Laboratory Medicine

September 2024 Vol 55 No 5 Pgs 535–676

labmedicine.com

Laboratory Medicine (ISSN 0007-5027), is published 6 times per year (bimonthly). Periodicals Postage paid at Chicago, IL and additional mailing offices. POSTMASTER: Send address changes to *Laboratory Medicine*, Journals Customer Service Department, Oxford University Press, 2001 Evans Road, Cary, NC 27513-2009.

SUBSCRIPTION INFORMATION: Annually for North America, $182 (electronic) or $241 (electronic and print); single issues for individuals are $32 and for institutions $71. Annually for Rest of World, £118/€167 (electronic) or £154/€220 (electronic and print); single issues for individuals are £21/€30 and for institutions £44/€63. All inquiries about subscriptions should be sent to Journals Customer Service Department, Oxford Journals, Great Clarendon Street, Oxford OX2 6DP, UK, Tel: +44 (0) 1865-35-3907, e-mail: jnls.cust.serv@oup.com. In the Americas, please contact Journals Customer Service Department, Oxford Journals, 4000 CentreGreen Way, Suite 310, Cary, NC 27513, USA. Tel: 800-852-7323 (toll-free in USA/Canada) or 919-677-0977, e-mail: jnlorders@oup.com.

MEMBERSHIP INFORMATION: The ASCP membership fees for pathologists are as follows: fellow membership is $399; fellow membership plus 1-year unlimited online CE is $529; 2-year fellow membership is $749; and 2-year fellow membership plus 2-year unlimited online CE is $1009. The ASCP membership fees for laboratory professionals are as follows: young professional membership is $59; annual membership is $119; annual membership plus 1-year unlimited online CE is $158; 3-year membership is $389. All inquiries about membership should be sent to American Society for Clinical Pathology, 33 West Monroe Street, Suite 1600, Chicago, IL 60603, Tel: 312-541-4999, e-mail: ascp@ascp.org.

CLAIMS: Publisher must be notified of claims within four months of dispatch/ order date (whichever is later). Subscriptions in the EEC may be subject to European VAT. Claims should be made to Laboratory Medicine, Journals Customer Service Department, Oxford University Press, 4000 CentreGreen Way, Suite 310, Cary, NC 27513, USA, Tel: 800-852-7323 (toll-free in USA/Canada) or 919-677-0977, e-mail: jnlorders@oup.com.

Laboratory Medicine is published bimonthly by Oxford University Press (OUP), on behalf of the ASCP, a not-for-profit corporation organized exclusively for educational, scientific, and charitable purposes. Devoted to the continuing education of laboratory professionals, *Laboratory Medicine* features articles on the scientific, technical, managerial, and educational aspects ofthe clinical laboratory. Publication of an article, column, or other item does not constitute an endorsement by the ASCP of the thoughts expressed or the techniques, organizations, or products described therein. *Laboratory Medicine* is indexed in the following: MEDLINE/PubMed, Science Citation Index, Current Contents—Clinical Medicine, and the Cumulative Index to Nursing and Allied Health Literature.

Printed in the USA

ASCP
Laboratory Medicine
33 West Monroe Street, Suite 1600
Chicago, IL 60603

T: 312-541-4999
F: 312-541-4750

Laboratory Medicine

September 2024
Vol 55, No 5
Pgs 535-676

OVERVIEW

SCIENCE

CASE STUDY

CORRECTION

ON THE COVER: The Bamboo Leaf Tree Snake is known by several names, including Bamboo Viper, Chinese Tree Viper, Bamboo Snake, Chinese Green Tree Viper, Chinese Bamboo Viper, Stejneger's Pit Viper, Stejneger's Palm Viper, Red Tail Snake, and Stejneger's Bamboo Pit Viper. Leonhard Stejneger was a Norwegian-born herpetologist who worked at the Smithsonian Institution for over 60 years. His eponymous snake belongs to the species *Trimeresurus stejnegeri* and is indigenous to northeast India and southern China. *T. stejnegeri* belongs to the subfamily of *Crotalinae*, commonly known as pit vipers, and its venom is a potent hemotoxin. In this issue of *Laboratory Medicine*, Dayong and colleagues describe a case of a 2-year-old Chinese boy who was bitten by a *Trimeresurus stejnegeri*, and the laboratory assessment of his subsequent coagulopathy was complicated by a hereditary Factor VII deficiency.

OVERVIEW

Noninvasive biomarkers for lupus nephritis

Ting Liu, MD, PhD,[1,2,3] *Yun-long Yang, MD,*[1,2] *Yan Zhou, MD,*[1,2] *Yong-mei Jiang, MD, PhD*[1,2]

[1]Department of Laboratory Medicine, West China Second University Hospital, Sichuan University, Chengdu, China; [2]Key Laboratory of Birth Defects and Related Diseases of Women and Children (Ministry of Education), West China Second University Hospital, Sichuan University, Chengdu, China; [3]State Key Laboratory of Biotherapy and Cancer Center/National Collaborative Innovation Center for Biotherapy, Sichuan University, Chengdu, China. Corresponding author: Yong-mei Jiang; jiangym_scu@163.com

Key words: lupus nephritis; laboratory medicine; noninvasive biomarker; urinary biomarker

Abbreviations: LN, lupus nephritis; SLE, systemic lupus erythematosus; ESRD, end-stage renal disease; dsDNA, double-stranded DNA; IFN-I, type I interferon; miRNA, microRNA; PBMCs, peripheral blood mononuclear cells; IL, interleukin; BLyS, B-lymphocyte stimulator protein; TNF, tumor necrosis factor; IFNAR1, IFN alpha/beta receptor 1; VCAM-1, vascular cell adhesion molecule-1; AECA, anti-endothelial cell antibodies; AXL, a receptor tyrosine kinase; IGFBP2, insulin like growth factor binding protein 2; TNFR, TNF receptor; TFH, T follicular helper cells; UFCR, urinary ferritin/creatinine ratio; NRP-1, neuropilin-1

Laboratory Medicine 2024;55:535-542; https://doi.org/10.1093/labmed/lmae015

ABSTRACT

Lupus nephritis (LN) is one of the most severe clinical manifestations of systemic lupus erythematosus (SLE). Notably, the clinical manifestations of LN are not always consistent with the histopathological findings. Therefore, the diagnosis and activity monitoring of this disease are challenging and largely depend on invasive renal biopsy. Renal biopsy has side effects and is associated with the risk of bleeding and infection. There is a growing interest in the development of novel noninvasive biomarkers for LN. In this review, we summarize most of the LN biomarkers discovered so far by correlating current knowledge with future perspectives. These biomarkers fundamentally reflect the biological processes of kidney damage and repair during disease. Furthermore, this review highlights the role of urinary cell phenotype detection in the diagnosis, monitoring, and treatment of LN and summarizes the limitations and countermeasures of this test.

Introduction

Lupus nephritis (LN) is one of the most severe clinical manifestations in patients with systemic lupus erythematosus (SLE), which has a significantly high morbidity and mortality.[1-3] Generally, about 30%-60% of adults and up to 70% of children with SLE will develop related nephritis as the disease progresses.[4-7] Additionally, 10%-30% of LN patients eventually develop end-stage renal disease (ESRD), requiring dialysis and transplantation.[8-10] As a chronic autoimmune disease, the pathogenesis of LN involves multiple factors, including the deposition of autoantibodies and complements in the glomerulus and the activation of the innate and adaptive immunity, which ultimately lead to glomerular, interstitial, tubular, and vascular damage. It is worth mentioning that the clinical manifestations of LN are not always consistent with the histopathological findings.[11,12] At present, the clinical diagnosis of LN mainly depends on renal histopathological changes. Renal biopsy is the gold standard for diagnosis and classification of the histopathological changes of the kidneys, but it is an invasive procedure that is unsuitable for continuous monitoring of patients.[13,14] Additionally, there are puncture-related side effects, such as bleeding and infection, associated with renal biopsy. Therefore, there is an urgent need to identify novel noninvasive biomarkers that can effectively reflect the activity and severity of LN.

Noninvasive biomarkers are laboratory indicators of biological, biochemical, or molecular substances obtained by noninvasive methods. They can be qualitatively and quantitatively detected by laboratory techniques, and their changes are closely related to the occurrence and development of diseases. Compared with renal biopsy, noninvasive biomarkers in blood and urine can significantly increase the frequency of kidney damage assessment, aid in monitoring disease activity, and guide clinical treatment in patients with LN.[15-17] A large number of studies have been conducted to develop novel noninvasive biomarkers for the biological processes in LN, especially those in urine, which fundamentally reflect the biological processes of kidney damage and repair during disease, such as inflammatory cell recruitment, immune cell activation and differentiation, and renal vascular damage and repair, etc.[18-20] In addition, it is well known that the choice of treatment strategy for LN patients mainly depends on nephritis severity, and late diagnosis of LN is associated with a high frequency of ESRD.[21-23] Therefore, early diagnosis, continuous monitoring, and early intervention of LN are important to significantly improve prognosis. To address these issues, there has been a growing interest in the development of novel noninvasive LN biomarkers over the past decade. The in-depth multidimensional study of this disease has led to the gradual incorporation of many noninvasive biomarkers, particularly urinary biomarkers, into the diagnostic strategy for LN. In this review, we mainly discuss the possible roles of potential noninvasive biomarkers in the early diagnosis, activity monitoring, and treatment guidance of LN by correlating the current knowledge with future perspectives (**TABLE 1**).

TABLE 1. Summary of biomarkers reviewed[a]

Biomarker	Sample type	Key points	Reference
Gene			
HLA-DR15 haplotype	Blood	Associated with diffuse proliferative glomerulonephritis	24
IFN-I polymorphisms	Blood	Associated with immune-mediated glomerular damage	25
miRNA	Blood	mir-125a-5p, mir-146a-5p, and mir-221-3p suggested therapeutic effect	26
Autoantibody			
Anti-NCS	Serum	Associated with renal disease and deserves further investigation	27
Anti-C1q	Serum	Associated with renal disease and deserves further investigation	28
Anti-α-actinin	Serum	Associated with renal disease and deserves further investigation	29
AECA	Serum	Associated with endothelial damage	30
Cytokines			
BLyS	Serum	Associated with the frequency of LN	31,32
IFNAR1	Serum	Associated with the frequency of LN	31
IL-16	Urine	Associated with the disease activity of LN	33,34
IFN-I	Urine	Associated with the extent of kidney damage	20
IL-35	Serum/Urine	Associated with renal involvement	35,36
Chemokines			
CCL2 (MCP-1)	Serum/Urine	Associated with active LN patients	37,38
CCL3 (MIP-1α)	Serum/Urine	Associated with renal disease activity	39,40
CCL5 (RANTES)	Serum/Urine	Associated with active LN patients	41,42
CCL7 (MCP-3)	Urine	Associated with renal disease activity	43
CCL8 (MCP-2)	Urine	Associated with renal disease activity	43
CCL19 (MIP-3β)	Serum	Associated with active LN patients	41
CXCL8 (IL-8)	Serum	Associated with active LN patients	44
CXCL9 (MIG)	Serum/Urine	Associated with active LN patients	41
CXCL10 (IP-10)	Serum/Urine	Associated with renal disease activity	45,46
CXCL11 (I-TAC)	Serum	Associated with renal disease activity	39
CXCL13 (BCA-1)	Serum	Associated with renal disease activity	47
CXCL16 (SR-PSOX)	Serum/Urine	Associated with renal disease activity	48
CX3CL1	Urine	Associated with renal disease activity	43
Other proteins			
TWEAK	Urine	Associated with renal involvement	49,50
OPG	Urine	Associated with renal involvement	49,51
UFCR	Urine	Significantly higher than that of SLE patients without LN	52
IGFBP-2	Serum	Associated with renal histology	53,54
TNFR1/TNFR2	Serum	Associated with renal histology	55,56
VCAM-1	Serum	Associated with damage and repair of renal vascular endothelium	57,58
NRP-1	Urine	Early prognostic biomarker in LN	59
AXL	Serum	Associated with renal histology	53,60
TGF-β	Urine	Associated with the disease activity of LN	33
Immune cells			
CD8+T cells	Urine	Associated with renal disease activity	61
CD4/CD8 ratio	Urine	Distinguishes LN from other inflammatory kidney diseases	62
CD14+ monocytes	Urine	Associated with class IV nephritis	63
TFH cells	Urine	Elevated in biopsies and deserves further investigation	64

AECA, anti-endothelial cell antibodies; anti-NCS, antinucleosome; anti-C1q, anti-complement 1q; AXL, a receptor tyrosine kinase; BCA-1, B cell attracting chemokine 1; BLyS, B-lymphocyte stimulator protein; CX3CL1, C-X3-C motif chemokine ligand 1; HLA, human leukocyte antigen; IFN-I, type I interferon; IFNAR1, interferon alpha/beta receptor 1; IGFBP2, insulin like growth factor binding protein 2; IP-10, IFN gamma inducible protein10; I-TAC, IFN inducible T cell α chemoattractant; MCP, monocyte chemoattractant protein; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; NRP-1, neuropilin-1; OPG, osteoprotegerin; RANTES, regulated upon activation, normal T cell expressed and secreted; SR-PSOX, scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein and bacteria; TFH cells, T follicular helper cells; TGF-β, transforming growth factor-β; TNFR, tumor necrosis factor receptor; TWEAK, TNF-like weak inducer of apoptosis; UFCR, urinary ferritin/creatinine ratio; VCAM-1, vascular cell adhesion molecule-1.
[a]*It must be taken into account that these data are only available in 1-2 studies, and these findings should be updated as more validation experiments are conducted.*

Conventional Laboratory Biomarkers for LN

The conventional laboratory markers used for LN diagnosis and monitoring mainly include proteinuria, protein/creatinine ratio, glomerular filtration rate, plasma complement levels (C3 and C4), anti-double-stranded (ds)DNA antibody, anti-cardiolipin antibody, rheumatoid factor, serum immunoglobulin, blood cell counts, as well as erythrocyte sedimentation rate and so on. Among these markers, the diagnostic specificity and sensitivity of the protein/creatinine ratio, plasma complement, anti-dsDNA antibody and serum immunoglobulins (IgG and IgA) for LN are relatively higher than those of the others. However these markers are still insufficient to diagnose renal damage or to monitor disease activity, and patients still need further renal biopsy.[25,65,66] For example, it is difficult to distinguish between irreversible glomerular capillary damage and ongoing LN activity in most patients with persistent proteinuria.[25] Moreover, Pinheiro et al[67] reported that LN may recur in the absence of an elevated level of anti-dsDNA. Likewise, Soliman et al[18] found that the diagnostic efficacy of anti-dsDNA varied across different assay platforms, making it difficult to achieve uniformity in clinical judgment. Based on these findings, we believe that LN can develop before the levels of any of these commonly used laboratory markers are altered and can continue to develop without obvious clinical signs. Correlations between these conventional laboratory biomarkers and LN are incomplete, and their utility in reflecting disease activity and predicting prognosis remains controversial.[68] Therefore, there is an urgent need to develop novel noninvasive biomarkers that can replace kidney biopsy and conventional biomarkers to better reflect the extent of kidney damage in patients with LN.

Blood Biomarkers for LN

The biggest problem with renal biopsy is that it can cause a variety of complications, including hematuria, kidney damage and loss, and even death.[69] Also, due to differences in infrastructure and personnel qualifications in different regions, not all the medical institutions can perform this test.[70,71] Compared with renal biopsy, side effects caused by venous blood collection are almost negligible. Of note, the detection of blood biomarkers requires relatively low infrastructure and personnel qualifications, making it easier to perform, especially in remote areas. Great effort has been made to identify novel noninvasive biomarkers in blood. To date, several studies have screened candidate biomarkers, including genes, cytokines, chemokines, autoantibodies, adhesion molecules, and factors involved in kidney damage and repair.[72,73]

Genes as Biomarkers for LN

Genetic factors are important in the pathogenesis of LN. Specifically, differences in levels of protein biomarkers, such as cytokines, chemokines, and autoantibodies in the serum and urine of LN patients can reflect genetic and epigenetic differences. In recent decades, numerous studies have evaluated the potential roles of genes in predicting LN susceptibility. On one hand, detection of changes in gene sequence, such as single nucleotide polymorphisms, is expected to be a novel biomarker indicating susceptibility to LN. For example, Marchini et al[24] found that the interactions of HLA-DQA and HLA-DR alleles in an Italian population confer a susceptibility to LN in SLE patients; the HLA-DR15 haplotype is especially associated with diffuse proliferative glomerulonephritis. Besides, Lodi et al[74] reported that type I interferon (IFN-I) apparently promoted immune-mediated glomerular damage in LN. Therefore, gene polymorphisms that enhanced IFN-I signaling were identified as risk factors for SLE and LN.[25] On the other hand, epigenetic modifications by DNA methylation, histone modification, and micro (mi)RNA can also influence gene expression and alter cell function without changing the genome sequence, thereby affecting LN susceptibility.[75] Su et al[26] used next-generation sequencing technology and real-time quantitative polymerase chain reaction technology to screen the different expression levels of miRNAs in the peripheral blood mononuclear cells (PBMCs) of SLE patients with and without LN. Ultimately, the study revealed that mir-125a-5p, miR-146a-5p, and mir-221-3p were significantly decreased in PBMCs of SLE patients with LN and then significantly increased after treatment, suggesting their role as biomarkers in early diagnosis and treatment guidance of LN.[26]

Serum Protein as Biomarkers for LN

In addition to cells, blood also contains a large number of bioactive substances, such as proteins. In recent decades, several studies have been conducted to screen serum biomarkers in LN patients based on proteomics.[18,39,76] Various soluble serum proteins, including cytokines, chemokines, adhesion molecules, and autoantibodies, were found to be correlated with LN disease severity and activity. For cytokines, Chun et al[77] found that SLE patients had higher serum levels of cytokines such as interleukin (IL)-6, IL-10, IL-12, and IFN-γ compared with normal healthy controls, but no significant differences in levels were observed between SLE patients with and without LN. These findings suggest that due to the common molecular pathways among various inflammatory diseases, the efficacy of serum cytokines in the diagnosis and differential diagnosis of LN is limited; more studies focusing on the correlations between cytokines in urine (or specific cells) and disease are being conducted. Notably, López et al[31] analyzed in depth the expression levels of pathogenic IFNα, B-lymphocyte stimulator protein (BLyS), and IL-17 axis in various blood cell types such as T and B lymphocytes, monocytes, dendritic cells, and neutrophils. They found that tumor necrosis factor (TNF) superfamily cytokine BLyS and related ligand IFN alpha/beta receptor 1 (IFNAR1) levels on monocytes or in the serum were associated with the frequency of nephritis.[31,32] For chemokines, numerous studies have shown that serum chemokines, including CCL3 (MIP-1α), CXCL10 (IP-10), CXCL11 (I-TAC), and CXCL13 (BCA-1), are strongly associated with disease activity of LN.[39,45,47] Also, Fu et al[41] reported that the transcriptional levels of interferon-inducible chemokines, including CCL2 (MCP-1), CCL5 (RANTES), CCL19 (MIP-3β), CXCL8 (IL-8), and CXCL9 (MIG), are also strongly associated with active LN patients.[37,41,44] Compared with cytokines, the levels of chemokines are more significantly changed in the blood of LN patients, and their potential as specific biomarkers is greater. For adhesion molecules, Yao et al[57] found that serum vascular cell adhesion molecule-1 (VCAM-1) was significantly increased in proliferative LN, suggesting that it can be used as a potential biomarker to assess the onset and remission of proliferative LN. It is reported that VCAM-1 is involved in the damage and repair of renal vascular endothelium during the disease course of LN patients.[58] For autoantibodies, a large number of studies have revealed the strong correlation between antinucleosome, anti-complement 1q, and anti-α-actinin and renal disease in SLE patients.[27-29] However, their role in assessing the severity and activity of LN remains to be further validated. Kondo et al[30] developed a solubilized cell surface protein capture enzyme-linked immunosorbent

assay to detect the serum antibodies in LN patients and further found a significant correlation between anti-endothelial cell antibodies (AECA) and glomerular hypercellularity, indicating that AECA, particularly IgA-AECA, are associated with endothelial damage. For other proteins, Wu et al[53] found that serum AXL (a receptor tyrosine kinase), insulin-like growth factor binding protein 2 (IGFBP2), and TNF receptor (TNFR) were significantly elevated in patients with active LN and showed significant positive correlations with the renal pathology activity index.[53] AXL and IGFBP2 are involved in regulating the innate immune response of monocytes and T cells, respectively, and seem to be biomarkers of particular interest in LN studies,[53,54,60] whereas TNFR1 and TNFR2 are found predominately in glomerular cells and tubular cells, respectively.[55] Once TNF-α binds to its receptors, a complex signaling cascade involving apoptosis and inflammation occurs.[56] Ultimately, the expression levels of specific serum proteins are closely related to the kidney damage, and its detection can be used to monitor the disease activity and guide treatment. However, the usefulness of serum protein detection in assessing LN susceptibility in SLE patients is limited.

Urinary Biomarkers for LN

Like blood, urine also contains a variety of components, such as epithelial cells, proteins, glucose, inorganic salts, and trace elements, which can be used as biomarkers for laboratory medicine. Arazi et al[20] used single cell transcriptomics to analyze kidney and urine samples from LN patients and healthy individuals. Their finding revealed similarities in the molecular signatures of urine and kidney tissues, highlighting the superiority of urine samples in understanding kidney damage. Additionally, numerous studies have demonstrated that the phenotype and composition of urine cells were similar to those cells observed in renal biopsies, suggesting that the immune cells examined in urine originated from the kidney.[18] Of note, urine collection is clinically very easy with few side effects; hence, it can be used to significantly improve the frequency of continuous monitoring in LN patients.[33,78,79] Therefore, urine testing has the potential to serve as a surrogate for renal biopsy in assessing the extent of kidney damage and inflammation level.

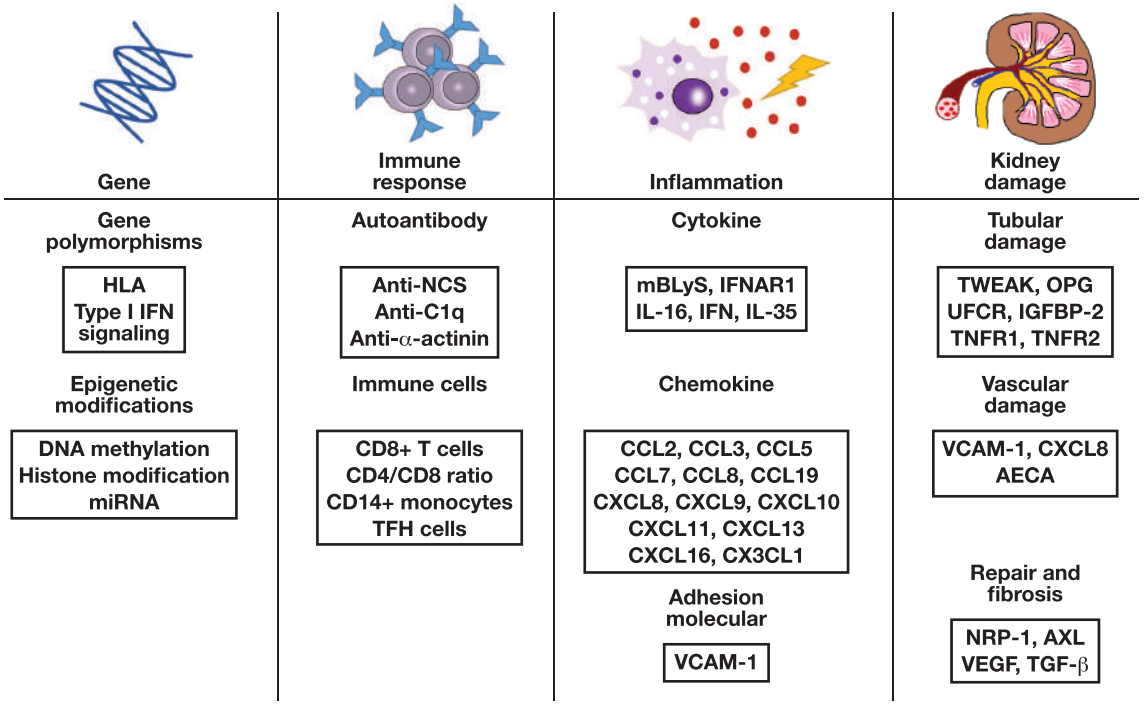
Urinary Immune Cells as Biomarkers for LN

Numerous studies have reported that urinary cell phenotype correlates with renal interstitial infiltration, which can reflect the disease activity and kidney damage.[80] Normally, there are no blood cells in the urine of healthy people, but some T cells, B cells, and macrophages may be observed in the urine of LN patients and other patients with proliferative renal diseases.[43,49,63] Therefore, the classification and counting of cells in urine may be a potential noninvasive biomarker for identifying and diagnosing of LN. Urine sediment analysis, including erythrocyte and leukocyte counts, as well as morphological analysis, can indicate the presence of renal disease and inflammation. However, this test has a very limited role in the differential diagnosis of renal diseases. Some researchers have introduced flow cytometry to further analyze the urine cell phenotype and preliminarily obtained a urinary cellular profile of SLE patients with LN. Abdelati et al[63] found that the numbers of CD3+CD4+, CD3+CD8+, and CD14+ cells in the urine of SLE patients with LN were significantly higher than those patients without LN. In particular, CD14+ monocyte counts were much higher in the urine of patients with class IV LN than those with class III LN and could serve as a potentially sensitive and specific biomarker for detecting proliferative LN.[63] In another study, a low CD4/CD8 ratio was found to be a specific manifestation of LN, suggesting that the shift of urinary T cells into CD8+ T cells is a marker that distinguishes LN from other inflammatory kidney diseases, such as diabetic nephropathy.[62] Therefore, both the absolute number and the proportion of urinary CD8+ T cells may be considered as promising biomarkers for assessing renal activity in patients with LN.[61] This finding was supported in the study by Kopetschke et al.[62] They found that the numbers of urinary T cells, B cells, and macrophages were correlated with the disease activity of LN; however, due to the low number of B cells and macrophages in urine, T cells remained the best cellular biomarker for LN. Of course, with the development of laboratory medicine technologies, it is conceivable that there will be more research focus on cells that are less abundant in urine, such as B cells and T follicular helper cells (TFH). Several studies have already confirmed that B cells and TFH cells are elevated in lupus biopsies and their number alterations in urine may be consistent with those in kidney tissue, which deserve further investigation.[64,81]

Urinary Proteins as Biomarkers for LN

It is well known that the activation of inflammatory pathways within the kidneys are early responses to kidney damage. During the inflammatory process, cytokines are secreted by immune cells such as T cells and macrophages and participate in the chemoattraction, migration, and activation of inflammatory cells. Fava et al[33] analyzed the urinary proteomic profiles in patients with LN and found that urinary IL-16, CD163, and TGF-β have the ability to mirror nephritis activity. Of note, IL-16 is a CD4 ligand with proinflammatory and chemotactic properties. Single cell RNA sequencing also showed that a large number of immune cells capable of producing IL-16 infiltrated at the inflammatory site of the kidney in LN patients, which supports the idea of IL-16 as a potential noninvasive marker for LN diagnosis and treatment monitoring.[33] Furthermore, Häyry et al[34] detected high levels of IL-16 expression in the plasma, urine, and kidney tissues in SLE patients, and only urinary IL-16 could differentiate patients with proliferative LN from those SLE patients without LN. Therefore, it is necessary to study the expression levels of cytokines and other inflammatory substances in the urine of LN patients. Similarly, Arazi et al[20] analyzed kidney samples and urinary cells from patients with LN using single cell RNA sequencing. The researchers found that a variety of immune cells, including myeloid cells, T cells, B cells, and natural killer cells in the kidney had a clear interferon response, and the gene expression of urine cells was highly consistent with that of kidney cells. Therefore, the IFN-I test in urinary cells can distinguish LN from other forms of nephritis and truly reflect the extent of kidney damage. Also, IL-35 has immunosuppressive and anti-inflammatory effects and is significantly elevated in the blood and urine of LN patients, which may aid in assessing renal involvement in SLE patients.[35] Cai et al[36] demonstrated that LAIR1 could be used as a novel potential target of the IL-35-regulated JAK/STAT signaling pathway, and its expression level in urine was significantly correlated with clinical inflammatory parameters of LN. For chemokines, Klocke et al[43] found that 9 urinary chemokines were significantly elevated in LN patients and correlated with disease activity and urinary cell counts. These chemokines include CCL2, CCL3, CCL5, CCL7, CCL8, CXCL9, CXCL10, CXCL16, and CX3CL1.[42,46,48] This finding was corroborated in the

FIGURE 1. **Summary of noninvasive biomarkers for lupus nephritis. AECA, anti-endothelial cell antibodies; anti-NCS, antinucleosome; anti-C1q, anti-complement 1q; AXL, a receptor tyrosine kinase; HLA, human leukocyte antigen; IFN-I, type I interferon; IFNAR1, interferon alpha/beta receptor 1; IGFBP2, insulin-like growth factor binding protein 2; IL, interleukin; mBLyS, membrane B-lymphocyte stimulator protein; miRNA, microRNA; NRP-1, neuropilin-1; OPG, osteoprotegerin; TFH, T follicular helper; TGF-β, transforming growth factor-β; TNFR, tumor necrosis factor receptor; TWEAK, TNF-like weak inducer of apoptosis; UFCR, urinary ferritin/creatinine ratio; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.**



study by Kulkarni and Anders.[40] Additionally, Kulkarni and Anders[40] and Singh et al[38] reported that CCL2 and CCL3 were highly expressed in both glomeruli and urine of LN patients, suggesting that the expression of chemokines in urine was consistent with that in kidney tissues. However, there was no significant correlation between urinary chemokine concentration and serum chemokine levels. These results indicate that elevated chemokines in urine mainly originate from the kidney rather than the blood. For other proteins, El-Shehaby et al[49] found that urinary levels of TNF-like weak inducer of apoptosis and osteoprotegerin were positively correlated with renal involvement as assessed by the renal disease activity index. These proteins were involved in the harmful physiological responses of cell survival, proliferation, differentiation, angiogenesis, migration and apoptosis, and then contributed to kidney damage in LN.[50,51] Kidney damage leads to the impairment of glomerular filtration function, which ultimately results in increased levels of protein substances in the urine. Qi et al[52] evaluated the clinical significance of urinary ferritin/creatinine ratio (UFCR) in LN, and found that the UFCR level in LN patients was significantly higher than that in patients without LN. Because ferritin cannot be filtered by normal glomeruli, damage of glomerular filtration should be the cause of elevated UFCR.[52] Moreover, Torres-Salido et al[59] found that the transmembrane receptor neuropilin-1 (NRP-1) is highly expressed in urine and renal tissue of LN patients, which could be used as an early prognostic biomarker of LN. In the kidney, NRP-1 could promote renal recovery through endothelial proliferation and migration, mesangial migration, and local T cell cytotoxicity.

Conclusion

A large number of potential noninvasive biomarkers targeting candidate genes, immune responses, inflammation, and kidney damage have been reported (**FIGURE 1**). Compared with renal biopsy, noninvasive biomarkers have many advantages. First, renal biopsy has high requirements for medical environment, facilities, equipment, and personnel qualification, and in addition to possible puncture-related complications, up to 35% of renal biopsies may fail to obtain adequate samples,[82] whereas the collection procedure for noninvasive biomarkers, especially from urine, is straightforward and efficient, with almost no unqualified specimen. Second, renal biopsy requires a clear clinical indication of kidney damage; therefore, renal biopsy is a lagging indicator. In contrast, the alterations signaled by noninvasive biomarkers can occur before kidney damage and therefore can be applied to assess and predict the risk of LN in patients with SLE. Third, the contraindications for renal biopsy include coagulation disorders, heart disease, mental illness, solitary kidney, and kidney atrophy, thus making it unsuitable for all patients. However, there are few contraindications for noninvasive biomarker tests, and urine tests in particular are suitable for almost everyone. Finally, histological classification artificially divides disease types based on a point in time, whereas the detection of noninvasive biomarkers can dynamically and continuously reflect the specific changes in the kidneys of LN patients. Based on these advantages, the development of noninvasive biomarkers has been very rapid in recent decades.

