500 (416.7/d) tests for hemostasis analytes in the pre-HemoCell implementation period and 12,324 (410.8/d) in the post-HemoCell implementation period (TABLE 2). The details of reruns are summarized in TABLE 2. Reruns were performed more than twice as often after implementation (18.1/d vs 44.7/d). Reruns for PT were mostly increased during the post-HemoCell period compared with the pre-HemoCell period (389 vs 48 reruns). The highest number of reruns during the study period were for aPTT.

Comparison of TATs and Rerun Times

Data for TATs and rerun times in the pre- and post-HemoCell periods for PT, aPTT, D-dimer, and fibrinogen are shown in TABLE 3. The TAT SDs and maximum TAT values decreased after HemoCell implementation, although the mean TATs for PT, aPTT, and D-dimer increased (TABLE 3). The mean TAT for fibrinogen decreased during the post-HemoCell period, and the TAT SD and maximum TAT were reduced from 18.409 to 11.549 min and from 117 to 56 min, respectively.

HemoCell implementation also reduced the mean rerun times and SDs for all analytes, and the mean rerun times were significantly decreased for PT and fibrinogen (both $P = .001$) (TABLE 3). The sample distributions of TATs and rerun times are presented as violin plots in FIGURE 1.

Discussion

Automation is a proven means of improving quality and efficiency in clinical laboratories.^{3,14} Most manufacturers of mainstream hemostasis analyzers expect modular workcells to provide for future needs and replace stand-alone analyzers in hemostasis testing, and they believe that the integration of automatic sample preparation and quality assessment is a valuable prospect.¹⁵ Compared with TLA, modular workcells better suit the needs of hemostasis laboratories because of sample-specific centrifugation and storage requirements.^{5,8,12,14} A study reported that the physical connection of coagulation analyzers to TLA systems, including clinical chemistry and immunochemistry analyzers, did not affect sample quality or test results.⁴ In this same study, a stand-alone centrifuge was used according to CLSI recommendations, but TATs and working

area requirements were not investigated. In our study, we analyzed TATs and rerun times pre- and post-HemoCell implementation in a hemostasis laboratory to evaluate its impact.

The total number of tests performed was similar before and after implementation (TABLE 2). Contrary to our expectations, the mean TAT of analytes, except fibrinogen, slightly increased after implementation (TABLE 3). The number of reruns increased significantly after implementation, which should have led to an increased TAT. Since there were only 21 reruns for fibrinogen in the post-HemoCell period (TABLE 2), fibrinogen TATs were not meaningfully affected by the increased number of reruns, and the mean TAT decreased slightly from pre- to post-HemoCell implementation (TABLE 3). A study on clinical chemistry and immunoassay tests using a MODULAR ANALYTICS Serum Work Area (Roche Diagnostics) that integrated multiple different analyzers reported that samples set to “auto-rerun” took 15 min longer to process than usual samples on stand-alone instruments.¹⁶ In our laboratory, HemoCell sample processing was performed using an 8-m track (100 mm/second) (FIGURE 2), which led to increased TATs compared with stand-alone analyzers. However, it is expected that ongoing optimization will further reduce TATs.¹⁷ Although TATs slightly increased after HemoCell implementation, the upper level as shown in the whiskers (upper end of the vertical line; maximum value of the data set excluding any outliers) of pre- and post-HemoCell TATs were under the 60-min TAT goal, except for fibrinogen TATs in the pre-HemoCell period (FIGURE 1). Moreover, the SDs of TATs and the maximum TAT values in the post-HemoCell period were lower than in the pre-HemoCell period (TABLE 3 and FIGURE 1), indicating that the HemoCell system minimizes TAT variations, thereby improving the service to clinicians and patients. Therefore, modular workcells for large volumes of hemostasis tests could improve the quality of tests through consistent TATs.

As mentioned above, the number of reruns increased by more than 2-fold after HemoCell implementation, especially reruns for PT (TABLE 2). During the pre-HemoCell period, laboratory technologists manually performed most of reruns. HemoCell implementation allowed for the automatic performance of reruns using the same rerun rules regarding delta change and dilution (TABLE 1). Before HemoCell implementation, a technologist was needed to check previous data from the LIS and decide whether or not a rerun should be performed. Consequently, human error could have resulted in some reruns not being performed, and the number of these mistakes could have been more than we anticipated. Some autovalidation rerun rules were only applied in the post-HemoCell period, and some tests were rerun automatically during the post-HemoCell period because previous results were unavailable in

TABLE 2. Number of Total Tests and Reruns

	PT	aPTT	D-dimer	Fibrinogen	Total
Total tests					
Pre-HemoCell	6010	5282	851	357	12,500 (416.7/d)
Post-HemoCell	6081	5035	975	233	12,324 (410.8/d)
Reruns					
Pre-HemoCell	48	391	96	9	544 (18.1/d)
Post-HemoCell	389	871	61	21	1342 (44.7/d)

aPTT, activated partial thromboplastin time; PT, prothrombin time.

TABLE 3. Comparison of Turnaround Times (TATs) and Rerun Times (Means $\pm$ SDs)

	Pre-HemoCell	Post-HemoCell	P Value ^a
TAT			
PT	19.66 $\pm$ 12.383 (4–117)	24.36 $\pm$ 12.267 (6–93)	.000
aPTT	20.24 $\pm$ 12.597 (6–117)	24.44 $\pm$ 12.136 (8–82)	.000
D-dimer	22.73 $\pm$ 15.436 (8–117)	25.56 $\pm$ 12.223 (8–66)	.000
Fibrinogen	24.36 $\pm$ 18.409 (4–117)	24.24 $\pm$ 11.549 (7–56)	.019
Rerun time			
PT	14.98 $\pm$ 10.614 (4–54)	10.42 $\pm$ 4.488 (6–40)	.001
aPTT	13.94 $\pm$ 5.825 (6–41)	13.08 $\pm$ 5.051 (6–42)	.043
D-dimer	7.70 $\pm$ 4.944 (5–30)	6.31 $\pm$ 1.639 (5–11)	.221
Fibrinogen	21.00 $\pm$ 10.100 (7–39)	8.43 $\pm$ 7.359 (3–36)	.001

aPTT, activated partial thromboplastin time; PT, prothrombin time; SD, standard deviation.

^aP < .05, statistically significant.

the HemoHub database, although we had 6-month run-in periods to accumulate patients' data. Unfortunately, we could not identify the detail information about rerun reasons of each sample from HemoCell or ACL TOP 750 instruments, and this represents a limitation of the study.

Rerun times were reduced after HemoCell implementation, although the number of reruns increased. The mean and SD of rerun times were reduced for all analytes, and the maximum values generally decreased from the pre- to the post-HemoCell period (TABLE 3 and FIGURE 1). Shorter and more consistent rerun times with a high number of sample reruns could bear testimony to the improvement of quality and standardization.

The implementation of modular workcells not only reduced rerun times and the possibility of human error¹⁸ but also reduced processing and hands-on times as well as workspace area requirements.¹⁶ During the post-HemoCell period, no reruns were manually performed by technologists. Furthermore, technologists did not need to spend time on sample tracking and retrieval or on manual sample predilution and reloading, thereby reducing personnel requirements and operator risk. Vall d'Hebron University Hospital Laboratory in Spain reported a 72% increase in the number of samples analyzed per technologist after HemoCell implementation.¹³ This could be a solution for laboratories where there are shortages of qualified laboratory technologists or the need to enable laboratory staff to focus on other work. Furthermore, HemoCell implementation at Vall d'Hebron University Hospital reduced workspace by 62% due to the use of fewer instruments, despite an increase in the number of samples analyzed.¹³ Modular workcells enable the laboratory area to be used more efficiently because they have a smaller footprint than TLA. We did not evaluate workspace requirements during our study. However, the working space was smaller than for the stand-alone systems used before HemoCell implementation (FIGURE 2). All instruments were arranged more compactly with the HemoCell setup, and laboratory technologists were able to check the statuses of all instruments using HemoHub without having to check individual instruments. All instrument data are displayed on a screen, and technologists could manage the instruments from a desk. Prior to HemoCell implementation, technologists sometimes needed to go to the analyzer to investigate problems flagged by error messages. There were 11,630 error messages in the pre-HemoCell implementation period and 3035 error messages in the post-HemoCell

implementation period. A total of 92.1% of error messages occurred due to misreading barcodes on the sample racks during the pre-HemoCell period. Although misreading increased by faded barcodes over time, misreading did not technically occur in the HemoCell system because HemoCell did not demand sample racks. Our laboratory performed more reruns with less manual work and a smaller working space after implementing the modular workcell system. Additionally, the use of a modular workcell system improved the efficiency of our laboratory although we were unable to perform statistical calculations to support this.

HemoHub implementation enabled us to standardize the validation process. Some auto-reruns, including simple dilution and rechecking in extended mode, were also installed in the ACL TOP 750 CTS; however, the HemoCell auto-rerun system using HemoHub was more specific and customizable than the stand-alone analyzers. Hemostasis laboratories require expert technologists who can interpret results based on their knowledge of the basic principles of hemostasis in healthy and diseased conditions. Recently, manufacturers of mainstream automated hemostasis analyzers have focused on data management systems and data analysis systems that incorporate artificial intelligence.¹⁵ HemoHub allows straightforward access to patient result histories and is a useful facility for autoverification.

Autoverification is a process that uses predefined computer-based rules to verify laboratory test results without human intervention in the clinical laboratory and is an efficient tool for improving TATs, reducing laboratory error detection rates and staff fatigue, minimizing technologist handling, and providing consistency in the testing and reporting processes when properly implemented.^{19,20} The process of verifying test results demands more manual review in hemostasis laboratories than in clinical chemistry or immunochemistry laboratories. Details of autoverification procedures for coagulation tests have been published.^{21–23} Hemostasis laboratories face many challenges regarding improving standardization and efficiencies by automation, and rule creation is the most critical step for ensuring autoverification efficiency. Laboratories should determine their own rules based on tests performed, patient population, and specializations.²⁴ In our study, we focused on the implementation of modular workcells rather than autoverification rules despite the inseparable nature of laboratory automation and autoverification. As

FIGURE 1. The violin plots show distributions for hemostasis analytes. Prothrombin time (PT) turnaround time (TAT) (A), activated partial thromboplastin time (aPTT) TAT (B), D-dimer TAT (C), fibrinogen TAT (D), PT rerun time (E), aPTT rerun time (F), D-dimer run time (G), and fibrinogen rerun time (H). Red line indicates median value.

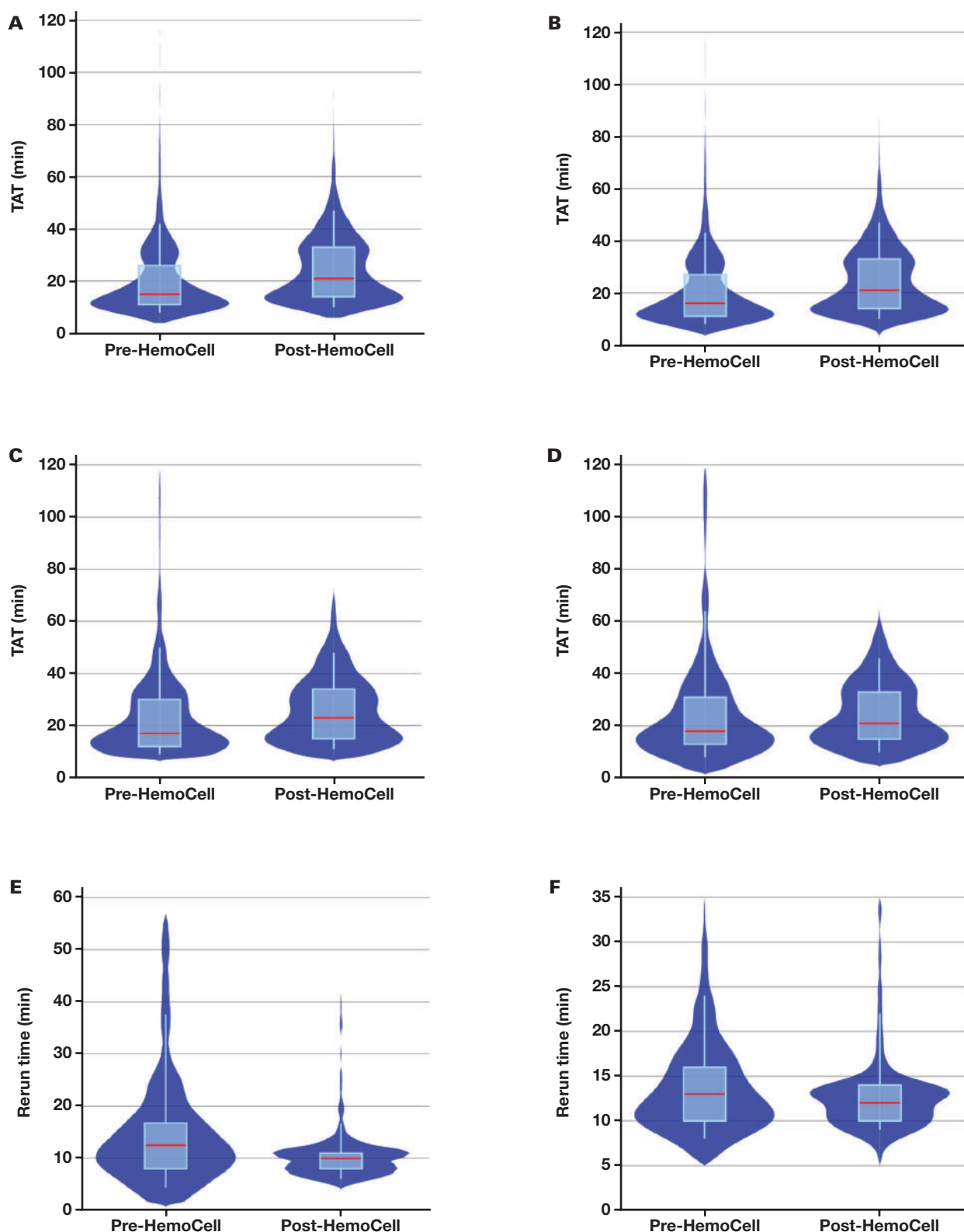
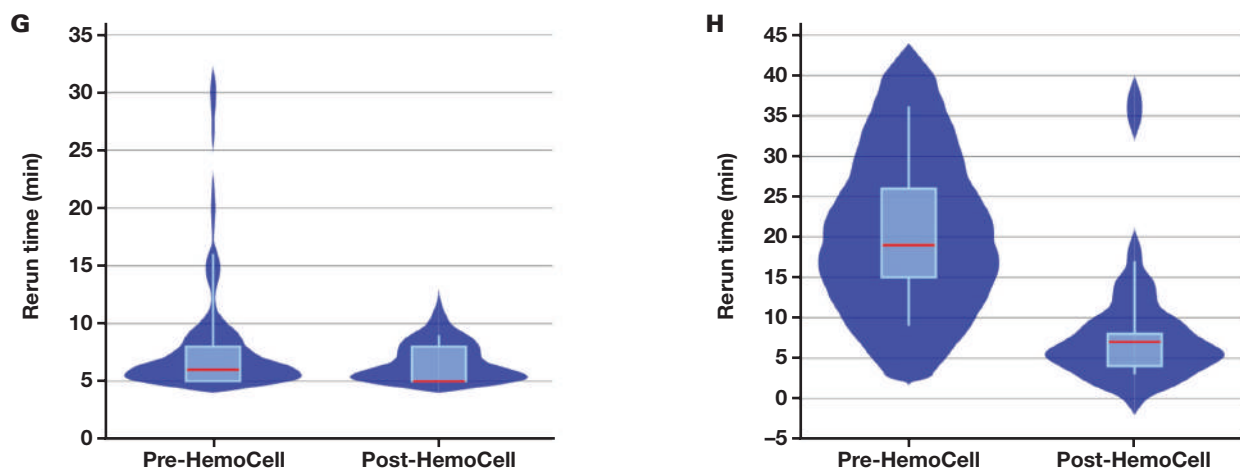
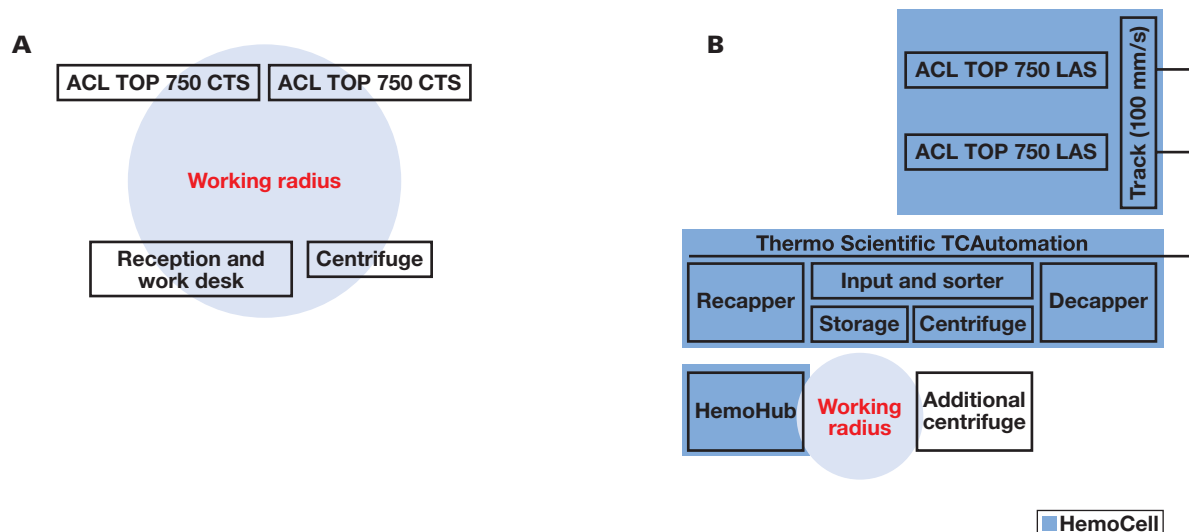


FIGURE 1. (cont)**FIGURE 2.** Working radii pre-HemoCell implementation (A) and post-HemoCell implementation (B). CTS, closed tube sample; LAS, laboratory automation system.

a result, post-HemoCell TATs increased slightly due to an increased number of rerun samples. Further studies are necessary to investigate the proper autoverification of modular workcells such as the HemoCell system.

Some study limitations should be mentioned. We did not compare costs during the pre- and post-HemoCell periods.²⁵ More reagents were used because of higher reruns in the post-HemoCell period, and additional expenditure was required for supplies specific to HemoCell use, such as cuvettes for aliquoting and caps for recapping. Furthermore, system installation costs were higher than for stand-alone systems, though they are likely to be offset in the long term. For example, efficient laboratory automation has been reported to lower the costs of laboratory diagnostic tests.²⁶ Also, the reduction of the workload of laboratory technologists due to autoverification might enable their deployment elsewhere.²¹ Long-term studies are necessary to better document the economic benefits of installing modular workcells in hemostasis laboratories.

TLA systems are at higher risk of bottlenecks that reduce laboratory productivity and increase TATs.^{3,27,28} In our laboratory, centrifugation within the HemoCell system caused some bottlenecks due to larger sample volumes during the mornings, and this problem was addressed by using an additional centrifuge that could also be used for stat tests (FIGURE 2). Although the 96-well plate centrifuge within the HemoCell system could process up to 400 tubes/h, all samples are automatically moved by a robotic arm from the rack to the centrifuge and from the centrifuge to the conveyor belt; this process can be time-consuming and cause a bottleneck when >400 samples/h arrive at the laboratory, as is usually the case in the mornings. More than 1 online centrifuge module or ES centrifuge (Werfen), which offers partially automated offline centrifugation using custom racks, could be added to the HemoCell system. Offline centrifugation did not substantially increase labor requirements in our laboratory. However, manufacturers of automated laboratory systems need to consider ways to overcome such bottleneck issues.

The functions of clinical laboratories depend on their size, the types of analytes being assessed, and other factors. Flexibility is a strength of modular workcells. Various autoverification systems can be applied by setting rerun rules that, when properly implemented, could improve TATs, increase repeatability, and reduce manual activities. Automated modular workcells also reduce working areas and increase the effectiveness of laboratory technologists compared with TLA or stand-alone systems.

Relatively little has been published on the experiences of modular workcell use in hemostasis laboratories. In this study, we presented our short-term practical experience of the HemoCell, which is a fully automated modular hemostasis workcell that includes a data manager. We conclude that modular workcell implementation in the hemostasis laboratory could improve laboratory quality, standardization, and efficiency by providing more consistent TATs and shorter and consistent rerun times with a higher number of reruns. Furthermore, the work area and personnel requirements can be reduced.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

- Markin RS, Whalen SA. Laboratory automation: trajectory, technology, and tactics. *Clin Chem*. 2000;46(5):764–771.
- Da Rin G. Pre-analytical workstations: a tool for reducing laboratory errors. *Clin Chim Acta*. 2009;404(1):68–74. doi:10.1016/j.cca.2009.03.024.
- Lippi G, Da Rin G. Advantages and limitations of total laboratory automation: a personal overview. *Clin Chem Lab Med*. 2019;57(6):802–811.
- Da Rin G, Lippi G. Total laboratory automation of routine hemostasis testing. *J Lab Autom*. 2014;19(4):419–422. doi:10.1177/2211068213511246.
- Graves S, Holman B, Felder RA. Modular robotic workcell for coagulation analysis. *Clin Chem*. 2000;46(5):772–777.
- CLSI document H21-A5. Edition AG-F. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
- Daves M, Giacomuzzi K, Tagnin E, et al. Influence of centrifuge brake on residual platelet count and routine coagulation tests in citrated plasma. *Blood Coagul Fibrinolysis*. 2014;25(3):292–295.
- Lippi G, Plebani M, Favaloro EJ. The changing face of hemostasis testing in modern laboratories: consolidation, automation, and beyond. *Semin Thromb Hemost*. 2015;41(3):294–299.
- Linskens EA, Devreese K. Pre-analytical stability of coagulation parameters in plasma. *Blood*. 2017;130:2348. doi:10.1182/blood.V130.Suppl_1.2348.2348.
- van Balveren JA, Huijskens MJ, Gemen EF, Péquériau NC, Kusters R. Effects of time and temperature on 48 routine chemistry, haematology and coagulation analytes in whole blood samples. *Ann Clin Biochem*. 2017;54(4):448–462. doi:10.1177/0004563216665868.
- Da Rin G, Lippi G. Sample rerun after short-term refrigerated storage: impact on routine coagulation testing. *Int J Lab Hematol*. 2014;36(5):e71–e73. doi:10.1111/ijlh.12185.
- Felder RA. Modular workcells: modern methods for laboratory automation. *Clin Chim Acta*. 1998;278(2):257–267. doi:10.1016/s0009-8981(98)00151-x.
- HemoCell workcell approach brings efficiencies to coag. <https://www.captodayonline.com/hemocell-workcell-approach-brings-efficiencies-to-coag/>
- Lippi G, Plebani M, Favaloro EJ. Technological advances in the hemostasis laboratory. *Semin Thromb Hemost*. 2014;40(2):178–185.
- Favaloro EJ, Lippi G. Recent advances in mainstream hemostasis diagnostics and coagulation testing. *Semin Thromb Hemost*. 2019;45(3):228–246.
- Mocarelli P, Horowitz GL, Gerthoux PM, et al. Increasing efficiency and quality by consolidation of clinical chemistry and immunochemistry systems with MODULAR ANALYTICS SWA. *J Autom Methods Manag Chem*. 2008;2008:1–14. doi:10.1155/2008/498921.
- Yu H-YE, Lanzoni H, Steffen T, et al. Improving laboratory processes with total laboratory automation. *Lab Med*. 2019;50(1):96–102.
- Ialongo C, Pieri M, Bernardini S. Artificial neural network for total laboratory automation to improve the management of sample dilution: smart automation for clinical laboratory timeliness. *SLAS TECHNOL: Transl Life Sci Innov*. 2017;22(1):44–49.
- Riley PW, Gallea B, Valcour A. Development and implementation of a coagulation factor testing method utilizing autoverification in a high-volume clinical reference laboratory environment. *J Pathol Inform*. 2017;8:25. doi:10.4103/jpi.jpi_95_16.
- Randell EW, Short G, Lee N, et al. Autoverification process improvement by Six Sigma approach: clinical chemistry & immunoassay. *Clin Biochem*. 2018;55:42–48. doi:10.1016/j.clinbiochem.2018.03.002.
- Mohammed S, Ule Priebsenow V, Pasalic L, Favaloro EJ. Development and implementation of an expert rule set for automated reflex testing and validation of routine coagulation tests in a large pathology network. *Int J Lab Hematol*. 2019;41(5):642–649. doi:10.1111/ijlh.13078.
- Onelöv L, Gustafsson E, Grönlund E, et al. Autoverification of routine coagulation assays in a multi-center laboratory. *Scand J Clin Lab Invest*. 2016;76(6):500–502. doi:10.1080/00365513.2016.1200135.
- Mlinaric A, Milos M, Herak DC, et al. Autovalidation and automation of the postanalytical phase of routine hematology and coagulation analyses in a university hospital laboratory. *Clin Chem Lab Med*. 2018;56(3):454–462.
- Topcu DI, Gulbahar O. A model to establish autoverification in the clinical laboratory. *Clin Biochem*. 2021;93:90–98. doi:10.1016/j.clinbiochem.2021.03.018.
- Kim K, Lee S-G, Kim TH, Lee SG. Economic evaluation of total laboratory automation in the clinical laboratory of a tertiary care hospital. *Ann Lab Med*. 2022;42(1):89–95. doi:10.3343/alm.2022.42.1.89.
- Hawker CD, Roberts WL, Garr SB, et al. Automated transport and sorting system in a large reference laboratory: part 2. Implementation of the system and performance measures over three years. *Clin Chem*. 2002;48(10):1761–1767.
- Tornel P, Ayuso E, Martinez P. Evaluation of the turnaround time of an integrated preanalytical and analytical automated modular system in a medium-sized laboratory. *Clin Biochem*. 2005;38(6):548–551. doi:10.1016/j.clinbiochem.2005.01.011.
- Lou AH, Elnenaei MO, Sadek I, Thompson S, Crocker BD, Nassar B. Evaluation of the impact of a total automation system in a large core laboratory on turnaround time. *Clin Biochem*. 2016;49(16-17):1254–1258. doi:10.1016/j.clinbiochem.2016.08.018.

Comparison of IgG Against COVID-19 Between Postmenopausal and Nonmenopausal Women Vaccinated With Sinopharm Vaccine

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Keywords: COVID-19, menopause, women, IgG against COVID-19, vaccine, inflammation

Abbreviations: Ig, immunoglobulin; IL, interleukin; BMI, body mass index; CBC, complete blood count; ELISA, enzyme-linked immunosorbent assay

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ABSTRACT

Objective: Since December 2019, the coronavirus disease has spread among the people of the world. Past studies have shown that viral diseases are more common and the immune response is stronger among menopausal women than nonmenopausal women. Therefore, in this study, we aimed to compare the amount of immunoglobulin (Ig) G against COVID-19 between postmenopausal and nonmenopausal women vaccinated with Sinopharm vaccine.

Methods: In this case-control study, 90 females vaccinated with the Sinopharm vaccine were randomly selected from February to April 2022: 45 menopausal participants as the case group and 45 nonmenopausal controls. Demographic characteristics were obtained and blood samples were taken from all subjects. A complete blood count was carried out and the levels of IgG against COVID-19 were measured by using the enzyme-linked immunosorbent assay method.

Results: The mean age was 33.3 ± 7.3 years and 60.2 ± 7.02 years for control and menopausal women, respectively. A significant difference was found between the 2 groups for the levels of IgG antibodies

against COVID-19 ($P = .002$, 17.2 ± 9.83 relative unit for case group and 10.2 ± 9.80 relative unit for control subjects). After adjusting, IgG against COVID-19 was significantly correlated to the menopausal state (odds ratio [confidence interval] = $1.08 [1.03-1.15]$; $P = .003$).

Conclusion: The results of this study showed that menopausal women had higher levels of IgG against COVID-19 in comparison with nonmenopausal females. However, more complementary studies are needed in this regard.

To date, the world has experienced many outbreaks of coronavirus-related infections, including severe acute respiratory syndrome coronavirus in 2002 to 2003 and Middle East respiratory syndrome coronavirus in 2011. However, none of them had ever become a global pandemic. As of January 7, 2020, the Chinese authorities announced that they had identified a new type of coronavirus and named it “2019 novel coronavirus”, which was changed to “The Corona Virus Disease 2019” (COVID-19) by the WHO on January 12.¹⁻³ This virus is a single-chain RNA with a diameter of 50 to 200 nm and has a strong tendency to reside in lung tissue for growth and environmental stimuli.^{4,5}

Studies have shown that men and women are different in terms of disease, onset time, and severity of underlying diseases, and probably the main reason for this discrepancy is the difference in sex hormones between men and women. According to studies done in the past, estradiol can prevent chronic diseases such as diabetes, blood pressure, atherosclerosis, and osteoporosis. Menopause is a stage in which the body undergoes many structural changes in hormones (including a decrease in estrogen and an increase in follicle-stimulation hormone), as well as a series of other physiological changes. A decrease in the function of the ovaries and sex hormones are seen during menopause.⁶⁻⁹ In addition, most of the body's immune cells have receptors for estradiol, and as a result, they can play an effective role in regulating the immune response against infections and even vaccination, changing the shape of immune cells and stimulating antibody production.¹⁰ In fact, sex hormones result in the shift of T helper lymphocytes from type 1 to type 2 and the production of T reg leading to regulating the immune response and resisting immunity. Therefore, when estrogen and progesterone reach their lowest levels, postmenopausal women are more susceptible to

infections and respond more strongly to viral infections.^{11–13} Among these inflammatory cytokines, interleukin (IL)-6 is effective in both immunity and inflammation. It has been established that the lack of regulation of IL-6 by estradiol will lead to the development and maintenance of diseases related to inflammation in postmenopausal women.¹⁴

To the best of our knowledge, no studies have evaluated the immune response caused by COVID-19 virus vaccination with Sinopharm vaccine in menopausal women. The current study was performed in menopausal and nonmenopausal women. Past studies have shown that viral diseases are more common in menopausal women than nonmenopausal women and the immune response is stronger among these women; these individuals are considered to be at high risk. Therefore, in this study, we aimed to compare the amount of IgG against COVID-19 between postmenopausal and nonmenopausal women vaccinated with Sinopharm vaccine.

Methods

Subjects and Research Design

This case-control study was conducted in Atabak Laboratory in Tehran, Iran. A total of 90 subjects, including 45 women of childbearing age and 45 postmenopausal women who had received their third dose of Sinopharm vaccine, were selected from January to April 2022. Ethics approval was obtained from the human research ethics committees of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1401.002). An informed consent form was signed by all subjects. Demographic characteristics were taken from the participants including age, gender, body mass index (BMI), stress level, sleep status, vocation, smoking, frequency of COVID-19, number of successful pregnancies, number of curettages, etc. Then, EDTA whole blood and clotted blood were obtained from the participants for complete blood count (CBC) and enzyme-linked immunosorbent assay (ELISA) tests, respectively.

Women in the 35- to 45-year-old age group who had at least 1 successful pregnancy without a history of miscarriage as well as menstruating in the last 12 months were selected as the control group. For the case group, women in the age range of 55 to 65 who had at least 12 months since their last menstrual period were included in the study. Pregnant women were excluded from the control group and persons with underlying diseases such as diabetes, high blood pressure, cardiovascular disease, cancer, and polycystic ovary syndrome as well as those undergoing dialysis or hormone treatment, persons with a history of hysterectomy, and those with genetic defects were excluded from both groups. None of the participants received medications such as insulin therapy, immunosuppressive drugs, anti-inflammatory drugs, or micronutrient and antioxidant supplements during the previous 80 days.

Clinical Laboratory Tests

To measure the IgG titer, the chemobind ELISA kit was used (Hayan pejoh pars, catalog number 202312). Briefly, the serum was first separated and 100 μ L of the diluted samples were added into the wells of microplates and incubated for 30 min at 37°C. During the incubation, the plate was covered with a plastic label according to the protocol of the kit. Then the plate was washed and 100 μ L of conjugated enzyme was added into each well and incubated for 30 min at 37°C. After washing, 100 μ L of substrate solution was added into each well and placed in the dark at room temperature for 15 min. In the next stage, 100 μ L

of stop solution was added into each well. Photometric measurement of color intensity at the 450 nm wavelength and wavelength between 620 nm and 650 nm was done 30 min after adding stop solution. Before measuring, the microplate was gently shaken to ensure a homogeneous distribution of the solution. The CBC test sample was measured with a Sysmex cell counter.

Statistical Analysis

Descriptive statistics such as mean and standard deviation for quantitative variables and frequency and frequency percentage for qualitative variables were reported. The logistic regression model under the bias reduction method was used to investigate the relationship between the variables in the case and control groups. All statistical calculations were done by R software and the *brglm* package along with the *glm* function. Finally, for the multivariate analysis, the multiple logistic regression model was used to evaluate the independent variables (marital status, BMI, smoking and IgG against COVID-19) that were significant in the bivariate analysis. A *P* value less than .05 was considered to be significant.

Results

In this case-control study, 45 cases and 45 controls were enrolled. The mean $\pm$ standard deviation of age was 60.2 ± 7.02 and 33.3 ± 7.3 for the menopausal and control groups, respectively ($P < .001$). The average BMI was 28.2 ± 4.2 and 25.6 ± 4.37 in the cases and control groups, respectively ($P = .011$). Regarding smoking, there was a significant relationship between the control group (11 cigarettes) and menopausal females (3 cigarettes) ($P = .038$). However, no significant differences were observed between the 2 groups in terms of parameters such as the frequency of COVID-19 infection, the time taken from last dose, red blood cells, white blood cells, hemoglobin, etc ($P > .05$). The comparison of demographic and clinical laboratory characteristics between the 2 groups is reported in **TABLE 1**. More importantly, a significant difference was found between the groups for the level of IgG antibodies against COVID-19 ($P = .002$, 17.2 ± 9.83 relative units for the case group and 10.2 ± 9.80 relative units for control subjects). After adjusting, IgG against COVID-19 was significantly correlated to the menopausal state (odds ratio [confidence interval] = 1.08 [1.03–1.15]; $P = .003$).

Discussion

It has been reported that immune responses to environmental factors like infections and vaccinations are sex-based responses.¹⁵ Women maintain a high immune reactivity after viral infections in comparison with men.¹⁶ Moreover, many changes in sex hormones occur after menopause in women.^{6–9} Because females, and especially menopausal women, generate higher antibody responses, vaccinations also result in higher antibody levels in women and provide efficient protection.¹⁵ However, this can lead to worse side effects due to the enhanced immune reactivity. In fact, this enhanced immune reactivity causes an effective resistance to infection but can develop immune-pathogenic effects and predisposition to autoimmunity due to hyperimmune responses.^{17,18} Therefore, our study was conducted with the aim of investigating the immune system status of menopausal and nonmenopausal women in response to the Sinopharm vaccine by measuring the IgG titer against COVID-19 in serum.

TABLE 1. Comparison of Demographic and Clinical Laboratory Characteristics Between Case and Control Groups

Characteristics		Group	No. (%) / Mean $\pm$ SD	OR (95% CI)	P Value	AOR (95% CI)	P Value
Marital status	Yes	Case	45 (53.57)	14.97 (1.67–Inf)	.026	10.3 (0.83–Inf)	.183
		Control	39 (46.43)				
	No	Case	0 (0.00)	Ref		Ref	
		Control	6 (100)				
Age (y)	—	Case	60.2 $\pm$ 7.02	1.62 (1.31–3.7)	<.001	—	—
		Control	33.3 $\pm$ 7.3				
BMI (kg/m ²)	—	Case	28.2 $\pm$ 4.2	1.15 (1.04–1.3)	.011	1.13 (1.02–1.3)	.035
		Control	25.6 $\pm$ 4.37				
Smoking	Yes	Case	3 (21.4)	0.25 (0.05–0.82)	.038	0.73 (0.13–3.01)	.686
		Control	11 (78.6)				
	No	Case	42 (55.3)	Ref		Ref	
		Control	34 (44.7)				
COVID-19 infection	Yes	Case	26 (59.1)	2.02 (0.89–4.82)	.101	—	—
		Control	27 (58.7)				
	No	Case	26 (59.1)	Ref			
		Control	18 (40.9)				
COVID-19 infection before vaccination	Yes	Case	9 (50)	1 (0.35–2.84)	1	—	—
		Control	9 (50)				
	No	Case	36 (50)	Ref			
		Control	36 (50)				
COVID-19 infection after vaccination	Yes	Case	7 (63.6)	1.79 (0.53–7.6)	.376	—	—
		Control	4 (36.4)				
	No	Case	38 (48.1)	Ref			
		Control	41 (51.9)				
Last infected COVID-19	—	Case	25.8 $\pm$ 27.8	1.02 (0.99–1.04)	.085	—	—
		Control	13.3 $\pm$ 23.6				
Time taken from last dose	>12 wk	Case	27 (54)	1.42 (0.62–3.33)	.407	—	—
		Control	23 (46)				
	>12 wk	Case	18 (45)	Ref			
		Control	22 (55)				
WBC ($\times 10^9$ /L)	—	Case	6.8 $\pm$ 2.39	0.93 (0.75–1.13)	.494	—	—
		Control	7.14 $\pm$ 1.89				
RBC ($\times 10^6$ / μ L)	—	Case	4.83 $\pm$ 0.49	1.19 (0.5–2.94)	.696	—	—
		Control	4.78 $\pm$ 0.47				
HGB (g/dL)	—	Case	13.6 $\pm$ 1.58	1.06 (0.82–1.38)	.654	—	—
		Control	13.4 $\pm$ 1.69				
HCT (%)	—	Case	41.1 $\pm$ 4.38	1.04 (0.95–1.56)	.416	—	—
		Control	40.3 $\pm$ 4.21				
MCV (fL)	—	Case	85.5 $\pm$ 7.86	1.02 (0.96–1.08)	.517	—	—
		Control	84.4 $\pm$ 6.87				
MCH (pg/cell)	—	Case	28 $\pm$ 3.35	0.988 (0.87–1.12)	.854	—	—
		Control	28.1 $\pm$ 3.18				
MCHC (g/dL)	—	Case	33 $\pm$ 1.06	0.914 (0.66–1.25)	.581	—	—
		Control	33.2 $\pm$ 1.54				

TABLE 1. (cont)

Characteristics		Group	No. (%) / Mean $\pm$ SD	OR (95% CI)	P Value	AOR (95% CI)	P Value
PLT (μ L)	—	Case	297 $\pm$ 79.9	1 (0.99–1.01)	.516	—	—
		Control	287 $\pm$ 58.7				
LYMPH (%)	—	Case	36 $\pm$ 9.29	0.99 (0.97–1.02)	.854	—	—
		Control	37.5 $\pm$ 32.3				
MXD (%)	—	Case	7.46 $\pm$ 2.8	0.9 (0.74–1.07)	.268	—	—
		Control	8.16 $\pm$ 2.38				
RDW-C (%)	—	Case	12.9 $\pm$ 1.81	1.02 (0.79–1.31)	.887	—	—
		Control	12.9 $\pm$ 1.62				
PDW (%)	—	Case	12.3 $\pm$ 2.17	1.06 (0.86–1.31)	.586	—	—
		Control	12 $\pm$ 1.94				
MPV (fL)	—	Case	9.72 $\pm$ 1.07	1.1 (0.72–1.71)	.664	—	—
		Control	9.63 $\pm$ 0.91				
P-LCR (%)	—	Case	24.5 $\pm$ 8.27	1.01 (0.96–1.07)	.618	—	—
		Control	23.7 $\pm$ 6.9				
IgG against COVID-19 (relative unit)	—	Case	17.2 $\pm$ 9.83	1.07 (1.03–1.12)	.002	1.08 (1.03–1.15)	.003
		Control	10.2 $\pm$ 9.80				

BMI, body mass index; HCT, hematocrit; HGB, hemoglobin; LYMPH, lymphocyte; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; MXD, mixed cell percent; OR, odds ratio; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; PLT, platelet; RBC, red blood cell; RDW-C, red cell distribution width; WBC, white blood cell.

The results of this study showed that menopausal women had higher levels of IgG against COVID-19 than nonmenopausal women. After adjusting, IgG against COVID-19 was significantly correlated to the menopausal state. Previous studies indicate that the production of IgG against COVID-19 in men and women was different and women had a higher titer of IgG in their blood circulation. It seems that this increased immune response leads to resistance against viral infection, but on the other hand it can expose women to autoimmune diseases and increase their susceptibility to disease.^{16,19–24} More importantly, a study in 2021 showed that menopausal females had higher incidences of predicted COVID-19. After finding a positive correlation between predicted COVID-19 with menopausal status, Costeira et al²¹ suggested that there is a protective effect of estrogen exposure on COVID-19.

Several factors and mechanisms may explain this discrepancy. Some may be related to lifestyle, genetics, and environmental situation of Iranian women and other people. Also, the ACE2 enzyme is regulated by estradiol and plays an important role in allowing the virus to enter the epithelial cells of the lung, gastrointestinal, brain, kidney and/or heart.²⁵ Accordingly, Stelzig et al²⁶ found that estradiol inhibited ACE2 in bronchial epithelial cells in females; however, another study has shown ACE2 stimulation on vascular endothelial cells of the heart.²⁷ Moreover, estradiol regulates B cells, produces T helper 2, and leads to an increase in titer of antibody.^{22,25} Estrogen hormone can enhance or reduce the immune response according to the concentration, distribution, and expression of α and β estrogen receptors in the brain, intestinal epithelial cells (macrophages), lymphatic tissue (dendritic cells), and lymphocytes.^{17,28,29} This hormone leads to the production of antiviral cytokines such as interferon- γ and tumor necrosis factor- α as well as toll-like receptor expressed on dendritic plasmacytoid. Estrogen also produces proinflammatory cytokines such as IL-12 (from macrophages) and reduces the production of IL-6, and this prevents the occurrence

of cytokine storm caused by IL-6.^{16,22,24,30} In fact, the lack of regulation of IL-6 by estradiol in postmenopausal women will lead to cytokine storm and the development and maintenance of diseases related to inflammation.¹⁴ These important changes probably explain the excessive production of IgG against COVID-19 in menopausal women despite the susceptibility to COVID-19.

In conclusion, the results of this study showed that menopausal women had higher levels of IgG against COVID-19 in comparison with nonmenopausal females. In addition, there was a correlation between IgG against COVID-19 and menopausal state. However, more complementary studies are needed in this regard.

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The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

- Sahin A-R, Erdogan A, Agaoglu PM, et al. 2019 novel coronavirus (COVID-19) outbreak: a review of the current literature. *EJMO*. 2020;4(1):1–7.
- Bedford J, Enria D, Giesecke J, et al. COVID-19: towards controlling of a pandemic. *Lancet*. 2020;395(10229):1015–1018. doi:10.1016/S0140-6736(20)30673-5.

3. Nabatchian F, Ashtiani M, Davoudi M, Teimourpour A, Davoudi N. A multivariate analysis model of changes in some laboratory parameters in response to COVID-19, diabetes, gender, and age. *Clin Lab*. 2021;67(1):1929–1938.
4. Gudbjartsson DF, Helgason A, Jonsson H, et al. Spread of SARS-CoV-2 in the Icelandic population. *N Engl J Med*. 2020;382(24):2302–2315. doi:10.1056/nejmoa2006100.
5. Lu R, Zhao X, Li J, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet*. 2020;395(10224):565–574. doi:10.1016/S0140-6736(20)30251-8.
6. Karvonen-Gutierrez CA, Leis A. Impact of menopause on women with systemic lupus erythematosus. *Maturitas*. 2021;154(1):25–30. doi:10.1016/j.maturitas.2021.09.004.
7. Khan N. Possible protective role of 17 β -estradiol against COVID-19. *J Allergy Infect Dis*. 2020;1(2):38–48. doi:10.46439/allergy.1.010.
8. Ghosh M, Rodriguez-Garcia M, Wira CR. The immune system in menopause: pros and cons of hormone therapy. *J Steroid Biochem Mol Biol*. 2014;142(1):171–175. doi:10.1016/j.jsbmb.2013.09.003.
9. Wang X-W, Hu H, Xu Z-Y, et al. Association of menopausal status with COVID-19 outcomes: a propensity score matching analysis. *Biol Sex Diff*. 2021;12(1):1–8.
10. Lovre D, Bateman K, Sherman M, Fonseca VA, Lefante J, Mauvais-Jarvis F. Acute estradiol and progesterone therapy in hospitalised adults to reduce COVID-19 severity: a randomised control trial. *BMJ Open*. 2021;11(11):e053684. doi:10.1136/bmjopen-2021-053684.
11. Ramírez-de-Arellano A, Gutiérrez-Franco J, Sierra-Díaz E, Pereira-Suárez AL. The role of estradiol in the immune response against COVID-19. *Hormones*. 2021;20(4):657–667. doi:10.1007/s42000-021-00300-7.
12. Arya V, Bajpai R, Bajpai A, Kathuria NS, Gupta A. Is menopause a potential risk factor for severity of COVID-19: a retrospective cross-sectional study? *Asian J Med Sci*. 2021;12(9):11–16. doi:10.3126/ajms.v12i9.37811.
13. Zafari Zangeneh F, Sarmast Shoushtari M. Estradiol and COVID-19: does 17-estradiol have an immune-protective function in women against coronavirus? *J Family Reprod Health*. 2021;15(3):150–159.
14. Mauvais-Jarvis F, Klein SL, Levin ER. Estradiol, progesterone, immunomodulation, and COVID-19 outcomes. *Endocrinology*. 2020;161(9):bqaa127. doi:10.1210/endocr/bqaa127.
15. Ruggieri A, Anticoli S, D'Ambrosio A, Giordani L, Viora M. The influence of sex and gender on immunity, infection and vaccination. *Ann Ist Super Sanita*. 2016;52(2):198–204. doi:10.4415/ANN_16_02_11.
16. Jin S, An H, Zhou T, et al. Sex-and age-specific clinical and immunological features of coronavirus disease 2019. *PLoS Pathog*. 2021;17(3):e1009420. doi:10.1371/journal.ppat.1009420.
17. Mangalam AK, Taneja V, David CS. HLA class II molecules influence susceptibility versus protection in inflammatory diseases by determining the cytokine profile. *J Immunol*. 2013;190(2):513–519. doi:10.4049/jimmunol.1201891.
18. Ngo ST, Steyn FJ, McCombe PA. Gender differences in autoimmune disease. *Front Neuroendocrinol*. 2014;35(3):347–369. doi:10.1016/j.yfrne.2014.04.004.
19. Qian J, Zhao L, Ye R-Z, Li X-J, Liu Y-L. Age-dependent gender differences in COVID-19 in mainland China: comparative study. *Clin Infect Dis*. 2020;71(9):2488–2494.
20. Taneja V. Sex hormones determine immune response. *Front Immunol*. 2018;9(1):1931. doi:10.3389/fimmu.2018.01931.
21. Costeira R, Lee KA, Murray B, et al. Estrogen and COVID-19 symptoms: associations in women from the COVID Symptom Study. *PLoS One*. 2021;16(9):e0257051. doi:10.1371/journal.pone.0257051.
22. Raza HA, Sen P, Bhatti OA, Gupta L. Sex hormones, autoimmunity and gender disparity in COVID-19. *Rheumatol Int*. 2021;41(8):1375–1386. doi:10.1007/s00296-021-04873-9.
23. Zeng F, Dai C, Cai P, et al. A comparison study of SARS-CoV-2 IgG antibody between male and female COVID-19 patients: a possible reason underlying different outcome between sex. *J Med Virol*. 2020;92(10):2050–2054. doi:10.1002/jmv.25989.
24. Gallman AE, Fassett MS. Cutaneous pathology of COVID-19 as a window into immunologic mechanisms of disease. *Dermatol Clin*. 2021;39(4):533–543. doi:10.1016/j.det.2021.05.008.
25. Cagnacci A, Xholli A. Change in Covid-19 infection and mortality rates in postmenopausal women. *Menopause*. 2021;28(5):573–575. doi:10.1097/GME.0000000000001731.
26. Stelzig KE, Canepa-Escaro F, Schiliro M, Berdnikovs S, Prakash YS, Chiarella SE. Estrogen regulates the expression of SARS-CoV-2 receptor ACE2 in differentiated airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2020;318(6):L1280–L1281. doi:10.1152/ajplung.00153.2020.
27. Bukowska A, Spiller L, Wolke C, et al. Protective regulation of the ACE2/ACE gene expression by estrogen in human atrial tissue from elderly men. *Exp Biol Med*. 2017;242(14):1412–1423. doi:10.1177/1535370217718808.
28. Von Haam E, Rosenfeld I. The effect of estrone on antibody-production. *J Immunol*. 1942;43(1):109–117.
29. Sherman B, Wysham W, Pfoh B. Age-related changes in the circadian rhythm of plasma cortisol in man. *J Clin Endocrinol Metab*. 1985;61(3):439–443.
30. Borgoni S, Kudryashova KS, Burka K, De Magalhães JP. Targeting immune dysfunction in aging. *Ageing Res Rev*. 2021;70(1):101410. doi:10.1016/j.arr.2021.101410.

Analysis of Specificity and Distribution Characteristics of Red Blood Cell Irregular Antibodies

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Keywords: alloantibody, unexpected antibody, autoantibody, hemolytic disease of fetus and newborn, delayed hemolytic transfusion reaction, blood transfusion

Abbreviations: RBC, red blood cell

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ABSTRACT

Objective: The presence of red blood cell (RBC) irregular antibodies can severely jeopardize mother and child and bring trouble to the treatment of anemia. The aim of this study was to analyze the specificity of RBC irregular antibody in inpatients.

Methods: An analysis was performed on samples from patients with RBC irregular antibodies. Antibody screening positive samples were analyzed.

Results: Among the 778 cases of irregular antibody positive samples, 214 were from males and 564 from females. History of blood transfusion accounted for 13.1% of the total. Of the women, 96.8% had a pregnancy. A total of 131 antibodies were identified. The antibodies included 68 Rh systematic antibodies, 6 MNS systematic antibodies, 6 Lewis systematic antibodies, 2 Kidd systematic antibodies, 10 autoantibodies, and 39 antibodies of uncertain specificity.

Conclusion: Patients with blood transfusion or pregnancy history are prone to produce RBC irregular antibodies.

Red blood cell (RBC) irregular antibodies, also known as unexpected antibodies, are antibodies other than anti-A and anti-B, including alloantibodies and autoantibodies, which are mainly produced by blood transfusion and pregnancy immune stimulation.^{1,2} Red blood cell irregular

antibodies affect the identification of the ABO blood group. Unexpected positive reactions resulting in an ABO discrepancy can occur if A1 or B reagent red cells, or both, used for serum grouping are positive for the antigen. In addition, the agglutination reaction in the main side and/or the secondary side easily occurs, which makes the determination of results difficult. Alloantibodies can interfere in cross-match testing and therefore can cause delay in obtaining compatible blood and is also sometimes associated with a delayed type of hemolytic transfusion reaction.³ Therefore, the importance of irregular antibody detection is beyond doubt.

This study analyzed the distribution characteristics and disease distribution characteristics of 778 patients with positive antibody screening in our hospital from January 2012 to January 2022. The analysis of 131 screening positive samples was conducted to study the specificity and distribution characteristics of RBC irregular antibodies in our hospital from April 2020 to January 2022.

Materials and Methods

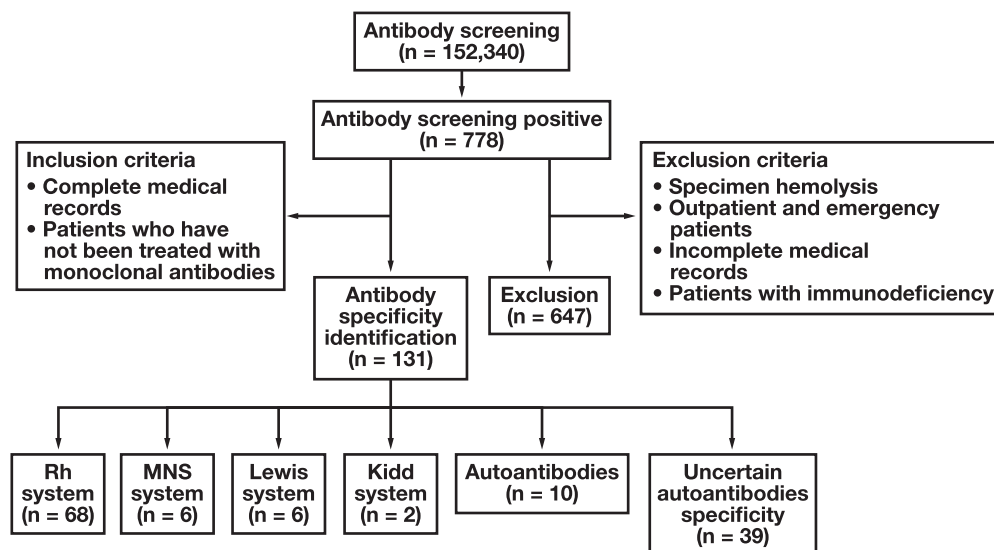
Data

A total of 778 patients with positive antibody screening in the Affiliated Hospital of Nantong University from January 2012 to January 2022 were selected as the research objects; 131 patients were identified as the research subjects from April 2020 to January 2022 (**FIGURE 1**). The cell pattern of RBC antibody identification spectrum cells is shown in **TABLE 1**. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) with the ethical approval from the Ethics Committee of the Affiliated Hospital of Nantong University (2022-K057-01).

Methods

The ABO and Rh(D) blood group identification and irregular antibody screening were performed by automatic blood group analysis with the ABO, Rh(D) blood typing reagent card (Suda Saier) and IgG + C3d anti-human globulin detection card (Suda Saier), respectively. The tests were carried out on the instrument (Aikang Aigel 400) according to established procedures. When positive samples of irregular antibodies were obtained, we used the anti-human globulin test card (Boxun) for retesting. If this was also positive, we used a saline indirect antiglobulin test method and anti-human globulin microcolumn gel method for antibody specific identification. The specificity of the antibody was judged based on the response of both the patient's plasma and spectrum cells. When the agglutination intensity is consistent with all the spectrum cells and autologous cells, it

FIGURE 1. Research object flow chart.



is judged as autoantibodies. Antibodies are defined to be of ambiguous specificity if they do not conform to the reaction pattern of cells, or if they react with all cells. If they react with all cells, then they are autoantibodies; if they react with all cells but the autocontrol, then they are antibodies to a high prevalence antigen. The plasma reacted with some cells, and the intensity of reaction could be strong or weak. We combined the results with medical history and analyzed the reaction results of the identified cell profile by negative exclusion and identified the specific alloantibodies with the spectrum cell results if possible.

Statistical Analysis

SPSS24.0 software was used for statistical analysis. Count data were expressed by frequency and percentage, which was assessed by using the χ^2 test. *P* values less than .05 were considered to be statistically significant.

Results

RBC Irregular Antibody Distribution Characteristics

Among the 152,340 patients in the study, 778 cases were positive for irregular antibodies, with a positive rate of 0.51%, including 214 males and 564 females. Among the 564 female patients, 464 had a record of pregnancy, and of these, 212 females had been pregnant once, accounting for the highest proportion; 100 (17.7%) had an unknown pregnancy history. Among the 564 females, 96.8% had been pregnant at least once. According to disease classification, the proportion of cancer patients was the highest, followed by pregnant women, and the proportion of tumors and pregnancy was relatively high, which accounted for 49.2%. The proportion of irregular antibodies in women who had one pregnancy was the highest, and with the increase of the number of pregnancies, the proportion of RBC irregular antibodies tended to decrease, as shown in **TABLE 2**.

Antibody-Specific Identification Results

The specific identification of 131 of the positive cases from April 2020 to January 2022 showed that there were 68 cases of Rh system antibodies,

accounting for 51.9%, and anti-E had the highest positive rate, accounting for 39.69%. In addition, 49 patients (37.40%) had autoantibodies (10 cases) or antibodies of uncertain specificity (39 cases) (**TABLE 3**).

Distribution Characteristics of Antibody Types in the 131 Cases

As shown in **TABLE 4**, among the 131 patients with RBC irregular antibodies, there were statistical differences in sex and pregnancy or transfusion, but no statistical differences in age and blood group.

Discussion

Among the 131 patients with RBC irregular antibodies, there were statistical differences in sex and pregnancy or transfusion in the study. The detection rate of irregular erythrocyte antibodies is higher in women than in men because women have been stimulated by alloantigens during pregnancy or abortion. In this study, it can be seen that the proportion of irregular antibodies in women who had 1 pregnancy was the highest, and with the increase of the number of pregnancies, the proportion of irregular antibodies tended to decrease. This is likely also due to the 1-, 2-, and 3-child policy in China over the years. It could be a function of the anamnestic response that naturally occurs. Red blood cell irregular antibodies decrease if not stimulated again. Therefore, it can be seen that the production mechanism of irregular antibodies during pregnancy is complex, and thus that further in-depth research is needed.

Judging from the disease distribution of the 778 cases of irregular antibody-positive patients, the proportion of tumors and pregnancy was relatively high, 49.2%, whereas blood system disease accounted for only 8.4% and the proportion of other diseases is lower. These results indicate that blood transfusion and pregnancy is the main cause of irregular antibody production in individuals.^{4,5}

Red blood cell irregular antibodies can lead to hemolytic disease of fetus and newborn.^{6–8} An increase in neonatal hemolytic cases is a warning that we need to constantly pay attention to the production of antibodies in pregnant women so as to ensure the safety of mothers

TABLE 1. Spectrum Cells

Number	Rh-hr			Kidd		MNS			Duffy		Diego		Kell		Lewis		P	D0		Yt					
	D	C	E	c	e	JK ^a	JK ^b	M	N	S	s	Mur	Fy ^a	Fy ^b	Di ^a	Di ^b	K	k	Le ^a	Le ^b	P1	D0 ^a	D0 ^b	Yt ^a	Yt ^b
1	+	0	+	+	0	+	+	+	0	0	+	0	+	0	+	/	0	+	+	+	+	+	/	/	/
2	+	+	0	0	+	+	0	+	+	+	+	0	+	0	0	/	0	+	0	+	+	+	/	/	/
3	+	+	+	+	+	+	0	0	+	0	+	0	+	+	+	/	0	+	+	+	+	+	/	/	/
4	+	+	+	+	+	+	0	0	+	+	+	0	+	0	0	/	0	+	0	+	0	0	/	/	/
5	+	0	+	+	+	0	+	+	+	0	+	0	+	0	0	+	0	+	0	+	+	0	+	+	0
6	+	0	+	+	0	0	+	+	+	0	+	0	+	0	0	/	0	+	0	+	0	/	/	/	/
7	0	0	+	+	+	+	0	0	+	0	+	0	+	0	0	/	0	+	0	+	+	/	/	/	/
8	+	+	+	+	+	+	0	+	+	+	+	0	+	0	0	/	0	+	0	+	0	/	/	/	/
9	0	0	0	+	+	+	+	0	+	0	+	0	+	0	0	+	0	+	0	0	0	0	+	+	0
10	+	+	0	0	+	+	0	+	+	0	+	+	+	0	+ ^w	/	0	+	0	+	+	/	/	/	/

^a+, a small cell agglutination mixed with numerous free red blood cells.

TABLE 2. Red Blood Cell Irregular Antibody Distribution

Classification	No. (%)
Sex	
Male	214 (27.5)
Female	564 (72.5)
Blood group	
A	268 (34.4)
B	225 (28.9)
O	196 (25.2)
AB	89 (11.4)
Age, y	
≤17	10 (1.29)
18–40	237 (30.5)
41–60	291 (37.4)
>60	240 (30.8)
Transfusion	
Yes	102 (13.1)
No	676 (86.9)
Pregnancy ^a	
0	18 (3.2)
1	212 (37.6)
2	123 (21.8)
3	71 (12.6)
≥4	58 (10.3)
Unknown times	82 (14.5)
Reason for medical treatment	
Pregnant ^a	145 (18.6)
Gynecological disease	40 (5.1)
Osteoarthritis	64 (8.2)
Digestive system disease	49 (6.3)
Respiratory disease	14 (1.8)
Cardiovascular disease	24 (3.1)
Blood system disease	65 (8.4)
Nervous system disease	22 (2.8)
Urinary system disease	21 (2.7)
Endocrine system disease	2 (0.3)
Trauma	22 (2.8)
Tumor	238 (30.6)
Burn	10 (1.3)
Pediatric disease	3 (0.4)
Eye disease	8 (1.0)
Autoimmune disease	12 (1.5)
Other	39 (5.0)

^a0: no pregnancy. Unknown times: a history of pregnancy but the number of pregnancies was not recorded in the medical records. Pregnant: hospitalized to give birth.

and babies. There are many references on the detection standards of irregular antibodies in pregnant women, and the British Committee for Standards in Haematology (BCSH) also gives suggestions.⁹ Medical units should screen and identify irregular antibodies according to the population characteristics of their own regions.

TABLE 3. Distribution of Red Blood Cell Irregular Antibodies

Blood Group System	Antibody Type	No. (%)
Rh system	Anti-E	52 (39.69)
	Anti-e	5 (3.82)
	Anti-D	3 (2.29)
	Anti-C	1 (0.76)
	Anti-c	4 (3.05)
	Anti-DC	1 (0.76)
MNS system	Anti-Ce	2 (1.53)
	Anti-M	6 (4.58)
Lewis system	Anti-Le ^a	6 (4.58)
Kidd system	Anti-Jk ^a	2 (1.53)
Autoantibody		10 (7.63)
Uncertain antibodies specificity		39 (29.77)

In this study, we collected 778 irregular antibody positive cases from January 2012 to January 2022. Due to the lack of patient information and other reasons, we tested the specificity of antibodies in only 131 patients from April 2020 to January 2022. Of the 131 detected antibodies, 68 cases were Rh system antibodies and 52 cases were anti-E. The detection rate of anti-E was the highest among Rh antibodies, which was consistent with the literature. The most common types of Rh antigens in clinical practice are D, C, c, E, and e. The strength of the antigen is as follows: D > E > C > c > e. However, in the “Technical Specifications for Clinical Blood Transfusion,” testing is mandatory for RhD and is not required for Rh other antigens, which may contribute to the higher proportion of anti-E positivity found in our study. In addition, the distribution frequency of E antigens in the Chinese Han population is 47%, so the probability of anti-E increased in alloimmunization. The distribution frequency of the E antigen and gene was also different in different regions. In addition, in the past, when patients with positive irregular antibodies were transfused, there was no condition for antibody-specific identification, and blind screening was mostly used for matching, which increased the probability of new antibodies and the difficulty of finding suitable blood. For these reasons, the positive rate of anti-E detection is higher than that of other antibodies. Therefore, it is recommended that in addition to detecting RhD antigen, RhE antigen should be used as a routine test before blood transfusion matching so as to reduce the positive rate of anti-E, which will reduce the occurrence of blood transfusion reactions.

In our research, we found 39 cases (29.77%) of antibodies with unclear specificity, second only to Rh system antibodies. The appearance of antibodies with unclear specificity may be attributed to the following reasons: (1) the concentration of antibodies in the patient will change and fade, (2) new antibodies, and (3) spectrum cells contain limited types of antigen.

In summary, the specific identification and the analysis of the relationship between irregular antibodies and sex, age, and disease can help to identify the pattern of antibody prevalence and provide experience for subsequent identification of antibodies. Some irregular antibodies will gradually diminish in vivo due to the lack of immune stimulation, which is why some antibodies in this experiment lacked specificity. As patients present in different hospitals and not all test results of RBC irregular antibodies can be tracked, we hope that along with the development of a blood transfusion medicine, an information-sharing platform for positive cases of irregular antibodies in different groups will be

TABLE 4. Population Distribution Characteristics of the 131 Cases of Red Blood Cell Irregular Antibodies

	Specific Antibody										Nonspecific Antibody		χ^2	P
	Anti-E	Anti-e	Anti-C	Anti-c	Anti-D	Anti-DC	Anti-Ce	Anti-Jk ^a	Anti-Le ^a	Anti-M	Autoantibody	Unclear		
Blood group														
A	16	2	0	1	1	0	0	2	0	1	1	14	1.311	.727
B	16	2	0	1	1	1	2	0	5	1	1	12		
O	16	0	1	1	1	0	0	0	0	4	6	9		
AB	4	1	0	1	0	0	0	0	1	0	2	4		
Sex														
Male	6	1	1	0	0	0	2	1	1	1	4	11	3.975	.046
Female	46	4	0	4	3	1	0	1	5	5	6	28		
Age, y														
≤17														
18-40	10	2	0	4	1	1	0	0	3	3	1	14		
41-60	26	3	0	0	1	0	1	1	2	2	3	18		
>60	16	0	1	0	1	0	1	1	1	1	6	5		
Immunization ^a														
Yes	46	5	0	4	3	1	1	2	5	5	8	28	4.355	.037
No	6	0	1	0	0	0	1	0	1	1	2	11		

^aImmunization: pregnancy or transfusion.

established to track the process of the generation, development, and subsidence of patients' irregular antibodies in real time, allowing the search for antigens even if antibody test results are negative. This study suggests that after the identification of clinically meaningful irregular antibodies, antigen-negative RBCs are always selected for transfusion, even if the patient's antibodies cannot be detected.¹⁰ Transfusion of antigen-negative RBCs against specific antibodies can reduce the occurrence of amnesia response, delayed hemolytic transfusion reaction, and delayed serological transfusion, which provides a theoretical basis to ensure the safety of blood transfusion.