We also are aware that the noninvasive biomarkers in these studies have some disadvantages. More effort should be made to apply

noninvasive biomarkers to clinical practice or incorporate them into clinical treatment guidelines for LN. For urinary cell detection, we find that the number of immune cells in LN patients varied slightly across different studies.[16,61-63] These differences could be explained by the differences in sample volume obtained, sample preparation techniques, gating strategy and patient ethnicity. Therefore, a standardized detection process must be established to apply urinary cell detection in clinical practice and ensure consistent results from different laboratories. In addition, assuming that urinary immune cells originated from the kidney, their role and clinical significance in renal disease remain unclear and must be evaluated. For serum protein detection, most studies have validated the efficacy of noninvasive biomarkers in SLE populations. If these biomarkers are to be applied in clinical practice, they need to be further validated in other types of nephritis to evaluate their potential in differential diagnosis. For urinary protein detection, it is necessary to consider the influence of urinary tract infection on the specificity of the detection biomarkers and further evaluate its efficacy. Because most inflammatory diseases of the urinary system share a common molecular pathway, it is conceivable that potential biomarkers in urine may not be specific to a particular disease.[83] Above all, more clinical trials are needed to further validate the diagnostic efficacy of novel noninvasive biomarkers and therefore promote the translation of basic research into clinical practice.

In conclusion, noninvasive biomarkers may provide a new turning point for disease diagnosis, monitoring, and treatment guidance in patients with LN, avoiding the hazards of repeat renal biopsy and untimely diagnosis. A better understanding of the distinct biological role and molecular mechanisms of noninvasive biomarkers in LN is also crucial for the development of possible therapeutic targets and strategies. Therefore, in addition to aiding in the diagnosis, monitoring, and treatment guidance of LN, noninvasive biomarkers may act as possible therapeutic targets for LN and must be investigated in detail in the future.

Funding

This work was supported by Sichuan Science and Technology Program (Grant No. 2023YFS0186 and 2023YFS0222) and the Clinical Research Foundation of West China Second University Hospital (Grant No. KL076).

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Roccatello D, Fenoglio R, Caniggia I, et al. Daratumumab monotherapy for refractory lupus nephritis. *Nat Med.* 2023;29(8):2041-2047.
2. Furie R, Rovin BH, Houssiau F, et al. Two-year, randomized, controlled trial of belimumab in lupus nephritis. *N Engl J Med.* 2020;383(12):1117-1128.
3. Hanly JG, O'Keeffe AG, Su L, et al. The frequency and outcome of lupus nephritis: results from an international inception cohort study. *Rheumatology.* 2016;55(2):252-262.
4. Furie RA, Aroca G, Cascino MD, et al. B-cell depletion with obinutuzumab for the treatment of proliferative lupus nephritis: a randomised, double-blind, placebo-controlled trial. *Ann Rheum Dis.* 2022;81(1):100-107.
5. Hiraki LT, Benseler SM, Tyrrell PN, Hebert D, Harvey E, Silverman ED. Clinical and laboratory characteristics and long-term outcome of pediatric systemic lupus erythematosus: a longitudinal study. *J Pediatr.* 2008;152(4):550-556.
6. Tarr T, Derfalvi B, Gyori N, et al. Similarities and differences between pediatric and adult patients with systemic lupus erythematosus. *Lupus.* 2015;24(8):796-803.
7. Cojocaru M, Cojocaru IM, Silosi I, Vrabie CD. Manifestations of systemic lupus erythematosus. *Maedica (Bucur)*. 2011;6(4):330-336.
8. Rovin BH, Teng YKO, Ginzler EM, et al. Efficacy and safety of voclosporin versus placebo for lupus nephritis (AURORA 1): a double-blind, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet.* 2021;397(10289):2070-2080.
9. Sule S, Fivush B, Neu A, Furth S. Increased risk of death in pediatric and adult patients with ESRD secondary to lupus. *Pediatr Nephrol.* 2011;26(1):93-98.
10. Jorge A, Wallace ZS, Lu N, Zhang Y, Choi HK. Renal transplantation and survival among patients with lupus nephritis: a cohort study. *Ann Intern Med.* 2019;170(4):240-247.
11. Yu F, Haas M, Glassock R, Zhao MH. Redefining lupus nephritis: clinical implications of pathophysiologic subtypes. *Nat Rev Nephrol.* 2017;13(8):483-495.
12. Malvar A, Pirruccio P, Alberton V, et al. Histologic versus clinical remission in proliferative lupus nephritis. *Nephrol Dial Transplant.* 2017;32(8):1338-1344.
13. Fanouriakis A, Kostopoulou M, Cheema K, et al. 2019 Update of the Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of lupus nephritis. *Ann Rheum Dis.* 2020;79(6):713-723.
14. Abujam B, Cheekatla S, Aggarwal A. Urinary CXCL-10/IP-10 and MCP-1 as markers to assess activity of lupus nephritis. *Lupus.* 2013;22(6):614-623.
15. Vanarsa K, Soomro S, Zhang T, et al. Quantitative planar array screen of 1000 proteins uncovers novel urinary protein biomarkers of lupus nephritis. *Ann Rheum Dis.* 2020;79(10):1349-1361.
16. Enghard P, Rieder C, Kopetschke K, et al. Urinary CD4 T cells identify SLE patients with proliferative lupus nephritis and can be used to monitor treatment response. *Ann Rheum Dis.* 2014;73(1):277-283.
17. Badot V, Luijten RK, van Roon JA, et al. Serum soluble interleukin 7 receptor is strongly associated with lupus nephritis in patients with systemic lupus erythematosus. *Ann Rheum Dis.* 2013;72(3):453-456.
18. Soliman S, Mohan C. Lupus nephritis biomarkers. *Clin Immunol.* 2017;185:10-20.
19. Hanaoka M, Gono T, Kawaguchi Y, et al. Urinary free light chain is a potential biomarker for ISN/RPS class III/IV lupus nephritis. *Rheumatology (Oxford).* 2013;52(12):2149-2157.
20. Arazi A, Rao DA, Berthier CC, et al; Accelerating Medicines Partnership in SLE Network. The immune cell landscape in kidneys of patients with lupus nephritis. *Nat Immunol.* 2019;20(7):902-914.
21. Jayne D, Rovin B, Mysler EF, et al. Phase II randomised trial of type I interferon inhibitor anifrolumab in patients with active lupus nephritis. *Ann Rheum Dis.* 2022;81(4):496-506.
22. Maria NI, Davidson A. Protecting the kidney in systemic lupus erythematosus: from diagnosis to therapy. *Nat Rev Rheumatol.* 2020;16(5):255-267.
23. Faurschou M, Starklint H, Halberg P, Jacobsen S. Prognostic factors in lupus nephritis: diagnostic and therapeutic delay increases the risk of terminal renal failure. *J Rheumatol.* 2006;33(8):1563-1569.
24. Marchini M, Antonioli R, Lleo A, et al. HLA class II antigens associated with lupus nephritis in Italian SLE patients. *Hum Immunol.* 2003;64(4):462-468.
25. Anders HJ, Lichtnekert J, Allam R. Interferon-alpha and -beta in kidney inflammation. *Kidney Int.* 2010;77(10):848-854.

26. Su YJ, Lin IC, Wang L, Lu CH, Huang YL, Kuo HC. Next generation sequencing identifies miRNA-based biomarker panel for lupus nephritis. *Oncotarget*. 2018;9(46):27911-27919.
27. Rodriguez-Jimenez NA, Perez-Guerrero EE, Gamez-Nava JI, et al. Anti-nucleosome antibodies increase the risk of renal relapse in a prospective cohort of patients with clinically inactive systemic lupus erythematosus. *Sci Rep.* 2020;10(1):12698.
28. Pickering MC, Botto M. Are anti-C1q antibodies different from other SLE autoantibodies? *Nat Rev Rheumatol.* 2010;6(8):490-493.
29. Tan Y, Yu F, Yang H, Chen M, Fang Q, Zhao MH. Autoantibodies against monomeric C-reactive protein in sera from patients with lupus nephritis are associated with disease activity and renal tubulointerstitial lesions. *Hum Immunol.* 2008;69(12):840-844.
30. Kondo A, Takahashi K, Mizuno T, et al. The level of IgA antibodies to endothelial cells correlates with histological evidence of disease activity in patients with lupus nephritis. *PLoS One.* 2016;11(10):e0163085.
31. Lopez P, Rodriguez-Carrio J, Caminal-Montero L, Mozo L, Suarez A. A pathogenic IFN alpha, BLyS and IL-17 axis in systemic lupus erythematosus patients. *Sci Rep.* 2016;6:20651.
32. Parodis I, Zickert A, Sundelin B, et al. Evaluation of B lymphocyte stimulator and a proliferation inducing ligand as candidate biomarkers in lupus nephritis based on clinical and histopathological outcome following induction therapy. *Lupus Sci Med*. 2015;2(1):e000061.
33. Fava A, Rao DA, Mohan C, et al; Accelerating Medicines Partnership in Rheumatoid Arthritis and Systemic Lupus Erythematosus Network. Urine proteomics and renal single-cell transcriptomics implicate interleukin-16 in lupus nephritis. *Arthritis Rheumatol.* 2022;74(5):829-839.
34. Hayry A, Faustini F, Zickert A, et al. Interleukin (IL) 16: a candidate urinary biomarker for proliferative lupus nephritis. *Lupus Sci Med.* 2022;9(1):e000744.
35. Nassif MA. Urine and serum interleukin 35 as potential biomarkers of lupus nephritis. *Cent Eur J Immunol*. 2021;46(3):351-359.
36. Cai Z, Zhang S, Wu P, et al. A novel potential target of IL-35-regulated JAK/STAT signaling pathway in lupus nephritis. *Clin Transl Med.* 2021;11(2):e309.
37. Abozaid MA, Ahmed GH, Tawfik NM, Sayed SK, Ghandour AM, Madkour RA. Serum and urine monocyte chemoattractant protein-1 as a markers for lupus nephritis. *Egypt J Immunol.* 2020;27(1):97-107.
38. Singh RG, Usha, Rathore SS, Behura SK, Singh NK. Urinary MCP-1 as diagnostic and prognostic marker in patients with lupus nephritis flare. *Lupus.* 2012;21(11):1214-1218.
39. Bauer JW, Baechler EC, Petri M, et al. Elevated serum levels of interferon-regulated chemokines are biomarkers for active human systemic lupus erythematosus. *PLoS Med.* 2006;3(12):e491.
40. Kulkarni O, Anders HJ. Chemokines in lupus nephritis. *Front Biosci.* 2008;13:3312-3320.
41. Fu Q, Chen X, Cui H, et al. Association of elevated transcript levels of interferon-inducible chemokines with disease activity and organ damage in systemic lupus erythematosus patients. *Arthritis Res Ther.* 2008;10(5):R112.
42. Lloyd CM, Dorf ME, Proudfoot A, Salant DJ, Gutierrez-Ramos JC. Role of MCP-1 and RANTES in inflammation and progression to fibrosis during murine crescentic nephritis. *J Leukoc Biol.* 1997;62(5):676-680.
43. Klocke J, Kopetschke K, Griessbach AS, et al. Mapping urinary chemokines in human lupus nephritis: potentially redundant pathways recruit CD4(+) and CD8(+) T cells and macrophages. *Eur J Immunol.* 2017;47(1):180-192.
44. Tsai CY, Wu TH, Yu CL, Lu JY, Tsai YY. Increased excretions of beta2-microglobulin, IL-6, and IL-8 and decreased excretion of Tamm-Horsfall glycoprotein in urine of patients with active lupus nephritis. *Nephron.* 2000;85(3):207-214.
45. Jakiela B, Kosalka J, Plutecka H, et al. Urinary cytokines and mRNA expression as biomarkers of disease activity in lupus nephritis. *Lupus.* 2018;27(8):1259-1270.
46. Marie MA, Abu Khalil RE, Habib HM. Urinary CXCL10: a marker of nephritis in lupus patients. *Reumatismo*. 2014;65(6):292-297.
47. Lee HT, Shiao YM, Wu TH, et al. Serum BLC/CXCL13 concentrations and renal expression of CXCL13/CXCR5 in patients with systemic lupus erythematosus and lupus nephritis. *J Rheumatol.* 2010;37(1):45-52.
48. Wen S, He F, Zhu X, Yuan S, Liu H, Sun L. IFN-gamma, CXCL16, uPAR: potential biomarkers for systemic lupus erythematosus. *Clin Exp Rheumatol.* 2018;36(1):36-43.
49. El-Shehaby A, Darweesh H, El-Khatib M, et al. Correlations of urinary biomarkers, TNF-like weak inducer of apoptosis (TWEAK), osteoprotegerin (OPG), monocyte chemoattractant protein-1 (MCP-1), and IL-8 with lupus nephritis. *J Clin Immunol.* 2011;31(5):848-856.
50. Susianti H, Hanggara DS, Lestari KD, Purnamasari P, Aprilia A. Analysis of TNF-like weak inducer of apoptosis for detecting lupus nephritis. *Comp Clin Path*. 2022;31(2):313-316.
51. Gupta R, Aggarwal A, Sinha S, et al. Urinary osteoprotegerin: a potential biomarker of lupus nephritis disease activity. *Lupus.* 2016;25(11):1230-1236.
52. Qi L, Xu J, Yang C, Hou X, Yang P. Urinary ferritin creatinine ratio, a potential biomarker for lupus nephritis activity. *Clin Rheumatol.* 2021;40(1):143-149.
53. Wu T, Ding H, Han J, et al. Antibody-array-based proteomic screening of serum markers in systemic lupus erythematosus: a discovery study. *J Proteome Res.* 2016;15(7):2102-2114.
54. Li H, Liang J, Gao Y, et al. IGFBP2 function as a novel biomarker for active lupus nephritis. *J Mol Med (Berl).* 2022;100(10):1479-1491.
55. Al-Lamki RS, Wang J, Skepper JN, Thiru S, Pober JS, Bradley JR. Expression of tumor necrosis factor receptors in normal kidney and rejecting renal transplants. *Lab Invest.* 2001;81(11):1503-1515.
56. Patel M, Oni L, Midgley A, et al. Increased concentration of plasma TNFR1 and TNFR2 in paediatric lupus nephritis. *Lupus.* 2016;25(9):1040-1044.
57. Yao GH, Liu ZH, Zhang X, et al. Circulating thrombomodulin and vascular cell adhesion molecule-1 and renal vascular lesion in patients with lupus nephritis. *Lupus.* 2008;17(8):720-726.
58. Mok CC, Soliman S, Ho LY, Mohamed FA, Mohamed FI, Mohan C. Urinary angiostatin, CXCL4 and VCAM-1 as biomarkers of lupus nephritis. *Arthritis Res Ther.* 2018;20(1):6.
59. Torres-Salido MT, Sanchis M, Sole C, et al. Urinary neuropilin-1: a predictive biomarker for renal outcome in lupus nephritis. *Int J Mol Sci*. 2019;20(18):4601.
60. Parodis I, Ding H, Zickert A, et al. Serum AXL predicts histology-based response to induction therapy and long-term renal outcome in lupus nephritis. *PLoS One.* 2019;14(2):e0212068.
61. Dolff S, Abdulahad WH, Arends S, et al. Urinary CD8+ T-cell counts discriminate between active and inactive lupus nephritis. *Arthritis Res Ther.* 2013;15(1):R36.
62. Kopetschke K, Klocke J, Griessbach AS, et al. The cellular signature of urinary immune cells in lupus nephritis: new insights into potential biomarkers. *Arthritis Res Ther.* 2015;17(1):94.
63. Abdelati AA, Eshak NY, Donia HM, El-Girby AH. Urinary cellular profile as a biomarker for lupus nephritis. *J Clin Rheumatol.* 2021;27(8):e469-e476.
64. Wing JB, Kitagawa Y, Locci M, et al. A distinct subpopulation of CD25(-) T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci U S A.* 2017;114(31):E6400-E6409.
65. Yu C, Li P, Dang X, Zhang X, Mao Y, Chen X. Lupus nephritis: new progress in diagnosis and treatment. *J Autoimmun.* 2022;132:102871.
66. Moroni G, Quaglini S, Radice A, et al. The value of a panel of autoantibodies for predicting the activity of lupus nephritis at time of renal biopsy. *J Immunol Res*. 2015;2015:106904.

67. Ho A, Magder LS, Barr SG, Petri M. Decreases in anti-double-stranded DNA levels are associated with concurrent flares in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2001;44(10):2342-2349.
68. Ho A, Barr SG, Magder LS, Petri M. A decrease in complement is associated with increased renal and hematologic activity in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2001;44(10):2350-2357.
69. Corapi KM, Chen JL, Balk EM, Gordon CE. Bleeding complications of native kidney biopsy: a systematic review and meta-analysis. *Am J Kidney Dis.* 2012;60(1):62-73.
70. De Rosa M, Azzato F, Toblli JE, et al. A prospective observational cohort study highlights kidney biopsy findings of lupus nephritis patients in remission who flare following withdrawal of maintenance therapy. *Kidney Int.* 2018;94(4):788-794.
71. Hill GS, Delahousse M, Nochy D, et al. Predictive power of the second renal biopsy in lupus nephritis: significance of macrophages. *Kidney Int.* 2001;59(1):304-316.
72. Zhou X, Zhang Y, Wang N. Systematic identification of key extracellular proteins as the potential biomarkers in lupus nephritis. *Front Immunol.* 2022;13:915784.
73. Maravillas-Montero JL, Reyes-Huerta RF. Update on novel blood-based biomarkers for lupus nephritis beyond diagnostic approaches. *Rev Invest Clin.* 2022;74(5):227-231.
74. Lodi L, Mastrolia MV, Bello F, et al. Type I interferon-related kidney disorders. *Kidney Int.* 2022;101(6):1142-1159.
75. Li Y, Fang X, Li QZ. Biomarker profiling for lupus nephritis. *Genom Proteom Bioinform.* 2013;11(3):158-165.
76. Tang C, Zhang S, Teymur A, et al. V-set immunoglobulin domain-containing protein 4 as a novel serum biomarker of lupus nephritis and renal pathology activity. *Arthritis Rheumatol.* 2023;75(9):1573-1585.
77. Chun HY, Chung JW, Kim HA, et al. Cytokine IL-6 and IL-10 as biomarkers in systemic lupus erythematosus. *J Clin Immunol.* 2007;27(5):461-466.
78. Li KY, Tam CHT, Liu H, et al; TRANSCEND Consortium. DNA methylation markers for kidney function and progression of diabetic kidney disease. *Nat Commun.* 2023;14(1):2543.
79. Liu C, Ma K, Zhang Y, et al. Kidney diseases and long non-coding RNAs in the limelight. *Front Physiol.* 2022;13:932693.
80. Enghard P, Humrich JY, Rudolph B, et al. CXCR3+CD4+ T cells are enriched in inflamed kidneys and urine and provide a new biomarker for acute nephritis flares in systemic lupus erythematosus patients. *Arthritis Rheum.* 2009;60(1):199-206.
81. Peterson KS, Huang JF, Zhu J, et al. Characterization of heterogeneity in the molecular pathogenesis of lupus nephritis from transcriptional profiles of laser-captured glomeruli. *J Clin Invest.* 2004;113(12):1722-1733.
82. Geldenhuys L, Nicholson P, Sinha N, et al. Percutaneous native renal biopsy adequacy: a successful interdepartmental quality improvement activity. *Can J Kidney Health Dis*. 2015;2:8.
83. Li Y, Tucci M, Narain S, et al. Urinary biomarkers in lupus nephritis. *Autoimmun Rev.* 2006;5(6):383-388.

OVERVIEW

Diagnostic and prognostic value of circulating exosomal glypican-1 in pancreatic cancer: a meta-analysis

Zengyun Qiao, [1,2,3] *Enbo Wang,* [1,3] *Boyang Bao,* [1,2] *Xiaodong Tan,* [13] *Hailong Chen,* [13] *Dong Wang, Liu Yuan,* [1,3]

[1]Department of Laboratory Medicine, West China Second University Hospital, Sichuan University, Chengdu, China; [2]Key Laboratory of Birth Defects and Related Diseases of Women and Children (Ministry of Education), West China Second University Hospital, Sichuan University, Chengdu, China; [3]State Key Laboratory of Biotherapy and Cancer Center/National Collaborative Innovation Center for Biotherapy, Sichuan University, Chengdu, China. Corresponding author: Yong-mei Jiang; jiangym_scu@163.com
Key words: lupus nephritis; laboratory medicine; noninvasive biomarker; urinary biomarker

Abbreviations: LN, lupus nephritis; SLE, systemic lupus erythematosus; ESRD, end-stage renal disease; dsDNA, double-stranded DNA; IFN-I, type I interferon; miRNA, microRNA; PBMCs, peripheral blood mononuclear cells; IL, interleukin; BLyS, B-lymphocyte stimulator protein; TNF, tumor necrosis factor; IFNAR1, IFN alpha/beta receptor 1; VCAM-1, vascular cell adhesion molecule-1; AECA, anti-endothelial cell antibodies; AXL, a receptor tyrosine kinase; IGFBP2, insulin like growth factor binding protein 2; TNFR, TNF receptor; TFH, T follicular helper cells; UFCR, urinary ferritin/creatinine ratio; NRP-1, neuropilin-1

Laboratory Medicine 2024;55:535-542; https://doi.org/10.1093/labmed/lmae015

ABSTRACT

Pancreatic cancer (PC) is usually detected in the advanced stages. Liquid biopsy has become a revolutionary strategy for cancer diagnosis and prognosis prediction. This study aims to investigate the diagnostic and prognostic value of circulating exosomal glypican-1 (GPC-1) in PC. We systematically searched relevant studies. For diagnostic accuracy, pooled sensitivity and specificity and the area under the summary receiver operating characteristic curve (AUC) were calculated. Regarding prognostic value, hazard ratios (HRs) and 95% CIs for overall survival (OS) were summarized by using a random-effects model. We found 8 studies that examined the diagnostic value of circulating exosomal GPC-1 in PC, and 3 studies that investigated its prognostic value. Pooled sensitivity and specificity were 0.88 (95% CI, 0.65-0.97) and 0.86 (95% CI, 0.72-0.94). The AUC was 0.93 (95% CI, 0.90-0.95). Prognostic analysis showed that higher levels of circulating exosomal GPC-1 were associated with poorer OS in PC patients, and the combined HR for OS was 4.59 (random-effects model, 95% CI = 1.17-18.03, P = .022). The results of both studies were robust and neither had publication bias. Circulating exosomal GPC-1 may be used as a diagnostic and prognostic biomarker for PC. However, this result needs to be validated by further research using a larger sample size

Introduction

Lupus nephritis (LN) is one of the most severe clinical manifestations in patients with systemic lupus erythematosus (SLE), which has a significantly high morbidity and mortality.[1-3] Generally, about 30%-60% of adults and up to 70% of children with SLE will develop related nephritis as the disease progresses.[4-7] Additionally, 10%-30% of LN patients eventually develop end-stage renal disease (ESRD), requiring dialysis and transplantation.[8-10] As a chronic autoimmune disease, the pathogenesis of LN involves multiple factors, including the deposition of autoantibodies and complements in the glomerulus and the activation of the innate and adaptive immunity, which ultimately lead to glomerular, interstitial, tubular, and vascular damage. It is worth mentioning that the clinical manifestations of LN are not always consistent with the histopathological findings.[11,12] At present, the clinical diagnosis of LN mainly depends on renal histopathological changes. Renal biopsy is the gold standard for diagnosis and classification of the histopathological changes of the kidneys, but it is an invasive procedure that is unsuitable for continuous monitoring of patients.[13,14] Additionally, there are puncture-related side effects, such as bleeding and infection, associated with renal biopsy. Therefore, there is an urgent need to identify novel noninvasive biomarkers that can effectively reflect the activity and severity of LN.

Noninvasive biomarkers are laboratory indicators of biological, biochemical, or molecular substances obtained by noninvasive methods. They can be qualitatively and quantitatively detected by laboratory techniques, and their changes are closely related to the occurrence and development of diseases. Compared with renal biopsy, noninvasive biomarkers in blood and urine can significantly increase the frequency of kidney damage assessment, aid in monitoring disease activity, and guide clinical treatment in patients with LN.[15-17] A large number of studies have been conducted to develop novel noninvasive biomarkers for the biological processes in LN, especially those in urine, which fundamentally reflect the biological processes of kidney damage and repair during disease, such as inflammatory cell recruitment, immune cell activation and differentiation, and renal vascular damage and repair, etc.[18-20] In addition, it is well known that the choice of treatment strategy for LN patients mainly depends on nephritis severity, and late diagnosis of LN is associated with a high frequency of ESRD.[21-23] Therefore, early diagnosis, continuous monitoring, and early intervention of LN are important to significantly improve prognosis. To address these issues, there has been a growing interest in the development of novel noninvasive LN biomarkers over the past decade. The in-depth multidimensional study of this disease has led to the gradual incorporation of many noninvasive biomarkers, particularly urinary biomarkers, into the diagnostic strategy for LN. In this review, we mainly discuss the possible roles of potential noninvasive biomarkers in the early diagnosis, activity monitoring, and treatment guidance of LN by correlating the current knowledge with future perspectives (**TABLE 1**).

TABLE 1. Summary of biomarkers reviewed[a]

Blood Type Compatibility									
		Blood type of donor							
		O-	O+	A-	A+	B-	B+	AB-	AB+
Blood type of recipient	AB+	√	√	√	√	√	√	√	√
	AB-	√		√		√		√	
	B+	√	√			√	√		
	B-	√				√			
	A+	√	√	√	√				
	A-	√		√					
	O+	√	√						
	O-	√							

Conventional Laboratory Biomarkers for LN

The conventional laboratory markers used for LN diagnosis and monitoring mainly include proteinuria, protein/creatinine ratio, glomerular filtration rate, plasma complement levels (C3 and C4), anti-double-stranded (ds)DNA antibody, anti-cardiolipin antibody, rheumatoid factor, serum immunoglobulin, blood cell counts, as well as erythrocyte sedimentation rate and so on. Among these markers, the diagnostic specificity and sensitivity of the protein/creatinine ratio, plasma complement, anti-dsDNA antibody and serum immunoglobulins (IgG and IgA) for LN are relatively higher than those of the others. However these markers are still insufficient to diagnose renal damage or to mon- itor disease activity, and patients still need further renal biopsy.[25,65,66] For example, it is difficult to distinguish between irreversible glomer- ular capillary damage and ongoing LN activity in most patients with persistent proteinuria.[25] Moreover, Pinheiro et al[67] reported that LN may recur in the absence of an elevated level of anti-dsDNA. Likewise, Soliman et al[18] found that the diagnostic efficacy of anti-dsDNA varied across different assay platforms, making it difficult to achieve uni- formity in clinical judgment. Based on these findings, we believe that LN can develop before the levels of any of these commonly used laboratory markers are altered and can continue to develop without obvious clinical signs. Correlations between these conventional laboratory biomarkers and LN are incomplete, and their utility in reflecting disease activity and predicting prognosis remains controversial.[68] Therefore, there is an urgent need to develop novel noninvasive biomarkers that can replace kidney biopsy and conventional biomarkers to better reflect the extent of kidney damage in patients with LN.

Blood Biomarkers for LN

The biggest problem with renal biopsy is that it can cause a variety of complications, including hematuria, kidney damage and loss, and even death.[69] Also, due to differences in infrastructure and personnel qualifications in different regions, not all the medical institutions can perform this test.[70,71] Compared with renal biopsy, side effects caused by venous blood collection are almost negligible. Of note, the detection of blood biomarkers requires relatively low infrastructure and personnel qualifications, making it easier to perform, especially in remote areas. Great effort has been made to identify novel noninvasive biomarkers in blood. To date, several studies have screened candidate biomarkers, including genes, cytokines, chemokines, autoantibodies, adhesion molecules, and factors involved in kidney damage and repair.[72,73]

Genes as Biomarkers for LN

Genetic factors are important in the pathogenesis of LN. Specifically, differences in levels of protein biomarkers, such as cytokines, chemokines, and autoantibodies in the serum and urine of LN patients can reflect genetic and epigenetic differences. In recent decades, numerous studies have evaluated the potential roles of genes in predicting LN susceptibility. On one hand, detection of changes in gene sequence, such as single nucleotide polymorphisms, is expected to be a novel biomarker indicating susceptibility to LN. For example, Marchini et al[24] found that the interactions of HLA-DQA and HLA-DR alleles in an Italian population confer a susceptibility to LN in SLE patients; the HLA-DR15 haplotype is especially associated with diffuse proliferative glomerulonephritis. Besides, Lodi et al[74] reported that type I interferon (IFN-I) apparently promoted immune-mediated glomerular damage in LN. Therefore, gene polymorphisms that enhanced IFN-I signaling were identified as risk factors for SLE and LN.[25] On the other hand, epigenetic modifications by DNA methylation, histone modification, and micro (mi)RNA can also influence gene expression and alter cell function without changing the genome sequence, thereby affecting LN susceptibility.[75] Su et al[26] used next-generation sequencing technology and real-time quantitative polymerase chain reaction technology to screen the different expression levels of miRNAs in the peripheral blood mononuclear cells (PBMCs) of SLE patients with and without LN. Ultimately, the study revealed that mir-125a-5p, miR-146a-5p, and mir-221-3p were significantly decreased in PBMCs of SLE patients with LN and then significantly increased after treatment, suggesting their role as biomarkers in early diagnosis and treatment guidance of LN.[26]

Serum Protein as Biomarkers for LN

In addition to cells, blood also contains a large number of bioactive substances, such as proteins. In recent decades, several studies have been conducted to screen serum biomarkers in LN patients based on proteomics.[18,39,76] Various soluble serum proteins, including cytokines, chemokines, adhesion molecules, and autoantibodies, were found to be correlated with LN disease severity and activity. For cytokines, Chun et

al[77] found that SLE patients had higher serum levels of cytokines such as interleukin (IL)-6, IL-10, IL-12, and IFN-γ compared with normal healthy controls, but no significant differences in levels were observed between SLE patients with and without LN. These findings suggest that due to the common molecular pathways among various inflammatory diseases, the efficacy of serum cytokines in the diagnosis and differential diagnosis of LN is limited; more studies focusing on the correlations between cytokines in urine (or specific cells) and disease are being conducted. Notably, López et al[31] analyzed in depth the expression levels of pathogenic IFNα, B-lymphocyte stimulator protein (BLyS), and IL-17 axis in various blood cell types such as T and B lymphocytes, monocytes, dendritic cells, and neutrophils. They found that tumor necrosis factor (TNF) superfamily cytokine BLyS and related ligand IFN alpha/beta receptor 1 (IFNAR1) levels on monocytes or in the serum were associated with the frequency of nephritis.[31,32] For chemokines, numerous studies have shown that serum chemokines, including CCL3 (MIP-1α), CXCL10 (IP- 10), CXCL11 (I-TAC), and CXCL13 (BCA-1), are strongly associated with disease activity of LN.[39,45,47] Also, Fu et al[41] reported that the transcrip- tional levels of interferon-inducible chemokines, including CCL2 (MCP- 1), CCL5 (RANTES), CCL19 (MIP-3β), CXCL8 (IL-8), and CXCL9 (MIG),
are also strongly associated with active LN patients.[37,41,44] Compared with cytokines, the levels of chemokines are more significantly changed in the blood of LN patients, and their potential as specific biomarkers is greater. For adhesion molecules, Yao et al[57] found that serum vascular cell adhesion molecule-1 (VCAM-1) was significantly increased in proliferative LN, suggesting that it can be used as a potential biomarker to assess the onset and remission of proliferative LN. It is reported that VCAM-1 is involved in the damage and repair of renal vascular endothelium during the disease course of LN patients.[58] For autoantibodies, a large number of studies have revealed the strong correlation between antinucleosome, anti-complement 1q, and anti-α-actinin and renal dis- ease in SLE patients.[27-29] However, their role in assessing the severity and activity of LN remains to be further validated. Kondo et al[30] developed a solubilized cell surface protein capture enzyme-linked immunosorbenassay to detect the serum antibodies in LN patients and further found a significant correlation between anti-endothelial cell antibodies (AECA) and glomerular hypercellularity, indicating that AECA, particularly IgA- AECA, are associated with endothelial damage. For other proteins, Wu et al[53] found that serum AXL (a receptor tyrosine kinase), insulin-like growth factor binding protein 2 (IGFBP2), and TNF receptor (TNFR) were significantly elevated in patients with active LN and showed signif- icant positive correlations with the renal pathology activity index.[53] AXL and IGFBP2 are involved in regulating the innate immune response of monocytes and T cells, respectively, and seem to be biomarkers of partic- ular interest in LN studies,[53,54,60] whereas TNFR1 and TNFR2 are found predominately in glomerular cells and tubular cells, respectively.[55] Once TNF-α binds to its receptors, a complex signaling cascade involving ap- optosis and inflammation occurs.[56] Ultimately, the expression levels of specific serum proteins are closely related to the kidney damage, and its detection can be used to monitor the disease activity and guide treatment. However, the usefulness of serum protein detection in assessing LN susceptibility in SLE patients is limited.

Urinary Biomarkers for LN

Like blood, urine also contains a variety of components, such as epithelial cells, proteins, glucose, inorganic salts, and trace elements, which can be used as biomarkers for laboratory medicine. Arazi et al[20] used single cell transcriptomics to analyze kidney and urine samples from LN patients and healthy individuals. Their finding revealed similarities in the molecular signatures of urine and kidney tissues, highlighting the superiority of urine samples in understanding kidney damage. Additionally, numerous studies have demonstrated that the phenotype and composition of urine cells were similar to those cells observed in renal biopsies, suggesting that the immune cells examined in urine originated from the kidney.[18] Of note, urine collection is clinically very easy with few side effects; hence, it can be used to significantly improve the frequency of continuous monitoring in LN patients.[33,78,79] Therefore, urine testing has the potential to serve as a surrogate for renal biopsy in assessing the extent of kidney damage and inflammation level.

Urinary Immune Cells as Biomarkers for LN

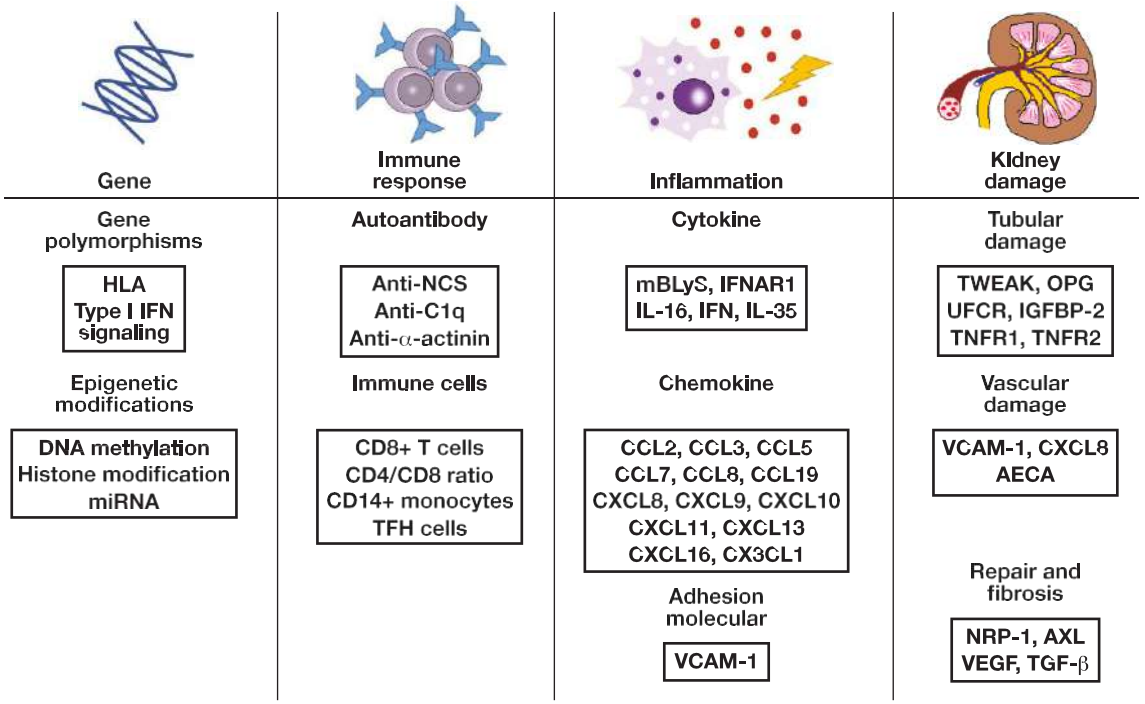
Numerous studies have reported that urinary cell phenotype correlates with renal interstitial infiltration, which can reflect the disease activity and kidney damage.[80] Normally, there are no blood cells in the urine of healthy people, but some T cells, B cells, and macrophages may be observed in the urine of LN patients and other patients with proliferative renal diseases.[43,49,63] Therefore, the classification and counting of cells in urine may be a potential noninvasive biomarker for identifying and diagnosing of LN. Urine sediment analysis, including erythrocyte and leukocyte counts, as well as morphological analysis, can indicate the presence of renal disease and inflammation. However, this test has a very limited role in the differential diagnosis of renal diseases. Some researchers have introduced flow cytometry to further analyze the urine cell phenotype and preliminarily obtained a urinary cellular profile of SLE patients with LN. Abdelati et al[63] found that the numbers of CD3+CD4+, CD3+CD8+, and CD14+ cells in the urine of SLE patients with LN were significantly higher than those patients without LN. In particular, CD14+ monocyte counts were much higher in the urine of patients with class IV LN than those with class III LN and could serve as a potentially sensitive and specific biomarker for detecting proliferative LN.[63] In another study, a low CD4/CD8 ratio was found to be a specific manifestation of LN, suggesting that the shift of urinary T cells into CD8+ T cells is a marker that distinguishes LN from other inflammatory kidney diseases, such as diabetic nephropathy.[62] Therefore, both the absolute number and the proportion of urinary CD8+ T cells may be considered as promising biomarkers for assessing renal activity in patients with LN.[61] This finding was supported in the study by Kopetschke et al.[62] They found that the numbers of urinary T cells, B cells, and macrophages were correlated with the disease activity of LN; however, due to the low number of B cells and macrophages in urine, T cells remained the best cellular biomarker for LN. Of course, with the development of laboratory medicine technologies, it is conceivable that there will be more research focus on cells that are less abundant in urine, such as B cells and T follicular helper cells (TFH). Several studies have al- ready confirmed that B cells and TFH cells are elevated in lupus biopsies and their number alterations in urine may be consistent with those in kidney tissue, which deserve further investigation.[64,81]

Urinary Proteins as Biomarkers for LN

It is well known that the activation of inflammatory pathways within the kidneys are early responses to kidney damage. During the inflammatory process, cytokines are secreted by immune cells such as T cells and macrophages and participate in the chemoattraction, migration,

and activation of inflammatory cells. Fava et al[33] analyzed the urinary proteomic profiles in patients with LN and found that urinary IL-16, CD163, and TGF-β have the ability to mirror nephritis activity. Of note, IL-16 is a CD4 ligand with proinflammatory and chemotactic properties. Single cell RNA sequencing also showed that a large number of immune cells capable of producing IL-16 infiltrated at the inflammatory site of the kidney in LN patients, which supports the idea of IL-16 as a potential noninvasive marker for LN diagnosis and treatment monitoring.[33] Furthermore, Häyry et al[34] detected high levels of IL-16 expression in the plasma, urine, and kidney tissues in SLE patients, and only urinary IL-16 could differentiate patients with proliferative LN from those SLE patients without LN. Therefore, it is necessary to study the expression levels of cytokines and other inflammatory substances in the urine of LN patients. Similarly, Arazi et al[20] analyzed kidney samples and urinary cells from patients with LN using single cell RNA sequencing. The researchers found that a variety of immune cells, including myeloid cells, T cells, B cells, and natural killer cells in the kidney had a clear interferon response, and the gene expression of urine cells was highly consistent with that of kidney cells. Therefore, the IFN-I test in urinary cells can distinguish LN from other forms of nephritis and truly reflect the extent of kidney damage. Also, IL-35 has immunosuppressive and anti-inflammatory effects and is significantly elevated in the blood and urine of LN patients, which may aid in assessing renal involvement in SLE patients.[35] Cai et al[36] demonstrated that LAIR1 could be used as a novel potential target of the IL-35-regulated JAK/STAT signaling pathway, and its expression level in urine was significantly correlated with clinical inflammatory parameters of LN. For chemokines, Klocke et al[43] found that 9 urinary chemokines were significantly elevated in LN patients and correlated with disease activity and urinary cell counts. These chemokines include CCL2, CCL3, CCL5, CCL7, CCL8, CXCL9, CXCL10, CXCL16, and CX3CL1.[42,46,48] This finding was corroborated in the

FIGURE 1. Summary of noninvasive biomarkers for lupus nephritis. AECA, anti-endothelial cell antibodies; anti-NCS, antinucleosome; anti-C1q, anti-complement 1q; AXL, a receptor tyrosine kinase; HLA, human leukocyte antigen; IFN-I, type I interferon; IFNAR1, interferon alpha/beta receptor 1; IGFBP2, insulin-like growth factor binding protein 2; IL, interleukin; mBLyS, membrane B-lymphocyte stimulator protein; miRNA, microRNA; NRP-1, neuropilin-1; OPG, osteoprotegerin; TFH, T follicular helper; TGF-β, transforming growth factor-β; TNFR, tumor necrosis factor receptor; TWEAK, TNF-like weak inducer of apoptosis; UFCR, urinary ferritin/creatinine ratio; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.



study by Kulkarni and Anders.[40] Additionally, Kulkarni and Anders[40] and Singh et al[38] reported that CCL2 and CCL3 were highly expressed in both glomeruli and urine of LN patients, suggesting that the expression of chemokines in urine was consistent with that in kidney tissues. However, there was no significant correlation between urinary chemokine concentration and serum chemokine levels. These results indicate that elevated chemokines in urine mainly originate from the kidney rather than the blood. For other proteins, El-Shehaby et al[49] found that urinary levels of TNF-like weak inducer of apoptosis and osteoprotegerin were positively correlated with renal involvement as assessed by the renal disease activity index. These proteins were involved in the harmful physiological responses of cell survival, proliferation, differentiation, angiogenesis, migration and apoptosis, and then contributed to kidney damage in LN.[50,51] Kidney damage leads to the impairment of glomerular filtration function, which ultimately results in increased levels of protein substances in the urine. Qi et al[52] evaluated the clinical significance of urinary ferritin/creatinine ratio (UFCR) in LN, and found that the UFCR level in LN patients was significantly higher than that in patients without LN. Because ferritin cannot be filtered by normal glomeruli, damage of glomerular filtration should be the cause of elevated UFCR.[52] Moreover, Torres-Salido et al[59] found that the transmembrane receptor neuropilin-1 (NRP-1) is highly expressed in urine and renal tissue of LN patients, which could be used as an early prognostic biomarker of LN. In the kidney, NRP-1 could promote renal recovery through endothelial proliferation and migration, mesangial migration, and local T cell cytotoxicity.

Conclusion

A large number of potential noninvasive biomarkers targeting candidate genes, immune responses, inflammation, and kidney damage have been reported (**FIGURE 1**). Compared with renal biopsy, noninvasive biomarkers have many advantages. First, renal biopsy has high requirements for medical environment, facilities, equipment, and personnel qualification, and in addition to possible puncture-related complications, up to 35% of renal biopsies may fail to obtain adequate samples,[82] whereas the collection procedure for noninvasive biomarkers, especially from urine, is straightforward and efficient, with almost no unqualified specimen. Second, renal biopsy requires a clear clinical indication of kidney damage; therefore, renal biopsy is a lagging indicator. In contrast, the alterations signaled by noninvasive biomarkers can occur before kidney damage and therefore can be applied to assess and predict the risk of LN in patients with SLE. Third, the contraindications for renal biopsy include coagulation disorders, heart disease, mental illness, solitary kidney, and kidney atrophy, thus making it unsuitable for all patients. However, there are few contraindications for noninvasive biomarker tests, and urine tests in particular are suitable for almost everyone. Finally, histological classification artificially divides disease types based on a point in time, whereas the detection of noninvasive biomarkers can dynamically and continuously reflect the specific changes in the kidneys of LN patients. Based on these advantages, the development of noninvasive biomarkers has been very rapid in recent decades.

We also are aware that the noninvasive biomarkers in these studies have some disadvantages. More effort should be made to apply noninvasive biomarkers to clinical practice or incorporate them into clinical treatment guidelines for LN. For urinary cell detection, we find that the number of immune cells in LN patients varied slightly across different studies.[16,61-63] These differences could be explained by the differences in sample volume obtained, sample preparation techniques, gating strategy and patient ethnicity. Therefore, a standardized detection process must be established to apply urinary cell detection in clinical practice and ensure consistent results from different laboratories. In addition, assuming that urinary immune cells originated from the kidney, their role and clinical significance in renal disease remain unclear and must be evaluated. For serum protein detection, most studies have validated the efficacy of noninvasive biomarkers in SLE populations. If these biomarkers are to be applied in clinical practice, they need to be further validated in other types of nephritis to evaluate their potential in differential diagnosis. For urinary protein detection, it is necessary to consider the influence of urinary tract infection on the specificity of the detection biomarkers and further evaluate its efficacy. Because most inflammatory diseases of the urinary system share a common molecular pathway, it is conceivable that potential biomarkers in urine may not be specific to a particular disease.[83] Above all, more clinical trials are needed to further validate the diagnostic efficacy of novel noninvasive biomarkers and therefore promote the translation of basic research into clinical practice.

In conclusion, noninvasive biomarkers may provide a new turning point for disease diagnosis, monitoring, and treatment guidance in patients with LN, avoiding the hazards of repeat renal biopsy and untimely diagnosis. A better understanding of the distinct biological role and molecular mechanisms of noninvasive biomarkers in LN is also crucial for the development of possible therapeutic targets and strategies. Therefore, in addition to aiding in the diagnosis, monitoring, and treatment guidance of LN, noninvasive biomarkers may act as possible therapeutic targets for LN and must be investigated in detail in the future.

Funding

This work was supported by Sichuan Science and Technology Program (Grant No. 2023YFS0186 and 2023YFS0222) and the Clinical Research Foundation of West China Second University Hospital (Grant No. KL076).

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Roccatello D, Fenoglio R, Caniggia I, et al. Daratumumab monotherapy for refractory lupus nephritis. *Nat Med.* 2023;29(8):2041-2047.
2. Furie R, Rovin BH, Houssiau F, et al. Two-year, randomized, controlled trial of belimumab in lupus nephritis. *N Engl J Med.* 2020;383(12):1117-1128.
3. Hanly JG, O'Keeffe AG, Su L, et al. The frequency and outcome of lupus nephritis: results from an international inception cohort study. *Rheumatology.* 2016;55(2):252-262.
4. Furie RA, Aroca G, Cascino MD, et al. B-cell depletion with obinutuzumab for the treatment of proliferative lupus nephritis: a randomised, double-blind, placebo-controlled trial. *Ann Rheum Dis.* 2022;81(1):100-107.
5. Hiraki LT, Benseler SM, Tyrrell PN, Hebert D, Harvey E, Silverman ED. Clinical and laboratory characteristics and long-term outcome of pediatric systemic lupus erythematosus: a longitudinal study. *J Pediatr.* 2008;152(4):550-556.
6. Tarr T, Derfalvi B, Gyori N, et al. Similarities and differences between

pediatric and adult patients with systemic lupus erythematosus. *Lupus.* 2015;24(8):796-803.

7. Cojocaru M, Cojocaru IM, Silosi I, Vrabie CD. Manifestations of systemic lupus erythematosus. *Maedica (Bucur)*. 2011;6(4):330-336.
8. Rovin BH, Teng YKO, Ginzler EM, et al. Efficacy and safety of voclosporin versus placebo for lupus nephritis (AURORA 1): a double-blind, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet.* 2021;397(10289):2070-2080.
9. Sule S, Fivush B, Neu A, Furth S. Increased risk of death in pediatric and adult patients with ESRD secondary to lupus. *Pediatr Nephrol.* 2011;26(1):93-98.
10. Jorge A, Wallace ZS, Lu N, Zhang Y, Choi HK. Renal transplantation and survival among patients with lupus nephritis: a cohort study. *Ann Intern Med.* 2019;170(4):240-247.
11. Yu F, Haas M, Glassock R, Zhao MH. Redefining lupus nephritis: clinical implications of pathophysiologic subtypes. *Nat Rev Nephrol.* 2017;13(8):483-495.
12. Malvar A, Pirruccio P, Alberton V, et al. Histologic versus clinical remission in proliferative lupus nephritis. *Nephrol Dial Transplant.* 2017;32(8):1338-1344.
13. Fanouriakis A, Kostopoulou M, Cheema K, et al. 2019 Update of the Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of lupus nephritis. *Ann Rheum Dis.* 2020;79(6):713-723.
14. Abujam B, Cheekatla S, Aggarwal A. Urinary CXCL-10/IP-10 and MCP-1 as markers to assess activity of lupus nephritis. *Lupus.* 2013;22(6):614-623.
15. Vanarsa K, Soomro S, Zhang T, et al. Quantitative planar array screen of 1000 proteins uncovers novel urinary protein biomarkers of lupus nephritis. *Ann Rheum Dis.* 2020;79(10):1349-1361.
16. Enghard P, Rieder C, Kopetschke K, et al. Urinary CD4 T cells identify SLE patients with proliferative lupus nephritis and can be used to monitor treatment response. *Ann Rheum Dis.* 2014;73(1):277-283.
17. Badot V, Luijten RK, van Roon JA, et al. Serum soluble interleukin 7 receptor is strongly associated with lupus nephritis in patients with systemic lupus erythematosus. *Ann Rheum Dis.* 2013;72(3):453-456.
18. Soliman S, Mohan C. Lupus nephritis biomarkers. *Clin Immunol.* 2017;185:10-20.
19. Hanaoka M, Gono T, Kawaguchi Y, et al. Urinary free light chain is a potential biomarker for ISN/RPS class III/IV lupus nephritis. *Rheumatology (Oxford).* 2013;52(12):2149-2157.
20. Arazi A, Rao DA, Berthier CC, et al; Accelerating Medicines Partnership in SLE Network. The immune cell landscape in kidneys of patients with lupus nephritis. *Nat Immunol.* 2019;20(7):902-914.
21. Jayne D, Rovin B, Mysler EF, et al. Phase II randomised trial of type I interferon inhibitor anifrolumab in patients with active lupus nephritis. *Ann Rheum Dis.* 2022;81(4):496-506.
22. Maria NI, Davidson A. Protecting the kidney in systemic lupus erythematosus: from diagnosis to therapy. *Nat Rev Rheumatol.* 2020;16(5):255-267.
23. Faurschou M, Starklint H, Halberg P, Jacobsen S. Prognostic factors in lupus nephritis: diagnostic and therapeutic delay increases the risk of terminal renal failure. *J Rheumatol.* 2006;33(8):1563-1569.
24. Marchini M, Antonioli R, Lleo A, et al. HLA class II antigens associated with lupus nephritis in Italian SLE patients. *Hum Immunol.* 2003;64(4):462-468.
25. Anders HJ, Lichtnekert J, Allam R. Interferon-alpha and -beta in kidney inflammation. *Kidney Int.* 2010;77(10):848-854.
26. Su YJ, Lin IC, Wang L, Lu CH, Huang YL, Kuo HC. Next generation sequencing identifies miRNA-based biomarker panel for lupus nephritis. *Oncotarget*. 2018;9(46):27911-27919.
27. Rodriguez-Jimenez NA, Perez-Guerrero EE, Gamez-Nava JI, et al. Anti-nucleosome antibodies increase the risk of renal relapse in a prospective cohort of patients with clinically inactive systemic lupus erythematosus. *Sci Rep.* 2020;10(1):12698.
28. Pickering MC, Botto M. Are anti-C1q antibodies different from other SLE autoantibodies? *Nat Rev Rheumatol.* 2010;6(8):490-493.
29. Tan Y, Yu F, Yang H, Chen M, Fang Q, Zhao MH. Autoantibodies against monomeric C-reactive protein in sera from patients with lupus nephritis are associated with disease activity and renal tubulointerstitial lesions. *Hum Immunol.* 2008;69(12):840-844.
30. Kondo A, Takahashi K, Mizuno T, et al. The level of IgA antibodies to endothelial cells correlates with histological evidence of disease activity in patients with lupus nephritis. *PLoS One.* 2016;11(10):e0163085.
31. Lopez P, Rodriguez-Carrio J, Caminal-Montero L, Mozo L, Suarez A. A pathogenic IFN alpha, BLyS and IL-17 axis in systemic lupus erythematosus patients. *Sci Rep.* 2016;6:20651.
32. Parodis I, Zickert A, Sundelin B, et al. Evaluation of B lymphocyte stimulator and a proliferation inducing ligand as candidate biomarkers in lupus nephritis based on clinical and histopathological outcome following induction therapy. *Lupus Sci Med*. 2015;2(1):e000061.
33. Fava A, Rao DA, Mohan C, et al; Accelerating Medicines Partnership in Rheumatoid Arthritis and Systemic Lupus Erythematosus Network. Urine proteomics and renal single-cell transcriptomics implicate interleukin-16 in lupus nephritis. *Arthritis Rheumatol.* 2022;74(5):829-839.
34. Hayry A, Faustini F, Zickert A, et al. Interleukin (IL) 16: a candidate urinary biomarker for proliferative lupus nephritis. *Lupus Sci Med.* 2022;9(1):e000744.
35. Nassif MA. Urine and serum interleukin 35 as potential biomarkers of lupus nephritis. *Cent Eur J Immunol*. 2021;46(3):351-359.
36. Cai Z, Zhang S, Wu P, et al. A novel potential target of IL-35-regulated JAK/STAT signaling pathway in lupus nephritis. *Clin Transl Med.* 2021;11(2):e309.
37. Abozaid MA, Ahmed GH, Tawfik NM, Sayed SK, Ghandour AM, Madkour RA. Serum and urine monocyte chemoattractant protein-1 as a markers for lupus nephritis. *Egypt J Immunol.* 2020;27(1):97-107.
38. Singh RG, Usha, Rathore SS, Behura SK, Singh NK. Urinary MCP-1 as diagnostic and prognostic marker in patients with lupus nephritis flare. *Lupus.* 2012;21(11):1214-1218.
39. Bauer JW, Baechler EC, Petri M, et al. Elevated serum levels of interferon-regulated chemokines are biomarkers for active human systemic lupus erythematosus. *PLoS Med.* 2006;3(12):e491.
40. Kulkarni O, Anders HJ. Chemokines in lupus nephritis. *Front Biosci.* 2008;13:3312-3320.
41. Fu Q, Chen X, Cui H, et al. Association of elevated transcript levels of interferon-inducible chemokines with disease activity and organ damage in systemic lupus erythematosus patients. *Arthritis Res Ther.* 2008;10(5):R112.
42. Lloyd CM, Dorf ME, Proudfoot A, Salant DJ, Gutierrez-Ramos JC. Role of MCP-1 and RANTES in inflammation and progression to fibrosis during murine crescentic nephritis. *J Leukoc Biol.* 1997;62(5):676-680.
43. Klocke J, Kopetschke K, Griessbach AS, et al. Mapping urinary chemokines in human lupus nephritis: potentially redundant pathways recruit CD4(+) and CD8(+) T cells and macrophages. *Eur J Immunol.* 2017;47(1):180-192.
44. Tsai CY, Wu TH, Yu CL, Lu JY, Tsai YY. Increased excretions of beta2- microglobulin, IL-6, and IL-8 and decreased excretion of Tamm- Horsfall glycoprotein in urine of patients with active lupus nephritis. *Nephron.* 2000;85(3):207-214.Jakiela B, Kosalka J, Plutecka H, et al. Urinary cytokines and mRNA expression as biomarkers of disease activity in lupus nephritis. *Lupus.* 2018;27(8):1259-1270.
45. Marie MA, Abu Khalil RE, Habib HM. Urinary CXCL10: a marker of nephritis in lupus patients. *Reumatismo*. 2014;65(6):292-297.
46. Lee HT, Shiao YM, Wu TH, et al. Serum BLC/CXCL13 concentrations and renal expression of CXCL13/CXCR5 in patients with systemic lupus erythematosus and lupus nephritis. *J Rheumatol.* 2010;37(1):45-52.
47. Wen S, He F, Zhu X, Yuan S, Liu H, Sun L. IFN-gamma, CXCL16, uPAR: potential biomarkers for systemic lupus erythematosus. *Clin Exp Rheumatol.* 2018;36(1):36-43.

48. El-Shehaby A, Darweesh H, El-Khatib M, et al. Correlations of urinary biomarkers, TNF-like weak inducer of apoptosis (TWEAK), osteoprotegerin (OPG), monocyte chemoattractant protein-1 (MCP-1), and IL-8 with lupus nephritis. *J Clin Immunol.* 2011;31(5):848-856.
49. Susianti H, Hanggara DS, Lestari KD, Purnamasari P, Aprilia A. Analysis of TNF-like weak inducer of apoptosis for detecting lupus nephritis. *Comp Clin Path*. 2022;31(2):313-316.
50. Gupta R, Aggarwal A, Sinha S, et al. Urinary osteoprotegerin: a potential biomarker of lupus nephritis disease activity. *Lupus.* 2016;25(11):1230-1236.
51. Qi L, Xu J, Yang C, Hou X, Yang P. Urinary ferritin creatinine ratio, a potential biomarker for lupus nephritis activity. *Clin Rheumatol.* 2021;40(1):143-149.
52. Wu T, Ding H, Han J, et al. Antibody-array-based proteomic screening of serum markers in systemic lupus erythematosus: a discovery study. *J Proteome Res.* 2016;15(7):2102-2114.
53. Li H, Liang J, Gao Y, et al. IGFBP2 function as a novel biomarker for active lupus nephritis. *J Mol Med (Berl).* 2022;100(10):1479-1491.
54. Al-Lamki RS, Wang J, Skepper JN, Thiru S, Pober JS, Bradley JR. Expression of tumor necrosis factor receptors in normal kidney and rejecting renal transplants. *Lab Invest.* 2001;81(11):1503-1515.
55. Patel M, Oni L, Midgley A, et al. Increased concentration of plasma TNFR1 and TNFR2 in paediatric lupus nephritis. *Lupus.* 2016;25(9):1040-1044.
56. Yao GH, Liu ZH, Zhang X, et al. Circulating thrombomodulin and vascular cell adhesion molecule-1 and renal vascular lesion in patients with lupus nephritis. *Lupus.* 2008;17(8):720-726.
57. Mok CC, Soliman S, Ho LY, Mohamed FA, Mohamed FI, Mohan C. Urinary angiostatin, CXCL4 and VCAM-1 as biomarkers of lupus nephritis. *Arthritis Res Ther.* 2018;20(1):6.
58. Torres-Salido MT, Sanchis M, Sole C, et al. Urinary neuropilin-1: a predictive biomarker for renal outcome in lupus nephritis. *Int J Mol Sci*. 2019;20(18):4601.
59. Parodis I, Ding H, Zickert A, et al. Serum AXL predicts histology-based response to induction therapy and long-term renal outcome in lupus nephritis. *PLoS One.* 2019;14(2):e0212068.
60. Dolff S, Abdulahad WH, Arends S, et al. Urinary CD8+ T-cell counts discriminate between active and inactive lupus nephritis. *Arthritis Res Ther.* 2013;15(1):R36.
61. Kopetschke K, Klocke J, Griessbach AS, et al. The cellular signature of urinary immune cells in lupus nephritis: new insights into potential biomarkers. *Arthritis Res Ther.* 2015;17(1):94.
62. Abdelati AA, Eshak NY, Donia HM, El-Girby AH. Urinary cellular profile as a biomarker for lupus nephritis. *J Clin Rheumatol.* 2021;27(8):e469-e476.
63. Wing JB, Kitagawa Y, Locci M, et al. A distinct subpopulation of CD25(-) T-follicular regulatory cells localizes in the germinal centers. *Proc Natl Acad Sci U S A.* 2017;114(31):E6400-E6409.
64. Yu C, Li P, Dang X, Zhang X, Mao Y, Chen X. Lupus nephritis: new progress in diagnosis and treatment. *J Autoimmun.* 2022;132:102871.
65. Moroni G, Quaglini S, Radice A, et al. The value of a panel of autoantibodies for predicting the activity of lupus nephritis at time of renal biopsy. *J Immunol Res*. 2015;2015:106904.
66. Ho A, Magder LS, Barr SG, Petri M. Decreases in anti-double-stranded DNA levels are associated with concurrent flares in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2001;44(10):2342-2349.
67. Ho A, Barr SG, Magder LS, Petri M. A decrease in complement is associated with increased renal and hematologic activity in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2001;44(10):2350-2357.
68. Corapi KM, Chen JL, Balk EM, Gordon CE. Bleeding complications of native kidney biopsy: a systematic review and meta-analysis. *Am J Kidney Dis.* 2012;60(1):62-73.
69. De Rosa M, Azzato F, Toblli JE, et al. A prospective observational cohort study highlights kidney biopsy findings of lupus nephritis patients in remission who flare following withdrawal of maintenance therapy. *Kidney Int.* 2018;94(4):788-794.
70. Hill GS, Delahousse M, Nochy D, et al. Predictive power of the second renal biopsy in lupus nephritis: significance of macrophages. *Kidney Int.* 2001;59(1):304-316.
71. Zhou X, Zhang Y, Wang N. Systematic identification of key extracellular proteins as the potential biomarkers in lupus nephritis. *Front Immunol.* 2022;13:915784.
72. Maravillas-Montero JL, Reyes-Huerta RF. Update on novel blood-based biomarkers for lupus nephritis beyond diagnostic approaches. *Rev Invest Clin.* 2022;74(5):227-231.
73. Lodi L, Mastrolia MV, Bello F, et al. Type I interferon-related kidney disorders. *Kidney Int.* 2022;101(6):1142-1159.Li Y, Fang X, Li QZ. Biomarker profiling for lupus nephritis. *Genom Proteom Bioinform.* 2013;11(3):158-165.
74. Tang C, Zhang S, Teymur A, et al. V-set immunoglobulin domain-containing protein 4 as a novel serum biomarker of lupus nephritis and renal pathology activity. *Arthritis Rheumatol.* 2023;75(9):1573-1585.
75. Chun HY, Chung JW, Kim HA, et al. Cytokine IL-6 and IL-10 as biomarkers in systemic lupus erythematosus. *J Clin Immunol.* 2007;27(5):461-466.
76. Li KY, Tam CHT, Liu H, et al; TRANSCEND Consortium. DNA methylation markers for kidney function and progression of diabetic kidney disease. *Nat Commun.* 2023;14(1):2543.
77. Liu C, Ma K, Zhang Y, et al. Kidney diseases and long non-coding RNAs in the limelight. *Front Physiol.* 2022;13:932693.
78. Enghard P, Humrich JY, Rudolph B, et al. CXCR3+CD4+ T cells are enriched in inflamed kidneys and urine and provide a new biomarker for acute nephritis flares in systemic lupus erythematosus patients. *Arthritis Rheum.* 2009;60(1):199-206.
79. Peterson KS, Huang JF, Zhu J, et al. Characterization of heterogeneity in the molecular pathogenesis of lupus nephritis from transcriptional profiles of laser-captured glomeruli. *J Clin Invest.* 2004;113(12):1722-1733.
80. Geldenhuys L, Nicholson P, Sinha N, et al. Percutaneous native renal biopsy adequacy: a successful interdepartmental quality improvement activity. *Can J Kidney Health Dis*. 2015;2:8.
81. Li Y, Tucci M, Narain S, et al. Urinary biomarkers in lupus nephritis. *Autoimmun Rev.* 2006;5(6):383-388.

SCIENCE

Differences in the levels of the appetite peptides ghrelin, peptide tyrosine tyrosine, and glucagon-like peptide-1 between obesity classes and lean controls

Gülşah Alyar, PhD, [1,*] *Fatma Zühal Umudum, PhD,* [1]

[1]Department of Transfusion Medicine, Sanjay Gandhi Postgraduate Institute Medical Sciences, Lucknow, India. *To whom correspondence should be addressed: rkcsgpgi@gmail.com.