In this study, there are some deficiencies, such as that the period of specimen collection was not long, and few cases were identified with positive antibodies when autoantibodies were present. Also there were no additional panel RBCs to further identify blood group antibody specificities, and Mia cells were lacking in spectrum cells. These are the directions that we must focus on in subsequent research. In addition, we will increase the number of centers used and constantly improve the sensitivity of specific antibody identification in the later studies to detect clinically meaningful antibodies that conform to regional characteristics.

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The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Tormey CA, Hendrickson JE. Transfusion-related red blood cell alloantibodies: induction and consequences. *Blood*. 2019;133(17):1821–1830. doi:10.1182/blood-2018-08-833962.
2. Dholakiya SK, Bharadva S, Vachhani JH, Upadhyay BS. Red cell alloimmunization among antenatal women attending tertiary care center in Jamnagar, Gujarat, India. *Asian J Transfus Sci*. 2021;15(1):52–56. doi:10.4103/ajts.AJTS_72_17.
3. Bhuvra DK, Vachhani JH. Red cell alloimmunization in repeatedly transfused patients. *Asian J Transfus Sci*. 2017;11(2):115–120. doi:10.4103/0973-6247.214347.
4. Li H, Xu HM, Zhang Y, Cui JX. Analysis of patients' irregular antibody screening and identification results before blood transfusion. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2015;23(3):861–865. doi:10.7534/j.issn.1009-2137.2015.03.050.
5. Li CY, Li YM, Huang F, Xiao J, Xu H. [Characteristics of blood type irregular antibodies in Han population of Chinese Sichuan area]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2015;23(2):533–536. doi:10.7534/j.issn.1009-2137.2015.02.046.
6. Chuang MT, Chang CH. Hemolytic disease of the fetus and newborn caused by irregular antibody: a mortality case report and case series during the past 15 years in NCKUH. *Taiwan J Obstet Gynecol*. 2022;61(5):896–899. doi:10.1016/j.tjog.2021.11.040.
7. Chen X, Feng J, Jiang Y. Hemolytic disease of the fetus and newborn caused by maternal autoantibody with mimicking anti-E specificity. *Lab Med*. 2021;52(4):399–402. doi:10.1093/labmed/lmaa096.
8. Iberahim S, Aizuddin MJ, Kadir NA, et al. Hemolytic disease of fetus and newborn in a primigravida with multiple alloantibodies involving anti-Jka and anti-E: a case report. *Oman Med J*. 2020;35(6):e206. doi:10.5001/omj.2020.135.
9. White J, Qureshi H, Massey E, et al; British Committee for Standards in Haematology. Guideline for blood grouping and red cell antibody testing in pregnancy. *Transfus Med*. 2016;26(4):246–263. doi:10.1111/tme.12299.
10. Noiret L, Slater A, Higgins JM. Determinants of red blood cell alloantibody detection duration: analysis of multiply alloimmunized patients supports peritransfusion factors. *Transfusion*. 2017;57(8):1930–1937. doi:10.1111/trf.14157.

3D Printing as an Effective Quality Assurance Implementation in Massive-Scale SARS-CoV-2 Testing at a SwabSeq Next-Generation Sequencing Laboratory

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Keywords: sequencing, genetics, SARS-CoV-2, virology, 3D printing, quality assurance

Abbreviations: UCLA, University of California, Los Angeles; PCR, polymerase chain reaction; QC, quality control; IQC, internal QC; MD, midday; NT, nighttime; 2D, 2-dimensional

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ABSTRACT

Massive-scale SARS-CoV-2 testing using the SwabSeq diagnostic platform came with quality assurance challenges due to the novelty and scale of sequencing-based testing. The SwabSeq platform relies on accurate mapping between specimen identifiers and molecular barcodes to match a result back to a patient specimen. To identify and mitigate mapping errors, we instituted quality control using placement of negative controls within a rack of patient samples. We designed 2-dimensional paper templates to fit over a 96-position rack of specimens with holes to show the control tube placements. We designed and 3-dimensionally printed plastic templates that fit onto 4 racks of patient specimens and provide accurate indications of the correct control tube placements. The final plastic templates dramatically reduced plate mapping errors from 22.55% in January 2021 to less than 1% after implementation and training in January 2021. We show how 3D printing can be a cost-effective quality assurance tool to mitigate human error in the clinical laboratory.

At the start of the COVID-19 pandemic, the University of California, Los Angeles (UCLA) SwabSeq COVID-19 clinical testing laboratory developed a novel protocol that used next-generation sequencing to per-

form rapid and highly scalable testing for COVID-19.¹ Using this assay, we are able to test thousands of specimens simultaneously using sample-specific molecular barcodes added to each patient's specimen at the time of polymerase chain reaction (PCR) amplification. We use a 96-well liquid handler to pipette heat-inactivated patient specimen into a 384-well PCR plate (the "primer plate") containing PCR primers to amplify the SARS-CoV-2 *S* gene as well as human *RPP30*, a housekeeping control gene. These primers are designed with both Illumina sequencing adapters as well as unique molecular barcodes, or indices, to allow for direct sequencing of the PCR amplicons without an additional fragmentation step.¹

These molecular barcodes enable thousands of samples to be combined, simultaneously sequenced, and analyzed for the presence or absence of SARS-CoV-2 within a single sequencing flow-cell.¹ A deconvolution step is performed during the analysis of sequencing reads to properly identify the patient specimen from which any given read originated and thus correctly identify the presence or absence of SARS-CoV-2 in the tested samples. A central aspect of the SwabSeq COVID-19 assay relies on a precise link of a specimen identifier with the molecular barcode associated with that well position in the 384-well PCR plate.

Given the throughput of the SwabSeq COVID-19 assay, quality control (QC) ensures robust, high-quality testing and minimizes the possibility for errors that could affect thousands of patients. The use of negative controls is a standard quality control method in laboratories and can be used to detect confounding bias and other sources of causal error.² This allows laboratory staff to analyze if the resulting associations are, in fact, causal.² Many labs also use internal QC (IQC) to monitor the quality of work being done during runs and analysis of results to ensure data released to patients are fit for purpose.³

The SwabSeq assay uses nuclease-free water-filled specimen tubes with unique tube identification barcodes, called control tubes, which serve multiple purposes, including serving as both negative controls and IQCs. Because the assay involves PCR amplification, there is a substantial risk for the spreading of amplification products that could affect our limit of detection for the virus.⁴ The water-filled control tubes, which should have no human or SARS-CoV-2 genetic material, can therefore be used to detect potential contamination from equipment, reagents, or aerosols, as well as cross-contamination between adjacent samples.

The control tubes are also used as IQCs to check the orientation of racks filled with patient samples. One of the potential errors that we recognized early in our clinical validations is the risk of improper orientation of the samples with respect to the plate containing PCR barcodes in fixed positions within the PCR plates. For example, a 180-degree rotation of a 384-well PCR plate containing unique molecular barcodes would lead to the misidentification of every patient specimen in the 384-well plate. To accommodate this, we reference the location of controls as a way of verifying proper orientation of the tube rack with the primer plate. Our molecular barcoding system currently relies on 16,384-well plates which contain unique combinations of molecular barcodes, meaning over 6,000 samples can be run within a single sequencing run. Therefore, for each 96-position rack of specimen tubes, we use control tubes that are placed in unique positions that encode (1) the identity of the quadrant within the 384-well PCR plate containing unique molecular barcodes, (2) the identity of the molecular barcode plate (1–16), and (3) which of the four daily sequencing runs the plate belongs to (AM, mid-day [MD], PM, and nighttime [NT]).

When analyzing the results of this assay, the reviewing scientist can easily and accurately confirm the location of control tubes using dual approaches: using the tube identifiers and the absence of sample material. If the location of the expected controls contains sample material, this indicates potential contamination or an orientation error during the preparation of the PCR plate. Orientation errors can occur at 2 levels: the 96-position rack and the 384-well PCR plate. A 96-position rack error can occur when the rack is erroneously pipetted into the 384-well PCR plate in the incorrect position, and this can be identified by the presence of one misoriented quadrant in the 384-well PCR plate. The reviewing scientist can also identify whether a plate was erroneously sequenced with the wrong sequencing run; for example, the orientation of the control tubes could show that a plate from the AM run was mistakenly swapped with a plate containing identical barcodes from the PM run, an error that could lead to incorrect results reported to 382 patients in the AM run and 382 patients in the PM run (a 384-well PCR plate contains 382 patient samples and 2 control tubes).

Because the placement of control tubes is an integral part of the QC metrics for the SwabSeq assay, it is critical that these tubes are placed in the correct positions in the 96-position rack of tubes. The UCLA SwabSeq laboratory adopted the Lean Six Sigma model, a system used by many clinical laboratories to improve efficiency and reduce errors.^{5,6} Using Lean Six Sigma principles, we followed the Design Measure Analyze Improve Control protocol⁷ to create a solution that reduced our control tube misplacement rate, first starting with 2-dimensional (2D) templates to visually check for control tube placement before any samples are pipetted into the PCR plate. The 2D templates proved to be a temporary solution and our laboratory sought a more robust, long-term quality assurance system that was specific to our unique problem.

Like many companies, hospitals, and researchers facing supply shortages due to the COVID-19 pandemic, our laboratory turned to 3D printing as a solution for our quality assurance problem. Three-dimensional printing has been used in hospitals and clinics for tissue manufacture, implants, and anatomical models for medical training.^{8,9} The equipment printed using this technology allows for improvements in both patient-based and research settings, in part because of its ability to rapidly create custom products. The vast use of 3D printing in the medical field led us to explore 3D template options to replace our 2D templates and bring 3D-printed technologies into our clinical laboratory.

Here, we present how we created and optimized 3D printed templates to significantly reduce quality errors for this assay, demonstrating the innovative opportunity to bring cutting-edge 3D-printing technologies into the clinical laboratory to improve quality.

Methods and Results

Our first attempt to reduce control tube placement errors was with 2D paper templates. Each template was made of 4 pieces of laminated colored paper the size of the specimen tube racks. Holes were cut out in specific locations to indicate where controls should be placed (**FIGURE 1A**). The template was designed to lay on top of 4 specimen tube racks, representing the 4 96-well quadrants of 1 384-well PCR plate, to show the technician the correct placement of the control tubes (**FIGURE 1B**). When we first designed these 2D templates, our sequencing runs consisted of 4 unique plates and 2 unique daily runs. Placement of the control tubes is contingent on 3 factors: rack quadrants (A, B, C, or D), plate number (1 to 4), and time of day (AM or PM), and thus we designed 32 unique positionings for the 2 control tubes that are placed in each specimen rack (**FIGURE 1C**). Each template covered 4 96-position specimen tube racks and was labeled A through D for the destination quadrant, with the color of the laminated paper corresponding to the plate number (for example, plate 1 used purple paper and plate 2 used blue). We created 2 sets of 4 templates each for the 2 daily runs and secured the templates together with a loose leaf binder ring labeled with the run name (AM or PM, **FIGURE 1D**).

Before instituting any kind of template to ensure proper control tube placement, laboratory personnel visually checked for placement of control tubes before pipetting samples from the 96-position racks to the 384-well PCR plates.⁶ To assess the effectiveness of our 2D-printed templates, we developed an automated script to count the number of 96-position specimen racks with misplaced tubes for each run. The control tube misplacement rate during the month of January 2021 was 22.55% (**TABLE 1**). After implementing the 2D paper templates on January 22, 2021, we saw a major decrease in the misplacement rate, down to 0.23% in February 2021, and continued to see a misplacement rate below 1% for the remainder of the first quarter of 2021 (**TABLE 1**).

To create the 3D-printed templates, laboratory staff measured specimen racks and designed covers to fit tightly over the 4 racks of sample tubes rather than sitting on top of the tubes (**FIGURE 2A**). This design allows the 4 specimen racks that will be pipetted into the same 384-well PCR plate containing molecular barcodes to have the control tubes inserted simultaneously without slipping, reducing errors. We created STL files (a type of design file compatible with many 3D printers) given these design parameters using web-based 3D design software SketchUp. We were careful to design the 3D templates to fit snugly enough to ensure the proper placement of the control tubes while still being loose enough to be removed from the rack of patient specimens without disrupting the tubes on the periphery of the rack.

The laboratory considered many options to manufacture the 3D templates, including buying a 3D printer; however, this solution was impractical due to the high cost and low yield of templates. We identified a local manufacturer that generated 2 sets of 4 templates (for AM and PM runs, 4 384-well PCR plates each containing 4 96-well quadrants) for \$1,925 (**FIGURE 2B**). These templates were printed with hard plastic, color-coordinated to represent the 384-well

FIGURE 1. A, Two-dimensional printed templates for the AM run with 4 primer plates. Holes in the templates correspond to the correct placement of the control tubes when the template is placed on top of specimen racks and the colors represent the 4 primer plates. B, The 2D template for primer plate 1 of the AM run in use on top of 4 specimen racks indicating the placement of control tubes (orange caps). C, Schematics for control tube placements for each of the 4 primer plates for AM and PM runs. Each color represents a plate number whereas the orange squares represent correct control tube placements. D, Two-dimensional templates for AM and PM runs for the 4 original primer plates. Each laminated sheet represents a 384 well plate consisting of 4 racks of 96 patient sample tubes and the templates for each of the 4 primer plates are bound with a metal ring.



plate number (1–4) with quadrant identification printed directly into the template (initially labeled as 1–4 but changed to A–D in future iterations of the templates).

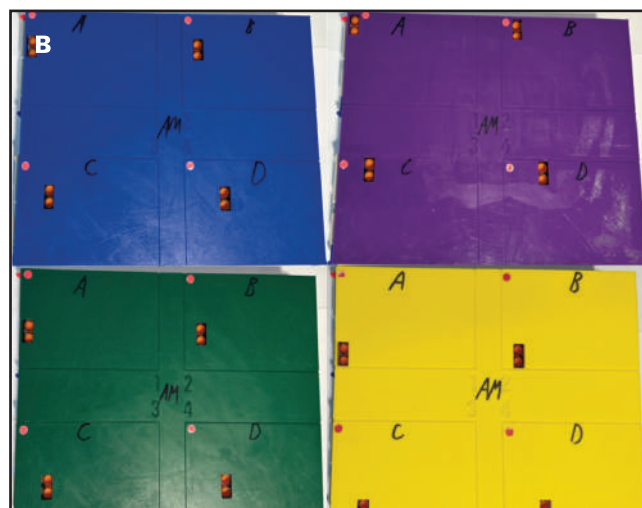
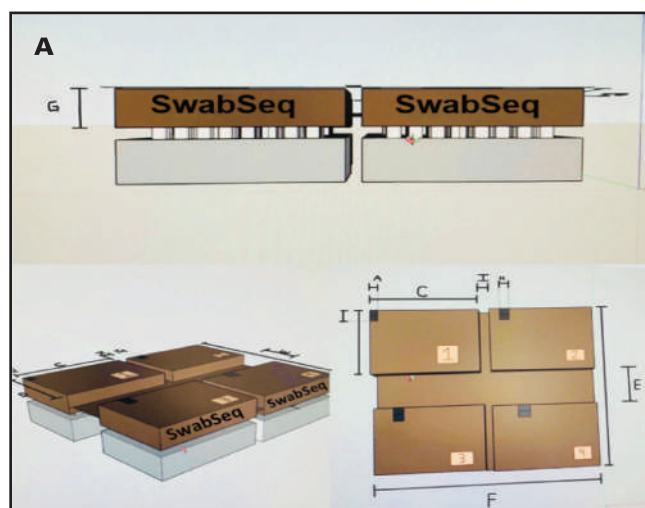
After implementing the 3D templates in late April 2021, we saw our control tube misplacement rate stabilize below 1%. There was a temporary increase in errors that represents a transition and training on

TABLE 1. Tube Misplacement Rates for January 2021 through October 2022^a

Year/Month	Total Racks Run	Correct	Incorrect	Percent Correct	Percent Incorrect
2021-01	366	304	62	83.06%	22.55%
2021-02	432	431	1	99.77%	0.23%
2021-03	352	350	2	99.43%	0.57%
2021-04	342	342	0	100.00%	0.00%
2021-05	249	242	7	97.19%	2.89%
2021-06	78	78	0	100.00%	0.00%
2021-07	176	176	0	100.00%	0.00%
2021-08	679	679	0	100.00%	0.00%
2021-09	1557	1557	0	100.00%	0.00%
2021-10	1525	1516	9	99.41%	0.60%
2021-11	1316	1315	1	99.92%	0.08%
2021-12	817	814	3	99.63%	0.37%
2022-01	1798	1796	2	99.89%	0.11%
2022-02	1860	1858	2	99.89%	0.11%
2022-03	1480	1479	1	99.93%	0.07%
2022-04	1689	1689	0	100.00%	0.00%
2022-05	1745	1744	1	99.94%	0.06%
2022-06	995	992	3	99.70%	0.30%
2022-07	650	650	0	100.00%	0.00%
2022-08	550	550	0	100.00%	0.00%
2022-09	528	528	0	100.00%	0.00%
2022-10	337	337	0	100.00%	0.00%

^aTube misplacement rates were calculated by counting the number of 96-position specimen racks with misplaced control tubes for each run. A rack is deemed to have misplaced control tubes if at least one of the control tubes is not in the proper place.

FIGURE 2. A, CADD file showing the design of our 3D templates designed to fit on top of 4 specimen racks. Importantly, the “G” dimension ensures that the template remains on top of the set of tubes without sliding. B, AM run 3D templates are shown on top of specimen racks with orange specimen tube caps representing the control tubes. The color of the template corresponds to the respective primer plate number 1-4.



the use of 3D templates (TABLE 1). However, as our staff was trained and became more comfortable with the 3D templates and QC was more stringently enforced, we observed the control tube misplacement rate drop and remain below 1% by June 2021 to the present time despite a 10-fold increase of sample volume.

In September 2021, the SwabSeq laboratory began to increase daily volume beyond 2 daily runs of 4 384-well plates each due to the return of students to in-person learning at UCLA. Initially, we added an MD run consisting of up to 4 384-well plates and designed and implemented a new control tube placement scheme and set of 3D templates

FIGURE 3. Scaling up required additional runs and primer sets. **A**, To increase our capacity, we added a midday (MD) run using this control tube placement scheme. **B**, Schematic showing control tube placement after introduction of third control tube due to increased sample volume for the “Winter” set of primer plates. Grid represents a 96-position rack of patient specimens and the control tube positions are shown in orange. Importantly, the third control tube (shown in the MD, PM, and nighttime [NT] runs) allows us to use the same control tube placements for all 4 daily runs while the third tube distinguishes the time of day.



(FIGURE 3A). Ultimately, due to much higher demand for testing, the laboratory increased our molecular barcode space from 1536 combinations (4 384-well plates) to 6144 combinations (16,384-well plates). We additionally added 1 additional sequencing run

(NT). To accommodate this increase in volume, we added an additional control tube to each rack of patient samples (FIGURE 3B). To avoid confusion, we divided our 16 primer plates into 4 groupings of 4 plates, denoted Winter, Spring, Summer and Fall, and

FIGURE 4. A, Current set of 3D printed templates for midday (MD) run configuration. The template colors (white, pink, orange, and gray) represent each of the 4 seasonal groupings whereas the tape color represents each of the 4 primer plates within that season. B, Shelves and bins containing all 16 3D printed templates organized for each season set. Each color of the 3D printed template represents the set of 4 plates for its respective season. Each 16-set of templates has unique control tube placements for MD, PM, and nighttime (NT) runs. This organizational system allows operating technicians to quickly and simply identify the proper templates for the run they are performing.



3D-printed new templates whose color corresponds to this grouping (FIGURE 4A). We also decided to organize these templates on a rack with bins indicating each of the seasons (FIGURE 4B). Ultimately, our 3D-printing strategy allowed for an extremely rapid scale-up of our control tube placement scheme and decreased control tube placement errors.

Discussion

Due to the high throughput nature of the SwabSeq assay, experimental controls and quality control safeguards are critical to the assay's integrity. Because we work on many samples, even a small error rate can represent tens or even hundreds of misidentified patient specimens, which is unacceptable. Errors would require the rerun of hundreds of

specimens, leading to delay in our turnaround time. One such error that poses a significant threat to the integrity of the assay is errors with the orientation of the sample rack with respect to the 384-well PCR plate. These errors result in misidentification of all patient specimens within the “flipped” rack(s).¹ To identify such issues before results are reported to patients, the SwabSeq assay uses the placement of negative control samples to orient a rack of patient specimen tubes within its proper run, 384-well PCR plate, and quadrant of the plate. Errors in the placement of these tubes reduce the effectiveness of this quality assurance safeguard, and so the laboratory sought an innovative way to prevent placement errors.

Here, we showed how an innovative and low-cost method of manufacturing a custom plastic product can be implemented in any clinical laboratory. Although our 2D laminated paper templates were helpful and modestly reduced placement errors, our 3D-printed control tube placement templates eventually were markedly better at preventing these errors and proved to be a major process improvement for our laboratory. We acknowledge the slight uptick in misplacement in June and July 2021, but due to the low number of plates being run and training new staff, we believe that the sharp decrease beginning in August 2021 and maintained to the present day is more indicative of the success of our 3D templates.

Even with this safeguard, we still see sources of error that do not result from misplacement of control tubes. For example, patient specimen racks can still be misoriented at the time of scanning individual tube barcodes using a flatbed scanner. Despite the samples being pipetted into the PCR plate in the correct orientation, this still results in a “plate flip,” as the specimen tube barcodes are still incorrectly associated with the molecular barcodes in the corresponding PCR plate wells. To account for this, our informatics pipeline cross-references specimen tube barcodes to a list of all the barcodes of our control tubes and thus will show scanning errors. Such automation strategies in tandem with our existing procedural quality safeguards will continue to keep the error rate for SwabSeq low so we can continue to provide accurate, high-quality testing for our clients.

The SwabSeq laboratory is encouraged by how quickly and efficiently we were able to identify this fault point in our testing procedure and design a custom solution that eliminated the issue. We are also encouraged by the scalability of this method: although we started with

templates for 2 daily runs with 4 384-well plates each, we were quickly able to scale to 3 daily runs with 16,384-well plates each by designing and manufacturing 3D templates for additional primer sets. SwabSeq plans to continue to innovate by using cutting-edge technologies like 3D printing to solve unique problems in the clinical laboratory.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Bloom JS, Sathe L, Munugala C, et al. Massively scaled-up testing for SARS-CoV-2 RNA via next-generation sequencing of pooled and barcoded nasal and saliva samples. *Nat Biomed Eng*. 2021;5:657–665. doi:10.1038/s41551-021-00754-5
2. Lipsitch M, Tchetgen E, Cohen T. Negative controls: a tool for detecting confounding and bias in observational studies. *Epidemiology*. 2010;21:383–388. doi:10.1097/ede.0b013e3181d61eeb
3. Kinns H, Pitkin S, Housley D, Freedman DB. Internal quality control: best practice. *J Clin Pathol*. 2013;66:1027–1032. doi:10.1136/jclinpath-2013-201661
4. Neumaier M, Braun A, Wagener C, Techniques B. Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics. International Federation of Clinical Chemistry Scientific Division Committee on Molecular Biology Techniques. *Clin Chem*. 1998;44:12–26. doi:10.1093/clinchem/44.1.12
5. de Koning H, Verver JPS, van den Heuvel J, Bisgaard S, Does RJMM. Lean six sigma in healthcare. *J Healthc Qual*. 2006;28:4–11. doi:10.1111/j.1945-1474.2006.tb00596.x
6. Jones J, Saul R, Sathe L, et al. Lean principles to improve quality in high-throughput COVID-19 testing using SwabSeq: a barcoded sequencing-based testing platform. *Lab Med*. 2022;53:e8–e13.
7. Inal TC, Goruroglu Ozturk O, Kibar F, et al. Lean Six Sigma methodologies improve clinical laboratory efficiency and reduce turnaround times. *J Clin Lab Anal*. 2018;32. doi:10.1002/jcla.22180
8. Tino R, Moore R, Antoline S, et al. COVID-19 and the role of 3D printing in medicine. *3D Print Med*. 2020;6:11.
9. Coakley M, Hurt DE. 3D Printing in the laboratory: maximize time and funds with customized and open-source labware. *J Lab Autom*. 2016;21:489–495.

An Assessment of Gender Equity and Parity Among “Nontraditional” Pathology Awards

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Keywords: gender equity, recognition awards, pathology, diversity, inclusion, equity

Abbreviations: ASCP, American Society for Clinical Pathology; AAMC, Association of American Medical Colleges

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ABSTRACT

Objective: The aim of this study was to assess the gender composition of nontraditional pathology recognition award recipients.

Methods: Cross-sectional analysis of American Society for Clinical Pathology (ASCP) Top Five 40 Under Forty and The Pathologist Power List award recipients' gender. Gender was independently analyzed by 2 authors using pronouns. Two analyses were performed: difference in gender parity and difference in gender equity for award recipients.

Results: From 2014 through 2022, 618 total awards were conferred. Significantly more men than women received an award overall (57.1% vs 42.9%; $P < .001$). Compared with population benchmarks, awards conferred to US-based nontrainee pathology physicians (men 56.2%, women 43.8%; $P = .091$) and US-based pathology physician trainees (men 60.5%, women 39.5%; $P = .15$) are equitable. Conversely, gender inequities exist among awards conferred to US-based nonphysician laboratory professionals (men 51.7%, women 48.3%; $P < .001$).

Conclusion: The Pathologist Power List and Top Five ASCP 40 Under Forty awards have not completely achieved gender parity, and gender inequities remain among subgroups.

Recently, Wobker and colleagues¹ documented differences in gender representation among pathology society recognition award recipients. These findings are disquieting, as recognition often directly contributes to opportunities for career advancement and academic promotion. Similarly, Parra-Herran et al² investigated the composition of editor/author gender for prominent anatomic pathology textbooks. The authors found that editors/authors overall comprised more men than women (59.5% vs 40.5%), but the proportion of women was approximately equal to the proportion of women in academic pathology in 2019 (40.5% vs 43.4%). However, the gender composition of the editors/author group varied widely among subspecialties, and in certain subspecialties, was not representative of the academic pathology physician workforce. This highlights the distinction between gender equity (ie, the proportion of a specific gender in a subgroup compared with the proportion of that gender in the entire population from which that subgroup is derived) and gender parity (ie, 50:50 gender balance).³

Although studies continue to illustrate inequities and disparities based on identifying demographics, including gender, among leadership positions, editorial boards, and official society recognition awards,³⁻⁷ little is known about recognition conferred via less traditional sources, such as magazines, websites, and social media-based groups. This is particularly true for specialties with an online or social media presence such as pathology, as this type of publicized recognition may also substantially contribute to reputation, prestige, career opportunities, career advancement, and compensation. Given that these nontraditional awards are conferred outside the typical academic setting, they could be more (or less) equitable with regard to gender. To probe this question, we assessed the gender composition of nontraditional pathology award recipients.

Methods

We investigated the perceived gender of recipients of the annual “The Pathologist Power List,” a nontraditional pathology recognition award conferred to “inspirational” laboratory professionals of all backgrounds

(eg, medical technologists, individuals with PhDs, pathologists) both within the United States (US) and internationally.⁸ This award is presented by *The Pathologist*, a magazine endorsed by the American Society for Clinical Pathology (ASCP). We also investigated the top 5 recipients of the ASCP 40 Under Forty recognition program, which “recognizes members under the age of 40 for their achievements and leadership qualities that are making an impact on pathology and laboratory medicine”.⁹

Award recipients’ perceived gender was independently analyzed by 2 authors using online pronouns in 3 categories (she/he/they) and coded as woman, man, or other. No individual identified as a third category; thus, we report gender as binary.

We analyzed all award recipients’ practice location (country), specialty, and training status at the time of award receipt and coded them into one of the following groups: (1) US-based nontrainee pathology physicians (ie, individuals who have completed pathology training), (2) US-based pathology physician trainees (ie, individuals in a pathology residency or fellowship program), (3) US-based nonphysician laboratory professionals (ie, individuals who have completed or are enrolled in a laboratory training program), and (4) non-US-based individuals (ie, non-US-based pathology physicians and laboratory professionals including trainees and those who have completed training). Of note, individuals with a PhD and board certification in a pathology specialty such as clinical chemistry or microbiology were included in group 3 (US-based laboratory professionals) for purposes of subgroup analysis.

Our analysis focused on total awards, not unique individual recipients. Thus, a single recipient may have received more than 1 award. We performed a gender parity analysis for all award recipients combined and all subgroups. The benchmark comparator for gender parity was 50:50 gender balance. We also performed a gender equity analysis with comparator benchmarks for the following award recipient subgroups: (1) US-based nontrainee pathologists compared with the gender/sex composition of the US pathology workforce reported by the Association of American Medical Colleges (AAMC),¹⁰ (2) US-based pathologists in training compared with the gender/sex composition of pathology residents and fellows as reported by the AAMC,¹¹ and (3) US-based nonphysician laboratory professionals compared with the gender/sex composition of the US laboratory workforce as reported by the US Bureau of Labor Statistics.¹² Gender equity subset analysis was not performed for non-US-based award recipients, as we were unable to obtain data regarding the gender/sex composition of this group, and therefore had no reliable comparator benchmarks.

Statistical analysis was performed using the χ^2 test to compare the actual observed proportion of award recipients’ gender to the expected proportion using the previously described comparator benchmarks for each specific population subgroup. Analyses were conducted using GraphPad PRISM version 9.2.0 (GraphPad Software). Two-sided *P* values <.05 were considered significant.

Results

From the inception of *The Pathologist* Power List in 2015 through 2022, 571 total awards were conferred. From 2014 through 2022, 47 ASCP Top Five 40 Under Forty awards were conferred. Significantly more men than women received an award overall (57.1%, 353/618 vs 42.9%, 265/618; *P* < .001), indicating gender parity was not achieved

FIGURE 1. Gender composition analysis of *The Pathologist* Power List and the American Society for Clinical Pathology Top Five 40 Under Forty award recipients. The solid line represents gender parity (50:50 gender balance). The dashed lines represent gender equity using benchmarks for active pathology physicians and pathology resident/fellow trainees derived from AAMC data, and for laboratory professionals derived from the US Bureau of Labor Statistics. All benchmark source data are reported as sex (male and female).^{10–12} AAMC, Association of American Medical Colleges; US BLS, United States Bureau of Labor Statistics.

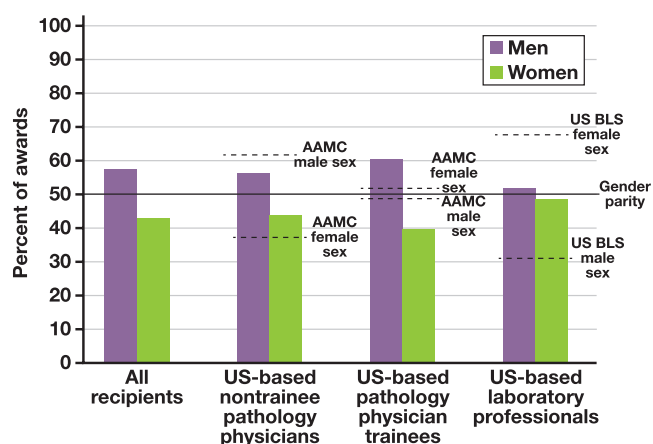
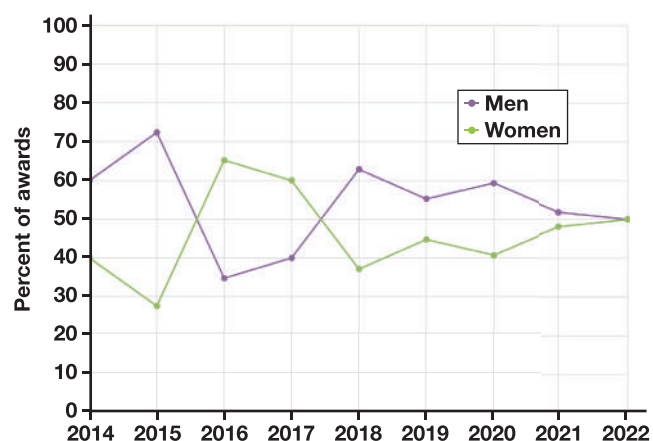


FIGURE 2. Gender composition of *The Pathologist* Power List and the American Society for Clinical Pathology Top Five 40 Under Forty award recipients by year.



(FIGURE 1). Although significantly more awards have been conferred to men, temporal analysis illustrates that this gap may be closing (FIGURE 2).

US-Based Nontrainee Pathology Physicians

Subset analysis revealed that 242 awards have been conferred to US-based nontrainee pathology physicians. More awards have been presented to men than women (56.2%, 136/242 vs 43.8%, 106/242; *P* = .054), although this difference was not significant. In comparison to the US pathology physician workforce, which comprises 61.5% males and 38.5% females (terms used by data source),¹⁰ the combined Power List and Top

Five 40 Under Forty awards are equitable with respect to gender/sex for US-based nontrainee pathologists ($P = .091$) (FIGURE 1).

US-Based Pathology Physician Trainees

Subset analysis revealed that 38 awards have been conferred to US-based pathology physician trainees. There is no difference in the proportion of awards presented to men compared with women (60.5%, 23/38 vs 39.5%, 15/38; $P = .19$). In 2019, US pathology trainees (residents and fellows) were composed of 49.0% males and 51.0% females (terms used by data source),¹¹ illustrating that awards conferred to US-based pathology physician trainees are equitable ($P = .15$) compared with the sex/gender composition of US pathology trainees (FIGURE 1).

US-Based Nonphysician Laboratory Professionals

Subset analysis revealed that 87 awards have been conferred to US-based nonphysician laboratory professionals. There is no difference in the proportion of awards presented to men compared with women (51.7%, 45/87 vs 48.3%, 42/87; $P = .75$). In 2021, the US laboratory professional workforce comprised 31.2% males and 68.8% females (terms used by data source).¹² When compared to this benchmark, women are inequitably represented as award recipients among US-based nonphysician laboratory professionals equitable ($P < .001$) (FIGURE 1).

Non-US-Based Pathology Physicians and Laboratory Professionals

Subset analysis revealed that 251 awards have been conferred to non-US-based pathology physicians and laboratory professionals currently in practice and training combined. Significantly more awards have been conferred to men than women (59.4%, 149/251 vs 40.6%, 102/251; $P = .003$). Gender equity analysis was not performed as there are no reliable benchmark comparator data.

Discussion

The annual Power List and ASCP Top Five 40 Under Forty, although not traditional academic awards, appear to carry significant influence in social circles. The *Pathologist* (@pathologistmag) Twitter account has over 16,000 followers, and tweets announcing the Power List averaged 107 retweets and 148 likes over the past 5 years. The Power List and 40 Under Forty awards are considered prestigious, with organizations, hospitals, and universities announcing award recipients among their news and honors press releases,^{13–18} and receiving this honor may contribute to professional advancement opportunities.

Our findings indicate that in aggregate, these awards have not reached parity, with a significantly greater number of awards being conferred to men overall. However, as most groups are not composed of an equal 50:50 balance of men and women, it is also important to analyze the composition of award recipients in relation to the subgroup from which they are selected. Although we found that significantly more awards have been conferred to men than women overall, in our subgroup analyses, we found that when the composition of the US pathology physician workforce is considered, the combined Power List and Top Five 40 Under Forty awards are equitable with respect to gender/sex for US-based nontrainee pathologists. Similar findings were also observed for the other subgroups with respect to gender equity, with the exception of US-based nonphysician laboratory professionals. This suggests that whereas, overall, awards may be presented to men more often than

women, if the composition of the populations from whom these award recipients are selected is considered, there is generally equitable distribution of nontraditional pathology awards based on gender. However, it is important to note that significantly more awards were conferred to men among US-based nonphysician laboratory professionals than would be expected given the gender composition of the field.

These findings also highlight the importance of appropriate and reliable benchmark data. Without these data, one is unable to accurately compare populations and assess whether inequities are present nor can one determine the effectiveness of diversity, equity, and inclusion initiatives. Additionally, although perhaps less important in the context of recognition awards, whether equity or parity is the most appropriate goal remains an open question.

The equity in the award conferral rate of the Power List and ASCP 40 Under Forty stands in contrast to pathology-based societal awards.¹ Although this study was neither designed nor intended to assess causation, the discrepancies observed between these studies may stem from how nominations are publicized, differing nomination criteria, selection committee composition, and selection committee voting opportunities. Additionally, observed differences between pathology societal awards and nontraditional awards may be influenced by the medium through which voting transpires. Given that these nontraditional awards are reliant on online voting, often via social media, the equity observed in award conferral rates may be partially explained by generational differences in social media usage, as well as familiarity with overall online voting practices.¹⁹

Compared with more traditional recognition awards, an additional unique aspect of the awards examined herein is the option for self-nomination. Both the Power List and the ASCP 40 Under Forty permit individuals to nominate themselves, which may influence the outcomes. Self-nomination removes a potentially discriminatory filter of another individual (eg, department chair or other senior person) performing the nomination, as nominations can be time-consuming and others may be unable or unwilling to make the time, consider it a priority, or may be unaware of the nomination window, influencing the pool of nominees. Individuals who desire nomination may believe it inappropriate to ask to be nominated; thus, self-nomination can remove that filter. Finally, an additional unique aspect of the ASCP 40 Under Forty award compared with many society awards is that individuals in training are included in the review and selection process for the award, which may also provide a broader perspective that influences the diversity of awardees.

These results have far-reaching implications not only from an awards standpoint but in assessing the field of pathology overall. Equitable practices by 1 pathology organization do not inherently counteract inequities propagated by other pathology organizations, particularly if the organizations collaborate or voluntarily associate with one another. This novel concept, termed interorganizational structural discrimination, was recently highlighted in the journal *Cell*, describing how organizations that work together must be jointly accountable for discriminatory practices.²⁰ This issue, which occurs when 2 or more organizations collaborate despite the presence of a structural discrimination issue that is known to be remediable, is thought to contribute to the numerous inequitable practices that occur throughout science and medicine. Therefore, pathology organizations should engage in collaborative monitoring and improvement of their practices to achieve synergistic equity.

Our study has several limitations, including a lack of data regarding the demographic composition of pathologists and laboratory professionals outside the US, and access to additional comparator benchmarks to analyze these subgroups would have been ideal. Furthermore, we analyzed award recipients' perceived gender, whereas our comparator benchmarks were reported as sex in the source data, and we recognize that gender and sex are not always congruent and ideally should not be used interchangeably.²¹ Additionally, we only analyzed gender, whereas inequities are far-reaching and not limited to gender but can be based on numerous identifying demographics including race, ethnicity, sexual orientation, disability status, etc. Furthermore, as is the case with all gender equity studies, potential misidentification of an individual's preferred gender and the inability to account for the entirety of the gender spectrum are inherent study limitations.

In summary, our analysis indicates that, compared with the US pathology and laboratory workforce, awardee distribution generally appears equitable among nontraditional pathology and laboratory medicine awards. Nonetheless, there is lack of gender parity overall, and awards presented to US-based nonphysician laboratory professionals are not equitable, illustrating that additional progress must be made. Nevertheless, a transparent nomination and selection process is critical if these types of awards are to be deemed representative of success, influence, and expertise, as opaque processes by anonymous authority figures have been demonstrated to perpetuate inequities. Additionally, these findings highlight the need for additional studies into other non-traditional awards and recognitions and how these might influence academic progress and whether they are indeed equitable with respect not only to gender but other demographics.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Wobker SE, Ginter PS, Parra-Herran C, et al. Recognition awards in pathology specialty societies. *Am J Clin Pathol*. 2022;158(4):499–505. doi:10.1093/ajcp/aqac076
2. Parra-Herran C, Khani F, Wobker SE. Gender distribution of editors and authors of reference textbooks in anatomic pathology: further edits are required. *Mod Pathol*. 2022. doi:10.1038/s41379-022-01153-0
3. Jacobs JW, Jagsi R, Stanford FC, et al. Gender representation among United States medical board leadership. *J Womens Health*. 2022. doi:10.1089/jwh.2022.0271
4. Mahmoudi M, Poorman JA, Silver JK. Representation of women among scientific Nobel Prize nominees. *Lancet*. 2019;394(10212):1905–1906. doi:10.1016/s0140-6736(19)32538-3
5. Jacobs JW, Adkins BD, Stephens LD, Woo JS, Booth GS. Gender inequities in transfusion medicine society recognition awards. *Transfus Med Rev*. 2022;36(2):82–86. doi:10.1016/j.tmr.2022.04.001
6. Lawson MA, Martin AE, Huda I, et al. Hiring women into senior leadership positions is associated with a reduction in gender stereotypes in organizational language. *Proc Natl Acad Sci U S A*. 2022;119(9):e2026443119. doi:10.1073/pnas.2026443119
7. Pinho-Gomes AC, Vassallo A, Thompson K, Womersley K, Norton R, Woodward M. Representation of women among editors in chief of leading medical journals. *JAMA Netw Open*. 2021;4(9):e2123026. doi:10.1001/jamanetworkopen.2021.23026
8. *The Pathologist*. <https://thepathologist.com/>. Accessed September 19, 2022.
9. ASCP 40 Under Forty Program. <https://www.qa2.ascp.org/content/get-involved/get-recognized/40-under-forty#>. Accessed October 28, 2022.
10. AAMC. Active Physicians by Sex and Specialty, 2019. <https://www.aamc.org/data-reports/workforce/interactive-data/active-physicians-sex-and-specialty-2019>. Accessed September 21, 2022.
11. AAMC. ACGME Residents and Fellows by Sex and Specialty, 2019. <https://www.aamc.org/data-reports/interactive-data/acgme-residents-and-fellows-sex-and-specialty-2019>. Accessed November 30, 2022.
12. US Bureau of Labor Statistics. Labor Force Statistics from the Current Population Survey, 2021. <https://www.bls.gov/cps/cpsaat11.htm>. Accessed December 2, 2022.
13. Institute of Biomedical Science. The Pathologist's Power List 2022. August 1, 2022. <https://www.ibms.org/resources/news/the-pathologists-power-list-2022/>. Accessed September 30, 2022.
14. Columbia University. Dr. Swikrity Upadhyay Baskota Named to The Pathologist's Power List for 2022. August 5, 2022. <https://www.pathology.columbia.edu/news/dr-swikrity-upadhyay-baskota-named-pathologists-power-list-2022>. Accessed October 15, 2022.
15. Crowe C. Williams Named to The Pathologist's Power List for 2022. July 29, 2022. <https://www.uab.edu/medicine/pathology/news-events/archive/williams-named-to-the-pathologist-s-power-list-for-2022>. Accessed September 30, 2022.
16. Loyola Medicine. Loyola Pathologist Named to The Pathologist Magazine's Power List. September 2, 2021. <https://www.loyolamedicine.org/about-us/news/power-list-2021>. Accessed September 28, 2022.
17. Association for Medical Education in Europe. Press Release: AMEE Associate Fellow selected for The Pathologist Power List 2022. September 2022. <https://amee.org/AMEE/News/AFAMEE-Power-list.aspx>. Accessed October 15, 2022.
18. Montgomery S. ASCP Announces Top Five of its 40 Under Forty Program to Recognize Future Generation of Laboratory Leaders. University of Michigan. <https://www.pathology.med.umich.edu/news/825/>. Accessed October 28, 2022.
19. Bowe BJ, Wohn DY. Are there generational differences? Social media use and perceived shared reality. In *Proceedings of the 2015 International Conference on Social Media & Society (SMSociety '15)*. Association for Computing Machinery, New York, 2015; Article 17, 1–5. doi:10.1145/2789187.2789200
20. Silver JK, Booth GS, Chatterjee A, et al. Organizations in science and medicine must hold each other accountable for discriminatory practices. *Cell*. 2022;185(17):3073–3078. doi:10.1016/j.cell.2022.06.043
21. Jacobs JW, Peabody Lever JE, Lee YS, et al. The importance of accurately defining gender and sex in pathology [published correction appears in *Am J Clin Pathol*. 2022 Jul 1;158(1):156]. *Am J Clin Pathol*. 2022;158(1):153–153. doi:10.1093/ajcp/aqac008

Lipoprotein-Associated Phospholipase A₂ Correlates with Reduced Left Ventricle Ejection Fraction in Hemodialysis Patients

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Keywords: hemodialysis, heart failure, left ventricular ejection fraction, lipoprotein-associated phospholipase A₂, N-terminal proBNP, C-reactive protein

Abbreviations: LVEF, left ventricular ejection fraction; HD, hemodialysis; Lp-PLA₂, lipoprotein-associated phospholipase A₂; CRP, C-reactive protein; HF, heart failure; Kt/V, urea clearance × time/volume; URR, urea clearance rate; PTH, parathyroid hormone; TG, triglyceride; TC, total cholesterol; NT-proBNP, N-terminal proBNP

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ABSTRACT

Objective: Reduced left ventricular ejection fraction (LVEF) is common in hemodialysis (HD) patients. Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is considered an important determinant of cardiovascular events. The aim of the study was to evaluate the relationship between Lp-PLA₂ and LVEF in HD patients.

Methods: Fifty-seven HD patients with coronary heart disease were enrolled. Predialysis and postdialysis venous whole blood samples were collected. The patients were divided into preserved and reduced LVEF groups. The relationship between Lp-PLA₂ and LVEF was assessed.

Results: A significant difference in C-reactive protein (CRP) and Lp-PLA₂ was observed, with higher levels noted in patients with reduced

LVEF ($P \leq .001$). Both Lp-PLA₂ and CRP were negatively correlated with LVEF in the HD patients. Only Lp-PLA₂ remained associated with LVEF in multiple regression analysis.

Conclusion: Lipoprotein-associated phospholipase A₂ levels are associated with LVEF and could potentially be used to evaluate chronic heart failure with reduced LVEF in HD patients for risk stratification management.

Heart failure (HF) is highly prevalent among the hemodialysis population, resulting in greater than half of all deaths. Heart failure risk factors, including fluid overload, anemia, atrial fibrillation, and uremic toxin accumulation are prevalent in maintenance HD patients.

Left ventricular (LV) dysfunction directly affects HF and is associated with poor survival in patients under hemodialysis. Routine assessment of LV ejection fraction (EF) has been recommended in patients with HF.¹ Zoccali et al² revealed an association between reduced LVEF and increased adverse events in 254 asymptomatic HD patients. Methods to improve EF help to decrease mortality in HD patients.

Lipoprotein-associated phospholipase A₂ (Lp-PLA₂), a 45-kDa protein composed of 441 amino acids,³ is a calcium-independent serine lipase that is produced by macrophages and other inflammatory cells. Lipoprotein-associated phospholipase A₂ enters the vessel wall and hydrolyzes platelet-activating factor-like oxidized lipids, resulting in the production of two pro-inflammatory mediators, lysophosphatidylcholine and oxidized nonesterified fatty acid, which promote plaque development in atherosclerosis.⁴ As a potential cardiovascular risk marker, Lp-PLA₂ plays an important role in cardiovascular diseases.^{5,6} Jenny et al⁷ found that subjects with HF exhibit elevated Lp-PLA₂ levels associated with an increase in mortality risk. In contrast, only a few studies have investigated Lp-PLA₂ in patients with renal diseases. Winkler et al⁸ showed that Lp-PLA₂ correlated with the dialysis vintage in diabetic HD patients, likely indicating a role in the progression of vascular damage.

The literature lacks studies on the role of Lp-PLA₂ in HD patients with reduced EF. This study sought to evaluate a possible relationship between serum Lp-PLA₂ activity and EF reduction among HD patients.

Methods

Study Design

Fifty-seven HD patients with coronary heart disease with a history of diagnostic coronary angiography at Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine were enrolled in this prospective cohort study. Patients over 18 years old who had been undergoing outpatient conventional hemodialysis 3 times a week with each session lasting 4 hours for at least 6 months were included in this study. Patients with malignancies, stroke, acute infections, autoimmune diseases, myocardial infarction, valvular disease, severe arrhythmia, hypertensive heart disease, cardiomyopathy, and acute HF were excluded. The study was approved by the local Ethics Committee of Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine (No. 2020049), and all patients provided signed informed consent in accordance with the Declaration of Helsinki principles.

Sample Collection

Demographic and clinical data were obtained at the time of enrollment and predialysis venous whole blood samples were collected. Hemoglobin, platelet count, D-dimer, albumin, triglyceride (TG), total cholesterol (TC), calcium, phosphorus, parathyroid hormone (PTH), N-terminal proBNP (NT-proBNP), C-reactive protein (CRP), and predialysis and postdialysis levels of Lp-PLA₂ were examined. The double antibody sandwich enzyme-linked immunoassay method was used to determine the serum Lp-PLA₂ with a Quantikine ELISA kit (R&D Systems).

Echocardiography

Echocardiographic studies by 2-dimensional M-mode, transthoracic, color Doppler echocardiography were performed on predialysis and postdialysis patients by a single trained cardiologist who was blinded to the clinical status. The echocardiography examination was performed in the left lateral decubitus position using standard parasternal, short-axis, and apical views. The LVEF was calculated and recorded.

The LVEF is an index of LV contractility that indicates the degree of change in LV volume from diastole to systole. The LVEF is calculated by subtracting the end-systolic LV volume from the end-diastolic LV volume and dividing it by the end-diastolic LV volume. Based on the guidelines of the American Society of Echocardiography, the lower limit of normal for LVEF was set at 50%, and accordingly, the patients were divided into 2 groups.

Statistical Analysis

Statistical analysis was performed by using SPSS 21.0. Continuous variables are expressed as the mean $\pm$ SD, and nonnormally distributed variables were expressed as medians (interquartile ranges). Student's *t*-test was used to compare groups, and categorical variables were analyzed by the χ^2 test. Univariate and multivariate logistic regression analyses were performed to determine significant factors associated with the categorical outcome variable LVEF. A *P* value $< .05$ was considered significant.

Results

Fifty-seven HD patients were enrolled (33 males and 24 females) in this study. The basic demographic and dialysis characteristics and laboratory data are summarized in **TABLE 1**. The HD patients were divided into

2 groups according to the LVEF cut-off value ($<50\%$ vs $\geq 50\%$). Group 1 included patients with LVEF $\geq 50\%$ ($n = 36$, 63%), whereas group 2 included patients with LVEF $<50\%$ ($n = 21$, 37%). No significant differences in age, sex, body mass index, or diabetes mellitus were found between the 2 groups. Dialysis data were also evaluated. The 2 groups were homogenous in terms of dialysis vintage, blood pressure, urea clearance $\times$ time/volume (Kt/V), urea clearance rate (URR), and parathyroid hormone (PTH).

With regard to biochemical markers, no significant differences in hemoglobin, platelet count, D-dimer, albumin, TC, TG, glucose, calcium, phosphate, or NT-proBNP were observed. A significant difference between groups was found for CRP, which was higher in the group of patients with reduced LVEF ($P < .001$). Serum Lp-PLA₂ levels were 351.24 ± 71.16 $\mu\text{g/L}$ in HD patients with reduced LVEF and 105.62 ± 22.31 $\mu\text{g/L}$ in patients with reserved LVEF. The difference in Lp-PLA₂ between the 2 groups was statistically significant ($P < .001$).

The Lp-PLA₂ and CRP levels were negatively correlated with LVEF in predialysis (**TABLE 2**) and postdialysis (**TABLE 3**) HD patients ($P \leq .05$). To determine whether such factors were independent predictors of LVEF, multiple linear regression analysis was performed, revealing that Lp-PLA₂ was significantly correlated with LVEF before dialysis ($P < .001$) and after dialysis ($P \leq .05$).

TABLE 1. Demographic and Clinical Characteristics of the HD Patients with Preserved ($\geq 50\%$) and Reduced ($<50\%$) LVEF^a

	Preserved LVEF (n = 36)	Reduced LVEF (n = 21)	P Value
Age, y	75 $\pm$ 8	67 $\pm$ 12	.095
Gender, No. male (%)	21 (58)	12 (57)	.960
BMI, kg/m ²	23.0 $\pm$ 2.4	23.1 $\pm$ 4.1	.978
Diabetes, No. (%)	18 (50)	15 (71)	.361
Dialysis vintage, mo	35.2 $\pm$ 10.1	30.7 $\pm$ 9.1	.352
SBP, mmHg	148.8 $\pm$ 14.8	143.9 $\pm$ 8.5	.431
DBP, mmHg	78.3 $\pm$ 7.4	82.4 $\pm$ 9.4	.299
Kt/V	1.49 $\pm$ 0.13	1.5 $\pm$ 0.14	.778
URR	0.66 $\pm$ 0.07	0.68 $\pm$ 0.07	.562
Hemoglobin, g/L	103.9 $\pm$ 12.8	100.6 $\pm$ 16.3	.625
Platelet count, 10 ⁹ /L	181.9 $\pm$ 62.7	204.7 $\pm$ 64.2	.459
D-dimer	0.73 $\pm$ 0.3	0.97 $\pm$ 0.47	.202
Albumin, g/L	36.5 $\pm$ 3.4	36.4 $\pm$ 4.8	.951
TC, mmol/L	3.8 $\pm$ 0.8	3.9 $\pm$ 0.6	.704
TG, mmol/L	1.5 $\pm$ 0.6	1.4 $\pm$ 0.8	.453
Glucose, mmol/L	6.7 $\pm$ 1.8	6.2 $\pm$ 1.5	.536
Calcium, mmol/L	2.10 $\pm$ 0.14	2.18 $\pm$ 0.21	.351
Phosphate, mmol/L	1.50 $\pm$ 0.39	1.46 $\pm$ 0.46	.866
PTH, pg/mL	289.4 $\pm$ 69.2	340.1 $\pm$ 93.0	.192
CRP, mg/L	3.4 $\pm$ 1.2	6.7 $\pm$ 1.8	<.001
NT-proBNP, pg/mL	16,671 $\pm$ 5790	19,410 $\pm$ 4296	.293
LVEF, %	60.9 $\pm$ 4.2	36.9 $\pm$ 8.7	<.001
Lp-PLA ₂ , $\mu\text{g/L}$	105.62 $\pm$ 22.31	351.24 $\pm$ 71.16	<.001

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; HD, hemodialysis; LVEF, left ventricle ejection fraction; PTH, parathyroid hormone; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; URR, urea clearance rate.

^aData are given as mean $\pm$ SD except where noted.

TABLE 2. Univariate and Multiple Linear Regression Analyses of the LVEF and Variables in HD Patients^a

Variable	R	P Value
Age	0.371	.118
Gender (male)	−0.047	.849
BMI	0.129	.599
Diabetes	0.130	.597
Dialysis vintage	0.324	.176
SBP	0.041	.866
DBP	−0.266	.271
Kt/V	−0.011	.966
URR	−0.255	.292
Hemoglobin	−0.067	.786
Platelet count	−0.074	.762
D-dimer	−0.238	.327
Albumin	−0.045	.855
TC	−0.106	.665
TG	0.120	.624
Glucose	0.121	.622
Calcium	−0.201	.409
Phosphate	−0.002	.993
PTH	−0.331	.167
CRP	−0.661	.002
NT-proBNP	−0.375	.114
LP-PLA ₂	−0.772	<.001
Variable	β	P
CRP	−0.199	.401
LP-PLA ₂	−0.079	<.001

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; HD, hemodialysis; LVEF, left ventricle ejection fraction; NT-proBNP, N-terminal proBNP; PTH, parathyroid hormone; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride; URR, urea clearance rate.

^aAdjusted $R^2 = 0.565$, $P < .001$. The independent variables used for multiple regression analysis were selected by univariate linear regression analysis ($P < .1$).

TABLE 3. Univariate and Multiple Linear Regression Analyses of the LVEF and Variables in Postdialysis HD Patients^a

Variable	Preserved LVEF (n = 36)	Reduced LVEF (n = 21)	P Value
CRP, mg/L	3.0 ± 0.9	5.6 ± 0.9	<.001
LP-PLA ₂ , μg/L	94.27 ± 19.02	333.06 ± 76.11	<.001
LVEF, %	63.1 ± 4.5	38.7 ± 8.9	<.001
Variable	R	P	
CRP, mg/L	−0.665	.002	
LP-PLA ₂ , μg/L	−0.731	<.001	
Variable	β	P	
CRP, mg/L	−0.171	.546	
LP-PLA ₂ , μg/L	−0.080	.043	

HD, hemodialysis; CRP, C-reactive protein; LVEF, left ventricle ejection fraction.

^aData are given as mean ± SD except where noted. Adjusted $R^2 = 0.536$, $P < .001$. The independent variables used for multiple regression analysis were selected by univariate linear regression analysis ($P < .1$).

Discussion

In this study, hemodialysis patients were divided into 2 groups based on LVEF. Patients with LVEF <50% were compared with those with LVEF ≥50%. Reduced LVEF is associated with adverse cardiovascular events in end-stage renal dysfunction patients,⁹ and even mild LV systolic dysfunction may be significantly associated with a worse prognosis in HD patients.¹ In a retrospective cohort study of 154 HD patients over 18 years, preserved LVEF occurred in most HD patients and was associated with better clinical outcomes.¹⁰

Inflammation is common among HD patients, suggesting that it is a major contributor to morbidity and mortality. As a widely used inflammatory biomarker, CRP is predictive of cardiovascular events, hospitalization, and all-cause and cardiovascular mortality.¹¹ Kim et al¹² found that persistent elevation of CRP may predict cardiac hypertrophy and dysfunction in patients maintained on hemodialysis. In our study, serum CRP levels were elevated in the reduced EF group. However, the CRP concentrations of the 2 groups were in the normal range. This observation may provide a plausible explanation for the lack of correlation between CRP and LVEF.

Due to its biological activity, Lp-PLA₂ has been identified as a predictor of cardiovascular morbidity and mortality in several cohort populations.¹³ Fewer studies have reported cardiovascular events in HD patients. In a cohort study with a 3-year follow-up, Lp-PLA₂ was potentially identified as an independent risk factor for acute cardiovascular events.¹⁴ In another study on HD patients with type 2 diabetes, Lp-PLA₂ was potentially predictive of cardiovascular events and mortality.⁸ More importantly, studies have shown that Lp-PLA₂ is strongly and independently associated with the mortality of patients with HF.^{15,16} However, no data are available to explore the relationship between Lp-PLA₂ and HD patients with HF, especially when combined with reduced LVEF. In this study, we first focused on chronic cardiac failure with reduced LVEF. Lipoprotein-associated phospholipase A₂ was elevated in both groups. Dialysis-induced local inflammation may increase Lp-PLA₂ production. Impaired cardiomyocytes may exert a higher inflammatory response,¹⁷ thereby inducing a greater increase in Lp-PLA₂ levels in the reduced LVEF group. To date, few studies have reported on the mechanism between Lp-PLA₂ and HF. Lipoprotein-associated phospholipase A₂-related inflammatory products leading to ischemic and nonischemic HF may be secondary to coronary endothelial dysfunction mediated by inflammatory mediators.¹⁸ In addition, Lp-PLA₂ is related to the severity of myocardial damage in patients with myocardial infarction.¹⁶ Thus, we hypothesize that Lp-PLA₂ directly participates in myocardial injury, resulting in HF. Markedly elevated Lp-PLA₂ levels may be helpful in providing scientific guidance for risk stratification management in HD patients. This was a single-center study with a relatively small cohort size. More studies are needed to investigate the relationship between Lp-PLA₂ and LVEF.

Conclusion

Our data demonstrate that Lp-PLA₂ levels are associated with LVEF, which could potentially be used as a tool for evaluating chronic HF with reduced LVEF in HD patients.

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Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Yamada S, Ishii H, Takahashi H, et al. Prognostic value of reduced left ventricular ejection fraction at start of hemodialysis therapy on cardiovascular and all-cause mortality in end-stage renal disease patients. *Clin J Am Soc Nephrol*. 2010;5(10):1793–1798. doi:[10.2215/cjn.00050110](https://doi.org/10.2215/cjn.00050110)
2. Zoccali C, Benedetto FA, Mallamaci F, et al. Prognostic value of echocardiographic indicators of left ventricular systolic function in asymptomatic dialysis patients. *J Am Soc Nephrol*. 2004;15(4):1029–1037.
3. Tjoelker LW, Wilder C, Eberhardt C, et al. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature*. 1995;374(6522):549–553. doi:[10.1038/374549a0](https://doi.org/10.1038/374549a0)
4. Xu C, Yu F, Mao S, et al. Lipoprotein-associated phospholipase A2 predicted cardiovascular disease in obstructive sleep apnea syndrome. *Respir Med*. 2020;163:105881. doi:[10.1016/j.rmed.2020.163.105881](https://doi.org/10.1016/j.rmed.2020.163.105881)
5. O'Donoghue M, Morrow DA, Sabatine MS, et al. Lipoprotein-associated phospholipase A2 and its association with cardiovascular outcomes in patients with acute coronary syndromes in the PROVE IT-TIMI 22 (PRavastatin Or atorVastatin Evaluation and Infection Therapy-Thrombolysis In Myocardial Infarction) trial. *Circulation*. 2006;113(14):1745–1752. doi:[10.1161/circulationaha.105.612630](https://doi.org/10.1161/circulationaha.105.612630)
6. Li N, Li S, Yu C, Gu S. Plasma Lp-PLA2 in acute coronary syndrome: association with major adverse cardiac events in a community-based cohort. *Postgrad Med*. 2010;122(4):200–205. doi:[10.3810/pgm.2010.07.2187](https://doi.org/10.3810/pgm.2010.07.2187)
7. Jenny NS, Solomon C, Cushman M, et al. Lipoprotein-associated phospholipase A(2) (Lp-PLA(2)) and risk of cardiovascular disease in older adults: results from the Cardiovascular Health Study. *Atherosclerosis*. 2010;209(2):528–532. doi:[10.1016/j.atherosclerosis.2009.09.021](https://doi.org/10.1016/j.atherosclerosis.2009.09.021)
8. Winkler K, Hoffmann MM, Krane V, et al. Lipoprotein-associated phospholipase A2 and outcome in patients with type 2 diabetes on haemodialysis. *Eur J Clin Invest*. 2012;42(7):693–701.
9. Yu WC, Lin YP, Chuang SY, et al. Cardiovascular determinants of prognosis in normotensive hemodialysis patients. *BMC Nephrol*. 2012;13:115. doi:[10.1186/1471-2369-13-115](https://doi.org/10.1186/1471-2369-13-115)
10. Girsberger M, Trinh E, Chan CT. Ventricular ejection fraction over time in patients on intensive home hemodialysis: a retrospective cohort study. *Hemodial Int*. 2020;24(3):290–298. doi:[10.1111/hdi.12838](https://doi.org/10.1111/hdi.12838)
11. Chawla LS, Krishnan M. Causes and consequences of inflammation on anemia management in hemodialysis patients. *Hemodial Int*. 2009;13(2):222–234. doi:[10.1111/j.1542-4758.2009.00352.x](https://doi.org/10.1111/j.1542-4758.2009.00352.x)
12. Kim BS, Jeon DS, Shin MJ, et al. Persistent elevation of C-reactive protein may predict cardiac hypertrophy and dysfunction in patients maintained on hemodialysis. *Am J Nephrol*. 2005;25(3):189–195. doi:[10.1159/000085585](https://doi.org/10.1159/000085585)
13. De Mauri A, Vidali M, Chiarinotti D, Dianzani U, Rolla R. Lipoprotein-associated phospholipase A2 predicts lower limb ischemia in hemodialysis subjects. *Ther Apher Dial*. 2020;24(5):548–553. doi:[10.1111/1744-9987.13465](https://doi.org/10.1111/1744-9987.13465)
14. De Mauri A, Vidali M, Chiarinotti D, et al. Lipoprotein-associated phospholipase A₂ predicts cardiovascular events in dialyzed patients. *J Nephrol*. 2019;32(2):283–288.
15. Van Vark LC, Kardys I, Bleumink GS, et al. Lipoprotein-associated phospholipase A2 activity and risk of heart failure: the Rotterdam study. *Eur Heart J*. 2006;27(19):2346–2352.
16. Gerber Y, Dunlay SM, Jaffe AS, et al. Plasma lipoprotein-associated phospholipase A2 levels in heart failure: association with mortality in the community. *Atherosclerosis*. 2009;203(2):593–598. doi:[10.1016/j.atherosclerosis.2008.07.035](https://doi.org/10.1016/j.atherosclerosis.2008.07.035)
17. Lachmet-Thebaud L, Marchandot B, Matsushita K, et al. Impact of residual inflammation on myocardial recovery and cardiovascular outcome in Takotsubo patients. *ESC Heart Fail*. 2021;8(1):259–269.
18. Low A, Mak E, Rowe JB, Markus HS, O'Brien JT. Inflammation and cerebral small vessel disease: a systematic review. *Ageing Res Rev*. 2019;53:100916. doi:[10.1016/j.arr.2019.53.100916](https://doi.org/10.1016/j.arr.2019.53.100916)

Urine Protein Immunofixation Electrophoresis: Free Light Chain Urine Immunofixation Electrophoresis Is More Sensitive than Conventional Assays for Detecting Monoclonal Light Chains and Could Serve as a Marker of Minimal Residual Disease

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Keywords: monoclonal gammopathy, urine immunofixation, free light chain immunofixation, multiple myeloma, minimal residual disease, serum free light chain

Abbreviations: MM, multiple myeloma; CMP, comprehensive metabolic profile; SFLC, serum free light chains; SPEP, serum protein electrophoresis; SIFE, serum immunofixation electrophoresis; UPEP, urine protein electrophoresis; UIFE, urine immunofixation electrophoresis; IMWG, International Myeloma Working Group; SFLCA, serum free light chain assay; MLCs, monoclonal light chains; LCMM, light chain multiple myeloma; LCPMM, light chain–predominant multiple myeloma; FLC-UIFE, free light chain urine immunofixation electrophoresis; MRD, minimal residual disease; TPr, total urine protein; I/U, involved/uninvolved; CAP, College of American Pathologists; MGUS, monoclonal gammopathy of undetermined significance; ENT, ear, nose, and throat

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ABSTRACT

Background: Immunoglobulin monoclonal light chains (MLCs) in serum and urine are markers for monoclonal gammopathy and could serve as markers of minimal residual disease (MRD) in multiple myeloma (MM). Excretion of MLCs in urine is known to result in renal damage and shorter survival in patients with LC-predominant MM.