Keywords: β thalassemia, DAT, ether elution, auto antibody, splenectomy, hepatitis C, serum ferritin, IgG

Abbreviations: DAT, direct antiglobulin test; IgG, immunoglobulin G; RBC, red blood cell; Hb, hemoglobin; AIHA, autoimmune hemolytic anemia; HCV, hepatitis C virus; AHG, antihuman globulin; ELISA, enzyme-linked immunosorbent assay; AOR, adjusted odds ratio

Laboratory Medicine 2023;54:406-410; https://doi.org/10.1093/labmed/lmac140

ABSTRACT

Objective: This study was conducted to estimate prevalence of direct antiglobulin test (DAT) positivity and its impact on transfusion support in patients with thalassemia.

Methods: The DAT testing was performed for patients with β-thalassemia who received transfusion from November 2021 to March 2022. Elution was done for DAT-positive samples.

Results: Of 180 patients, 21 (11.6%) were DAT positive. Immunoglobulin G (IgG) was present in 4 (19%) and IgG+C3d was present in 8 (38%). Only complement was present in 9 (42.8%) patients. The IgG-reactive DATs were associated with pan-reactive eluate. Patients who were DAT-positive had significantly higher levels of serum bilirubin, ferritin, and IgG than those who were DAT-negative.

Conclusion: Autoantibody formation in multiply transfused thalassemia patients is common and merits equal attention as alloimmunization. It is particularly important as DAT-positive red blood cells may undergo clinically significant hemolysis, which may increase the transfusion requirements with associated sequalae such as increased serum ferritin and splenomegaly.

Thalassemia is an inherited hemolytic disorder caused by partial or complete defect in α- or β-globin chain synthesis. The curative treatment for thalassemia is stem cell transplant, in the absence of which severe disease is managed by regular and lifelong red blood cell (RBC) transfusion to keep the hemoglobin (Hb) level between 9 and 11.5 g/dL.[1] Gene therapy by autologous hematopoietic stem cells modified with a lentiviral vector expressing the β-globin gene is a promising approach to completely cure β thalassemia.[2]

A major complication of transfusion therapy in thalassemia is the development of anti-RBC antibodies (alloantibodies and/or autoantibodies). Although formation of alloantibodies occurs due to phenotypic differences between donor and patient RBCs, immunologic disturbances arising from chronic overstimulation of the immune system caused by the joint effect of allogeneic transfusions and splenectomy are responsible for the development of autoantibodies.[3] It is hypothesized that the missing role of the spleen in filtering damaged and conformationally changed RBCs after splenectomy may further augment the immune response and lead to the formation of autoantibodies.[4] Thus, it is not uncommon to find positive direct antiglobulin tests (DATs) in thalassemic patients even when phenotypically matched RBCs have been transfused.

The prevalence of RBC autoantibodies has been estimated to be 6.5% in chronically or intermittently transfused patients[3] as compared to 1.4% in the general population.[5] The autoantibodies can remain silent or cause accelerated clearance of RBCs and autoimmune hemolytic anemia (AIHA),[5] resulting in clinical hemolysis. Autoantibodies may also cause difficulty in cross-matching blood and shorten the duration of RBC survival, requiring immunosuppressive therapy or splenectomy. In some patients, there are anti-RBC autoantibodies present that give a positive DAT without notable RBC destruction.[5] The antibody eluted from the RBCs in such cases often reacts with all RBCs except Rh null RBCs. This prospective study was conducted to estimate the prevalence of DAT positivity and its impact on transfusion support in thalassemia patients. We also studied the association of various clinical and laboratory parameters with DAT positivity.

Materials and Methods

This study was conducted prospectively on participants with β thalassemia for a period of 5 months (from November 2021 to March 2022) after

obtaining written informed consent and a waiver from the institutional ethical review board. Patients with connective tissue and autoimmune diseases were excluded from the study.

We used EDTA samples received for routine compatibility testing. No extra sample was taken. Plasma was separated and stored at −80°C in aliquots, which were thawed at room temperature later and used for antibody screening, anti-hepatitis C (HCV) antibody testing, and total serum immunoglobulin G (IgG) estimation. The RBCs were used for performing the DAT.

The DAT was performed by conventional tube technique using AABB standard.[6] Briefly, 1 drop of 2% to 5% suspension of RBCs was dispensed into test tubes and washed 3 times with normal saline, the final wash decanted completely. Two drops of polyspecific antihuman globulin (AHG) reagent (Eryclone Anti-Human Globulin, Tulip Diagnostics) were then added, mixed well, tube-centrifuged at 1000*g* for 30 s, and cells were examined for agglutination. All reactions were graded and recorded.[4] Further distinction of autoantibody was done using monospecific gel cards (IgG/C3cC3d) (DC-Screening I, DiaMed)

Ether elution was performed on all DAT positive samples following the standard method[7] and eluate was tested for the presence of antibody using reagent RBC panel in AHG phase. Antibody screening and identification was performed by the column agglutination technique using commercially available 3-cell (ID-Diacell I-II-III DiaMed) and 11-cell panels (ID-Diapanel, DiaMed), respectively.

Serum IgG concentrations were quantified using the enzyme-linked immunosorbent assay (ELISA) method (Invitrogen, ThermoFisher Scientific). Maximum detection limit of this assay is 0.105 µg/mL (52,500 IU/µL).

Screening for anti-HCV antibody was performed by an enzyme-linked fluorescent assay using a VIDAS Hepatitis panel (bioMérieux Clinical Diagnostics).

Clinical details such as age, sex, age at first transfusion, splenectomy, and transfusion records including number of units transfused and the interval between transfusions, were obtained from the hospital information system. Other biochemical parameters such as hemoglobin, serum total bilirubin, serum conjugated bilirubin, and serum ferritin were obtained from patient files.

Statistical Analysis

All statistical tests were performed using IBM SPSS software for Windows version 20 (IBM). Categorical variables were presented as number and proportions, and continuous variables were presented as median and quartile (χ^2 test). Association was shown by regression (binary logistic). A *P* value of <.05 was considered significant.

Results

A total of 180 participants with β thalassemia who were on regular transfusion therapy over a period of November 2021 to March 2022 at our center were included. Of the 180 patients, 161 (90%) were diagnosed with β thalassemia major and 19 (10%) were diagnosed with β thalassemia intermedia. The majority of these patients were treated exclusively at our institution for thalassemia for the entirety of the study period. Of the 180 patients, 21 (11.6%) were positive for DAT using polyspecific AHG reagent. Patient clinical and demographic details are given in **TABLE 1**. A majority of the patients were male (125/180, 69.4%) with a median age of 13 years. A total of 29 patients (29/180, 16.1%) had undergone splenectomy.

TABLE 2 shows laboratory features in DAT-positive and DAT-negative patients. Alloantibody was present in only 1 DAT-positive patient compared to 9 (5.7%) DAT-negative patients; the difference was not significant (*P* = .866). Only IgG was present in 4 (19%), IgG+C3d was present in 8 (38%), and in the remaining 9 (42.8%) patients, RBCs were coated with complement only. Eluate was pan-reactive in all IgG-coated RBCs and negative in complement-coated RBCs. All DAT-positive patients had significantly higher levels of total bilirubin, serum ferritin, and total serum IgG level compared to DAT-negative patients (*P* < .05). The rate of HCV positivity was also found to be higher in the DAT-positive group than in the DAT-negative group (28.6% vs 1.2%) (*P* < .05).

TABLE 3 shows transfusion requirements in the patients. It was found that total number of units (median) transfused per year and annual consumption of RBCs in milliliters per kilogram per year were higher in DAT-positive patients, 31 vs 24 units and 228.5 vs 152 mL, respectively (*P* < .05).

Although the interval between 2 transfusions was significantly less in DAT-positive patients, alloantibodies were detected in 9 (5%) DAT-negative patients and 1 (4.7%) DAT-positive patient. The specificity of the alloantibodies mostly belonged to RH, Kell, and Kidd specificities (**TABLE 4**).

A positive DAT was associated with splenectomy (*P* = .018), elevated IgG level (*P* = .021), positive HCV Ab (*P* = .018), increased serum ferritin (*P* = .036), decreased transfusion interval (*P* = .018), and increased consumption of RBCs per year (*P* = .013) on multivariate analysis (**TABLE 5**).

Discussion

Although alloimunization is a known complication in multiply transfused thalassemia patients, autoantibody formation is less commonly reported in the literature. Autoantibodies in these patients cause

TABLE 1. Clinical Details of Thalassemia Patients with and without DAT Positivity[a]

Variable	All Patients (n = 180)	DAT-Positive Patients (n = 21)	DAT-Negative Patients (n = 159)	*P* Value
Age, median (quartile), y	13 (9-21)	15 (12.5-22.5)	12 (9-21)	.082
Male	125 (69.4)	16 (76.2)	109 (68.6)	.457
Female	55 (30.6)	5 (23.8)	50 (31.4)	
Thalassemia major	161 (89.4)	20 (95.2)	141 (88.7)	
Thalassemia Intermedia	19 (10.6)	1 (4.8)	18 (11.3)	
Age at first transfusion, median (quartile), y	0.58 (0.41-1.5)	0.75 (0.57-1.13)	0.58 (0.41-1.1.50)	.459
Splenectomy	29 (16.1)	10 (47.6)	19 (11.9)	<.001

DAT, direct antiglobulin test.
[a]*Data are given as No. (%) except where indicated.*

TABLE 2. Laboratory Features of Thalassemia Patients with and without DAT Positivity

Laboratory Feature	DAT-Positive (n = 21)	DAT-Negative (n = 159)	*P* Value
Alloimmunization, No. (%)	1 (4.8)	9 (5.7)	.866
Auto antibody, No. (%)			
IgG+C3d	8 (38)	NA	
IgG only	4 (19)		
C3d only	9 (42.8)		
Total bilirubin, median (quartile), mg/dL	1.7 (1.1-2.2)	1.1 (0.7-1.87)	.011
Conjugated bilirubin, median (quartile), mg/dL	0.75 (0.57-1.13)	0.6 (0.44-0.86)	.109
Serum ferritin, median (quartile), ng/mL	3300 (2627-4466)	2090 (1746-2598)	<.001
Total serum IgG[a] level >52,500 IU/dL, No. (%)	16 (76.2)	28 (48.3)	.027
Anti-HCV antibody positive, No. (%)	6 (28.6)	2 (1.2)	<.001

DAT, direct antiglobulin test; HCV, hepatitis C virus; IgG, immunoglobulin G; NA, not applicable.
[a]Measured in all DAT-positive and 58 DAT-negative patients

TABLE 3. Transfusion Support in Thalassemia Patients

Variable	DAT-Positive (n = 21)	DAT-Negative (n = 159)	*P* Value
No. of units transfused/y, median (quartile)	31 (25-38)	24 (21-26)	<.001
Average interval between 2 consecutive transfusions, median (quartile), d	22 (15-28)	27 (24-30)	.002
Annual consumption of RBCs, median (quartile), mL/kg/y	228.5 (138.9-297.1)	152 (115-208)	.013

DAT, direct antiglobulin test.

TABLE 4. Specificity of Alloantibodies in DAT-Positive and DAT-Negative Patients

Antibody	DAT-Positive (n = 21)	DAT-Negative (n = 159)	Total
Anti-E	0	1	1
Anti D + Anti C	0	1	1
Anti C + Anti K	0	1	1
Anti E + Anti K	0	2	2
Anti-K	1	3	4
Anti Jk[a]	0	1	1

DAT, direct antiglobulin test.

hemolysis of the patient's own RBCs as well as transfused RBCs. The DAT is the most sensitive diagnostic tool for detection of in vivo sensitization of RBCs.

A DAT positivity in thalassemia patients can either be due to autoantibodies or residual donor RBCs coated with alloantibodies. However, in our study, DAT positivity was due only to autoantibodies and not alloantibodies.

In the current study, the rate of DAT positivity among patients with β-thalassemia was 11.6% (21/180). The prevalence of autoantibody formation in patients with thalassemia in other countries has been reported as 28.8% in Egypt,[8] 23% in Hong Kong,[9] 22.8% in Albania,[10] and 6.5% in the United States.[11]

In a study from India, Dhawan et al[1] reported a very high rate of autoantibody formation (28.2 %) among 319 multiple transfused patients with β thalassemia major. Similarly, another study from India reported a 15.9% rate of autoantibody formation in this group of patients.[12]

In our study, DAT positivity due to IgG+ complement was found to be an independent predictor of clinically significant hemolysis in patients with β thalassemia with underlying RBC autoimmunity.[5] Amen et al[13] noted that 52% of the autoantibodies detected in their subjects were of IgG in nature; however, none of them caused significant hemolysis. Singer et al[4] found autoantibodies in 25% of those with thalassemia, having IgG specificity in 68.8% and C3d specificity in 32.2% of patients, respectively. Clinically significant AIHA developed in 3 patients: IgG-induced in 1 and IgG with complement in the other 2.[4] In a study from India, IgG only was detected on 56.3% of DAT-positive RBCs.[12] It has been reported that patients with thalassemia major show a significant lymphocytosis, with mainly B-cell changes consistent with ongoing B-cell stimulation associated with chronic exposure to RBC antigens.[14] This B-cell stimulation results in an increase in serum immunoglobulin, immune complexes, and RBCs expressing surface immunoglobulin.

Age at the start of transfusion plays a role in the alloantibody and autoantibody formation. Previous studies have reported a lower frequency of autoantibody formation associated with the early onset of transfusion.[15,16] Consistent with the previous studies, we also observed that early onset of transfusion (0.58 year) was associated with low frequency of autoantibody formation (159/180, 88.3%) compared to late onset (0.75 year) (21/180, 11.6%), however, the difference was not statistically significant ($P = .459$) (**TABLE 1**). Some authors reported no association between autoantibodies and age of start of transfusion ($P = .5$, $P = .8$, respectively).[8,17]

In our study, 47.6% of DAT-positive patients had undergone splenectomy compared to 11.9% of the DAT-negative participants ($P < .001$). Singer et al[4] observed that 56% of the patients with autoantibodies had undergone splenectomy in their group of patients.

TABLE 5. Multivariate Analysis of DAT-Positive Patients

Variable	Regression Coefficient (β)	Adjusted Odds Ratio (95% Confidence Interval)	*P* Value
Serum IgG	−6.237	0.002 (0.000-0.387)	.021
Serum bilirubin conjugated	−3.115	0.044 (0.002-1.180)	.061
Serum ferritin	−0.002	0.998 (0.997-1.000)	.036
Interval between 2 consecutive transfusions	0.584	1.794 (1.106-2.9098)	.018
Annual consumption of packed RBCs	−0.054	0.948 (0.909-0.989)	.013
HCV reactivity	−7.62	0.000 (0.000-0.264)	.018
Splenectomy	−10.3	0.000 (0.000-0.169)	.018

DAT, direct antiglobulin test; HCV, hepatitis C virus; IgG, immunoglobulin G.

Arinsburg et al[3] reported an even higher number of patients with splenectomy (88.1%).

Persons with thalassemia who have undergone splenectomy have higher levels of serum IgG, which is not unexpected, as the spleen is not clearing the RBCs coated with autoantibodies.[18] In our study, 76.2% of DAT-positive patients had serum IgG levels greater than 52,500 IU/dL in contrast to 48.3% of DAT-negative patients (*P* = .027, **TABLE 2**). Arinsburg et al[3] noted elevated IgG levels in 88.1% of DAT-positive patients. Multiple studies in general patient populations have shown that increased serum IgG levels are associated with a positive DAT. [19,20] Immunological abnormalities in those with thalassemia, including an expansion of circulating B cells and a modest polyclonal gammopathy, are thought to play a role in the abnormal IgG levels.

The HCV positivity among DAT-positive patients was 28.6% in our study, which was significantly higher than that in DAT-negative patients (*P* < .001, **TABLE 2**). Arinsburg et al[3] reported 60% HCV positivity among DAT-positive subjects. The HCV has been implicated both in the autoantibody formation and autoimmune diseases. The HCV might be involved in the breakdown of self-tolerance triggering autoreactivity.[21] Bhattachrya et al[22] reported very high prevalence of anti-HCV antibodies in multiply transfused thalassemia patients in India. Of 300 patients,75(25%) were found to be HCV positive by ELISA. Among them, 49 (65%) were HCV RNA positive with significant viral load in their blood.

Serum ferritin levels were significantly higher in DAT-positive patients in our study (3300 ng/mL vs 2090 ng/mL, *P* < .001). This could be due to the combined effect of more transfusions and the higher rate of splenectomy in DAT-positive patients[22] or simply an increased number of transfusions in DAT-positive patients.[23]

The transfusion response in terms of number of units transfused per year, the interval between 2 transfusions, and annual consumption of RBCs was significantly higher among the DAT-positive than the DAT-negative patients (**TABLE 3**). This indicates that DAT-positive RBCs may be undergoing hemolysis more profoundly than DAT-negative RBCs, which is also corroborated by increased levels of serum conjugated bilirubin in DAT-positive patients. However, a previous study reported similar transfusion response among DAT-positive and DAT-negative patients.[3]

On multivariate analysis, a positive DAT in thalassemia was associated with elevated serum IgG levels (adjusted odds ratio [AOR] 0.002, *P* = .021), elevated serum ferritin (AOR 0.998, *P* = .036), decreased interval between transfusions (AOR 1.794, *P* = .018), increased annual consumption of RBCs (AOR 0.948, *P* = .013), HCV positivity (AOR 0.00, *P* = .018), and splenectomy (AOR 0.000, *P* = .018).

We did not find any association between alloimmunization and DAT positivity, which is consistent with Arinsburg et al.[3] In contrast, Jain et al[12] reported significantly higher alloimmunization in patients with positive DAT.

Raised serum conjugated bilirubin level coupled with increased transfusion requirements and decreased transfusion interval in DAT-positive thalassemia patients indicates the development of AIHA. However, due to lack of all the clinical and baseline laboratory information, the DAT-positive subjects in our study cannot be confirmed to have AIHA. This is one of the limitations of this study. Khaled et al[5] reported 25 cases of AIHA among 87 DAT-positive patients. However, it is important for the clinician to closely monitor DAT-positive thalassemia patients for any signs of worsening clinical and laboratory features of hemolysis to rule out the possibility of development of AIHA so that therapeutic interventions can be planned.

Thus, the phenomenon of autoantibody formation in multiply transfused thalassemia patients is not uncommon and merits the same attention as alloimmunization. It is particularly important, as DAT-positive RBCs may undergo clinically significant hemolysis, which may increase the transfusion requirements with associated sequalae such as increased serum ferritin and splenomegaly.

REFERENCES

1. Dhawan HK, Kumawat V, Marwaha N, et al. Alloimmunization and autoimmunization in transfusion dependent thalassemia major patients: study on 319 patients. *Asian J Transfus Sci.* 2014;8(2):84–88. doi:10.4103/0973-6247.137438.
2. Rattananon P, Anurathapan U, Bhukhai K, Hongeng S. The future of gene therapy for transfusion-dependent beta-thalassemia: the power of the lentiviral vector for genetically modified hematopoietic stem cells. *Front Pharmacol.* 2021;12:730873. doi:10.3389/fphar.2021.730873.
3. Arinsburg SA, Skerrett DL, Kleinert D, Giardina PJ, Cushing MM. The significance of a positive DAT in thalassemia patients. *Immunohematology.* 2010;26(3):87–91.
4. Singer ST, Wu V, Mignacca R, Kuypers FA, Morel P, Vichinsky EP. Alloimmunization and erythrocyte autoimmunization in transfusion-dependent thalassemia patients of predominantly Asian descent. *Blood.* 2000;96(10):3369–3373.
5. Khaled MB, Ouederni M, Sahli N, et al. Predictors of autoimmune haemolytic anemia in beta-thalassemia patients with underlying red blood cells autoantibodies. *Blood Cells Mol Dis.* 2019;79:102342.

6. Cohn CS, Delaney M, Johnson ST and Katz LM. *Technical Manual*. 20th ed. Bethesda, MD: AABB; 2020.
7. Rubin H. Antibody elution from red blood cells. *J Clin Pathol.* 1963;16(1):70–73.
8. Ahmed AM, Hasan NS, Ragab SH, Kuypers FA, Morel P, Vichinsky EP. Red cell alloimmunization and autoantibodies in Egyptian transfusion-dependent thalassaemia patients. *Arch Med Sci.* 2010;6(4):592–598.
9. Cheng CK, Lee CK, Lin CK. Clinically significant red blood cell antibodies in chronically transfused patients: a survey of Chinese thalassemia major patients and literature review: RBC antibodies in thalassemia major. *Transfusion.* 2012;52(10):2220–2224. doi:10.1111/j.1537-2995.2012.03570.x.
10. Seferi I, Xhetani M, Face M, Burazeri G, Nastas E, Vyshka G. Frequency and specificity of red cell antibodies in thalassemia patients in Albania. *Int J Lab Hematol.* 2015;37(4):569–574. doi:10.1111/ijlh.12362.
11. Vichinsky E, Neumayr L, Trimble S, et al. CDC thalassemia investigators. transfusion complications in thalassemia patients: a report from the Centers for Disease Control and Prevention (CDC). *Transfusion.* 2014;54(4):971–972.
12. Jain A, Agnihotri A, Marwaha N, Sharma RR. Direct antiglobulin test positivity in multi-transfused thalassemics. *Asian J Transfus Sci.* 2016;10(2):161–163. doi:10.4103/0973-6247.164268.
13. Amen R, Shemmari SA, Humood SA, Chawdhury RI, Eyaadi OA, Bashir AA. RBC alloimmunization and autoimmunization among transfusion-dependent thalassemia patients. *Transfusion*. 2003;43(11):1604–1610.
14. Hodge G, Lloyd JV, Hodge S, Story C, Han P. Functional lymphocyte immunophenotypes observed in thalassaemia and haemophilia patients receiving current blood product preparations. *Br J Haematol.* 1999;105(3):817–825. doi:10.1046/j.1365-2141.1999.01385.x.
15. Michail-Merianou V, Pamphili-Panousopoulou L, Piperi-Lowes L, Pelegrinis E, Karaklis A. Alloimmunization to red cell antigens in thalassemia: comparative study of usual versus better-match transfusion programmes. *Vox Sang.* 1987;52(1-2):95–98.
16. Spanos T, Karageorga M, Ladis V, Peristeri J, Hatziliami A, Kattamis C. Red cell alloantibodies in patients with thalassemia. *Vox Sang.* 1990;58(1):50–55. doi:10.1111/j.1423-0410.1990.tb02055.x.
17. Noor HM, Ariffin N, Illuni HI, Rosline H. Red cell autoantibodies among thalassaemia patients in Hospital Universiti Sains Malaysia. *Singapore Med J.* 2007;48(10):922–925.
18. Tovo PA, Miniero R, Barbera C, Sacchetti L, Saitta M. Serum immunoglobulins in homozygous β-thalassemia. *Acta Haematol.* 1981;65(1):21–25. doi:10.1159/000207144.
19. Huh YO, Liu FJ, Rogge K, Chakrabarty L, Lichtiger B. Positive direct antiglobulin test and high serum immunoglobulin G values. *Am J Clin Pathol.* 1988;90(2):197–200. doi:10.1093/ajcp/90.2.197.
20. Szymanski IO, Odgren PR, Fortier NL, Snyder LM. Red blood cell associated IgG in normal and pathologic states. *Blood.* 1980;55(1):48–54.
21. McMurray RW, Elbourne K. Hepatitis C virus infection and autoimmunity. *Semin Arthritis Rheum.* 1997;26(4):689–701. doi:10.1016/s0049-0172(97)80005-4.
22. Bhattacharya KK, Biswas A, Gupta D, Sadhukhan PC. Experience of hepatitis C virus seroprevalence and its genomic diversity among transfusion dependent thalassemia patients in a transfusion centre. *Asian J Transfus Sci.* 2018;12(2):112–116.
23. Shah R, Trehan A, Das R, Marwaha RK. Serum ferritin in thalassemia intermedia. *Indian J Hematol Blood Transfus.* 2014;30(4):281–285. doi:10.1007/s12288-013-0267-y.

Evaluation of some nonroutine cardiac biomarkers among adults and children with beta-thalassemia major

Abdulkareem M Jewad [1], Ameer J Shwayel [1]

[1]Connective Tissue Diseases Research Center, [2]Biotechnology Research Center, [3]Tuberculosis and Lung Disease Research Center, [4]Rahat Breathe and Sleep Research Center, [5]Department of Internal Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. *To whom correspondence should be addressed: dr_khabbazi@yahoo.com. #First authors.

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

Laboratory Medicine 2024;54:469-472; https://doi.org/10.1093/labmed/lmac146

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 ED[9] 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

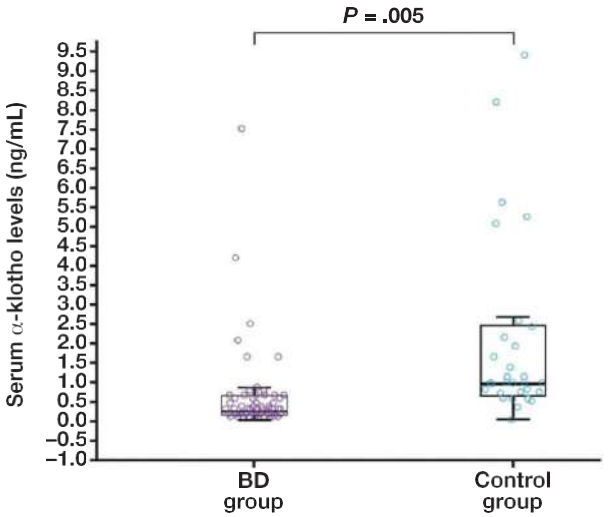
ED is characterized by decreased vasodilation and predisposes patients to prothrombotic and proinflammatory states.[16] Several study reports

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 peroxide[24]; upregulating endothelial nitric oxide synthase, the enzyme that controls nitric oxide production in endothelial cells[5]; 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).

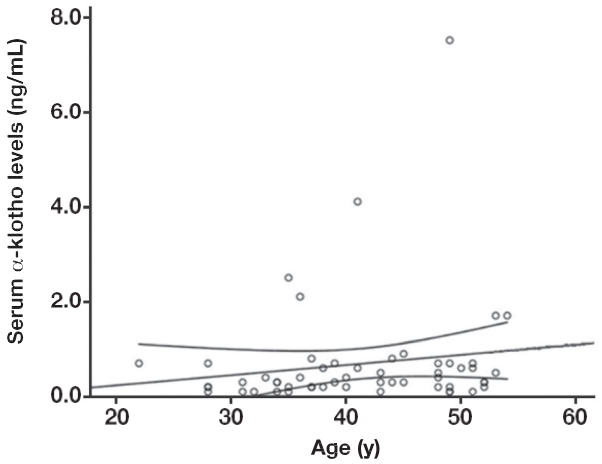


TABLE 2. Serum α-klotho Levels in Patients with BD (n = 55)

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.
[a]n = 2 were considered to be too few patients for analysis.

activation of NF-κB and suppresses the expression of TNF-α, IL-6, and IL-8.[27,28] Ushigusa et al[12] 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 al[29] reported underdevelopment of the immune system and impairment of B-cell differentiation in klotho-knockout mice. Liu et al[28] reported an anti-inflammatory property of α-klotho. It has been shown[30] 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 al[12] 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 al[13] and Hajialilo 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.

Acknowledgments

We thank L. Khabbazi who helped us in editing of the text.

Funding

This research was funded by the Connective Tissue Diseases Research Center of Tabriz University of Medical Sciences (grant No. 63671).

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. 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
2. Demirelli S, Degirmenci H, Inci S, Arisoy A. Cardiac manifestations in Behcet's disease. *Intractable Rare Dis Res.* 2015;4(2):70–75. doi:10.5582/irdr.2015.01007
3. Sadeghi A, Davatchi F, Shahram F, et al. Serum profiles of cytokines in Behcet's disease. *J Clin Med.* 2017;6(5):49.
4. 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
5. Lucherini OM, Lopalco G, Cantarini L, et al. Critical regulation of Th17 cell differentiation by serum amyloid-A signalling in Behcet's disease. *Immunol Lett.* 2018;201:38–44. doi:10.1016/j.imlet.2018.10.013
6. Kayikçioğlu 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
7. 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/s11239-022-02637-1
8. 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
9. 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/wjc.v6.i12.1262
10. 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

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.
13. Talotta R, Atzeni F, Bongiovanni S, et al. Measurement of serum alpha-klotho insystemic sclerosis patients: results from a pivotal study. *Ann Rheum Dis.* 2016;75(2):10.1136/annrheumdis-2016-eular.1683.
14. Hajialilo 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
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
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
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
19. Ozdemir R, Barutcu I, Sezgin AT, et al. Vascular endothelial function and plasma homocysteine levels in Behcet's disease. *Am J Cardiol.* 2004;94(4):522–525. doi: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
21. Caliskan M, Yilmaz S, Yildirim E, et al. Endothelial functions are more severely impaired during active disease period in patients with Behcet's disease. *Clin Rheumatol.* 2007;26(7):1074–1078. doi: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
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
25. Cui W, Leng B, Wang G. Klotho protein inhibits H_2O_2-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
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-κB activation. *Endocrine.* 2009;35(3):341–346. doi: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
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
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

Assessment of appropriate use of amylase and lipase testing in the diagnosis of acute pancreatitis at an academic teaching hospital

Valerie Ryholt, MLS, MS(ASCP)[CM1], Julie Soder, DCLS, MLS(ASCP)[CM1,2], Janet Enderle, PhD, MLS(ASCP)[CM1], Rajkumar Rajendran, DCLS, MLS(ASCP)[CM1,2]

Departments of [1]Clinical Laboratory Sciences and [2]Pathology, University of Texas Medical Branch, Galveston, TX, US. Corresponding author: Rajkumar Rajendran; rarajend@utmb.edu

Key words: amylase; lipase; overutilization; underutilization; unnecessary tests; cost savings; acute pancreatitis

Abbreviations: AP, acute pancreatitis; ACG, American College of Gastroenterology; ASCP, American Society for Clinical Pathology

Laboratory Medicine 2024;55:566-570; https://doi.org/10.1093/labmed/lmae008

ABSTRACT

Objective: Despite evidence-based guidelines stating that lipase alone should be used in the diagnosis of suspected acute pancreatitis, health care providers continue to order amylase or amylase and lipase together. The purpose of this study was to assess the utilization of appropriate laboratory testing related to the diagnosis of acute pancreatitis.

Methods: The study used a retrospective cross-sectional design. The timeframe was from January 1, 2020, to December 31, 2020. A retrospective chart review was used to collect data for the following: patient-provider encounter notes, patient demographics, provider demographics, differential and final diagnosis, and laboratory test results. Data analysis include stratification of categorical variables and calculation of cost savings.

Results: For the 12-month period, this study found 2567 (9.3%) of all amylase and lipase tests to be unnecessary. Amylase tests (1881; 73.2%) made up the most unnecessary tests followed by lipase tests (686; 26.7%). An analysis of test-ordering behavior by providers revealed that 81.5% of all unnecessary tests were ordered by MDs. Finally, this study estimated a total cost savings of $128,350 if all unnecessary tests were eliminated.

Conclusion: Our study demonstrated that amylase and lipase tests have been overutilized in the diagnosis of acute pancreatitis.