Methods: Retrospective review of urine immunofixation in 1738 specimens at 3 medical centers was conducted to assess the utility of urinalysis for diagnosis and monitoring of monoclonal gammopathy. We tested 228 stored urine specimens via the modified urine immunofixation method, using antisera to assay free LCs (FLCs).

Results: Our review of urine immunofixation results and medical records validated the theory that the only meaningful value-added finding was detection of monoclonal free light chains. Examination of 228 urine specimens using our novel method revealed 18.4% additional positive results. The rate of incremental findings for lambda LCs was nearly 3-fold higher than for kappa LCs.

Conclusions: The new method of urine immunofixation is significantly more sensitive and more efficient than the conventional method for detecting MLCs in urine. The new assay appears to be sensitive enough to prove that MLCs serve as a marker of MRD in MM.

Plasma cell myeloma/multiple myeloma (MM) is a malignant tumor of terminally differentiated B lymphocytes/plasma cells. MM constitutes the second-most-common hematologic malignant neoplasm in adults and accounts for approximately 2% of cancer deaths.¹ The diagnostic workup for MM includes routine laboratory tests, such as CBC and comprehensive metabolic profile (CMP), as well as immunoglobulin quantification, serum free light chain (SFLC) concentration, serum protein electrophoresis (SPEP) and serum protein immunofixation electrophoresis (SIFE), and urine protein electrophoresis (UPEP) and urine protein immunofixation electrophoresis (UIFE). These tests are recommended by the International Myeloma Working Group (IMWG) for the diagnosis and monitoring of monoclonal gammopathic disorders.^{2–5} UPEP by itself is not useful for detection of monoclonal immunoglobulins in urine but has traditionally been performed along with UIFE. UPEP may provide useful information regarding kidney disorders, but we believe that it does not add value in the diagnosis and follow-up of patients with monoclonal gammopathy.

It has been proposed^{6,7} that quantification of SFLCs eliminates the need for urinalysis. However, serum free light chain assay (SFLCA) is neither sensitive nor specific for monoclonal gammopathy despite grossly abnormal SFLC levels and markedly abnormal kappa/lambda

ratios.^{8–10} SFLCs are elevated in patients with inflammation and/or chronic renal failure. More than half of the patients with polyclonal hypergammaglobulinemia display an abnormal kappa/lambda ratio—almost always a kappa-dominant ratio.^{8–10} The variability in results from different testing methods is also concerning.^{11,12} The development of an oligoclonal pattern in patients receiving stem cell transplants limits the value of the kappa/lambda ratio due to the dominance of kappa LC-associated clones.⁹

In approximately 85% of MM cases, the tumors secrete intact immunoglobulins. Approximately 15% of MM secrete only LCs.¹⁰ However, in almost all instances of MM secreting intact immunoglobulins, excess MFLCs are also secreted. The excess MFLCs may be detectable in serum via conventional SIFE and are detectable with greater sensitivity by FLC-modified SIFE.¹³ Excess MFLCs can also be detected in urine, and detection of monoclonal light chains (MLCs) is the main reason for performing UIFE.

SFLC quantification is useful in the diagnosis and monitoring of light chain multiple myeloma (LCMM). Approximately 18% of the intact immunoglobulin-secreting MM tumors produce a markedly greater abundance of MFLCs, and this group has been designated as light chain-predominant MM (LCPMM). Patients with LCPMM and patients with LCMM, with tumors secreting higher levels of MFLCs, exhibit greater renal injury and 2 years shorter survival, compared with patients with conventional MM.^{14–16}

Urinalysis is an underutilized test, despite being a noninvasive means of collecting specimens that is critical in diagnosis and monitoring of monoclonal gammopathic disorders. Specifically, ascertainment of complete response to treatment of MM requires the absence of monoclonal immunoglobulins in serum and urine.¹⁷ Urine displays MLCs in all cases of LCMM.⁶ UIFE with antisera specific for free light chains (FLC-UIFE) has the potential to improve the detection of malignant MLCs in patients after treatment (as we describe in this report). The results of FLC-modified SIFE and the data presented in this article support the use of FLC-UIFE for detection of minimal residual disease (MRD).¹³

FLC-modified SIFE has been shown¹³ to be effective in detecting MLCs in serum, with better sensitivity than conventional SIFE and MASS-FIX MALDI. We applied a similar approach to urinalysis to test for improvement in sensitivity in detection of MLCs.

Methods

This study was conducted at 3 medical school-affiliated hospitals in the southeastern (institution A), midwestern (institution B), and northern (institution C) United States. The investigation consisted of retrospective review of UIFE and SPEP + SIFE results to ascertain the value added by UIFE examination. Total urine protein (TPr) and SFLC levels were also documented and analyzed for diagnostic utility. Stored, concentrated urine specimens, previously tested by routine UIFE, were examined via the FLC-UIFE method, as described later in this article, at institution A. The SPIFE Touch System (Helena Laboratories) was used for SPEP, SIFE, UPEP, and UIFE at institution A. Institutions B and C used the CAPILLARYS system (Sebia) for SPEP and IFE. SFLC quantification was conducted by using binding-site reagents and Optilite (The Binding Site Group). The study protocol was reviewed by relevant institutional review boards, which considered the proposal to be exempt.

Data from the 3 institutions were analyzed separately. The involved LC identity of the relevant monoclonal immunoglobulin was ascertained from the results of SPEP/SIFE testing and designated as LC in the data tables. The levels of SFLC at the time of UIFE were recorded as mg/L. TPr concentration, before further concentration, in the specimens analyzed was recorded as mg/dL.

The UIFE results were scrutinized for additional information gleaned from UIFE that was not available from SPEP/SIFE and UPEP examination. In particular, the presence of a distinct band of MFLCs was noted as such. In specimens in which MFLC bands were detectable, comparison with specimens without such bands for the concentration of TPr, cognate SFLC concentration, and ratio of involved to uninvolved SFLC concentration, the lowest level of cognate SFLC, lowest ratio of involved to uninvolved SFLC, and lowest TPr concentration associated with the presence of MFLC in urine by conventional UIFE were recorded. Similarly, the highest level of cognate SFLC, the highest ratio of involved to uninvolved SFLC, and the highest concentration of urine protein associated with lack of MFLC in UIFE were determined.

For FLC-UIFE, urine specimens submitted for routine patient testing were concentrated by membrane filtration with Millipore Concentrators (Merck) and tested by conventional UPEP and UIFE. Residual specimens were stored at 4°C, and selected specimens were evaluated via FLC-UIFE. Only specimens from patients with monoclonal gammopathy or a history of monoclonal gammopathy were selected. Concentrated urine was applied to UIFE gels procured from Helena Laboratories, and the SPIFE Touch instrument was used to electrophorese the specimens, as is usually performed in conventional UIFE. Instead of antisera provided by the manufacturer with the UIFE/SIFE kits, the gels were stained with antisera to SFLCs. The antikappa and antilambda antisera to FLCs were obtained from Sebia. We applied 50 µL of undiluted antisera in the slots in the UIFE/SIFE template. Other than using antisera specific to FLCs, the FLC-UIFE protocol was similar to conventional UIFE. Incubation with antibody and staining were conducted per the standard protocol for UIFE using the Helena platform. The stained gels were evaluated visually and the results compared with those yielded by previously conducted conventional UIFE.

We also compared the results of conventional UIFE with the results from FLC-UIFE at one of the institutions. Relative detection of MFLCs by the 2 methods was evaluated. Urine specimens collected from January 2020 through March 2022 at institution A, for which sufficient volume for testing was available, were evaluated for testing via FLC-UIFE. Patients with extant monoclonal immunoglobulins or a history of positive SPEP/SIFE results or UIFE results for monoclonal Ig were included. We tested a total of 228 specimens via FLC-UIFE and compared the results with the recorded results of conventional UIFE. We analyzed rates of detection of additional positive results between kappa and lambda LCs via χ^2 testing.

Results

In this review of 1738 UIFE examinations, the only meaningful additional information in UIFE was the presence of MFLCs in a variable proportion of specimens. The proportion of specimens with MFLCs was markedly different among the 3 institutions. At institution A, only 17.6% of the specimens displayed a band of MFLCs; at institution B, the corresponding figure was 88.7%; and at institution C, the corresponding

value was 39.8%. The distribution of diagnoses at institution A is given in **TABLE 1**.

We note that the vast majority of the patients had multiple diagnoses. A prototypic pattern was for the patient to have hypertension, diabetes, and chronic kidney disease, as well as having a body mass index in the overweight category. Patients with multiple pathologies are listed in the multimorbid category. The diagnosis listed in the table represents the likely diagnosis prompting the UIFE study. Patients with monoclonal gammopathy disorders were often tested multiple times, whereas other patients were usually tested only once.

A summary of the findings with respect to the relationship between the presence and absence of MFLCs in urine, detected by conventional UIFE, at the different institutions relative to other parameters is shown in **TABLE 2**. The lowest concentration of the cognate LC with a positive result for MFLCs was 0.56 mg/L for kappa and 1.03 mg/L for lambda. The highest concentration of cognate LC with a negative UIFE result was 502.71 mg/L for kappa and 94.39 mg/L for lambda. The lowest ratio of concentration of involved and uninvolved (I/U) SFLC in urine testing positive for MFLCs via conventional UIFE was 0.0018 for kappa and 0.0073 for lambda. The highest ratio of concentration of I/U SFLC in patients with urine that tested negative for MFLCs by conventional UIFE was 609.56 for kappa and 222.68 for lambda. The lowest concentration of TPr in a specimen containing MFLCs of either type was 2.0 mg/dL. The highest concentration of TPr in a specimen testing negative for MFLCs of either type was 7428.0 mg/dL. The proportions of urine

specimens testing positive for MLCs, via the FLC UIFE method, at total protein concentration of <5.0 mg/dL, <10.0 mg/dL, <15.0 mg/dL, <20.0 mg/dL, and <30.0 mg/dL are shown in **TABLE 3**. The proportion of urine specimens with detectable MFLCs detected via conventional UIFE is also noted in **TABLE 2**, and it varies markedly among institutions.

Comparison of conventional UIFE with FLC-UIFE probed with antisera for kappa and lambda FLCs is shown in **TABLE 4**. In a single instance of a patient with IgM kappa monoclonal immunoglobulin, the conventional UIFE testing yielded a positive result for kappa MLCs, but negative results were derived via FLC-UIFE. The specimen was retested by conventional UIFE to exclude deterioration of the specimen being responsible for the negative result on FLC-UIFE. Repeat testing confirmed the presence of kappa MLCs determined via conventional UIFE and a negative result via FLC-UIFE (**FIGURE 1**). This finding was observed despite the fact that FLC-UIFE had higher sensitivity, as demonstrated by a positive result in a patient with an SFLC level of 0.48 mg/dL, whereas the lowest SFLC concentration with a positive result on conventional UIFE for kappa MFLCs had an SFLC level of 1.19 mg/dL.

FLC-UIFE detected MLCs in approximately 18% more specimens vs conventional UIFE. The higher positive rate is statistically significant, at $P < .001$. As noted earlier herein, there was only 1 instance in which FLC-UIFE failed to detect kappa MLCs in urine, and conventional UIFE detected them. Also, lambda MLCs were detected by FLC-UIFE at almost 3 times the rate at which additional kappa MLCs were detected—29% vs 11%. The higher rate of detection for lambda MLCs, compared with kappa LCs, was significant, at $P < .001$. The higher rate of detection of MFLCs is partly due to comigration of MLCs and corresponding monoclonal intact immunoglobulin and the MLC band being obscured by intact monoclonal immunoglobulin (**FIGURES 2 and 3**). FLC-UIFE also detected MFLCs not stained by conventional UIFE reagents (**FIGURES 2 and 3**).

The relationship of the kappa/lambda LC ratio recommended by The Binding Site, with respect to the presence and absence of detectable MLCs with FLC-UIFE, is shown in **TABLE 5**. A normal ratio does not exclude MLCs, and an abnormal ratio is not diagnostic of monoclonal gammopathy yielding high false-negative and high false-positive rates.

Discussion

Monoclonal immunoglobulins are present in the serum and/or urine of virtually all cases of neoplastic monoclonal gammopathy, with the possible exception of nonsecretory MM.¹⁸ Monoclonal immunoglobulins are also present in bodily fluids in many other benign and malignant disorders.⁵ Although the detection of monoclonal immunoglobulins does not diagnose MM, evidence of monoclonal proliferation of plasma cells is essential for the diagnosis of MM. Monoclonal intact immunoglobulins and/or MLCs serve as markers of the presence of clonal plasma cells.¹⁹

Examination of serum levels of FLCs is recommended by the IMWG and by an expert panel of the College of American Pathologists (CAP).^{3,10} It is generally accepted that SFCLA is not a valid test for establishing the monoclonality of immunoglobulins or LCs.⁵ Quantification of SFLC is essential for establishing a diagnosis of LCPMM and constitutes a myeloma-defining condition based on the concentration of involved LCs and/or the ratio of involved to uninvolved LC concentration.^{4,15}

TABLE 1. Clinical Diagnoses, Affected Organ Systems, and Distribution of Diagnoses in the Patient Population at the Main Study Site (Institution A)

Clinical Diagnosis/Diagnoses	%
Neurologic disorders	22.7
MM	13.6
Chronic kidney disease	12.5
Hematological disorders without monoclonal gammopathy	9.2
Multimorbid/polymorbid	7.0
Rheumatologic disorder	5.6
Cardiac disorders	5.5
MGUS	4.7
Osteoporosis	4.5
Infections, including HIV and sepsis	3.6
Amyloidosis	3.2
Gastroenterology lesions	3.1
Endocrine disorders	3.0
Bone lesions	2.7
Other renal disorders	2.5
Psychiatric disorders	1.1
Smoldering MM	0.7
Skin disorders	0.7
Gynecologic disorders	0.6
Urologic disorders	0.4
ENT lesions	0.2
Respiratory disorders	0.2

ENT, ear, nose, and throat; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma.

TABLE 2. Urine and SFLC Parameters Regarding Presence or Absence of Detectable MLCs in Urine via Conventional UIFE^a

Conventional UIFE Results	Institution A	Institution B	Institution C
	LC Conc, mg/L		
Lowest conc at which kappa positive	1.19	0.56	0.57
Lowest conc at which lambda positive	1.03	1.23	1.44
Highest conc at which kappa negative ^b	502.71	213.27	54.12
Highest conc at which lambda negative ^b	75.4	94.39	15.73
Urine protein lowest conc positive	4	5	2
Urine protein highest conc negative	7428 ^c	352	524.4
I/U Ratio			
Lowest K/L conc at which kappa positive	0.059	0.002	0.129
Lowest L/K conc at which lambda positive	0.007	0.114	0.806
Highest K/L conc at which kappa negative	274.7	609.56	82
Highest L/K conc at which lambda negative	119.68	222.68	62.5
	Institution A	Institution B	Institution C
	No. (%)		
No monoclonal LCs detected in urine	1128 (82.0)	17 (11.3)	86 (39.8)
Kappa monoclonal LCs	143 (10.4)	87 (58.0)	88 (40.7)
Lambda monoclonal LCs	99 (7.2)	44 (29.3)	40 (18.5)
Undetermined monoclonal LCs, No.	2	2	0
Total No. of specimens	1372	150	216

conc, concentration; I/U, involved/uninvolved; LC, light chain; MLC, monoclonal light chain; SFLC, serum free light chain; TPr, total urine protein; UIFE, urine immunofixation electrophoresis.

^aSFLC is expressed as mg/L, and TPr is measured as mg/dL.

^bThe high SFLC values with negative results via conventional UIFE are due to comigration of the relevant MLC with the intact immunoglobulin and do not imply the absence of MLCs. FLC-UIFE would identify MLCs in such circumstances.

^cThis unusually high TPr value was from a patient with nephrotic syndrome, when seen in the emergency department. Follow-up specimens were not available. The next 2 highest urine protein values with negative UIFE results, from different patients, were 2965 mg/L and 2226 mg/dL.

TABLE 3. TPr Values for Specimens Testing Positive for MLCs^{a,b}

Total Positive at TPr Value	No. (%)
≤5 mg/dL	13 (5.1)
≤15 mg/dL	47 (18.5)
≤20 mg/dL	58 (22.8)
≤30 mg/dL	79 (31.1)
Total	254 (100)

MLCs, monoclonal light chains; TPr, total urine protein; UIFE, urine immunofixation electrophoresis.

^aIncluding the specimen that tested positive via UIFE only.

^bApproximately one-third of the specimens displayed MLCs despite having urine protein values in the normal range.

The validity of assigning SFLC concentration as a myeloma-defining condition has been questioned because the criteria are not LC-specific, despite strong evidence of greater production of kappa FLCs than lambda FLCs.^{20–22} Quantification of SFLC is also useful in prognostication and monitoring of the course of disease in LCMM.^{14–16} Despite this caveat, it has been proposed that SFLCA can replace urine examination.^{6,7}

It has also been documented¹⁰ that an abnormal kappa/lambda ratio is not diagnostic of monoclonality, and a normal ratio does not exclude monoclonal gammopathy or MM. A small but significant number of cases of lambda chain-associated MM—IgG lambda and IgA lambda—do not produce/secrete enough lambda MFLCs to render the kappa/

TABLE 4. Comparison of Performance of Conventional UIFE and FLC-UIFE in Detecting MLCs in Urine

Conventional UIFE vs FLC-UIFE Testing for MLCs	Kappa	Lambda
Specimens with LC type per SPEP/SIFE, No.	140	88
Specimens testing positive via conventional UIFE, No.	52	24
Specimens testing positive via FLC-UIFE, No.	67	51
Lowest LC conc with positive results, mg/L	0.48	1.03
Highest LC conc with negative results, mg/L	38.27	37.92
Lowest I/U ratio with positive results	0.82	0.396
Highest I/U ratio with negative results	105.29	35
Urine protein lowest conc with positive results	4 mg/dL	
Urine protein highest conc with negative results	7428 mg/dL	
Additional cases of MLC identified via FLC-UIFE, No.	15	27
Additional FLC-UIFE positive results, % ^a	10.7%	30.7%
Total additional FLC-UIFE positive results, No. (%)	42 (18.4%)	

FLC, free light chain; I/U, involved/uninvolved; LC, light chain; MLCs, monoclonal light chains; SIFE, serum immunofixation electrophoresis; SPEP, serum protein electrophoresis; UIFE, urine immunofixation electrophoresis.

^aThe greater number of lambda-positive specimens, compared with kappa-positive specimens, was statistically significant, at $P < .001$. The greater rate of positivity for lambda MLCs was predominantly due to greater sensitivity of antiserum to lambda FLCs than the antilambda antiserum in Helena Laboratories kits, and only partly due to comigration of lambda MLCs with intact monoclonal immunoglobulin.

^b $P < .001$.

lambda ratio abnormal.¹⁰ The requirement for normal kappa/lambda ratio as a condition for stringent complete response has also been challenged due to a high incidence of false-positive results in patients after stem cell transplantation.^{9,23} In fact, as supported by the data in this communication, it could be posited that FLC-UIFE is more suited for diagnosing monoclonal gammopathy than SFLCA.

In this article, we specifically address the utility of conventional UIFE and examine the improvement of urinalysis by utilizing FLC-UIFE. Observations have suggested that the only information added by UIFE was the detection of MLCs in urine.²⁴ The monoclonal heavy chains and intact monoclonal immunoglobulins are readily detectable in SPEP/SIFE. Detection of MLCs in urine is an important diagnostic feature to document and address in treatment, due to the known nephrotoxic nature of MLCs, aka Bence-Jones proteins.

A systematic examination of UIFE results in 1738 urine specimens validated the impression that the only meaningful information gleaned

from UIFE is the documentation of MFLCs. Thus, we propose that conventional UIFE testing for gamma, alpha, and mu chains could be eliminated because it does not provide any value-added information. A separate electrophoresis for UPEP could also be eliminated because conventional UIFE includes a lane for TPr. Therefore, UIFE could be revised from 6 lanes of total protein, antigamma, antialpha, antimu, antikappa, and antilambda to 3 lanes for total proteins and kappa and lambda FLCs. The vendors of electrophoresis equipment could design templates to accommodate this more efficient and more sensitive method. The increased sensitivity of this method could allow detection of residual disease that would be missed by conventional UIFE, and this method may qualify as a test for MRD in MM.

The refinement of UIFE examined in this report strongly favors replacing the conventional antikappa and antilambda sera in UIFE with antisera specific to kappa and lambda FLCs. The 18% additional cases of MLCs detected by FLC-UIFE were in consonance with the expectation from clinical data presented in [TABLE 1](#). As noted in the FLC-modified SIFE, the additional MLCs detected by the FLC immunofixation electrophoresis is partly due to the detection of MFLCs that have electrophoretic mobility similar to the coexisting intact monoclonal immunoglobulins and are thus not detectable by conventional UIFE.¹³ An additional factor in the improved detection rate is likely to be greater sensitivity of FLC-UIFE, as evidenced by the detection of MLCs in urine with a serum concentration of 0.48 mg/L, compared with the corresponding level of 1.19 mg/L detected by conventional UIFE for kappa LCs when both methods were used in parallel.

The markedly different rates of positive findings on UIFE at the 3 institutions reflect the wide variation in utilization of UIFE. At institution A, the neurology and nephrology services are prominent users of UIFE, whereas the oncology service does not routinely order UIFE due to clinician preference. At institution B, the hematology/oncology service is the dominant user of UIFE. At institution C, the hematology/oncology service was the largest user, with sizeable contributions from rheumatology, neurology, and nephrology service. Despite the markedly different rates of finding MLCs in urine, the low SFLC levels and the low ratio of involved to uninvolved SFLC concentrations associated with detection of MLCs in urine are remarkably similar at the 3 institutions. Moreover, the negative results at high levels of SFLC and the ratio of involved to uninvolved SFLC concentrations also show similar trends at the 3 institutions. These findings highlight the previously documented

FIGURE 1. Urine from a patient with IgM kappa monoclonal immunoglobulin in serum was tested via conventional urine immunofixation electrophoresis reagents. Staining with antikappa antibody is presented in lane A and shows a low-intensity kappa monoclonal light chain (LC). No kappa LC was detected with antiserum to kappa free LCs, in lane B.

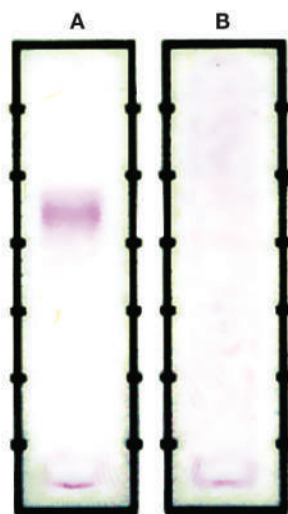
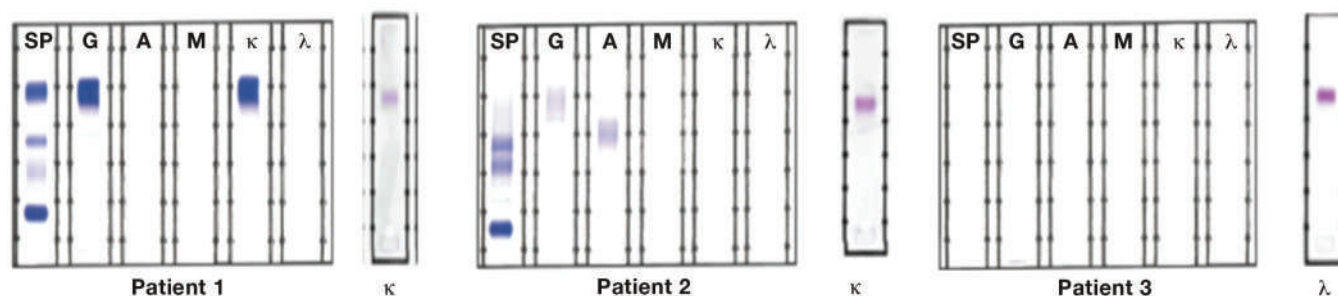


FIGURE 2. Conventional urine immunofixation electrophoresis (UIFE) and free light chain (FLC)-UIFE from 3 patients. The lanes marked SP, G, A, M, K, and L represent conventional UIFE. The unmarked separate lane in the first 2 patients was stained with antiserum to kappa FLCs and in the third patient for lambda LCs. The lack of detection of kappa monoclonal free light chains (MFLCs) in patient 1 by conventional UIFE is due to overlap in the location of intact monoclonal IgG kappa and kappa MFLC band. The detection of monoclonal kappa and lambda LC bands in patients 2 and 3 reflects the greater sensitivity of FLC-UIFE over conventional UIFE.



observation of poor correlation between SFLC levels and the presence of MLCs.⁵

Detection of MLCs in urine is important in patient evaluation because MLCs are nephrotoxic. The FLC-UIFE is a promising means of promoting detection of MLCs in urine, facilitating the diagnosis of

FIGURE 3. Conventional serum immunofixation electrophoresis (SIFE) and free light chain (FLC)–SIFE from the patient with the highest level of lambda FLCs at institution A. Lane A represented staining for IgA; lane L represents staining for lambda LCs by conventional reagents in the Helena IFE kit (Helena Laboratories); lane FL represents staining with Sebia antiserum to lambda FLCs. The conventional UIFE was interpreted to contain monoclonal IgA lambda. The lambda monoclonal free light chains (MFLCs) comigrate with the intact monoclonal IgA lambda. Although staining in the L lane is darker, that fact by itself was considered insufficient to conclude the presence of lambda MFLCs. The upper, darker band in lane FL represents lambda MFLCs migrating at the same location as intact IgA lambda. The lower, fainter band in the FL lane represents a separate, lower concentration of lambda MLCs with different mobility, likely due to alterations in serum or urine. The altered lambda MFLCs did not react with the antilambda antiserum in the Helena IFE kits. This case illustrates the 2 mechanisms of higher sensitivity of FLC-UIFE: monoclonal light chains (MLCs) comigrating with intact monoclonal Ig, which can be identified as MFLCs due the specificity of the antiserum for free light chains (FLCs); altered MFLCs in urine were detectable with antiserum to FLCs but not via the conventional reagents. These 2 mechanisms were variably applicable in cases in which FMLCs were detected by Sebia antisera to FLCs but were not detectable by conventional antisera in Helena IFE kits.

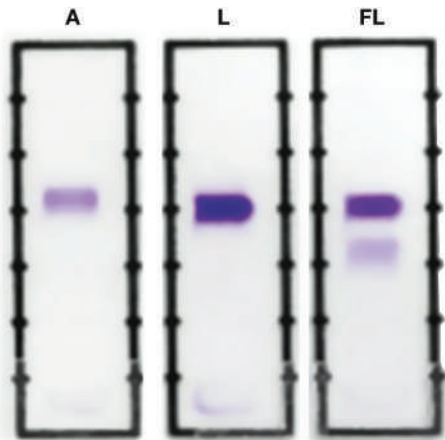


TABLE 5. Performance of FLC-UIFE with Respect to K/L Ratio

Result	No. (%)
Kappa positive at K/L <1.65	12 (16.0)
Kappa negative at K/L >1.65	45 (63.3)
Lambda negative at K/L <0.26	6 (16.2)
Lambda positive at K/L >0.26	28 (59.6)

FLC, free light chain; K/L, kappa/lambda; UIFE, urine immunofixation electrophoresis.

monoclonal gammopathy of renal significance (MGUS), and facilitating the detection of MRD.²⁵

The lack of detection of kappa MLCs in urine via FLC-UIFE in a patient with IgM kappa monoclonal immunoglobulin in serum likely reflects the lack of appropriate epitopes in the immunogen used for production of the reagent antibodies. A similar negative result was also documented by The Binding Site¹⁸ and was corrected by changes to the immunogen. Kappa MLCs were detected by conventional UIFE at the initial examination. Testing via conventional and FLC-UIFE was repeated to ensure that the lack of reactivity with FLC-UIFE was not due to deterioration of the specimen during storage.

As an alternative to the recommendations by the IMWG and the CAP panel, we propose that diagnostic work-up for monoclonal gammopathy should consist of SPEP, SIFE, and FLC-UIFE only.^{3,4} SFLCA may be carried out to diagnose LCPMM and for monitoring of LCMM. We do not have sufficient data to comment on the usefulness of SFLCA in amyloidosis and nonsecretory MM. The criteria for myeloma-defining condition based on LC concentration have been challenged because the current criteria are not LC specific, despite documentation of a marked excess of kappa FLCs over lambda FLCs.^{21,22} Although we did not study this facet, it is feasible that the more-sensitive FLC-UIFE test could serve a marker of MRD, especially in patients with LCMM and LCPMM.

Conclusions

The only useful information added by UIFE, over SPEP and SIFE, is detection of MFLCs. All instances in which intact monoclonal immunoglobulins could be detected by UIFE were in patients with positive SIFE results for the cognate immunoglobulin. UIFE with antisera to free LCs detects 18% more instances of MLCs in urine than conventional UIFE.

Based on these findings, we suggest the following diagnostic algorithms for screening for monoclonal gammopathy:

- Screening for monoclonal gammopathy should consist of SPEP, SIFE, and FLC-UIFE only. SFLCA does not add value due to the large number of false-negative and false-positive results it yields.
- FLC-UIFE should be performed in the work-up for diagnosis of monoclonal gammopathy and for detection of residual disease in patients treated for MM.
- FLC-UIFE should be performed in the aforementioned circumstance irrespective of the serum level of FLCs and the ratio of I/U LC concentration.
- FLC-UIFE should be performed in all specimens, notwithstanding TPr in the normal range.
- FLC-UIFE promotes better utilization of resources and provides a more-sensitive method for detection of MLCs in urine.
- The role of FLC-UIFE as a test for MRD should be explored, as has been suggested for FLC-modified SIFE.

Conflict of Interest Disclosure

Dr Singh serves a consultant to Beckman-Coulter, Sebia, Diazyme, and HealthTap. Dr Singh has applied for a United States patent for “Free Light Chain Immunofixation Electrophoresis” (patent application No. 63/357,801). Dr Bollag holds United States patent US6191268 B1. Dr Plapp has provided expert testimony for the law firm of Newman, Comley & Ruth, Jefferson City, MO.

REFERENCES

- Islami F, Ward EM, Sung H, et al. Annual report to the nation on the status of cancer, part 1: national cancer statistics. *J Natl Cancer Inst*. 2021;113(12):1648–1669. doi:10.1093/jnci/djab131
- Dispenzieri A, Kyle R, Merlini G, et al. International Myeloma Working Group. International Myeloma Working Group guidelines for serum-free light chain analysis in multiple myeloma and related disorders. *Leukemia*. 2009;23(2):215–224. doi:10.1038/leu.2008.307
- Keren DF, Bocsi G, Billman BL, et al. Q. Laboratory detection and initial diagnosis of monoclonal gammopathies. *Arch Pathol Lab Med*. 2022;146(5):575–590. doi:10.5858/arpa.2020-0794-CP
- Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. *Lancet Oncol*. 2014;15(12):e538–e548. doi:10.1016/S1470-2045(14)70442-5
- Singh G. Serum and urine protein electrophoresis and serum-free light chain assays in the diagnosis and monitoring of monoclonal gammopathies. *J Appl Lab Med*. 2020;5(6):1358–1371.
- Dejoie T, Corre J, Caillon H, et al. Serum free light chains, not urine specimens, should be used to evaluate response in light-chain multiple myeloma. *Blood*. 2016;128(25):2941–2948. doi:10.1182/blood-2016-07-726778.
- Katzmann JA, Dispenzieri A, Kyle RA, et al. Elimination of the need for urine studies in the screening algorithm for monoclonal gammopathies by using serum immunofixation and free light chain assays. *Mayo Clin Proc*. 2006;81(12):1575–1578. doi:10.4065/81.12.1575
- Singh G. Serum free light chain assay and κ/λ ratio performance in patients without monoclonal gammopathies: high false-positive rate. *Am J Clin Pathol*. 2016;146(2):207–214. doi:10.1093/ajcp/aqw099
- Singh G. Oligoclonal pattern/abnormal protein bands in post-treatment plasma cell myeloma patients: implications for protein electrophoresis and serum free light chain assay results. *J Clin Med Res*. 2017;9(8):671–679. doi:10.14740/jocmr3049w
- Singh G. Serum free light chain assay and κ/λ ratio: performance in patients with monoclonal gammopathy-high false negative rate for κ/λ ratio. *J Clin Med Res*. 2017;9(1):46–57. doi:10.14740/jocmr2802w
- Cotten SW, Shajani-Yi Z, Cervinski MA, Voorhees T, Tuchman SA, Korpi-Steiner N. Reference intervals and diagnostic ranges for serum free κ and free λ immunoglobulin light chains vary by instrument platform: implications for classification of patient results in a multi-center study. *Clin Biochem*. 2018;58:100–107. doi:10.1016/j.clinbiochem.2018.06.003
- Morales-García LJ, Pacheco-Delgado MS. Serum free light chain reference intervals in an Optilite and their influence on clinical guidelines. *Clin Biochem*. 2021;92:54–60. doi:10.1016/j.clinbiochem.2021.02.008
- Wilhite D, Arfa A, Cotter T, Savage NM, Bollag RJ, Singh G. Multiple myeloma: detection of free monoclonal light chains by modified immunofixation electrophoresis with antisera against free light chains. *Pract Lab Med*. 2021;27:e00256. doi:10.1016/j.plabm.2021.e00256
- Jin Y, Savage NM, Bollag RJ, Xu H, Singh G. Light chain multiple myeloma: high serum free light chain concentrations portend renal damage and poorer survival. *J Appl Lab Med*. 2021;6(6):1592–1600. doi:10.1093/jalm/jfab090
- Singh G, Xu H. Light chain predominant intact immunoglobulin monoclonal gammopathy disorders: shorter survival in light chain predominant multiple myelomas. *Lab Med*. 2021;52(4):390–398. doi:10.1093/labmed/lmaa057
- Singh G, Savage NM, Jillella AP, Bollag RJ. Light chain-predominant multiple myeloma subgroup: impaired renal function correlates with decreased survival. *Lab Med*. 2022;53(2):145–148. doi:10.1093/labmed/lmab054
- Dimopoulos M, Kyle R, Fermand J-P, et al; International Myeloma Workshop Consensus Panel 3. Consensus recommendations for standard investigative workup: report of the International Myeloma Workshop Consensus Panel 3. *Blood*. 2011;117(18):4701–4705. doi:10.1182/blood-2010-10-299529
- Badwell AR. *Serum Free Light Chain Analysis Plus Hevylite*. 7th ed. Birmingham, UK: The Binding Site; 2015.
- Kyle RA, Durie BGM, Rajkumar SV, et al. International Myeloma Working Group. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia*. 2010;24(6):1121–1127. doi:10.1038/leu.2010.60.
- García de Veas Silva JL, Bermudo Guitarte C, Menéndez Valladares P, Rojas Noboa JC, Kestler K, Duro Millán R. Prognostic value of serum free light chains measurements in multiple myeloma patients. *PLoS One*. 2016;1:e0166841.
- Lee WS, Singh G. Serum free light chains in neoplastic monoclonal gammopathies: relative under-detection of lambda dominant kappa/lambda ratio, and underproduction of free lambda light chains, as compared to kappa light chains, in patients with neoplastic monoclonal gammopathies. *J Clin Med Res*. 2018;10(7):562–569. doi:10.14740/jocmr3383w
- Lee WS, Singh G. Serum free light chain assay in monoclonal gammopathic manifestations. *Lab Med*. 2019;50(4):381–389. doi:10.1093/labmed/lmz007
- Kumar S, Paiva B, Anderson KC, et al. International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *Lancet Oncol*. 2016;17(8):e328–e346. doi:10.1016/S1470-2045(16)30206-6
- Lahuerta J-J, Jiménez-Ubieto A, Paiva B, et al. Role of urine immunofixation in the complete response assessment of MM patients other than light-chain-only disease. *Blood*. 2019;133(25):2664–2668. doi:10.1182/blood.2019000671
- Leung N, Bridoux F, Nasr SH. Monoclonal gammopathy of renal significance. *N Engl J Med*. 2021;384(20):1931–1941. doi:10.1056/NEJMr1810907

Evaluation of Vitamin D Status and the Analysis of Risk Factors of Vitamin D Deficiency in Twin Pregnancies

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Keywords: twin pregnancy, 25-hydroxyvitamin D, nutrition, vitamin D deficiency, singleton pregnancy, pregnant Chinese women

Abbreviations: VD, vitamin D; TP, twin pregnancies; 25(OH)D, 25-hydroxyvitamin D; VDBP, VD binding protein; epi-25(OH)D, C-3 epimer of 25-hydroxyvitamin D; SP, singleton pregnancies; VDD, VD deficiency; ART, assisted reproductive technologies; LC-MS/MS, liquid chromatography-tandem mass spectrometry; IOM, Institute of Medicine; ALB, albumin; BMI, body mass index; OR, odds ratio

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ABSTRACT

Objective: Optimization of maternal vitamin D (VD) status has beneficial effects on pregnancies, but little is known about it of twin pregnancies (TP). Our aim was to promote the current understanding of VD status and its associated factors in TP.

Methods: We performed liquid chromatography-tandem mass spectrometry to quantify 25-hydroxyvitamin D [25(OH)D] and used the enzyme-linked immunosorbent assay method to detect vitamin D binding protein (VDBP) in 218 singleton pregnancies (SP) and 236 TP.

Results: Levels of 25(OH)D and VDBP were higher in TP than SP. The 25(OH)D, free 25(OH)D, C-3 epimer of 25-hydroxyvitamin D [epi-25(OH)D], and VDBP all increased with gestational progress. Age, body mass index, and hemoglobin level were associated with VD deficiency (VDD). Analysis of covariance demonstrated that the 25(OH)D and VDBP of TP and SP still showed differences after adjusting for the above associated factors.

Conclusion: Differences in VD status were found in SP and TP, suggesting that the assessment of VD status in TP should be treated with caution. High VDD prevalence is observed among pregnant Chinese women, and it is recommended to promote evaluation for VDD.

The prevalence of twin births has been growing due to the increasing

use of fertility drugs, artificial insemination, and assisted reproductive technologies (ART).¹ However, maternal/fetal morbidity/mortality in twin pregnancies (TP) are strikingly higher than in singleton pregnancies (SP).¹ Additionally, most multiple pregnancy-related diseases are resistant to technological or pharmaceutical interventions, whereas optimization of maternal nutritional status can have beneficial effects on the course and outcome of multiple pregnancies.² Vitamin D (VD) plays an essential role in cellular metabolism, fetal intrauterine growth, and the maternal oxidative stress balance.³ In turn, VD deficiency (VDD) could increase risk of preeclampsia, gestational diabetes, caesarean section, and bacterial vaginosis in pregnant women and intrauterine growth retardation and low birth weight in the fetus.^{4,5} Accordingly, VD status and supplement recommendations for SP have been well established, whereas specific trials for TP await. To our knowledge, limited studies have evaluated 25-hydroxyvitamin D [25(OH)D] level in TP,⁶⁻¹³ and consensus has not yet been reached (Supplemental Table S1). Such disparities may be attributed to the application of improper quantification strategies and ethnic differences among study populations. Most of these studies quantified 25(OH)D by immunoassay (Supplemental Table 1), and studies using liquid chromatography-tandem mass spectrometry (LC-MS/MS) did not resolve epi-25(OH)D₃ (the physiologically inactive isomer of 25(OH)D₃). Such circumstances are likely to lead to overestimation of 25(OH)D.¹⁴ Ethnicity is an important factor related to VDD. However, few studies have focused on Asian populations, especially Chinese TP women. Therefore, in the present study, by using a convincing LC-MS/MS method, we aim to (1) evaluate and compare 25(OH)D in TP and SP among Chinese pregnant women, (2) test relationships between gestational weeks and 25(OH)D/vitamin D binding protein (VDBP), and (3) analyze VDD-related factors in TP.

Materials and Methods

Study Participants

We recruited 300 SP and 300 TP from ethnically identical pregnant women attending Renmin Hospital of Wuhan University (Wuhan, China) for routine prenatal screening for aneuploidy from May to September 2019. Maternal clinical characteristics and medical histories were obtained from computerized medical records

and well-trained clinicians. Histories of spontaneous abortions, malformed fetuses, and stillbirths were considered to be adverse pregnancy and childbirth history. Gestational age was between 11 and 25 weeks determined by their last menstrual period and the results of ultrasonography. The number of fetuses was determined by the results of B-mode ultrasonography. Chorionicity of TP was confirmed by examining the inter-twin membrane at its junction with the placenta.¹⁵ Participants were excluded when stillbirths, fetal chromosomal abnormalities, fetal malformations, terminated gestation, or miscarriage before 24 weeks occurred, or when obstetric complications (such as gestational diabetes and intrahepatic cholestasis of pregnancy) and other major diseases (such as thyroid disease or diabetes before pregnancy) were found. The final study population consisted of 218 SP and 236 TP. Of the TP, 191 (81.0%) were dichorionic twins and 45 (19.0%) were monochorionic twins. Written informed consent was obtained from all participants before study entry. The study was conducted according to the guidelines in the Declaration of Helsinki and supervised and approved by the Ethics Committee of Renmin Hospital of Wuhan University (approval reference number: WDRY2021-K042).

Blood Sample Collection and Routine Laboratory Analyses

Blood samples were collected by venipuncture after overnight fasting. Serum was immediately separated into a polypropylene conical centrifuge tubes after blood collection (centrifuged at 1000g for 5 min at 4°C) and promptly frozen at -80°C until determinations were made. Siemens Advia 2400 automatic biochemistry analyzer was used to determine biochemical indexes. Blood indicators were analyzed by Sysmex XN-9000. The 25(OH)D was determined by an LC-MS/MS method described previously.¹⁶ Chromatogram output, data processing, and quantitation were performed using AB SCIEX Analyst 1.6.2 software (Applied Biosystems). Related details such as sample preparation steps, liquid chromatography instruments, and mass spectrometry detection can be found in the supplementary materials. The 25(OH)D was calculated as the sum of 25(OH)D₂ and 25(OH)D₃; epi-25(OH)D₃ was not included. The human Vitamin D BP Quantikine ELISA Kit (DVDBP0B) (R&D Systems) was used to detect VDBP following the manufacturer's protocol (wavelength: 450 nm). Samples from SP or TP were measured simultaneously at the end of the study in the same analytical batches. The laboratory technicians were blinded to sample types and any expected outcomes.

Statistical Analysis

All women with data available were included in the analysis. All VDD was defined as 25(OH)D concentration <12.0 ng/mL (30 nmol/L) according to the Institute of Medicine (IOM).¹⁷ Free serum 25(OH)D concentration was calculated from measured total 25(OH)D, serum albumin (ALB), VDBP, the affinity constant of ALB ($K_{aALB} = 6 \times 10^5$ L/mol) and the affinity constant of VDBP for 25(OH)D ($K_{aVDBP} = 6 \times 10^8$ L/mol) using the following equation¹⁸:

$$\text{Free serum 25(OH)D} = \frac{\text{measured total 25(OH)D}}{(1 + \text{ALB} \times K_{aALB} + \text{VDBP} \times K_{aVDBP})}$$

Age, height, weight, and body mass index (BMI) of all study subjects were measured before pregnancy. Age was categorized as <25, 25-34, and ≥35 years. Weight was categorized as ≤60 and >60 kg. According to the World Health Organization standard, BMI was categorized as normal weight, overweight, and obesity (18.5-24.9, 25-29.9 and >30 kg/m²).¹⁹

Gestational age was categorized as 11 to 17 and 17 + 1 to 25 weeks. Education level was described as ≤9 years (less than or equivalent high school), and >9 years (greater than high school). Parity was classified as 0 (primipara) and ≥1 (multipara). To identify anemia, the Centers for Disease Control and Prevention threshold of hemoglobin concentration (<105 g/L) was used.^{20,21}

Normally distributed parameters are reported as the means ± standard error. Frequencies are expressed as number of cases/percentage. Skewed data are expressed as median and interquartile ranges. Normality of distribution was performed by Shapiro-Wilk test. If data were continuous and normally distributed, independent *t* testing was employed to evaluate the comparison, otherwise, Mann-Whitney *U* test was used. The χ^2 test was applied for the comparison of categorical variables. Linear analyses were used to test relationships between gestational weeks and 25(OH)D/VDBP levels. Pearson correlation analysis was used to evaluate the correlation coefficients between variables. Univariate and multivariate logistic regression analyses were used to estimate crude and adjusted odds ratio (OR), respectively. Analysis of covariance was used to analyze differences in VD status between SP and TP before and after controlling for other potential influencing factors. The statistical analyses were performed using IBM SPSS, version 22.0. A two-tailed *P* < .05 was considered statistically significant.

Results

Participant Characteristics

Six hundred pregnancies were assessed for eligibility and 146 (24.3%) cases were excluded due to stillbirths (*n* = 7), fetal chromosomal abnormalities (*n* = 6), fetal malformations (*n* = 11), terminated gestation (*n* = 18) or miscarriage (*n* = 12) before 24 weeks, preeclampsia (*n* = 32), gestational diabetes (*n* = 21), or incomplete information (*n* = 39). Hence, the study population included 218 SP and 236 TP. Of the TP, 191 (81.0%) were dichorionic twins and 45 (19.0%) were monochorionic twins. **TABLE 1** summarizes the clinical characteristics of TP and SP. Compared with SP, increased maternal age, weight, BMI, heart rate, total cholesterol, triacylglycerol, and high incidence of ART, advanced educational level, and primipara were observed in TP (*P* < .05). In contrast, decreased systolic pressure, diastolic pressure, ALB, glucose, calcium, phosphorus, hemoglobin, and hematocrit levels were found (*P* < .05).

Maternal 25(OH)D Status in Second Trimester

TABLE 1 and **FIGURE 1** depict maternal 25(OH)D status of the 454 participants. The 25(OH)D was calculated as the sum of 25(OH)D₂ and 25(OH)D₃. The level of 25(OH)D₂ and epi-25(OH)D₃ showed no significant differences between the 2 groups. We detected 25(OH)D₂ in 31.2% (68/218) SP and 33.5% (79/236) TP, and epi-25(OH)D₃ in 67.4% (147/218) SP and 74.6% (176/236) TP. Epi-25(OH)D₃ concentration can make up 0% to 37.56% of the total 25(OH)D concentration. Furthermore, the Pearson correlation coefficient between epi-25(OH)D₃ and 25(OH)D was 0.602 (*P* < .001) in SP and 0.638 (*P* < .001) in TP. 25(OH)D₃ could be detected in every participant, and significant differences were found in 25(OH)D₃ (SP: 10.29 [5.30-13.09] ng/mL, TP: 14.53 [8.14-18.58] ng/mL, *P* < .001) and 25(OH)D (SP: 11.07 [6.47-15.15] ng/mL, TP: 15.40 [9.98-22.13] ng/mL, *P* < .001) between the 2 groups. Also, VDBP concentration (SP: 347.53 ± 74.26 μg/mL, TP: 378.91 ± 86.01 μg/mL, *P* < .001) differed significantly. Differences in VD nutritional status between TP and SP

TABLE 1. Maternal Clinical Characteristics of Twin and Singleton Pregnancies^a

Study Variables	Singleton (n = 218)	Twins (n = 236)	P Value
Age (y)	27.18 ± 4.14	30.49 ± 5.06	<.001
Han ethnicity	216 (99.1)	233 (98.7)	.718
Height (cm)	160.37 ± 5.40	159.41 ± 5.46	.060
Weight (kg)	58.27 ± 8.08	61.02 ± 7.62	<.001
BMI (kg/m ²)	22.85 ± 3.61	24.21 ± 3.77	<.001
Gestational age (wk)	18.22 ± 4.39	17.88 ± 4.87	.120
ART	44 (20.2)	90 (38.1)	<.001
Cigarette smokers	27 (12.4)	20 (8.5)	.172
APCH	41 (18.8)	55 (23.3)	.252
Vitamin D supplementation	20 (9.2)	34 (14.3)	.072
Marital status			
Partner	171 (78.4)	199 (84.3)	.107
No partner	47 (21.6)	37 (15.7)	
Education level (y)			
≤9	120 (55.0)	80 (34.9)	<.001
>9	98 (45.0)	156 (66.1)	
Parity			
0	71 (32.6)	132 (55.9)	<.001
≥1	147 (67.4)	104 (44.1)	
Systolic pressure (mmHg)	128.42 ± 25.84	122.80 ± 26.65	.023
Diastolic pressure (mmHg)	72.94 ± 18.88	64.38 ± 20.38	<.001
Heart rate (beats/min)	90.77 ± 13.73	95.45 ± 16.13	.001
ALT (U/L)	23 (18–30)	24.0 (14–33)	.685
AST (U/L)	22 (16–29)	23.00 (16–34)	.768
Serum albumin (g/L)	37.62 ± 4.71	34.75 ± 5.54	<.001
Urea (mmol/L)	3.99 ± 1.17	4.06 ± 1.57	.595
Creatinine (μmol/L)	101.50 ± 44.43	98.67 ± 52.44	.538
Glucose (mmol/L)	5.67 ± 1.17	5.02 ± 1.02	<.001
Calcium (mg/dL)	9.12 ± 0.18	8.92 ± 0.20	<.001
Phosphorus (mg/dL)	3.42 ± 0.10	3.02 ± 0.09	<.001
Total Cholesterol (mmol/L)	3.91 ± 1.16	4.26 ± 1.24	.002
Triacylglycerol (mmol/L)	1.23 ± 0.59	1.55 ± 0.52	<.001
HDL (mmol/L)	1.08 ± 0.42	1.12 ± 0.40	.306
LDL (mmol/L)	2.31 ± 0.79	2.39 ± 0.92	.374
Hemoglobin (g/L)	105.08 ± 19.65	100.12 ± 20.14	.008
Hematocrit (%)	35.63 ± 3.92	34.36 ± 3.21	.001
Epi-25(OH)D ₃ (ng/mL)	6.33 (3.13–9.35)	5.72 (3.10–9.50)	.645
25(OH)D ₂ (ng/mL)	3.67 (1.75–5.04)	3.46 (1.75–4.96)	.586
25(OH)D ₃ (ng/mL)	10.29 (5.30–13.09)	14.53 (8.14–18.58)	<.001
25(OH)D (ng/mL)	11.07 (6.47–15.15)	15.40 (9.98–22.13)	<.001
Free 25(OH)D (pg/mL)	3.20 (2.28–3.83)	3.29 (2.78–3.96)	.123
VDBP (μg/mL)	347.53 ± 74.26	378.91 ± 86.01	<.001

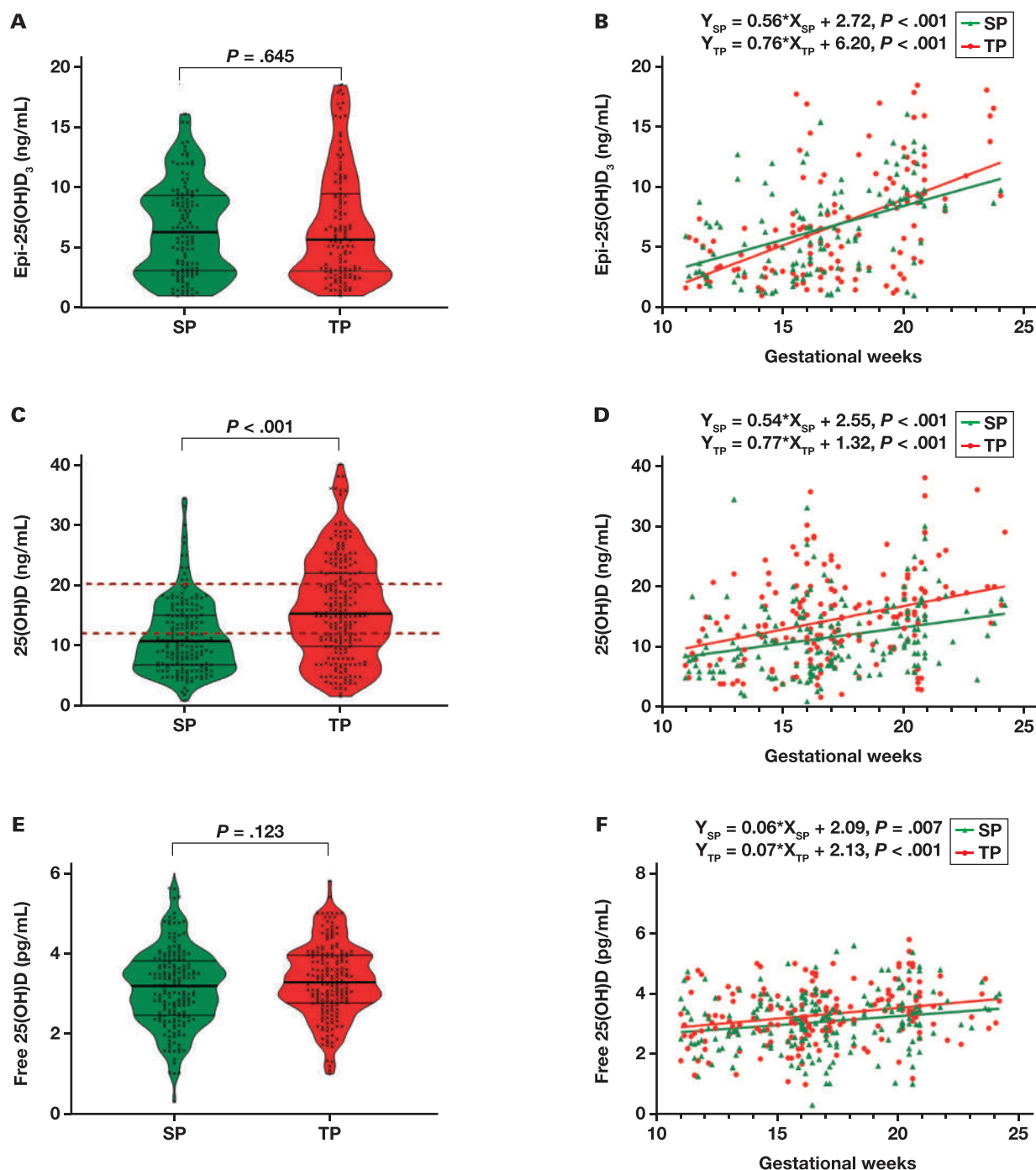
APCH, adverse pregnancy and childbirth history; ART, assisted reproductive technologies; VDBP, vitamin D binding protein.

^aAge, height, weight, and BMI of all study subjects were measured before pregnancy. P < .05 was considered significant.

were found to persist in 25(OH)D ($P < .001$) and VDBP ($P < .001$) when adjusted for VDD-related factors (including age, BMI, and hemoglobin) using analysis of covariance (TABLE 2). Calculated free serum 25(OH)D in TP (3.29 [2.78–3.96] pg/mL) was slightly higher than that of SP (3.20 [2.28–3.83] pg/mL) with no significance. The data show 25(OH)D

(FIGURE 1C) epi-25(OH)D₃ (FIGURE 1A) represent a skewed distribution, and free 25(OH)D (FIGURE 1E) and VDBP (FIGURE 1G) were distributed in a nearly normal fashion. The concentration of epi-25(OH)D₃ (FIGURE 1B), 25(OH)D (FIGURE 1D), and VDBP (FIGURE 1H) increased with gestational progress in the second trimester.

FIGURE 1. Comparison of epi-25(OH)D (A), 25(OH)D (C), free plasma 25(OH)D (E) and vitamin D binding protein (VDBP) (G), and their changing trends with gestational weeks (B, D, F, H) in women with singleton pregnancy (SP) and twin pregnancy (TP) in the cross-sectional study. The upper and lower horizontal lines in C represent cut-off values for vitamin D deficiency (< 12.0 ng/mL) and sufficiency (> 20 ng/mL).



ter ($P < .05$), whereas free 25(OH)D (FIGURE 1F) did not show a significant upward trend.

Association Factors of VDD

Notably, 58.2% (264/454) of participants had VDD according to the IOM standard, and the prevalence of VDD was significantly greater in SP (154/218, 70.7%) than in TP (110/236, 46.6%, $P < .001$). Crude and adjusted odds of VDD-associated factors for all eligible subjects were

analyzed in univariate and multivariate logistic regression, as shown in TABLE 3. Meanwhile, we also performed the analysis of VDD-related factors for singleton and twin mothers, respectively, as shown in Supplementary Table 2. In general, the odds of VDD obtained from the subjects in different clusters with TP closely paralleled the results obtained from SP. Multivariate logistic regression analysis (TABLE 3) adjusted for several variables that were statistically significant in univariate logistic regression analysis, including pregnancy type, age,

FIGURE 1. (cont)

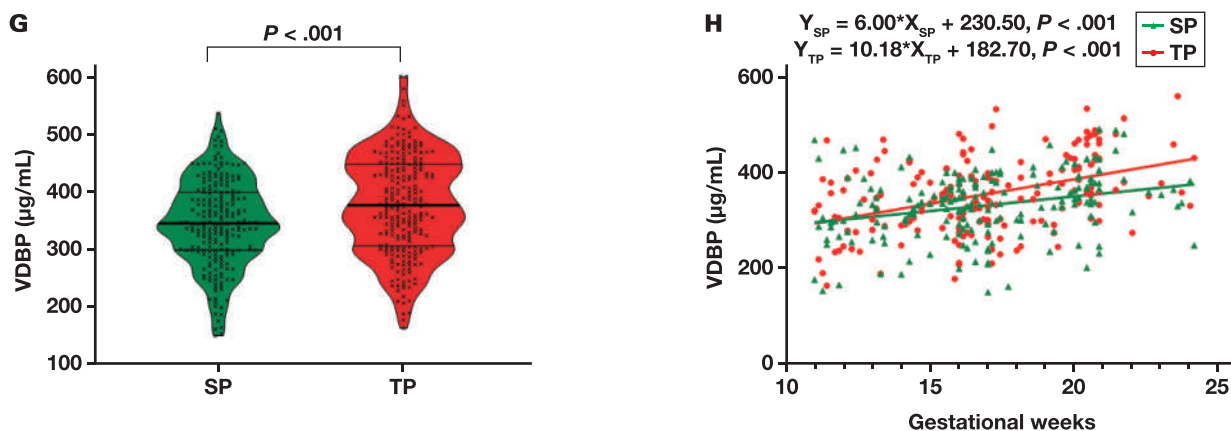


TABLE 2. Adjusted and Unadjusted Vitamin D Levels in Singleton and Twin Mothers¹

Variable	Group	Unadjusted		Adjusted ^{a,b}	
		Mean	SD	Mean	SE
25(OH)D ₂ (ng/mL)	Singleton (n = 68)	3.678	1.72	3.65	0.21
	Twin (n = 79)	3.49	1.62	3.51	0.20
Epi-25(OH)D ₃ (ng/mL)	Singleton (n = 147)	6.84	5.91	6.79	0.39
	Twin (n = 176)	6.59	3.9	6.64	0.39
25(OH)D (ng/mL)	Singleton (n = 218)	11.61	5.91	11.32	0.57
	Twin (n = 236)	16.28	8.30	16.52	0.52
VDBP (µg/mL)	Singleton (n = 218)	347.53	74.26	331.11	5.52
	Twin (n = 236)	378.91	86.01	351.17	5.52

^aCovariate in adjusted model include age, body mass index, and hemoglobin.

^bIn the adjusted model, there were also significant differences in 25(OH)D and VDBP (vitamin D binding protein) levels between the singleton and twin groups ($P < .001$).

BMI, education level, parity, and hemoglobin. Compared with SP, TP appears to be a protective factor for VDD, with an OR of 0.36 (0.18–0.57, $P < .001$) and an adjusted OR of 0.39 (0.15–0.65, $P < .001$). Compared with pregnant women <25 years of age, those who were 25 to 34 years old or ≥35 years old had increased risk of VDD, with ORs of 2.34 (1.23–4.67, $P = .010$) and 2.67 (1.67–6.34, $P = .001$), respectively. Risk of VDD for women ≥35 years persisted in multivariable models with an adjusted OR of 2.36 (1.13–5.35, $P = .003$). Compared with women with BMI between 18.5 and 24.9 kg/m², OR (95% confidence interval [CI]) and adjusted OR (95% CI) of VDD for women with obesity (BMI >30 kg/m²) were 1.67 (1.18–3.23, $P = .002$) and 1.63 (1.16–3.39, $P = .001$), respectively. When stratified by educational level, a higher proportion of women who had received advanced education had VDD, resulting in an unadjusted OR (95% CI) of 1.48 (1.12–4.28, $P = .001$) for VDD risk. However, adjustments for other factors made these correlations insignificant ($P = .107$). Also, multiparas were more than 3 times as likely to have VDD than primiparas (OR = 3.87, 95% CI = 1.37–7.39, $P = .001$). In the multivariable model, these correlations disappeared ($P = .087$). In unadjusted models, a serum hemoglobin concentration <105 g/L was associated with increased odds of VDD (OR = 2.98, 95%CI = 1.21–5.23, $P = .001$). Decreased risk of anemia with VDD presented in adjusted models (adjusted OR = 2.28, 95% CI = 1.23–4.98, $P = .001$).

Discussion

It is common to observe TP representing a state of dramatic physiologic changes and perinatal pathologies that increase the risk of perinatal morbidity and neonatal mortality.²² It is also assumed that the presence of twin fetuses magnifies nutritional demands and causes greater nutritional drain,²³ so the potential beneficial effect of optimal VD status on TP outcomes should not be underestimated. Nevertheless, there is still a paucity of specific VD studies for TP so that few recommendations can be made at present. To the best of our knowledge, this is the first study precisely presenting the quantification of 25(OH)D after excluding epi-25(OH)D₃ interference in Chinese TP. We believe this data will be useful to improve the efficacy of nutrition programs and prevent unfavorable outcomes of TP.

Interpretations of Clinical Characteristics of TP and SP

Compared with SP, we found more frequent use of ART, older average age, greater weight, and elevated BMI in TP, which was consistent with previous reports.^{1,24,25} In ART decision-making, it is important or even preferential to consider the possibility of TP.²⁵ Age alone can account for 25% to 30% of the observed increase in multiple pregnancies.²⁶ Also, older gestation age is associated with higher educational level, as women nowadays are postponing pregnancies due to educational opportunities. As the maternal metabolic rate in TP has been found to be approximately 10% higher than SP previously,¹⁹ the TP group in our study exhibited faster heart rate and lower glucose concentrations. Additionally, TP increases cardiac output and cardiac index during the second

TABLE 3. Crude and Adjusted Odds of Vitamin Deficiency in Pregnant Women

Variable	No. (%)	Pregnant Women (n = 454)			
		Crude OR ^a	P	Adjusted OR ^b	P
Pregnancy type					
Singleton	218 (48.0)	Reference		Reference	
Twin	236 (52.0)	0.36 (0.18–0.57)	<.001	0.39 (0.15–0.65)	<.001
Age (y)					
<25	89 (19.6)	Reference		Reference	
25–34	269 (59.3)	2.34 (1.23–4.67)	.010	2.12 (0.87–6.45)	.690
≥35	96 (21.1)	2.67 (1.67–6.34)	.001	2.36 (1.13–5.35)	.003
Weight (kg)					
≤60	194 (42.7)	Reference		—	—
>60	260 (57.3)	2.56 (0.52–6.30)	.493	—	—
BMI (kg/m²)					
18.5–24.9	157 (34.6)	Reference		Reference	
25–29.9	193 (42.5)	1.34 (0.75–4.35)	.392	1.21 (0.52–4.22)	.362
>30	104 (22.9)	1.67 (1.18–3.23)	.002	1.63 (1.16–3.39)	.001
Gestational age (wk)					
≤17	274 (60.4)	Reference		—	—
>17	180 (39.6)	1.21 (0.74–2.98)	.239	—	—
Cigarette smokers					
No	407 (89.6)	Reference		—	—
Yes	47 (10.4)	1.75 (0.48–6.26)	.246	—	—
APCH					
No	232 (51.1)	Reference		—	—
Yes	222 (48.9)	3.67 (0.35–7.26)	.578	—	—
Educational level (y)					
≤9	200 (44.1)	Reference		Reference	
>9	254 (55.9)	1.48 (1.12–4.28)	.001	1.87 (0.32–5.34)	.007
Parity					
0	169 (37.2)	Reference		Reference	
≥1	218 (48.0)	3.87 (1.37–7.39)	.001	2.87 (0.96–6.39)	.087
Hemoglobin (g/L)					
≥105	152 (33.5)	Reference		Reference	
<105	302 (66.5)	2.98 (1.21–5.23)	.001	2.28 (1.23–4.98)	.001

APCH, adverse pregnancy and childbirth history.

^aCrude odds and 95% CI in parentheses (all such values) of univariate logistic regression. $P < .05$ indicates significance.

^bAdjusted OR and 95% CI in parentheses (all such values) for multivariate logistic regression (the confounders adjusted in logistic regression include pregnancy type, age, body mass index, educational level, parity, and hemoglobin). $P < .05$ indicates significance.

trimester, consistent with our observations of decreased diastolic pressure. Decreased systolic/diastolic pressure, ALB, and hematocrit in TP implies peripheral resistance reduction and plasma volume expansion, which echoes the conclusion of previous studies.^{19,21,27} Moreover, ALB, hemoglobin, glucose, calcium, and phosphorus levels were reduced and triglyceride and cholesterol were increased in TP. Such results may be ascribed to the greater blood volume increase in TP than in SP.²⁷

Maternal VD Metabolism in SP and TP

The change of VD metabolism during pregnancy has long been an issue of concern in nutritional and clinical science. We detected epi-25(OH)D₃ in over 70% of the enrolled subjects, accounting for 0% to 37% of 25(OH)D concentration. In other research, Bailey and Di Mao²⁸ found

epi-25(OH)D₃ in 77% of Korean and 94.5% of Hong Kong pregnant women,²⁹ and in Ireland, O'Callaghan et al³⁰ found that epi-25(OH)D₃ was detectable in all maternal samples. We found epi-25(OH)D₃ was not only proportional to 25(OH)D₃, but also increased as gestational progress (FIGURE 1B). This result indicated the effect of pregnancy on the production of epi-25(OH)D₃. However, many immunological and mass spectrometry methods cannot exclude the interference and influence of epi-25(OH)D₃ in the detection of the 25(OH)D level, which often leads to overestimation of VD levels.¹⁴ Therefore, identifying epi-25(OH)D₃ with proper methodologies is of great significance for accurate evaluation of the “real” 25(OH)D status in pregnancy.

Currently, the change in maternal 25(OH)D during pregnancy is still controversial with suggestions of either increase,³¹ decrease,³² or

no change.³³ Based on our data, a mild but steady increase of 25(OH)D was observed with week of gestation (**FIGURE 1D**) for both TP and SP. To further clarify this phenomenon, we investigated the concentrations of the other 2 critical 25(OH)D-related components, VDBP and free 25(OH)D, from the same sample pool. In serum, 25(OH)D combined with VDBP accounts for the majority (85%–90%) of 25(OH)D. VDBP stabilizes the concentration and prolongs the half-life of 25(OH)D. Free 25(OH)D has been regarded as the bioavailable form of 25(OH)D, which functions the related activity in vivo.³⁴ As shown in **FIGURE 1**, these targets also revealed the increasing trend as 25(OH)D. We believed such results show that there is a gradual activation of 25(OH)D metabolism in the maternal biosystem. The mechanism behind this may be partly due to sex hormone variation and placenta development. It has been reported that estrogen can stimulate the synthesis of VDBP, and high estrogen level during pregnancy makes the concentration of VDBP in pregnant women 40% to 50% higher than that in a nonpregnant group.³⁴ In addition, human placental trophoblast in vitro could synthesize and release 25(OH)D₃ using its precursor, cholecalciferol,³⁵ and placental fragments incubated ex utero could convert vitamin D₂ to 25(OH)D₂.³⁶ Meanwhile, 24-hydroxylase mitochondrial cytochrome p450 enzyme CYP24A1 (converting 25(OH)D to inactive metabolites) expressed in the placenta is methylated during pregnancy, which may decelerate 25(OH)D consumption.³⁷

It is natural to think that the 25(OH)D level of TP would be lower than that of SP because TP seems to have a higher nutritional requirement that can result in depletion of 25(OH)D reserves. However, we were surprised to witness a significantly greater level of 25(OH)D in TP than in SP (15.18 [9.98–19.72] vs 11.05 [6.47–13.17] ng/mL, $P < .001$). Combining the experimental results discussed in the previous section, we speculate that there are 3 key factors attributed to this phenomenon. The first is that higher VDBP level in TP may lead to elevated 25(OH)D level. As shown in **FIGURE 1**, the increase of VDBP with gestational weeks seemed more efficient for TP than SP. Second, placentas in TP may play an important role in 25(OH)D level because placentas in TP are generally much heavier (69%) and mature within a shorter time than those of SP.^{38,39} Third, higher metabolic rates in TP (110%) can cause 40% greater increase in caloric requirements than in SP,⁴⁰ so that increased dietary intakes in TP may provide more VD resources.

According to our results, the baseline serum concentration of 25(OH)D was significantly higher in TP than SP and TP would be much less likely to incur VDD than SP (as discussed above) when using the currently established uniform VDD criteria. The VDBP is the major 25(OH)D carrier protein, and VDBP appears to inhibit some of the effects of 25(OH)D because the binding moiety may not be able to act on target cells.⁴¹ Low levels of VDBP in SP appear to result in equivalent free 25(OH)D levels to those in TP, so low total 25(OH)D levels may not be indicative of functional VDD. That is, despite lower 25(OH)D levels in the SP group, low levels of VDBP in SP may provide protection against manifestations of VDD. Therefore, free 25(OH)D may be a more appropriate marker of VDD. As shown in **TABLE 1**, when 25(OH)D and VDBP significantly differed between TP and SP, free 25(OH)D remained relatively similar. In this regard, apart from 25(OH)D, free 25(OH)D seems to be a promising indicator for VDD diagnosis. Certainly, further research is still needed to determine the actual reference range of 25(OH)D and free 25(OH)D in TP.

General Interpretations of VDD for All Enrolled Subjects

The prevalence of VDD in pregnant women in Asia is often reported to be highest globally,⁴² especially in Chinese women (>90%),^{5,43,44} as compared with the United States (27%–91%), Canada (39%–65%), Europe (19%–96%), Australia and New Zealand (25%–87%), Turkey (95.6%), Iran (89.4%), and Pakistan (89.0%).^{5,45} Meanwhile, as the pregnant mother is the only source of VD for fetuses, fetal (umbilical cord) serum 25(OH)D concentrations correlate well with maternal 25(OH)D concentrations, always being 50% to 89% of the mother's.³⁷ Given that fetuses are supposed to require 25(OH)D >12.0 ng/mL to prevent nutritional rickets,⁴⁶ prevention of maternal VDD at the threshold of 20.0 ng/mL (also known as the threshold of the Endocrine Society⁴⁷) may be adequate to ensure fetuses obtain sufficient 25(OH)D. Using this threshold, we found a high prevalence of VDD in both TP (149/236, 63.1%) and SP (198/218, 90.8%). Considering that China is a large country with diverse diet habits, such results may specifically represent the situation in the central area, where this study was conducted. As the sample collection period was confined to the summer (May to September) to minimize the effects of seasonal changes on 25(OH)D level, the situation could be worse in winter.

After stratified analysis, women aged ≥35 years were 2 to 3 times more likely to suffer from VDD for both SP and TP. The reason may be that 7-dehydrocholesterol (the precursor of vitamin D₃) level decreases with age, thus weakening capacity to produce vitamin D₃.⁴⁸ Advanced education level was associated with older mothers, which may be a dependent variable, as the correlation between VDD and education level was no longer significant in multivariable model. It has been well described that obesity is a risk factor for VDD,⁴⁹ which was confirmed in this study for both groups. Additionally, we found that anemia (hemoglobin <105 g/L) was associated with the risk of VDD in pregnant women. This may be ascribed to reduced outdoor activity and insufficient sun exposure caused by anemia-related illnesses and fatigue.^{20,21} We noticed that the use of VD supplements is not popular in China. Only a limited number of pregnant women used VD supplements during pregnancy in SP (9.2%) and TP (14.3%); another study in China reported only 10.04% of the participants used VD supplements,⁴⁴ compared with 31% in Canada and less than 40% on average.⁵⁰ As the use of VD supplements can effectively increase the 25(OH)D level of pregnant women and prevent VDD,^{51,52} it is urgent to promote and popularize VD supplementation for pregnant women in China.

Conclusion

This population-based study evaluated maternal 25(OH)D status in SP and TP. Differences in VD status were found in SP and TP, suggesting that the assessment of VD nutritional status in TP should be treated with caution. Also, this study found important determinants of VDD, including age, BMI, and hemoglobin levels. We demonstrated that epi-25(OH)D₃ was widespread in Chinese pregnant women, so it is important to eliminate its interference when evaluating 25(OH)D levels. Also, in addition to serum 25(OH)D, free 25(OH)D level should be assessed to make a comprehensive and accurate evaluation of VD status in TP. Furthermore, as the overall VDD rate is high among pregnant Chinese women, it is critical to understand the important determinants of VDD and take prompt corrective measures when VDD was confirmed. Future studies should consider the reference range of free 25(OH)D level and VD interventions in TP to achieve optimal healthy pregnancy and outcomes.

Limitations

Limitations of this study included a lack of data about sun exposure, type of clothing worn by study participants, levels of physical activity/exercise, and dietary VD intake. Also, the descriptive cross-sectional analysis was unable to determine the causal relationship between VDD and associated factors.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Santana DS, Silveira C, Costa ML, et al; WHO Multi-Country Survey on Maternal and Newborn Health Research Network. Perinatal outcomes in twin pregnancies complicated by maternal morbidity: evidence from the WHO Multicountry Survey on Maternal and Newborn Health. *BMC Pregnancy Childbirth*. 2018;18(1):449. doi:10.1186/s12884-018-2082-9
2. Luke B. Nutrition for multiples. *Clin Obstet Gynecol*. 2015;58(3):585–610. doi:10.1097/GRF.0000000000000117
3. Cyprian F, Lefkou E, Varoudi K, Girardi G. Immunomodulatory effects of vitamin D in pregnancy and beyond. *Front Immunol*. 2019;10:2739. doi:10.3389/fimmu.2019.02739
4. Owens NJ. Vitamin D supplementation for women during pregnancy: summary of a Cochrane review. *Explore (NY)*. 2019;16(1):73–74.
5. van der Pligt P, Willcox J, Szymlek-Ga EA, et al. Associations of maternal vitamin D deficiency with pregnancy and neonatal complications in developing countries: a systematic review. *Nutrients*. 2018;10(5):640.
6. Hillman LS, Haddad JG, Haddad MD. Human perinatal vitamin D metabolism I: 25hydroxyvitamin D in maternal and cord blood. *J Pediatr*. 1974;84(5):742–749.
7. Reddy GS, Norman AW, Willis DM, et al. Regulation of vitamin D metabolism in normal human pregnancy. *J Clin Endocrinol Metab*. 1983;56(2):363–370.
8. Okah FA, Tsang RC, Sierra R, Brady KK, Specker BL. Bone turnover and mineral metabolism in the last trimester of pregnancy: effect of multiple gestation. *Obstet Gynecol*. 1996;88(2):168–173. doi:10.1016/0029-7844(96)00159-7
9. Nakayama S, Yasui T, Suto M, et al. Differences in bone metabolism between singleton pregnancy and twin pregnancy. *Bone*. 2011;49(3):513–519. doi:10.1016/j.bone.2011.05.016
10. Bodnar LM, Rouse DJ, Momirova V, et al., Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Maternal-Fetal Medicine Units (MFMU) Network. Maternal 25-hydroxyvitamin d and preterm birth in twin gestations. *Obstet Gynecol*. 2013;122(1):91–98. doi:10.1097/AOG.0b013e3182941d9a
11. De La Calle M, García S Duque M, Bartha JL. Vitamin D concentrations are decreased in singleton and twin pregnancies. *Med Clin (Barc)*. 2016;147(8):371–372.
12. Goswami D, Rani R, Saxen A, et al. Maternal and neonatal vitamin-D status in twin versus singleton pregnancies. *J Obstet Gynaecol Res*. 2016;42(10):1250–1257.
13. Li X, Yu J, Wen L, et al. Vitamin D status in women with dichorionic twin pregnancies and their neonates: a pilot study in China. *BMC Pregnancy Childbirth*. 2021;21(1):279. doi:10.1186/s12884-021-03707-7
14. Singh RJ, Taylor RL, Reddy GS, Grebe SKG. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. *J Clin Endocrinol Metab*. 2006;91(8):3055–3061. doi:10.1210/jc.2006-0710
15. Vieira LA, Warren L, Pan S, et al. Comparing pregnancy outcomes and loss rates in elective twin pregnancy reduction with ongoing twin gestations in a large contemporary cohort. *Am J Obstet Gynecol*. 2019;221(3):253.e1253–253253.e8. doi:10.1016/j.ajog.2019.04.001
16. Le J, Yuan TF, Zhang Y, et al. New LC-MS/MS method with single-step pretreatment analyzes fat-soluble vitamins in plasma and amniotic fluid. *J Lipid Res*. 2018;59(9):1783–1790.
17. Ross AC, Taylor CL, Yaktine AL, et al. *Dietary Reference Intakes for Calcium and Vitamin D, Institute of Medicine (US) Committee*. Washington, DC: National Academies Press (US). 2011;2:54–96.
18. Hektoen HH, Rødsahl TE, Stenehjem JS, et al. Vitamin D and vitamin D-binding protein and risk of bladder cancer: a nested case-control study in the Norwegian Janus Serum Bank Cohort. *Cancer Med*. 2021;10(12):4107–4116. doi:10.1002/cam4.3960
19. Kominarek MA, Rajan P. Nutrition recommendations in pregnancy and lactation. *Med Clin North Am*. 2016;100(6):1199–1215. doi:10.1016/j.mcna.2016.06.004
20. James AH. Iron deficiency anemia in pregnancy. *Obstet Gynecol*. 2021;138(4):663–674. doi:10.1097/AOG.0000000000004559
21. Shinar S, Shapira U, Maslovitz S. Redefining normal hemoglobin and anemia in singleton and twin pregnancies. *Int J Gynaecol Obstet*. 2018;142(1):42–47. doi:10.1002/ijgo.12506
22. Santana DS, Souza RT, Surita FG, et al. Twin pregnancy in Brazil: a profile analysis exploring population information from the national birth e-registry on live births. *Biomed Res Int*. 2018;1:9189648. doi:10.1155/2018/9189648
23. Gandhi M, Gandhi R, Mack LM, et al. Estimated energy requirements increase across pregnancy in healthy women with dichorionic twins. *Am J Clin Nutr*. 2018;108(4):775–783. doi:10.1093/ajcn/nqy184
24. Gandhi M, Gandhi R, Mack LM, et al. Impact of changes in maternal body composition on birth weight and neonatal fat mass in dichorionic twin pregnancies. *Am J Clin Nutr*. 2018;108(4):716–721. doi:10.1093/ajcn/nqy180
25. McLennan AS, Gyamfi-Bannerman C, Ananth CV, et al. The role of maternal age in twin pregnancy outcomes. *Am J Obstet Gynecol*. 2017;217(1):80.e180–8080.e8. doi:10.1016/j.ajog.2017.03.002
26. Luke B, Martin JA. The rise in multiple births in the United States: who, what, when, where, and why. *Clin Obstet Gynecol*. 2004;47(1):118–133. doi:10.1097/00003081-200403000-00016
27. Umazume T, Yamada T, Furuta I, et al. Morphofunctional cardiac changes in singleton and twin pregnancies: a longitudinal cohort study. *BMC Pregnancy Childbirth*. 2020;20(1):750. doi:10.1186/s12884-020-03452-3
28. Bailey D, Perumal N, Yazdanpanah M, et al. Maternal-fetal-infant dynamics of the C3-epimer of 25-hydroxyvitamin D. *Clin Biochem*. 2014;47(9):816–822. doi:10.1016/j.clinbiochem.2014.01.015
29. Mao D, Yuen LY, Ho CS, et al. Maternal and neonatal 3-epi-25-hydroxyvitamin D concentration and factors influencing their concentrations. *J Endocr Soc*. 2022;6(1):170.
30. O'Callaghan KM, Hennessy A, Hull G, et al. Estimation of the maternal vitamin D intake that maintains circulating 25-hydroxyvitamin D in late gestation at a concentration sufficient to keep umbilical cord sera ≥25–30 nmol/L: a dose-response, double-blind, randomized placebo-controlled trial in pregnant women at northern latitude. *Am J Clin Nutr*. 2018;108(1):7–91.
31. Jones KS, Meadows SR, Schoenmakers I, Prentice A, Moore SE. Vitamin D status increases during pregnancy and in response to vitamin D supplementation in rural Gambian women. *J Nutr*. 2020;150(3):492–504. doi:10.1093/jn/nxz290
32. Zhang JY, Lucey AJ, Horgan R, Kenny LC, Kiely M. Impact of pregnancy on vitamin D status: a longitudinal study. *Br J Nutr*. 2014;112(7):1081–1087. doi:10.1017/S0007114514001883
33. Gustafsson MK, Romundstad PR, Stafne SN, et al. Alterations in the vitamin D endocrine system during pregnancy: a longitudinal study of 855 healthy Norwegian women. *PLoS One*. 2018;13(4):e0195041.

doi:[10.1371/journal.pone.0195041](https://doi.org/10.1371/journal.pone.0195041)

34. Jassil NK, Sharma A, Bikle D, Wang X. Vitamin D binding protein and 25-hydroxyvitamin D levels: emerging clinical applications. *Endocr Pract.* 2017;23(5):605–613. doi:[10.4158/EP161604.RA](https://doi.org/10.4158/EP161604.RA)
35. Park H, Wood MR, Malysheva OV, et al. Placental vitamin D metabolism and its associations with circulating vitamin D metabolites in pregnant women. *Am J Clin Nutr.* 2017;106(6):1439–1448. doi:[10.3945/ajcn.117.153429](https://doi.org/10.3945/ajcn.117.153429)
36. Slominski AT, Kim TK, Shehabi HZ, et al. In vivo production of novel vitamin D2 hydroxy-derivatives by human placentas, epidermal keratinocytes, Caco-2 colon cells and the adrenal gland. *Mol Cell Endocrinol.* 2014;383(1-2):181–192. doi:[10.1016/j.mce.2013.12.012](https://doi.org/10.1016/j.mce.2013.12.012)
37. Dovnik A, Mujezinović F. The association of vitamin D levels with common pregnancy complications. *Nutrients.* 2018;10(7):867. doi:[10.3390/nu10070867](https://doi.org/10.3390/nu10070867)
38. Filipecka-Tyczka D, Jakiel G, Kajdy A, Rąbiejewski M. Is growth restriction in twin pregnancies a double challenge? A narrative review. *J Mother Child.* 2021;24(4):24–30. doi:[10.34763/jmotherandchild.20202404.d-20-00016](https://doi.org/10.34763/jmotherandchild.20202404.d-20-00016)
39. Roselló-Soberón ME, Fuentes-Chaparro L, Casanueva E. Twin pregnancies: eating for three? Maternal nutrition update. *Nutr Rev.* 2005;63(9):295–302. doi:[10.1111/j.1753-4887.2005.tb00144.x](https://doi.org/10.1111/j.1753-4887.2005.tb00144.x)
40. Koletzko B, Godfrey KM, Poston L, et al; Early Nutrition Project Systematic Review Group. Nutrition during pregnancy, lactation and early childhood and its implications for maternal and long-term child health: the early nutrition project recommendations. *Ann Nutr Metab.* 2019;74(2):93–106. doi:[10.1159/000496471](https://doi.org/10.1159/000496471)
41. Powe CE, Evans MK, Wenger J, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. *N Engl J Med.* 2013;369(21):1991–2000. doi:[10.1056/NEJMoa1306357](https://doi.org/10.1056/NEJMoa1306357)
42. Choi R, Kim S, Yoo H, et al. High prevalence of vitamin D deficiency in pregnant Korean women: the first trimester and the winter season as risk factors for vitamin D deficiency. *Nutrients.* 2015;7(5):3427–3448. doi:[10.3390/nu7053427](https://doi.org/10.3390/nu7053427)
43. Hong-Bi S, Yin X, Xiaowu Y, et al. High prevalence of vitamin D deficiency in pregnant women and its relationship with adverse pregnancy outcomes in Guizhou, China. *J Int Med Res.* 2018;46(11):4500–4505. doi:[10.1177/0300060518781477](https://doi.org/10.1177/0300060518781477)
44. Yun C, Chen J, He Y, et al. Vitamin D deficiency prevalence and risk factors among pregnant Chinese women. *Public Health Nutr.* 2017;20(10):1746–1754. doi:[10.1017/S1368980015002980](https://doi.org/10.1017/S1368980015002980)
45. Cashman KD, Dowling KG, Škrabáková Z, et al. Vitamin D deficiency in Europe: pandemic? *Am J Clin Nutr.* 2016;103(4):1033–1044. doi:[10.3945/ajcn.115.120873](https://doi.org/10.3945/ajcn.115.120873)
46. O'Callaghan KM, Kiely ME. Ethnic disparities in the dietary requirement for vitamin D during pregnancy: considerations for nutrition policy and research. *Proc Nutr Soc.* 2018;77(2):164–173. doi:[10.1017/S0029665117004116](https://doi.org/10.1017/S0029665117004116)
47. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited. *J Clin Endocrinol Metab.* 2012;97(4):1153–1158. doi:[10.1210/jc.2011-2601](https://doi.org/10.1210/jc.2011-2601)
48. Wyskida M, Wiecezowska-Tobis K, Chudek J. Prevalence and factors promoting the occurrence of vitamin D deficiency in the elderly. *Postepy Hig Med Dosw.* 2017;71(0):0198–0190. doi:[10.5604/01.3001.0010.3804](https://doi.org/10.5604/01.3001.0010.3804)
49. Larqué E, Morales E, Leis R, Blanco-Carnero JE. Maternal and foetal health implications of vitamin D status during pregnancy. *Ann Nutr Metab.* 2018;72(3):179–192. doi:[10.1159/000487370](https://doi.org/10.1159/000487370)
50. Cashman KD. Vitamin D deficiency: defining, prevalence, causes, and strategies of addressing. *Calcif Tissue Int.* 2020;106(1):14–29. doi:[10.1007/s00223-019-00559-4](https://doi.org/10.1007/s00223-019-00559-4)
51. Palacios C, Trak-Fellermeier MA, Martinez RX, et al. Regimens of vitamin D supplementation for women during pregnancy. *Cochrane Database Syst Rev.* 2019;10(10):CD013446.
52. Maugeri A, Barchitta M, Blanco I, Agodi A. Effects of vitamin D supplementation during pregnancy on birth size: a systematic review and meta-analysis of randomized controlled trials. *Nutrients.* 2019;11(2):442. doi:[10.3390/nu11020442](https://doi.org/10.3390/nu11020442)

Autosomal Recessive Renal Hypouricemia Type 2 Presenting with Childhood Stroke in a Sri Lankan Child

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Abbreviations: MRA, magnetic resonance angiography; ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; HRH, hereditary renal hypouricemia; AKI, acute kidney injury

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Patient History

A previously healthy 14-year-old girl who was the youngest of 4 siblings with no parental consanguinity presented with sudden onset right-sided upper limb and lower limb weakness, unsteady gait, and slurred speech. On examination, there was right-sided upper motor facial nerve palsy. A brain MRI revealed a left-sided parietal lobe infarction.

In the complete blood count and peripheral blood smear there was a neutrophil leukocytosis with mild left shift maturation. More than 5% of neutrophils displayed hypersegmentation. Platelets and red blood cells were normal, both quantitatively and morphologically, except for a few teardrop-shaped cells. The overall picture indicated vitamin B₁₂ deficiency. There were no features suggestive of iron deficiency. Renal function and electrolytes were within age-matched intervals. Plasma glucose level was 4.8 mmol/L. The infection markers, including cerebrospinal fluid analysis, were normal. The coagulation screen performed as international normalized ratio/prothrombin time, activated partial thromboplastin time, and fibrinogen were unremarkable (TABLES 1 and 2).

Investigations to find the etiology of the stroke included a 2-dimensional echocardiogram, carotid duplex, and brain magnetic resonance angiography (MRA); all were normal except in the MRA of the circle of Willis, a minimal narrowing of the distal left middle cerebral artery was noted when compared with the right, querying a focal vasculitis. The screen for lupus anticoagulant did not give evidence of the stroke etiology. The lipid profile performed on the follow-up clinic visit was within age and gender matched reference intervals.

With the impression favoring vitamin B₁₂ or folate deficiency in the initial blood smear, investigation progressed with plasma

homocysteine found to be 9.57 µmol/L (4.6–8.1) and serum vitamin B₁₂ was 404 pmol/L (140–650). Sequence analysis of the *MTHFR* gene revealed a heterozygous variant C > T (*MTHFR* 677 C > T) and was considered unlikely to cause hyperhomocysteinemia by itself.

However, in a follow-up visit as part of the metabolic screening performed at the laboratory, the general biochemistry results indicated a very low uric acid level, 26 µmol/L (119–327), and subsequent analysis confirmed the finding with a value of 8 µmol/L (119–327). Urine uric acid to creatinine ratio was 0.33 mmol/mmol (0.2–0.4) and fractional excretion of uric acid was 268% (6%–20%). The repeated urine studies confirmed values of 0.27 mmol/mmol and 576%. Hereditary renal hypouricemia was suspected and genetic testing using exome sequencing revealed a homozygous variant in the *SLC2A9* gene: c.646G > A p.(Gly216Arg), classified as likely pathogenic (class 2) according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) in addition to ClinGen SVI and therefore confirming a diagnosis of hereditary renal hypouricemia (HRH) type 2.¹

Discussion

Hereditary renal hypouricemia is an underdiagnosed disease. Isolated defects in renal urate transporters cause hyperuricosuria and ultimately result in hypouricemia. Two transporters are mainly involved, URAT1 transporter coded by the *SCL22A12* gene, resulting in hereditary renal hypouricemia type I (OMIM: 220150), and GLUT 9 transporter coded by the *SLC2A9* gene, causing HRH type 2 (OMIM:612076).²

Two isoforms are coded by the *SLC2A9* gene, the long GLUT 9 isoform in the proximal tubule basolateral membrane and the short isoform in the collecting tubule apical membrane, both having slight functional differences.³ The GLUT 9 transporter, which is a type of facilitated glucose transporter, is considered the most potent transporter of uric acid.⁴ Hereditary renal hypouricemia type 2 can have both autosomal recessive and dominant pattern of inheritance. The most severe form results from the homozygous loss-of-function variant, which completely prevents uric acid reabsorption by all apical transporters, including URAT 1.⁵

Most patients are asymptomatic, especially in the pediatric group. Some manifest as having micro- and macroscopic hematuria due to nephrolithiasis, and another category of patients presents with exercise or dehydration-induced acute kidney injury (AKI). Association

TABLE 1. Hematological Investigations

Test	Result	Reference Interval
Total white blood cell count, cells/ μ L	36,700	4000–11,000
Neutrophils, %	72.6	50–70
Hemoglobin, g/dL	13.5	11–16
Mean corpuscular volume, fL	85.3	80–100
Mean corpuscular hemoglobin, pg	28.4	27–34
Platelet count, cells/ μ L	383,000	150,000–450,000
Erythrocyte sedimentation rate, mm/h	11	<20
Prothrombin time, sec	10.8	Control 10.6
Fibrinogen (antigen), g/L	2.9	1.5–4
Fibrinogen Clauss assay, g/dL	270	200–400
Prothrombin time, sec	10.8	Control 10.6
Sickling test	Negative	
Thrombophilia screening (ANA, DS DNA, PANCA, CANCA)	Negative	

of posterior reversible encephalopathy was also documented with the AKI.⁶

In children above the age of 1 year, hypouricemia is defined as less than 119 μ mol/L (2 mg/dL). Hypouricemia results from impaired production, increased oxidation due to uricase treatment, or reduced reabsorption due to hereditary or acquired conditions.⁷ In our patient, hypouricemia resulted from impaired absorption of uric acid due to loss of function of GLUT 9.

Hereditary renal hypouricemia type 2 patients show marked hypouricemia with increased fractional excretion of uric acid compared with type 1 patients. Our patient also had a lowest value of 8 μ mol/L, in line with the documented values ranging from 0.6 to 40 μ mol/L (0.01–0.67 mg/dL) and a highest fractional excretion of uric acid value of 576% as compared with the 17% to 296% range.⁶

The missense variant c.646G > A p.(Gly216Arg) in the *SLC2A9* gene identified in our patient resulted in an amino acid change from highly conserved glycine to arginine at position 216. This has been previously reported in the literature in affected patients in homozygosity^{8,9} and compound heterozygosity^{9,10} where patients presented with severe exercise-induced AKI. Maalouli et al⁸ reported on a family of four individuals harboring the variant in homozygous state, of whom only the index patient was affected and presented with AKI after exercise secondary to familial renal hypouricemia type 2. The siblings and mother were asymptomatic and not further evaluated.⁸ In vitro functional studies have also revealed reduced urate uptake efficiency in the GLUT 9 with Gly216Arg defect.¹¹ According to ACMG, AMP, and ClinGen SVI, the variant is considered likely pathogenic (PP3, PP4, PM3).

Hyperuricemia is a recognized marker of obesity, metabolic syndrome, and renal disease due to the proinflammatory action of urate radicals produced when interacting with oxidizing agents.¹² However, a J-shaped relationship has been demonstrated with uric acid levels and cardiovascular events, indicating an association of hypouricemia in the pathogenesis.¹³

Oxidative stress plays a main role in the development of cardiovascular disease. Uric acid, on the other hand, is proposed as a prominent low-molecular-mass antioxidant in the body, accounting for about 60% in plasma. The antioxidant action as an electron donor may provide protection to the tissue in equilibrium with blood as well. Thus, a

TABLE 2. Biochemical Investigations

Test	Result	Reference Interval
Total cholesterol, mg/dL	166	111–202
Triglyceride, mg/dL	63	45–210
High density lipoprotein, mg/dL	50	31–68
Low density lipoprotein (indirect), mg/dL	103	<110
Plasma glucose (fasting), mmol/L	5	3.8–5.6
Total protein, g/L	62	64–83
Albumin, g/L	36	34–50
Plasma homocysteine, μ mol/L	9.57	4.8–8.1
Vitamin B ₁₂ , pmol/L	404	140–650
Serum uric acid, μ mol/L	8	119–327
Urine uric acid: creatinine, mmol/mmol	0.33	0.2–0.4
Fractional excretion of uric acid, %	268	6–20

protective role of uric acid in oxidative stress is suggested.¹⁴ Histological and imaging results of renal biopsies suggestive of repeated vasoconstriction in GLUT 9 mutation patients with AKI are evidence for the oxidative stress and vasoconstriction hypothesis.^{15,16}

In a Japanese study, hypouricemic patients with HRH type 1 showed endothelial dysfunction, which is an initiating step in atherosclerosis leading to cardiovascular events.¹⁷ Considering the more severe hypouricemia seen in HRH type 2, a far more significant endothelial dysfunction is to be expected in these patients.

To the best of our knowledge, ischemic stroke in a patient with HRH type 2 has not been reported. Extensive evaluation was done on our patient to find an etiology for the ischemic stroke, with no significant finding. With the evidence of endothelial dysfunction associated with severe hypouricemia, HRH type 2 could be hypothesized as the etiology. However, according to the external database gnomAD, the 0.5% frequency of the variant reported in the South Asian population,¹⁸ and some among them being asymptomatic, other unknown genomic variants or environmental factors should also be considered as the cause.

Conclusion

This case report discusses an unusual presentation in a patient with HRH type 2 and a rare probable causal relationship of ischemic stroke. This also stresses the importance of performing general biochemistry, including uric acid, in evaluating inborn errors of metabolism.

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Conflict of Interest Disclosure

S.S. and C.B. are employees of Centogene GmbH (Rostock, Germany).

REFERENCES

- Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med Off J Am Coll Med Genet*. 2015;17(5):405–424.

2. Sperling O. Hereditary renal hypouricemia. *Mol Genet Metab.* 2006;89(1):14–18. doi:[10.1016/j.ymgme.2006.03.015](https://doi.org/10.1016/j.ymgme.2006.03.015).
3. Anzai N, Ichida K, Jutabha P, et al. Plasma urate level is directly regulated by a voltage-driven urate efflux transporter URATv1 (SLC2A9) in humans. *J Biol Chem.* 2008;283(40):26834–26838. doi:[10.1074/jbc.c800156200](https://doi.org/10.1074/jbc.c800156200).
4. Vitart V, Rudan I, Hayward C, et al. SLC2A9 is a newly identified urate transporter influencing serum urate concentration, urate excretion and gout. *Nat Genet.* 2008;40(4):437–442.
5. Dinour D, Gray NK, Campbell S, et al. Homozygous SLC2A9 mutations cause severe renal hypouricemia. *J Am Soc Nephrol.* 2010;21(1):64–72.
6. Peris Vidal A, Marin Serra J, Lucas Sáez E, et al. Hereditary renal hypouricaemia type 1 and 2 in three Spanish children: review of published paediatric cases. *Nefrol Engl Ed.* 2019;39(4):355–361.
7. Maesaka JK, Fishbane S. Regulation of renal urate excretion: a critical review. *Am J Kidney Dis Off J Natl Kidney Found.* 1998;32(6):917–933.
8. Maalouli C, Dahan K, Devresse A, Gillion V. Mutation in the SLC2A9 gene: a new family with familial renal hypouricemia type 2. *Case Rep Nephrol.* 2021;4751099. doi:[10.1155/2021/4751099](https://doi.org/10.1155/2021/4751099).
9. Stiburkova B, Taylor J, Marinaki AM, Sebesta I. Acute kidney injury in two children caused by renal hypouricaemia type 2. *Pediatr Nephrol Berl Ger.* 2012;27(8):1411–1415.
10. Jeannin G, Chiarelli N, Gaggiotti M, et al. Recurrent exercise-induced acute renal failure in a young Pakistani man with severe renal hypouricemia and SLC2A9 compound heterozygosity. *BMC Med Genet.* 2014;15:3. doi:[10.1186/1471-2350-15-3](https://doi.org/10.1186/1471-2350-15-3).
11. Ruiz A, Gautschi I, Schild L, Bonny O. Human mutations in SLC2A9 (Glut9) affect transport capacity for urate. *Front Physiol.* 2018;9:476. doi:[10.3389/fphys.2018.00476](https://doi.org/10.3389/fphys.2018.00476).
12. Sharaf El Din UAA, Salem MM, Abdulazim DO. Uric acid in the pathogenesis of metabolic, renal, and cardiovascular diseases: a review. *J Adv Res.* 2017;8(5):537–548.
13. Verdecchia P, Schillaci G, Reboldi G, Santeusano F, Porcellati C, Brunetti P. Relation between serum uric acid and risk of cardiovascular disease in essential hypertension: the PIUMA study. *Hypertens Dallas Tex* 1979. 2000;36(6):1072–1078.
14. Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc Natl Acad Sci U S A.* 1981;78(11):6858–6862.
15. Windpessl M, Ritelli M, Wallner M, Colombi M. A novel homozygous SLC2A9 mutation associated with renal-induced hypouricemia. *Am J Nephrol.* 2016;43(4):245–250. doi:[10.1159/000445845](https://doi.org/10.1159/000445845).
16. Nishida H, Kaida H, Ishibashi M, Baba K, Kouno K, Okuda S. Evaluation of exercise-induced acute renal failure in renal hypouricemia using Tc-99m DTPA renography. *Ann Nucl Med.* 2005;19(4):325–329. doi:[10.1007/bf02984627](https://doi.org/10.1007/bf02984627).
17. Sugihara S, Hisatome I, Kuwabara M, et al. Depletion of uric acid due to SLC22A12 (URAT1) loss-of-function mutation causes endothelial dysfunction in hypouricemia. *Circ J Off J Jpn Circ Soc.* 2015;79(5):1125–1132.
18. 4-9982251-C-T | gnomAD v2.1.1 | gnomAD. Available at: <https://gnomad.broadinstitute.org/variant/4-9982251-C-T>.

Comprehensive Next-Generation Sequencing Testing in a Patient with TEMPI Syndrome

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Keywords: plasma cells, paraproteinemia, monoclonal gammopathy, MGUS, polycythemia, erythropoietin, erythrocytosis

Abbreviations: TEMPI, telangiectasia-erythrocytosis with elevated erythropoietin-monoclonal gammopathy-perinephric collections-intrapulmonary shunting; JAK2, Janus kinase 2; NGS, next-generation sequencing; BFU-E, bone marrow burst-forming unit-erythroid; EPO, erythropoietin; PCR, polymerase chain reaction; MGUS, monoclonal gammopathy of uncertain significance; HIF, hypoxia-inducible factor.

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ABSTRACT

TEMPI syndrome is a new and poorly understood disease that is currently considered a type of plasma cell neoplasm with paraneoplastic manifestations. The TEMPI acronym defines the hallmarks of the syndrome: T for telangiectasia; E for erythrocytosis with elevated erythropoietin; M, monoclonal gammopathy; P, perinephric collections; and I, intrapulmonary shunting. Due to the marked erythrocytosis as the most common presenting feature, TEMPI is often misdiagnosed as polycythemia vera. However, unlike polycythemia vera, TEMPI is not associated with a *JAK2* mutation. The pathogenesis of TEMPI syndrome is unknown, although a few hypothetical disease mechanisms have been previously discussed. Here we present a new case of TEMPI syndrome, discuss results of a next-generation sequencing (NGS) panel covering 1,425 known cancer-related genes, and review the current literature with focus on an update of the genetics of TEMPI syndrome. This is the first report of TEMPI that includes results of comprehensive NGS testing.

Case Report

A 53-year-old nonsmoking male was first referred to a hematologist in 2006 to evaluate for unexplained erythrocytosis. The initial complete

blood cell count showed a hemoglobin level of 19.8 g/dL with normal leukocyte and platelet counts. After an initial hematological work-up and the identification of positive spontaneous erythroid colony formation in vitro (bone marrow burst-forming unit-erythroid [BFU-E] and colony-forming unit-erythroid), a diagnosis of polycythemia vera (PV) was made. The patient initiated treatment with phlebotomies every 2 to 3 months and aspirin 100 mg daily. In March 2007, the patient developed thrombosis of the left subclavian artery, which was treated with percutaneous transluminal angioplasty and stent implementation. After 3 weeks, he developed thrombosis of the left brachialis artery with ischemia in the left-hand fingers and was treated by surgical thrombectomy followed by anticoagulation.

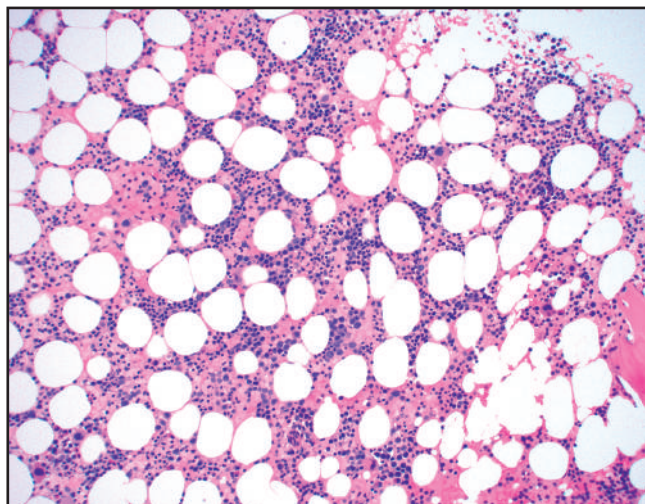
After 12 years, the patient presented for a repeat hematologic work-up. Facial and trunk telangiectasias were noted on examination (**FIGURE 1**). Laboratory evaluation of peripheral blood revealed a normal complete blood cell count, a regular mean corpuscular volume despite frequent phlebotomies, and a normal lactate dehydrogenase level. An IgG kappa monoclonal gammopathy was detected on serum protein electrophoresis (M-spike of 2.3 g/dL) and an extremely elevated level of serum erythropoietin (EPO) at 444 IU/mL. The *JAK2* mutation analysis by polymerase chain reaction (PCR) did not reveal *JAK2* variants. An abdominal ultrasound showed no hepatosplenomegaly. Abdominal and pelvic computed tomography scan revealed the existence of bilateral perirenal fibrosis and small fluid collections. Spirometry was normal ($SO_2 = 98\%$), whereas diffusing capacity for carbon monoxide showed reduced oxygen diffusion (71% of predicted). A positron emission tomography scan showed an increased accumulation of fluorodeoxyglucose around the proximal right femur (SUV4.2), moderate accumulation of fluorodeoxyglucose in the bones of axial skeleton, and a fluid collection in the perirenal left anterior dimension of 27 × 73 × 77 mm. Magnetic resonance imaging showed an osteolytic lesion of the proximal edge of the right femur measuring 32 mm in diameter.

A bone marrow biopsy was performed. A Wright Giemsa bone marrow aspirate smear demonstrated maturing trilineage hematopoiesis without dysplasia and a normal percentage of plasma cells. Histologic examination of H&E-stained sections of the bone marrow clot revealed normocellular marrow particles (~40% cellularity) with a slight erythroid predominance (**FIGURE 2**). Immunohistochemical stains showed an increase in CD138-positive plasma cells at 20% (**FIGURE 3**). Culture of hematopoietic progenitors from peripheral blood indicated the presence of BFU-E colonies. However, when the colonies

FIGURE 1. Telangiectasias, noted bilaterally over the patient's face, neck, and trunk.



FIGURE 2. Bone marrow biopsy findings. The bone marrow is overall normocellular for age (40% cellularity, 65 years of age) with a predominance of erythroids (mononuclear cells with round nuclei and condensed chromatin) (H&E, $\times 200$).

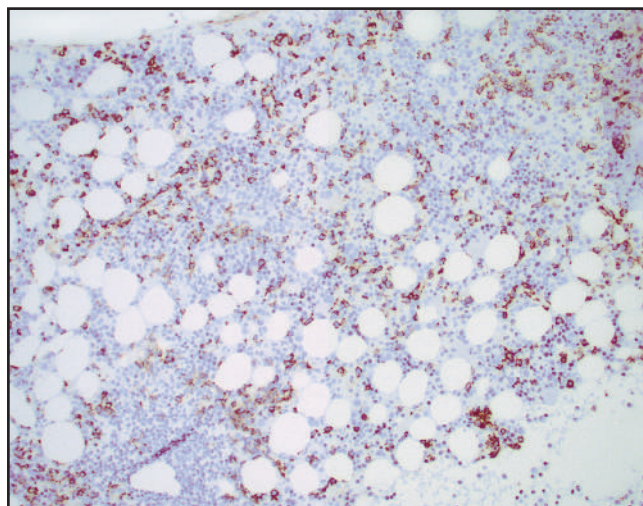


were “picked” (separated from the culture) and May-Grünwald Giemsa-stained, they were shown to consist of mature mutually agglutinated erythrocytes and not the erythroblasts typical of BFU-E colonies.

Chromosome analysis of bone marrow aspirate material showed the reference male karyotype (46, XY). A fluorescence in situ hybridization panel to detect the presence of myeloma-associated cytogenetic abnormalities t(4;14), t(14;16), del(1p), del(11q), del(13q), del(17p), dup(1q) was performed and was normal. In light of the above clinical and laboratorial findings, the diagnosis of TEMPI syndrome was established.

In an attempt to further characterize the genetics of TEMPI, pre-treatment bone marrow material was sent for targeted next-generation sequencing (NGS) analysis. Patient consent was obtained on an institutional review board-approved study (protocol #STU122013-023). A total of 5 unstained slides were cut at 10 μ m thickness from nondecalcified formalin-fixed paraffin-embedded (FFPE) bone marrow

FIGURE 3. A CD138 immunostain is positive in plasma cells, demonstrating an increase at approximately 20% of the marrow cellularity ($\times 200$).



clot preparation. The specimen passed DNA quality control metrics preestablished for clinical practice in the NGS laboratory. The NGS study used a custom panel of DNA probes covering 1425 known cancer-related genes, which includes genes commonly involved in the pathogenesis of myeloproliferative neoplasms and other hematolymphoid neoplasms (see supplemental figure). DNA was extracted using Qiagen Allprep kit, the library preparation was done using Roche Kapa Hyperprep kits, and gene selection was performed by hybridization using Roche Nimblegen baits. Gene sequencing was performed on an Illumina NextSeq 550 and bioinformatics used a custom pipeline as previously described.¹ Variants were reviewed and curated in an inhouse software suite.² Quality metric targets were >90% of all exons in the panel have at least 100 $\times$ coverage and a mean coverage of $\geq 300\times$. This study revealed no genetic alterations in the 1425 genes. Of note, specific germline genetic testing was not performed for our patient.

In August 2019, the patient received radiotherapy (30 Gy in 10 sessions) to treat the femoral osteolytic lesion. Annual bone marrow evaluations revealed no evidence of disease progression with a stable level of plasma cell bone marrow infiltration (20%–25%). Levels of serum paraprotein (2.1–2.3 g/mL) and EPO (200–300 mU/mL) remained stable. Treatment with daratumumab is now planned due to a decrease in glomerular filtration, an increase in the left-sided perirenal fluid collection to 105 $\times$ 64 $\times$ 63 mm, and a newly formed right-sided fluid collection of 35 $\times$ 30 mm.

Discussion

TEMPI syndrome was described in 2011 and is defined by the presence of telangiectasia, erythrocytosis with elevated EPO, monoclonal gammopathy, perinephric fluid collections, and intrapulmonary shunting.³ It has been recently listed as a provisional entity under “plasma cell neoplasms with paraneoplastic manifestations” in the revised 4th edition and upcoming 5th edition of the WHO Classification of Hematopoietic Tumors.^{4,5} To date there are at least 26 patients identified with TEMPI worldwide.^{6–14} The plasma cell burden in the majority of patients with TEMPI is generally at the level of monoclonal

gammopathy of uncertain significance (MGUS) (serum M-spike <3 g/dL and <10% clonal marrow plasma cells), but may increase over time.^{3,10} The TEMPI syndrome patients described to date have had negative *JAK2* V617 and exon 12 mutation analyses by PCR.^{6,7,9–12,14}

Whereas most reports of TEMPI include negative *JAK2* mutation analysis, this is the first report of a NGS comprehensive panel covering 1425 known cancer-related genes in TEMPI. Our analysis of paraffin-embedded nondecalfied bone marrow material reveals no pathogenic variants. A *JAK2* mutation analysis is helpful in suspected PV when a positive *JAK2* mutation analysis could be diagnostic even when other signs of TEMPI are present.¹⁵ However, some authors indicate that in the setting of erythrocytosis with known coexisting monoclonal gammopathy, *JAK2* mutation analysis is of questionable utility.¹⁶ The rare (<5%) cases of PV negative for *JAK2* V617F and exon 12 mutations are likely positive for other activating mutations in genes commonly involved in PV, such as *TET2*.¹⁷ None of these mutations were detected in our study, effectively ruling out the possibility of myeloid neoplasms.

In addition to PV, the differential diagnosis of TEMPI may include other diseases in which erythrocytosis is a prominent feature. In such instances, the level of serum EPO can be helpful to narrow down the possibilities. In PV, primary congenital erythrocytosis, and types of secondary congenital erythrocytosis caused by *PHD2* mutations, EPO is at normal to decreased levels.^{18–20} In TEMPI, the level of serum EPO correlates with severity of hypoxemia and may reach levels as high as ~8000 mU/mL (median EPO level in TEMPI, 500 mU/mL).²¹ Although a few TEMPI patients have shown EPO levels of <100 mU/mL,¹³ the strikingly high level of EPO seen in most TEMPI patients is a distinctive sign of TEMPI when compared with many disorders with elevated EPO. In secondary hypoxemia and iatrogenic cases related to phlebotomy, for example, EPO is typically not higher than 30 mU/mL.²¹ In secondary congenital erythrocytosis, patients characteristically present with elevated EPO levels but the level of EPO appears to be <400 mU/mL.²² A notable exception are disorders associated with mutations in the *VHL* gene. In these disorders, there is upregulation and stabilization of hypoxia-inducible factor (HIF) resulting in levels of EPO as high as 3000 mU/mL, similar to levels seen in TEMPI.²³ A detailed history is also an important part of the clinical evaluation because, unlike congenital erythrocytosis,¹⁸ TEMPI patients identified so far tend to be older and do not have a family history of the disease. The NGS panel we performed for our patient did not include genes associated with congenital erythrocytosis.

In this patient, cytogenetics shows no structural or numerical cytogenetic abnormalities or cancer-related mutations (eg, *MyD88*, *NRAS*, *KRAS*, or *BRAF*). Similar to our results, mutations and cytogenetic abnormalities associated with MGUS and/or symptomatic plasma cell myeloma (PCM), including abnormalities related to disease progression, have not been identified in TEMPI in the literature.^{5,12} Mirroring the genetic findings, the quantification of monoclonal protein and percentage of plasma cells in TEMPI are typically at the level of MGUS/asymptomatic PCM.^{3,6,21} There are few reports of TEMPI-related signs and symptoms, specifically erythrocytosis and/or perinephric collections, in patients with presumed diagnosis of “multiple myeloma”; however, it was unclear based on the reports whether the patients had definitive end-organ damage and high burden disease.^{24,25} Cases of erythrocytosis coexisting with “myeloma” had been described in the literature prior to the recogni-

tion of TEMPI,^{25,26} but it is unclear how many of these patients had symptomatic PCM as per current diagnostic criteria.⁵ Also unclear is the pathogenetic relationship between TEMPI and other plasma cell neoplasms such as light chain amyloidosis or POEMS (polyneuropathy, organomegaly, endocrinopathies, monoclonal protein, skin changes). Given that the majority of well-characterized TEMPI patients have had asymptomatic PCM/MGUS and normal cytogenetics, an alternative etiology for any TEMPI-related signs may be considered when arising in patients with clinical and/or genetic profiles of symptomatic PCM. Interestingly, a recent publication describes the presence of TEMPI symptoms in a patient with *MyD88*-mutated, IgM-positive lymphoplasmacytic lymphoma,¹¹ suggesting that TEMPI symptoms may be seen in a broader range of plasmacytic neoplasms and B-cell lymphomas with plasmacytic differentiation.

Given the low number of TEMPI patients, detailed studies of the genetics of the disease are limited. In the literature, postmortem whole exome sequencing of peripheral blood showed no abnormalities.⁸ More recently, a study of genomic alterations in TEMPI using whole genome sequencing monoclonal plasma cells revealed interesting results of potential significance in the understanding of TEMPI pathophysiology.¹² The study described complex single nucleotide variants within chromosome 2 involving cancer-related genes (other than those associated with myeloma and myeloproliferative neoplasms). It also reported genetic alterations in chromosome 22 potentially altering the expression of cytokine *MIF*, previously linked to increased vascular endothelial growth factor and *HIF*. These findings were hypothesized to contribute to increased vascular formation and EPO in TEMPI.¹² The possibility that plasma cells secrete substances that act as *HIF* factors or EPO-like proteins is another interesting hypothesis that requires further investigation.

In conclusion, this is the first report of TEMPI to include results of a comprehensive NGS mutation analysis using a panel of 1425 genes performed in paraffin-embedded marrow material, in which no genetic abnormalities were identified. Similar to several patients described in the literature, *JAK2* mutation analysis was negative and cytogenetics was normal, indicating that TEMPI is not associated with a known pathogenic or likely pathogenic *JAK2* mutation. To date, a single study of whole genome sequencing of bone marrow revealed genomic alterations in a TEMPI patient.¹⁰ These alterations were different from those seen in advanced myeloma and were not diagnostic of any specific disease. There are rare reports of a patient with diagnosis of *MyD88*-mutated lymphoplasmacytic lymphoma and a *JAK2*-mutated PV who also had symptoms of TEMPI. It remains critical to distinguish TEMPI syndrome from PV due to the remarkably different treatment approaches for these diagnoses. As the recognition of TEMPI increases, it may become more important to use mutation panels to exclude mimickers as well as to identify a potential association of TEMPI with other clonal plasmacytic disorders.

Supplementary Data

Supplemental figures and tables can be found in the online version of this article at www.labmedicine.com

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

- Raulerson CK, Villa EC, Mathews JA, et al. SCHOOL: software for clinical health in oncology for omics laboratories. *J Pathol Inform.* 2022;13:1. doi:10.4103/jpi.jpi_20_21.
- Raulerson C, Jimenez G, Wakeland B, Villa E, Sorelle J, Malter J, Gagan J, Cantarel B. ANSWER: annotation software for electronic reporting. *JCO Clin Cancer Inform.* 2022 Jan;6:e2100113. doi:10.1200/CCI.21.00113. Erratum in: *JCO Clin Cancer Inform.* 2022 Mar;6:e2200043.
- Sykes DB, Schroyens W, O'Connell C. The TEMPI syndrome—a novel multisystem disease. *N Engl J Med.* 2011;365(5):475–477. doi:10.1056/NEJMc1106670.
- Alaggio R, Amador C, Anagnostopoulos I, et al. The 5th edition of the World Health Organization classification of haematolymphoid tumours: lymphoid neoplasms. *Leukemia.* 2022;36(7):1720–1748. doi:10.1038/s41375-022-01620-2.
- McKenna RW, Kyle RA, Kuehl WM, et al. Plasma cell neoplasms. In: Swerdlow SH, Campo E, Harris NL, et al (eds). *WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues*. Revised 4th ed. Lyon, France: WHO Press; 2017: 241–258.
- Zhang X, Fang M. TEMPI syndrome: erythrocytosis in plasma cell dyscrasia. *Clin Lymphoma Myeloma Leuk.* 2018;18(11):724–730. doi:10.1016/j.clml.2018.07.284.
- Lor M, Cheng M, Liang B, Cheng CE. TEMPI syndrome with progressive telangiectasias associated with pulmonary deterioration. *JAMA Dermatol.* 2020;156(12):1379–1380. doi:10.1001/jamadermatol.2020.2668.
- Diral E, Parma M, Renso R, et al. A fatal case of TEMPI syndrome, refractory to proteasome inhibitors and autologous stem cell transplantation. *Leuk Res.* 2020;97:106441. doi:10.1016/j.leukres.2020.106441.
- Shizuku T, Matsui K, Yagi S, Iwabuchi S. The first case of TEMPI syndrome in Japan. *Intern Med.* 2020;59(14):1741–1744. doi:10.2169/internalmedicine.3547-19.
- Sykes DB, O'Connell C, Schroyens W. The TEMPI syndrome. *Blood.* 2020;135(15):1199–1203. doi:10.1182/blood.2019004216.
- Kubik T, Minoo P. TEMPI syndrome associated with IgM monoclonal gammopathy. *Blood.* 2022;139(8):1254. doi:10.1182/blood.2021014393.
- Sun C, Xu J, Zhang B, et al. Whole-genome sequencing suggests a role of MIF in the pathophysiology of TEMPI syndrome. *Blood Adv.* 2021;5(12):2563–2568. doi:10.1182/bloodadvances.2020003783.
- Ruan Y, Zhao X, Pan M. Diffuse telangiectasia: a clue to the TEMPI syndrome. *JAAD Case Rep.* 2021;10:99–101. doi:10.1016/j.jdc.2021.02.022.
- Strobl J, Sinz C, Heil P, Stary G, Weninger W. Cutaneous ulceration as primary presentation of TEMPI syndrome. *J Eur Acad Dermatol Venereol.* 2021;35(12):e891–e894.
- Khan J, Sykes DB. Case report: a 37-year-old male with telangiectasias, polycythemia vera, perinephric fluid collections, and intrapulmonary shunting. *BMC Hematol.* 2014;14(1):11. doi:10.1186/2052-1839-14-11.
- Langabeer SE. The JAK2 V617F mutation in plasma cell neoplasms with co-existing erythrocytosis. *J Clin Diagn Res.* 2015;9(12):EL01. doi:10.7860/JCDR/2015/16407.6934.
- Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia.* 2010;24(6):1128–1138. doi:10.1038/leu.2010.69.
- McMullin MF. Genetic background of congenital erythrocytosis. *Genes (Basel).* 2021;12(8):1151. doi:10.3390/genes12081151.
- Gangat N, Szuber N, Pardanani A, Tefferi A. JAK2 unmutated erythrocytosis: current diagnostic approach and therapeutic views. *Leukemia.* 2021;35(8):2166–2181. doi:10.1038/s41375-021-01290-6.
- Lappin TR, Lee FS. Update on mutations in the HIF: EPO pathway and their role in erythrocytosis. *Blood Rev.* 2019;37:100590. doi:10.1016/j.blre.2019.100590.
- Rosado FG, Oliveira JL, Sohani AR, et al. Bone marrow findings of the newly described TEMPI syndrome: when erythrocytosis and plasma cell dyscrasia coexist. *Mod Pathol.* 2015;28(3):367–372. doi:10.1038/modpathol.2014.117.
- Percy MJ, McMullin MF, Jowitt SN, et al. Chuvash-type congenital polycythemia in 4 families of Asian and Western European ancestry. *Blood.* 2003;102(3):1097–1099. doi:10.1182/blood-2002-10-3246.
- Bond J, Gale DP, Connor T, et al. Dysregulation of the HIF pathway due to VHL mutation causing severe erythrocytosis and pulmonary arterial hypertension. *Blood.* 2011;117:3699–3701. doi:10.1182/blood-2010-12-327569.
- Liang SH, Yeh SP. Relapsed multiple myeloma as TEMPI syndrome with good response to salvage lenalidomide and dexamethasone. *Ann Hematol.* 2019;98(10):2447–2450. doi:10.1007/s00277-019-03761-4.
- Hutchison EJ, Taverna JA, Yu Q, Yeager AM. Polycythemia: an unusual presentation of multiple myeloma. *BMJ Case Rep.* 2016;2016:bcr2016216686.
- Beguín Y, Yerna M, Loo M, Weber M, Fillet G. Erythropoiesis in multiple myeloma: defective red cell production due to inappropriate erythropoietin production. *Br J Haematol.* 1992;82(4):648–653. doi:10.1111/j.1365-2141.1992.tb06939.x.

Transfusion-Related Acute Lung Injury Caused by HLA-II Antibodies: A Case Report

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Abbreviations: AFB, acid-fast bacillus; ARDS, acute respiratory distress syndrome; BNP, Brain Natriuretic Peptide; CT, computed tomography; HLA, human leukocyte antigen; HNA, human neutrophil antigen; IL, interleukin; MFI, Mean Fluorescence Intensity; PEEP, positive end expiratory pressure; TACO, transfusion-associated circulatory overload; TRALI, transfusion-related acute lung injury.

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ABSTRACT

Objective: The aim of this study was to retrospectively analyze the etiology of a case of suspected transfusion-related acute lung injury (TRALI) occurring after blood transfusion.

Methods: The clinical symptoms, signs, imaging examinations, and laboratory test results of a patient with suspected TRALI after blood transfusion were retrospectively analyzed, and human leukocyte antigen (HLA) genotyping of the patient and HLA antibodies of the plasma donors were performed.

Results: The clinical manifestations of the patient were consistent with those of TRALI after blood transfusion. This TRALI was treated by timely ventilator support. The patient results of high-resolution HLA genotyping were HLA-A* 01:01, 11:01; HLA-B* 15:02, 37:01; HLA-C* 06:02, 08:01; DRB1* 10:01, 12:02; DRB3* 03:01, 03:01; DQA1* 01:05, 06:01; DQB1* 03:01, 05:01; DPA1* 01:03, 02:01; and DPB1* 02:01, 09:01. Of the 6 plasma donors tested, 3 were found to have HLA-II antibodies, which were HLA-DPA1*01:03, HLA-DQB1*03:01, and HLA-DQB1*03:01 antibodies.

Conclusion: We described a case of TRALI caused by HLA-DQB1*03:01 antibody and DPA1*01:03 antibody.

Transfusion-related acute lung injury (TRALI) is a serious adverse transfusion reaction leading to patient mortality.^{1,2} TRALI is characterized by the sudden onset of noncardiogenic pulmonary edema and acute hypoxemia after transfusion, mostly within 1 to 2 h after transfusion. It is characterized by the sudden onset of dyspnea, severe hypoxemia, decreased oxygen saturation, and bilateral pulmonary infiltrates. Most patients require emergency oxygenation or even ventilator support. The mechanism of TRALI is not fully understood. Two primary mechanisms have been proposed; one is the antibody hypothesis implicating anti-leukocyte antibodies (anti-human leukocyte antigen [HLA] and/or anti-human neutrophil antigen [HNA]), and the other is the 2-hit hypothesis. In this theory, the patient has an inflammatory, traumatic, surgical, or other condition that causes the initial activation of neutrophils, and the subsequent transfusion containing anti-leukocyte antibodies leads to a massive activation and aggregation of neutrophils that damages the endothelium, resulting in pulmonary edema.^{1,2}

At present, most of the reported antibodies causing TRALI are HLA-I and HNA antibodies, whereas there are few reports of HLA-II antibodies. In this study, HLA-DQB1*03:01 antibody and DPA1*01:03 antibodies against the patient's antigens were detected in the serum of 3 plasma donors. We report a case of TRALI caused by these 2 HLA-II antibodies.

Case Report

A 19-year-old O positive male presented with the chief complaint of hematemesis with black stools for 6 days and ongoing dyspnea. He was admitted and diagnosed with gastrointestinal bleeding and anemia. The patient was treated with blood transfusion in Dandong People's Hospital, which is a general hospital. After dyspnea, the patient was transferred to Shengjing Hospital of China Medical University, "AAA" and general hospital. A total of 1200 ml of plasma and 8 U of red blood cells were infused. After transfusion, the anemia symptoms improved. Three hours later, a fever with a maximum temperature of 38.1°C developed, accompanied by dyspnea, subxiphoid pain, and expectoration of white sputum. The symptoms were not alleviated by oxygenation; the patient was then transferred to our hospital. The patient's vital signs were heart rate 109 bpm, blood pressure 104/70 mmHg, SO₂ 93%, and T 37.1°C. Physical exam showed unstable breathing, pallor, no rash, red pharynx, wet rales in both lungs, and light tenderness in the upper abdomen, without rebound tenderness. After admission, blood analysis indicated the following: arterial partial pressure of carbon dioxide (PaCO₂), 37.2 mmHg; arterial partial pressure of oxygen (PaO₂), 52.5 mmHg; white blood cell (WBC), 7.74 × 10⁹/L; neutrophil, 61.4%; hemoglobin level, 76 g/L; C-reactive protein level, 1.94 mg/L (0–8 mg/L); and brain natriuretic peptide level,

268.9 pg/mL (0–80 pg/mL). Enhanced chest computed tomography (CT) indicated bilateral infiltrates, interstitial edema, a mediastinal effusion, bilateral pleural effusions, and an interlobar effusion. The diagnoses were acute upper gastrointestinal bleeding, type I respiratory failure, moderate anemia, and myocardial injury. The patient was placed on fluid restriction and proton-pump inhibitors, a hemostatic drug, empiric antibiotic therapy, furosemide, and facemask oxygen therapy, but respiratory symptoms did not improve.