Introduction

Acute pancreatitis (AP) is an inflammatory condition of the pancreas resulting in local or systemic complications ranging from mild to severe. Acute pancreatitis is responsible for over 275,000 hospital admissions, resulting in over $2.5 billion in health care costs each year.[1] Most patients with AP have a mild form; however, those with a more severe form can experience organ failure or infected necrosis, resulting in a mortality rate of 30%.[2] In 2018, the National Confidential Enquiry into Patient Outcome and Death found that 20% of patients admitted to the hospital with AP had been previously admitted with the same condition.[3] Patients who are not properly diagnosed and assessed quickly are at risk for hospital readmissions, prolonged length of stay, intensive care unit admission, and death.[4]

According to the American College of Gastroenterology (ACG), 2 or more of the following criteria must be met for diagnosis: (1) abdominal pain suggestive of pancreatitis, (2) serum amylase or lipase level greater than 3 times the upper normal value, or (3) characteristic imaging findings.[5] Amylase is an enzyme produced primarily by the acinar cells of the pancreas and salivary glands. In AP, serum amylase rises rapidly 3 to 6 hours from onset, has a half-life of 10 to 12 hours, and remains elevated for 3 to 5 days before excretions by the kidneys.[6] Hyperamylasemia is often seen in AP; however, it can also occur in many other pancreatic and nonpancreatic conditions, including malignant conditions of the pancreas and other organs, cystic fibrosis, burns, acidosis, pregnancy, chronic alcoholism, diseases involving salivary glands, and other disease states.[7] Lipase is also a pancreatic enzyme produced primarily by pancreatic acinar cells. In AP, serum lipase will rise from 3 to 6 hours following onset, peak around 24 hours, and stay elevated for 1 to 2 weeks.[6] Although lipase can be elevated in other conditions, such as trauma, appendicitis, diabetic ketoacidosis, malignant tumors, and other disease states, it still serves as a sensitive biomarker for AP when compared with amylase due to the relative amounts of lipase produced in the pancreas over other areas of the body.[7]

Laboratory measurements of serum lipase and serum amylase have been the "gold standard" for the diagnosis of AP.[8] Previous research has established that lipase testing should be favored over amylase testing primarily because it provides a longer diagnostic window and greater sensitivity when diagnosing AP.[5] A systematic review of studies comparing the sensitivity and specificity of amylase vs lipase found

that lipase was significantly more sensitive in detecting AP in 8 out of 9 studies reviewed in the analysis.[7] Furthermore, studies have also demonstrated that ordering amylase and lipase concurrently provides no additional diagnostic or prognostic value.[7,9] In 2013, the ACG made the official recommendation to order lipase alone when AP is suspected and not to continue to order lipase or amylase after a diagnosis has been made.[5] Despite these recommendations, current research has demonstrated that health care providers continue to order amylase tests, or amylase and lipase tests concurrently, when AP is suspected. According to the National Pathology Quality Registry, published by the American Society for Clinical Pathology (ASCP), lipase should be preferred over total and pancreatic amylase for the initial diagnosis of AP and that testing should not be repeated over time to monitor disease prognosis.[10] Evidence of demonstrated opportunity for quality improvement at other university-affiliated hospital systems has led us to the question whether laboratory tests for serum amylase and lipase are used appropriately by providers in the diagnosis of AP. To test this hypothesis, our study determined whether serum amylase and lipase tests are being ordered appropriately by evaluating the ordering of amylase alone, lipase alone, amylase and lipase together, and ordering unnecessary repeat tests. We also identified provider demographics and settings that demonstrate the greatest opportunity for future intervention studies. A cost analysis was performed to identify the financial impact of inappropriate test utilization to justify future interventional studies aimed at quality improvement.

Methods

We performed a retrospective chart review at a 750-bed academic teaching hospital using the hospital's electronic medical records. Data was collected over a 12-month period from January 1, 2020, to December 31, 2020. Data collected include patient-provider encounter notes, provider demographics, setting information, patient demographics, and laboratory test results obtained during the encounter for AP. Timestamps were used to determine whether tests were repeated within 48 hours of a diagnostic amylase or lipase value. Concurrent amylase and lipase testing were defined as amylase and lipase tests being performed within 24 hours of each other, and unnecessary repeat tests were identified as those being performed after a diagnostic serum amylase or lipase test (3 times the upper limit of normal) was documented. The determination of diagnostic test results was based on serum lipase and amylase values of greater than 3 times the upper limit of normal (630 U/L for lipase and 330 U/L for amylase). To analyze the appropriateness of laboratory test utilization by providers, provider demographic and setting information was collected. Collected provider demographic information included provider level (MD/DO faculty, resident, nurse practitioner, or physician assistant), specialty, and subspecialty. Provider setting data information was captured to identify tests ordered in emergency departments, outpatient clinics, or inpatient hospital settings. Finally, a cost analysis to identify the financial impact of inappropriate test utilization was performed based on published literature. Patients under 18 years of age, pregnant patients, and patients who were prisoners seen at the Texas Department of Criminal Justice hospital were excluded from this study. Data were deidentified and each patient was assigned a unique identifier in Microsoft Excel by the primary investigator. Regarding data analysis, categorical variables, such as the occurrence of inappropriate lipase tests, inappropriate amylase tests, and inappropriate concurrent tests, provider demographics, and patient demographics, were presented as frequencies. Descriptive statistics were used to compare continuous variables between study groups. A P value of $<.05$ was deemed statistically significant. Our institutional review board considered this study to be a quality assessment/quality improvement study that did not require institutional review board approval or oversight.

Results

Initial data included 27,559 amylase and lipase tests, which were categorized into 5 categories: lipase alone, repeat lipase after diagnosis, amylase alone, repeat amylase after diagnosis, and concurrent amylase ordered with lipase. The count and categorization of the total amylase and lipase tests is shown in **FIGURE 1**. The overall occurrence of unnecessary amylase and lipase testing for the study period was found to be 2567 (9.3%) out of 27,559 tests analyzed (**FIGURE 2**). Of the 2567 unnecessary tests, the amylase test (1881; 73.2%) was the most unnecessary test in the diagnosis of AP followed by lipase tests (686; 26.7%) (**FIGURE 3**).

An analysis of test-ordering behavior by provider level revealed that 2092 (10%) tests ordered by MDs or DOs were considered unnecessary, followed by nurse practitioners (377 tests; 6%), physician assistants (77 tests; 13%), and residents (21 tests; 19%), as listed in **TABLE 1**.

Regarding provider specialty, (952; 31%) amylase and lipase tests ordered by internal medicine providers were unnecessary, followed by surgery (556; 26%), family medicine (342; 23%), and emergency medicine (385; 2%) (**TABLE 2**).

Within internal medicine subspecialties, 248 tests out of 515 (48%) ordered by the nephrology subspeciality were unnecessary, followed by neurology with 15 tests (41%) and endocrinology with 49 (35%). In terms of total unnecessary tests ordered, providers from the general medicine subspecialty ordered the greatest number of unnecessary tests (329; 35%) (**TABLE 3**).

Based on a study by Hammami et al,[11] the cost of an amylase or lipase test can be conservatively estimated at $50 per test. When this cost

FIGURE 1. Amylase and lipase tests. Count and categorization of the total 27,550 amylase and lipase tests. Concurrent amylase with lipase is amylase ordered along with lipase prior to diagnosis; repeat lipase/amylase is repeat tests ordered within 48 hours of a diagnostic amylase or lipase test.

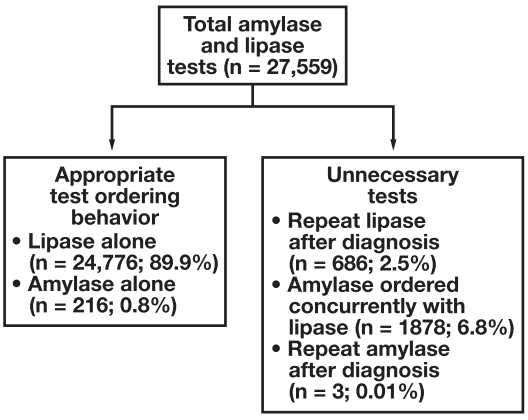


FIGURE 2. **Total amylase and lipase tests ordered. Count and rate of unnecessary amylase and lipase tests ordered from the total 27,559 amylase and lipase tests.**

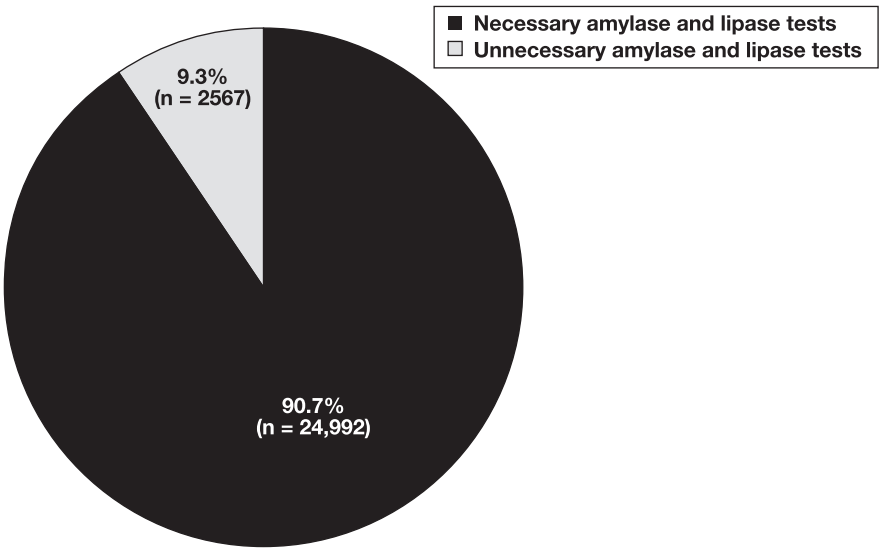


FIGURE 3. **Unnecessary amylase and lipase tests. Differentiation of amylase and lipase from 2567 unnecessarily ordered tests.**

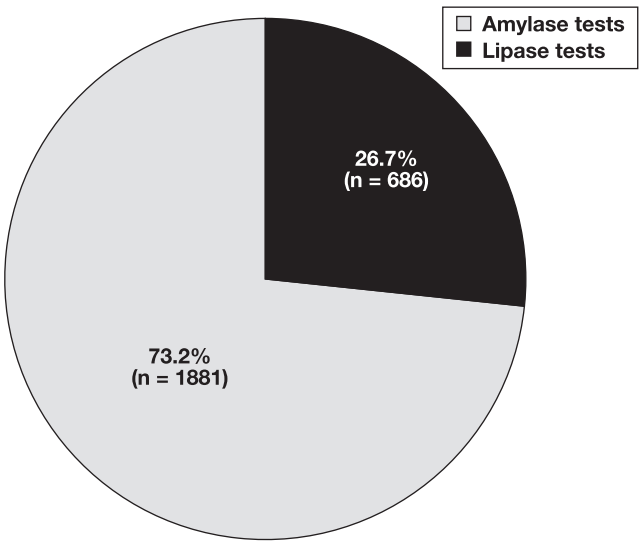


TABLE 1. **Unnecessary amylase and lipase tests ordered by provider level**

Provider level	Total tests	Unnecessary tests	Frequency of unnecessary tests[a]
MD/DO	20,443	2092	10%
Nurse practitioner	6412	377	6%
Physician assistant	592	77	13%
Resident	112	21	19%
Total	27,559	2567	9%

[a]*Frequency of unnecessary tests = unnecessary tests/total tests × 100.*

per test was applied to our study's findings regarding unnecessary tests, potential total cost savings if all unnecessary tests were eliminated was $128,350 ($50 × 2567).

TABLE 2. **Unnecessary amylase and lipase tests ordered by provider specialty**

Specialty	Total tests	Unnecessary tests	Frequency of unnecessary tests[a]
Emergency medicine	20,082	385	2%
Family medicine	1495	342	23%
Internal medicine	3027	952	31%
Surgery	2150	556	26%
Total	26,754	2235	8%

[a]*Frequency of unnecessary tests = unnecessary tests/total tests × 100.*

TABLE 3. **Unnecessary amylase and lipase tests ordered by internal medicine subspecialty**

Internal Medicine Department	Total tests	Unnecessary tests	Frequency of unnecessary tests[a]
General	1106	329	30%
Nephrology	515	248	48%
Gastroenterology	406	129	32%
Pulmonary	389	105	27%
Endocrinology	140	49	35%
Cardiology	257	33	13%
Geriatric	105	30	29%
Neurology	37	15	41%
Infectious disease	72	14	19%
Total	3027	952	31%

[a]*Frequency of unnecessary tests = unnecessary tests/total tests × 100.*

Discussion

Despite current recommendations that lipase alone should be ordered in the differential diagnosis of AP, many university-affiliated hospital systems have demonstrated a lack of adherence to current guidelines.

From our study's findings, we identified the overall rate of unnecessary testing is 9.4% (2567 of 27,559 tests) whereas other academic health care systems have demonstrated varying unnecessary testing rates. For example, a study conducted at a university-affiliated hospital found that 45% of the 8801 amylase and lipase tests ordered were considered unnecessary, and a 2019 study identified superfluous testing in 30.6% of 23,950 amylase/lipase tests and questionably superfluous testing in 12.6%.[11,12] These findings suggest that even with recommendations offered by organizations such as ACG and ASCP and research findings that demonstrate ordering amylase and lipase concurrently provides no additional diagnostic or prognostic value, health care providers continue to order amylase tests or amylase and lipase tests concurrently when AP is suspected.[5,7,9,13] It is important to note that these recommendations apply to the diagnostic stage of AP only.

Out of 2567 unnecessary tests, the amylase test (1881; 73.2%) made up most unnecessary tests in the diagnosis of AP followed by lipase tests (686; 26.7%). In patients suspected of AP, serum lipase alone is the preferred test because it has been found to have greater diagnostic sensitivity than amylase (64%-100% vs 45%-87%).[14] Data regarding the analytical sensitivity and specificity of amylase, lipase, and amylase + lipase has been published in many research studies; however, health care providers continue to unnecessarily use amylase as a tool in the diagnosis of AP. One hypothesis to consider is the fact that the amylase test has been historically used in the diagnosis of AP and it is continued to be seen as a valuable diagnostic tool, but it is only valuable when the result of the test is used in conjunction with lipase and appropriate clinical signs and symptoms. For example, it is clinically important to differentiate pancreatic amylase from other amylase isoforms, and an elevated amylase with normal lipase may indicate a problem outside of pancreas.[15] Furthermore, the lipase-to-amylase ratio may be helpful in distinguishing between the etiology of pancreatitis but not in initial assessment.[16] In summary, results from dual measurement of both amylase and lipase show no improvement in sensitivity (83.3%) and a minor increase in specificity (97.4%) compared with lipase alone.[17,18] For future test-ordering practice in the diagnosis of AP, providers should consider the ACG and ASCP guidelines and avoid using these tests in clinical scenarios such as initial assessment for diagnosis of acute AP, following serial trends on a daily basis on a patient that has a diagnosis of AP, and short-term monitoring of disease for clinical improvement.[5,19]

Regarding unnecessary test ordering by provider type, our study's analysis revealed that 2092 (10%) of unnecessary tests were ordered by MDs or DOs, followed by nurse practitioners (377; 6%), physician assistants (77; 13%) and residents (21; 19%). Of note, residents had the highest percent of unnecessary tests ordered; however, they represent only ~1% of ordered unnecessary tests when compared with other provider types. The common reasons that providers order unnecessary laboratory tests were discussed in several studies, and these include defensive behavior and fear or uncertainty, lack of experience, use of protocols and guidelines, routine clinic practice, inadequate educational feedback, and clinician's unawareness about the cost of examinations.[20-22] Furthermore, providers from the internal medicine department had the highest frequency of unnecessary tests (952 of 3027 tests; 31%) compared with emergency medicine (385 of 20,082 tests; 2%). These findings were similar to those in a study by Ritter et al[23] that analyzed 103,682 tests performed in multiple settings over a 1-year period, showing that the percentage of unnecessary amylase and lipase tests varied greatly between ambulatory (45%), emergency (8%), and inpatient (28%) settings. The authors reported a high rate of compliance in emergency settings, with unnecessary testing at less than 1%, whereas inpatient and ambulatory compliance demonstrate higher rates of unnecessary testing at 30%-45%. Our academic teaching hospital performed similarly, with the emergency department being the most compliant with current regulations, with only 2% of tests ordered being classified as inappropriate or unnecessary, whereas the other departments have inappropriate test percentages up to 31%. One possible explanation to the variation in unnecessary test ordering across various settings could be the difference in skillset between providers that are generalists vs those who are specialists. An article comparing generalist and specialty care hypothesized that generalists perform slightly better on standardized tests of general medical knowledge than specialists and do a little better with respect to test ordering.[24]

Finally, the cost analysis in this study estimated a savings of $128,350 ($50 × 2567) per year by eliminating all unnecessary testing. The $50 cost per test was based on the study by Hammami et al,[11] which found that 45% of the 8801 amylase and lipase tests ordered were considered unnecessary and elimination of these tests could offer a cost-savings of nearly $200,000 over a 6-month period and another $39,350 if superfluous tests were considered as well. A study conducted at a university-affiliated teaching hospital that implemented an intervention (deselection of repeat amylase tests) showed a decrease of 87% in unnecessary amylase tests, resulting in an annualized cost savings of $719,000 per year.[25] In an emergency department of a psychiatric facility, by educating providers on the minimal utility of amylase and the subsequent removal of amylase from an abdominal pain laboratory panel, monthly charges decreased from an average of $27,860 to $6664—an annual decrease of $254,352.[26] In a 2022 study, a multidisciplinary team approach (Plan, Do, Study, Act) was used in phases where the initial phase decreased repeat amylase and lipase tests by 73%, phase 2 involved educating residents and the emergency department regarding laboratory testing guidelines, and a subsequent order set change in the electronic medical record (phase 3) led to a 48% reduction in amylase orders, resulting in cost savings of $1645 direct costs and a yearly cost savings of $3290; the cost per test was $35.[27] Finally, in a 2019 study, an organization discovered that they were spending $48,100 on unnecessary amylase orders during a 2-month study period where, if the lipase test only had been ordered, no cases of AP would have been missed.[28]

Overall, the results of our study support the hypothesis that unnecessary lipase and amylase tests have been ordered in the diagnosis of AP; however, most providers were following current recommendations. A rate of 9% unnecessary amylase and lipase testing warrants action to improve quality at this study's setting. Focusing on ambulatory and inpatient settings will provide the most impact when designing an intervention, although most tests to evaluate AP were performed in the emergency department.

Limitations

This study was limited by the fact that data represent a single university-affiliated hospital system serving a population of residents in Southeast Texas. Another limitation to this study is that serum amylase and lipase results that are borderline for an AP diagnosis could influence the

number of tests that are categorized as unnecessary, as it may be unclear how health care providers are interpreting and making decisions based on these results. Finally, appropriate and inappropriate test-ordering behavior may provide the same diagnostic capabilities to health care providers if the inappropriate test ordering includes both amylase and lipase concurrently.

Conclusion

This pilot study demonstrated that, whereas most test-ordering providers were ordering amylase and lipase tests appropriately, a significant number of tests were still considered unnecessary. Future intervention studies should be designed to target ambulatory and inpatient settings across all campuses to reduce the greatest amount of unnecessary testing. Furthermore, studies should first evaluate whether a simple modification to existing test-ordering sets may provide a reduction in unnecessary amylase testing, as this strategy demonstrated benefits with little disruption to providers. Educational interventions should also be considered; however, no claims can be made regarding an association between test-ordering behavior and adverse patient outcomes addressed in this study.

Acknowledgments

We are thankful for the support and expertise provided by the faculty in the Clinical Laboratory Science Committee at the University of Texas Medical Branch, Galveston, TX.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Forsmark Ch E, Vege SS, Wilcox CM. Acute pancreatitis. *N Engl J Med.* 2017;1(6):598-599. doi:10.1056/NEJMra1505202
2. Foster BR, Jensen KK, Bakis G, Shaaban AM, Coakley FV. Revised Atlanta Classification for acute pancreatitis: a pictorial essay-erratum. *Radiographics.* 2019;39(3):912. doi:10.1148/rg.2019194003
3. O'Reilly DA, McPherson SJ, Sinclair MT, Smith N. "Treat the cause": lessons from the NCEPOD report on acute pancreatitis. *Pancreatology*. 2017;17(3):329-333. doi:10.1016/j.pan.2017.02.010
4. Shafiq F, Khan MF, Asghar MA, Shamim F, Sohaib M. Outcome of patients with acute pancreatitis requiring intensive care admission: a retrospective study from a tertiary care center of Pakistan. *Pak J Med Sci*. 2018;34(5):1082-1087. doi:10.12669/pjms.345.15575
5. Tenner S, Baillie J, DeWitt J, Vege SS; American College of Gastroenterology. American College of Gastroenterology guideline: management of acute pancreatitis. *Am J Gastroenterol.* 2013;108(9):1400-1415; 1416. doi:10.1038/ajg.2013.218
6. Lippi G, Valentino M, Cervellin G. Laboratory diagnosis of acute pancreatitis: in search of the Holy Grail. *Crit Rev Clin Lab Sci.* 2012;49(1):18-31. doi:10.3109/10408363.2012.658354
7. Ismail OZ, Bhayana V. Lipase or amylase for the diagnosis of acute pancreatitis? *Clin Biochem.* 2017;50(18):1275-1280. doi:10.1016/j.clinbiochem.2017.07.003
8. Leppaniemi A, Tolonen M, Tarasconi A, et al. 2019 WSES guidelines for the management of severe acute pancreatitis. [review]. *World J Emerg Surg.* 2019;14(27):1-20. doi:10.1186/s13017-019-0247-0
9. Akhtar A, Sarode R, Agrawal D. Measuring both serum amylase and lipase for acute pancreatitis lowers quality and raises cost. *Cleve Clin J Med.* 2017;84(9):670-672. doi:10.3949/ccjm.84a.16103
10. National Pathology Quality Registry. American Society for Clinical Pathology. https://www.ascp.org/content/docs/default-source/get-involved-pdfs/istp_npqr/npqr-ensuring-privacy-and-security-for-you-and-your-patients.pdf?
11. Hammami MB, Khoury M, Sule SS, Haire HM. Superfluous amylase/lipase testing at a university-affiliated teaching hospital: a retrospective review. *Ochsner J.* 2019;19(2):102-106. doi:10.31486/toj.18.0058
12. Aljomah AS, Hammami MM. Superfluous amylase/lipase testing at a tertiary care hospital: a retrospective study. *Ann Saudi Med.* 2019;39(5):354-358. doi:10.5144/0256-4947.2019.354
13. Testing for amylase. American Society for Clinical Pathology. https://www.choosingwisely.org/clinician-lists/american-society-clinical-pathology-testing-for-amylase/?highlight=amylase
14. Alter D, Koch DD. A review of acute pancreatitis. *JAMA.* 2021;325(23):2402-2402. doi:10.1001/jama.2021.6012
15. Otsuki M. Usefulness of amylase isoenzyme determination for the diagnosis of pancreatic diseases. *Nihon Rinsho*. 1995;53(5):1184-1191.
16. Akinfemiwa O, Zubair M, Muniraj T. *Amylase*. 2023. In: *StatPearls*. StatPearls Publishing; 2024. Accessed January 25, 2024. https://www.ncbi.nlm.nih.gov/books/NBK557738/
17. Lankisch PG, Petersen M. Lipase/amylase ratio: not helpful in the early etiological differentiation of acute pancreatitis. *Z Gastroenterol.* 1994;32(1):8-11.
18. Cook AE, Jalavu TP, Zemlin AE. Audit of amylase and lipase requests in suspected acute pancreatitis and cost implications, South Africa. *Afr J Lab Med*. 2022;11(1):1834. doi:10.4102/ajlm.v11i1.1834
19. Banks PA. Practice guidelines in acute pancreatitis. *Am J Gastroenterol.* 1997;92(3):377-386.
20. Miyakis S, Karamanof G, Liontos M, Mountokalakis TD. Factors contributing to inappropriate ordering of tests in an academic medical department and the effect of an educational feedback strategy. *Postgrad Med J.* 2006;82(974):823-829. doi:10.1136/pgmj.2006.049551
21. Axt-Adam P, van der Wouden JC, van der Does E. Influencing behavior of physicians ordering laboratory tests: a literature study. *Med Care.* 1993;31(9):784-794. doi:10.1097/00005650-199309000-00003
22. Why doctors order so many unnecessary tests. 2023. Accessed June 15, 2023. https://www.hhmglobal.com/knowledge-bank/news/why-doctors-order-so-many-unnecessary-tests.
23. Ritter JP, Ghirimoldi FM, Manuel LSM, et al. Cost of unnecessary amylase and lipase testing at multiple academic health systems. *Am J Clin Pathol.* 2020;153(3):346-352. doi:10.1093/ajcp/aqz170
24. Donohoe MT. Comparing generalist and specialty care: discrepancies, deficiencies, and excesses. *Arch Intern Med.* 1998;158(15):1596-1608. doi:10.1001/archinte.158.15.1596
25. Jaeger C, Sullivan P, Waymack J, Griffen DG. Effectively reducing amylase testing using computer order entry in the emergency department: quality improvement without eliminating physician choice. *J Innov Health Inform.* 2017;24(3):907.
26. Reducing patient charges by eliminating amylase from lab panels. 2013. Accessed June 15, 2023. https://hbr.org/2013/11/reducing-patient-charges-by-eliminating-amylase-from-lab-panels.
27. Khanna V, Taha A, Chamseddin R, Eshman Y, Tegeltija V. S67 overutilization of amylase and lipase testing in acute pancreatitis. *Am J Gastroenterol*. 2022;117(10S):e48-e49. doi:10.14309/01.ajg.0000856908.41762.2f
28. Hupp M, Peterson N, Buchner S, Karger AB. Amylase and lipase co-ordering in the workup of pancreatic disorders is unnecessary and costly: an institutional lab utilization study. *Am J Clin Pathol.* 2019;152(suppl 1):S25. doi:10.1093/ajcp/aqz112.048

Laboratory stewardship perceptions and testing patterns at a pediatric tertiary care center

Tejas S Desai[1,2], Ken Tang[3], Viveak Kaul[4], Ivan M Blasutig[1,5,6], Melanie Buba[1,2,4]

[1]Department of Infectious Disease, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2]Department of Infectious Disease, Chengdu First People's Hospital, Chengdu, China, [3]Emergency Department, Chengdu First People's Hospital, Chengdu, China. *To whom correspondence should be addressed: zhongsen6606@163.com.

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

Laboratory Medicine 2024;54:479-488; https://doi.org/10.1093/labmed/lmac151

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 al[6] 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

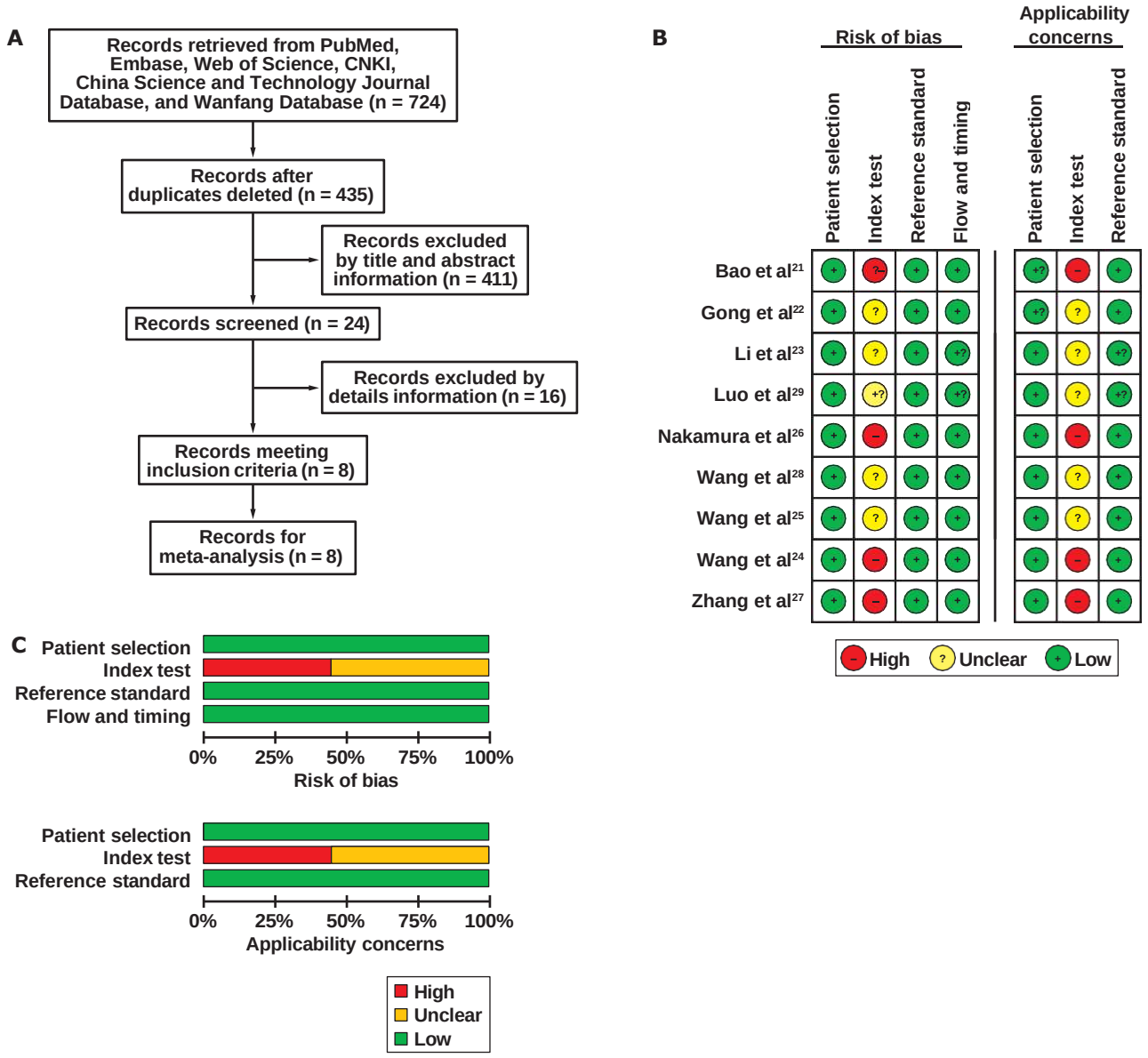
Search Strategy

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.**



2024;54:479-488 | https://doi.org/10.1093/labmed/lmac151

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	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 ± 14.9, S1: 33.8 ± 9.4, S2: 38.0 ± 8.4, S3: 39.4 ± 13.2, S4: 50.1 ± 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 ± 9.5, S2: 37.7 ± 9.7, S3: 36.2 ± 9.3, S4: 37.5 ± 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 ± 12.8, F1: 34.8 ± 13.0, F2: 40.2 ± 10.1, F3: 38.0 ± 10.2, F4: 45.5 ± 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 ± 12.0	48/25
Wang Y	2018	Chinese	China	Serum	RT-qPCR	Scheuer	miR-122	U6	138	37.0 ± 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 ± 10.4	66/25
Luo K	2022	Chinese	China	Serum	RT-qPCR	Scheuer	miR-21, miR-148b	U6	134	32.4 ± 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 ≤F2, Scheuer score of ≤S1 or S2, or Fibrosis-4 index of ≤F1. Significant fibrosis was defined as a METAVIR score of ≥F3, Scheuer score of ≥S2 or S3, or Fibrosis-4 index of ≥F2. The basic characteristics of included studies are shown in **TABLES 1** and **2**.