On day 2 after admission, the patient's dyspnea worsened and was accompanied by decreased blood oxygen saturation, decreased consciousness, and recurrent fever. The patient was sedated and mechanically ventilated without significant improvement. On day 3, blood culture, sputum culture, as well as mycoplasma pneumoniae antibody titer and acid-fast bacillus (AFB) smear results were negative. On day 4, the patient's blood oxygen saturation improved and the PaO₂ increased. Therefore, the fraction of oxygen concentration in the inspired air (FiO₂) was decreased to 45%, and the positive end expiratory pressure (PEEP) was gradually lowered to 6 cm H₂O. After the PEEP was lowered, the oxygen saturation decreased to 90%, and blood gas analysis indicated a PaO₂ of 68 mmHg and an alveolar-arterial oxygen difference of 237. The PEEP was again raised to 8 cm H₂O. Reexamination indicated no obvious improvement in lung radiolucency, and mechanical ventilation was continued. On day 5 after admission, the patient was febrile, still drowsy, and exhibited coarse breathing sounds. A chest reexamination was performed. On day 6 after admission, the patient was no longer febrile or obviously wheezing. The ventilator parameters and SIMV mode were adjusted and maintained for 1 h and then switched to oxygenation via endotracheal intubation. Half an hour later, the patient was extubated and switched to high-flow oxygen through a nasal cannula at a flow rate of 60L/min and an oxygen concentration of 46%. The patient had no significant dyspnea, and finger pulse oximetry indicated 99% oxygen saturation. Oxygen was discontinued 10 days after admission, and CT indicated that the bilateral lung infiltrates were significantly absorbed. The patient was discharged 17 days after admission. The imaging on 5 different days is shown in **FIGURE 1**.

Without jugular vein engorgement, heart volume increased and there was only a slight increase in Brain Natriuretic Peptide (BNP) and no improvement after diuresis; a transfusion-associated circulatory overload (TACO) was unlikely, so TRALI was diagnosed. To further investigate, high-resolution HLA genotyping was performed. The results were HLA-A* 01:01, 11:01; HLA-B* 15:02, 37:01; HLA-C* 06:02, 08:01; DRB1* 10:01, 12:02; DRB3* 03:01, 03:01; DQA1* 01:05, 06:01; DQB1* 03:01, 05:01; DPA1* 01:03, 02:01; and DPA1* 02:01, 09:01. This patient received blood transfusions from a total of 10 donors, including 6 plasma donors and 4 red blood cell donors. The HLA antibody testing was performed for 6 plasma donors. The antibody specificity testing was done with 2 different methods. Three plasma donors were detected as positive for antibodies HLA-DPA1* 01:03 (Mean Fluorescence Intensity [MFI] 1094), HLA-DQB1* 03:01 (MFI 664), and HLA-DQB1* 03:01 (MFI 628). We suggest that the HLA-DQB1* 03:01 and DPA1*01:03 antibodies in 3 donors' plasma caused TRALI in the patient.

Discussion

A TRALI is a serious adverse transfusion reaction, with a general mortality rate of 5% to 10%³ that increases to 60% in critically ill patients.^{1,3} The typical clinical presentation of TRALI is acute

respiratory distress during or within 6 h after transfusion, which may be accompanied by chills and fever, tachycardia, and hypoxemia, with patients having an oxygenation index $\leq$ 300 mmHg or blood oxygen saturation $<$ 90%.³ In our case, the patient had a sudden onset of dyspnea after a blood transfusion. After symptomatic treatment, preliminary diagnosis was acute respiratory distress syndrome (ARDS). Further testing of the patient and donors revealed HLA-II antibodies (HLA-DQB1*03:01,DPA1*01:03) from 3 female donors against the patient's HLA antigens. It has been shown that total quantity of HLA-II antibodies (MFI $>$ 1500) is a TRALI risk factor.⁴ In our case, the total MFI was 2386, which might indicate TRALI. Considering the clinical manifestations, laboratory test results, and diagnostic findings, a diagnosis of TRALI was confirmed.

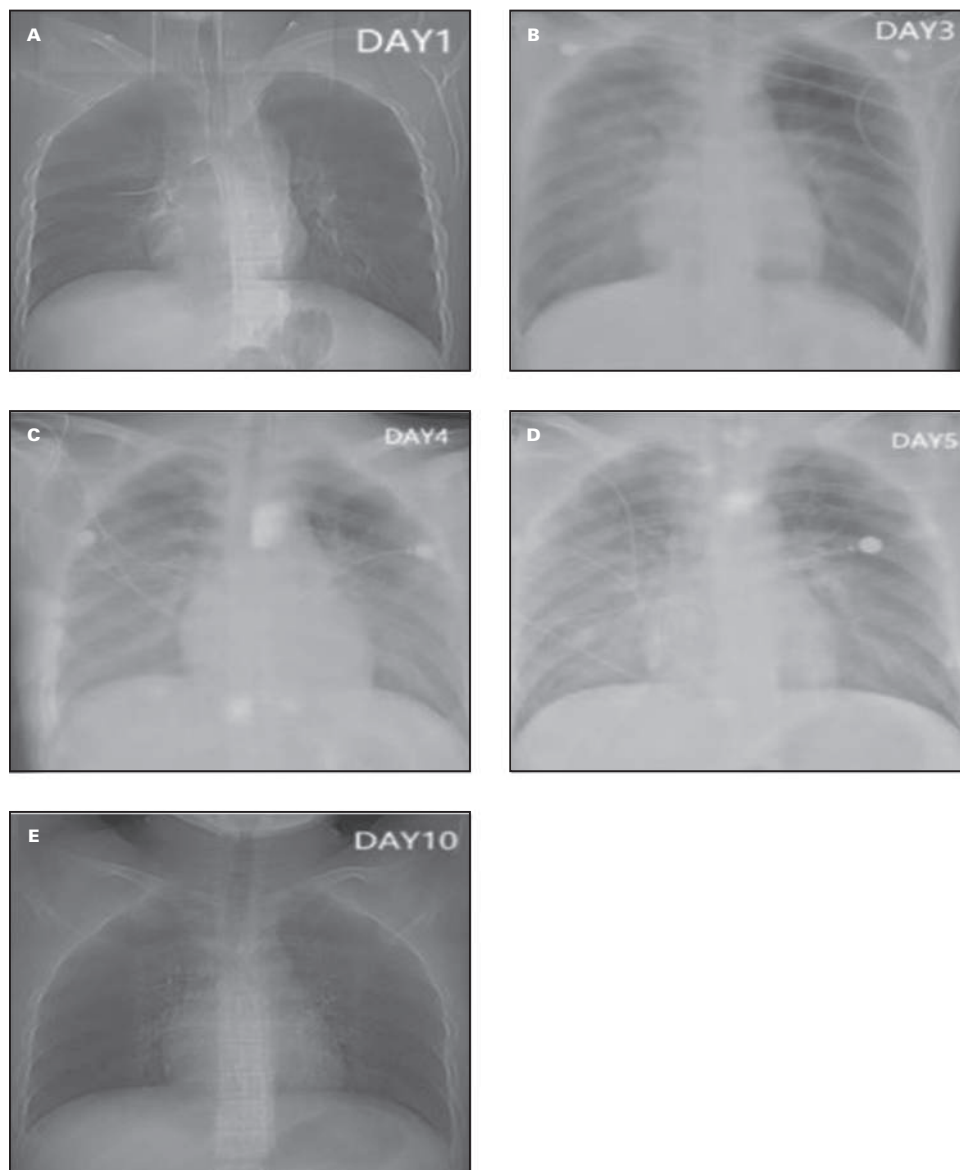
Most reports suggest that the pathophysiological process of TRALI is an immune reaction between the recipient and donor, which activates neutrophils and leads to their accumulation in the pulmonary capillaries, where granulocytes degranulate and release proteases and reactive oxygen species. This causes endothelial damage and increased permeability leading to pulmonary edema.^{5,6} However, HLA-II antibodies have been increasingly reported in recent years. Studies have shown that TRALI caused by HLA-II antibodies operates through a different mechanism.⁷ In TRALI induced by anti-HLA-II, the antibodies first activate monocytes, inducing the release of tumor necrosis factor- α , interleukin (IL)-1 β , and leukotriene B₄. These cytokines continue to recruit and to activate neutrophils, which continue to produce and to release cytotoxic reactive oxygen species and enzymes that increase endothelial cell permeability, leading to pulmonary edema.^{5–7} The pathogenesis of TRALI in the absence of homologous leukocyte antibodies may be an important research direction in the future.

The HLA-II antibodies that have been shown to potentially cause TRALI include HLA-DR7, -DR8, -DR9, -DR13, -DR14, -DR15, -DR51, -DR52, and -DR53. There have been fewer reports of HLA-DQ, and those that have been reported include HLA-DQ3, -DQ4, -DQ5, and -DQ9.^{6,8–12} In this study, HLA-DQB1* 03:01 and DPA1* 01:03 antibodies against recipient antigens were detected in donor sera. Antibody to HLA-DQB1* 03:01 is serologically classified as HLA-DQ7, and antibody to HLA-DPA1* 01:03 is serologically classified as HLA-DP19. A TRALI caused by these two antibodies has not been reported. The frequency of positive HLA-DQB1* 03:01 genotype in the Chinese blood donor population is 21.0942%; however, the positivity rate of the antibodies has not been reported.¹³

The majority of HNA and HLA antibodies occur in female blood donors with a history of multigravidity.¹⁴ In 2014, the Association for the Advancement of Blood and Biotherapies recommended the use of plasma from males, females with no detectable HLA or HNA antibodies, and females with no history of pregnancy. Several countries that have implemented a change in strategy have achieved significant results, with a marked decrease in the incidence of TRALI.^{1,5,7,13} At present, blood donors are not screened for HLA and HNA antibodies in China, which may lead to the transfusion of blood containing HLA or HNA antibodies. In this case, antibodies were tested after suspected TRALI in 6 plasma donors whose blood was transfused to the patient, and HLA-II antibodies were detected in 3 donors, all of whom were multiparous.

In addition, according to the newly revised guidelines for the diagnosis of TRALI in 2019, patients are divided into type I and type II TRALI according to whether they have risk factors for ARDS before

FIGURE 1. Imaging on 5 different days. A, day 1: Bilateral lungs infiltrates, interstitial edema, mediastinal effusions, bilateral pleural effusion, and interlobar effusion. B, day 3: The findings are generally similar to those on day 1. C, day 4: No significant improvement in bilateral lung field radiolucency. D, day 5: Slight improvement in bilateral lung field radiolucency. E, day 10: Obvious absorption of bilateral lung infiltrates.



transfusion.⁷ In this case, there were no risk factors for ARDS, such as trauma and aspiration before gastrointestinal bleeding, but the patient had sudden dyspnea after massive blood transfusion. According to the revised guidelines in 2019, we considered this case to be TRALI type I. Despite the serious clinical symptoms of TRALI, accurate and timely treatment results in a good prognosis, with approximately 80% of patients improving within 48 to 96 h. Approximately 20% of patients require continuous ventilation support, and a few patients develop hypoxemia and lung infiltrates that can last for more than 7 days. In this case, the patient developed respiratory distress 3 h after transfusion and could not be maintained by face mask oxygenation; thus, he was transferred after 72 h of active symptomatic treatment. Dyspnea improved after 5 days of mechanical ventilation, and the patient was discharged after being hospitalized for 17 days.

This was a typical case of TRALI caused by HLA-II antibodies HLA-DQB1* 03:01 and DPA1* 01:03. The patient's clinical and imaging manifestations were consistent with clinical TRALI, and the patient's condition improved after treatment. The specificity of the antibodies causing TRALI was elucidated through blood group serological examination and genetic analysis. One limitations of the treatment approach in the present case is the failure to collect pulmonary edema fluid from the patient for laboratory testing. Also, testing showed the patient's genotype and likely antigen corresponded to antibodies in donor serum but did not demonstrate direct agglutination between donor serum and patient WBCs. In addition, it has been suggested in the literature that the levels of cytokines, such as IL-8, and IL-6, are significantly elevated in patients with TRALI at onset¹; however, cytokines were not tested in this study.

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Personal and Professional Conflicts of Interest

All authors declare that they have no conflict of interests.

REFERENCES

1. Looney MR, Roubinian N, Gajic O, et al., Transfusion-Related Acute Lung Injury Study Group. Transfusion related acute lung injury study group: prospective study on the clinical course and outcomes in transfusion-related acute lung injury. *Crit Care Med*. 2014;42(7):1676–1687. doi:10.1097/CCM.0000000000000323.
2. Liunbruno GM, Franchini M. Solvent/detergent plasma: pharmaceutical characteristics and clinical experience. *J Thromb Thrombolysis*. 2015;39(1):118–128. doi:10.1007/s11239-014-1086-1.
3. Kim J, Na S. Transfusion related acute lung injury; clinical perspectives. *Korean J Anesthesiol*. 2015;68(2):101–105. doi:10.4097/kjae.2015.68.2.101.
4. Toy P, Gajic O, Bacchetti P, et al., TRALI Study Group. Transfusion-related acute lung injury: incidence and risk factors. *Blood*. 2012;119(7):1757–1767. doi:10.1182/blood-2011-08-370932.
5. Kopko PM, Paglieroni TG, Popovsky MA, Muto KN, MacKenzie MR, Holland PV. TRALI: correlation of antigen-antibody and monocyte activation in donor-recipient pairs. *Transfusion*. 2003;43(2):177–184. doi:10.1046/j.1537-2995.2003.00307.x.
6. Wakamoto S, Fujihara M, Sakagawa H, et al. Endothelial permeability is increased by the supernatant of peripheral blood mononuclear cells stimulated with HLA class II antibody. *Transfusion*. 2008;48(10):2060–2068. doi:10.1111/j.1537-2995.2008.01809.x.
7. Vlaar APJ, Toy P, Fung M, et al. An update of the transfusion-related acute lung injury (TRALI) definition. *Transfus Clin Biol*. 2019;26(4):354–356. doi:10.1016/j.traccli.2019.05.007.
8. Sachs UJ, Wasel W, Bayat B, et al. Mechanism of transfusion-related acute lung injury induced by HLA class II antibodies. *Blood*. 2011;117(2):669–677. doi:10.1182/blood-2010-05-286146.
9. Clippel DD, Emonds MP, Compennoll V. Are we underestimating reverse TRALI? *Transfusion*. 2019;59(9):2788–2793.
10. Win N, Brown C, Navarrete C. TRALI associated with HLA class II antibodies. *Transfusion*. 2003;43(4):545–546. doi:10.1046/j.1537-2995.2003.00363.x.
11. Nishimura M, Hashimoto S, Takanashi M, et al. Role of anti-human leucocyte antigen class II alloantibody and monocytes in development of transfusion-related acute lung injury. *Transfus Med*. 2007;17(2):129–134.
12. Kopko PM, Popovsky MA, Mackenzie MR, et al. HLA class II antibodies in transfusion-related acute lung injury. *Transfusion*. 2001;41(10):1244–1248. doi:10.1046/j.1537-2995.2001.41101244.x.
13. Zhou XY, Zhu FM, Li JP, et al. High-resolution analyses of human leucocyte antigens allele and haplotype frequencies based on 169,995 volunteers from the China Bone Marrow Donor Registry Program. *PLoS One*. 2015;10(9):e0139485. doi:10.1371/journal.pone.0139485.
14. Kelher MR, Banerjee A, Gamboni F, Anderson C, Silliman CC. Antibodies to major histocompatibility complex class II antigens directly prime neutrophils and cause acute lung injury in a two-event in vivo rat model. *Transfusion*. 2016;56(12):3004–3011. doi:10.1111/trf.13817.

The Importance of Medical Laboratory Scientists and the Number of Doctoral Scientists that Began Their Career by Working on the Front Lines of Laboratory Medicine

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Keywords: survey, doctoral scientists, medical laboratory scientists, employment opportunities, recruitment, staffing shortages, microbiologists and chemists.

Abbreviations: MLS, medical laboratory scientists; ASM, American Society of Microbiology; AACC, American Association of Clinical Chemists; ASCP, American Society for Clinical Pathology; CLIA, Clinical Laboratory Improvement Amendments; HCLD, High Complexity Clinical Laboratory Director; ABB, American Board of Bioanalysis

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ABSTRACT

Background: Due to dwindling numbers of medical laboratory scientists (MLS), we are faced with staffing issues due to lack of visibility, recognition, low wages, and perceived lack of opportunities for upward career mobility. A brief survey of doctoral-level clinical microbiologists showed a significant number were certified, worked “the bench,” and used this experience as a steppingstone to become a laboratory director or consultant.

Objectives: To help dispel the notion working as an MLS is a dead-end job, the authors developed an expanded national survey.

Methods: The survey was sent to 815 board-certified microbiologists and chemists to determine their career path. Results indicated that of 243 respondents, 41.3% were MLS certified, 47.1% had worked “the bench,” and 51.9% and 63.5% became laboratory or technical directors, respectively.

Conclusion: Survey results provide evidence to dispel the notion that MLS and bench working jobs lack upward career mobility and may inspire students for a career in laboratory medicine.

In 2021, the American Society of Microbiology (ASM) Personnel Standards and Workforce Subcommittee of the Clinical and Public Health Microbiology Committee released a comprehensive supplement based

on the results from a workforce survey distributed on ClinMicroNet and DivCNet ASM email distribution programs. The document provides data that highlights laboratory demographics and vacancy rates in the workforce with the hope that the information would be helpful in revealing just how critical staffing shortages have become and supporting efforts to change the staffing trajectory.¹ In 2022, a commentary was released² that in many respects is complementary to the authors’ publication³ that emphasized the root causes for the shortages, that is, noncompetitive compensation, lack of visibility, recognition, benefits, staffing flexibility, career ladders and opportunities, and potential solutions. Since students may not be attracted to the medical laboratory scientists (MLS) field because they are unaware of career opportunities that may be available to an MLS, we took an approach that focused on addressing one of the root causes for staffing shortages by emphasizing opportunities that are available to laboratory professionals. After an initial brief survey for doctoral-level clinical microbiologists that showed a significant number were certified, worked “the bench,” and used their experience as a steppingstone to become a laboratory director or consultant, an expanded national survey was developed that focused on one of the solutions outlined in the 2022 commentary,² “Expand innovative and alternative career routes.” Our goal was to confirm our initial survey observations using an expanded survey that was sent by email and to participants on the ASM and American Association of Clinical Chemists (AACC) email distribution programs to determine the percent of doctoral-level microbiologists and chemists that were MLS and used their laboratory experience for advanced opportunities.

Materials and Methods

The authors performed an expanded national survey of board-certified physicians, microbiologists, and chemists to confirm an earlier observation reported in the article entitled “A day in the life of a consultant microbiologist,”⁴ that a notable number of doctoral level microbiologists were American Society for Clinical Pathology (ASCP)-certified or the equivalent and had worked on the bench early in their career before or during graduate school as a stepping stone to becoming a board-certified, Clinical Laboratory Improvement Amendments (CLIA) laboratory or technical director, and/or consultant.⁵ Confirmatory results could help lay the foundation for students to consider a career as an MLS and the potential opportunities for career growth.

TABLE 1. Results from a national survey of 243 microbiologists and chemists along with their career pathways.

Participants	Board Certified	ASCP Certified or Equivalent	Worked as Bench MLS	Boarded/ASCP/MLS/CLIA Laboratory Director	CLIA Laboratory Director	CLIA Technical Supervisor, Director, or Consultant
Doctoral (206) MD/DO (32) PharmD (1) Other (4)	Doctoral (171) MD/DO (22)	Doctoral (86) MD/DO (3) Other (1)	Doctoral (98) MD/DO (6) +ASCP (67)	Doctoral (51) MD/DO (3)	Doctoral (108) MD/DO (11)	Doctoral (132) MD/DO (16)
PhD (201)	(167)	(81)	(92)	(48)	(105)	(128)
DrPH (5)	(2)	(4)	(5)	(2)	(3)	(3)
ScD (2)	(2)	(1)	(1)	(1)	(0)	(1)
PharmD (1)	(0)	(0)	(0)	(0)	(0)	(0)
MD (28)	(21)	(2)	(4)	(2)	(10)	(15)
DO (2)	(1)	(1)	(2)	(1)	(1)	(1)
Nondoctoral or Not Indicated (4)	(0)	(1)	(0)	(0)	(0)	(0)
Total (243)	(193)	(90)	(104)	(54)	(119)	(148)

Results

TABLE 1 provides the results from our national survey of medical and doctoral level microbiologists and chemists and their career pathway. Of the 243 responders, 208 were doctoral-level microbiologists or chemists. If the PharmD, MD, DO, and nondoctoral/not indicated responders are excluded, 41.3% of participants were ASCP-certified or the equivalent, 47.1% had worked at some point in their career on the bench, 51.9% were a CLIA laboratory director during their career, and/or 63.5% were a technical supervisor, director, or consultant. In contrast, data from ASCP showed only about 1% of MLSS went on to obtain work as a doctoral-level MLS (PhD, DCLS), (personal communication with E. Garcia, ASCP).

Of the 243 total responders, 143 (81.3%) were doctoral level microbiologists; 110 (76.9%) were board-certified D(ABMM) or HCLD(ABB); 69 (48.3%) were ASCP-certified or the equivalent; 68 (47.6%) had worked as an (MLS) during their career; 63 (44.1%) were CLIA laboratory directors during their career; 82 (57.3%) were a CLIA technical supervisor, director, or consultant; and 39 (27.3%) were board-certified, ASCP-certified, had worked on the bench, and were CLIA laboratory directors. Of 65 (26.7%) chemist responders out of 243 total responders, 62 of the 65 (95.4%) responders were board-certified D(ABCC); 17 (26.2%) were ASCP certified; 26 (40.0%) had worked on the bench; 45 (69.2%) were CLIA laboratory directors during their career; and 50 (76.9%) were a CLIA technical supervisor, director, or consultant.

A notable number of survey participants were certified and/or worked the bench as an MLS before or after they obtained their PhD or MD/DO (as the authors had done). In addition, several participants stated that working on the bench really helped to promote their careers, and one participant had enrolled in an MLS training program after they received their doctorate to gain knowledge of all areas of the laboratory, which led to an advancement as a director of the laboratory system.

Discussion

Recent publications have outlined reasons why we are experiencing staffing shortages and solutions to address these shortages.^{2,3} Strategies to encourage students can best be developed using a collaboration of stakeholders, for example, health science educators, K-12, magnet and precollege educational program administrators,

mentors and role models, certification board members, legislators, and county school supervisors responsible for business partnerships that help increase recruitment of individuals into the field of MLS. The information gleaned from our national survey may be incorporated into educational programs to stimulate the interest of students in a career in laboratory medicine by emphasizing the opportunities that would be available for those taking the path to becoming an MLS.

Conclusion

Staffing shortages can best be addressed by a collaboration of stakeholders working together to develop strategies that highlight the role of MLS, what it takes to become a laboratory professional, and the potential opportunities afforded them. Results from our study show a sizable number of individuals were certified medical laboratory scientists early in their career. They gained laboratory experience and then took the opportunity to move up the ladder in their career to positions of more responsibility and remuneration. These results highlight opportunities students can aspire to once they become an MLS.

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REFERENCES

- 2021 Clinical Microbiology Workforce Survey § From the Personnel Standards and Workforce Sub-Committee of the Clinical and Public Health Microbiology Committee, *American Society for Microbiology*.

Available from: <https://asm.org/getmedia/df01720e-4d74-4535-bb0a-7bb7ad677960/Supplemental-Material-Survey-docx-37.pdf> Accessed November 3, 2022.

2. Leber AL, Peterson E, Bard JD. The hidden crisis in the times of Covid-19: Critical shortages of medical laboratory professionals in clinical microbiology. *J Clin Microbiol*. 2022;60(8):1–11. doi:10.1128/jcm.00241-22.
3. Halstead DC, Sautter RL. Special Report. A Literature Review on How We Can Address Medical Laboratory Scientist Staffing Shortages. *Lab*

Med June 19, 2022;XX:e0–e0. doi:10.1093/labmed/lmac090.

4. Halstead DC, Sautter RL. A day in the life of a consultant microbiologist. *Clin Micro News* 2021;43(6):33–38.
5. Clinical Laboratory Improvement Amendments (CLIA). *Laboratory Director Responsibilities*. Centers for Disease Control and Prevention (CDC) and the Centers for Medicare and Medicaid Services (CMS). Available from: <https://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/Downloads/brochure7.pdf> Accessed August 28, 2022.

Laboratory Diagnosis of Primary Amoebic Meningoencephalitis

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Keywords: primary amoebic meningoencephalitis, PAM, *Naegleria fowleri*, diagnosis, amoebic meningoencephalitis, free-living amoebas

Abbreviations: PAM, primary amoebic meningoencephalitis; IHC, immunohistochemistry; IIF, indirect immunofluorescence; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; RBC, red blood cell; WBC, white blood cell; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; CDC, Centers for Disease Control and Prevention; Ig, immunoglobulin; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectra; ITS, internal transcribed spacer; C_T, cycle threshold; LAMP, loop-mediated isothermal amplification; NGS, next-generation sequencing; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; rRNA, ribosomal ribonucleic acid; SCGYEM, serum-casein-glucose-yeast extract medium; PYNFH, peptone-yeast extract-nucleic acid-folic acid-hemin.

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ABSTRACT

Primary amoebic meningoencephalitis (PAM) is a fulminant fatal human disease caused by the free-living amoeba *Naegleria fowleri*. Infection occurs after inhalation of water containing the amoeba, typically after swimming in bodies of warm freshwater. *N. fowleri* migrates to the brain where it incites meningoencephalitis and cerebral edema leading to death of the patient 7 to 10 days postinfection. Although the disease is rare, it is almost always fatal and believed to be underreported. The incidence of PAM in countries other than the United States is unclear and possibly on track to being an emerging disease. Poor prognosis is caused by rapid progression, suboptimal treatment, and underdiagnosis. As diagnosis is often performed postmortem and testing is only performed by a few laboratories, more accessible testing is necessary. This article reviews the current methods used in the screening and confirmation of PAM and makes recommendations for improved diagnostic practices and awareness.

Primary Amoebic Meningoencephalitis

Primary amoebic meningoencephalitis (PAM) is a rapidly progressing, almost always fatal disease caused by the free-living amoeba *Naegleria*

fowleri. A total of 381 PAM cases have been reported worldwide, with the greatest number of case exposures reported in the United States, Pakistan, and Mexico.¹ The majority of cases have been registered after the year 2000, with most patients being male and around the age of 14 years. The increased number of cases in the last decade is suggested to be the result of improved diagnostic methods and climate change that is facilitating thriving conditions of the amoeba.² Although PAM is a rare disease, *N. fowleri* infection has been described as an emerging infectious disease and a medical concern, with the forecast of an increased number of reported cases in the future.^{2,3}

N. fowleri is distributed in soil and warm freshwater deposits such as in lakes, rivers, swimming pools, sewage, and hot springs worldwide, with the route of infection being inhalation of water contaminated by the amoeba^{4,5} (FIGURE 1).

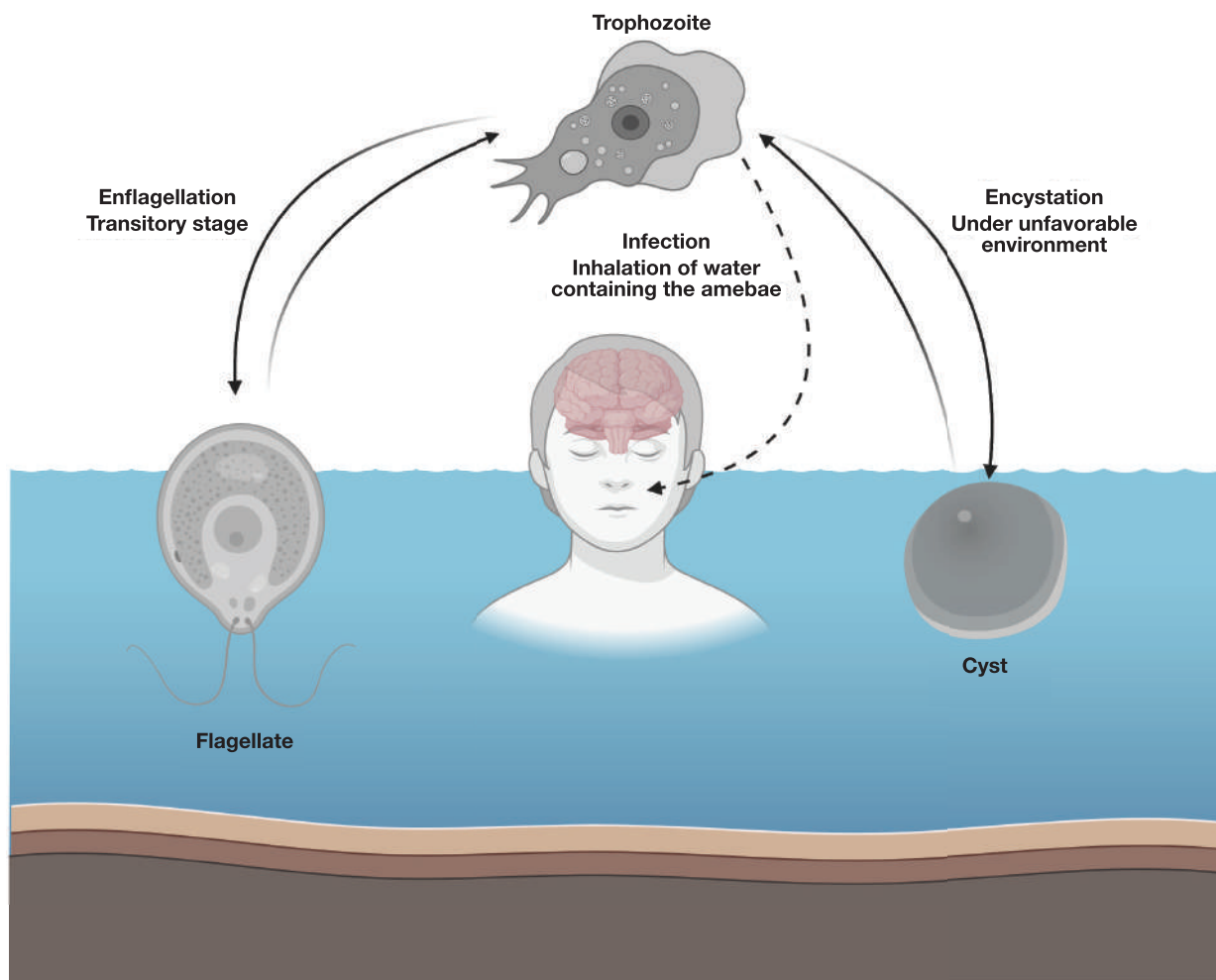
After infection, *N. fowleri* attaches to the nasal mucosa and migrates to the olfactory nerve then crosses the cribriform plate to reach the olfactory bulbs, where it causes necrotizing meningoencephalitis. Direct brain tissue damage is the result of *N. fowleri* forming food cups that ingest human tissue and by the release of cytolytic substances such as hydrolases, neuraminidase, and phospholipases.⁶ In addition, tissue damage is exacerbated by activation of innate immune system responses, primarily by the complement cascade, neutrophils, and macrophages.⁷

Clinical onset typically occurs 2 to 8 days postinfection, with signs and symptoms similar to those of meningitis, characterized by intense headache, stiff neck, fever, chills, photophobia, seizures, and confusion.^{8,9} Death of the patient 3 to 7 days after initial clinical presentation is the most frequent outcome, as only 32 of 381 patients have survived, and only 7 of the surviving patients had a confirmed *N. fowleri* diagnosis.^{1,6}

Poor prognosis of the disease is multifactorial, as rapid progression of the infection, underdiagnosis, suboptimal treatment, and lack of awareness play a role. Current chemotherapeutic intervention for PAM consist of a mixture of antimicrobial, antifungal, and anticancer medications, with miltefosine being recognized as the most effective treatment option.^{10–12}

Diagnosis of PAM is often performed postmortem following histopathological microscopic examination of brain tissue revealing *N. fowleri* trophozoites and confirmation by immunohistochemistry (IHC), indirect immunofluorescence (IIF), or polymerase chain reaction (PCR). Cases that were diagnosed before the death of the patient have been primarily done by direct visualization of the amoebas in cerebrospinal fluid (CSF) and confirmation by IHC, IIF, or PCR.¹ Successful diagnosis of PAM relies on prompt identification and awareness of available testing methodologies.

FIGURE 1. Life cycle of *Naegleria fowleri*. Free-living amoebas thrive in temperate bodies of freshwater. The cyst stage allows the amoebas to survive harsh environmental conditions. A nonfeeding nondividing transitory flagellate stage can also form under certain conditions. Infection occurs via inhalation of ameboid trophozoites in water. Figure created with BioRender.com.



To this end, the purpose of this review is to summarize the current clinical laboratory methods used in the diagnosis of PAM (TABLE 1).

Direct Visualization

Initial CSF findings usually reveal elevated opening pressure, increased red blood cell (RBC) and white blood cell (WBC) counts with marked neutrophilia. The CSF chemistry analysis, as in cases of bacterial meningitis, is characterized by cloudy CSF with increased protein and decreased glucose levels.^{1,13}

Direct wet mount microscopic examination of fresh unrefrigerated CSF specimens can reveal numerous motile *N. fowleri* amoebas.¹⁴ However, amoebas may be missed at first examination and repeat CSF analysis may be required for successful observation by wet mount preparations. Amoebas most often appear as actively moving cells in the trophozoite stage measuring 15 to 30 μm in diameter (FIGURE 2).

Alternatively, cytospin preparations of CSF at 5000 g for 5 min can be used to concentrate amoebas followed by staining with Wright-Giemsa, hematoxylin and eosin (H&E), or modified trichrome stains to increase contrast and ease visualization.¹⁵

To maximize viability and observation of *N. fowleri* trophozoites, CSF preparations must be evaluated as soon as possible after

lumbar puncture. Oil-immersion examination of CSF cytospin preparations stained with Wright-Giemsa reveals prominent presence of neutrophils and observation of *N. fowleri* cells, which are distinguished by vacuolated trophozoites with a single large pale nucleus.^{16,17}

In the microbiology department, caution should be taken when examining Gram stain CSF slide preparations of suspected *N. fowleri* cases, as Gram stain is not useful for diagnosis as it does not properly stain nuclear morphology and amoebas may be mistaken for WBCs.^{13,18}

Special precautions should be taken when making slide preparations for *N. fowleri*. Refrigerated CSF should be avoided as cold kills the amoebas, causing loss of motility, which will make them more difficult to observe. Likewise, heat-fixing CSF preparations should be avoided, as heat can destroy the amoebas, leading to a possible false-negative interpretation.¹⁹

Postmortem diagnosis of *N. fowleri* is usually performed on examination of brain tissue biopsy. Ideally, fresh unfixed brain tissue is recommended for histological examination. If examination is delayed or specimen must be transported to a reference or state laboratory, it is recommended to use biopsy material fixed in 10% neutral buffered formalin. Tissue sections can then be stained with H&E, trichrome stain, periodic acid-Schiff (PAS), or Wright-Giemsa.^{13,20} Similar to CSF examinations,

TABLE 1. Methods used in the detection of *Naegleria fowleri*

Method	Techniques	Notes
Microscopy	Wet mount	Observation of motile trophozoites
	Cytospin	Marked neutrophilia Wright-Giemsa, H&E, or modified trichrome
	Tissue biopsy	Observation of trophozoites
		Wright-Giemsa, H&E, trichrome stain, or PAS
Antigen detection	IIF	Uses FITC-conjugated secondary antibodies
	IHC	Uses HRP-conjugated secondary antibodies
	Flow cytometry	Uses N3B monoclonal antibody
		Research setting only
Nucleic acid detection	MALDI-TOF	Research setting only
		Research setting only
	Triplex real-time PCR	Tests for <i>Acanthamoeba</i> , <i>B. mandrillaris</i> , and <i>N. fowleri</i>
		Targets 18S rRNA gene sequences
Culture	LAMP	Targets virulence-related protein gene region Research setting only
	NGS	Used in clinical and research settings
	Monoxenic	Uses non-nutrient agar supplemented with <i>E. coli</i>
	Axenic	Nelson, SCGYEM, or PYNFH media
Serology	ELISA	Detection of antibody titers
		Not used for diagnosis of PAM
	Immunofluorescence	Used for detection of <i>N. fowleri</i> -binding antibodies
		Not used for diagnosis of PAM

CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; H&E, hematoxylin and eosin; HRP, horseradish peroxidase; IHC, immunohistochemistry; IIF, indirect immunofluorescence; LAMP, loop-mediated isothermal amplification; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NGS, next-generation sequencing; PAM, primary amoebic meningoencephalitis; immunofluorescence; PAS, periodic acid-Schiff; PCR, polymerase chain reaction; PYNFH, peptone-yeast extract-nucleic acid-folic acid-hemin; rRNA, ribosomal ribonucleic acid; RBC, red blood cell; SCGYEM, serum-casein-glucose-yeast extract medium; WBC, white blood cell.

amoebas in tissue appear in the trophozoite stage measuring 10 to 35 μ m in diameter with granular and vacuolated plasma and a single large nucleus with a dense karyosome.^{21,22} Staining using antibodies can also be performed for IHC and IIF studies as described below. Care must be taken when examining tissue biopsies as other amoebas such as *Balamuthia mandrillaris* and *Acanthamoeba* spp. resemble *N. fowleri*, making it difficult to differentiate them based on morphology alone.²³ Therefore, confirmatory tests are required for proper identification (FIGURE 3).

As a resource for laboratory professionals, the Centers for Disease Control and Prevention (CDC) offers an image bank of phase contrast, cytopsin, tissue biopsy, and immunofluorescence photographs of *N. fowleri*.²⁴

Antigen Detection Methods

Identification of *N. fowleri* from brain tissue samples and CSF can be accomplished by the use of immunohistochemical staining techniques. The two main methods used are IIF staining and IHC using antibodies

specific to *N. fowleri*. These methodologies employ polyclonal or monoclonal antibodies that can identify amoebas in tissue sections and CSF.

The IIF protocols typically use rabbit immunoglobulin (Ig)G incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG as secondary antibody. The IIF preparations can then be observed using a fluorescence microscope at 493 nm excitation and 528 nm emission.^{25,26} Similarly, IHC protocols employ immunoperoxidase staining by using secondary antibodies conjugated with horseradish peroxidase (HRP) and the addition of substrate for visualization.

The IIF and IHC methods are performed at the CDC but are not widely available methodologies in clinical laboratories as commercial kits are not readily available. The primary antibodies are mostly prepared in-house by immunizing animals with *N. fowleri* to produce antisera. These methods are impractical for the clinical laboratory setting and only performed when antisera or monoclonal antibodies are already available by the reference laboratory or in research settings.

Detection of *N. fowleri* by flow cytometry has been accomplished by the use of N3B monoclonal antibody and from polyclonal sera.^{15,27,28} These flow cytometry protocols were tested from culture preparations and environmental water samples, but the protocol may be modified as an investigational approach to detect the presence of amoebas from clinical specimens.

A promising approach for the rapid diagnosis of *N. fowleri* is the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. Mass spectra (MS) databases can be built for the identification of *N. fowleri* from CSF or brain tissue samples. Thus, reproducible MS fingerprinting patterns have been produced from patients diagnosed with PAM and from water samples containing the amoebas.

Nucleic Acid Detection

Detection of *N. fowleri* nucleic acids in CSF and fresh tissue samples is considered the gold standard and the method of choice for confirmation of PAM. Various PCR protocols are available for the detection of *N. fowleri*, with most assays targeting the 5.8S ribosomal DNA gene, the internal transcribed spacer (ITS)1/ITS2, or the 18S rRNA gene sequences.¹⁹ Comparison of four PCR protocols in terms of thermal settings, assay sensitivity and specificity, and limit of detection has been studied from environmental samples, but no similar study has been completed from clinical specimens.²⁹

The CDC uses a triplex real-time PCR protocol to detect *Acanthamoeba* spp., *B. mandrillaris*, and *N. fowleri* in a single PCR run.³⁰ Following DNA extraction, the triplex real-time PCR uses 9 primers, 2 primers that target a variable region in the 18S rRNA gene and a TaqMan probe for each species, that simultaneously detect *N. fowleri*, *Acanthamoeba* spp., and *B. mandrillaris*. The CDC multiplex protocol has been tested in CSF, brain tissue, and culture samples with a turnaround time of less than 5 h. The CDC protocol can be adapted to test for *N. fowleri* only. The CDC assay has a detection limit of 1 amoeba per sample and specificity for 6 strains of *N. fowleri* without cross reactivity with other CNS pathogens, including *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *S. agalactiae*, *Toxoplasma gondii*, and in cysticercosis-positive patients. This assay produced cycle threshold (C_T) values of 22 from brain tissue samples and a C_T value of 23 from CSF specimens.³⁰ Requesting a test order for *N. fowleri* PCR identification at the CDC requires submitting at least 0.1 g of fresh unfixed tissue or at least 0.2 mL of CSF. Routine

FIGURE 2. Morphology of *Naegleria fowleri*. (A) Diagram of *N. fowleri* ameboid form depicting nucleus and vacuoles. (B) Ameboid cell with prominent nucleus and multiple vacuoles (unstained, 100×). (C) Two ameboid cells with their marked nucleus (unstained, 40×). Care should be taken to not mistake amoebas for artifacts or epithelial cells. (D) Magnified field of view showing 2 ameboid cells (unstained, 100×). E) Unstained *N. fowleri* (100×) F) H&E-stained *N. fowleri* (100×).

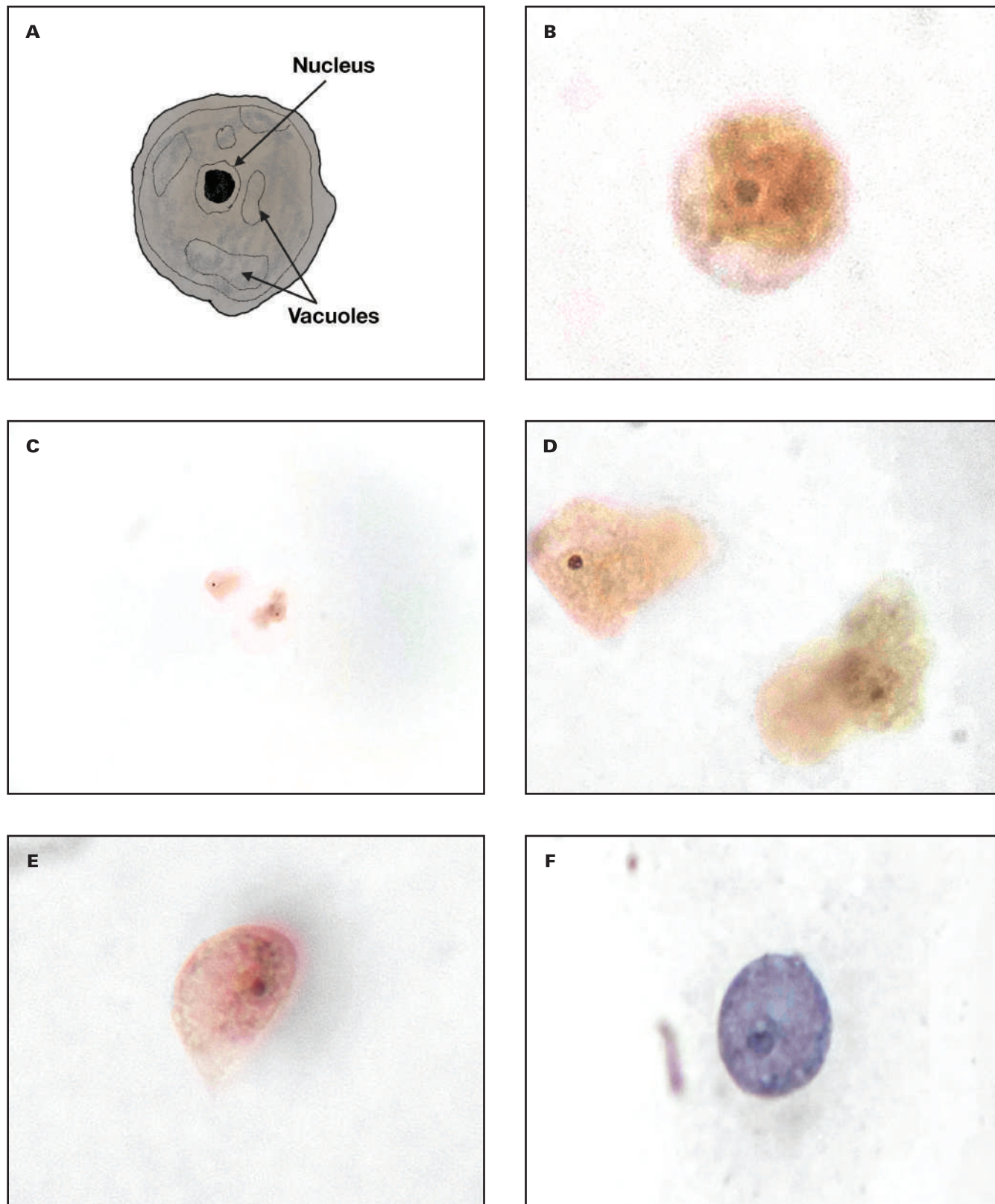
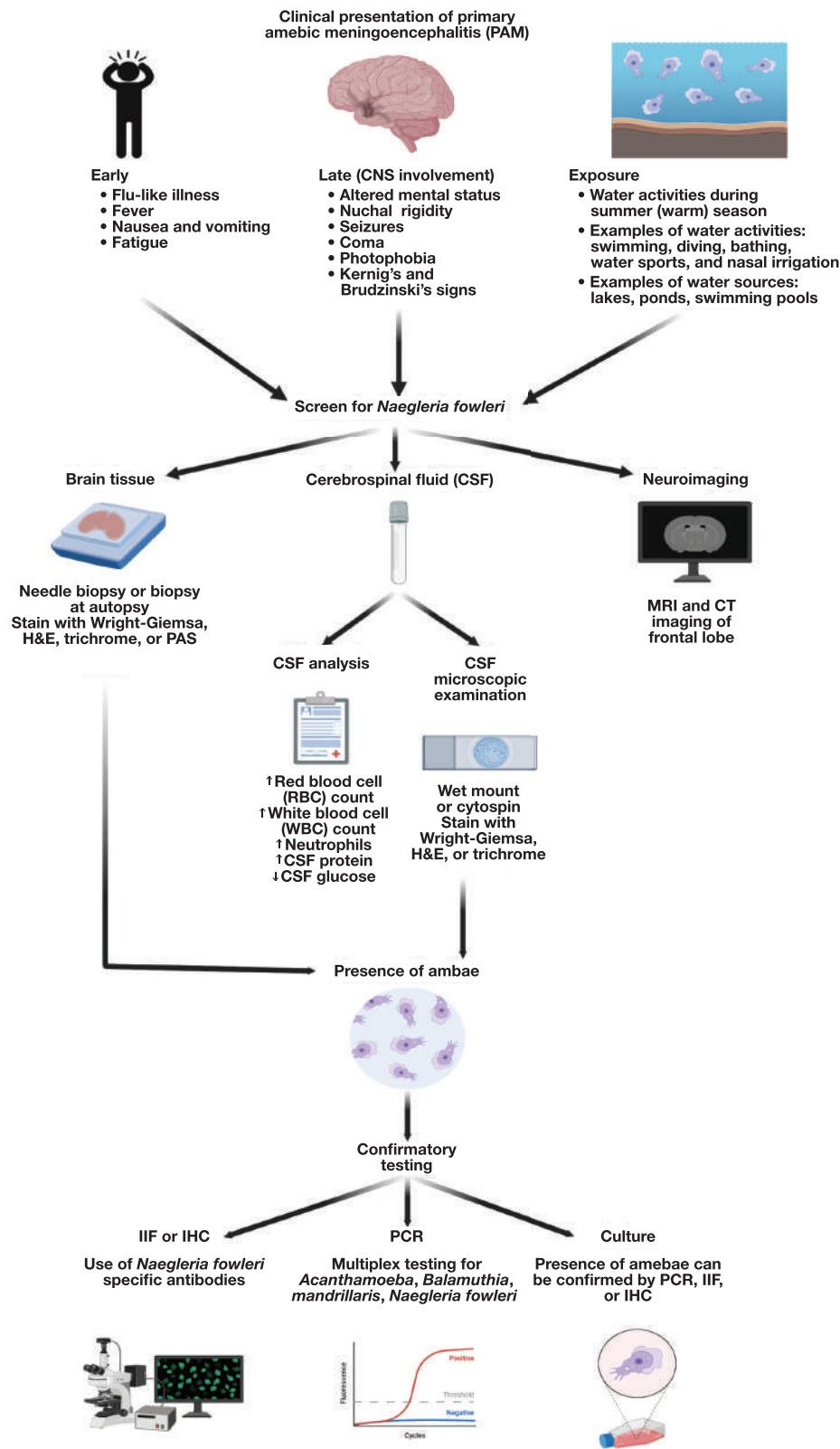


FIGURE 3. Identification of *Naegleria fowleri*. Diagnosis of primary amoebic meningoencephalitis is first considered in patients presenting encephalitis or meningitis. Neuroimaging reveals frontal lobe damage. Microscopic examination of brain tissue and cerebrospinal fluid that reveals presence of amoeba must undergo confirmatory testing to identify the etiology. Figure created with BioRender.com.



turnaround time of 7 days is expected, but with proper communication with the CDC and prompt shipment of specimens, the CDC can provide same-day PCR results when critical for clinical decision making.^{19,30,31}

An alternative amplification test for *N. fowleri* diagnosis is the loop-mediated isothermal amplification (LAMP) assay that uses a set of 5 primers that target an *N. fowleri* virulence-protein gene region. Although the LAMP assay has not been evaluated in the clinical setting, it provides a promising approach that may be incorporated in diagnostic medicine, as it circumvents the need for PCR instrumentation and reagents as positive reactions are detected by a color change using hydroxy naphthol blue in a single reaction tube. As not all clinical laboratories are capable of performing in-house PCR testing, the LAMP test provides an appealing alternative for PAM testing in field studies or in laboratories without PCR equipment. The LAMP test has a sensitivity comparable to that of PCR as it can detect 1 *N. fowleri* trophozoite in CSF and water samples. The test demonstrates high specificity, as it can discriminate from other amoebas, *Toxoplasma gondii*, *Cryptococcus neoformans*, *Escherichia coli*, and *Mycobacterium tuberculosis*.³²

Next-generation sequencing (NGS) can also be performed to diagnose PAM. One case of pediatric PAM was diagnosed using NGS from CSF. In this case, successful identification of *N. fowleri* by NGS was accomplished from nucleic acid extraction and purification followed by cDNA library preparation for high-throughput sequencing on Illumina NextSeq 550 DX.³³ The resulting reads were mapped against the *N. fowleri* genome (ASM1484362v1) and nucleic acid sequencing results were confirmed by PCR and gel electrophoresis.³³ Similarly, another case report from an adult patient used CSF for nucleic acid extraction followed by NGS using the BGISEQ-500 platform. The sequencing reads mapped to the 18S rRNA of *N. fowleri* and were confirmed by PCR.³⁴ Thus, NGS presents an appealing approach for rapid confirmation of PAM cases granted that sequencing capabilities are available at the diagnostic lab to ensure quick turnaround time.

Culture

Isolation of *N. fowleri* from clinical specimens is a common procedure performed to confirm amoebic infection in cases of PAM. Likewise, isolation of this pathogen from environmental samples is performed to establish the source of *N. fowleri* infection or to screen soil or water samples for the presence of *Naegleria*. From clinical specimens, *N. fowleri* has been isolated and cultured from brain tissue biopsies and CSF.^{35,36} The procedure to culture *N. fowleri* from human specimen samples involves monoxenic growth of *N. fowleri* at 37°C in nonnutrient agar supplemented with a spread of *E. coli*. This approach allows recovery of *N. fowleri* after 1 to 2 days incubation. The presence of amoebas in culture can be confirmed by using a dissecting microscope and 10× objective.³⁷ In culture, *N. fowleri* can be transformed into flagellated and cyst stages by altering the environmental conditions and nutrients. When isolating and culturing *N. fowleri* from environmental samples, one of the major challenges is to select for *N. fowleri* growth while inhibiting the growth of other soil or water organisms such as bacteria, fungi, other protozoa, and helminths. In this instance, *N. fowleri* can be recovered by first using nonnutrient agar with *E. coli* as food source, then switching to axenic culture with antibiotics to prevent bacterial growth.³⁷

Culture of *N. fowleri* can also be accomplished in axenic conditions using enriched nutrient medium with antibiotics (penicillin, streptomycin, and gentamicin). Different types of media, such as Nelson, serum-casein-glucose-yeast extract medium (SCGYEM), and peptone-

yeast extract-nucleic acid-folic acid-hemin (PYNFH), can be used for the axenic cultivation of *N. fowleri*.^{37,38} As the presence of bacteria can be a major variable in certain experiments, the growth of *N. fowleri* in axenic conditions is the method of choice in experiments involving host-microbe interactions, proteomics, and transcriptomics.

State and reference laboratories that perform culture of *N. fowleri* have a turnaround time of 3 to 10 days. As one of the major limitations of culture assays is viability of the microorganism, unpreserved and unrefrigerated clinical specimens are recommended. The CSF specimens for culture may be refrigerated at 4°C for no longer than 24 hours. The required CSF volume ranges from 1 to 2 mL to be transported in a sterile leak-proof container. Interpretation of culture results is indicated as negative when no parasites are observed with the note that no viable amoebas may be present in the specimen.

As *N. fowleri* is highly pathogenic, proper handling is critical for safe culture manipulation of this microorganism. Laboratory safety is an important consideration when deciding to perform *Naegleria* culture. As of now, there are no reported cases of laboratory-acquired infections with free-living amoebas. The handling of *N. fowleri* must adhere to BSL2 practice guidelines, in which laboratory personnel must be properly trained to follow BSL2 standard procedures, including the use of personal protective equipment, restricted access to the laboratory and handling of specimens, and proper waste disposal. If available, the use of a biological safety cabinet to handle cultures of *N. fowleri* is recommended to minimize inhalation of aerosols and prevent contamination of cultures with fungi or bacteria.^{15,37}

Serology

Detection of anti-*N. fowleri* antibodies from clinical specimens is not typically performed for the diagnosis of PAM as most patients die before a strong humoral immune response is developed. Rather, serologic testing is mainly used to assess previous exposure or for population studies that assess the prevalence of *N. fowleri* exposure in endemic areas.

Serum samples of healthy humans have been demonstrated to have antibody titers positive for *N. fowleri*, indicating previous exposure to *N. fowleri* or related nonpathogenic strains.⁷ Previous studies have demonstrated that in regions where PAM cases have occurred, such as the southeastern United States, Australia, and New Zealand, healthy young adults have IgM antibodies that agglutinate *N. fowleri* cells.³⁹

In another study, serum samples from patients living in different regions of the United States demonstrated differences in antibody titers. In this study, the serum from 30 healthy adult humans in North Carolina was assayed for agglutinating ability against 4 *Naegleria* species. Most of the serum samples of the subjects were able to agglutinate *N. fowleri* trophozoites at a titer of 1:16 compared to sera of patients from Pennsylvania who demonstrated a titer level of 1:4 *N. fowleri*-agglutinating antibodies. Thus, it is suggested that the difference in titers is the result of the population living in North Carolina being exposed to *N. fowleri*-related antigens more frequently than the population in Pennsylvania.⁴⁰

Another study demonstrated that sera from patients with “humidifier fever” reacts with antigenic material from *Naegleria*, suggesting that *N. fowleri* and other microorganisms could be possible causative agents of humidifier fever.⁴¹ In such cases, humidifier fever did not progress to PAM. In another study, 1 PAM survivor was demonstrated to have specific antibodies to *N. fowleri* at 7, 10, and 42 days postadmission. Interestingly, the father of the patient had a low antibody titer to

N. fowleri as well, but did not present any clinical manifestations, whereas the mother of the patient demonstrated a negative titer.⁴²

Another study looked at the prevalence of antibody titers to *N. fowleri* in hospitalized patients, which demonstrated presence of IgG and IgM titers in 101 out of 115 patients ages 15 to 98 years old.⁴³ Similarly, IgA and IgM antibodies to *N. fowleri* have been detected in serum and saliva samples from patients with upper respiratory tract infections in parasite-endemic areas, healthy individuals, and healthy individuals living in a parasite-nonendemic region.⁴⁴ A more recent study demonstrated seropositivity to *N. fowleri* to a 1:100 titer level in all test subjects, children and adolescents living in northwest Mexico, and 1:500 seropositivity in 98% of all test subjects.⁴⁵

Detection of antibody titers to *N. fowleri* is most commonly performed by enzyme-linked immunosorbent assay (ELISA) protocols. An ELISA is usually performed by coating microtiter plates with whole-cell lysates of *N. fowleri* followed by the addition of serial dilutions of patient sera and the use of animal anti-human IgG secondary antibody conjugated with horseradish peroxidase for detection.^{40,45}

Alternatively, Western blot protocols that use transfer of electrophoresed *N. fowleri* lysate to nitrocellulose membranes for testing patient sera with secondary antibody detection systems have been used mostly in research settings.^{26,46,47} Immunofluorescence using *N. fowleri* trophozoites fixed on coverslips and incubated with patient sera and FITC-conjugated animal anti-human antibodies has demonstrated the ability to detect antibodies specific to *N. fowleri* from human and animal specimens.^{26,40}

Although the presence of anti-*Naegleria* antibodies does not represent previous disease, a hypothesis that may explain the presence of antibodies binding to *N. fowleri* antigens is the exposure to other free-living amoebas that are antigenically related. Thus, the role humoral immune response in protection to *N. fowleri* should be further evaluated, and the diagnostic value of antibody titers should be used to track the presence of amoebas in the population or to track the foci of infection.

Environmental Detection

There are over 30 *Naegleria* species members, and although *N. fowleri* is the main pathogenic amoeba of the *Naegleria* genus, two other species, *N. italica* and *N. australiensis*, have been described as potential pathogenic species. Thus, identification and differentiation of these 3 species is considered when monitoring potable water and recreational water supplies.⁴⁸ However, testing of water associated with cases of PAM is not routinely performed in clinical investigations as *N. fowleri* and other free-living amoebas are ubiquitous and not routinely screened from water or soil sources. The methodology used to test environmental water samples is similar to the methods described in the previous sections of this review, with nucleic acid identification and culture being the main methods for screening water. Other methods have been developed to identify pathogenic and potentially pathogenic *Naegleria* spp. from environmental water sources by single primer real-time PCR followed by melting-curve analysis of the 5.8S ribosomal DNA gene and flanking noncoding spacers.⁴⁸

Laboratories that offer environmental testing services include the CDC, state laboratories, and private laboratories that provide aquatic microbiology testing, such as ESML Analytical, Inc. and Biological Consulting Services of North Florida, Inc., have protocols available for testing domestic, recreational, and environmental water.

Conclusion

Diagnosis of PAM is usually performed postmortem following histopathological microscopic examination of brain tissue revealing the presence of trophozoites. Other methods used to diagnose PAM are cytospin preparations of CSF, PCR, antigen detection by IHC and IIF, and culture. Although *N. fowleri* infections are extremely rare, there has been an increase in the number of cases over the last 10 years and the incidence is expected to rise in the future. Although many laboratories will not encounter a case of PAM, it is important to emphasize that fast diagnosis is critical to increase the chances of patient survival. Thus, physicians and laboratory personnel should be familiar with the symptomatology, diagnostic tests available, and treatment options, as the aggressive nature of the disease makes prompt treatment crucial.

To improve treatment outcomes, it is advised that hospitals located in regions where most *Naegleria* cases have occurred keep miltefosine readily available. Previously, the CDC had provided miltefosine for the treatment of PAM and other free-living amebae infections. However, distribution of miltefosine is currently administered by commercial pharmaceutical companies. To this end, Impavido (miltefosine) capsules can be obtained free of charge through the Profunda and CDC consignment program of orphan drugs. As of now, only 26 hospitals carry Impavido on-site, but this program seeks to expand the at-hand supply of miltefosine to hospitals.

To improve diagnostic outcomes, laboratory personnel should be informed about the diagnostic tests available for PAM. Because the clinical presentation and initial CSF values resemble those of bacterial meningitis, it is of great importance to properly screen cases of meningitis to rule out amoebic meningoencephalitis, as treatment against bacterial and viral meningitis is ineffective against *Naegleria*. Furthermore, it is advised that diagnosticians consider PAM as the cause for meningitis in pediatric and young adult patients, especially if there is history of recent exposure to recreational water.

Moreover, the prevalence of the disease may not be completely clear, as cases may be underreported due to misdiagnosis or mischaracterization with other meningitis etiologies. Continuous training of laboratory personnel for the prompt identification of *N. fowleri* from CSF specimens is critical for successful diagnosis of PAM. It is recommended that laboratory sites participate in proficiency testing that includes prepared slides of *N. fowleri*, and standard operating procedures should be in place so that laboratory personnel are familiar with the testing procedures used at the CDC and have a readily available plan for expedited shipping of samples suspected of being *N. fowleri* or other pathogenic free-living amoebas.

In addition, there is room for the development of additional rapid PAM diagnostic tests, as confirmatory results for *N. fowleri* have an estimated turnaround time of 3 to 10 days. Methods with faster turnaround time are needed to rule out PAM in patients presenting with meningoencephalitis. The development and implementation of rapid tests that can be widely available in all laboratory settings for quick screening of patients with meningitis will be beneficial to improve incidence reporting and to initiate prompt treatment.

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REFERENCES

- Gharpure R, Bliton J, Goodman A, Ali IKM, Yoder J, Cope JR. Epidemiology and clinical characteristics of primary amebic meningoencephalitis caused by *Naegleria fowleri*: a global review. *Clin Infect Dis*. 2021;73(1):e19–e27. doi:10.1093/cid/ciaa520.
- Maciver SK, Piñero JE, Lorenzo-Morales J. Is *Naegleria fowleri* an emerging parasite? *Trends Parasitol*. 2020;36(1):19–28. doi:10.1016/j.pt.2019.10.008.
- Siddiqui R, Khan NA. Primary amoebic meningoencephalitis caused by *Naegleria fowleri*: an old enemy presenting new challenges. *PLoS Negl Trop Dis*. 2014;8(8):e3017. doi:10.1371/journal.pntd.0003017.
- Trabelsi H, Dendana F, Sellami A, et al. Pathogenic free-living amoebas: epidemiology and clinical review. *Pathol Biol (Paris)*. 2012;60(6):399–405. doi:10.1016/j.patbio.2012.03.002.
- De Jonckheere JF. Origin and evolution of the worldwide distributed pathogenic amoeboflagellate *Naegleria fowleri*. *Infect Genet Evol*. 2011;11(7):1520–1528. doi:10.1016/j.meegid.2011.07.023.
- Grace E, Asbill S, Virga K. *Naegleria fowleri*: pathogenesis, diagnosis, and treatment options. *Antimicrob Agents Chemother*. 2015;59(11):6677–6681. doi:10.1128/AAC.01293-15.
- Marciano-Cabral F, Cabral GA. The immune response to *Naegleria fowleri* amoebas and pathogenesis of infection. *FEMS Immunol Med Microbiol*. 2007;51(2):243–259. doi:10.1111/j.1574-695X.2007.00332.x.
- Naegleria fowleri*: Sources of infection, pathophysiology, diagnosis, and management; a review. <https://onlinelibrary.wiley.com/doi/epdf/10.1111/1440-1681.13192>. Accessed July 22, 2022.
- Venkatesan A, Tunkel AR, Bloch KC, et al. Case definitions, diagnostic algorithms, and priorities in encephalitis: consensus statement of the international encephalitis consortium. *Clin Infect Dis*. 2013;57(8):1114–1128. doi:10.1093/cid/cit458.
- Ali A, Ortiz JF, Morillo Cox A, Armas M, Orellana VA. Miltefosine: a miracle drug for meningoencephalitis caused by free-living amoebas. *Cureus*. 2021;13(3):e13698. doi:10.7759/cureus.13698.
- Kaminsky R. Miltefosine Zentaris. *Curr Opin Investig Drugs*. 2002;3(4):550–554.
- Schuster FL, Guglielmo BJ, Visvesvara GS. In-vitro activity of miltefosine and voriconazole on clinical isolates of free-living amoebas: *Balamuthia mandrillaris*, *Acanthamoeba* spp., and *Naegleria fowleri*. *J Eukaryot Microbiol*. 2006;53(2):121–126. doi:10.1111/j.1550-7408.2005.00082.x.
- da Rocha-Azevedo B, Tanowitz HB, Marciano-Cabral F. Diagnosis of infections caused by pathogenic free-living amoebas. *Interdiscip Perspect Infect Dis*. 2009;2009(Special Issue):1–14. doi:10.1155/2009/251406.
- Jain R, Prabhakar S, Modi M, Bhatia R, Sehgal R. *Naegleria* meningitis: a rare survival. *Neurol India*. 2002;50(4):470–472.
- Siddiqui R, Ali IKM, Cope JR, Khan NA. Biology and pathogenesis of *Naegleria fowleri*. *Acta Trop*. 2016;164(December):375–394. doi:10.1016/j.actatropica.2016.09.009.
- Dunn AL, Reed T, Stewart C, Levy RA. *Naegleria fowleri* that induces primary amoebic meningoencephalitis: rapid diagnosis and rare case of survival in a 12-year-old Caucasian girl. *Lab Med*. 2016;47(2):149–154. doi:10.1093/labmed/lmw008.
- Anjum SK, Mangrola K, Fitzpatrick G, et al. A case report of primary amebic meningoencephalitis in North Florida. *IDCases*. 2021;25(June):e01208. doi:10.1016/j.idcr.2021.e01208.
- Martinez AJ, Visvesvara GS. Laboratory diagnosis of pathogenic free-living amoebas: *Naegleria*, *Acanthamoeba*, and *Leptomyxid*. *Clin Lab Med*. 1991;11(4):861–872.
- Cope JR, Ali IK. Primary amebic meningoencephalitis: what have we learned in the last 5 years? *Curr Infect Dis Rep*. 2016;18(10):31. doi:10.1007/s11908-016-0539-4.
- Barnett ND, Kaplan AM, Hopkin RJ, Saubolle MA, Rudinsky MF. Primary amoebic meningoencephalitis with *Naegleria fowleri*: clinical review. *Pediatr Neurol*. 1996;15(3):230–234. doi:10.1016/s0887-8994(96)00173-7.
- Visvesvara GS. Amebic meningoencephalitis and keratitis: challenges in diagnosis and treatment. *Curr Opin Infect Dis*. 2010;23(6):590–594. doi:10.1097/QCO.0b013e32833ed78b.
- Sugita Y, Fujii T, Hayashi I, et al. Primary amebic meningoencephalitis due to *Naegleria fowleri*: an autopsy case in Japan. *Pathol Int*. 1999;49(5):468–470. doi:10.1046/j.1440-1827.1999.00893.x.
- Hara T, Yagita K, Sugita Y. Pathogenic free-living amoebic encephalitis in Japan. *Neuropathol Off J Jpn Soc Neuropathol*. 2019;39(4):251–258. doi:10.1111/neup.12582.
- Photos and Videos of *Naegleria fowleri*. *Naegleria fowleri*. CDC. Published October 17, 2022. <https://www.cdc.gov/parasites/naegleria/naegleria-fowleri-media.html>. Accessed November 2, 2022.
- Réveiller FL, Marciano-Cabral F, Pernin P, Cabanes PA, Legastelois S. Species specificity of a monoclonal antibody produced to *Naegleria fowleri* and partial characterization of its antigenic determinant. *Parasitol Res*. 2000;86(8):634–641. doi:10.1007/pl00008544.
- Sparagano O, Drouet E, Brebant R, Manet E, Denoyel GA, Pernin P. Use of monoclonal antibodies to distinguish pathogenic *Naegleria fowleri* (cysts, trophozoites, or flagellate forms) from other *Naegleria* species. *J Clin Microbiol*. 1993;31(10):2758–2763. doi:10.1128/jcm.31.10.2758-2763.1993.
- Muldrow LL, Tyndall RL, Fliermans CB. Application of flow cytometry to studies of pathogenic free-living amoebas. *Appl Environ Microbiol*. 1982;44(6):1258–1269. doi:10.1128/aem.44.6.1258-1269.1982.
- Flores BM, Garcia CA, Stamm WE, Torian BE. Differentiation of *Naegleria fowleri* from *Acanthamoeba* species by using monoclonal antibodies and flow cytometry. *J Clin Microbiol*. 1990;28(9):1999–2005. doi:10.1128/jcm.28.9.1999-2005.1990.
- Streby A, Mull BJ, Levy K, Hill VR. Comparison of real-time PCR methods for the detection of *Naegleria fowleri* in surface water and sediment. *Parasitol Res*. 2015;114(5):1739–1746. doi:10.1007/s00436-015-4359-5.
- Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. *J Clin Microbiol*. 2006;44(10):3589–3595. doi:10.1128/JCM.00875-06.
- Test Order. Submitting Specimens to CDC. Infectious Diseases Laboratories. CDC. Published July 22, 2022. <https://www.cdc.gov/laboratory/specimen-submission/detail.html>. Accessed August 3, 2022.
- Mahittikorn A, Mori H, Popruk S, et al. Development of a rapid, simple method for detecting *Naegleria fowleri* visually in water samples by loop-mediated isothermal amplification (LAMP). *PLoS One*. 2015;10(3):e0120997. doi:10.1371/journal.pone.0120997.
- Huang S, Liang X, Han Y, Zhang Y, Li X, Yang Z. A pediatric case of primary amoebic meningoencephalitis due to *Naegleria fowleri* diagnosed by next-generation sequencing of cerebrospinal fluid and blood samples. *BMC Infect Dis*. 2021;21(1):1251. doi:10.1186/s12879-021-06932-9.
- Wang Q, Li J, Ji J, et al. A case of *Naegleria fowleri* related primary amoebic meningoencephalitis in China diagnosed by next-generation sequencing. *BMC Infect Dis*. 2018;18(1):349. doi:10.1186/s12879-018-3261-z.
- Yadav D, Aneja S, Dutta R, Maheshwari A, Seth A. Youngest survivor of *Naegleria* meningitis. *Indian J Pediatr*. 2013;80(3):253–254. doi:10.1007/s12098-012-0756-2.
- Sood A, Chauhan S, Chandel L, Jaryal SC. Prompt diagnosis and extraordinary survival from *Naegleria fowleri* meningitis: a rare case report. *Indian J Med Microbiol*. 2014;32(2):193–196. doi:10.4103/0255-0857.129834.
- Schuster FL. Cultivation of pathogenic and opportunistic free-living amoebas. *Clin Microbiol Rev*. 2002;15(3):342–354. doi:10.1128/CMR.15.3.342-354.2002.
- Zaongo SD, Shaio MF, Ji DD. Effects of culture media on *Naegleria fowleri* growth at different temperatures. *J Parasitol*. 2018;104(5):451–456. doi:10.1645/18-6.

39. Reilly MF, Marciano-Cabral F, Bradley DW, Bradley SG. Agglutination of *Naegleria fowleri* and *Naegleria gruberi* by antibodies in human serum. *J Clin Microbiol.* 1983;17(4):576–581. doi:[10.1128/jcm.17.4.576-581.1983](https://doi.org/10.1128/jcm.17.4.576-581.1983).
40. Marciano-Cabral F, Cline ML, Bradley SG. Specificity of antibodies from human sera for *Naegleria* species. *J Clin Microbiol.* 1987;25(4):692–697. doi:[10.1128/jcm.25.4.692-697.1987](https://doi.org/10.1128/jcm.25.4.692-697.1987).
41. Finnegan MJ, Pickering CA, Davies PS, Austwick PK, Warhurst DC. Amoebas and humidifier fever. *Clin Allergy.* 1987;17(3):235–242. doi:[10.1111/j.1365-2222.1987.tb02008.x](https://doi.org/10.1111/j.1365-2222.1987.tb02008.x).
42. Seidel JS, Harmatz P, Visvesvara GS, Cohen A, Edwards J, Turner J. Successful treatment of primary amebic meningoencephalitis. *N Engl J Med.* 1982;306(6):346–348. doi:[10.1056/NEJM198202113060607](https://doi.org/10.1056/NEJM198202113060607).
43. Dubray BL, Wilhelm WE, Jennings BR. Serology of *Naegleria fowleri* and *Naegleria lovaniensis* in a hospital survey. *J Protozool.* 1987;34(3):322–327. doi:[10.1111/j.1550-7408.1987.tb03183.x](https://doi.org/10.1111/j.1550-7408.1987.tb03183.x).
44. Rivera V, Hernández D, Rojas S, et al. IgA and IgM anti-*Naegleria fowleri* antibodies in human serum and saliva. *Can J Microbiol.* 2001;47(5):464–466.
45. Lares-Jiménez LF, Borquez-Román MA, Alfaro-Sifuentes R, Meza-Montenegro MM, Casillas-Hernández R, Lares-Villa F. Detection of serum antibodies in children and adolescents against *Balamuthia mandrillaris*, *Naegleria fowleri* and *Acanthamoeba* T4. *Exp Parasitol.* 2018;189(June):28–33. doi:[10.1016/j.exppara.2018.04.011](https://doi.org/10.1016/j.exppara.2018.04.011).
46. Powell EL, Newsome AL, Allen SD, Knudson GB. Identification of antigens of pathogenic free-living amoebas by protein immunoblotting with rabbit immune and human sera. *Clin Diagn Lab Immunol.* 1994;1(5):493–499. doi:[10.1128/cdli.1.5.493-499.1994](https://doi.org/10.1128/cdli.1.5.493-499.1994).
47. Shin HJ, Cho MS, Jung SU, et al. Molecular cloning and characterization of a gene encoding a 13.1 kDa antigenic protein of *Naegleria fowleri*. *J Eukaryot Microbiol.* 2001;48(6):713–717. doi:[10.1111/j.1550-7408.2001.tb00211.x](https://doi.org/10.1111/j.1550-7408.2001.tb00211.x).
48. Robinson BS, Monis PT, Dobson PJ. Rapid, sensitive, and discriminating identification of *Naegleria* spp. by real-time PCR and melting-curve analysis. *Appl Environ Microbiol.* 2006;72(9):5857–5863. doi:[10.1128/AEM.00113-06](https://doi.org/10.1128/AEM.00113-06).

Correction: The Importance of Medical Laboratory Scientists and the Number of Doctoral Scientists that Began Their Career by Working on the Front Lines of Laboratory Medicine

Laboratory Medicine 2023;54:e133; <https://doi.org/10.1093/labmed/lmad007>

This is a correction to: The Importance of Medical Laboratory Scientists and the Number of Doctoral Scientists that Began Their Career by Working on the Front Lines of Laboratory Medicine, *Laboratory Medicine*, 2022, <https://doi.org/10.1093/labmed/lmac143>

In the originally published version of this manuscript, there was an error in the title of the paper which should read: “The Importance of Medical Laboratory Scientists and the Number of Doctoral Scientists that Began Their Career by Working on the Front Lines of Laboratory Medicine” instead of “Special Report The Importance of Medical Laboratory Scientists and the Number of Doctoral Scientists that Began Their Career by Working on the Front Lines of Laboratory Medicine”.

This error has been corrected.

Clinical Educators' Teaching Approaches and Attributes in Laboratory Medicine

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Abbreviations: SOP, standard operating procedure.

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ABSTRACT

The quality of clinical experiences for students in health care professions relies heavily on clinical educators. Hence the quest to be informed on qualities that make great clinical educators in medical laboratory professions as well as teaching methods. A 48-question survey was developed, validated, and distributed to laboratory professionals in the American Society for Clinical Pathology database. Four questions pertaining to teaching and evaluation as well as clinical educators' attributes were evaluated in the study. Responses were analyzed using the Statistical Package for the Social Sciences. Descriptive statistics were performed with the *P* value set at .05. The results revealed that communication and motivation to teach were most valued among clinical educators and empathy was the least valued attribute. Educators reported on different approaches to teaching and evaluating students. Clinical educators could benefit from training that highlights these attributes and teaching methods for great clinical experiences both for educators and students.

Clinical educators are an integral part of the clinical education received by students in any medical field, and some researchers argue that clinical education is the most important factor in achieving clinical outcomes.¹ Clinical educators are the major players responsible for the clinical education received in a clinical setting, such as a medical center, hospital, or outpatient clinic.² The characteristics and roles of clinical educators cannot be underestimated as they have an influence on students' clinical experiences. They influence the training environment, professional behavior, critical thinking, and psychomotor skills, among many others. Seasoned clinical educators have exceptional abilities that smoothly facilitate learning. The following questions are relevant to what makes a great clinical educator: What are their teaching styles and attributes? Which attributes help students succeed? Previous studies indicate that an educator's attributes and teaching style impact student's clinical education experience. Irby³ indicated that attributes such as arrogance and insensitivity could negatively affect a student's clinical education.

Although research in other medical fields indicates fundamental attributes and teaching styles that clinical educators use to guide student success, these styles and attributes may vary from one field to the next. Sutkin and colleagues⁴ explained that clinical educators in medical schools have an understanding of what makes a good clinical educator, but this is less delineated in other health professions. Fifteen health science clinical educators who were interviewed in an Australian study found role modeling, scholarly activity, and professionalism to be the most desired attributes of a clinical educator.⁵ Clinical educators associated with students in medical school found knowledge, clinical and technical competence, enthusiasm, positive relationship with students, and communication skills to be key attributes of a good educator.⁴

It is important to evaluate what makes a good clinical educator in the laboratory sciences to gain new insights into student success. There is a false belief that every educated individual or expert is a suitable instructor.⁶ It is not surprising that individuals with years of work experience still need to be trained in pedagogy for better outcomes. The purpose of this study was to understand the attributes and teaching styles that make a quality clinical educator for laboratory professionals from the perception of clinical educators. Studies for the most part focus on the clinical educators' attributes from the students' perspective. Ilic et al⁷ explained that students focus on attributes such as the clinical educators' ability to transfer knowledge. In different studies, students' perceptions of what a clinical educator believes to fuel a learning environment vary greatly. Student's published perspectives appear over broad areas of medicine, including nursing. There are limited studies that involve the perception of the clinical educator. Although various studies do involve the student's perception in various areas of medicine, Ilic's⁷ study proved that clinical educators and students do not have the same perceptions of what makes a good clinical educator. In studies that focused on clinical educators in other medical fields, the attributes concentrated more on characteristics like professionalism. This attribute was not valued by students in similar studies.⁷

This study aimed to examine what attributes and teaching styles make a good clinical educator, through the perceptions of clinical educators. Educators were surveyed through a series of questions using multiple choice, the Likert scale, and open-ended questions. This study did not use objective measurements to determine how attributes or teaching styles affect student learning or clinical outcomes.

Materials and Methods

A comprehensive methodology can be found in *Establishing the Need for Clinical Educator Training for Laboratory Professionals in Laboratory Medicine*. This is the third publication from this dataset.

Research Design

Our research team developed 48 questions directed at clinical educators, designed from a detailed literature review and comments made by previous students regarding their clinical experiences. After validation, the questions were put on an electronic platform for electronic distribution by the American Society of Clinical Pathology (ASCP). The survey was distributed to laboratory professionals who are members of ASCP. The survey was distributed on March 8, 2021, and was closed on April 16, 2021. Common themes and categories were found in data that were later analyzed.

Data Analysis

Cronbach's alpha of 0.935 determined the reliability of the electronic questionnaire. Cronbach's alpha is used to measure the internal consistency among the designed questions. Survey questions included multiple choice, Likert scale ranking, and open-ended question formats. The Institutional Review Board at Austin Peay State University approved this research as IRB # 0-066.

Results

Two hundred and seven individuals participated in the survey. When assessing clinical educators teaching style, 99% of clinical educators stated they share their personal experiences as laboratory professionals with their students.

Clinical educators were asked to rank the most essential qualities a clinical educator should possess, with 1 being the most important and 8 being the least important. Effective communication and a desire to train were rated as the most important attributes of a clinical educator. The least important attribute, as determined by survey participants,

was empathy. **FIGURE 1** shows the most and least valued characteristics and provide details for each characteristic. Participants were asked if there were any additional characteristics that they felt should be added to the list of favorable characteristics. The comprehensive list is provided in **TABLE 1**.

A total of 82% of survey participants explained that they changed their training approach based on the student's performance. Of this 82%, only 7% of participants further elaborated with an open-ended response. Seven of 9 open-ended responses explain that flexibility in a student's clinical education is important to enhance the student's learning. Clinical educators explain that flexibility allows more time to be spent on areas of student struggle. A χ^2 test was performed to determine whether a relationship existed between a clinical educator changing their approach to training based on the student's performance and whether the clinical educator compared the current student to previous students. A significant relationship did exist ($P < .05$). A χ^2 analysis was also performed to determine whether the clinical educator's approach to training changed based on a student's performance and whether the clinical educator uses student errors as a teaching tool. A significant relationship does exist ($P < .05$).

Of survey participants, 96% explained they use student mistakes and errors as a teaching tool. Of the 4% of participants who explained that they do not use students' errors as a teaching tool, 16% of those participants further explained why they chose not to in an open-ended response. These clinical educators fear this teaching style may jeopardize student confidence. One clinical educator explains, "just assume errors will occur with students prior to them finishing training. Just watch for them and correct the mistakes." A χ^2 test was performed to determine whether a relationship exists between the clinical educator who uses student errors as a teaching tool and those clinical educators who share their personal experiences while training students. There is a significant relationship ($P < .05$).

A total of 99% of clinical educators stated they give verbal instructions when demonstrating procedures or techniques and 97% of the survey participants stated they explained the theory or principle behind what they are demonstrating to the students. Clinical educators were asked whether they provide the student with a comprehensive picture of disease processes by integrating laboratory results from other departments. Participant responses can be seen in **FIGURE 2**. Clinical educators who did not provide theory or principle were given the opportunity to respond in an open-ended format. There were 3 responses indicating that students read the theory and principles in the standard operating procedure (SOP), one explained that students should be taught this in their didactic education, and the last explained that due to the limitations on clinical time, educators only explain when time permits.

Survey participants were asked whether multiple clinical educators train the same student in the same section and whether there was flexibility in methods of training. "Do you allow for flexibility in training methods or techniques among the various clinical educators?" Participants responded by selecting 1 of 3 answers: always, sometimes, or never. The results of these selections can be seen in **FIGURE 3**. Participants had the opportunity to further explain their position in an open-ended format. Most respondents explained that their flexibility heavily depends on the flexibility of the program and facility. Many participants further explained, "there are many ways in teaching to get to the same endpoint."

Clinical educators were asked what their backup plan was when their job required their focus to shift to responsibilities other than the student.

FIGURE 1. Most and least valued characteristics.

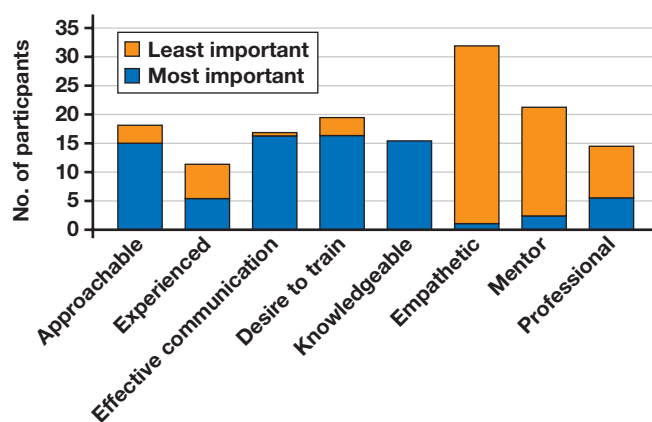


TABLE 1. Participants' Suggested Clinical Educator Characteristics

Characteristic	Frequency
Able to teach to different learning styles (flexible)	23
Enthusiasm	19
Patience	15
Polite	4
Follows through/dependable	4
Organized	4
Attentive	3
Knowledgeable	3
Honest	3
Professional	3
Team player	3
Unbiased/fairness in assessments	2
A leader	2
Sets boundaries with students	2
Attention to detail	2
Humorous	2
Approachable	2
Open minded	1
Up to date	1
Teaching experience	1
Administration supports clinical educator	1
Admits when wrong	1
Motivated	1
Willing to learn	1
Motivated	1
Confidence	1
Available for questions after rotation	1
Respected by peers	1
Communicates well	1
Time management	1
Has compassion	1
Teaches grass-root regulations (CLIA regulations)	1

They were asked to select all that applied from the following responses: students observe the work of the clinical educator, student studies, someone else works with the student, the time is used as a mentoring opportunity, or the student is sent home. The choices that participants selected for each backup plan used for students can be seen in **FIGURE 4**. Participants were allowed to provide additional open-ended responses for the backup plan they used with their students. Ten participants responded to the open-ended questions with various responses. Two clinical educators provided an assigned project that students worked

on throughout their rotation. Two different clinical educators provided students with extra specimens with known results for them to practice test performance. One educator indicated that students help with activities in the laboratory, such as inventory, and one educator explained that students read SOPs. Students watch supplemental videos with one clinical educator. One educator explained they were hired solely as a clinical educator and no backup plan exists. One educator stated their students do personal projects like origami and another educator stated that students were moved to evenings or weekends.

Clinical educators were further asked if a student left early, how were lost training hours recaptured? A total of 92 participants responded in an open-ended format. Most commonly, students do not make up time that is lost, according to 35 clinical educators. Twenty other clinical educators indicated that students reschedule for an additional day of training. Nineteen educators stated that students are not allowed to leave early from training. Students are expected to arrive early or stay late in addition to their regular shift, according to 9 survey participants. There were 8 clinical educators who would send the student home with an assignment, case studies, a book review, or journal article reviews. One clinical educator stated that the student would be assigned a "boot camp" to make up hours missed.

Survey participants were asked how they ensured their students had achieved competence for a given procedure or test. Participants were asked to select all that applied and were given the following list to choose from: by student observation of the clinical educator (26%), by the clinical educator's observation of the student (88%), by having the student work up mock specimens (75%), or using written or oral tests (74%).

Discussion

This study lists various teaching styles and attributes as preferred traits of a good clinical educator by clinical educators. Research on the clinical educators' perception of what makes a good clinical educator is generally limited and is nonexistent, to the best of our knowledge, in laboratory medicine. Traits that were favorably rated are discussed below.

Communication

Clinical educators who participated in this study identified 2 attributes that they believed were the most important: effective communication and having a desire to train. Other studies that involve the perspective of the clinical educators have not identified effective communication as the most important attribute of being a good clinical educator. However, in several studies where students listed the worst attributes in a clinical educator, poor communication was listed as the worst attribute. The sentinel study, Characteristics of Best and Worst Clinical Teachers as Perceived by University Faculty and Students,⁸ ranked deficiency in communication skills as the second worst quality and poor discussions on expectations was the third worst quality of an ineffective clinical

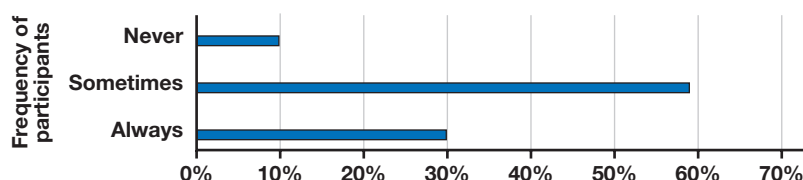
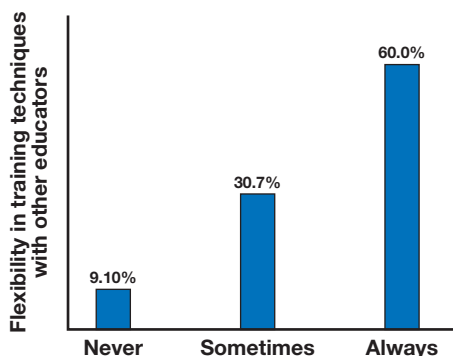
FIGURE 2. A Clinical educator's communications of comprehensive disease processes.

FIGURE 3. Flexibility in training techniques with other educators.



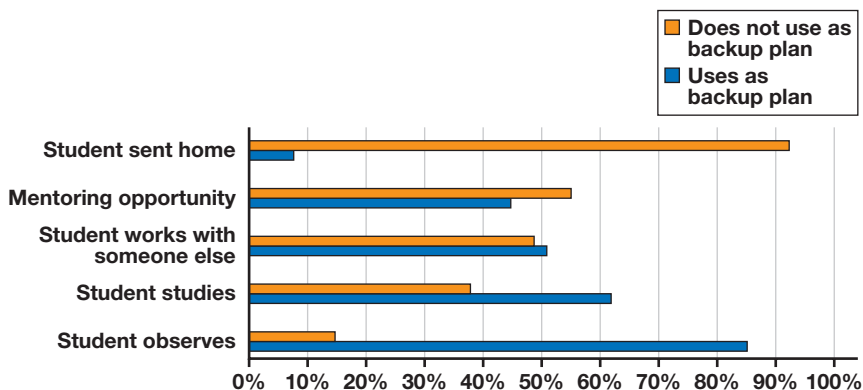
educator. In the Ilic et al⁷ study, students conveyed that communication in the form of feedback skills was the second most important attribute of a good educator. In contrast, of the 15 clinical educators that participated in the study, none indicated that feedback skills were an important attribute of a good clinical educator.

Communication is an important quality of a clinical educator. Communication is a broad term that encompasses feedback, communicating progress, providing positive reinforcement, enhancing engagement, and providing one's pearls of wisdom. Clinical educators can define expectations, identify areas that students should improve, and provide evaluations, but if the clinical educator cannot communicate these expectations, and provide critiques or feedback through effective communication, then obtaining the best materials for a student's clinical education is a moot point. Effective communication can curtail many problems and can alleviate a multitude of misunderstandings.⁹

Motivation

Internal motivation was also tied to the best attribute of a clinical educator. Only 3 clinical educators who participated in this study selected motivation as the least important quality. Darvishpour and Javadi-Pashaki⁶ explained that students in their study believed that internal motivation was one of the characteristics of an effective clinical instructor. They indicate that a good clinical educator who is willing to teach must make clinical learning enjoyable and must be able to motivate students to get them involved in the learning.⁶ This makes the clinical environment attractive.

FIGURE 4. Backup plans for students.



In the Soroush et al¹⁰ study, students in the nursing program ranked internal motivation as the most important attribute of a good clinical educator. A student in that study stated, "A clinical educator who has energy and motivation with the up-to-date knowledge of a younger teacher but the experience of an older one, would be the perfect clinical educator."¹⁰ Another student explained that their educator's motivation was what they needed to continue their education.¹⁰ These sentiments are similar to the perspectives of clinical educators in the present study.

On the opposite end of the spectrum, a clinical educator that is not motivated or energetic in their profession transmits this apathy to their student. In the worst case, unmotivated clinical educators may be overworked and burned out. It would not come as a surprise that during the staffing shortage seen during the COVID-19 pandemic, many health professionals are dealing with a lack of motivation from being overworked. In Soroush's study, a nursing student stated, "The interest and the energy of the clinical educators are important. A clinical educator must love their job."¹⁰ Another student stated, "If the clinical educator always complains about the nursing profession, the student begins to ask themselves why are we trying to become a nurse? Clinical educators complain that it's not a good job, it's hard, and the salary is low."¹⁰

Knowledgeable

The third most desirable trait as selected by the clinical educators in this study was knowledge. None of the clinical educators in this study felt that this was the least-desired trait in a clinical educator. Monk's¹¹ study explained that an educator's knowledge of the subject matter has been shown to influence the student's achievement positively. Clinical educators should be aware of their professional responsibility and should make every attempt to advance their theoretical and clinical knowledge.¹⁰

In the Ilic et al⁷ study, 50% of health science students felt that knowledge was the most important attribute of clinical educators, whereas only 13.3% of health science clinical educators in the study felt knowledge was important. However, scholarly activity was not deemed to be an attribute that was important in making a good clinical educator.⁷

Clinical competence is also applied knowledge. Clinical competency fills the gap between practical and theoretical training. Inadequate clinical competence of the clinical educator reduces the student's trust in the learning environment.¹² In the Kol and İnce¹² study, it was clear that nursing students wanted the preceptors to be knowledgeable and competent in their field and agreed that these attributes are an essential component of effective teaching. In the Eiroa and Konin¹³ study that

looked at the physical training of clinical educators, it was determined that the clinical educator should always be able to provide a sound explanation as to why the treatment being applied is the best treatment available. In the Soroush et al¹⁰ study, clinical competence was the main point that students continued to point out in regards to what makes a good clinical educator.

Approachable

The approachability of the clinical educator was tied for the second-most desirable trait in a good clinical educator in this study. Three participants in this study ranked this attribute as the least desirable trait in a clinical educator. A thorough literature review demonstrated that this may be an overlooked attribute that may provide clues for whether a person is a good clinical educator.^{1,9,13} Other studies that looked at the perception of students or clinical educators rarely mentioned approachability as a reviewed attribute.

In the Morgan and Knox study,⁸ students perceived unapproachability as one of a few traits that make a clinical educator ineffective. The student should be able to feel comfortable approaching the preceptor with any issue that arises without feeling afraid or uncomfortable.¹³ Approachability is fostered in an environment where the student feels they can practice and learn in a judgment-free environment.¹³

Experience

The clinical educator's experience was the fifth-most desirable trait of a good clinical educator. It is the clinical experience, along with other attributes, that contributes to an effective clinical educator capable of effective teaching.¹⁰ In the Soroush et al study,¹⁰ a student stated they felt it was important for a clinical educator to have at least 10 years of experience in different clinical areas to be a good clinical educator. However, the number of years of clinical experience in laboratory medicine required to be an effective clinical educator has not been determined.

In the Darvishpour and Javadi-Pashaki⁶ study, it was determined that if clinical educators did not have sufficient experience, their academic status was questioned by the students. The study also showed that without clinical experience, clinical educators were not effective at meeting teaching outcomes. That study highly recommended that clinical educators have sufficient experience when being selected for that role.⁶

Professional Demeanor

Professional demeanor was determined to be the sixth-most desirable trait of a good clinical educator. Professional demeanor refers to the clinical educator's behavior in the workplace. This includes attitude, appearance, and manners. In the Ilic et al study,⁷ 20% of students felt professionalism was an important attribute of a good clinical educator. This was compared to only 5.3% of clinical educators who felt this was an important attribute in the same study. In the Soroush et al¹⁰ study, nursing students felt professionalism was the second-most important attribute of a clinical educator. When clinical educators accept the role of teaching students, they should consider the principles of professional ethics.¹²

In the Astle⁹ study, 3500 pharmaceutical students felt professionalism and being a good role model were the most important attributes of being a good clinical educator. The author indicates that it is within the practice setting that students acquire professional behaviors and learn how to positively interact with other professionals. It was clear from this study that disgruntled pharmacists who had a negative attitude toward their em-

ployer or the profession discouraged students when the student needed encouragement most.⁹ Students are often taught about liability and ethics in their clinical education, but after graduation, these concepts are probably not high on any healthcare professional's to-do list.¹³

Mentor

The seventh-most important attribute of a good clinical educator was mentorship. A study reviewing physiotherapy students' perception of clinical education determined that mentorship was the most important attribute of a good clinical educator. This was the only study found that ranked mentorship this high. This may be an attribute specific to physiotherapy students; therefore, the perception of laboratory medicine students should be evaluated. Other studies that reviewed the perception of clinical educators did not mention mentorship as an evaluated attribute.

Empathetic

Clinical educators ranked empathy as the least important attribute of a good clinical educator. This varies greatly from students' perceptions seen in other health care professions. The Morgan and Knox⁸ study that focused on nursing students found that students felt the worst clinical educators were those who did not empathize with them. It is not clear what nursing students are expecting their clinical educators to empathize with, and future studies should consider determining the focus of the desired empathy in laboratory medicine students. Possible areas of focus may be the difficulty of the medical laboratory program, social challenges between the educator and student, empathy because they are a student and may be slower or make mistakes, or personal issues.

Other Attributes

Participants were able to list other attributes they felt made a good clinical educator in an open-ended format. Twenty-three individuals felt flexibility in their teaching style was an important attribute. This was not an attribute listed in any other study but should be reviewed as a possible attribute in future studies. Enthusiasm was listed by 19 participants. In other studies, enthusiasm was included as part of motivation. In this study, these were kept separate because enthusiasm is the excited energy one exudes and motivation is the reasoning one has for behaving in a certain way. Patience was also listed by 15 participants but was not listed as an attribute of a good clinical educator in any other study. This attribute should also be considered in future studies.

Negative Attributes

A literature review indicated that students in other fields of medicine had selected attributes they felt made the worst clinical educator. The worst clinical educator was generally defined as an individual that did not enable the success of the clinical student and did not aid the student in meeting teaching outcomes for their clinical education. The Morgan and Knox⁸ study indicated that nursing students perceived the worst clinical educator as an unapproachable person who lacked empathy, belittled students regarding mistakes, and did not communicate their expectations. The Morgan and Knox⁸ study also incorporated nursing faculty's perspective. The faculty felt the worst clinical educator did not like nursing, could not identify a student's strengths and weaknesses, and is deficient in communication skills.⁸ This study also indicated that both faculty and students feel that the worst clinical educators are also judgmental and not open minded.⁸

Teaching Styles

In addition to the preceptor having the ability to share their knowledge, it is important for the preceptor to be interested in not only advancing their clinical knowledge but to want to advance their teaching skills.¹³ Teaching styles as used in this study refer to strategies used to facilitate learning.

Ninety-nine percent of clinical educators in this study state that they share their personal experiences with their students. Do students feel that their clinical educators are sharing these experiences as much as they say they are? Laboratory medicine students' perception of a good clinical educator should be incorporated into future research. This is a form of mentorship that is thought to be important for clinical students' success.

A large portion (82%) of clinical educators indicated that they change their training approach based on the student's performance. Only 9 participants chose to further elaborate on the importance of flexibility in the clinical educator training approach. The 18% of clinical educators who choose not to be flexible with their training approach should reconsider. In this study, 11% of clinical educators felt this attribute was important to be a good clinical educator.

About 4% of participants who explained that they do not use student errors as a teaching tool further explained that they attempt not to jeopardize student confidence. Using students' errors as a teaching tool is an important part of the feedback. Students need to understand how they are performing throughout their clinical course.⁹ It is frustrating for students to discover for the first time they did not meet the expectations of the preceptor at the end of the rotation.⁹ Students should understand areas that need improvement as the clinical course progresses to give the students time to make the necessary improvements.

Survey participants were asked how they ensured their students had achieved competence for a given procedure or test. Evaluation of students is a complex task required of clinical educators. Twenty-six percent of participants evaluated students simply by having the student observe the clinical instructor. Eighty-eight percent of clinical educators watch the student evaluate their performance. Seventy-five percent of clinical educators expect the student to appropriately test a mock sample whereas 74% require students to pass a written or an oral test.

Some students complained that their clinical educators do not provide the theories or principles for testing. Some students have stated that their clinical educators do not remember those theories in practice. When clinical educators were asked about explaining theories or principles, 97% of participants explained that they do take the time to explain those theories and principles. Future research should focus on students' responses to determine whether students feel their clinical educators explained the theory or principles and if they effectively cover those theories or principles to the students' satisfaction.

Limitations

This research lists teaching styles and attributes indicated by current clinical educators who are members of ASCP. The teaching styles listed in this study may differ from those outside this membership. Future research should be conducted to determine the array of teaching styles that may exist from various cohorts within the field of laboratory medicine.

In this study, students' results from areas outside of laboratory medicine were used to explain the results seen by clinical educators in this study. It would be best to compare the results of students who are in the laboratory medicine field. Sutkin and colleagues⁴ explained it best when they stated, "attributes of a good clinical educator are not well understood" with regard to those who are not medical or nursing students. Currently, no studies exist that review laboratory medicine students' perceptions of a good clinical educator. Future research should focus on the laboratory medicine student's perception to gain a full perspective of what attributes may make a good clinical educator.

This study did not use objective measurements to determine how attributes or teaching styles affect student learning. Future research should focus on the success of students in laboratory medicine who study under a clinical educator who has been identified to have these preferred traits to determine whether they are truly valuable for student success.

Conclusion

It is difficult to describe the attributes of a good clinical educator. This research focuses on the perception of the clinical educator as opposed to student perception, a topic on which current research is limited. Communication, motivation, knowledge, approachability, experience, professional demeanor, mentorship, and empathy were determined to be the leading attributes to make a good clinical educator from perception of clinical educators.

There are significant differences between the perceptions of clinical educators and those of students as to what makes a good clinical educator. According to the Ilıc et al⁷ study, students have a greater focus on the clinical educator's knowledge and their ability to transfer that knowledge to the student. Clinical educators focus on professional attributes, which are not valued by the student.⁷

Teaching styles varied greatly from one clinical educator to the next. Commonalities among clinical educators were flexibility in their teaching, sharing personal experiences, and using student mistakes as a teaching tool.

Communication and the motivation to teach were ranked as the most important attributes whereas empathy was the least important. Other attributes that were ranked high on the list include approachability, knowledge, experience, and mentorship. This information could help in training clinical educators to modify or enhance attributes that can provide a great clinical experience for students.

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REFERENCES

1. Niederriter JE, Eyth D, Thoman J. Nursing students' perceptions on characteristics of an effective clinical instructor. *SAGE Open Nurs*. 2017;3:2377960816685571.
2. Saarikoski M. The main elements of clinical learning in healthcare education. *CLES-Scale*. 2018:7–15.
3. Irby DM. Clinical teacher effectiveness in medicine. *Acad Med*. 1978;53(10):808–815. doi:10.1097/00001888-197810000-00003.

4. Sutkin G, Wagner E, Harris I, Schiffer R. What makes a good clinical teacher in medicine? A review of the literature. *Acad Med*. 2008;83(5):452–466. doi:[10.1097/acm.0b013e31816bee61](https://doi.org/10.1097/acm.0b013e31816bee61).
5. Gibson SJ, Porter J, Anderson A, et al. Clinical educators' skills and qualities in allied health: a systematic review. *Assoc Study Med Educ*. 2019;53.5:432–442. doi:[10.1111/medu.13782](https://doi.org/10.1111/medu.13782).
6. Darvishpour A, Javadi-Pashaki N. Good clinical instructor from the perspective of nursing students: a qualitative study. *J Qual Res Health Sci*. 2019;7(4):372–382.
7. Ilic D, Harding J, C Allan, Diug B. "What are the attributes of a good health educator?" *Int J Med Educ*. 2016;7:206–211. doi:[10.5116/ijme.5765.0b6a](https://doi.org/10.5116/ijme.5765.0b6a).
8. Morgan J, Knox JE. Characteristics of "best" and "worst" clinical teachers as perceived by university nursing faculty and students. *J Adv Nurs*. 1987;12(3):331–337. doi:[10.1111/j.1365-2648.1987.tb01339.x](https://doi.org/10.1111/j.1365-2648.1987.tb01339.x).
9. Astle J. 10 preceptor qualities that create a great learning environment. *Patient Care Retail Clinic Healthcare Solutions*. 2(4) 2014. <http://www.insidepatientcare.com/issues/2014/august-september-2014-vol-2-no-4/68-10-preceptor-qualities-that-create-a-great-learning-environment>
10. Soroush A, Andaieshgar B, Vahdat A, et al. The characteristics of an effective clinical instructor from the perspective of nursing students: a qualitative descriptive study in Iran. *BMC Nurs* 2021;20:36. doi:[10.1186/s12912-021-00556-9](https://doi.org/10.1186/s12912-021-00556-9).
11. Monk D, King J. *Multilevel Teacher Resource Effects on Pupil Performance in Secondary Mathematics and Science: The Case of Teacher Subject-matter Preparation*. Ehrenberg R, ed. Ithaca, NY: ILR Press; 1994.
12. Kol E, İnce S. Determining the opinions of the first-year nursing students about clinical practice and clinical educators. *Nurse Educ Pract*. 2018;31:35–40. doi:[10.1016/j.nepr.2018.04.009](https://doi.org/10.1016/j.nepr.2018.04.009).
13. Eiroa D, Konin J. How to be an effective athletic training preceptor: A literature review. *Internet J Allied Health Scie Practice*. 19(1):4. doi:[10.46743/1540-580X/2021.1980](https://doi.org/10.46743/1540-580X/2021.1980).

Pathology – The Beginnings of Laboratory Medicine

Second in a Series

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Abbreviations: AMA, American Medical Association; ACS, American College of Surgeons; ASCP, American Society for Clinical Pathology; BOR, Board of Registry; NAACLS, National Accrediting Agency for Clinical Laboratory Sciences; MT, Medical Technologist; LT, Laboratory Technician; ASMT, American Society of Medical Technologists; CAP, College of American Pathologists; LAP, Laboratory Accreditation Program; AMT, American Medical Technologists; MLT, Medical Laboratory Technician; ISCLT, International Society for Clinical Laboratory Technology; NCA, National Credentialing Agency for Laboratory Personnel; AAM, American Academy of Microbiology; ASC, American Society of Cytology; AABB, American Association of Blood Banks; NSH, National Society of Histotechnology; CMP, Certification Maintenance Program; ANSI, American National Standards Institute; ASCLS, American Society for Clinical Laboratory Science; BOC, Board of Certification; AGT, Association of Genetic Technologists; CLIA, Clinical Laboratory Improvement Act; HEW, Department of Health Education and Welfare; HHS, Department of Health and Human Services; POLs, physician office laboratories; COLA, Commission on Office Laboratory Accreditation; CDC, US Centers for Disease Control and Prevention; NLRB, National Labor Relations Board; HRSA, Health Resources and Services Administration; BLS, Bureau of Labor Statistics.

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The first article in this series followed the historical timeline of pathology and laboratory medicine from ancient times, with the earliest development of laboratory tests through the centuries, to the beginnings of various laboratories as recognized today.

Various early developments of “laboratories” (pharmaceutical, physical, chemical, biological, forensic, microbiological) for early scientists were for experimental study and scientific research purposes. None in the beginning were specifically medically oriented for testing/analysis for direct diagnosis for patient care. Clinical laboratories would eventually evolve into existence but not originally be affiliated with hospitals. Using clinical laboratories distinctly for medical testing to aid in the detection, diagnose, and treatment of disease would be forthcoming as an offspring of these research laboratories. It would take some time for pathology and laboratory medicine to become recognized as a specialty established in hospitals. Historians generally demonstrate that

laboratories were first created as independent rooms separate from hospitals to eventually be part of university hospitals.

How did pathology develop to become an important discipline of healthcare at all hospitals? Who became the workforce managing these laboratories?

Timeline of Hospital Laboratories

Only as recently as 2015—nearly 200 years after Thomas Jefferson (1743–1826) designed the University of Virginia Rotunda—chemistry equipment was discovered hidden behind a brick wall. At that time, laboratories associated with teaching institutions were still used purely for the purposes of experimental research and teaching.

Laboratories in the 1800s were kept distinct and separate from the mostly rudimentary hospitals, which at the time were mostly “charitable” institutions to tend to the sickly poor (collectively called almshouses in colonial America). Eventually, “voluntary” hospitals that were dependent on donations would be created.

The establishment of the early clinical laboratories can be traced first to central Europe. Ironically, the development of the laboratory would be slow, because the consensus of the medical field at this time was to focus on the sick patient as the center of the physician’s work, not the laboratory.

In 1828, Guy’s Hospital in England was set up to treat the incurables discharged from St. Thomas Hospital, with 2 clinical wards designed for medical student internships for chemical diagnostics of the sick at bedside. It was not until 1842 that a very small laboratory was even associated for clinical use.

In the late 19th century, the United States began to give some attention to the practice of pathology. It was during the Civil War (1861–1865) that the Surgeon General Office instructed all medical officers to forward all specimens, both surgical and medical, for laboratory testing. It has been reported that over 7,630 specimens were collected during the Civil War.

The first reported medical laboratory in the United States is attributed to Victor C. Vaughan, MD (1851–1929), but it was not affiliated with any hospital. Following the Civil War, Vaughan discovered a chemistry laboratory in a locked room at Mount Pleasant College in Missouri where he was an instructor, became fascinated, and began teaching

chemistry. During his medical training at the University of Michigan College of Medicine, he worked as a “laboratory assistant” examining the 2 major body fluids, urine and blood.

Although the laboratory was still not hospital-connected at the time, the department of pathology was gaining some attention. Hospital facilities were not available until 1875. Vaughan is quoted for the “pride” in performing laboratory testing, his primary interest. Despite an active practice in medicine, his interest in the laboratory continued and he became the first editor of the *Journal of Laboratory and Clinical Medicine* in 1915, expressing the view that “no physician should practice medicine without the laboratory.”

Dr. William H. Welch (1850–1934) is recognized for studying pathology and bacteriology in Germany. Deeply influenced by the European microscope and chemical methods and bacteriology work in European hospitals, he returned to America in 1877 and implored institutions to build laboratories on site. He firmly believed that a laboratory was an integral part of a hospital for the study of the origin of disease.

At first he was denied, as space and funding were not available for any laboratories at hospitals. Bellevue Hospital Medical College (the first medical college affiliated with a hospital) finally agreed, and it has been reported he was originally only afforded 3 rooms and a \$25.00 budget. Carnegie Laboratory was recognized as the nation’s first pathology bacteriology laboratory in 1884. The general consensus was to improve patient care.

The newly forming Johns Hopkins Hospital & Medical School thereafter appointed Dr. Welch as the first physician to become Professor of Pathology. In 1884, a pathological laboratory was actually opened on the corner of the hospital grounds as the morgue. By 1886, a 2-story building with bacteriology on the ground floor and pathological anatomic morgue laboratory on the 2nd floor was in use. However, the laboratory was not connected to any hospital buildings at that time. Most materials were brought in from other hospitals, including the Bay View Asylum.

The hospital itself opened in 1889 and Dr. Welch became head of a department of pathology. Welch is credited for both graduating highly respected “academic” physicians through teaching as well as research. He’s noted for the discovery of *Clostridium*, the organism that causes gas gangrene.

Perhaps the most highly regarded and admired of physicians in the history of medicine is the Canadian, Sir William Osler (1849–1919), who became the physician-in-chief at Johns Hopkins. He performed autopsies and was a very strong vocal supporter of the view that clinical laboratories were necessary and played an eminent role in medicine. By 1894, the Board of Trustees of the hospital recognized the Department of Pathology as part of the hospital and approved appointment of a resident pathologist.

With a \$10,000 bequest, the first clinical laboratory was constructed by 1896 at Johns Hopkins Hospital for both the teaching and practice of clinical pathology. Some references claim the room was 12 × 12 and the laboratory equipment was valued at \$300.00 with an annual maintenance of \$50.00. This was a simple laboratory for bacterial and chemistry testing.

Hospitals throughout the United States began to open clinical laboratories. Notable is the William Pepper Laboratory of Clinical Medicine at the University of Pennsylvania in 1895, considered the first of its kind equipped for research, teaching, and routine analytical work. Dr. Welch delivered the opening address, stating that a hospital connected with a medical school should serve both

for training students as well as the treatment of patients. However, laboratory medicine as a discipline was still not identified as its own specialty; it remained directly under the Department of Medicine.

By 1899, William Osler supported the creation of “ward laboratories,” small laboratories directly off hospital floors, at Johns Hopkins Hospital, which were for use of and by the practicing physician. Routine laboratory testing was then performed by the attending physicians (clinicians). The more complex laboratory testing and research, however, would still be performed by the pathologists.

Objections began circulating throughout the medical community as well as in public and political arenas regarding the creation of laboratories in hospitals. Major concerns were that they were too expensive, required space, testing was too time consuming, and testing was unsafe. Bedside availability to the patient was also still preferred for any laboratory testing for the clinician at this time. Ironically, the concern was to maintain the patient as the center of medical attention, not laboratory tests.

With equipment at that time relatively lacking, issues of space and budget were still major drawbacks to the functioning of the newly created clinical laboratories. By the mid-1910s, however, the increased complexity and magnitude of laboratory testing assays were finally being acknowledged as directly correlating and relating to diseases. Laboratory testing became too sophisticated for most medical interns to perform in a ward. Therefore, hospitals had begun to make room for the hospital-based clinical pathologist and began providing space for pathology clinical laboratories.

Pathology as a specialty would eventually evolve from medical university-based autopsies and research/teaching facilities and early investigation of public safety developments covering health diseases into the clinical laboratories we know today.

The Emergence of the Laboratory Practitioner

For centuries, physicians (mostly men) performed the laboratory testing available. With growth of hospitals for patient care, laboratory techniques also became more exact, complicated, and sophisticated. The physicians assigned laboratory testing to their medical interns. These interns (mostly men) predominantly performed laboratory testing on their own patients.

With the growing sophistication of laboratory testing, physicians in the late 1800s and early 1900s eventually found the need to hire “specialists” to assist with clinical laboratory testing. A 1900 census documents the first official reference to laboratory workers in the United States: 100 “technicians,” all of whom were male.

The transition of the laboratory worker had begun, from physician to intern to specialist to technician, and would continue.

World War I (1914–1918): The Great War

There was considerable growth in pathology during World War I, and clinical laboratories would play a major role. In Europe, Base Hospital #28, with a one room laboratory, was built in 1 month by the French and Americans. The equipment of this laboratory provided microscopic pathology, autopsies, hematology, bacteriology, serology, and chemistry. It has been documented that this well-equipped laboratory performed over 16,000 tests in 7 months. Most astounding was the creation of the

gas gangrene incubator. The laboratory personnel built their own anaerobic incubator using a 10-gallon gasoline can to isolate the deadly anaerobes that did not survive in ordinary oxygen incubators.

At this time, this laboratory was considered state-of-the-art. All routine tests performed at the time in mainland American hospitals were readily available, including Wassermann complement fixation tests for syphilis, Widal agglutination tests for typhoid, bacterial cultures, parasite exams, blood hematocrit and differentiation/morphology, blood iron levels, glucose measurement in blood and urine, surgical pathology, and autopsies. All of these diagnostic tools aided in elevating the image of the laboratory in the eyes of the military, the medical community, and the public.

World War I also focused attention on the critical shortage of qualified laboratory technicians to perform the newly developed complex testing. The Army and Navy hospitals required skilled and experienced technicians to assist pathologists and bacteriologists in the newly created medical departments. Meanwhile, back in the States, clinical laboratories finally found a niche in hospitals. However, hospitals found it difficult to staff with the reduction of medical personnel both during the war and shortages of medically trained men after the war.

Women, however, were now socially permitted to pursue careers in scientific disciplines. Doing “more with less” was achieved by hiring young women at lower salaries for laboratory assistant positions. These positions were trained on-the-job.

The war had introduced thousands of medical doctors to the use of laboratories directly for patient care, and the demand for hospital laboratories grew. Physicians returning from military service expected availability of laboratories, high standards for diagnostics, and expert competency of laboratory staff in local community hospitals. World War I brought much attention to the need for training programs to meet the laboratory personnel shortage.

Birth of Regulatory Agencies, Accreditations of Educational Programs, and Certification of Laboratory Workers

Self-regulating professional associations emerged for entry-level laboratory workers employed by hospitals. Pathology societies, as well as pathology journals, began to flourish. Pathology was developing both a professional identity and educational support.

The American Medical Association (AMA), created in 1847 in Philadelphia, was established specifically for a medical code of ethics. This became the largest association of physicians (both MDs and DOs) and accepted qualified female physicians beginning in 1869. The association began publishing the *Journal of the American Medical Association* in 1883.

The American College of Surgeons (ACS) was created in 1913 just before the United States entered WWI. After the war ended, ACS collected and gathered valuable feedback from surgeons returning from abroad who expected and demanded standardization of pathology and laboratory services. The ACS Minimum Standards were initiated in 1918 and were generally accepted. The simple one page document, with one section dedicated to laboratories, covered surgical pathology autopsies and required all surgical specimens be examined. It also mentions that the clinical laboratory should provide various services. Although brief with no definitive staffing requirements or minimum qualifications, this marked the very beginning of a national initiative in healthcare for standardization of hospitals and laboratories.

In 1915, Pennsylvania enacted a law requiring all hospitals and institutions to contain an adequate laboratory employing full-time laboratory technicians. This became a burden for hospitals, as there were too few technicians available to perform the required laboratory testing. It has been documented that Dr. John Kolmer, a Philadelphia pathologist, began training technicians. In 1918, it was reported that he trained 75 young women in 2 years.

Training programs emerged in hospitals under the direction of pathologists to prepare individuals to work in clinical laboratories. Practicing pathologists with some level of knowledge of laboratory work began to teach their “assistants” (nurses, secretaries, clerks, all of whom were female). The allied health professional emerged as these technical assistants became trained to perform the required ancillary healthcare laboratory testing.

By the Roaring Twenties, pathology and laboratory medicine would become a highly dynamic medical field. Over 3,000 hospitals reported incorporating clinical laboratories. Statistics indicate an increase of technicians to 3,500 with approximately 2,000 of these being women.

The year 1922 also marked a major development to advance pathology and laboratory medicine. Pathologists seeking professional recognition distinct from other physicians initiated the creation of ASCP (American Society of Clinical Pathologists, renamed American Society for Clinical Pathology in 2001). The ASCP was to be the organization to attain greater scientific proficiency in clinical pathology as well as to maintain the status of pathologists as equal to that of other medical specialists. The ASCP was also established to oversee and standardize the training of laboratory technicians.

The ASCP strategically aligned and worked with AMA and ACS to standardize hospital laboratory design. By 1926, ASCP formed the Committee on Registration of Technicians, the function of which was to define and classify technicians. This committee’s first official report indicated the need for a registry. Dr. Kano Ikeda, secretary of the committee, formulated the “Rules and Regulations of the American Registry of Medical Technicians.”

In 1928, ASCP officially created the subcommittee of the Board of Registry (BOR). Six pathologists administered review of primary objectives for minimum entry-level competency standards of education, technical skills, and code of ethics for certification of the practitioner. The BOR extended into competency assessment for an accreditation of educational programs, which would become the Board of Schools (precursor to the National Accrediting Agency for Clinical Laboratory Sciences [NAACLS]) in 1946.

A “professional” status was being created for the ancillary health care laboratory practitioner. This was a milestone towards recognition of the education and skills required of a qualified laboratory worker. This would standardize laboratory services with a competency of qualified laboratory workforce, all under the supervision of a pathologist who determined written or oral diagnosing based on laboratory testing.

In 1931, ASCP continued with definitive professional protocols. The *American Journal of Clinical Pathology* became its first academic journal. The ASCP BOR instituted the MT (Medical Technologist) and LT (Laboratory Technician) initials after the registrant’s name and subsequently added the parenthetical “(ASCP)” to distinguish those who met the rigid requirements of the BOR, which included the first MT certification.

By 1933, the Board of Registry published its first list of approved schools and had its first conference with AMA to discuss joint approval

of schools for laboratory technicians. Colleges and universities instituted a 4-year course and recommended that the fourth year of the program be entirely practical and take place in an approved laboratory. The BOR would not approve any commercial school for laboratory technicians.

After April 1, 1933, candidates were required to apply and successfully pass both written and practical examinations and meet certain personal qualifications. Pretechnical educational requirements were increased to 1 year of college with specific science coursework requirements. The first examination was offered in October 1933 and was originally a written essay. There were twelve questions on the examination, which covered each of the laboratory disciplines. The examination was 50% oral/practical, 25% written, and 25% personal/psychological.

In 1933, the American Society of Medical Technologists (ASMT) was formed. The members were registrants of the Registry of Medical Technologists.

By 1935, ASCP BOR designated the title of MT to registrants who were certified LTs with a college degree. The LT title was discontinued the following year. In 1935, Dr. A. W. Freshman was appointed as the official examiner. Until this time, board members were grading the increasing number of written examination papers and there was concern regarding the uniformity of grading among several examiners.

Initially, technicians were deemed to be high school graduates with 18 months of laboratory didactic and clinical training, whereas technologists held a degree from a university with at least 1 year of basic sciences and 1 year of clinical training in a recognized approved laboratory.

In 1936, pathologists advocating for pathology to be a distinct and separate specialty from medicine eventually brought forth the establishment of the American Board of Pathology (ABPath), granting certification in anatomic and/or clinical pathology to qualified medical doctors. The evaluation of schools was then transferred to the Council on Medical Education of AMA.

In 1937, the "Model Curriculum for Training Students in Medical Technology" was published. Later retitled "A Curriculum for Schools of Medical Technology," it served as the benchmark for medical technology. Pretechnical educational requirements were increased to 2 years of college including major sciences.

World War II (1939–1945)

Major advances to the clinical laboratory discipline came on behalf of the US Armed Forces (Army/Navy). The medical laboratory (part of the 2nd and 3rd units) performed bacteriology/microbiology, hematology, chemistry, and serology testing as well as postmortem examination. Clinical pathology played a major role in medicine during this time, particularly with the introduction of transfusion and blood bank as a distinct laboratory discipline. A mobile laboratory was created and performed over 918 separate procedures. World War II significantly affected the increased importance of technicians in public health and military hospitals.

In 1945, there were a recorded 26 Medical Laboratory Technicians (MOS 858) enlisted in the military. After 13 weeks of basic training, the training of laboratory technicians commenced with men being assigned to the Medical Field School in Texas. All reserve officers were trained at the Medical Field Service Training School in Pennsylvania. It was recognized and recommended that MOS 858 be adequately trained prior to being sent overseas since the laboratory field was so comprehensive.

Twentieth Century Expansion of Quality Standards

Once the AMA officially recognized pathology as a practice of medicine in the 1920s, steps continued forward to expand the standardization of quality in pathology and laboratory medicine. In many states, medical licensure was not required to practice pathology, and nonmedical professionals were operating laboratories. Furthermore, relations between hospital administrations and pathologists were often complicated and not economically favorable to pathologists.

Therefore, in 1946, a group of pathologists created the College of American Pathologists (CAP) a national organization, to pursue recognition and respect for the specialty of pathology and receive reasonable reimbursement.

The College of American Pathologists conducted the first comprehensive surveys in 1963 and accredited the first laboratories by 1964. Becoming the largest organization of board-certified pathologists, CAP committed to improve standards of laboratory medicine in the United States through the Laboratory Accreditation Program (LAP) with discipline-specific CAP accreditation checklists.

Today, CAP accredits nearly 8000 laboratories, nearly 600 of which are located outside the United States. On-site inspections of government-licensed laboratories are conducted every 2 years by CAP inspectors. These inspection teams are composed of practicing pathologists and laboratory technologists qualified through CAP inspector training programs.

Although the ACS Minimum Standards for Hospitals would remain the regulatory guideline for hospitals for approximately 32 years, the Joint Commission on Accreditation of Hospitals (JCAH) was founded in 1951. Renamed the Joint Commission on Accreditation of Healthcare Organizations (JCAHO) in 1987 and shortened to Joint Commission in 2007, it was originally created through merging the Hospital Standardization Programs with quality standards from the American College of Physicians, the American Hospital Association and the AMA.

The Joint Commission today remains an independent not-for-profit organization and the nation's oldest and largest accreditation commission in healthcare. Evaluating and accrediting more than 22,000 health care organizations and programs in the US, the Joint Commission sets national standards requiring on-site surveys. The internationally recognized Gold Seal of Approval demonstrates that an organization has achieved performance standards in delivery quality and safety.

The ASCP continued to definitively expand quality standards of certification of personnel performing laboratory testing along with decisive revisions to the Registry. In 1940, as the membership of ASMT grew, the BOR recommended to ASCP that they appoint an advisory committee of medical technologists to be consultants on the BOR. Two ASMT members and 3 registrants-at-large were appointed to attend the board meeting.

Created in 1933 as the ASCLT (American Society of Clinical Laboratory Technicians), by 1936, this association of nonphysician laboratory professionals became known as ASMT. The ASMT created an independent journal, established state chapters, and held national conventions. By 1947, ASCP Board of Registry passed a resolution for 3 representatives from ASMT with full voting rights and changes in constitution and bylaws effective by 1949.

American Medical Technologists (AMT) was founded in 1939 by a group of medical technologists as a nonprofit national and international professional society for allied health. Starting with the designation of

Medical Technologist (MT[AMT]), credentials for other professional roles were added such as Medical Laboratory Technician (MLT[AMT]), Registered Phlebotomy Technician (RPT[AMT]), Molecular Diagnostics Technologist (MT[AMT]), Certified Medical Laboratory Assistant (CMLA[AMT]) and Certified Laboratory Consultant (CLC[AMT]). The AMT further offers certification for medical and dental assistants. Today, AMT provides continuing education, advocacy, and scholarships.

Other organizations that also developed certification examinations and professional nomenclature included the International Society for Clinical Laboratory Technology (ISCLT) founded in 1962. This organization ceased to exist in 1999 when it merged with American Association of Bioanalysts (AAB). Founded in 1956, AAB includes laboratories as members through the National Independent Laboratory Association (NILA).

By 1948, the ASCP BOR essay examinations were revised to a true/false and multiple choice question examination format. Within a year, the examination consisted exclusively of 200 multiple choice questions as the standard questions. The grading of multiple choice questions can be done by machine and thus, the examinations were available for machine analysis, enabling statistical feedback to the individual and to the medical technology teaching programs.

Over the 1950s and 1960s, various specialist certification categories were added such as microbiology (SM), cytotechnologist (SCT), blood banking (SBB), chemistry (SC) and hematology (SH).

The first Examination Content Guidelines was developed in 1974 to establish 5 categories of certification: MT, MLT, SH, HT, and CLA (Certified Laboratory Assistant would be discontinued in 1982).

By the end of December 1973, ASCP agreed to dissolve the BOS (Board of Schools) and turned over the operation and functions of accreditation for educational institutions to the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS), an autonomous non-profit independent organization.

NAACLS was established to accredit educational programs in clinical laboratory science (university-based and hospital-based programs). Sponsored by various participating professional organizations, NAACLS offers a process of external peer review, granting public recognition to the educational programs that meet set standards in education for clinical laboratory science. Recognized by the Council for Higher Education Accreditation (CHEA), the mission statement continues to be commitment to review and approve quality in educational programs in the clinical laboratory sciences and related health care disciplines. The NAACLS programs unfortunately trended downward from the 1970s to mid-2000s. However, by 2023 - NAACLS celebrating 50 years anniversary with a very welcoming trend upward as presented at the 2022 Clinical Laboratory Educators Conference (CLEC).

By 1977, ASMT withdrew its representation from the ASCP BOR to sponsor the independent National Credentialing Agency for Laboratory Personnel (NCA). Independent of pathologists' oversight, NCA was charted as a nonprofit agency for peer review certification of laboratory personnel. This independent agency also certified applicants who passed comprehensive cognitive examination as either Clinical Laboratory Scientist (CLS NCA) or Clinical Laboratory Technician (CLT NCA).

Also in 1977, ASCP BOR board membership was revised to include 6 pathologists, 6 medical technologists, 2 lay members, and a representative from each of 6 participating organizations of specialists: American Academy of Microbiology (AAM), American Society of Cytology (ASC), Advancement of Blood & Biotherapies (AABB), National Registry of

Clinical Chemistry (NRCC), National Society of Histotechnology (NSH), and Society of Nuclear Medicine.

The 1980s noted the development of additional certifications such as those of histotechnologist (HTL) and immunology (I). In 1989, the phlebotomy technician (PBT) certification was established.

By 1994 all certification examinations were administered using computer adaptive testing (CAT). The ASCP and Board of Registry were on the internet in 1996.

The Certification Maintenance Program (CMP) was implemented in 2004 requiring participation by earning minimum number of points in certain areas. These points could be earned through a number of activities including continuing education, college coursework, publications, presentations, and advanced certification. In this manner, the laboratory practitioner would maintain their ASCP certification by completing CMP every 3 years.

International certifications were created by ASCP BOR in 2006 and denoted by the credential with parenthetical ASCPi (ie, MT(ASCPi)). In 2007, ASCP BOR achieved accreditation by the American National Standards Institute (ANSI, now known as ANSI National Accreditation Board [ANAB]) for MT, MLT, and PBT certifications. In addition, 2 states (California and New York) approved the MT(ASCP) and MLT(ASCP) examinations for state licensure. Expansion of specialties continued in 2017 with the specialist in cytometry (SCYM) and in 2018, with the specialist in molecular biology (SMB).

In 1993, ASMT was renamed to American Society for Clinical Laboratory Science (ASCLS). Today, ASCLS continues nationally as a professional society with state chapters to promote aspects of clinical laboratory practice, education, and management with standards setting, position papers, conferences, and political advocacy for clinical laboratory practitioners with a code of ethics of duty to patient, colleagues, the profession, and society.

October 2009 brought a momentous event with the uniting of the ASCP BOR and the NCA (sponsored by ASCLS) forming a single certification agency: the ASCP Board of Certification (BOC). In July 2009, the ASCP, ASCP BOR, NCA, ASCLS, and Association of Genetic Technologists (AGT) signed an agreement for a single certification agency. This agreement was viewed as a cooperative effort by professional societies to consolidate certification for both graduating students as well as for the over 500,000 board-certified medical laboratory professionals.