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

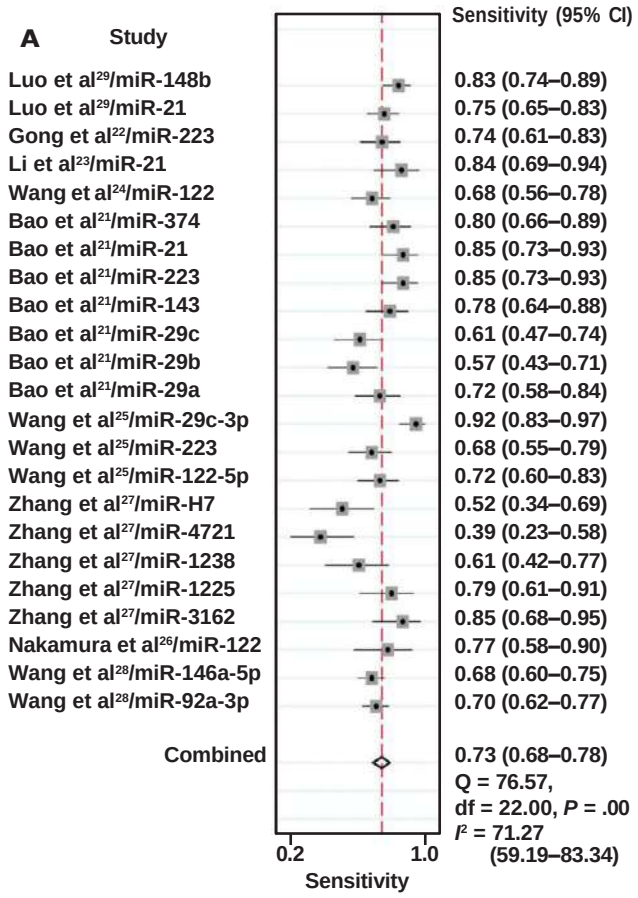
The data of the 9 included articles were extracted and integrated. The pooled sensitivity was 0.73 (95% CI, 0.68–0.78) with an I^2 value of 71.27% (**FIGURE 2A**). The pooled specificity was 0.78 (95% CI, 0.70–0.84) with an I^2 value of 83.40% (**FIGURE 2B**). The pooled PLR was 3.32 (95% CI, 2.52–4.37) 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

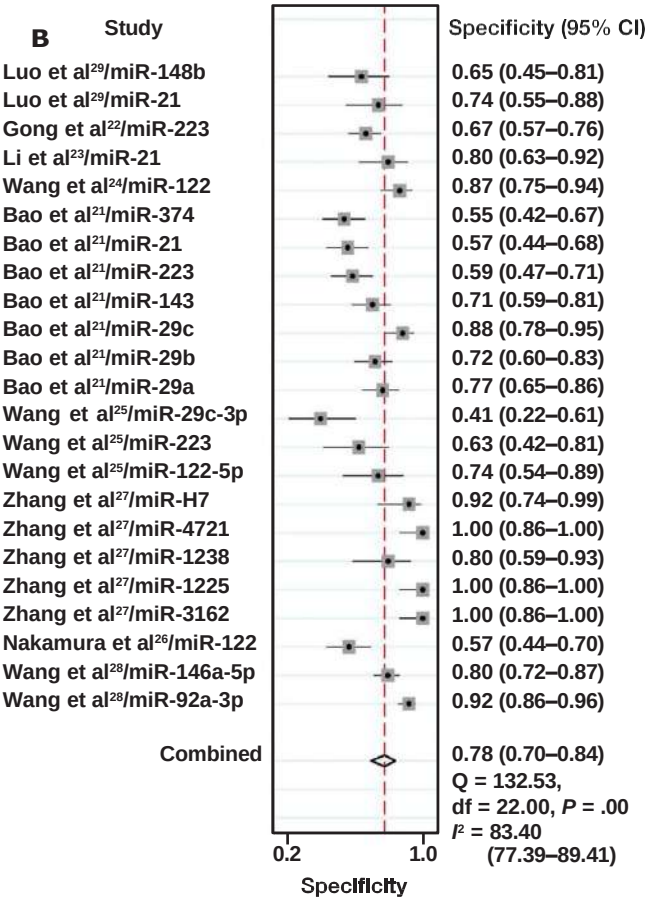
TABLE 2. Diagnostic Accuracy and Cut-Off Values

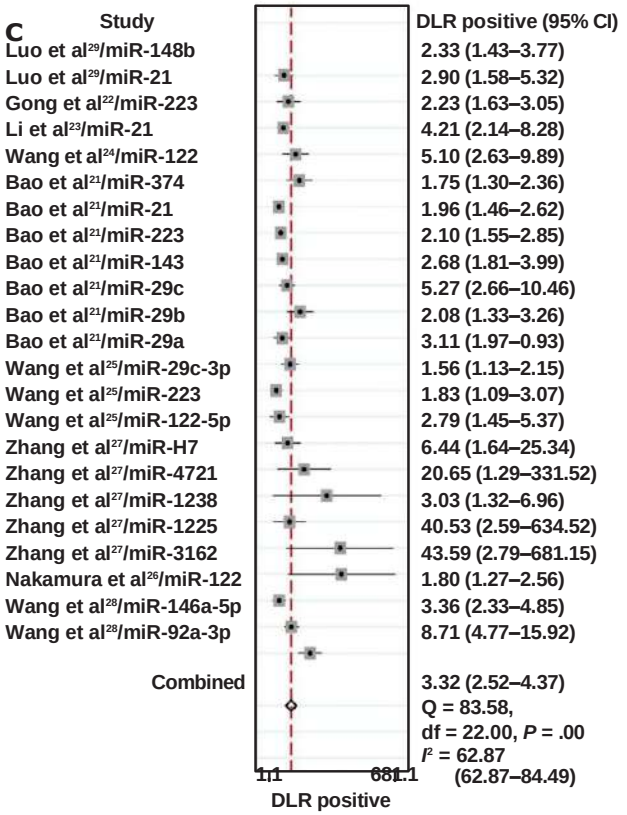
Author, Year	TP	FP	FN	TN	Cut-off Value	Regulation Mode
Wang Q, 2021	111	10	47	114	2.1	Upregulated
Wang Q, 2021	107	25	51	99	1.5	Upregulated
Nakamura M, 2017	23	26	7	35	Unknown	Downregulated
Zhang Q, 2021	28	0	5	25	Unknown	Upregulated
Zhang Q, 2021	26	0	7	25	Unknown	Upregulated
Zhang Q, 2021	20	5	13	20	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.

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.**







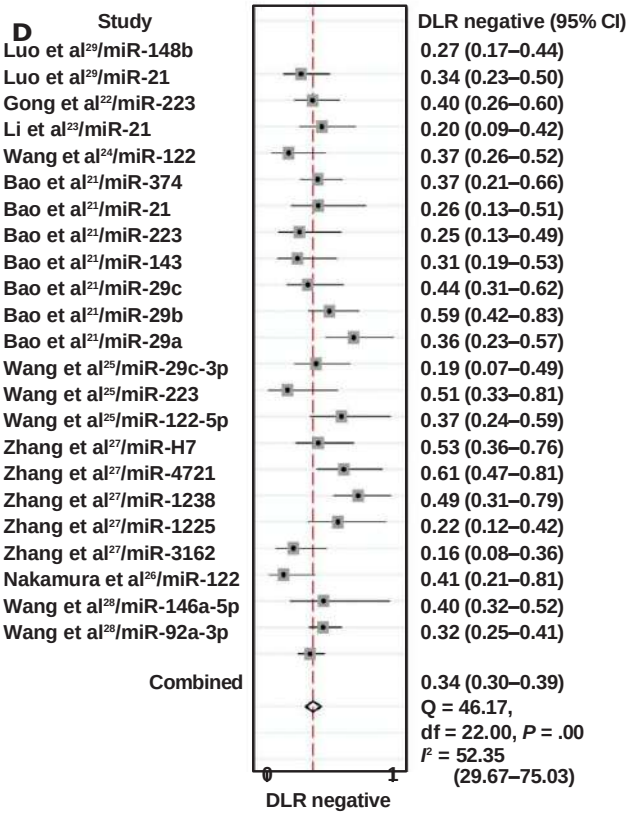
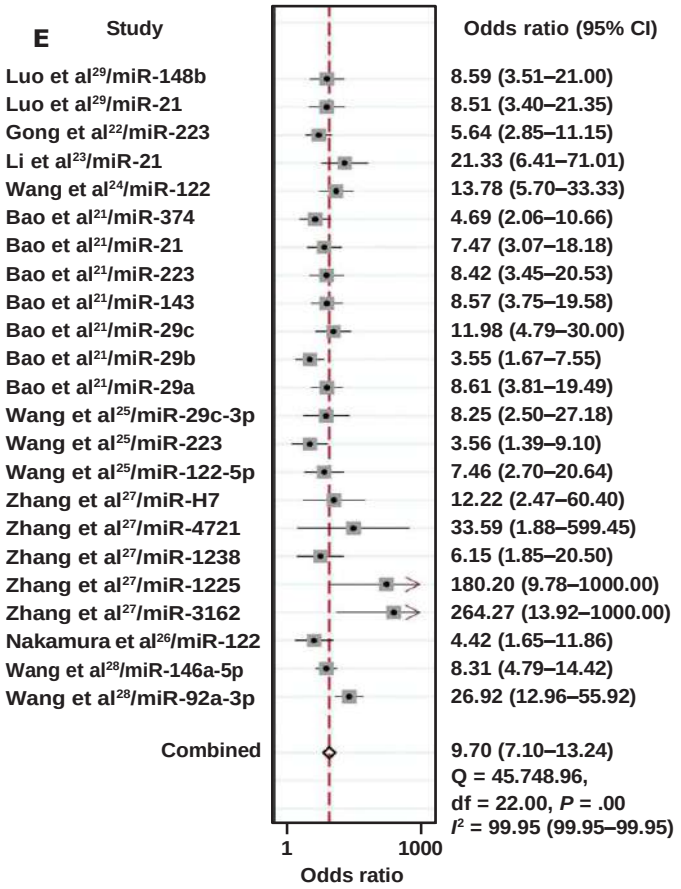
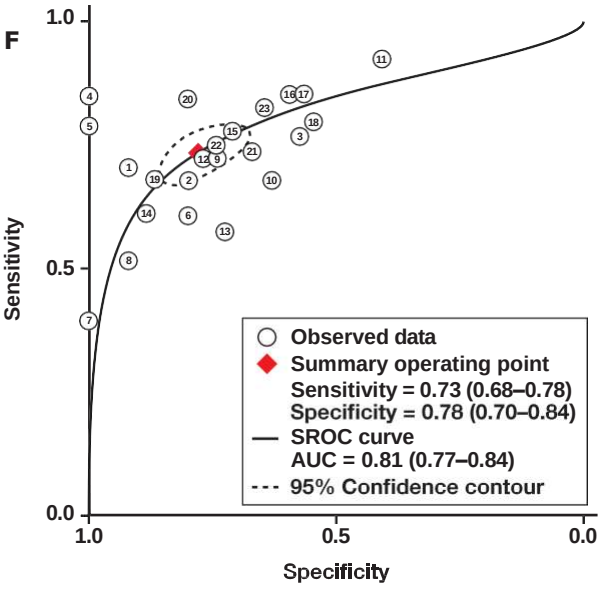


FIGURE 2. (cont)





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 I^2 value of 52.95%, pooled specificity was 0.75 (95% CI, 0.68–0.81) with an I^2 value of 80.38%, pooled PLR was 2.97 (95% CI, 2.38–3.69) with an I^2 value of 51.98%, pooled NLR was 0.35 (95% CI, 0.31–0.39) with an I^2 value of 0, and pooled DOR was 8.52 (95% CI, 6.60–10.99) with an I^2 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.

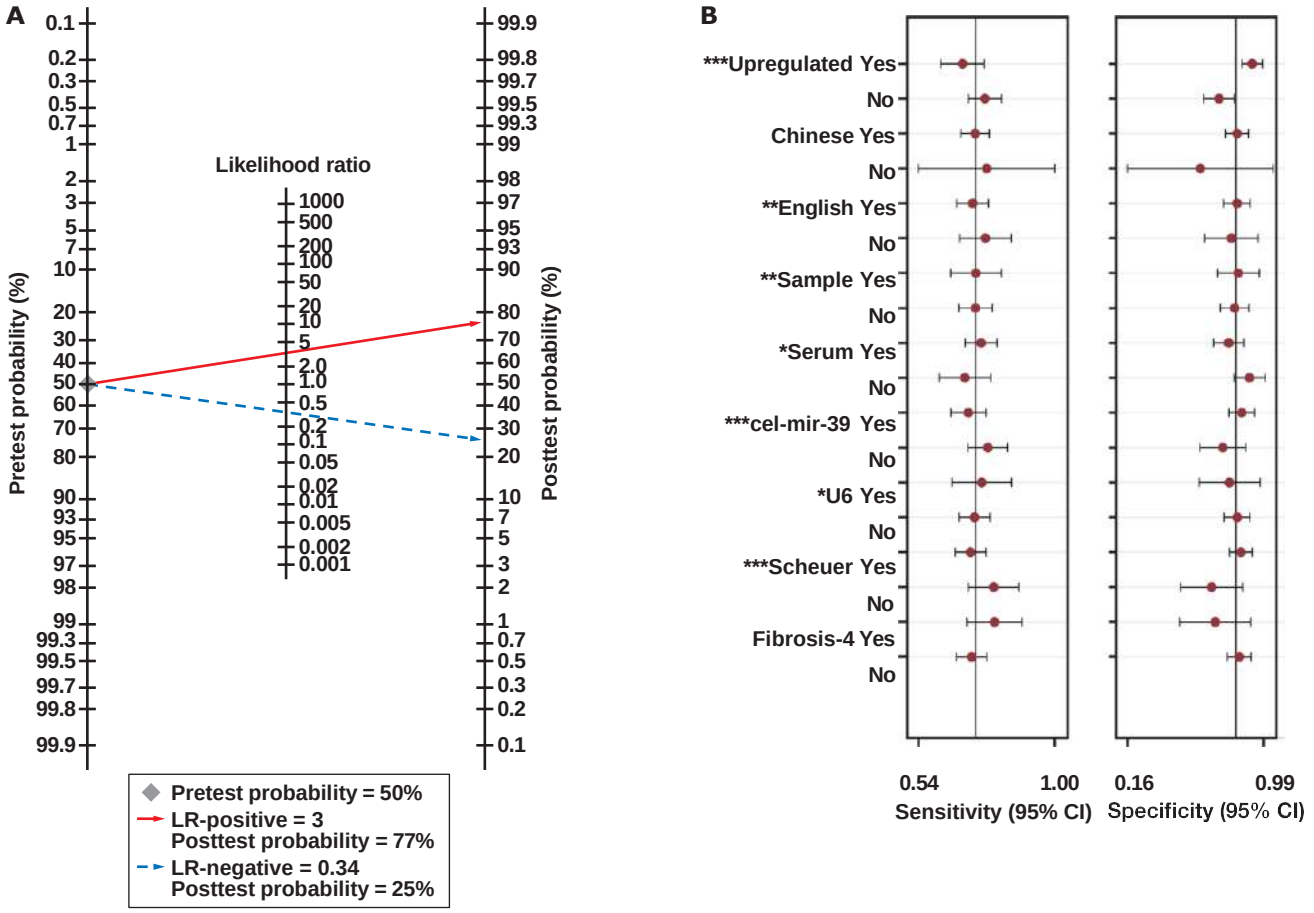
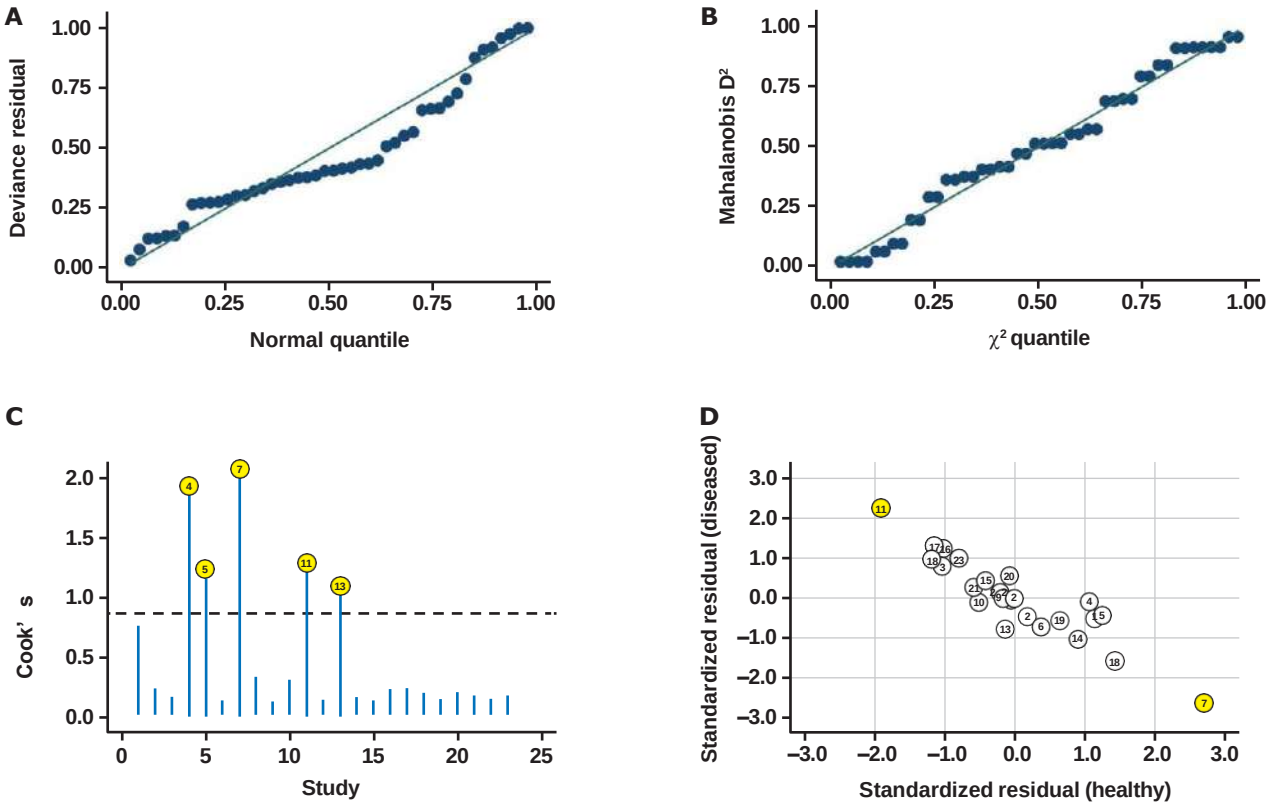


TABLE 3. Results of Univariable Meta-Regression and Subgroup Analyses

Parameter	Category	No. of Studies	Sensitivity	*P*1	Specificity	*P*2
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.

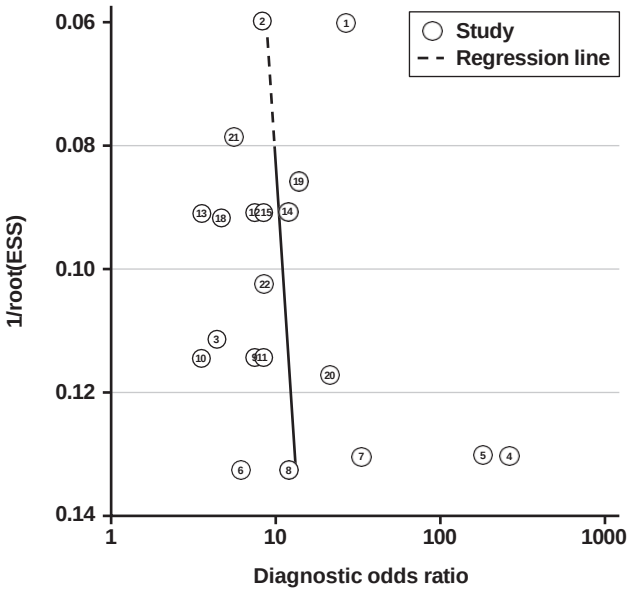
Serological markers of liver fibrosis mainly include direct and indirect markers. The indirect markers include transaminase, bilirubin, coagulation function test, and platelet count. Most factors affect the formation of liver fibrosis during the progression of liver disease, which can represent the change 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 al[36] 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 cardiomyocytes[37] 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-β1 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

1. Aydın MM, Akçalı KC. Liver fibrosis. *Turk J Gastroenterol*. 2018;29:14–21. doi: 10.5152/tjg.2018.17330
2. 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.
3. van der Meer AJ, Sonneveld M, Schouten JN, Janssen HL. [Reversibility of hepatic fibrosis]. *Ned Tijdschr Geneeskd.* 2014;158:A6790.
4. Roehlen N, Crouchet E, Baumert TF. Liver fibrosis: mechanistic concepts and therapeutic perspectives. *Cells.* 2020;9:875. doi:10.3390/cells9040875
5. 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
6. Yasaka K, Akai H, Kunimatsu 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
7. 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
8. Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol*. 2007;23:175–205. doi:10.1146/annurev.cellbio.23.090506.123406
9. 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
10. 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
11. 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
12. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology.* 1994;19:1513–1520.
13. 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
14. 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.
15. 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
16. 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
17. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med*. 2002;21:1539–1558. doi:10.1002/sim.1186
18. 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
19. 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/eurrev_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

Cystic fibrosis–related diabetes screening at a large pediatric center

Anil K. Chokkalla, PhD,[1,2] Pamela Tuley, MSHA, RN,[3] Miray Kurtca, MD,[1,2] Herda Ona, BS,[2] Fadel E. Ruiz, MD,[3] Sridevi Devaraj, PhD[1,2]

[1]Department of Pathology & Immunology, Baylor College of Medicine, Houston, TX, US; [2]Department of Pathology, Texas Children's Hospital, Houston, TX, US; [3]Section of Pediatric Pulmonology, Department of Pediatrics, Baylor College of Medicine and Texas Children's Hospital, Houston, TX, US. Corresponding author: Sridevi Devaraj; sxdevara@texaschildrens.org

Key words: diabetes; glucose tolerance test; quality improvement; cystic fibrosis

Abbreviations: OGTT, oral glucose tolerance test; CF, cystic fibrosis; CFRD, cystic fibrosis–related diabetes; CFF, Cystic Fibrosis Foundation; HbA1c, hemoglobin A1c; QI, quality improvement

Laboratory Medicine 2024;55:580-584; https://doi.org/10.1093/labmed/lmae009

ABSTRACT

Objective: Cystic Fibrosis Foundation guidelines recommend annual diabetes screening by oral glucose tolerance test (OGTT) in pediatric patients with cystic fibrosis (CF) starting at the age of 10 years. Adherence to these guidelines proves to be challenging, and the nationwide screening rates are still considered suboptimal. The aim of this study was to assess and improve the screening rates at our large pediatric center.

Methods: A 4-year retrospective audit of OGTT completion among pediatric patients with CF of age ≥10 years who are not yet diagnosed with diabetes was conducted. A collaborative working group was formed to identify the barriers to screening and formulate a quality improvement plan, which was monitored and evaluated for a 9-month period.

Results: Diabetes screening rates determined by OGTT completion at our center showed a gradual decline during the COVID-19 pandemic from 2019 to 2022. Following the implementation of the quality improvement plan during the summer of 2023, there was a marked increase in OGTT ordering compliance by providers as well as test completion by patients. Notably, the fractional OGTT completion rate rose from 45% during the preintervention phase (January-April 2023) to 70% during the postintervention phase (May-September 2023).

Conclusion: Diabetes screening in pediatric patients with CF can be effectively improved by refining practices related to patient experience, care coordination, and laboratory testing strategies.

Introduction

Diabetes is a common comorbidity in cystic fibrosis (CF) patients, with an estimated prevalence of 2% in children, 19% in adolescents, and 40%-50% in adults.[1] Cystic fibrosis–related diabetes (CFRD) is recognized as type 3c diabetes, which is clinically distinct from type 1 and 2 diabetes, as both insulin insufficiency and transient insulin resistance prevails.[2] Of note, CFRD patients do not display macrovascular complications, unlike type 1 or 2 diabetes.[1] Pancreatic islet impairment and ductal obstruction caused by hyperviscous exocrine secretions, fatty infiltrations, and progressive fibrosis drive the pathophysiology of CFRD.[3] The major risk factors associated with the development of CFRD include pancreatic insufficiency, female sex, liver disease, G-tube feeding, and a history of allergic bronchopulmonary aspergillosis.[1] Cystic fibrosis–related diabetes is associated with adverse clinical outcomes such as increased mortality, decreased lung function, and poor nutritional status.[4] More importantly, CFRD onset is insidious, and patients may not show typical diabetic symptoms like polydipsia and polyuria.[5] Therefore, it is crucial to screen and identify the individuals at high risk for CFRD.

The American Diabetes Association, Cystic Fibrosis Foundation (CFF), and Pediatric Endocrine Society all recommend a 2-hour 75-g oral glucose tolerance test (OGTT) for annual CFRD screening in children starting from age ≥10 years.[6] Importantly, hemoglobin A1c (HbA1c) is not recommended for screening due to its low sensitivity in CF patients.[7] Furthermore, CF patients undergoing organ transplantation should obtain OGTT preoperatively if they have not been screened within the prior 6 months.[6] The clinical spectrum of OGTT screening results falls into 4 groups: (1) normal glucose tolerance (1 h glucose <200 mg/dL and 2 h glucose <140 mg/dL), (2) indeterminate glucose tolerance (1 h glucose ≥200 mg/dL and 2 h glucose <140 mg/dL), (3) impaired glucose tolerance (2 h glucose ≥140 and <200 mg/dL), and (4) CFRD (2 h glucose ≥200 mg/dL).[6] Furthermore, CFRD diagnosis requires abnormal OGTT results confirmed on 2 separate days unless the patient shows symptoms like polydipsia and polyuria and a random plasma glucose ≥200 mg/dL.[6] In acutely ill patients receiving intravenous antibiotics

and glucocorticoids, persistently high fasting plasma glucose ≥126 mg/dL or 2-hour postprandial glucose ≥200 mg/dL for up to 48 hours is diagnostic of CFRD.[6]

Cystic fibrosis–related diabetes screening rates have trended upward over the past decade. For instance, the national average for CFRD screening almost doubled from 31.5% in 2011 to 61.5% in 2019.[8] However, this trend was interrupted by the COVID-19 pandemic. According to the CFF's 2020 report, only 49.9% of eligible patients of age <18 years with CF completed annual OGTTs. To assess the CFRD screening rates at our large pediatric CFF-accredited care center, we conducted a retrospective cohort study using data from 2019 to 2022. Importantly, we identified the barriers to suboptimal CFRD screening and developed a comprehensive quality improvement (QI) plan in collaboration with CF center leadership. Finally, after implementing the QI initiative, we tracked OGTT ordering and test completion rates until September 2023.

Methods

Study Cohort

This study was conducted in accordance with the protocol approved by the Baylor College of Medicine Institutional Review Board. We performed a retrospective cohort study involving CF patients of age ≥10 years who had not yet been diagnosed with diabetes. Patients previously diagnosed with CFRD were excluded. Patient demographic information (age, sex, ethnicity, and geographical location) and laboratory data related to OGTT and HbA1c testing was retrieved from the electronic medical records. Eligible CF patients underwent OGTT and HbA1c testing at the Texas Children's Hospital or an outpatient reference laboratory. For the OGTT, the patient ingested 75 g/10 oz glucola solution orally within 5 minutes. A fasting specimen prior to ingestion and a 2-hour postingestion specimen were collected in a gray-top tube, and glucose levels were measured on a Vitros XT 7600 analyzer (Ortho Clinical Diagnostics). Testing was discontinued when (1) the patient vomited while ingesting the glucose solution, (2) the patient was unable to ingest the entire amount of glucose solution in 5 minutes, (3) the timed specimen was unable to be collected within the 5 minute collection window, and (4) the patient was not fasting for 8-14 hours prior to the test. A thorough chart review was performed to capture and retrieve OGTT test results from both hospital and external laboratories. National averages for OGTT screening for the years 2019, 2020, and 2021 were obtained from the CFF patient registry on request. The national data for the years 2022 and 2023 was not available at the time of this article preparation.

Quality Improvement Initiative

Texas Children's Hospital is 1 of more than 130 CFF-accredited care centers with a large team comprising pediatric pulmonologists, genetic counselors, clinical and research coordinators, dieticians, and mental health coordinators. We launched a collaborative working group with CF center leadership to assess and improve the state of diabetes screening among CF patients. Broadly, this study was conducted in 2 phases. In phase 1, we assessed the CFRD screening rates for the years 2019, 2020, 2021, and 2022. Before phase 2, the core members of the QI working group, including the CF center program director, clinical chemistry laboratory director, CF center nurse coordinator, and clinical chemistry fellow, examined each step of the CFRD screening process and identified potential barriers centered around patient experience, physician order entry, and the laboratory testing process. Root causes for these barriers were identified based on literature and discussions during the CF center meetings. In phase 2, we devised a QI plan for 2023 with 3 key interventions: (1) scheduling annual summer laboratory testing for all eligible patients, (2) a best-practice advisory to guide providers with OGTT ordering, and (3) a streamlined process for OGTT appointments at the outpatient laboratory. These interventions were carried out in collaboration with a multidisciplinary team, involving CF center scheduling staff for the annual summer laboratory screenings, a laboratory information system analyst for designing the best-practice advisory, and an outpatient laboratory supervisor for increasing the number of OGTT appointments and assigning a float phlebotomist. Throughout this process, the team participated in monthly CF meetings to brainstorm and identify practical solutions. Postimplementation of the QI plan in May 2023, the 2 outcome measures, including OGTT ordering and completion rates, were assessed until September 2023.

Statistical Analysis

A 2-tailed Fisher's exact test was used to compare the screening rates between 2019 and 2022. Results with $P < .05$ were considered statistically significant.

Results

Our study cohort included a total of 151 eligible CF patients without diabetes of age ≥10 years in 2019, 155 in 2020, 153 in 2021, 168 in 2022, and 174 in 2023 (**TABLE 1**). Prior to the QI interventions, OGTT completion rates showed a descending trend: 67.5% in 2019, 61.9% in 2020, 60.8% in 2021, and 56% in 2022 (**TABLE 1**). Cystic fibrosis–related diabetes screening at our center declined by ~11.5% ($P = .0383$) over the 4-year period from 2019 to 2022. Of note, our screening rates were still higher than the national average in 2019, 2020, and 2021 (**TABLE 1**). More importantly, electronic order requisitions for OGTT were absent for 11.3% of the eligible patients in 2019, 15.5% in 2020, 19% in 2021, and 21.4% in 2022 (**TABLE 1**). Altogether, this suggests that the CFRD screening based

TABLE 1. Overview of cystic fibrosis–related diabetes screening from 2019 to 2022

Variable	2019	2020	2021	2022
CF patients (age ≥10 years) without diabetes	151	155	153	168
OGTT completion, %	67.5	61.9	60.8	56.0
National average for OGTT compliance, %	61.5	49.9	56.5	—
Patients without OGTT order requisitions, %	11.3	15.5	19.0	21.4
Patients with abnormal OGTT (>200 mg/dL)	5	1	0	1
Patients without OGTT results				
Males, %	46.9	44.1	50	51.4
Females, %	53.1	55.9	50	48.6
Hispanics/Latinos, %	83.7	78	73.3	78.4
Non-Hispanics/non-Latinos, %	16.3	22	26.7	21.6
Patients with HbA1c results, %	42.9	35.6	41.7	24.3

CF, cystic fibrosis; HbA1c, hemoglobin A1c; OGTT, oral glucose tolerance test.

on OGTT-ordering compliance and test completion is suboptimal, highlighting room for improvement.

Among eligible CF patients without OGTT screening, there is essentially an equal split between male and female sex, with more Hispanic than non-Hispanic population (**TABLE 1**). The geocoding analysis did not reveal any specific pattern related to the location of noncompliant CF patients. In fact, a majority of noncompliant patients originated in Houston and not the suburbs. Of the CF patients who did not undergo OGTT, 42.9% had HbA1c results in 2019, 35.6% in 2020, 41.7% in 2021, and 24.3% in 2022 (**TABLE 1**). Notably, HbA1c-based screening also dropped by 18.6% ($P = .047$) over the 4-year period from 2019 to 2022. Upon overlapping the noncompliant patients for OGTT screening each year from 2019 to 2022, the majority of them were found to be recurring. Only 10 patients in 2019, 15 in 2020, 19 in 2021, and 37 in 2022 were uniquely noncompliant for OGTT screening (**FIGURE 1**).

Next, we performed root cause analysis using the Ishikawa diagram to visualize the potential barriers for suboptimal CFRD screening at our center (**FIGURE 2**). The core members of the QI working group, including the CF center program director, clinical chemistry laboratory director, CF center nurse coordinator, and clinical chemistry fellow, examined each step of the CFRD screening process and identified 4 barriers related to patient participation, the procedure for physician order entry, communication lapses, and the hurdles within the testing process itself (**FIGURE 2**). The core working group then created a plan-do-study-act framework with 3 key interventions (**TABLE 2**). The first intervention was to improve patient participation; eligible patients are given an option for annual super visits during the summer block. Importantly, uncompleted OGTTs during the preintervention period were linked to patients for whom order requests had been sent to the external reference laboratory. Second, to guide the physicians in entering appropriate OGTT orders, a best practice advisory with test priority was created. Third, to facilitate a higher volume of OGTTs during the annual super visits at the outpatient clinic, we expanded laboratory appointment slots (5 per day) and allocated a float phlebotomist. The core working group also ensured that other CF care providers were well aware of these newly implemented strategies.

After implementation of the QI initiative, an electronic dashboard was used to track the OGTT ordering and test completion rates in real time. Of the 174 eligible CF patients for 2023, 165 patients had OGTT order requisitions from the provider in the electronic medical records, and 107 patients completed their annual OGTTs by September 2023. Of the 67 patients without annual OGTT completion in 2023, 41 were unique and 26 recurred from 2022. Of note, 4 patients refused testing due to acute illness during the appointment or other contradictions and 3 patients from Louisiana preferred to get tested locally. Excluding these patients, the OGTT order requisitions were pending for only 2 patients by September 2023. Among the patients who completed OGTTs, only 1 patient was diagnosed with CFRD from the screening. Overall, physician ordering compliance showed significant improvement ($P < .00001$) from 79.6% in 2022 to 95% by September 2023, whereas the OGTT completion rate rose from 56% in 2022 to 60% by September 2023.