The federal government was also involved in regulation of laboratories, establishing minimal quality requirements through the Clinical Laboratory Improvement Act of 1967 (CLIA '67). Revisions proposed were realized through various amendments and by 1972, established quality control and proficiency testing as well as rules for personnel competency. An examination administered by the Department of Health Education and Welfare (HEW, created in 1953) was used to qualify nonbachelor medical technologists. When a separate federal Department of Education was created in 1979, HEW was renamed the Department of Health and Human Services (HHS).

After years of proposals to update CLIA '67 to include all laboratories in the United States, major legislation was finally passed with the bill known as the Clinical Laboratory Improvement Act of 1988 as drafted by the Public Health Service Act. Numerous delays involved laboratory industry feedback, including physician office laboratories (POLs) lobbying for lesser requirements. The CLIA '88 was finally implemented in 1992 with additional amendments in 1997 and 2012. Every clinical laboratory in the United States, including POLs, would now be required to have a certificate

issued by the HHS establishing quality standards for all laboratory testing. The CLIA defined a clinical laboratory as any facility performing laboratory testing on specimens obtained from humans for the purpose of providing information for the diagnosis, prevention, and treatment of disease.

Quality assurance and quality control programs were put in place, involving the entire testing process: preanalytical, analytical, and postanalytical processes. The CLIA also mandated successfully passing proficiency tests (treating and testing unknown samples the same as patient samples). Three specific classifications of testing levels were established: waived (no complexity requiring no supervision), moderate complexity, and high complexity. The more complicated the test, the more strict the mandatory requirements.

Laboratory professionals were now categorized and designated as “testing personnel” with specific educational and supervisory requirements for each level outlined. Although CLIA ’88 had a significant impact for high standards of quality in laboratory testing, the personnel standards unfortunately lowered education requirements for personnel performing complex testing. Concerns were expressed by medical laboratory professionals that the personnel standards designated did not match the regulated quality standards of testing established.

States and organizations were permitted to be waived or achieved “deemed status” if demonstrating they meet or exceed the federal government’s national CLIA standards; that is, performance is equal to or greater than expectations in a particular area of expertise. To be a recipient of federal payments from Medicare or Medicaid federal programs, healthcare organizations must meet the government requirements for program participation set forth in federal government regulations.

Founded in 1965 as Health Care Financing Administration (HCFA until 2001), the Centers for Medicare and Medicaid Services (CMS) is the federal agency within the HHS responsible for clinical laboratory quality standards under CLIA and determines deemed status on the federal level. Granting “deeming authority” to an accrediting agency gives that agency the ability to determine that a healthcare organization meets Medicare and Medicaid accrediting requirements.

For example, in 1968, the CAP Basic Survey (previously known as the “Small Hospital Survey”) was accepted equivalency under CLIA -67 by the HEW and in 1969, the CAP Inspection and Accreditation program was declared equivalency to CLIA-67 standards. The CAP standards to maintain accuracy of laboratory testing meets or often exceed CLIA, Food and Drug Administration, and Occupational Safety and Health Administration requirements.

By 1988, CAP expanded quality assessments by initiating the Q-Probes as short-term studies for laboratories to offer comprehensive assessment providing quality improvement of laboratory processes. The CAP achieved deemed status for LAP under CLIA ’88 in 1995.

The Joint Commission was also recognized with deemed equivalency by CMS by 1993 for hospital and laboratory inspections. The Joint Commission in turn reached a formal agreement with CAP on equivalency for Joint Commission hospital accreditation.

Over the years, a private nonprofit organization developed to meet federal requirements for oversight of laboratory testing. The Commission on Office Laboratory Accreditation (COLA) was originally founded in 1988 as an alternative to assist POLs with CLIA regulatory compliance. The CMS granted deeming authority in 1993. Using a standardized and educational approach, COLA further expanded to include hospitals and independent laboratories. In 1997, COLA’s accreditation program received recognition from the Joint Commission (cooperative agree-

ment expiration with an extended transition period to December 31, 2024). Implementing a quality management system, COLA obtained certification to International Organization for Standardization 9001 in 2011. Today, COLA accredits over 7000 medical laboratories.

With rapid growth of laboratory technology expanding particularly in the 1960s, 31 clinicians and laboratory scientists representing 15 organizations came together in 1968 to develop a formalized standardization of laboratory chemistry testing. Jointly, the US Centers for Disease Control and Prevention (CDC) and CAP pursued the need to form such a committee. The National Committee for Clinical Laboratory Standards (NCCLS) was formally organized in 1968 and published its first documentation in 1969. Accredited in 1977 by ANSI, it was renamed Clinical and Laboratory Standards Institute (CLSI) in 2005 and remains a global association of over 1500 organizations and over 2000 volunteers representing a balance of professional, industry, and government for standardization of laboratory testing.

Status of Professionalism

Accreditation of medical facilities and of educational programs is separate and distinct from certification of personnel. *Accreditation* refers to a facility achieving official approval by an objective independent peer appraisal—a process of validation of standards set by an appropriate organization. Accreditation, which is voluntary, requires an extensive evaluation and review of quality standards and demonstrating a commitment for continuous quality assurance. This involves site visits in compliance with established standards. Hospitals are accredited by The Joint Commission. Laboratories are accredited by CAP. Colleges and universities are accredited by NAACLS.

Certification is a voluntary process of quality standards of the individual and is a distinguished and professional recognition by a national professional organization. Sitting for the national boards by ASCP, laboratory professionals acquire certification in their field and the right to use the appropriate certification title.

When directed by the government, accreditation and certification become law and mandatory.

Despite laboratory work being viewed as a profession and those who work in laboratories being viewed as professionals by professional organizations all these decades, medical technologists weren’t deemed professionals by government standards and regulations until 1995.

One laboratory professional opposed the nonprofessional classification of laboratory professionals at his facility by the local union and was granted request to petition for review by the National Labor Relations Board (NLRB). There was overwhelming support by the national professional associations, particularly ASCP, as well as AMS volunteers and coordinated support via amicus briefs filed by a number of other national professional societies such as AACC, CLMA, CAP, ISCLT, AABB and ASCLS. April 28, 1995, brought the decisive ruling considered a major landmark decision for laboratory professionals. The NLRB officially ruled that medical technologists are professional employees within the National Labor Relations Act Section 2(12).

To be deemed “professional” by NLRB, the following 4 criteria must be met regarding much of the work performed:

1. predominantly intellectual in character
2. requires consistent exercise of discretion and independent judgment

3. results of work performed cannot be standardized in relation to a given period of time
4. requires knowledge and education of a specialized and advanced nature

The NLRB went further to clarify that there had been overemphasis in the role of automation, indicating that equipment is simply tools to perform automated analysis and do not eradicate the need for judgment and discretion in the performance of testing. The NLRB also noted that educational requirements and certification procedures for medical technologists were equal to or greater than those for RNs. In summary, many professional societies and associations as well as laboratory professionals believed this major legal ruling was the foundation for many legislative avenues to pursue, including state licensure.

Licensure is to legally authorize via legislation enacted through the government with the principle objective to protect the public. Licensure establishes entry level educational standards, determines body of knowledge/skills, sets full scope of practice, and enforces disciplinary standards and actions of moral character. In contrast to private sector certification, licensure is required to be able to work in a field of study; it is unlawful to engage in work in the designated profession without a license. The US DHS Health Resources and Services Administration (HRSA) designated licensure as “right to practice.” A person not properly licensed is prohibited by statute from working in the designated profession. Licensure safeguards both the profession and the professional, which in turn safeguards the public served. However, by 2022, fewer than a dozen states maintained state licensure for medical laboratory professionals, whereas every state requires licensure for all medical professionals such as nurses, doctors, pharmacists, physician assistants, therapists, etc.

Automation of the Laboratory

Before WWII, laboratory testing was basically a manual process. Medical technologists had to manually sample, process, pipette, mix, aliquot, and incubate as well as manually report findings. However, after WWII, with greater reliance on laboratory testing for patient care, came increased volume ordering and laboratory testing. Instrumentation automation came on the scene but was originally designed only for specific tasks such as washing and filtration. By the 1950s, automatic titration was being used for processes such as photometric, coulometric, potentiometric processes.

As volume of testing assay menus were made available and test ordering increased proportionately with reliance on laboratory results for patient care, the first stand-alone automation for the laboratory was introduced in 1957, with clinical chemistry analyzers. The Autoanalyzer was a continuous flow analysis to increase sampling. By the 1960s came an unprecedented expansion of laboratory tests developed and subsequently, cutting edge advancement of automation. The sequential multiple analyzer was developed in 1969 and eventually developed into what was at that time the most innovative diagnostic instrument, the sequential multiple analyzer with computer, in 1974.

Hematology laboratory automation followed. Medical technologists/technicians originally predominantly relied solely on manual review using microscopes, counting hundreds of samples while manually measuring hemoglobin; that is, until the development of the electronic blood cell impedance technology by Coulter in 1953. This discovery eventu-

ally led to fully automated hematology analyzers performing complete blood counts and today's automated instrumentation that perform and reports over 40 hematological parameters.

Perhaps the most interesting instrumentation advancements in the historical perspective came with automated analytical systems for one of the oldest laboratory techniques: urinalysis. In 1956, industrially produced commercial dipsticks (Clinistix or Clinistrip) were introduced. This major breakthrough invention became a National Historic Chemical Landmark in 2010. Eventually, urinalysis came into the 21st century, starting with the first urine sediment particle analyzer, Yellow Iris, in 1985. Today's fully automated analyzers include microscopy and flow cytometry.

Automation combined with management oversight would drive the performance of some of the most commonly ordered laboratory tests, as well as specialty testing, from preanalytical (collection) to analysis (testing) to postanalytical (reporting). Automation with instrumentation and robotics interfaced with computers became the necessary tool to assist the laboratory professional with workload and workflow. These analyzers improved turnaround times and reduced errors and overall health care costs to hospital administration.

Each step with advanced automation platforms shifted laboratory medicine from procedure-based to value-based, patient-centered health-care and brought the laboratory professional from repetitive labor technician to executing cognitive oversight and directly collaborating with clinicians to improve patient care. Technological and computer communications advanced along with the science behind the medical expansions for an evidence-based laboratory medicine as a practice.

With greater reliance on laboratory testing came the understanding of the amount of testing being performed. Most laboratory professionals are well aware of the claim mentioned in numerous articles promoting the significant role of laboratory medicine, that 70% to 80% of decisions in diagnosis in a patient's chart (electronic medical record) are influenced by and based on laboratory testing. The earliest published claim is by Forsman of the Mayor Clinical in the United States from 1996 suggesting that 60% to 70% of all critical decisions are based on laboratory results. Forsman went further in 2004 to claiming that 80% of the objective data in a patient's clinical record is contributed by the laboratory. Although some sources dispute the statements for statistical validation, citing the importance of value over volume, the fact remains that the use of laboratory medicine is undisputed for patient care outcomes.

According to the CDC, over 14 billion laboratory tests are ordered annually and 70% of medical decisions depend on laboratory testing results. The CDC concluded that laboratory medicine is an essential and integral part of clinical decisions for detection, diagnosis and treatment, and prevention and management of disease.

Transformation to Today's Laboratory Professional

What is important to recognize is the transformation and progression of the laboratory practitioner. The laboratory practitioner skill set developed and expanded from a psychomotor orientation to critical thinking. Historically, early technician assistants were manual laborers with repetitive psychomotor skills. This evolved in the 21st century to laboratory professionals as critically independent judgment thinkers. Psychomotor behavioral manual techniques were eventually replaced by higher cognitive levels of interpretation. Accuracy (how close to the true value) and precision

(reproducing that value) became the key components driving quality control and quality assurance in the laboratory and relies on the laboratory professional to deliver.

Today's laboratory professionals with advanced degrees have expanded career opportunities beyond working at the bench at healthcare facilities, such as in infection control, safety, risk, Laboratory Information System, Point of Care Testing, quality assurance, and management, in industry as technical consultants, customer support, sales/marketing, and other, into education as clinical advisors, professors, program directors, and instructors. Some have continued careers as consultants or negotiators in POLs and public laboratories or have become regulatory inspectors or writers and editors or researchers and even legal counsel. Doctorate of Clinical Laboratory Science (DCLS) degree-holders have also become acutely involved with laboratory direct patient care, assisting with ordering and reporting of laboratory testing and results.

The Choosing Wisely campaign was launched on a national level in 2012 to engage physicians in dialogue with the laboratory professionals to avoid unnecessary medical tests. This program is supported today by 70 medical societies, including ASCP, representing pathology and laboratory medicine.

Laboratory medicine began from the most simplistic tasting of urine to today's sophisticated complex instrumentation. Diagnostic testing has evolved from mostly male clinicians and practitioners to the predominantly female workforce we have today. The transformation of the clinical laboratory and its technologist/technician from ancillary and auxiliary support in healthcare to a disciplined profession took some time to evolve as a separate and distinct career. Today it has developed and grown in modern society as a critical field of study in healthcare.

Standardizing the Name of the Professional

What is today's laboratory professional called? A medical laboratory scientist? Medical technologist? Clinical laboratory scientist? Clinical laboratory technologist? Lab tech? So many names defining one practitioner is part of a dilemma for our professional identity.

The ASCP BOC has collaborated on a position paper entitled "Standardizing the Professional Title of Medical Laboratory Professionals." The sponsoring and participating professional laboratory societies include ASCP, ASCLS, AABB, American Association of Pathologists' Assistants (AAPA), AGT, ASC, American Society for Microbiology (ASM), CLMA, and NSH. In September of 2022, AMT also joined these societies. The intention is to offer a standardization of the titles of medical laboratory professionals. The purpose is to acquire a familiarity for improved recognition with the public, media, healthcare teams, facility administrations and government agencies to gain support for the profession and the professional.

Laboratory Workforce Shortages

In its annual survey of high-demand careers in 2011, the US News & World Report declared medical technologists/clinical laboratory scientists among "The 50 Best Careers" as the "unsung heroes of the healthcare industry."

The Bureau of Labor Statistics (BLS) continue to report staggering growth for laboratory professionals, especially those certified, about 25,000 projected each year on average and predicts a 7% increase from 2021 to 2031 (this is an estimate due to lack of state licensure to confirm

practitioners). The HRSA National Center for Health Workforce Analysis had estimated an increase demand for laboratory professionals in the coming years (22% between 2012 and 2025 and 19% by 2030).

Today, ASCP celebrates 100 years as the largest professional membership organization for pathologists and laboratory professionals and continues to conduct a Vacancy Survey every 2 years, acquiring data on workforce shortages and focusing on how to address workforce issues that hospital and laboratory facilities continue to face.

At present, the value of laboratory medicine as essential in healthcare for patient care cannot be denied. Beyond reporting accurate and reliable test results in a timely manner, laboratories are also moving to collaborate for an overall value-based healthcare system. It is therefore critical now more than ever to attain one strong voice for advocacy to acquire the media attention and public awareness and industry respect and legislative support needed to maintain the highly regulatory compliance of quality standards of laboratory testing for patient care by highly qualified medical laboratory professionals.

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Suggested References

1. AACC Laboratory Medicine: Advancing Quality in Patient Care. [Care AACC.org](#); Oct 2015:1–17
2. About John Hopkins Medicine: The Four Founding Physicians. 2020:1–3
3. Adeli K. *Taskforce: IFCC TF-ICO: Searching for the Evidence that Laboratory Medicine Matters*. 2015:1–23
4. Albertson, D. Washington Report: Federal labor panel declares MTs 'professionals'. 1995;MLO:17–18
5. Altman J, Lavelle L, McKenna & Cuneo. Group Health Associates/ NLRB Decision Memo. May 4, 1995.
6. American Medical Technologists (AMT) Homepage [americanmedtech.org](#); 2020
7. American Society for Clinical Pathology (ASCP): History [www.ascp.org](#); 2020:1–2
8. American Society for Clinical Pathology (ASCP) BOC (Board of Certification) Newsletter Mar 2021:2–11
9. American Society for Clinical Pathology (ASCP) Board of Certification Timeline (1920s to 2010s) June 2020:1–4
10. American Society for Clinical Pathology (ASCP). *Fifty Years of Service - the History of ASCP*. Lab Medicine Oxford Academic; June 1971:17–26
11. ASCP.org: 70% of Today's Medical Decisions Depend on Laboratory Results - Lab Professionals Save Lives. April 2019:1
12. ASCP State Licensure of Laboratory Personnel Policy Number 05-02 [www.ascp.org](#)

13. ASCP Task Force on the Laboratory Professionals Workforce 2012.
14. ASCLS & ASCP BOC Position Paper: Standardizing the Professional Title of Medical Laboratory Professionals. 2020:1–8.
15. ASCLS Position Paper: Value of Medical Laboratory Science Personnel and Clinical Laboratory Services in Healthcare www.ascls.org; Jan 2021:1–2.
16. ASCLS-CLMA-ASCP-AMT-AGT-NSH. Workforce Issue Brief Position: Growing Crisis in the Clinical Laboratory Workforce; 2019
17. ASCLS.org: Addressing the Clinical Laboratory Workforce Shortage. Aug 2018
18. American Society for Clinical Laboratory Science (ASCLS) Career Center: Becoming a Clinical Laboratory Professional. ascls.org; 2021:1–3
19. ASCLS Code of Ethics ascls.org; 2021:1–3
20. ASCLS TODAY; June/July 1995:IX(6):1–7
21. American Journal of Medical Technology Letter to the Editor. 1936
22. Armbruster DA, Overcash DR, Reyes J. Clinical chemistry laboratory automation in the 21st century - Amat Victoria curam. *Clin Biochem Rev.* 2014;35(3):143–153
23. Badrick T. Guest editorial: evidence-based laboratory medicine. *Clin Biochem Rev.* 2013;34:43–46
24. Beck SJ, Laudicina RJ. Focus: passing the torch: mentoring the next generation of laboratory professionals. *Clin Lab Sci* 2001;14(1):33
25. Berger D. A brief history of medical diagnosis and the birth of the clinical laboratory Part 2 - Laboratory Science and professional certification in the 20th century. *MLO Med Lab Obs* 1999;31(8):32–34, 36, 38
26. Berger D. A brief history of medical diagnosis and the birth of the clinical laboratory Part 3 - Medicare, government regulation, and competency certification. *MLO Med Lab Obs* 1999:41–42, 44
27. Berger D. A brief history of medical diagnosis and the birth of the clinical laboratory Part 4 - Fraud and abuse, managed care and lab consolidation. *MLO Med Lab Obs.* 1999:1–5
28. Bureau of Labor Statistics. Clinical Laboratory Technologists and Technicians. BLS.gov; Sept 2020:1–2
29. Bureau of Labor Statistics. The Economic Daily. BLS.gov; April 2020:1–3
30. Bushnell J. Defining Our Identity ASCLS Today 2021;34(1):1–2
31. Butina M, Kenimer Leibach E. From technical assistants to critical thinkers: from World War II to 2014. *Clin Lab Sci* 2014;27(4):209–219
32. Buttner, J. The origin of clinical laboratories. *Eur J Clin Chem Clin Biochem.* 1992;30(10):585–593
33. CAP TODAY: Guide to urinalysis instrumentation; 2015:1–4
34. Centers for Medicare & Medicaid Services. Services History. www.cms.gov; 2020:1–14
35. CCS Healthcare Informatics: Total Laboratory Automation. 2020:1–2
36. CDC Division of Laboratory Systems (DLS): Strengthening Clinical Laboratories. Nov 2018:1–2
37. Check W. Urinalysis finally catches up. CAP Today. Sept 2002:1–7
38. Customerservice@CLSI.org: Crosswalk of Documents Referenced within CAP Accreditation Checklists. 2018:1–8
39. Clinical Laboratory Improvement Amendment (CLIA). Feb 2020:1–2
40. CLIA Overview. www.cms.gov; 2013:1–3
41. CLSI.org The History of CLSI; 2021
42. College of American Pathologists (CAP): Laboratory Accreditation Program (LAP); 2021:1–4
43. College of American Pathologists: Q-Probes: 1990s Enhancing the Profession in the Digital World. 2021:14–17
44. College of American Pathologists Historical Timeline. 2021:1–17
45. Commission on Accreditation. CAAHEP. www.caahep.org; 2021:1–4
46. Commission on Office Laboratory Accreditation (COLA) Inc. www.cola.org; 2021
47. Dark Daily: MT & CLS Laboratory Certification Agencies Agree to Unite. Sep 5, 2008:1–4
48. Dark Daily: Single Certification Means Good-bye to Med Techs (MTs) and Clinical Lab Scientists (CLSs)! Aug 3, 2009:1–3
49. Dark Daily: Medical Laboratory Technologists a US News & World Report “Best Career” for 2011 Jan 5, 2011:1–2
50. Delwhiche FA. Mapping the literature of clinical laboratory science. *J Med Libr Assoc.* 2003;91(3):303–310.
51. Dood M, VanHess R, Grenache D. Beyond test results: defining the role of clinical laboratories in value-based population health equity. AACC Clinical Laboratory News. March 2021:1–6.
52. Family Physician: Clinical Laboratory Improvement Amendments (CLIA). 2020:1–3
53. FDA.gov: Clinical Laboratory Improvement Amendments (CLIA). 2020:1–2
54. Felder R. Advances in clinical laboratory automation. AACC. Dec 2014:1–4
55. Fishbein M. *The American Medical Association 1847–1947.* (Biography of Victor C. Vaughan Written by Bierring MD, Walter). W.B. Saunders Co, 1947:1–4
56. Fritsma, GA. NCA and BOR consider new credentialing agency: American Society for Clinical Laboratory Science. July 2006;19(3):132–133:1–4
57. Futrell K. The Value of the Laboratory in the New Healthcare Model Oxford Software. July 2013:1–23
58. Garcia E, Kundu I, Fong K. American Society for Clinical Pathology's 2019 wage survey of medical laboratories in the United States. *AJCP* 2020:1–25
59. Garcia E, Kundu I, Schultz M. ASCP Wage 2019 wage survey report: a call for increased and stronger advocacy. *Critical Values* April 2021:18–22
60. Garcia E, Kundu I, Kelly M, Soles R. The American Society for Clinical Pathology's 2018 vacancy survey of medical laboratories in the United States. *Am J Clin Pathol.* 2019;152(2):115–168.
61. Guterl GO. CAHEA to CAAHEP evolution nearly complete. *ADVANCE for Medical Laboratory Professionals.* Feb 1996:19
62. Guterl, GO. NCA changes name, adds certification. *ADVANCE for Medical Laboratory Professionals.* Dec 1997:29
63. Hallworth MJ. Editorial: The 70% claim: what is the evidence base? *Ann Clin Biochem.* 2011;48:487–488
64. Hauss O. Bringing urinalysis into the 21st century: from uroscopy to automated flow cytometry. *Sysmex J Int.* 2008;18(2):38–43
65. Hermansen, JM A pandemic response creates laboratory opportunities. *Med Lab Manage* 2021;10(1):1–3
66. Hertel, NG. Covid-19 highlights behind-the-scenes work of critical laboratory staff in St. Cloud, beyond. St. Cloud Times. Jan 2021
67. Hiltunen M. Dispelling the 70% claim with laboratory's true value. *Med Lab Manage.* 2017:1–6
68. Hoffmann, JML. Laboratory hematology in the history of Clinical Chemistry and Laboratory Medicine CCLM 2013;51(1):119–127
69. Holmes GEF. *Medicine in the First World War: Laboratory Medicine - Gas Gangrene.* University of Kansas Medical Center. May 2019:1–2
70. HRSA Health Workforce: Allied Health Workforce Projections 2016–2030. Dec 2020:1–6
71. Islin H. Interpretation of laboratory results. acutearetesting.org; Jan 2010:1–7
72. John Hopkins Medicine: The History of John Hopkins Medicine. 2020:1–2
73. Journal of Urban Health. Camac, Charles Nicoll Bancker. *Bull N.Y. Acad. Med* 1941;17(1):80
74. Karni K. ASCLS History American Society for Clinical Laboratory Science (ASCLS). www.ascls.org; 2020:1–2
75. Kohler, R. *From Medical Chemistry to Biochemistry: The Making of a Biomedical Discipline.* Cambridge University Press. Oct 2011 (1982)

76. Kotlarz, V. The quest for professional recognition. *Clin Lab Sci*. 1998;11(1):35–48
77. Kotlarz, VR. Tracing our roots: the beginnings of a profession. *Clin Lab Sci*. 1998;11(3):161–166
78. Kotlarz V. History of medical technology in the United States. *Clin Lab Sci*. 1991;4(4):233–236
79. Kotlarz, VR. Tracing our roots: origins of clinical laboratory *Clin Lab Sci*. 1998;11(1):5–7
80. Kotlarz V. The quest for professional recognition. *Clin Lab Sci*. 1998;11(1):35–42
81. Kotlarz, VR. Tracing our roots: the first clinical laboratory scientist. *Clin Lab Sci*. 1998;11(2):97–100
82. Kotlarz V. Tracing our roots: the beginnings of a profession. *Clin Lab Sci*. 1998;11(3):161–166
83. Kotlarz, VR. Tracing our roots: early clinical laboratory scientists and their work -- myth and reality. *Clin Lab Sci*. 1998;11(4):209–213
84. Kotlarz, VR. Tracing our roots: a professional identity emerges: 1928–1945. *Clin Lab Sci*. 1998;11(5):275–279
85. Kotlarz V. Tracing our roots: the broadening horizons of clinical laboratory practice (1945–62). *Clin Lab Sci*. 1998;11(6):339–345
86. Kotlarz V. Tracing our roots: progress in advancing the profession (1945–62). *Clin Lab Sci*. 1999;12(2):91–96
87. Kotlarz VR. Tracing our roots: a new era in clinical laboratory science education. *Clin Lab Sci*. 1999;12(4):213–219
88. Kotlarz, VR. Tracing our roots: years of turmoil (1962–1977). *Clin Lab Sci*. 1999;12(6):336–341
89. Kotlarz, VR. Tracing our roots: the rocky road toward recognition of clinical laboratory science's professional status (1962–1977). *Clin Lab Sci*. 2000;13(2):166–171
90. Kotlarz, VR. Tracing our roots: new opportunities and new challenges in clinical laboratory science (1977–1992) *Slin Lab Sci*. 2001;14(1):13–18
91. Kristopalitis T, Wantuch E. Introduction to the principles of laboratory medicine. *AAMC*. Oct 2013:1–3
92. Lab Medicine: Impact of Time Lapse on ASCP Board Examination Scores. 2015;26(3):e53–e58
93. Laboratory Science Careers. What is Laboratory Science? 2020:1–7
94. Larkin Nolen, JD The power of laboratory automation. *MLO Jan* 2014:1–7
95. Leclair, SJ Editorial: history is the present. *Clin Lab Sci*. 2006;10(1):4
96. Lewis, D. The Oldest Chemistry Irab in America? Smithsonian Magazine website. <http://www.smithsonianmag.com/smart-news/university-of-virginia-rotunda-jefferson-chemistry-lab-180956929/>. Published October 14, 2015. Accessed December 3, 2020.
97. Library of Congress LC Linked Data Service: Camac, C.N.B. (Charles Nicoll Banacker) 1868–1940. 2015:1–2
98. Lindberg DS, Britt MS, Fisher FW. *Williams' Introduction to the Profession of Medical Technology*. 4th ed. Philadelphia PA: Lea & Febiger; 1984
99. MacCallum WG. *John Hopkins Pathology: Our History 1885–1931*: 1–6
100. Mayo Clinic: Medical Laboratory Scientist: What does a medical laboratory scientist do? 2020:1–8
101. Medical Technology Schools AMT (American Medical Technologists) 2020:1–6
102. Medical Lab Technician AMT 2014:1–2
103. Medicare and Medicaid Background. [cms.gov](https://www.cms.gov); 2014:1–7
104. McKay DR. What is a Medical Laboratory Technologist? The balance Careers. July 2020:1–8
105. MLO A Journey from tube to patient focused solutions: The Evolution of Laboratory Automation. Nov 2019:1–12
106. Montgomery S. 100 years of advocating for laboratory medicine, pathology, & quality patient care. *Crit Values*. June 29, 2022
107. NAACLS: Mission, Vision, and Value Statements of the National Accrediting Agency for Clinical Laboratory Sciences (NAACLS). www.naaccls.org; 2016:1–2
108. Nadeau, K. State of Industry: lab administrators prioritize accurate and timely financial and operational performance. *MLO online.com*; 2021:27–35
109. National Labor Relations Board - National Relations Act - NLRB Guidance. Menu 2020:1–40
110. National Labor Relations Board - Decisions Section 2(12) April 28, 1995:1–7
111. NCCA: Mission and Vision NCCA (National Commision for Certifying Agencies) 2020:1–3
112. Nevins K. An Introduction to Laboratory Regulations: Part 1. Lablogatory *LabMedicine* July 2019:1–5.
113. Novis DA, Nelsen S, Blond BJ, et al. Laboratory staff turnover: a college of american pathologists Q-probes study of 23 clinical laboratories. 2020;144(3):1–11
114. Nolen, JDL. The power of laboratory automation. *Automation MLO Jan* 2014:1–7
115. Ngo A, Gandhi P, Miller WG. Frequency that laboratory tests influence medical decisions. *JALM Jan* 2017:410–414
116. Olsen, K The first 110 years of laboratory automation: technologies, applications, and the creative scientist. *J Lab Autom* 2012;17(6):469–480
117. Pearson L. *CLIA Regulations ARUP Health*. University of Utah; Aug 2020
118. Prakash M, Carlton Johnny J. Things you don't learn in medical school: Caduceus. *J. Pharm Bioallied Sci*. 2015;(Suppl 1):S49–S50.
119. Quest Diagnostics: 70% of medical decisions are based on lab results. 2020:1–7
120. Rector C. What's in a name? *ASCLS Today*. 33(4):1
121. Reiser, SJ. *Medicare and the Reign of Technology*. Cambridge University Press; 1981
122. Riedl, J. Hematology: challenges in hematology diagnostics. *MLO* 2014:1–11
123. Rohde RE, Falleur DM, Ellis JR. Almost anyone can perform your medical laboratory tests - wait, what? [Elsevier.com](https://www.elsevier.com); March 2015:1–9
124. Rohde, RE, Who is doing all those COVID-19 tests? Why you should care about medical laboratory professionals. *The Conversation*. Dec 2020:1–5
125. Rosenfeld, L. *Four Centuries of Clinical Chemistry*. CRC Press; Aug 1999
126. Schubert J. LabVine:Laboratory Medicine at "Point of Health" A future perspective and call to action for Lab Medicine. March 2021:1–14
127. Scott, K. The laboratory workforce shortage demands new solutions. *CLN Nov* 2015:14–5
128. Soniak, M. What Do Snakes and Sticks Have to Do with Doctors? *Mental Floss*. May 2012:1–5
129. [Stago.us.com](https://stago.us.com). Hemostatis: History of Coagulation. 2020
130. Tobin P. CLIA Categorization Processes. www.fda.gov; July 2019:1–15
131. The History of CCLSI (NCCLS). [clsi.org](https://www.clsi.org); 2021:4–9
132. The Joint Commission: FACT SHEET. 2018:1–2
133. The Joint Commission: History of The Joint Commission. www.jointcommission.org; 2020:1–2
134. The Joint Commission: What is Deemed Status and Who Grants it? [Home.akitabox.com](https://www.home.akitabox.com); 2021:3–8
135. The Joint Commission: Over a century of quality and safety. 2020:1–18
136. The Joint Commission Newsletter Issue 1–2023
137. Uncyclopedia: Medical technologist. 1–10
138. University of Pennsylvania: Perelman School of Medicine: Pathology and Laboratory Medicine. 2020:1–2

139. US Bureau of Health & Human Services HRSA. The Clinical Laboratory Workforce: The Changing Picture of Supply, Demand, Education, and Practice July 2005:1–68.
140. US Bureau of Labor Statistics. Occupational Employment and Wages. May 2016
141. US News & World Report: Medical Laboratory Technologists “Best Career” for 2011
142. Walz, SE. Education & Training in Laboratory Medicine in the United States. JIFCC (Journal of the International Federation of Clinical Chemistry) April 2017:1–4
143. Wians FH. Clinical Laboratory Tests: Which, Why, and What Do the Results Mean? *LabMedicine*. 2009;40(2):105–113
144. Wikipedia: American Medical Association. 1–8
145. Wikipedia: American Board of Pathology. 1–2
146. Wikipedia: American Society for Clinical Pathology. 2020:1
147. Wikipedia: Bellevue Hospital. 1–5
148. Wikipedia AABB 1–2.
149. Wikipedia: Centers for Medicare & Medicaid Services (CMS). 2020:1–6
150. Wikipedia: Clinical and Laboratory Standards Institute (CLSI). 1–3
151. Wikipedia: Clinical Urine tests. 1–17
152. Wikipedia: HCFA. 1
153. Wikipedia: Laboratory. 1–7
154. Wikipedia: Louis B. Wilson. 1–2
155. Wikipedia: Medical Laboratory 2020:1–6
156. Wikipedia: United States Department of Health and Human Services. 2020:1–5
157. Wikipedia: William Osler. 1–9
158. Wikipedia: William H. Welch. 1–4
159. Williams MR, Lindberg DS. *An Introduction to the Profession of Medical Technology*. 3rd ed. Philadelphia PA: Lea & Febiger; 1979.
160. Wright Jr MD PhD. In the Wake of the Great War: How Pathology Became a Clinical Discipline in America? [USCap.org](https://academic.oup.com/labmed/article/54/5/e141/7117538); United States & Canadian Academy of Pathology; 2017:1–9
161. WW2 US Medical Research Centre: 3rd Medical Laboratory - Unit History. 2007–2021. July 2020:1–13151.

A rare occurrence of *Anaplasma*-associated peritonitis

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Key words: anaplasmosis; peritonitis; morulae; WBC differential; granulocytes; neutrophil

Abbreviations: ED, emergency department; CBC, complete blood count; CT, computed tomography scan; WBC, white blood cell; UTI, urinary tract infection; CCPD, continuous cycling peritoneal dialysis; PAP, Papanicolaou; PCR, polymerase chain reaction; BUN, blood urea nitrogen; AST, aspartate aminotransferase.

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ABSTRACT

Anaplasmosis or human granulocytic anaplasmosis is a tick-borne illness caused by the bacteria, *Anaplasma phagocytophilum*, resulting from an infected tick bite. Examination of a blood smear within the first week of exposure may show microcolonies of anaplasmae (morulae) in the cytoplasm of neutrophils that are highly suggestive of anaplasmosis but not definitive. We present the first case describing *Anaplasma*-related peritonitis and morulae in peritoneal fluid granulocytes in a peritoneal dialysis patient who developed anaplasmosis.

Clinical History

An 81-year-old woman on peritoneal dialysis for end-stage renal disease presented to a community hospital emergency department (ED) in June 2022 complaining of generalized abdominal pain, nausea, vomiting, and diarrhea. Review of symptoms was additionally notable for fatigue, chills, and fever, which she self-treated with acetaminophen. A complete blood count (CBC), comprehensive metabolic panel, lactic acid, lipase, serum magnesium high sensitivity troponin I, urinalysis, and a computed tomography scan (CT) of the abdomen without contrast were ordered. The initial ED visit laboratory findings were normal or considered chronic and nonactionable, except for an abnormal urinalysis. The urinalysis was positive for blood, leukocyte esterase that reflexed to culture. Urine culture subsequently grew >100,000 colonies/mL *E. coli*. Additionally, the CBC with manual differential at this time was also significant for 85% neutrophils with 2% lymphocytes in the presence of a normal white

blood cell (WBC) count, and thrombocytopenia (82,000 cells/ μ L). The patient was prescribed antiemetics and an antibiotic for a urinary tract infection (UTI) and discharged to home. There was no clinical comment for the thrombocytopenia.

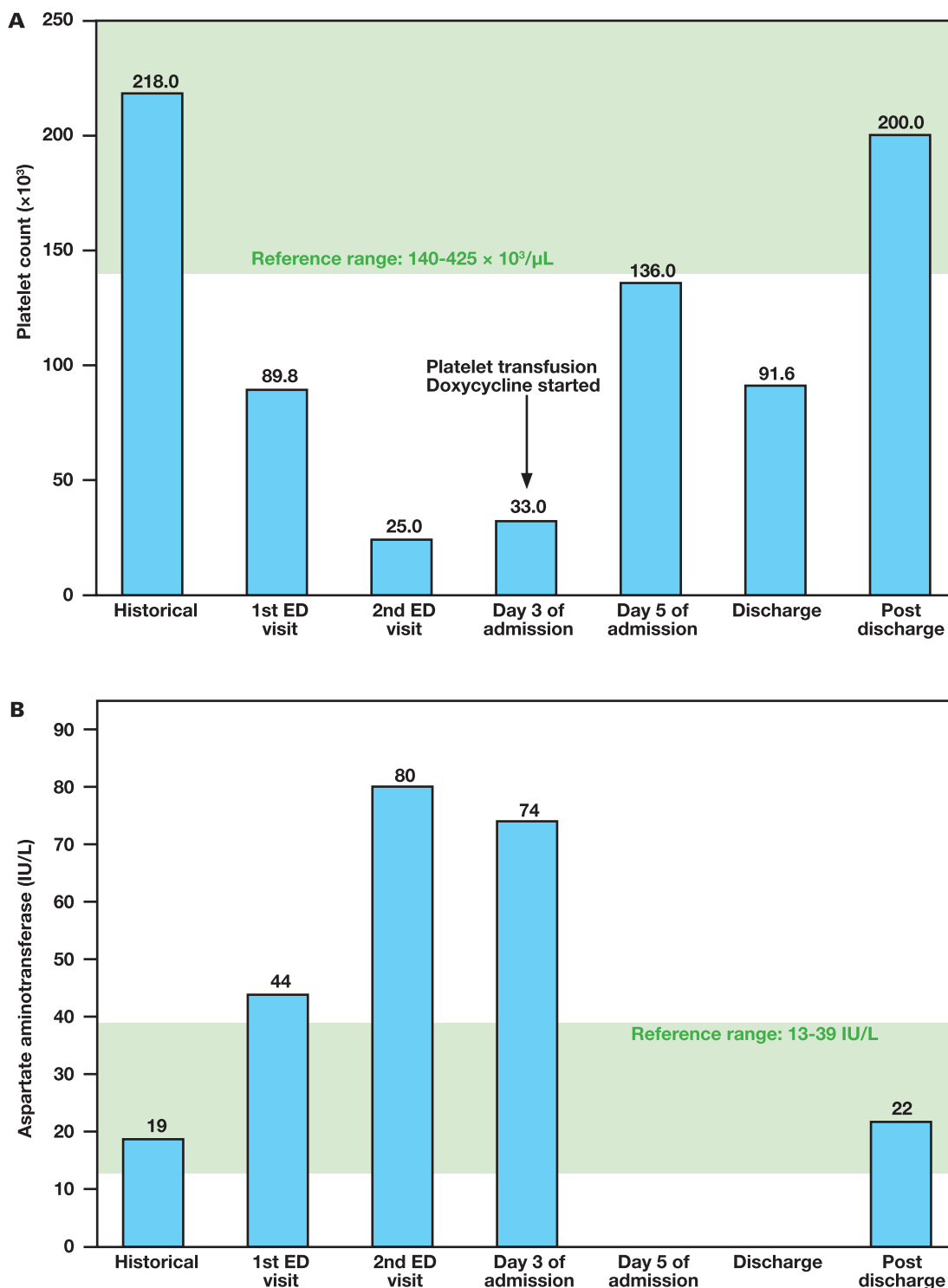
Four days later, the patient presented to the medical center ED with worsening symptoms and distended and tender abdomen. A CBC with differential, basic metabolic panel, hepatic panel, urinalysis, blood cultures, and stool molecular pathogen panel were ordered. Clinically, she had developed peritonitis, prompting collection of peritoneal fluid for cell count, differential, and cultures. The UTI had resolved. The stool molecular pathogen panel and blood cultures were negative. Another CT of the abdomen without contrast was unremarkable. The CBC at this ED admission, compared to the community hospital CBC, showed the platelet count dropped to 25,000 cells/ μ L and hemoglobin and hematocrit dropped to 10.8 g/dL and 30.9%, respectively. There remained a relative and absolute neutrophilia and lymphocytopenia. Leukocytosis was observed in the peritoneal fluid. Peritonitis is potentially life threatening, so the patient was admitted to the medical center for treatment. The hospital staff obtained a more in-depth clinical history, in which the patient admitted to living in the woods and removing a couple of ticks from her leg prior to the first ED visit. Peritoneal dialysis continued throughout her hospital stay.

Results

The patient's comprehensive metabolic panel and hemoglobin were stable prior to the initial ED visit. Biochemical analytes were consistent with a patient being treated with continuous cycling peritoneal dialysis (CCPD) for end-stage renal disease. Her anemia was being monitored with hemoglobin measurements monthly and treated with erythropoietin as needed.

Initial community hospital ED laboratory results were significant for CBC with a low hemoglobin (10.8 g/dL) and platelet count (82,000/ μ L) and urinary tract infection. Despite a platelet count below 100,000 cells/ μ L with a delta flag, the patient was treated for UTI and discharged. The second medical center ED presentation laboratory test results were significant for hyponatremia, elevated blood urea nitrogen (BUN) and creatinine, a doubling of aspartate aminotransferase (AST), and further declines in hemoglobin and platelet count (25,000/ μ L). The decrease in the platelet count and increase in AST were remarkable at an acute-care ED visit.

FIGURE 1 shows the platelet count and AST values over the patient's hospital encounters. Historically, the patient's platelet count and AST

FIGURE 1. Platelet count (A) and AST timeline from admission to discharge (B).

values were within the respective reference ranges. Platelet count continued to drop during the hospital stay until the patient was transfused with 1 platelet apheresis unit and doxycycline was administered on day 2 of admission. When the hemoglobin level was 7.4 g/dL (on day 3 of admission), 1 unit of packed red blood cells was administered to treat anemia. These transfusions and doxycycline ameliorated the cell counts that

subsequently improved such that the platelet count returned to normal at discharge. Likewise, AST levels continued to rise then began to decline posttreatment, eventually returning to normal. Her AST was within the reference range within 1 week from her return home.

Although the WBC count was within the reference range, the auto-differential flagged a delta check for neutrophilia, lymphopenia,

FIGURE 2. Wright's stain peripheral blood smear, 100x magnification, showing morulae and vacuoles in neutrophils (A and B).

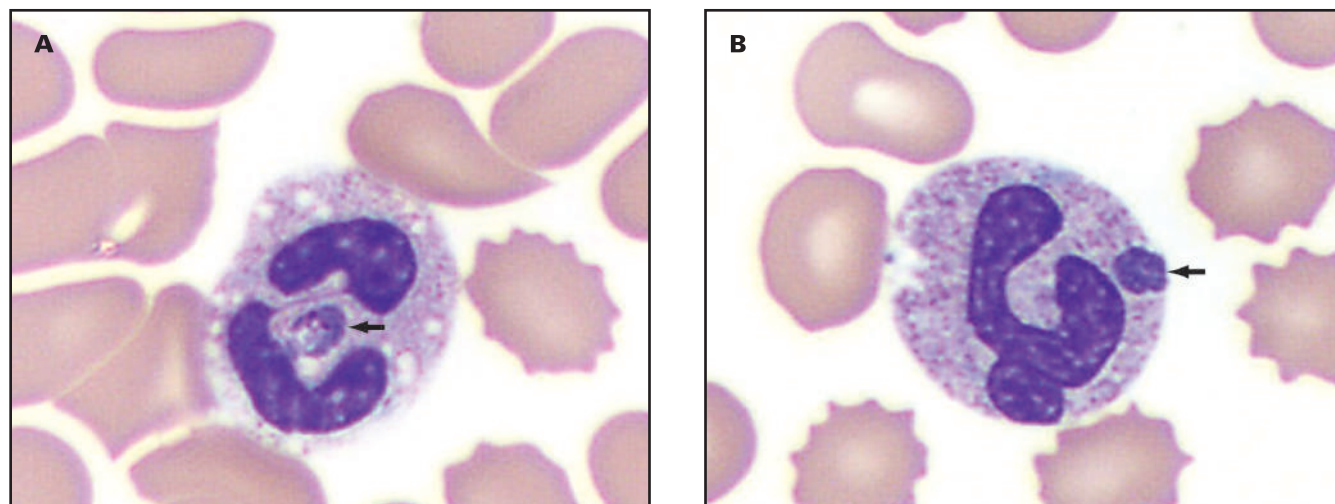


TABLE 1. Body Fluid Cell Counts and Differentials

Cell component	Day 1	Day 1	Day 10 discharge
	Peritoneal fluid	Ascites fluid	Peritoneal dialysate
WBC (cells/ μ L)	691	644	18
RBC (cells/ μ L)	0	0	0
% Neut	41	39	0
% Lymph	38	26	67
% Mono	16	27	33
% Unidentified	5	8	0

WBC=white blood cell; RBC=red blood cell. The automated cell counter body fluid channel has a lower limit of quantitation of 2000 cells/ μ L. Actual RBC counts were retrieved from the analyzer software.

and monocytopenia. A manual differential using Wright's stain was performed within minutes of the CBC. The manual differential was notable for neutrophilia with vacuolated neutrophils containing intra-cytoplasmic inclusions or morulae, as shown in **FIGURE 2**, along with thrombocytopenia. The medical center laboratory requested the differential slide from the community hospital laboratory to see whether the morulae were present from the initial ED visit. This was confirmed by the medical center hematology section, and education was provided to the community hospital laboratory staff.

The body fluid cell automated counts and manual differentials are shown in **TABLE 1**. The elevated leukocyte count is indicative of infection or inflammation in the abdomen. Similar to the peripheral blood smear, the body fluid cytospin differential showed neutrophils containing intra-cytoplasmic inclusions (**FIGURE 3**). Incidentally, no fluid morulae containing neutrophils were observed on cytology prepared slides on either initial exam or retrospectively. This was confirmed by pathologist review by another laboratory. Body fluids submitted for cytology are processed with Papanicolaou (PAP) stain and are generally screened using a 10 $\times$ objective with high dry magnification and review at 40 $\times$ objective for fine nuclear detail and the presence of malignant cells. Cytological examination with PAP is an insensitive method for detection of intra-cytoplasmic inclusions or infectious agents in a 12 to 15 micron neutrophil.

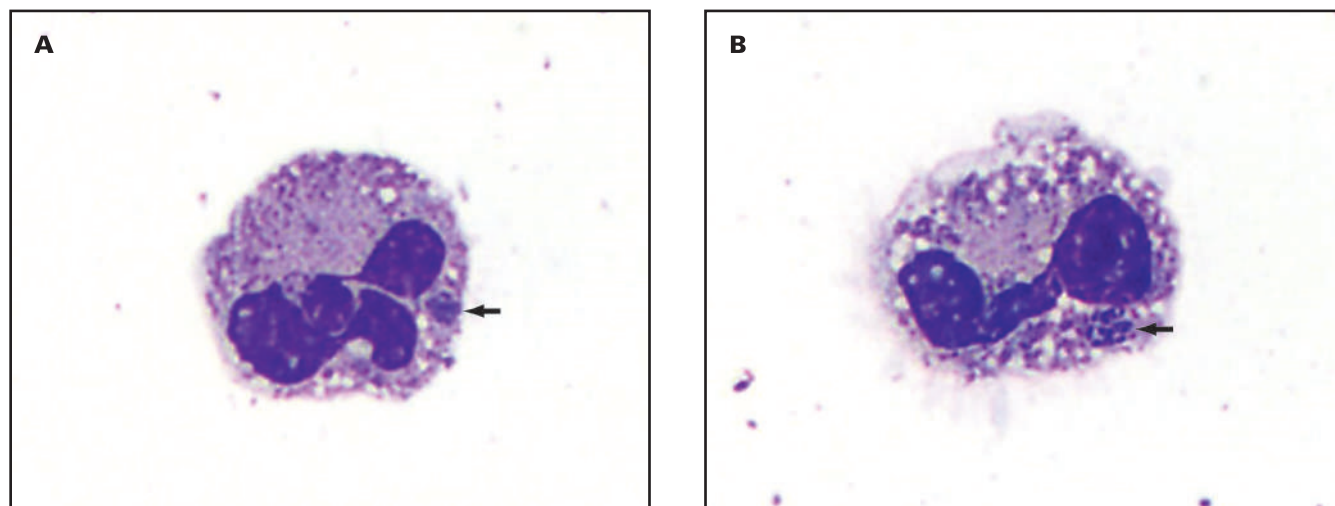
The peripheral blood CBC with differential results and clinical presentation of the patient led us to suspect anaplasmosis. The patient's practitioners were alerted to the laboratory findings and the suggestion for additional testing. Orders were placed for tickborne illness serology and a tick polymerase chain reaction (PCR) panel; both were sent to the reference laboratory for confirmation testing. Tick serology by immunofluorescence assay (IFA) microscopy was positive for *Anaplasma phagocytophilum* immunoglobulin G at 1:128 titer and negative for Lyme disease, *Babesia microti*, and *Ehrlichia chaffeensis*. The tick PCR panel was positive for *A. phagocytophilum* and negative for *B. microti*, *duncani*, *miyamotoi*; *E. chaffeensis*, *ewingii/canis*, *muris*-like. Once blood confirmation of anaplasmosis was obtained, specific molecular testing for *A. phagocytophilum* in peritoneal and ascites fluids was also tested at the state Department of Health laboratory and yielded positive results in both fluids. Fluid and blood bacterial, fungal, and mycobacterial cultures were all negative. These findings confirmed the diagnosis of *Anaplasma*-associated peritonitis.

Discussion

Anaplasma phagocytophilum is an intra-granulocytic bacteria transmitted to humans by an infected tick bite. The ticks most commonly transmitting *Anaplasma* in the United States are the *Ixodes scapularis* or *Ixodes pacificus* black-legged ticks. There has been a 10-fold increase in anaplasmosis cases nationally since 2000.^{1,2} The New England, mid-Atlantic, and upper Midwest states have the highest incidence compared to other parts of the country.² New York state has documented a 33% increase in cases from 2009 to 2018.³ Nonspecific flu-like symptoms and unnoticed tick bites may lead to absence of medical care and underreporting of true cases. Unlike Lyme disease, there is no rash accompanying anaplasmosis. It is important for hematology laboratories to be aware of the increased prevalence of anaplasmosis and other tickborne diseases when reviewing CBC results and differential cells.

A CBC and comprehensive metabolic panel are ordered for patients presenting with nonspecific symptoms of fatigue, fever, and other flu-like symptoms to the ED. Symptoms develop within the first 1 to 2 weeks after an infected tick bite. Within the first 5 days, an individual

FIGURE 3. Wright's stain, 100x magnification, of peritoneal fluid cytospin showing morulae in neutrophils (A and B).



may develop flu-like symptoms of fever, chills, muscle aches, headache, and loss of appetite. However, older persons or individuals with a compromised immune system may develop severe and life-threatening illness. Co-infections with other tickborne diseases such as Lyme disease can also occur. In this case, the patient is an older person who presented with nonspecific symptoms in June, a time of year when tickborne illnesses increase. She had several co-morbidities (UTI, CCPD, anemia) that can complicate diagnosis. Without the recognition of an unexplained thrombocytopenia and neutrophilic morulae by an astute technologist, treatment may have been delayed. In fact, the inclusion bodies were missed by the community hospital laboratory at the initial ED visit, creating an educational opportunity. The Centers for Disease Control and Prevention recommend treating for *Anaplasma* infection if high suspicion exists and not waiting for confirmation test results.¹

A CBC with differential may be used as early surrogate markers for a presumptive diagnosis of *Anaplasma* infection before molecular testing is completed.^{4,5} Other cases of anaplasmosis have documented leukopenia with neutrophilia, lymphopenia, and thrombocytopenia.⁴⁻⁶ In fact, Turbett et al⁴ have proposed using a CBC to reduce unnecessary *Anaplasma* PCR testing. The WBC count in our patient remained within the reference range but did exhibit neutrophilia, lymphopenia, and a significant thrombocytopenia.

Thrombocytopenia has been shown in other rickettsia tickborne diseases such as ehrlichiosis and Rocky Mountain spotted fever.¹ The cause of tickborne thrombocytopenia is not fully understood and is likely multifactorial. It is known that rickettsia target endothelial cells, leading to platelet adhesion and loss of platelets from circulation.⁷ In some systemic infections, this can lead to hypercoagulability with increases in von Willebrand factor and disseminated intravascular coagulation or tickborne hemorrhagic fever.⁷ Fortunately, our patient did not progress to these conditions.

Turbett et al⁴ also observed elevated AST and alanine transaminase (ALT) as was seen in our case. Elevated liver enzymes have been observed in tickborne diseases that may be related to hepatic injury, although not always.⁸ Gastrointestinal symptoms in tickborne disease are nonspecific and may include nausea, vomiting, or diarrhea along with elevated transaminases,⁸ the same symptoms our patient presented at the initial ED visit.

Examination of a blood smear during acute illness may show intracytoplasmic inclusions or morulae of anaplasmae in neutrophils in up to 80% of anaplasmosis cases.⁹ Monocytic morulae are also found, but to a lesser extent, in the human monocytic ehrlichiosis. Hence, the observance of morulae in a particular cell type cannot conclusively differentiate *Anaplasma* from *Ehrlichia* species.¹ Granulocytes respond to infection and inflammation by complex chemotactic signals.¹⁰ Neutrophilic granulocytes contain a large number of granules in the cytoplasm that carry several digestive enzymes responsible for degrading pathogens. Neutrophils are capable of locomotion, traveling between circulation and tissues.¹⁰ In the event of infection, the cell directs its locomotion to the area of infection, where it engulfs the bacteria into a phagocytic vacuole. These phagocytized organisms will be digested and destroyed. As the bacteria clear, neutrophils become vacuolated and Döhle bodies may appear. It is important not to confuse Döhle bodies with morulae. Staining characteristics, location, and size of inclusions differ. Morulae are larger, darker round inclusions in the cytoplasm. Döhle bodies stain pale bluish-gray with Wright's stain, appear rod-shaped and are typically located near the cell membrane, whereas morulae appear darker, are round, oval, or irregular in shape.

Anaplasma molecular testing within the first week of exposure is the most sensitive method for confirming the diagnosis of anaplasmosis. The sensitivity of PCR declines after the second week after exposure, when serology testing may be more beneficial.¹ This patient was unable to determine exactly when she was bitten by the ticks and did not mention this at either ED visit, underscoring the importance of a thorough clinical history. Thus, both PCR and serology panels were ordered and both were positive, suggesting she was exposed within the previous 2 weeks of the initial ED visit.

This case highlights the value of close communication of acute laboratory findings with physicians to facilitate early treatment and early discharge. Although this patient's length of stay was 10 days, her treatment initiated on day 1 of admission after discussion with the covering practitioner. She recovered from anaplasmosis while being treated for chronic kidney disease with dialysis. Nephrology continues to follow her CCPD treatment. This case also provided an educational opportunity for the community hospital laboratory to increase their clinical laboratory

scientist's awareness of neutrophilic morulae. Additionally, we believe this case is the first documentation of anaplasmosis causing peritonitis in a human and the first description of morulae in granulocytes from the peritoneal fluid.

The authors have no conflicts of interest or external funding sources to declare.

REFERENCES

1. CDC. Anaplasmosis. <https://www.cdc.gov/anaplasmosis/>. Accessed Sep 14, 2022.
2. Pritt BS, Dumler JS. *Ehrlichia*, *Anaplasma* and related intracellular bacteria. In: *Manual of Clinical Microbiology*, 12th ed. Washington, DC: ASM Press; 2019:1163–1179.
3. NYS Dept of Health Communicable Disease Annual Reports and Related Information. 2021. NYS Communicable Diseases Statistics. <https://www.health.ny.gov/statistics/diseases/communicable/2018/docs/select.pdf>. Accessed Sep 9 2022.
4. Turbett S, Anahtar MN, Pattanayak V, et al. Use of routine complete blood count results to rule out Anaplasmosis without the need for specific diagnostic testing. *Clin Infect Dis*. 2020;70:1215–1221. doi:10.1093/cid/ciz346.
5. Patnaik MM. Inclusion bodies in tick-borne diseases diagnosed in patients from northern Wisconsin. *Clin Med Res*. 2009;7(1/2):45–47.
6. Stokes W, Lisboa LF, Lindsay LR, Fonseca K. Case report: anaplasmosis in Canada: locally acquired *Anaplasma phagocytophilum* infection in Alberta. *Am J Trop Med Hyg*. 2020;103(6):2478–2480. doi:10.4269/ajtmh.20-0603.
7. Pantanowitz L. Mechanisms of thrombocytopenia in tick-borne diseases. *Internet J Infect Dis*. 2002;2(2):1–6.
8. Ziadi SA, Singer C. Gastrointestinal and hepatic manifestations of tickborne diseases in the United States. *Clin Inf Dis*. 2002;34(9):1206–1212.
9. Bakken JS, Krueth J, Wilson-Nordskog C, Tilden RL, Asanovich K, Dumler JS. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. *JAMA*. 1996;275(3):199–205.
10. Rosales CN. A cell with many roles in inflammation or several cell types? *Front Physiol*. 2018;9(113):1–17.

Heparin Concentration in Evacuated Tubes and Its Effect on pH, Ionized Calcium, Lactate, and Potassium in Venous Blood Gas Analysis

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Keywords: lithium heparin, sodium heparin, venous blood gas, ionized calcium, pH, evacuated tubes

Abbreviations: CLSI, Clinical & Laboratory Standards Institute; USP, United States Pharmacopeia; iCa, ionized calcium; ... , nonapplicable

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ABSTRACT

Arterial blood specimens collected in evacuated tubes are unacceptable for blood gas analysis. However, evacuated tubes are routinely used for venous blood-gas analysis. The impact of the blood to heparin ratio on venous blood in evacuated tubes is unclear. Venous blood was drawn into lithium and sodium heparin evacuated tubes that were 1/3 full, 1/2 full, 2/3 full, and fully filled. Specimens were analyzed for pH, ionized calcium (iCa), lactate, and potassium on a blood-gas analyzer. The results for specimens filled only 1/3 full for lithium and sodium heparin tubes revealed a significant increase in pH and a significant decrease in the iCa. Underfilling the lithium and sodium heparin evacuated tubes did not significantly impact the lactate or potassium results. Venous whole-blood specimens should be filled to at least 2/3 full for accurate pH and iCa results.

Blood gas testing and result interpretation are common practice in the ED and ICU. The preanalytical phase accounts for most errors reported in clinical laboratories.¹ A high degree of quality throughout the total testing process is critical to provide reliable test results. The Clinical & Laboratory Standards Institute (CLSI) guideline C46-A2 states that heparin should be the anticoagulant of choice for preparation of specimens for blood-gas analysis.² The correct amount of heparin is vital in the prevention of coagulation in blood and in obtaining accurate test results.

According to CLSI guideline C46-A2 the evacuated tube is an unacceptable specimen type for arterial blood gas analysis due to the fact that gas assays cannot be run anaerobically on the blood gas analyzer.² However, evacuated tubes, along with syringes, are used for the collection of venous blood gas for nongas analysis.³

There are multiple reasons why evacuated tubes are used: to save time, to avoid duplicate blood draws, and to reduce pain.⁴ Underfilling the heparinized evacuated tubes may result in erroneous results due to dilutional or chemical effects. The dilutional effects of heparin are avoided by the use of preloaded lyophilized heparin in BD Vacutainer Lithium and Sodium Heparin Tubes (Becton, Dickinson and Company). However, the effects resulting from various concentrations of heparin due to underfilling are still unclear for the assays of pH, iCa, lactate, and potassium. The aim of our study was to determine the effects on accuracy for underfilled, heparinized evacuated tubes of venous blood for the assay of pH, iCa, lactate, and potassium.

Materials and Methods

Peripheral venous blood specimens were collected from 8 healthy volunteer employees at The Ohio State University, Columbus. This study was used for quality improvement; we collected no identified private information from the volunteers. Therefore, this study was deemed exempt by the university hospital IRB. The venous blood donated by each volunteer was drawn into 8 separate evacuated tubes, which were 1/3 full, 1/2 full, 2/3 full, and fully filled BD Vacutainer Heparin Tubes (sodium heparin tubes, Cat# 367878, spray-coated sodium heparin 95 United States Pharmacopeia [USP] units, 6 mL in full) and BD Vacutainer PST Tubes (lithium heparin tubes, Cat# 367962, spray-coated lithium heparin 84 USP units with polymer gel, 4.5 mL in full) (Becton, Dickinson and Company). All tubes were mixed by gentle inversion. The corresponding heparin concentrations in each group are listed in **TABLE 1**.

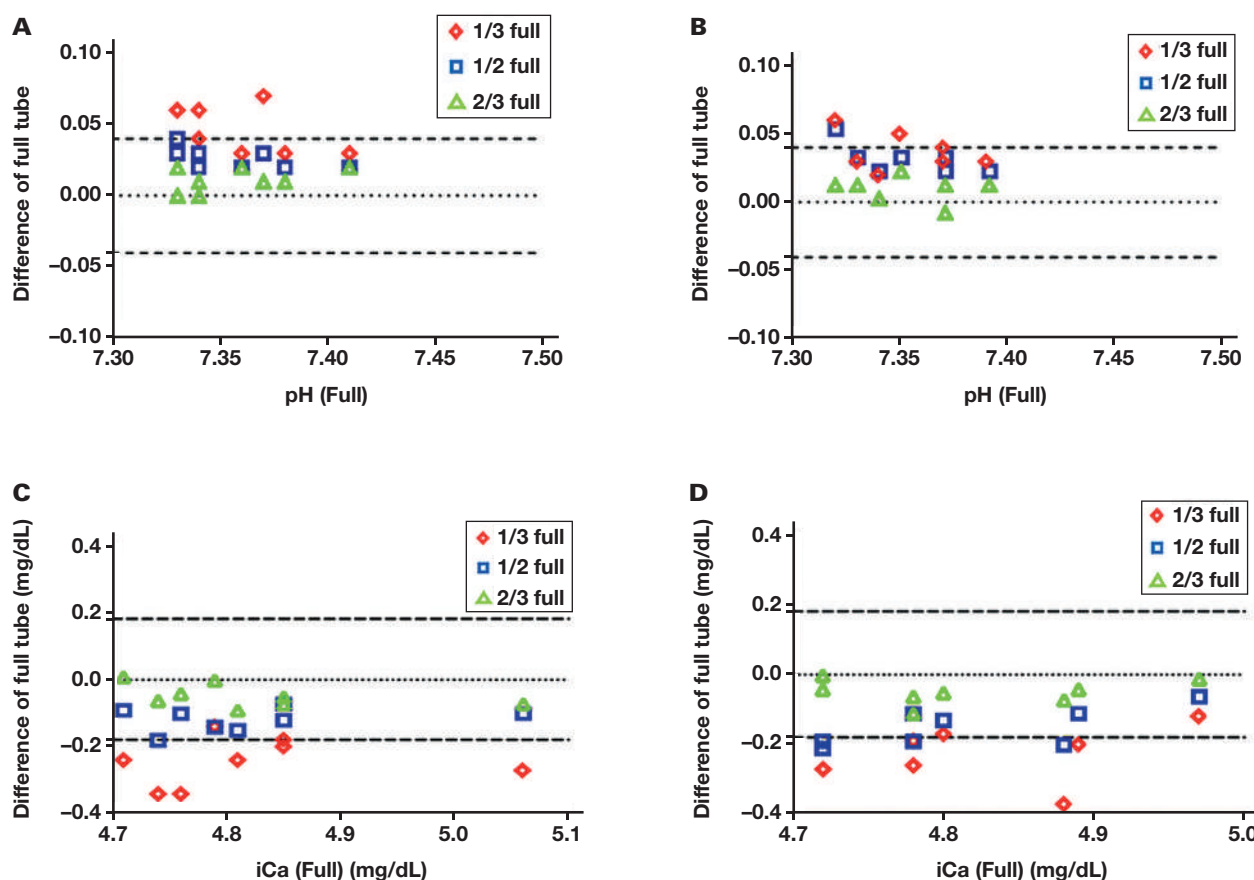
Assays for pH, iCa, lactate, and potassium for each specimen were run on the ABL800 blood gas analyzer (Radiometer Medical ApS) consecutively. All assays were completed within 15 minutes of blood drawing. The data were analyzed using a paired *t*-test ($\alpha = .05$) and Bland-Altman analysis and compared by linear regression (GraphPad Prism 9.0, GraphPad Software). The acceptability criteria, listed in **FIGURE 1**, were defined according to the latest College of American Pathologists survey (AQ-A 2022).

TABLE 1. Calculated Heparin Concentrations in Each Group

Measurement	Sodium Heparin				Lithium Heparin			
	1/3 Full	1/2 Full	2/3 Full	Full	1/3 Full	1/2 Full	2/3 Full	Full
Volume	2.0 mL	3.0 mL	4.0 mL	6.0 mL	1.5 mL	2.3 mL	3.0 mL	4.5 mL
Heparin concentration (USP units/mL)	47.50	31.67	23.75	15.83	56.00	36.52	28.00	18.67

Abbreviation: USP, United States Pharmacopeia.

FIGURE 1. Bland-Altman plots for comparison of results in each underfilled-tube group, compared with fully filled tubes. The dash line represents the acceptable criteria—pH, 0.04; ionized calcium (iCa), 0.18 mg/dL. A, Plot 1, lithium heparin tube. B, Plot 2, sodium heparin tube. C, Plot 3, lithium heparin tube. D, Plot 4, sodium heparin tube.



Results

The results showed significant differences in iCa and pH in the evacuated tubes that were filled only 1/3 full (sodium/lithium heparin tubes), when compared with the fully filled tubes (TABLE 2; $P < .05$). There was a positive mean bias of 0.047 for pH in specimens that were filled only 1/3 full in lithium heparin tubes, compared with fully filled tubes. There was a positive mean bias of 0.036 for pH in specimens that were only 1/3 full in sodium heparin tubes, compared with the fully filled tubes (TABLE 2).

Bland-Altman plots for each analyte are presented in FIGURE 1. In total, 4 of the 8 results (50%) from the 1/3-full lithium heparin tubes exceeded the acceptability criteria, and 2 of 8 results (25%) from the 1/3-full sodium heparin tubes exceeded the acceptability criteria. All results from the 1/2- and 2/3-full lithium heparin tubes were within the acceptability criteria. Of the 8 results from the 1/2-full sodium heparin tubes, 7 results were within the acceptability criteria (FIGURES 1A and 1B).

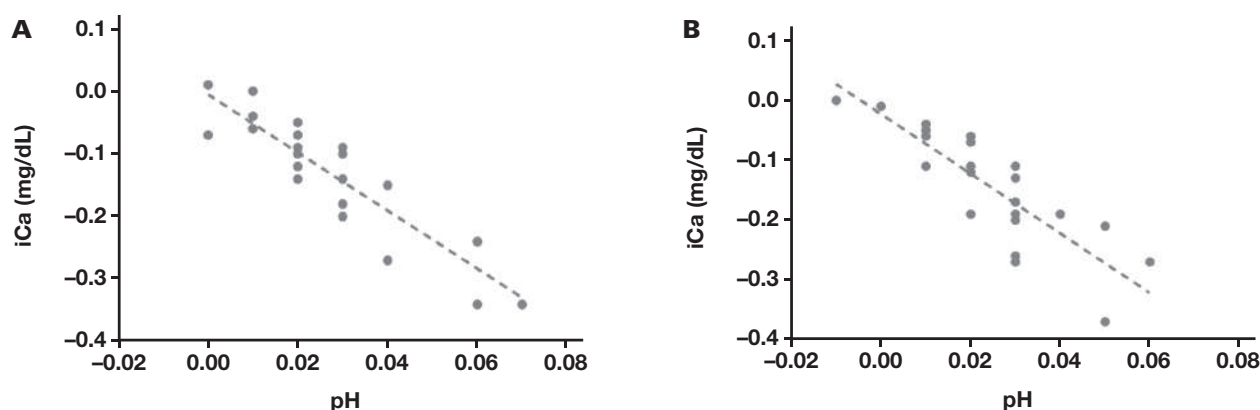
There was a negative mean bias of 0.24 mg/dL for ionized calcium (iCa) in the 1/3-full lithium heparin tubes and a 0.23-mg/dL negative mean bias in the 1/3-full sodium heparin tubes, compared with the fully filled tubes (TABLE 2). FIGURES 1C and D, show 6 of 8 results (75%) of the 1/3-full lithium heparin tubes, and 6 of 8 results (75%) of the 1/3-full sodium heparin tubes exceeded the acceptable criteria. Also, 3 of 8 results (38%) of the 1/2-full sodium heparin tubes exceeded the acceptable criteria. There were no outliers among the 1/2-full and 2/3-full lithium heparin tubes.

All but one of the lactate results were within acceptable criteria in the 1/3-full lithium and sodium heparin tubes (Supplemental Figures 1A and 1B). All potassium results in each of the tube fill groups were within acceptable criteria (Supplemental Figures 1C and 1D). The correlation between differences in pH (underfilled tube to fully filled tube) vs the difference in iCa (underfilled tube to fully filled tube) were performed in lithium and sodium heparin tubes (FIGURE 2), showing good correlation between negatively biased iCa and positively biased pH.

TABLE 2. Results of Venous pH and iCa

Analyte		Sodium Heparin				Lithium Heparin			
		1/3 Full	1/2 Full	2/3 Full	Full	1/3 Full	1/2 Full	2/3 Full	Full
pH	Specimen 1	7.38	7.38	7.36	7.35	7.40	7.37	7.35	7.34
	Specimen 2	7.40	7.38	7.37	7.35	7.39	7.38	7.38	7.36
	Specimen 3	7.36	7.36	7.34	7.34	7.38	7.36	7.34	7.34
	Specimen 4	7.42	7.41	7.40	7.39	7.44	7.43	7.43	7.41
	Specimen 5	7.36	7.36	7.34	7.33	7.39	7.37	7.35	7.33
	Specimen 6	7.38	7.37	7.33	7.32	7.39	7.36	7.33	7.33
	Specimen 7	7.41	7.40	7.38	7.37	7.41	7.40	7.39	7.38
	Specimen 8	7.40	7.39	7.36	7.37	7.44	7.40	7.38	7.37
	Mean (SD)	7.389 (0.022)	7.381 (0.018)	7.360 (0.023)	7.353 (0.023)	7.405 (0.023)	7.384 (0.024)	7.369 (0.033)	7.358 (0.028)
	P value (compared with full tube)	.007	.02	.53	...	.002	.066	.47	...
iCa (mg/dL)	Specimen 1	4.52	4.59	4.67	4.78	4.40	4.56	4.68	4.74
	Specimen 2	4.51	4.68	4.81	4.88	4.67	4.78	4.78	4.85
	Specimen 3	4.85	4.91	4.96	4.97	4.79	4.96	4.99	5.06
	Specimen 4	4.69	4.78	4.85	4.89	4.65	4.73	4.80	4.85
	Specimen 5	4.63	4.67	4.75	4.80	4.57	4.66	4.72	4.81
	Specimen 6	4.45	4.51	4.68	4.72	4.47	4.62	4.72	4.71
	Specimen 7	4.59	4.67	4.72	4.78	4.65	4.65	4.79	4.79
	Specimen 8	4.45	4.53	4.72	4.72	4.42	4.66	4.72	4.76
	Mean (SD)	4.586 (0.136)	4.668 (0.132)	4.770 (0.098)	4.818 (0.088)	4.578 (0.137)	4.703 (0.123)	4.775 (0.097)	4.821 (0.109)
	P value (compared with full tube)	.001	.018	.33	...	.001	.06	.38	...

Abbreviations: iCa, ionized calcium; ... , nonapplicable.

FIGURE 2. Correlation between the difference in pH (underfilled tube vs full tube) vs the difference in iCa (underfilled tube vs full tube). A, Lithium heparin tube. B, Sodium heparin tube. iCa indicates ionized calcium.

Discussion

In this study we evaluated the effects of different concentrations of lithium and sodium heparin on pH, iCa, lactate, and potassium on venous blood in evacuated tubes on a blood gas analyzer. Our results revealed that heparin concentrations at approximately 50 USP units/mL led to significant increases in pH and were accompanied

by a significant decrease in iCa. Underfilling Vacutainer tubes did not affect lactate or potassium results. Therefore, the laboratory must establish a fill requirement as part of its acceptable-collection criteria, to provide the most accurate results by avoiding the potential interference caused by the lithium/sodium heparin anticoagulant.

TABLE 3. Precision of pH and iCa on Radiometer ABL 800 Blood Gas Analyzer^a

Analyte	Measurement	Level 1	Level 2	Level 3
pH	Mean	7.09	7.39	7.56
	CV %	0.04%	0.03%	0.04%
	Points	80	73	81
iCa	Mean (mg/dL)	4.29	2.29	1.64
	CV %	1.48%	2.92%	3.07%
	Points	80	73	80

Abbreviation: iCa, ionized calcium.

^aRadiometer Medical ApS.

Heparin binds with calcium, and the use of calcium-balanced heparin can help minimize the effect of calcium binding.² In our study, the heparin in the evacuated tubes is measured by USP units, which contain calcium-balanced heparin and so, therefore, the calcium-heparin binding is minimized.⁵ Our results revealed a strong relationship between iCa and pH changes, which was likely due to the iCa reduction in an alkaline environment. Calcium binding to proteins is pH-dependent. Calcium binds to negatively charged sites on proteins. We found that the negative bias of iCa in concert with the positive bias of pH explains one of the potential reasons of decrease iCa in high concentrations of heparin (approximately 50 USP units/mL).

The observation of positive bias in pH in specimens that were filled only 1/3 full in lithium and sodium heparin tubes might be explained by the hypothesis of air pressure. When the evacuated tubes are underfilled, higher vacuum suction might cause more pCO₂ to be released from the blood, resulting in lower pH, compared with the fully filled evacuated tubes. Further studies are needed to explore this hypothesis.

Another possibility for the variations in iCa or pH might be imprecision of the analyzer. The precision of the Radiometer ABL 800 blood-gas analyzer in our laboratory is listed in **TABLE 3**. The CV of iCa at the concentration of approximately 4.3 mg/dL was 1.48%, and the iCa ranges in all the specimens in this study was 4.4–5.0 mg/dL. The estimated bias from the analyzer was 0.07 mg/dL, which is by using the acceptability criteria of 0.18 mg/dL. Therefore, we believe the decreased iCa was caused by the more alkaline environment in specimen-collections tubes that were not entirely filled, rather than by any imprecision of the analyzer.

We were only able to evaluate a limited range of pH due to the fact that the blood specimens were donated from healthy volunteers. Further studies should include a range of healthy to critically ill patients, to assess the impact of heparin on iCa in a wider pH range.

Supplementary Data

Supplemental figures and tables can be found in the online version of this article at www.labmedicine.com.

REFERENCES

1. Carraro P, Plebani M. Errors in a stat laboratory: types and frequencies 10 years later. *Clin Chem*. 2007;53(7):1338–1342. doi:10.1373/clinchem.2007.088344
2. D'Orazio P, Clinical and Laboratory Standards Institute (CLSI). *Blood Gas and pH Analysis and Related Measurements: Approved Guideline, (C46-A2)*. 2nd Ed. CLSI; 2009.
3. Røys EÅ, Husøy A-M, Brun A, Aakre KM. Impact of different sampling and storage procedures on stability of acid/base parameters in venous blood samples. *Clin Chem Lab Med*. 2021;59(9):e370–e373. doi:10.1515/cclm-2021-0202
4. Chauvin A, Javaud N, Ghazai A, et al. Reducing pain by using venous blood gas instead of arterial blood gas (VEINART): a multicentre randomised controlled trial. *Emerg Med J*. 2020;37(12):756–761. doi:10.1136/emered-2019-209287
5. Vickers MD, Morgan M, Spencer RM. *Drugs in Anaesthetic and Intensive Care Practice*. 8th ed. Butterworth-Heinemann; 1999.

Effects of the Noise Reduction and Communication Management Headset System SLOS on Noise and Stress of Medical Laboratory Workers

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Keywords: noise reduction, headphone system, stress reduction, SLOS, technology acceptance, exhaustion reduction, supporting work technology, medical laboratory

Abbreviations: WHO, World Health Organization; SOTOS, Silent Operating Theatre Optimisation System; OT, operating theatre; SLOS, Silent Laboratory Optimization System; SANA, sofortanalytik; MQ, Monthly Questionnaire; DQ, Daily Questionnaire; STAI, State-Trait Anxiety Inventory; PSS-10, Perceived Stress Scale 10; LSB, Leipziger StimmungsBogen; TAM2, Technology Acceptance Model

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ABSTRACT

Objective: To investigate the effects of the Silent Laboratory Optimization System (SLOS), a technical-noise reduction and communication-management system, on noise load and stress among medical-laboratory workers.

Methods: We conducted a quasiexperimental field study (20 days with SLOS as the experimental condition, and 20 days without SLOS as the control condition) in a within-subjects design. Survey data from 13 workers were collected before and after the shift. Also, a survey was conducted after the control and experimental conditions, respectively. Noise was measured in dBA and as a subjective assessment. Stress was operationalized via a stress composite score (STAI and Perkhofers Stress Scale), the Perceived Stress Scale (PSS), an exhaustion score (Leipziger StimmungsBogen in German [LSB]), and salivary cortisol values in µg/L.

Results: SLOS users perceived significantly less noise ($V = 76.5$; $P = .003$). Multilevel models revealed a stress reduction with the

SLOS on the composite score, compared with a stress increase in the control condition ($F[1, 506.99] = 6.00$; $P = .01$). A lower PSS score ($F[1, 13] = 4.67$; $P = .05$) and a lower exhaustion level ($F[1, 508.72] = 9.057$; $P = .003$) in the experimental condition were found, whereas no differences in cortisol ($F[1, 812.58.6] = 0.093$; $P = .76$) were revealed.

Conclusion: The workers showed reduced noise perception and stress across all criteria except cortisol when using SLOS.

Noise load is a problem in many workplaces and an important health issue^{1–3} for the workers exposed to noise that exceeds the thresholds set by the ergonomic standards of the World Health Organization (WHO). The WHO recommendations vary depending on specific target contexts, with the highest threshold being 50 dBA.^{4–6} The health sector itself is a sensitive work domain because noise, which potentially impairs performance,^{7,8} is a critical factor not only for workers but also for patients. Noise levels exceed recommendations not only in workplaces such as operating theatres^{1,7,9} but also in medical laboratories—the target context of this article—where lab devices by themselves produce between 66 dBA and 80 dBA.^{10,11}

Performance in general,^{7,8} as well as productivity and concentration, are diminished by increased noise.¹² Noise leads to higher exhaustion, lower well-being, and more stress.¹² Also, a positive correlation has been found between exposure to noise and cardiovascular diseases.^{1–3} This is likely to occur because noise is a stressor.^{4,11–14} According to the transactional stress model, a person perceives stress when 2 appraisals are made. First, the person appraises the situation to be potentially harmful to their well-being (primary appraisal) and second, the person does not think that they have enough resources to deal with the situation or to solve the problem (secondary appraisal).¹⁵

Earplug solutions, as suggested by the WHO,⁶ do not solve the noise problem optimally. Use of earplugs would change the situation-person transaction insofar as they reduce the perceived noise and thus offer a resource regarding the secondary appraisal. However, in most workplaces, workers still need to communicate, which is made more difficult by earplugs.

The Silent Operating Theatre Optimisation System (SOTOS), which functions as a noise reduction and communication management

tool,¹⁶ promises a solution for the suboptimal situation of noisy work environments. Initially, it was developed for the operating theatre (OT). Systematic evaluation yielded mostly positive effects on noise perception, stress, exhaustion, and technical acceptance of the system.^{13,16–18} For this study we adapted the SOTOS to the necessities of the medical lab, creating the Silent Laboratory Optimization System (SLOS). This headphone system allows communication between workers and reduces environmental noise, while allowing the option to listen to music and to communicate via microphones. A strength of the system is its communicational matrix which, depending on the structure of the tasks and subtasks, allows users to connect certain workers or groups of workers with each other.¹⁶

This field study evaluates the effects of SLOS on noise and stress, as well as user acceptance of the system and comfort in a medical lab, by comparing the lab workers during the experimental condition with the SLOS and the control condition without the SLOS, during a total of 40 workdays. We were interested in determining whether the system would objectively reduce in-room noise at the workplace, as was found in OTs,¹⁸ and subjectively for the lab workers, as the findings of a technical study on SOTOS suggest.¹⁶ Therefore, a replication of these findings was one of the goals of this study.

According to the transactional stress model,¹⁵ the SLOS system could positively impact the primary stress appraisal provided by workers by changing the situation through noise reduction, while still allowing communication with colleagues. The system offers an additional resource relating to the secondary appraisal—ie, workers now have an option to deal with the noise issue. This is why we expected to observe stronger stress reduction benefits during the experimental condition, compared with the control condition, based on the transactional stress model.¹⁵ These assumptions are supported by the job demand-control model,¹⁹ which postulates that psychological strain results from the joint effects of the demands of a work situation (stressors) and the range of decision-making freedom (control or job decision latitude). Workers experience high job strain when job demands are high and job decision latitude is low.¹⁹

Higher noise is associated with higher job demands.^{20–22} Accordingly, working with the SLOS reduces job demands directly. At the same time, doing so grants lab workers higher job decision latitude because they are now able to control noise by using the SLOS and to control communication by implementing their own choices in the communication matrix. Lower job demands and higher job decision latitude reduce the likelihood of high job strain.¹⁹ Also, the stress-buffering effects of work-related social support on work-related stress have been empirically confirmed.^{23–25}

Due to the greater communicative range offered by the communication matrix, more work-related social support can be received from work colleagues, and the physical distance between colleagues can be overcome, which creates more communicational opportunities for social support. Derived from both theories, the second goal of this study was to replicate the findings from the OT, in which the novel communication system reduced stress,^{13,17} but this time, in the context of a medical lab.

Also, we were interested in evaluating the SLOS concerning its acceptance level among users and the user level of comfort with the system. To implement the SLOS in a workplace, it is necessary that the workers feel comfortable with the technical device and accept it as a useful tool. Besides the investigation of the effects of the SLOS on noise and stress outcomes, it was therefore also our goal to research the subjective

acceptance of the system through the technical system evaluation. For this purpose, the test subjects were asked about their perceived comfort with and acceptance of the SLOS. Additionally, we were interested in whether the system would enable communication in technical terms and whether the users would perceive it as nonobstructive and always usable, so they could stay informed about the work of their collaborators, when needed.

Materials and Methods

Study Design and Participants

To evaluate the effects of the SLOS in a lab environment, we conducted a field study with an experimental and a control condition. The study took place in the immediate analysis section (in German, *sofortanalytik* [SANA]) of a large-scale medical lab in Goettingen, Germany, during the early shift of regular workdays, with weekends excluded.

The test subjects were all 13 of the lab assistants of the SANA. On each weekday a maximum of 7 individuals were working the early shift and therefore were integrated into the study. Each worker fulfilled the tasks of a certain role (**FIGURE 1**). In general, some workstations need to be operated dependently with one another, whereas others are independent. The lab managers claimed that communication takes place irregularly between all roles, which was confirmed by randomly conducted observations.

The main function of the stations COBAS 1 to 5 and KRYPTOR is the clinical, chemical and immunological analysis of specimens sent in by local clinics. These stations differ in their specific clinical, chemical, and immunological analysis approaches or by the test assignments. The Residual List group deals with more complex material than the aforementioned roles and offers a manual validation of results. Because of the fixed shift plan, the randomized assignment of test subjects to treatment conditions was practically unfeasible. The lab crew worked together in their regular composition. All participants signed a declaration of consent for their participation and allowed the anonymous usage of data for research. The ethics committee of the Georg-Elias-Mueller Institute of Psychology in Goettingen, Germany approved the study (no. 295) on January 1, 2022.

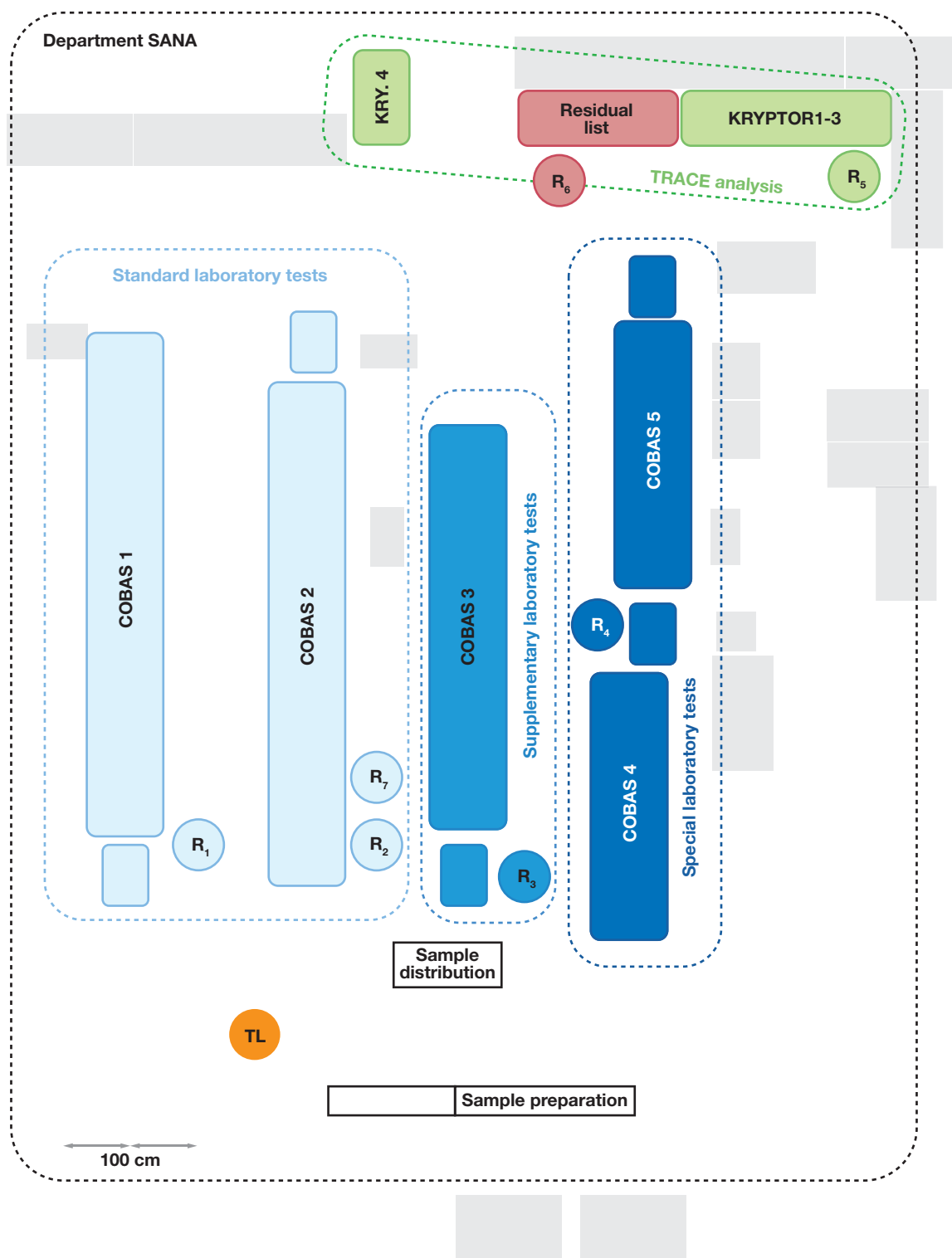
The dependent variables of this study are noise and stress. The latent variable noise is operationalized by the objective measurement of dBA in the lab and subjectively perceived noise. The latent variable of stress is operationalized by 3 subjective stress-level variables, cortisol level, and a subjective exhaustion measurement as a stress outcome. The independent variables (IV) are *treatment* (experimental, control), *time of measurement* (preshift, postshift), and *specimen number* of the cortisol specimen (1, 2, or 3).

Procedures

The data collection for the control condition was conducted between January 10, 2022, and February 4, 2022. The experimental condition started on February 7, 2022 and lasted until March 4, 2022, during which the test subjects worked with the SLOS.

Dependent variables were measured via paper-and-pencil questionnaires, which included items for all psychological and demographic variables. The Monthly Questionnaire (MQ) was conducted after the control condition and after the experimental condition. The Daily Questionnaire (DQ) was given daily before and after each shift.

FIGURE 1. Workplace of the immediate analysis section (in German, *sofortanalytik* [SANA]) of the medical laboratory. R1-7: role 1–role 7. TL=team leader.



To minimize measurement biases, all surveys were conducted right before starting or right after finishing the daytime shift. The time of the before-shift and after-shift measurements depended on the arrival and departure of the workers, usually between 6:15 AM and 8:30 AM, and between 2:30 PM and 4:00 PM.

We used a decibel meter was used to measure the noise level in the lab between 9:30 AM and 2:00 PM. In both study conditions, test subjects were asked to provide saliva specimens to create a daily profile of their cortisol levels. Due to technical problems such as battery issues in the SLOS on February 8, 11, and 17, these days were excluded—not all team

members of the lab crew worked the whole shift with the system on these days. The excluded data were replaced by 3 additional buffer days on March 7, 8, and 9, 2022. In the end, each condition lasted 4 weeks, with 5 survey days per week, leading to 20 survey days per condition.

Noise Measurement

Noise in the lab room was operationalized by the sound-pressure level that was measured by a VOLTcraft SL-451 instrument (Conrad Electronic International GmbH & CoKG) once per second as dBA. The VOLTcraft was used when available, which led to 28 measurement days, of which half were conducted in the control condition and half in the experimental condition. Because no special noise events were mentioned in the daily protocols of the test coordinators and because the availability of the VOLTcraft was random, we assumed that a representative specimen was collected for the noise measurement. The data for each daily measurement was saved by the software Voltsoft PRO, version 1 (Conrad Electronic International GmbH & CoKG). The subjective perception of noise was measured via 2 items in the second MQ ("Did you perceive the noise level in general (without the SLOS) in the lab as loud and/or disturbing?"; "Did you perceive the noise level in general (with the SLOS) as loud and/or disturbing?").

Stress Measurement

Daily subjective stress levels were measured using the German short version of the State-Trait Anxiety Inventory (STAI) for state anxiety²⁶ and the Perkhofers Stress Scale.²⁷ The subjective stress level relating to the experienced stress during the past 4 weeks were measured with the German version of the Perceived Stress Scale 10 (PSS-10).²⁸ We analyzed cortisol specimens to assess physiological stress. The stress outcome exhaustion was operationalized by the exhaustion subscale of the Leipziger Stimmungsbogen (LSB) in German.²⁹

The STAI includes 8 items. The items ask for agreement concerning statements about the present perception of stress. Answers were given on an 8-point Likert-type scale (1 = not at all through 8 = completely).²⁶ The Perkhofers Stress Scale is a combined single-item questionnaire of visual analogous scales and face-rating scales. It can be assumed that both measurements assess the same construct because their convergent validity was reported, with the variables correlating significantly ($r = .67$).²⁷ This was the reason for calculating a composite stress score.

The PSS-10²⁸ measures the subjective stress level during the past 4 weeks, which matches the length of the treatment conditions. The PSS-10 consists of 10 items, including questions about the frequency of subjectively stressful experiences during the past 4 weeks. Answers were given on a 5-point Likert-type scale (1 = never through 5 = very often).

Cortisol saliva specimens were taken right after waking (specimen 1), half an hour after waking (specimen 2), and before the after-shift questionnaire (specimen 3), using the test specimen kit Elecsys Cortisol II (Roche Diagnostics GmbH). All specimens were collected and protocolled by the test administrators, resulting in 3 cortisol specimen per subject per day measured in µg/L.

Saliva cortisol is an established biomarker for stress in human beings.^{30–32} In contrast to serum cortisol, the measurement of saliva cortisol is noninvasive,³³ which makes it more practical for studies. Also, this method allows researchers to control for the possible biasing effects of stress regarding the venipuncture used in collecting serum cortisol specimens.^{33,34}

Exhaustion at work is an immediate result of workplace stressors³⁵ and therefore represents a stress outcome.³⁶ Hence, we added exhaustion as a further stress-related measure, to expand the multimodal approach. The exhaustion subscale of the LSB²⁹ is a questionnaire consisting of 6 items asking for the extent to which adjectives associated with exhaustion, such as being tired or worn out, are applicable to the present perception of a subject, on a 5-point Likert-type scale (1 = not at all through 5 = very much).

Technical System Evaluation

Concerning the Technical System Evaluation, 3 variables were integrated into the MQ2 (all items can be found in TABLES 1–3). The first variable is the technical acceptance of the SLOS in general and consists of 4 self-developed items asking about the perceived support provided by the system, the comfort of the headphones and the microphone, and the overall audio quality. The second variable is the specific acceptance of the SLOS and consists of 12 items. This construct was derived from the extended Technology Acceptance Model (TAM2),³⁷ with the target technology being the SLOS. The TAM2 integrates 4 facets, namely, *intention to use*, *perceived usefulness*, *perceived ease of use*, and *subjective norm*. The third variable is concerned with the perceived effects of the SLOS

TABLE 1. Descriptive Statistics of SLOS Technical Acceptance^a

Item	Mean (SD)
In general, work with the SLOS was ...	4.85 (0.90)
Until the end of the workday, the earphones were ...	4.23 (0.73)
If you wore a neckband microphone, this was...	3.31 (1.32)
The overall audio quality was...	5.15 (0.69)

Abbreviation: SLOS, Silent Laboratory Optimization System.

^aAnswers were provided on a Likert-type scale: Item 1: 1 = extremely disturbed, 2 = pretty disturbed, 3 = mildly disturbed, 4 = little supported, 5 = pretty supported, 6 = extremely supported. Item 2 and Item 3: 1 = extremely uncomfortable, 2 = pretty uncomfortable, 3 = mildly uncomfortable, 4 = mildly comfortable, 5 = pretty comfortable, 6 = extremely comfortable. Item 4: 1 = extremely bad, 2 = pretty bad, 3 = reasonably bad, 4 = reasonably good, 5 = pretty good, 6 = extremely good.

TABLE 2. Descriptive Statistics of SLOS Acceptance^a

Item	Mean (SD)
Assuming I have access to the SLOS, I intend to use it.	4.38 (1.04)
Given that I have access to SLOS, I predict that I would use it.	4.46 (1.05)
Using the SLOS improves my performance in my job.	3.92 (1.26)
Using the SLOS enhances my effectiveness in my job.	3.77 (1.30)
I find the SLOS to be useful in my job.	4.23 (1.17)
Using SLOS increases my effectiveness at work.	3.92 (1.12)
My interaction with the SLOS is clear and understandable.	4.38 (0.87)
Interacting with the SLOS does not require a lot of my mental effort.	4.31 (1.11)
I find the SLOS to be easy to use.	3.85 (1.07)
I find it easy to get the system to do what I want it to do.	3.92 (0.86)
People who are important to me think I should use the SLOS.	3.92 (0.95)
People who influence my behavior think I should use the SLOS.	3.69 (1.03)

Abbreviation: SLOS, Silent Laboratory Optimization System.

^aAnswers were provided on a Likert-type scale: 1 = not at all, 2 = a little bit true, 3 = partially true, 4 = pretty much true, 5 = absolutely true.

TABLE 3. Descriptive Statistics of SLOS Communication^a

Item	Mean (SD)
I could communicate unhindered with my colleagues via SLOS.	4.85 (0.90)
Communication with the SANACrew was possibly at all times.	5.15 (1.21)
I was always sufficiently informed about what my colleagues were doing.	5.31 (0.86)

Abbreviation: SLOS, Silent Laboratory Optimization System; SANA, sofortanalytik.

^aAnswers were provided on a Likert-type scale: 1 = Does not apply at all, through 6 = Applies fully.

on communication via 3 self-developed items. Because this study is not concerned with communicational analyses, we were only interested in whether the SLOS would technically allow for clear communication.

Statistical Analysis

Statistical analysis and graphical representation of the data were performed using R software, version 4.0.3.³⁸ Descriptive statistics of demographic characteristics and Technical System Evaluation of acceptance and comfort are reported according to their respective levels of measurement as mean (SD) or as absolute and relative frequencies. Noise-exposure-level dBA values are reported as energetic mean (SD) for the experimental and control conditions. Analysis of the difference in noise-exposure dBA between treatment conditions was performed using Wilcoxon signed-rank testing. To avoid overpowering the test due to the high number of data points of decibel measurements (each survey day: 1 per second from 9:30 AM through 2:00 PM), we compared the energetic means of survey days. We also performed analysis of the difference in subjective perception of noise between treatment conditions using a Wilcoxon signed-rank test. The effect size of these tests is reported as *r*.

The analysis of the effect of the SLOS on stress was conducted using multiple linear mixed models, with subject ID included as a random effect. In the analyses of the effects of SLOS on the stress composite score and exhaustion, time of measurement and treatment were included as fixed effects. Because a high correlation was present between the STAI and the Perkhofers Stress Scale ($r = .67$),²⁷ a composite score was calculated with equal weighting of the 2 scales to test the influence of the system on the state stress. For the analysis of the SLOS on the PSS, only 1 data point per participant was available for each treatment condition. So, we included only treatment as a fixed effect in this model.

For the analysis of the effect of SLOS on the concentration of salivary cortisol, the specimen number was included as a fixed effect along with treatment. The effect sizes of the models are reported as conditional R^2 , and the effect sizes of the individual predictors are reported as partial η^2 . In addition to model parameters, the simple effects are reported for the models investigating the stress composite score and exhaustion. For all statistical tests, $P < .05$ was considered statistically significant. All tests were 2-sided.

Results

In total, 13 individuals participated during 40 workdays. The mean age of the participants was 36.6 years (SD = 8.12). The necessary adaptation of the procedure to the shift plan meant that some subjects participated more often than others, which resulted in repeated measurements in different frequencies for different individuals. In addition to the shift

TABLE 4. Mean (SD) Values of Each Dependent Variable

Dependent Variable	Control Group	Experimental Group
	Mean (SD)	
Noise, dBA	67.40 (1.57)	69.7 (2.92)
Subjective noise	5.08 (1.50)	1.92 (1.12)
Composite stress score before work shift	16.20 (5.66)	17.70 (5.49)
Composite stress score after work shift	17.40 (5.69)	17.00 (6.06)
Perceived stress score	28.40 (6.33)	25.20 (5.74)
Cortisol specimen 1, $\mu\text{g/L}$	4.27 (2.36)	4.09 (2.35)
Cortisol specimen 2, $\mu\text{g/L}$	6.70 (3.19)	6.48 (3.07)
Cortisol specimen 3, $\mu\text{g/L}$	1.32 (0.58)	1.44 (0.80)
Exhaustion before work shift	2.32 (0.94)	2.23 (0.88)
Exhaustion after work shift	2.57 (1.01)	2.32 (0.95)

plan, holiday periods, sick days, and COVID-19 quarantine periods were further reasons for an unequal frequency of participation per person. In total, 4 participants were male (31%) and 9 were female (69%); none were nonbinary. Concerning all dependent variables, **TABLE 4** gives an overview with mean (SD) values.

Concerning the Technical System Evaluation, 3 variables, namely, SLOS Technical Acceptance, SLOS Acceptance (based on the TAM2³⁷), and SLOS Communication, were investigated descriptively. The results are displayed in table form: **TABLE 1** offers the results for the technical acceptance of the SLOS (mean [SD], 4.38[0.64]). **TABLE 2** includes the results for the SLOS acceptance according to the TAM2³⁷ (4.06 [0.75]) with the 4 facets *intention to use* (4.42 [1.04]), *perceived usefulness* (3.96 [1.12]), *perceived ease of use* (4.12 [0.91]), and *subjective norm* (3.81 [0.93]). **TABLE 3** displays the results for the technical effects on communication (5.10 [0.75]).

In the analysis of objective noise in the lab and subjective noise, treatment was included as a fixed effect. The results of Wilcoxon signed-rank test in 28 individuals showed no difference in dBA between the experimental condition, with an energetic mean (SD) of 69.70 (2.92) dBA and the control condition, with 67.40 (1.57) dBA ($V = 37$, $P = .36$; $r = 0.11$). However, there was a significant association between the treatment condition and whether the lab workers would perceive the noise as being loud and annoying ($V = 76.5$; $P = .003$; $r = 0.75$). Test subjects in the control condition scored higher (mean [SD], 5.08 [1.50]) than workers during the experimental condition (1.92 [1.12]). **FIGURE 2** presents the results as a bar chart.

We observed a significant correlation between the STAI and the Perkhofers Stress Scale ($r = .49$; 95% CI, 0.43–0.56; $P < .001$). In the analysis of the composite stress score, treatment and time of measurement were included as fixed effects, whereas the subject ID was integrated as a random effect (conditional $R^2 = .334$). There was no significant main effect of treatment on the stress composite score ($F[1, 510.93] = 0.17$; $P = .68$; partial $\eta^2 < .001$), and also no significant main effect of time of measurement ($F[1, 506.99] = 0.29$; $P = .59$; partial $\eta^2 < .001$). However, there was a significant interaction between treatment and time of measurement on the composite score ($F[1, 506.99] = 6.00$; $P = .02$; partial $\eta^2 = .01$).

The experimental condition started with a higher mean (SD) value (17.70 [5.49]) than the control condition (16.20 [5.66]) before the shift. This difference was significant ($t[509.11] = 2.00$; $P = .046$). The experimental condition showed a decrease in stress, leading to a lower

FIGURE 2. Bar chart of subjective noise perception ratings for the experimental and control conditions.

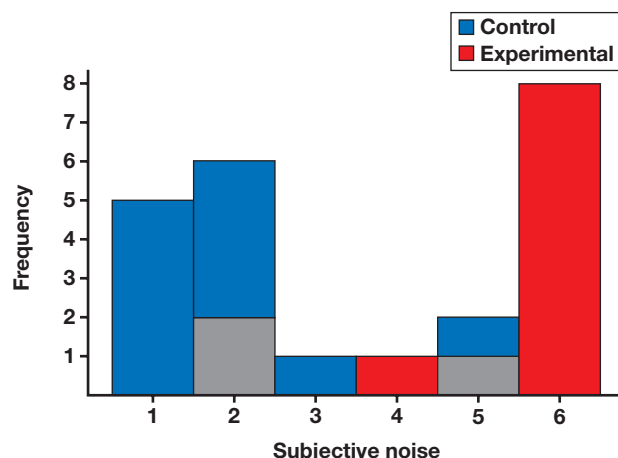
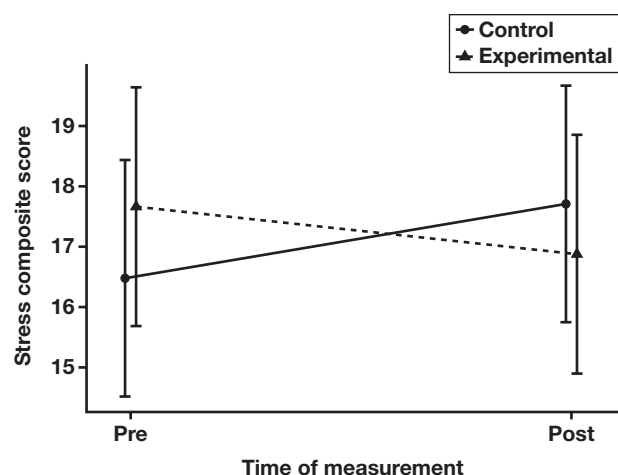


FIGURE 3. Time of measurement $\times$ treatment effect plot for the composite stress score. Means before and after shift for the experimental and control condition. Error bars represent 95% CIs.

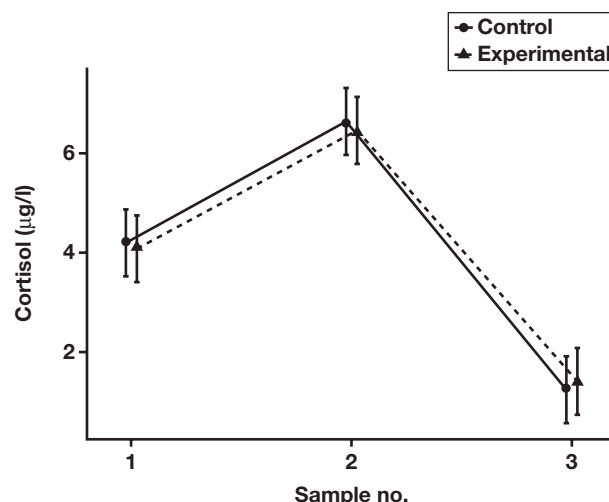


mean (SD) value (17.00 [6.06]) compared with the control condition (17.40 [5.69]), after the shift. This difference was not significant ($t[509.11] = 1.40$; $P = .16$). The course from before to after the shift was significantly different between the treatment conditions. The results are graphically represented in **FIGURE 3**.

In the analysis of the perceived stress scale, treatment was included as a fixed effect, whereas the subject ID was integrated as a random effect (conditional $R^2 = .609$). There was a significant main effect of treatment ($F[1, 13] = 4.67$; $P > .049$; partial $\eta^2 = .26$). The mean (SD) of the control condition (28.4 [6.22]) was significantly higher than that recorded during the experimental condition (25.2 [5.74]).

In analyzing the cortisol levels, treatment and specimen number were included as fixed effects, whereas the subject ID was integrated as a random effect (conditional $R^2 = .596$). There was no significant main effect of treatment on the cortisol level ($F[1, 806.35] = 0.09$; $P = .76$; partial $\eta^2 < .001$). However, there was a significant main effect of the specimen number ($F[2, 806.35] = 480.58$; $P < .001$; partial $\eta^2 = .54$). There was

FIGURE 4. Specimen number $\times$ treatment effect plot for the cortisol level in $\mu\text{g/L}$. Means for each specimen number (after waking up, half an hour after waking up, and before the after-shift questionnaire) for the experimental and control condition. Error bars represent 95% CIs.



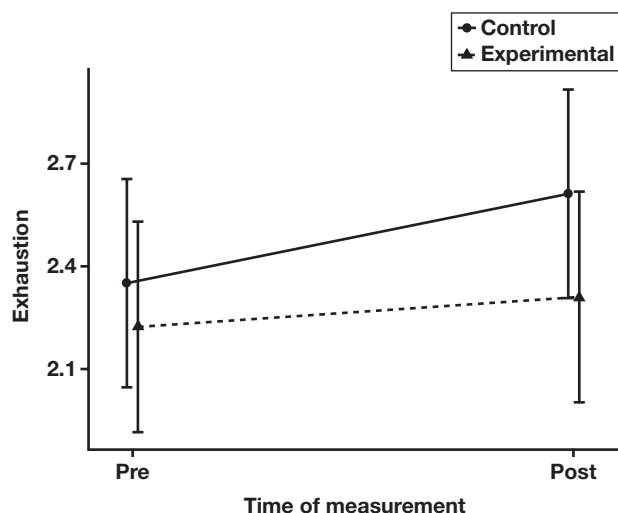
no significant interaction between treatment and specimen number on the cortisol level ($F[2, 806.35] = 0.61$; $P = .54$; partial $\eta^2 < .001$). In both conditions, a similar level was measured for the first specimen (experimental: mean (SD) = 4.09 [2.35]; control: 4.27 [2.36]), followed by a peak at the second specimen (experimental: 6.48 [3.07]; control: 6.70 [3.19]) and a decrease leading to a lower cortisol level in specimen 3 (experimental: 1.44 [0.80]; control: 1.32 [0.58]), compared with the first specimen. The results are graphically represented in **FIGURE 4**.

In the analysis of exhaustion, treatment and time of measurement were included as fixed effects, whereas the subject ID was integrated as a random effect (conditional $R^2 = .309$). There was a significant main effect of treatment on exhaustion ($F[1, 508.72] = 9.06$; $P = .003$; partial $\eta^2 = .01$), and also a significant main effect of time of measurement ($F[1, 504.23] = 6.40$; $P = .01$; partial $\eta^2 = .02$). However, there was no significant interaction between treatment and time of measurement on exhaustion ($F[1, 504.23] = 1.59$; $P = .21$; partial $\eta^2 = .003$). We found a similar exhaustion mean (SD) in both conditions, in which the control condition (2.32 [0.94]) was reporting a slightly higher mean (SD) in the premeasurement condition than during the experimental condition (2.23 [0.88]). This difference was not significant ($t[506.64] = 1.29$; $P = .20$). Both conditions showed a rise from the before-shift condition to the after-shift condition, leading to significantly higher mean (SD) values after the shift than before. The rise of the control condition was stronger, leading to a higher exhaustion mean (SD) value in the control condition (2.57 [1.01]) compared with the experimental condition (2.32 [0.95]), at the after-shift time. This difference was significant ($t[506.69] = 3.03$; $P = .003$). Less exhaustion was found in the experimental condition compared with the control condition, in general. The results are graphically represented in **FIGURE 5**.

Discussion

Besides the investigation of the SLOS effects on noise and stress, our goal was to evaluate the SLOS concerning its acceptance among the staff,

FIGURE 5. Time of measurement $\times$ treatment effect plot for exhaustion. Means before and after shift for the experimental and control condition. Error bars represent 95% CIs.



because low acceptance could change the interpretation of the findings. The 3 constructs (SLOS technical acceptance, SLOS acceptance, and SLOS communication) that were built to investigate this topic, which was termed Technical System Evaluation, show means that are all above the center of the scale—that is, 4.38 (center at 3.50) concerning technical acceptance, 4.03 (center at 3.00) for SLOS acceptance based on the TAM2,³⁷ and 5.10 (center at 3.50) for SLOS acceptance concerning communication. These results show that the system is well accepted by its users.

Further, the users wanted to continue working with the SLOS once they had learned about its features. The score of the corresponding facet of SLOS acceptance—intention to use—is 4.42 (center at 3.00) and therefore near the maximum of 5.00. Only the comfort of the neckband microphone is inconsistent with the consistently positive acceptance scores, because the test subjects judged its comfort with a rating just under the center of the scale of 3.50, which is between a little uncomfortable and a little comfortable. Possibly, when considering future use, the separate microphone set-up should be reconsidered, because the users were not strongly convinced in its value. In general, the results for acceptance and comfort suggest that the SLOS is integrated well into the working day and is not perceived as disturbing the workday or being awkward but as being a useful source of support.

The replication of the noise-reduction effect in the working area¹⁸ failed because there was no difference between the conditions concerning the dBA measurement in the lab. The SLOS does not change the noise in the room. Nevertheless, in both conditions, the dBA levels were exceeding the mentioned WHO recommendations,^{4–6} confirming the noise issue in the context of medical labs. This finding implies that helpful solutions are needed, because noise consequences are detrimental to the performance^{7,8,12} and health^{1–3} of the medical-lab staff.

However, the SLOS reduces noise for the user objectively, via its technical headphone properties, such as its active and passive noise-canceling effects.¹⁶ This means that although the noise level in the room is objectively not changed by the SLOS, it is changed subjectively for the SLOS user.¹⁶ This objective noise reduction is confirmed by the subjective noise-perception results of this field study. The effect size of $r = 0.75$

displays a large effect, which shows that SLOS causes an appreciable improvement in working conditions for the lab crew. The result indicates that the noise level in the medical lab is primarily generated through machine and environmental sounds. Because the noise consequences are mostly relevant to the people working in this context, the H1 can be accepted as demonstrating that the SLOS lowered noise perception for the users. Hence, equally to the SOTOS, the SLOS is confirmed as a tool to reduce noise for the users, which they also subjectively perceive.

Because the lab crew is exposed to notable noise, it seems to be in line with the illustrated stress theories^{15,19} that the measured stress level should be beneficially moderated by the SLOS. For the stress analysis, subjective and objective measures were considered. Regarding the subjective measures, the claimed correlation between the STAI and the Perkhofers Stress Scale²⁷ was confirmed in this study, which substantiated the decision to integrate both measures into 1 composite score. A stress-reducing effect of the SLOS is consistently suggested by the results of the analysis of all subjective measurements, whereas this concept is not supported by the analysis of the objective stress criterion cortisol.

The relationship between salivary cortisol and self-reported stress remains unclear. The results of a meta-analysis³⁹ showed inconsistent findings on the relationship between salivary cortisol and self-reported stress. Consistent with our results, 2 study reports^{40,41} found no significant correlation between salivary cortisol and perceived stress in a healthy population at work.

It seems that unexpected work-related stressors trigger a significant salivary cortisol response, whereas work-related stressors to which workers are accustomed are not associated with evoking a salivary cortisol response, possibly because of habituation processes.⁴⁰ It has to be mentioned that no significant cross-sectional and longitudinal relations between salivary cortisol and perceived stress measured via the PSS have been found.⁴² Also, associations of salivary cortisol and self-reported stress via STAI are not always found.⁴³

In addition, salivary cortisol levels are influenced by light exposure. Greater exposure to daylight results in a higher morning-cortisol peak.⁴⁴ The salivary cortisol specimens in the control condition were mainly collected in January. The data collection of the experimental condition ran until March, with greater daylight exposure in the morning. This information could introduce bias in discerning the potential SLOS effects on the salivary cortisol levels of medical-lab workers. Because psychosocial stress is only 1 potential trigger for cortisol release,⁴⁵ a different variable, such as heart-rate variability,¹⁴ may have served as a more valid stress marker.

An alternative explanation for the results could be that the cortisol analysis is a valid representation of stress, and that the subjective measurements are biased or confounded, for example, by social desirability or acquiescence. However, a particular strength of this field study might be the fact that multiple stress measurements were integrated. Because all subjective measurements lead to matching implications, it seems likely that the multiple subjective measures have a higher informative value than the debatable variable cortisol level.

Due to the assumed supremacy of the subjective indicators, we conclude that the SLOS provides a benefit for the medical-lab crew. A reduction in the stress composite score during the course of the shift was found when using the SLOS, compared with an increase for the control condition, which underlines the positive impact of the system regarding the stress composite score. Because the significant interaction is disordinal, the main effects are not to be interpreted.

In contrast to our expectations, we found a significant difference at the premeasurement stage, in which a higher stress composite mean (SD) value was found in the experimental condition, whereas at the postmeasurement point, no difference was found between treatment conditions. One possible explanation for the higher premeasurement level in the experimental condition could be that the unfamiliarity of the staff with the SLOS triggers a stress response. It has been shown⁴⁶ that unknown technologies can lead to an activation of the sympathetic stress response. However, the results of experimental and field research display that new technology is not necessarily a source of stress.⁴⁷

An alternative explanation for the difference we observed before the shift may be found through a methodical reflection of the field study setting. For practical reasons, the first 4 weeks of the field study were conducted outside the SLOS, followed by a 4-week period with the SLOS. This set-up comes with the disadvantage that global changes in the environment can systematically influence the stress outcome—for instance, political changes or intensification of global conflicts can affect the everyday life of large segments of the population. Individual stressors were supposed to be balanced out throughout the study; however, global events in the last 4 weeks of the study period could account for the observed higher stress levels. Ultimately, these explanations remain speculative. Because there is no definitive answer to this question, the focus of this study was on the development of the level of the composite stress score from before to after measurements. This focus was found to be positively modified by SLOS as the significant interaction, leading to a stress reduction in the experimental condition, compared with a rise in stress in the control condition.

In terms of the PSS, this effect seems to be confirmed, because the mean (SD) value in the experimental condition is significantly lower than the mean (SD) value in the control condition. The stress-relieving effect of the SLOS does not only seem to be relevant on a daily basis, as the analysis of the stress composite score showed, but also on a monthly scale, for which the PSS was integrated into the study. The effect size of the construct representing the daily stress—namely, the stress composite score—is small, whereas a large effect was found for the long-term stress measurement of the PSS. Possibly, the stress-reducing effect accumulates during the period of daily measurements, leading to a larger effect for the PSS.

Concerning the stress outcome variable exhaustion, it was shown³⁶ that the staff was significantly more exhausted after work than before, although the observed effects were small. Although both conditions started from a similar level at the premeasurement time point, the workers in the control condition were significantly more exhausted after the shift than the test subjects working with the SLOS. Also, the significant main effect shows that the SLOS causes a lower exhaustion level for the workers in general and not only after work.

Because all subjective stress measures conform to the hypothesis, we conclude that the system is a tool that enhances daily working comfort in providing stress reduction for the medical-lab staff. The measurement of the perceived noise also supports the conclusion of the SLOS being a helpful working tool. Because the multimeasurement approach of this study leads to consistent findings, it can be strongly presumed that the results are valid.

In this study the SLOS was evaluated, for the first time in the literature to our knowledge, in the context of a medical lab, which means that more field studies in other labs and other noise-polluted work settings would deepen the understanding of the working mechanisms. In our

study results, it was shown that the noise levels in the medical lab are higher than the standards given by the WHO. The SLOS did not reduce the noise levels in the room. However, because its technical properties reduce noise for the user via active and passive noise canceling,¹⁶ it could be shown that subjective noise reduction was successful. Concerning stress, all measures except cortisol reacted beneficially to the system, which leads to the conclusion that the SLOS is an effective noise- and stress-reducing tool.

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Personal and Professional Conflicts of Interest

Coauthor Friedrich invented and technically developed the SLOS. The system is patented by the University Medical Center Goettingen (EP20213914.3).

REFERENCES

- Katz JD. Noise in the operating room. *Anesthesiology*. 2014;121(4):894–898. doi:10.1097/ALN.0000000000000319
- Babisch W. Cardiovascular effects of noise. *Noise Health*. 2011;13(52):201–204. doi:10.4103/1463-1741.80148
- Münzel T, Schmidt FP, Steven S, et al. Environmental noise and the cardiovascular system. *J Am Coll Cardiol*. 2018;71(6):688–697. doi:10.1016/j.jacc.2017.12.015
- Clark C, Paunovic K. WHO Environmental Noise Guidelines for the European Region: a systematic review on environmental noise and quality of life, wellbeing and mental health. *IJERPH*. 2018;15(11):2400. doi:10.3390/ijerph15112400
- de Lima Andrade E, da Cunha e Silva DC, de Lima EA, et al. Environmental noise in hospitals: a systematic review. *Environ Sci Pollut Res*. 2021;28(16):19629–19642. doi:10.1007/s11356-021-13211-2
- Berglund B, Lindvall T, Schwela DH, World Health Organization. *Guidelines for Community Noise*. 1999.
- Szalma JL, Hancock PA. Noise effects on human performance: a meta-analytic synthesis. *Psychol Bull*. 2011;137(4):682–707. doi:10.1037/a0023987
- Keller S, Tschan F, Beldi G, et al. Noise peaks influence communication in the operating room. An observational study. *Ergonomics*. 2016;59(12):1541–1552. doi:10.1080/00140139.2016.1159736
- Hasfeldt D, Laerkner E, Birkelund R. Noise in the operating room—what do we know? A review of the literature. *J Perianesth Nurs*. 2010;25(6):380–386. doi:10.1016/j.jopan.2010.10.001
- Griffiths PD, Kell RL, Taylor W. Noise levels in a clinical chemistry laboratory. *J Clin Pathol*. 1970;23(5):445–449. doi:10.1136/jcp.23.5.445
- Gultekin E, Yener M, Develioglu ON, et al. Noise pollution in biochemistry laboratories of different hospitals in Istanbul/Turkey. *Turk Arch Otolaryngol*. 2013;51:67–69. doi:10.5152/tao.2013.17

12. Bergefurt L, Weijs-Perrée M, Appel-Meulenbroek R, et al. The physical office workplace as a resource for mental health – a systematic scoping review. *Build Environ*. 2022;207A:108505. doi:[10.1016/j.buildenv.2021.108505](https://doi.org/10.1016/j.buildenv.2021.108505)
13. Lehrke J, Boos M, Cordes A, et al. Effects of a technical solution on stress of surgical staff in operating theatres. *Thorac Cardiovasc Surg*. 2022;70(5):392–400. doi:[10.1055/s-0041-1741059](https://doi.org/10.1055/s-0041-1741059)
14. Waterland P, Khan FS, Ismaili E, Cheruvu C. Environmental noise as an operative stressor during simulated laparoscopic surgery. *Surg Laparosc Endosc Percutan Tech*. 2016;26(2):133–136. doi:[10.1097/SLE.0000000000000250](https://doi.org/10.1097/SLE.0000000000000250)
15. Lazarus RS, Launier R. Stressbezogene Transaktionen zwischen Person und Umwelt. In: *Stress Theorien, Untersuchungen, Maßnahmen*. Huber;1981:213–259.
16. Friedrich M, Boos M, Pagel M, et al. New technical solution to minimise noise exposure for surgical staff: the 'silent operating theatre optimisation system'. *BMJ Innov*. 2017;3(4):196–205. doi:[10.1136/bmjinnov-2016-000188](https://doi.org/10.1136/bmjinnov-2016-000188)
17. Meyer-Lamp I, Boos M, Schugmann LS, et al. Silent operating theatre optimisation system for positive impact on surgical staff-members' stress, exhaustion, activity and concentration in urological da Vinci surgeries. *BMJ Innov*. 2021;7(1):175–184. doi:[10.1136/bmjinnov-2019-000413](https://doi.org/10.1136/bmjinnov-2019-000413)
18. Leitsmann C, Uhlig A, Popeneciu IV, et al. The Silent Operation Theatre Optimisation System (SOTOS[®]) to reduce noise pollution during da Vinci robot-assisted laparoscopic radical prostatectomy. *J Robot Surg*. 2021;15(4):519–527. doi:[10.1007/s11701-020-01135-x](https://doi.org/10.1007/s11701-020-01135-x)
19. Karasek RA. Job demands, job decision latitude, and mental strain: implications for job redesign. *Adm Sci Q*. 1979;24(2):285. doi:[10.2307/2392498](https://doi.org/10.2307/2392498)
20. Abbasi M, Yazdanirad S, Habibi P, et al. Relationship among noise exposure, sensitivity, and noise annoyance with job satisfaction and job stress in a textile industry. *Noise Vib Worldw*. 2019;50(6):195–201. doi:[10.1177/0957456519853812](https://doi.org/10.1177/0957456519853812)
21. Abbasi M, Yazdanirad S, Mehri A, et al. Noise exposure and job stress – a structural equation model in textile industries. *Arch Acoust*. 2020;45(4):601–611. doi:[10.24425/AOA.2020.135248](https://doi.org/10.24425/AOA.2020.135248)
22. Chau N, Choquet M, Falissard B, Lorhandicap Group. Relationship of physical job demands to initiating smoking among working people: a population-based cross-sectional study. *Ind Health*. 2009;47(3):319–325. doi:[10.2486/indhealth.47.319](https://doi.org/10.2486/indhealth.47.319)
23. Cohen S, Wills TA. Stress, social support, and the buffering hypothesis. *Psychol Bull*. 1985;98(2):310–357. doi:[10.1037/0033-2909.98.2.310](https://doi.org/10.1037/0033-2909.98.2.310)
24. Karasek RA, Triantis KP, Chaudhry SS. Coworker and supervisor support as moderators of associations between task characteristics and mental strain. *J Organiz Behav*. 1982;3(2):181–200. doi:[10.1002/job.4030030205](https://doi.org/10.1002/job.4030030205)
25. LaRocco JM, House JS, French JRP. Social support, occupational stress, and health. *J Health Soc Behav*. 1980;21(3):202–202. doi:[10.2307/2136616](https://doi.org/10.2307/2136616)
26. Grimm J, ed. *State-Trait-Anxiety Inventory nach Spielberger*. Deutsche Lang- und Kurzversion; 2009.
27. Buchberger W, Rungg C, Neururer S, et al. Self-assessment of psychic Stress using a single-item scale [in German]. *Pflegewissenschaft*. 2019;21:24–29. doi:[10.3936/1691](https://doi.org/10.3936/1691)
28. Schneider EE, Schönfelder S, Domke-Wolf M, et al. Measuring stress in clinical and nonclinical subjects using a German adaptation of the Perceived Stress Scale. *Int J Clin Health Psychol*. 2020;20(2):173–181. doi:[10.1016/j.ijchp.2020.03.004](https://doi.org/10.1016/j.ijchp.2020.03.004)
29. Hinz A, Hessel A, Brähler E. The Leipzig atmosphere [in German]. *Zeitschrift für Differentielle und Diagnostische Psychologie*. 2002;23:55–65. doi:[10.1024/0170-1789.23.1.55](https://doi.org/10.1024/0170-1789.23.1.55)
30. Hellhammer DH, Wüst S, Kudielka BM. Salivary cortisol as a biomarker in stress research. *Psychoneuroendocrinology*. 2009;34(2):163–171. doi:[10.1016/j.psyneuen.2008.10.026](https://doi.org/10.1016/j.psyneuen.2008.10.026)
31. Kirschbaum C, Hellhammer DH. Salivary cortisol in psychoneuroendocrine research: recent developments and applications. *Psychoneuroendocrinology*. 1994;19(4):313–333. doi:[10.1016/0306-4530\(94\)90013-2](https://doi.org/10.1016/0306-4530(94)90013-2)
32. Smyth N, Hucklebridge F, Thorn L, Evans P, Clow A. Salivary cortisol as a biomarker in social science research. *Soc Personal Psychol Compass*. 2013;7(9):605–625. doi:[10.1111/spc3.12057](https://doi.org/10.1111/spc3.12057)
33. Pollard TM. Use of cortisol as a stress marker: practical and theoretical problems. *Am J Hum Biol*. 1995;7(2):265–274. doi:[10.1002/ajhb.1310070217](https://doi.org/10.1002/ajhb.1310070217)
34. Bozovic D, Racic M, Ivkovic N. Salivary cortisol levels as a biological marker of stress reaction. *Med Arh*. 2013;67(5):374371–374374. doi:[10.5455/medarh.2013.67.374-377](https://doi.org/10.5455/medarh.2013.67.374-377)
35. Demerouti E, Bakker AB, Nachreiner F, Schaufeli WB. The job demands-resources model of burnout. *J Appl Psychol*. 2001;86(3):499–512. doi:[10.1037/0021-9010.86.3.499](https://doi.org/10.1037/0021-9010.86.3.499)
36. Stordeur S, D'Hoore W, Vandenberghe C. Leadership, organizational stress, and emotional exhaustion among hospital nursing staff. *J Adv Nurs*. 2001;35(4):533–542. doi:[10.1046/j.1365-2648.2001.01885.x](https://doi.org/10.1046/j.1365-2648.2001.01885.x)
37. Venkatesh V, Davis FD. A theoretical extension of the technology acceptance model: four longitudinal field studies. *Manage Sci*. 2000;46(2):186–204. doi:[10.1287/mnsc.46.2.186.11926](https://doi.org/10.1287/mnsc.46.2.186.11926)
38. R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing; 2020. <https://www.R-project.org/>
39. Hjortskov N, Garde AH, Ørbæk P, Hansen AM. Evaluation of salivary cortisol as a biomarker of self-reported mental stress in field studies. *Stress Health*. 2004;20(2):91–98. doi:[10.1002/smi.1000](https://doi.org/10.1002/smi.1000)
40. Karlson B, Lindfors P, Riva R, Mellner C. Psychosocial work stressors and salivary cortisol. In: Kristenson M, Garvin P, Lundberg U, eds. *The Role of Saliva Cortisol Measurement in Health and Disease*. Bentham Science Publishers; 2012:43–66. doi:[10.2174/9781608053421120101](https://doi.org/10.2174/9781608053421120101)
41. Sjörs A, Ljung T, Jonsdottir IH. Diurnal salivary cortisol in relation to perceived stress at home and at work in healthy men and women. *Biol Psychol*. 2014;99:193–197. doi:[10.1016/j.biopsycho.2014.04.002](https://doi.org/10.1016/j.biopsycho.2014.04.002)
42. Mikkelsen S, Forman JL, Fink S, et al. Prolonged perceived stress and saliva cortisol in a large cohort of Danish public service employees: cross-sectional and longitudinal associations. *Int Arch Occup Environ Health*. 2017;90(8):835–848. doi:[10.1007/s00420-017-1241-z](https://doi.org/10.1007/s00420-017-1241-z)
43. González-Cabrera J, Fernandez-Prada M, Molina-Ruano R, Blazquez A. Psychosocial risk at work, self-perceived stress, and salivary cortisol level in a sample of emergency physicians in Granada. *Emergencias*. 2012;24(2):101–106.
44. Scheer FAJL, Buijs RM. Light affects morning salivary cortisol in humans. *J Clin Endocrinol Metab*. 1999;84(9):3395–3398. doi:[10.1210/jcem.84.9.6102](https://doi.org/10.1210/jcem.84.9.6102)
45. Ellison PT. Endocrinology, energetics, and human life history: a synthetic model. *Horm Behav*. 2017;91:97–106. doi:[10.1016/j.yhbeh.2016.09.006](https://doi.org/10.1016/j.yhbeh.2016.09.006)
46. Iffat R, Sami LK. Impact of electronic services on users : a study. *JLIS*. 2010;1(2):263–276. doi:[10.4403/jlis-it-4523](https://doi.org/10.4403/jlis-it-4523)
47. Hamborg KC, Greif S. New technologies and stress. In: Schabracq MJ, Winnubst JAM, Cooper CL, eds. *The Handbook of Work and Health Psychology*. John Wiley & Sons, Ltd; 2004:207–235. doi:[10.1002/0470013400.ch11](https://doi.org/10.1002/0470013400.ch11)

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