As expected, month-wise OGTT ordering and completion among eligible patients showed peak during the summer months, particularly from May to July. During the preintervention phase from January to April 2023, only 24 patients completed the OGTT out of 53 order requisitions (**FIGURE 3**). However, after implementing QI between

FIGURE 1. Yearwise overlap of cystic fibrosis (CF) patients without oral glucose tolerance tests (OGTTs).

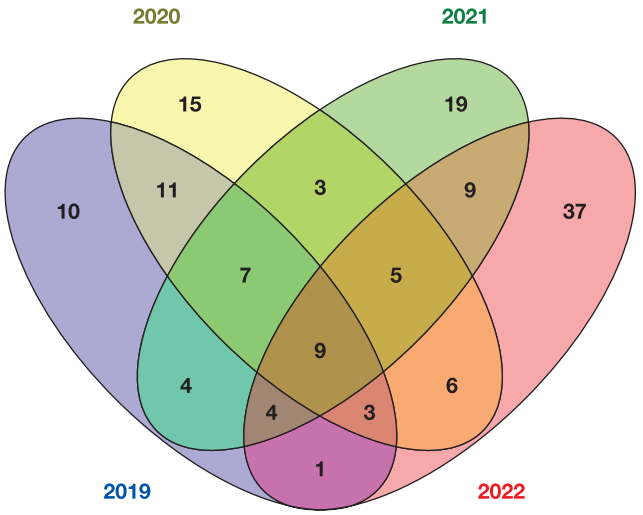


FIGURE 2. Potential causes for suboptimal cystic fibrosis–related diabetes (CFRD) screening. OGTT, oral glucose tolerance test.

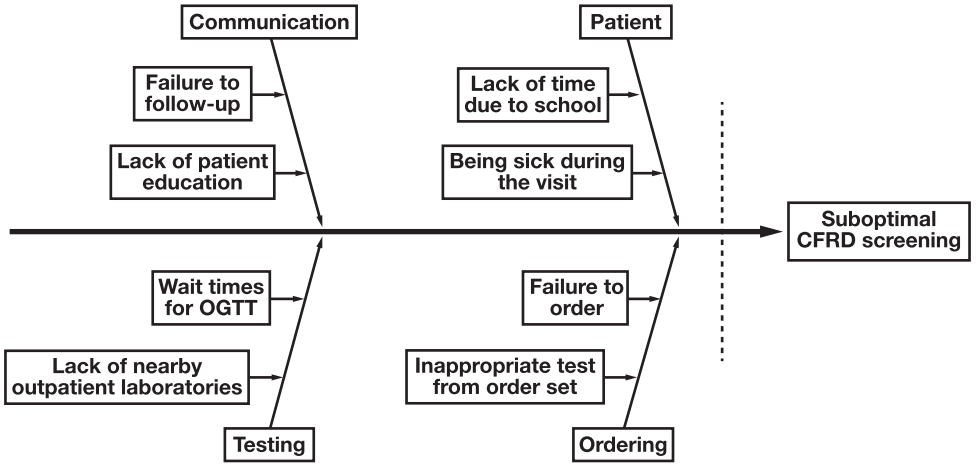


TABLE 2. Key interventions administered in the quality improvement initiative

Barriers	Root causes	Interventions
Patient no-show	Lack of time due to school	Annual super visits during the summer block
	Sick during the visit	Appropriate follow-up and reminders
Physician order entry	Inappropriate test from order set	Best practice advisory for the test priority
	Missed ordering OGTT	Physician reminders during the monthly CF meetings
Patient communication	Lack of patient education	Annual newsletters
	Lack of follow-up	Telephone reminders to patient families
Lab testing	Long wait times for OGTT	Incentives like free parking for patient families (future)
	Limited slots at the outpatient lab	Increased the number of appointments

CF, cystic fibrosis; OGTT, oral glucose tolerance test.

May and September 2023, 79 patients completed the OGTT out of 112 order requisitions (**FIGURE 3**). Therefore, our QI initiative improved the fractional OGTT completion from 45% during January-April to 70% during May-September. Altogether, our data highlights the need for CF center–specific strategies to reverse the suboptimal OGTT screening in pediatric patients.

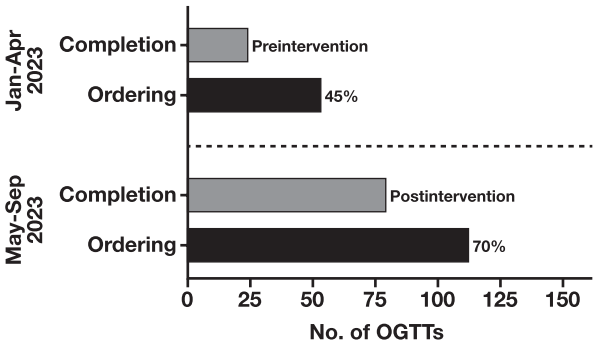
Discussion

Over 30% of CF patients suffer from 5 or more comorbidities, including diabetes, liver disease, bone disease, anxiety/depression, pancreatic insufficiency, and reproductive dysfunction.[9] Of note, CF patients with diabetes display a mortality rate ~6-fold higher than those without diabetes.[10] As per the multisociety guidelines, annual diabetes screening by 2-hour OGTT is recommended for all CF patients starting at the age of 10 years.[6] However, there are numerous challenges in compliance with these guidelines. Primarily the testing itself, with the need for fasting and multiple blood draws over a period of 2 hours, especially during busy multidisciplinary CF clinic visits, is the most challenging factor.[8,11,12] Although CFRD screening rates improved nationwide in the past decade, it is still considered suboptimal. Many CF centers across the US have already adopted QI strategies to tackle this issue.[8,11-13] However, none of the studies involving these strategies were performed post-COVID-19 pandemic, which had a significant overall impact on health care workflows and practices.

The CFF-accredited program at Texas Children's Hospital provides comprehensive CF care to ~386 pediatric patients from various regions within Texas and nearby states. A retrospective analysis of CFRD screening rates between 2019 and 2022 at our center highlighted a downtrend, which was still higher than national averages. Notably, during the peak of the COVID-19 pandemic in 2020, the CFRD screening rate of our center was 61.9%, in contrast to a national average of just 49.9%. In an effort to halt the declining CFRD screening rates, we used a 3-pronged approach, refining practices specific to patient visits, physician order entry, and laboratory testing.

Several studies have recognized the value of annual super visits where CF patients attend a multidisciplinary CF clinic and undergo

FIGURE 3. Oral glucose tolerance test (OGTT) ordering and completion rates after quality improvement initiative.



various laboratory tests, such as pulmonary function tests, chest X-rays, vitamins, lung function tests, pancreatic enzymes, OGTT, and HbA1c, on the same day.[12] This approach is thought to provide family-centered holistic care and guidance for self-management. However, OGTT is known for its high within-individual variation, with a coefficient of variation of 25.3% among CF patients. Hence, the effect of the overall laboratory testing burden during the super visit on the OGTT results should be considered.[14] In our QI plan, we also incorporated patient education–centered practices, such as annual newsletters and telephone reminders before the appointment. Moreover, we are considering incentives, such as free parking, for the families on the day of the super visit.

Inappropriate test ordering by the provider was determined to be another major barrier to suboptimal CFRD screening at our center. For instance, a majority of the patients without OGTT results have order requests placed at Quest Diagnostics. This finding caused us to look into the CF care provider's order sets and modify them appropriately. Specifically, we created a best practice advisory assigning priority to test codes. To accommodate the influx of OGTTs at the hospital laboratory, we undertook a major effort to optimize blood draw scheduling, personnel allocation, laboratory workflow, and testing inventory by constant communication with laboratory managers and administrators. Clearly, these QI measures showed a tangible outcome by significantly improving the OGTT-ordering compliance rate and slightly improving the OGTT completion rate.

A major limitation of this study is the lack of longitudinal OGTT compliance data post-QI implementation. However, we intend to sustain these efforts long-term with continuous quality improvement, ultimately enhancing CF patient outcomes. Furthermore, we plan to incorporate additional outcome measures, such as the frequency of acute pulmonary exacerbations and body mass index, on an ad hoc basis. Although we did not notice any difference in the number of patients with abnormal OGTT results (2 h glucose >200 mg/dL) after implementation of QI, it will be valuable to monitor this parameter longitudinally to assess the clinical significance of such efforts. Because >90% of patients with CF are eligible for CFTR modulation therapy with Trikafta, it will be important to consider its effects on diabetes screening and incidence for future studies. Although there is no clear evidence regarding the role of CFTR modulators in improving long-term glycemic control, they have been shown to enhance overall outcomes.[15] Overall, we believe that the scope of our QI initiative will likely be further benefited by fostering collaborations and knowledge exchange with other large pediatric CF centers across the US.

Acknowledgments

A.K.C. was supported by Ching Nan Ou Fellowship Endowment in Clinical Chemistry. The Cystic Fibrosis Foundation provided the national averages related to CFRD screening in the US on request. The authors acknowledge the feedback provided by other CF center members during the meetings.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Khare S, Desimone M, Kasim N, Chan CL. Cystic fibrosis-related diabetes: prevalence, screening, and diagnosis. *J Clin Transl Endocrinol.* 2022;27:100290.
2. Iafusco F, Maione G, Rosanio FM, Mozzillo E, Franzese A, Tinto N. Cystic fibrosis-related diabetes (CFRD): overview of associated genetic factors. *Diagnostics (Basel).* 2021;11(3):572.
3. Coderre L, Debieche L, Plourde J, Rabasa-Lhoret R, Lesage S. The potential causes of cystic fibrosis-related diabetes. *Front Endocrinol (Lausanne).* 2021;12:702823.
4. Mason KA, Marks BE, Wood CL, Le TN. Cystic fibrosis-related diabetes: the patient perspective. *J Clin Transl Endocrinol*. 2021;26:100279.
5. Moran A. Cystic fibrosis-related diabetes: an approach to diagnosis and management. *Pediatr Diabetes.* 2000;1(1):41-48.
6. Moran A, Brunzell C, Cohen RC, et al; CFRD Guidelines Committee. Clinical care guidelines for cystic fibrosis-related diabetes: a position statement of the American Diabetes Association and a clinical practice guideline of the Cystic Fibrosis Foundation, endorsed by the Pediatric Endocrine Society. *Diabetes Care.* 2010;33(12):2697-2708.
7. Lanng S, Hansen A, Thorsteinsson B, Nerup J, Koch C. Glucose tolerance in patients with cystic fibrosis: five year prospective study. *BMJ.* 1995;311(7006):655-659.
8. Kern AS, Prestridge AL. Improving screening for cystic fibrosis-related diabetes at a pediatric cystic fibrosis program. *Pediatrics.* 2013;132(2):e512-e518.
9. Dickinson KM, Collaco JM. Cystic fibrosis. *Pediatr Rev.* 2021;42(2):55-67.
10. Zirbes J, Milla CE. Cystic fibrosis related diabetes. *Paediatr Respir Rev.* 2009;10(3):118-123.
11. Abdulhamid I, Guglani L, Bouren J, Moltz KC. Improving screening for diabetes in cystic fibrosis. *Int J Health Care Qual Assur.* 2015;28(5):441-451.
12. Liou TG, Jensen JL, Allen SE, et al. Improving performance in the detection and management of cystic fibrosis-related diabetes in the Mountain West Cystic Fibrosis Consortium. *BMJ Open Diabetes Res Care.* 2016;4(1):e000183.
13. Lisa Read MAG, Gandrud L, Sachs MA, et al. Impact of standardized screening protocols for cystic fibrosis–related diabetes in a pediatric population. *J Clin Outcomes Manag.* 2015;22(8).
14. Scheuing N, Holl RW, Dockter G, et al. High variability in oral glucose tolerance among 1128 patients with cystic fibrosis: a multicenter screening study. *PLoS One.* 2014;9(11):e112578.
15. Merjaneh L, Hasan S, Kasim N, Ode KL. The role of modulators in cystic fibrosis related diabetes. *J Clin Transl Endocrinol.* 2022;27:100286.

Rainbow phlebotomy collection and urine aliquots for emergency department add-on testing in the era of pandemic-driven supply shortages

Scott Potter, MD,[1,2] *Joseph W. Rudolf, MD,*[1,2,*] *Lauren N. Pearson, DO, MPH*[1,2,*]

[1]Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT, US; [2]ARUP Laboratories, University of Utah, Salt Lake City, UT, US. Corresponding author: Lauren N. Pearson; lauren.pearson@aruplab.com. *Contributed equally.

Key words: rainbow; rainbow draw; supply chain; urine aliquot; rainbow phlebotomy; emergency department add-on testing

Abbreviations: ED, emergency department; LIS, laboratory information system

Laboratory Medicine 2024;55:585-589; https://doi.org/10.1093/labmed/lmae011

ABSTRACT

Background: Rainbow blood draws for add-on testing in the emergency department (ED) are a common practice at our institution. We sought to determine the prevalence of this practice among reference laboratory clients and characterize the impact of pandemic-driven supply shortages.

Methods: This cross-sectional study surveyed 354 client laboratories to understand specimen collection practices in specific clinical environments and how these practices may have been affected by supply chain shortages. Data analysis by descriptive statistics was performed in Qualtrics.

Results: A total of 138 laboratories took the survey (39% response rate) with 57% indicating that their ED performed rainbow draws. Of these, 16% have a formal policy regarding rainbow draws, and 76% of respondents indicated that their institution was required to modify practices due to pandemic-driven supply shortages. A total of 19% indicated they routinely collect multiple urine aliquots for add-on testing.

Conclusion: Rainbow draws and collection of urine aliquots in the ED for add-on testing are relatively common practices, with few institutions maintaining formal policies regarding the practice. Pandemic-driven supply chain shortages affected a majority of respondent laboratories and local cost-benefit analysis regarding extra specimen collection is recommended to limit waste of laboratory resources.

Introduction

A rainbow draw, named for the colors of the outer protective caps of blood specimen vacutainer tubes collected, is often perceived as an essential part of operations workflows in certain clinical settings, such as emergency department (ED) care. Theoretically, this allows for the full spectrum of add-on testing without the harm of a second venipuncture "poke" and perceived subsequent delay associated with specimen collection and processing. Limitations in laboratory operations imposed by integrating a new laboratory information system (LIS) in our hospital-based clinical laboratory necessitated discussion at our institution regarding the practice of rainbow draws and urine aliquots collected at ED admission. These discussions prompted the following questions: Is this the standard of care at other institutions? Given recent supply chain limitations, is this practice sustainable? What are the benefits of this practice? What are the risks and unintended consequences? To inform discussions at our institution, a survey regarding collection of rainbow draws and urine aliquots in the ED was distributed to our reference laboratory clients. We sought to understand the prevalence of this practice in the context of recent supply shortages affecting laboratories and health care systems.

Methods

The goal of the cross-sectional survey was to determine whether and how institutions perform rainbow draws (defined as the practice of collecting a predefined set of blood tubes) and urine aliquot collections. The survey questions were intended to understand specimen collection practices in specific clinical environments and how these practices may have been affected by supply chain shortages. A total of 354 ARUP Laboratories clients who previously completed the 2021 Client Satisfaction Survey (distributed October 2021) were invited electronically by email to participate in the survey on March 24, 2022. ARUP Laboratories is a national nonprofit academic clinical reference laboratory based in Salt Lake City, UT. Survey questions (Supplement 1) were administered in the English language in Qualtrics. The survey was open for 3 weeks until April 15, 2022. Reminder emails were sent out each week (March 31, April 6, and April 13). The survey invitation stated that the survey findings may be published but that individual responses would be analyzed in aggregate and remain confidential. Data analysis by descriptive statistics was performed in Qualtrics.

TABLE 1. Rainbow draw respondent demographics (n = 138)

Facility type	No. (%) of respondents	United States region		
		East	Central	West
Community 1 (0-100 beds)	31(22.5)	7	16	8
Community 2 (101-250 beds)	32 (23.2)	8	14	10
Community 3 (251-500 beds)	33 (23.9)	10	11	12
Community 4 (>500 beds)	4 (2.9)	0	3	1
Reference lab: regional	4 (2.9)	1	0	3
Reference lab: specialty	2 (1.4)	0	0	2
Reference lab: national	1 (0.7)	0	0	1
Reference lab: research	1 (0.7)	0	0	1
Academic	18 (13.0)	6	7	5
Veterans Affairs/federal	2 (1.4)	0	1	1
Pathology group or clinic	3 (2.2)	0	3	0
Children's	7 (5.1)	2	5	0

Results

A total of 138 clients took the survey (39% response rate) from a variety of facilities across 3 geographical regions (TABLE 1). Of these, 78 participants (57%) selected "yes" to their ED performing "rainbow draws" and were asked additional questions about rainbow draws. A total of 60 participants (43%) selected "no" (36%) or "don't know" (8%) and were then directed to questions about urine aliquot collections. A total of 16% of the institutions have a formal policy governing the use of rainbow draws (46% did not and 38% of respondents did not know).

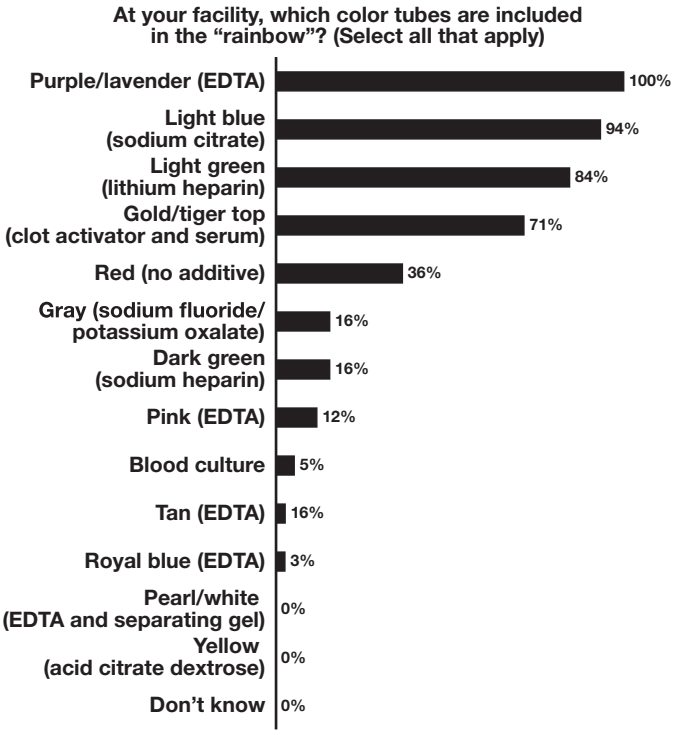
Most institutions (>70%) who collect rainbow draws include purple/lavender EDTA, light blue sodium citrate, light green lithium heparin, and gold/tiger top serum tubes in the collection set (FIGURE 1). Approximately one-third also include a red top with no additive. A small minority of institutions include the remaining types of tubes queried in the survey types. Most institutions collect blood into 1 tube of each tube type in the set, although a few collect multiple tubes of each type.

A total of 78% of respondents indicated that their facility was required to modify tubes included in the rainbow draws due to supply chain shortages. A total of 13% did not make modifications and 9% did not know. Light green lithium heparin, light blue sodium citrate, blue top, and purple/lavender EDTA tubes were most affected by supply chain issues (FIGURE 2). Free response comments showed that for many who have changed their practices regarding rainbow draws, nationwide vacutainer shortage, limited number of "extra" tubes actually being used for add-on testing, and a desire to decrease cost, medical waste, and iatrogenic anemia were reasons driving policy change. Respondents also mentioned conversion to a new LIS as a driving factor in discontinuing the practice due to limited informatics capability to support the practice safely.

Approximately one-third of institutions use rainbow draws in specific clinical scenarios (FIGURE 3A), with facilitation of add-on testing and trauma workflows being the most indicated reasons to default to rainbow collection (FIGURE 3B).

Of 135 total, 26 participants (19%) who answered questions about urine aliquot practice indicated that their ED routinely collects multiple aliquots of urine in case of unanticipated testing and were asked additional questions about urine aliquots (3 participants omitted the question). A total of 109 participants (81%) selected no (61%) or didn't know (20%) and were directed to the end of the survey. Gray top (preservative), yellow top (no additive), sterile cups, and clear tubes were used to collect urine aliquots in the ED (FIGURE 4A). FIGURE 4B shows the clinical laboratory tests for which urine aliquots are collected; urine culture, toxicology testing, and urinalysis were the most frequently utilized tests.

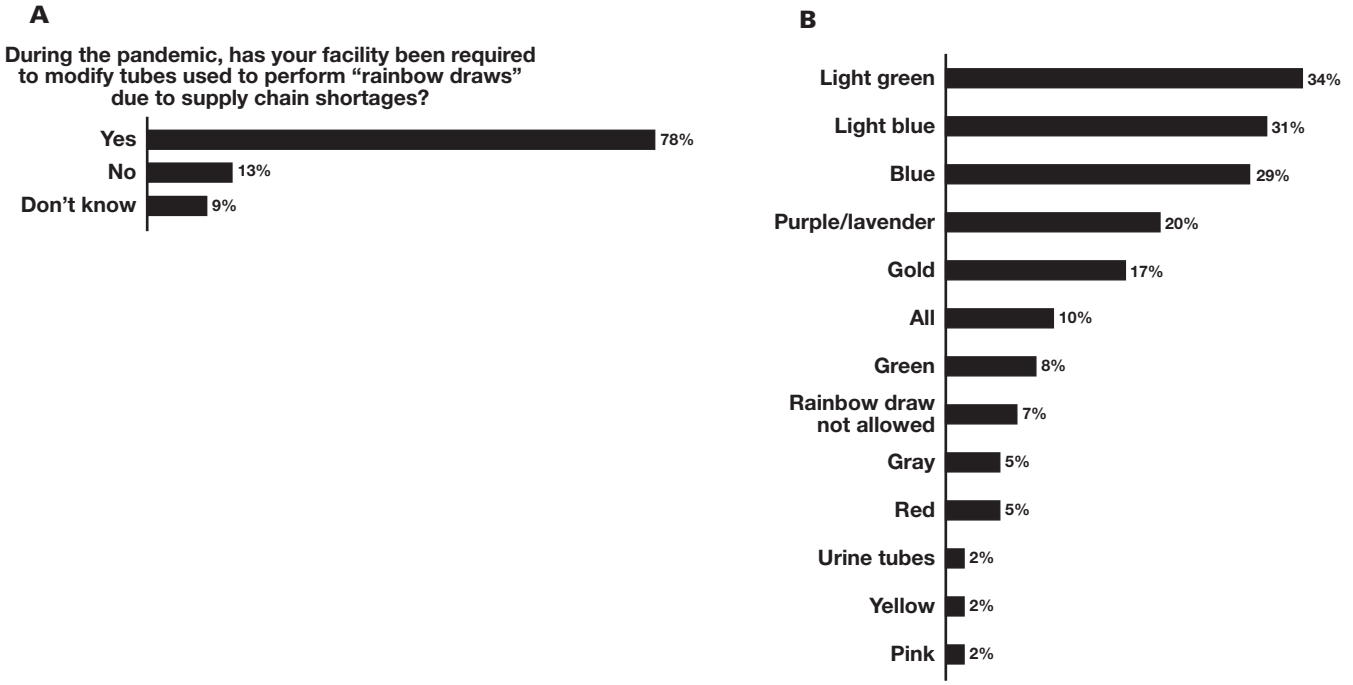
FIGURE 1. Tubes included in the "rainbow" at surveyed institutions. The percentages in the chart do not add up to 100% because participants could select more than 1 option.



Discussion

Implementation of a new LIS system was the catalyst for reexamining our institution's practices; however, patient safety, cost, medical waste,

FIGURE 2. Impacts of supply chain limitations on the practice of rainbow draws (A) and venipuncture blood collection tubes (B).



and specimen quality concerns were perceived by the laboratory for many years prior. The laboratory built custom processes and procedures to accommodate the rainbow collection to add-on testing workflow desired by the ED specifically. In our workflow, specimens are received and stored unprocessed as extra specimens and later retrieved from storage after a technician approves add-on testing that is requested. Prior to laboratory automation, this was a manual process and is now partially automated. Specimens are received on the track, centrifuged on the automated line if appropriate, and sent to refrigerated storage. Unpublished internal data at our hospital laboratory showed a low frequency of add-on requests for the stored specimens collected during a rainbow draw. Our anecdotal experience is corroborated by 1 survey respondent who indicated that <10% of stored specimens were being used for add-on testing at their facility. This is similar to reported rates of 4% and 7% in the limited literature addressing rainbow draws.[1,2] Snozek et al[3] indicate that of 5 standard tubes in their rainbow, the fluoride oxalate, citrate, and serum separator tubes were often discarded unused; however, the rapid serum tube and EDTA were used nearly 100% of the time for initial or add-on testing.

Prior laboratory studies addressed the rainbow process from the standpoint of eliminating waste. They evaluated cost, risk of iatrogenic anemia, human resources burden, laboratory operations burden (staffing, space, and infrastructure), and rate of use of the extra tubes.[1-5] The most recent study incorporated rates of tube use compared with staff perception of benefit from the practice to provide insight to encourage systematic change.[3] Among all of these studies, the actual prevalence of the practice of rainbow collections has not been estimated. Our survey is unique in that it estimates a prevalence for rainbow collections (57% of our respondents) as well as impact of recent supply chain shortages (78% modified practice) across a spectrum of facility types and in all regions of the United States.

Is This the Standard of Care at Other Institutions?

With just over half of our surveyed clients indicating they perform rainbow collections and only 19% affirming collection of urine aliquots in the ED, it may be argued that these traditional practices do not constitute a standard of care. It was noted that 1 survey respondent shared that trauma level I best practice is to collect all required draws during initial phlebotomy procedure; however, compliance with this purported best practice may not necessarily mean collecting 1 of every tube type at the initial phlebotomy. We were also unable to find a best practice regarding phlebotomy in level I trauma centers. It has been suggested that a chief complaint or specific clinical scenario approach may be appropriate to use rainbow collections and urine aliquots without adding undue burden or increasing the aforementioned sources of waste.[3,4] It has also been suggested that future studies should be geared toward identifying appropriate clinical scenarios in which a rainbow collection, and we would add urine aliquot collection, may be appropriate.[2]

Is This Practice Sustainable?

At our institution, local factors—laboratory resources, LIS limitations, and storage—along with global factors, such as vacutainer shortages and the recent pandemic, have affected discussions about the practice of rainbow and urine aliquot collections. As previously indicated, a majority of those who perform rainbow collections were forced to alter their practices due to supply chain limitations. In their comments, many respondents indicated that the practice of rainbow collections had been tolerated but discouraged at their institution, and the vacutainer shortage was the final push to abandon the practice. With 78% of respondents who perform rainbow draws indicating they had to change or modify their practice due to pandemic-related workflow issues, this suggests that the practice may not be sustainable in the current environment. A few respondents even indicated that a single extra tube was

FIGURE 3. Clinical scenarios in which rainbow collections are performed (A) and why (B). Respondents could select more than 1 reason. Of the 26% who selected "other" (A), 80% said they perform rainbow collections for emergency department (ED) patients. TAT, turnaround time.

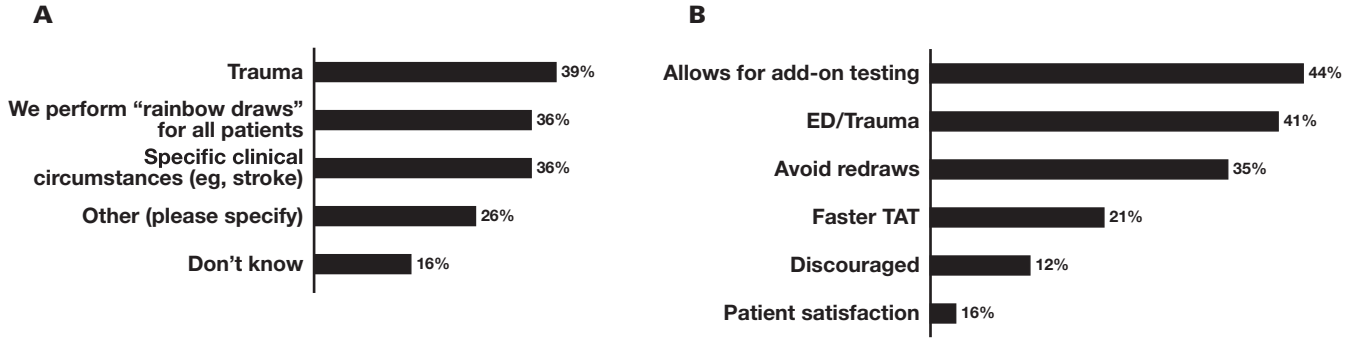
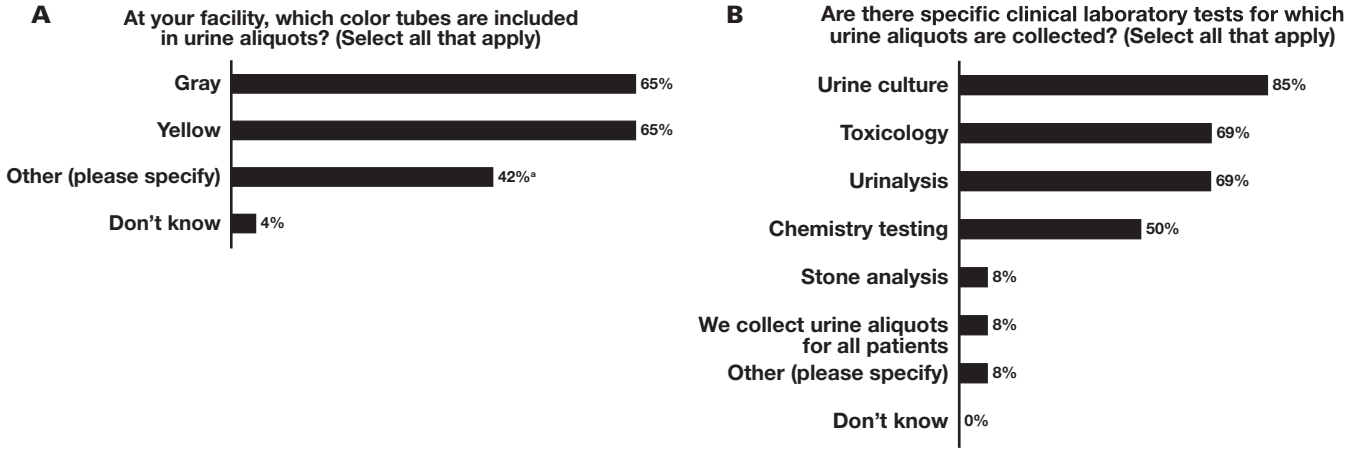


FIGURE 4. Tubes included in urine aliquots (A) and specific clinical laboratory tests for which urine aliquots are collected in the ED (B). [a]Of those 42% who selected "other (please specify)," 50% said they use sterile cups for urine aliquots and 20% said clear tubes.



not tolerated in their systems, and others discussed tracking of unused specimens collected as part of their quality and monitoring processes to eliminate waste. With ongoing disruption in global supply chains, extra tubes to be collected, stored, and discarded without use for testing may be unsustainable in some settings.

What Are the Benefits to This Practice?

Perceived benefits of this practice inevitably center on the decreased turnaround time for clinically actionable test results and greater patient satisfaction with avoidance of additional phlebotomy collections.[3,4] Snozek et al[3] showed a high perceived value of rainbow draws among their surveyed ED staff, and perception of utilization rates of tubes, add-on rates of tests, and averted additional venipunctures was overestimated compared with the true rates of these practices. One example is that averted venipunctures were perceived by staff at a rate of >11 patients/day (79% of staff) vs the calculated rate of averted venipunctures as 7 patients/day.[3] Although this example could be interpreted to show there is not as much benefit from the practice of rainbow collections as traditionally believed, this does show that our colleagues in the ED place a great deal of importance on these practices to achieve their desired standard of patient care. Individual institutions will potentially need internal studies to elucidate the true value of this practice. Again, further work to determine the appropriate clinical scenarios for add-on testing would be beneficial to the broad community of ED and trauma care. One scenario where rainbow collections could show benefit is in circumstances where there are large numbers of patients in the ED waiting room or triage area awaiting full medical evaluation. Having basic laboratory results reported for these patients could, in theory, expedite diagnosis and disposition.

What Are the Risks and Unintended Consequences?

In 2003 the College of American Pathologists released results from a survey of 140 laboratories addressing the overcollection of blood for routine laboratory testing. They estimated that more than 8.5 times the blood volume necessary for the indicated tests was collected, leading to waste and potentially iatrogenic anemia. It was concluded that most institutions could decrease blood volume while not affecting the reliability and turnaround time for their testing procedures.[6] This was a landmark report that showed the potential for systematic change in laboratory practices to improve patient care. In 2010, Loh et al[5] were the first to discuss the problem of extra blood tubes from the perspective of waste, including loss of blood from excess phlebotomy and the financial cost of the additional consumables, processing time, and storage of tubes in the laboratory process. Seaver and Gray[1] focused on true cost

analysis, eliminating waste, and improving staff efficiency as an essential laboratory function due to increasing costs and decreasing reimbursement for laboratory tests. Mullen[4] indicated an opportunity for cost savings by focusing on the medical waste reduction alone as well as decreased risk of exposure inherent to all steps of collection, processing, testing, storage, and disposal of potentially hazardous samples. Anecdotal experience as well as comments from respondents suggests that storage of extra tubes often requires a deviation from standard practice compared with specimens drawn with active orders. These deviations can introduce opportunities for error and may lead to suboptimal conditions for testing when add-on requests are made. Additional assessment of risk from the clinical workflow could be enlightening, and a study incorporating ED metrics and viewpoints would add to the body of literature.

When a larger than necessary number of blood specimens is collected, there is a risk of patient specimen labeling and identification errors, which is a patient safety concern. Additionally, failure to follow appropriate collection practices for order of draw can result in spurious results being reported, which, if unrecognized, could result in misdiagnosis and inappropriate treatment. Because some specimens collected during rainbow draws will be stored as extra specimens without consideration for the specimen processing and storage conditions required for the myriad of tests that may be subsequently ordered, there is a risk of spurious, poor quality results being reported for the tests that are done using the stored extra specimen. For example, electrolytes and common enzyme tests are affected by ambient vs cold storage conditions and whether the plasma or serum is separated from cells. Although of perceived benefit, the unintended consequences of potentially spurious results and reduced quality is a risk for patient care that should be carefully considered at every institution.

Conclusion

Our study shows that rainbow draws and urine aliquot collections happen at many institutions in the United States but that there is heterogeneity among institutional practices. Few institutions have a policy governing the use of these workflows. Given the quality, cost, and laboratory operations impacts, each institution should determine local prevalence of the practice, assess benefits vs risks, and quantify waste and the cost of maintaining this process. Variables affecting an institution's ability to accommodate this workflow include (but are not limited to) availability of phlebotomists to collect specimens from patients in the ED, acuity of care offered in the ED, volume and wait times for ED care, the ability of a laboratory LIS to accommodate rainbows and urine aliquots, laboratory staffing limitations, and the efficiency of manual vs automated processes in the laboratory for receiving, storing and retrieving specimens. This study only determined the prevalence of rainbow draws and urine aliquots in the ED; future studies could examine prevalence in other clinical areas where there may be perceived benefit, such as inpatient acute care and obstetrics. Perceived patient satisfaction associated with reduced venipuncture draws was not included in our study. An additional area for future research includes collaboration with ED physicians and other clinicians to identify potential use-case scenarios where rainbow draws and urine aliquots could be clinically and operationally beneficial. This survey showed many laboratories were affected by pandemic-driven supply constraints, which represent another reason to revisit the practice of rainbow phlebotomy collections and urine aliquots for optimal areas of use.

Acknowledgments

The authors thank Leslie Smith for her expertise in drafting survey questions, facilitating the survey, and providing preliminary data analysis of responses.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. Seaver C, Gray AJ. Drawing extra blood tubes in the ED re-examining a common practice. *Med Lab Obs.* 2012;44(12):38+.
2. Humble RM, Hounkponou HG, Krasowski MD. The "rainbow" of extra blood tubes—useful or wasteful practice? *JAMA Intern Med*. 2017;177(1):128-129. https://doi.org/10.1001/jamainternmed.2016.6834
3. Snozek CLH, Hernandez JS, Traub SJ. "Rainbow draws" in the emergency department: clinical utility and staff perceptions. *J Appl Lab Med*. 2019;4(2):229-234. https://doi.org/10.1373/jalm.2018.027649
4. Mullen M. Our hospital is over the rainbow. *Am J Clin Pathol.* 2012;138(suppl 2):A353. https://doi.org/10.1093/ajcp/138.suppl2.149
5. Loh TP, Saw S, Sethi SK. Extra blood tubes — an affordable excess? *Clin Chim Acta*. 2010;411(19-20):1544-1545. https://doi.org/10.1016/j.cca.2010.06.007
6. Dale JC, Ruby SG. Specimen collection volumes for laboratory tests: a College of American Pathologists study of 140 laboratories. *Arch Pathol Lab Med.* 2003;127(2):162-168. https://doi.org/10.5858/2003-127-162-SCVFL

The Arg/Arg genotype of leptin receptor gene Gln223Arg polymorphism may be an independent risk factor for nonalcoholic fatty liver disease

Mahsa Navari [1], Fatemeh Zarei [2], Shiva Sayedsalehi [1], Touraj Mahmoudi [1], Mitra Rostami [1], Aidin Mahban [3], Gholamreza Rezamand [4 5], Asadollah Asadi [6], Reza Dabiri [7], Hossein Nobakht [7], Hamid Farahani [8], Seidamir Pasha Tabaeian [4 5], Mohammad Reza Zali [1]

[1]Department of Clinical Laboratory Sciences, Faculty of Allied Medicine, Tehran University of Medical Sciences, Tehran, Iran, [2]Student Research Committee, Jahrom University of Medical Sciences, Jahrom, Iran, [3]Student Research Committee, Kashan University of Medical Sciences, Kashan, Iran, [4]Department of Anesthesiology, Faculty of Paramedical Sciences, Kashan University of Medical Sciences, Kashan, Iran, [5]Student Research Committee, Guilan University of Medical Sciences, Guilan, Iran, [6]Department of Anesthesiology, Faculty of Paramedical Sciences, Guilan University of Medical Sciences, Guilan, Iran. *To whom correspondence should be addressed: rafrisham@sina.tums.ac.ir.

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

Laboratory Medicine 2024;54:502-506; https://doi.org/10.1093/labmed/lmac159

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 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 202412). Briefly, the serum was first separated and 100 µL 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 µL of conjugated enzyme was added into each well and incubated for 30 min at 37°C. After washing, 100 µL of substrate solution was added into each well and placed in the dark at room temperature for 15 min. In the next stage, 100 µL 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 ± 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 ± 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 ± 7.02	1.62 (1.31–3.7)	<.001	—	—
		Control	33.3 ± 7.3				
BMI (kg/m$^{2)}$	—	Case	28.2 ± 4.2	1.15 (1.04–1.3)	.011	1.13 (1.02–1.3)	.035
		Control	25.6 ± 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 ± 27.8	1.02 (0.99–1.04)	.085	—	—
		Control	13.3 ± 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 (× 10^{9}/L)	—	Case	6.8 ± 2.39	0.93 (0.75–1.13)	.494	—	—
		Control	7.14 ± 1.89				
RBC (× 10^{6}/μL)	—	Case	4.83 ± 0.49	1.19 (0.5–2.94)	.696	—	—
		Control	4.78 ± 0.47				
HGB (g/dL)	—	Case	13.6 ± 1.58	1.06 (0.82–1.38)	.654	—	—
		Control	13.4 ± 1.69				
HCT (%)	—	Case	41.1 ± 4.38	1.04 (0.95–1.56)	.416	—	—
		Control	40.3 ± 4.21				
MCV (fL)	—	Case	85.5 ± 7.86	1.02 (0.96–1.08)	.517	—	—
		Control	84.4 ± 6.87				
MCH (pg/cell)	—	Case	28 ± 3.35	0.988 (0.87–1.12)	.854	—	—
		Control	28.1 ± 3.18				
MCHC (g/dL)	—	Case	33 ± 1.06	0.914 (0.66–1.25)	.581	—	—
		Control	33.2 ± 1.54				

TABLE 1. (cont)

Characteristics		Group	No. (%)/Mean ± SD	OR (95% CI)	*P* Value	AOR (95% CI)	*P* Value
PLT (/μL)	—	Case	297 ± 79.9	1 (0.99–1.01)	.516	—	—
		Control	287 ± 58.7				
LYMPH (%)	—	Case	36 ± 9.29	0.99 (0.97–1.02)	.854	—	—
		Control	37.5 ± 32.3				
MXD (%)	—	Case	7.46 ± 2.8	0.9 (0.74–1.07)	.268	—	—
		Control	8.16 ± 2.38				
RDW-C (%)	—	Case	12.9 ± 1.81	1.02 (0.79–1.31)	.887	—	—
		Control	12.9 ± 1.62				
PDW (%)	—	Case	12.3 ± 2.17	1.06 (0.86–1.31)	.586	—	—
		Control	12 ± 1.94				
MPV (fL)	—	Case	9.72 ± 1.07	1.1 (0.72–1.71)	.664	—	—
		Control	9.63 ± 0.91				
P-LCR (%)	—	Case	24.5 ± 8.27	1.01 (0.96–1.07)	.618	—	—
		Control	23.7 ± 6.9				
IgG against COVID-19 (relative unit)	—	Case	17.2 ± 9.83	1.07 (1.03–1.12)	.002	1.08 (1.03–1.15)	.003
		Control	10.2 ± 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 al[21] 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 al[26] 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.

Acknowledgments

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

This study was funded by Tehran University of Medical Science with the code 1400-3-102-55956.

Conflict of Interest Disclosure

The authors have nothing to disclose.

REFERENCES

1. 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.
2. 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 17β-estradiol against COVID-19. *J Allergy Infect Dis.* 2020;1(2):38–4810.46439/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):e053684e053684. doi:10.1136/bmjopen-2021-053684.
11. Ramírez-de-Arellano A, Gutiérrez-Franco J, Sierra-Diaz 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):573–57510.1097/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.

Microbiological profile of long COVID and associated clinical and radiological findings: a prospective cross-sectional study

Monalisa Dey [1], Baijayantimala Mishra [1], Prasanta Raghab Mohapatra [2], Sudipta Mohakud [3], Bijayini Behera [1]

[1]Department of Blood Transfusion, Affiliated Hospital of Nantong University, Nantong, China. *To whom correspondence should be addressed: huicjs@163.com.

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

Abbreviations: RBC, red blood cell

Laboratory Medicine 2024;54:507-511; https://doi.org/10.1093/labmed/lmac160

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

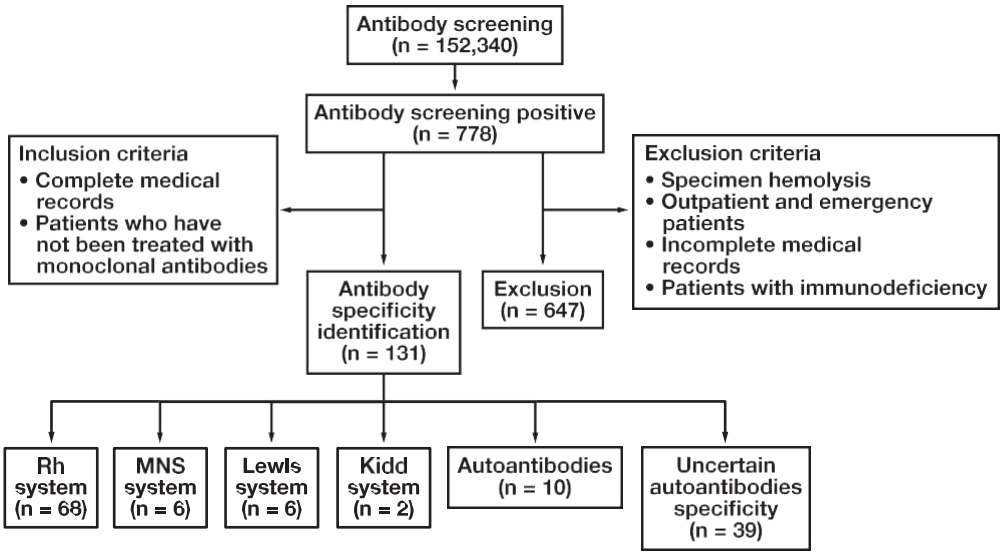
Data

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	DO		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	DO^a	DO^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	/	/	/	/
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	+	0	/	/	/	/
9	0	0	0	+	+	+	+	0	+	0	+	0	+	0	0	+	0	+	+	0	0	0	+	+	0
10	+	+	0	0	+	+	0	+	+	0	+	+	+	0	$+^w$	/	0	+	0	+	+	/	/	/	/

$+^w$, 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)

[a]0: 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)
	Anti-Ce	2 (1.53)
MNS system	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													1.311	.727
A	16	2	0	1	1	0	0	2	0	1	1	14		
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													3.975	.046
Male	6	1	1	0	0	0	2	1	1	1	4	11		
Female	46	4	0	4	3	1	0	1	5	5	6	28		
Age, y													3.607	.307
≤17												2		
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]													4.355	.037
Yes	46	5	0	4	3	1	1	2	5	5	8	28		
No	6	0	1	0	0	0	1	0	1	1	2	11		

[a]*Immunization: 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.

Acknowledgments

This study was supported by the Nantong Commission of Health Project (Program No. MB2020080), Nantong Technology Project (Program No. JCZ20012), and the Jiangsu Transfusion Association "InTec PRODUCTS.INC" Project (Program No. JS2020015).

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. Bhuva 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.

Bone marrow findings post allogeneic transplant for myeloproliferative neoplasms and chronic myelomonocytic leukemia with increased fibrosis

Srishti Gupta [1], Elizabeth L Courville [1]

[1]Department of Computational Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, US, [2]UCLA SwabSeq COVID19 Laboratory, David Geffen School of Medicine at UCLA, Los Angeles, CA, US, [3]Department of Human Genetics, David Geffen School of Medicine at UCLA, Los Angeles, CA, US, [4]Department of Pathology and Lab Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, US. *To whom correspondence should be addressed: VArboleda@mednet.ucla.edu. [a]Contributed equally.

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

Laboratory Medicine 2024;54:512-518; https://doi.org/10.1093/labmed/lmac161

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 perform 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 tagmentation 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, midday [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 protocol[7] 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.**

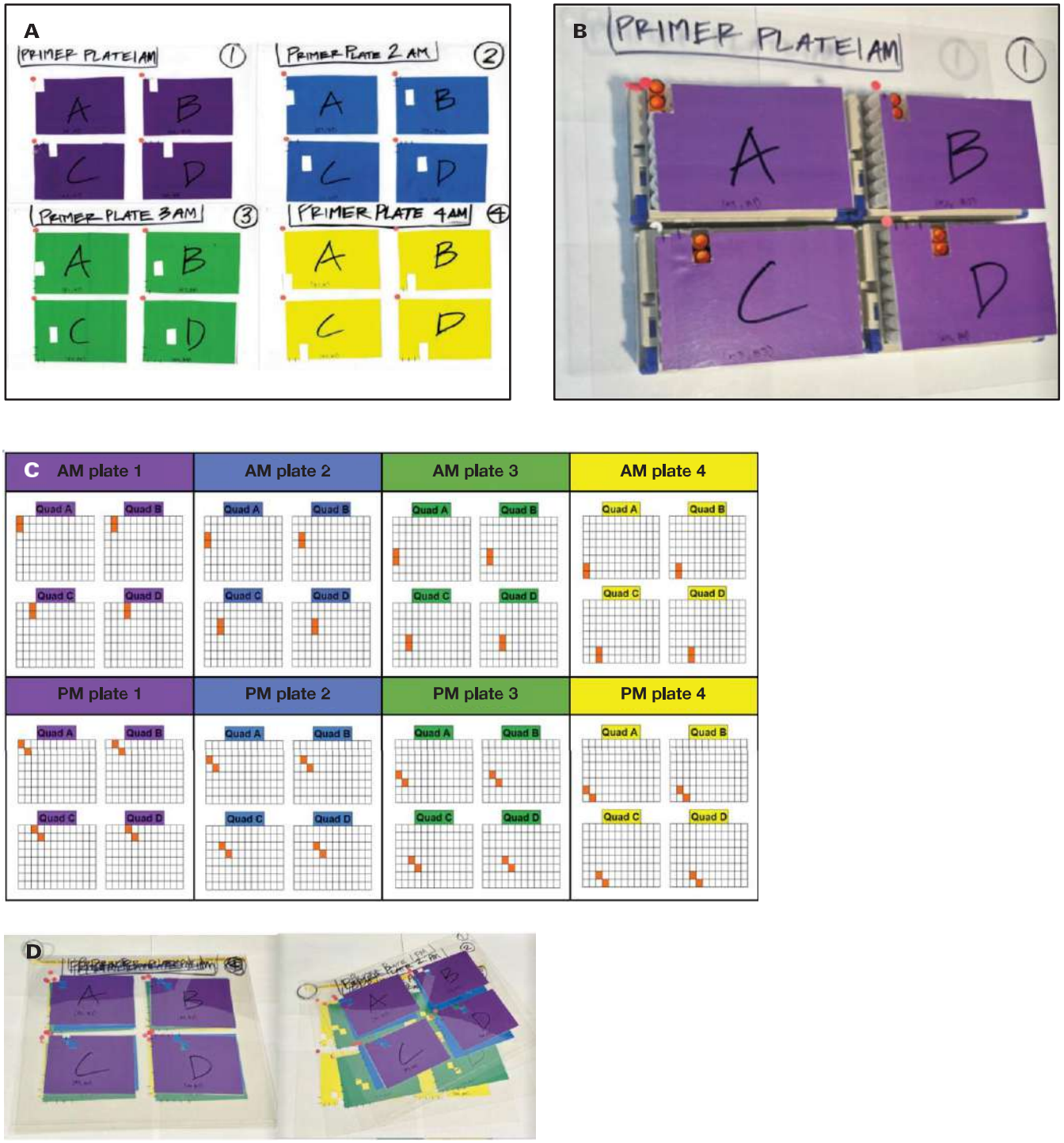


plate number (1–4) with quadrant identification printed directly into the template (initially labeled as 1–4 but changed to A–D in future iterations of the templates).

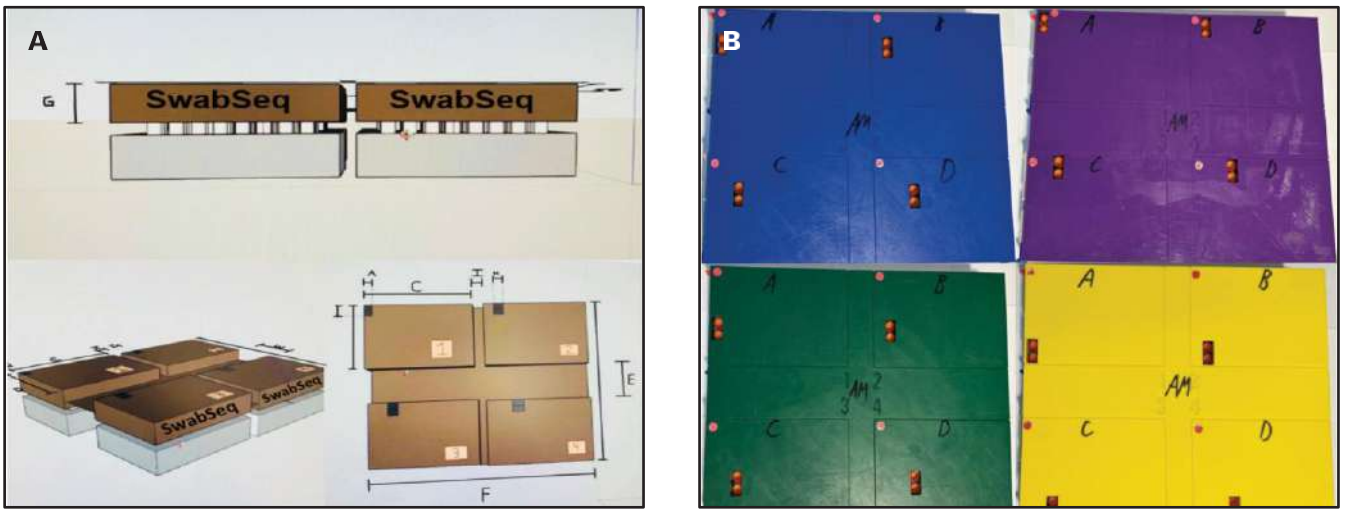
After implementing the 3D templates in late April 2021, we saw our control tube misplacement rate stabilize below 1%. There was a temporary increase in errors that represents a transition and training on

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%

[a]*Tube 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.

Serum microRNA-122 for assessment of acute liver injury in patients with extensive skeletal muscle damage

Yu Zhang [1], Chui Mei Ong [2], Kara Lynch [3,4], Javier Waksman [3], Alan H B Wu [4]

[1]Department of Laboratory Medicine, Yale School of Medicine, New Haven, CT, USA, [2]Department of Pathology, Division of Transfusion Medicine and Hemostasis, University of Texas Southwestern, Dallas, TX, USA, [3]Department of Pathology, University of California San Diego, La Jolla, CA, USA, [4]Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA, [5]Department of Pathology, City of Hope National Medical Center, Irvine, CA, USA, [6]Department of Pathology, Microbiology & Immunology, Vanderbilt University Medical Center, Nashville, TN, USA. *To whom correspondence should be addressed: Jeremy.jacobs@yale.edu.

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

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

Laboratory Medicine 2024;54:519-522; https://doi.org/10.1093/labmed/lmac166

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 colleagues[1] 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 al[2] 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.

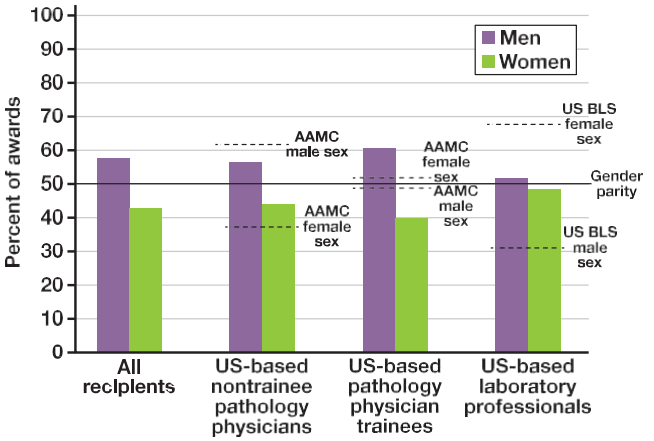
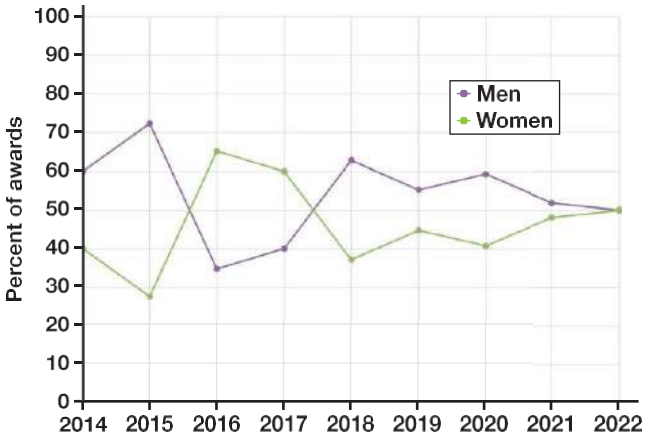


FIGURE 2. Gender composition of The Pathologist Power List and the American Society for Clinical Pathology Top Five 40 Under Forty award recipients by year.



(**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

2024;54:519-522 | https://doi.org/10.1093/labmed/lmac166

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 nontraditional 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.tmrv.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

A clinical update of compound heterozygosity for hemoglobin Hekinan II [a27(B8)Glu-Asp; HBA1: c.84G>T] variant in China

Liqiu Pan[1,2], Yuling Qiu[3], Lihua Ye[4], Linlin Li[1,2], Yuanyuan Huang[5], Wuning Mo[1,2], Faquan Lin[1,2]

[1]Department of Nephrology, Shidong Hospital, University of Shanghai for Science and Technology, Shanghai, China, [2]Department of Cardiology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China, [3]Department of Nephrology, Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, Nanjing, China, [4]Department of Nephrology, Affiliated Hospital of Jiangsu University, Jiangsu University, Zhenjiang, China, [5]Department of Central Lab, Affiliated Hospital of Jiangsu University, Jiangsu University, Zhenjiang, China, [6]Department of Laboratory Medicine, Affiliated Hospital of Jiangsu University, Jiangsu University, Zhenjiang, China. *To whom correspondence should be addressed: windcloud82@163.com. [a]These authors contributed equally to this work.

Keywords: hemodialysis, heart failure, left ventricular ejection fraction, lipoprotein-associated phospholipase A_2, N-terminal proBNP, C-reactive protein

Abbreviations: LVEF, left ventricular ejection fraction; HD, hemodialysis; Lp-PLA_2, lipoprotein-associated phospholipase A_2; 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

Laboratory Medicine 2024;54:523-526; https://doi.org/10.1093/labmed/lmac162

ABSTRACT

Objective: Reduced left ventricular ejection fraction (LVEF) is common in hemodialysis (HD) patients. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is considered an important determinant of cardiovascular events. The aim of the study was to evaluate the relationship between Lp-PLA_2 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-PLA_2 and LVEF was assessed.

Results: A significant difference in C-reactive protein (CRP) and Lp-PLA_2 was observed, with higher levels noted in patients with reduced LVEF ($P \leq .001$). Both Lp-PLA_2 and CRP were negatively correlated with LVEF in the HD patients. Only Lp-PLA_2 remained associated with LVEF in multiple regression analysis.

Conclusion: Lipoprotein-associated phospholipase A_2 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 al[2] 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 A_2 (Lp-PLA_2), 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 A_2 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-PLA_2 plays an important role in cardiovascular diseases.[5,6] Jenny et al[7] found that subjects with HF exhibit elevated Lp-PLA_2 levels associated with an increase in mortality risk. In contrast, only a few studies have investigated Lp-PLA_2 in patients with renal diseases. Winkler et al[8] showed that Lp-PLA_2 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-PLA_2 in HD patients with reduced EF. This study sought to evaluate a possible relationship between serum Lp-PLA_2 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-PLA_2 were examined. The double antibody sandwich enzyme-linked immunoassay method was used to determine the serum Lp-PLA_2 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 ± 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 ≥50%). Group 1 included patients with LVEF ≥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 × 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-PLA_2 levels were 351.24 ± 71.16 μg/L in HD patients with reduced LVEF and 105.62 ± 22.31 μg/L in patients with reserved LVEF. The difference in LP-PLA_2 between the 2 groups was statistically significant (P < .001).

The LP-PLA_2 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 predicators of LVEF, multiple linear regression analysis was performed, revealing that LP-PLA_2 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 (≥50%) and Reduced (<50%) LVEF[a]

	Preserved LVEF (n = 36)	Reduced LVEF (n = 21)	*P* Value
Age, y	75 ± 8	67 ± 12	.095
Gender, No. male (%)	21 (58)	12 (57)	.960
BMI, kg/m^2	23.0 ± 2.4	23.1 ± 4.1	.978
Diabetes, No. (%)	18 (50)	15 (71)	.361
Dialysis vintage, mo	35.2 ± 10.1	30.7 ± 9.1	.352
SBP, mmHg	148.8 ± 14.8	143.9 ± 8.5	.431
DBP, mmHg	78.3 ± 7.4	82.4 ± 9.4	.299
Kt/V	1.49 ± 0.13	1.5 ± 0.14	.778
URR	0.66 ± 0.07	0.68 ± 0.07	.562
Hemoglobin, g/L	103.9 ± 12.8	100.6 ± 16.3	.625
Platelet count, 10^9/L	181.9 ± 62.7	204.7 ± 64.2	.459
D-dimer	0.73 ± 0.3	0.97 ± 0.47	.202
Albumin, g/L	36.5 ± 3.4	36.4 ± 4.8	.951
TC, mmol/L	3.8 ± 0.8	3.9 ± 0.6	.704
TG, mmol/L	1.5 ± 0.6	1.4 ± 0.8	.453
Glucose, mmol/L	6.7 ± 1.8	6.2 ± 1.5	.536
Calcium, mmol/L	2.10 ± 0.14	2.18 ± 0.21	.351
Phosphate, mmol/L	1.50 ± 0.39	1.46 ± 0.46	.866
PTH, pg/mL	289.4 ± 69.2	340.1 ± 93.0	.192
CRP, mg/L	3.4 ± 1.2	6.7 ± 1.8	<.001
NT-proBNP, pg/mL	16,671 ± 5790	19,410 ± 4296	.293
LVEF, %	60.9 ± 4.2	36.9 ± 8.7	<.001
LP-PLA2, μg/L	105.62 ± 22.31	351.24 ± 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.
[a]Data are given as mean ± 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-PLA2	−0.772	<.001
Variable	**β**	***P***
CRP	−0.199	.401
LP-PLA2	−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.
[a]Adjusted $R^2 = 0.565$, $P < .001$. 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.
[a]Data 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 al[12] 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-PLA_2 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-PLA_2 was potentially identified as an independent risk factor for acute cardiovascular events.[14] In another study on HD patients with type 2 diabetes, Lp-PLA_2 was potentially predictive of cardiovascular events and mortality.[8] More importantly, studies have shown that Lp-PLA_2 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-PLA_2 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 A_2 was elevated in both groups. Dialysis-induced local inflammation may increase Lp-PLA_2 production. Impaired cardiomyocytes may exert a higher inflammatory response,[17] thereby inducing a greater increase in Lp-PLA_2 levels in the reduced LVEF group. To date, few studies have reported on the mechanism between Lp-PLA_2 and HF. Lipoprotein-associated phospholipase A_2-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-PLA_2 and LVEF.

Conclusion

Our data demonstrate that Lp-PLA_2 levels are associated with LVEF, which could potentially be used as a tool for evaluating chronic HF with reduced LVEF in HD patients.

Acknowledgments

This study was supported by the Medical Science and Technology Development Foundation, Nanjing Department of Health (No. QRX17063).

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
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
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
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
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
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
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
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
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
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
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
14. De Mauri A, Vidali M, Chiarinotti D, et al. Lipoprotein-associated phospholipase A_2 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
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

Feasibility of opportunistic colorectal cancer screening of hospitalized patients in tertiary care and community hospitals

Ivan Stevic[1,2], *Harminder Singh*[3], *AbdulRazaq Sokoro*[3,4,5]

Department of Pathology, [1]Medical College of Georgia at Augusta University, Augusta, GA, [2]University of Michigan Ann Arbor, MI, and [3]University of Kansas Medical Center, Kansas City, KS. *To whom correspondence should be addressed: gurmukhsinghmdphd@yahoo.com.

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 electrophorsis; 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

Laboratory Medicine 2024;54:527-533; https://doi.org/10.1093/labmed/lmac155

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 shown[13] 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 FMLCs 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.

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.

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 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.
[a]SFLC is expressed as mg/L, and TPr is measured as mg/dL.
[b]The 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.
[c]This 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.
[a]Including the specimen that tested positive via UIFE only.
[b]Approximately one-third of the specimens displayed MLCs despite having urine protein values in the normal range.

TABLE 4. Comparison of Performance or 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.
[a]The 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.

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]

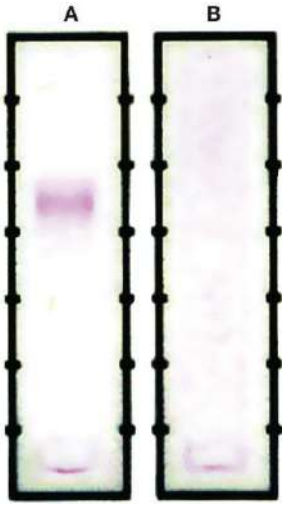
It has also been documented[10] that an abnormal kappa/lambda ratio is not diagnostic of monoclonality, and a normal ratio does not exclude monoclonal gammopathy or MM. A small but significant number of cases of lambda chain–associated MM—IgG lambda and IgA lambda—do not produce/secrete enough lambda MFLCs to render the kappa/

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.

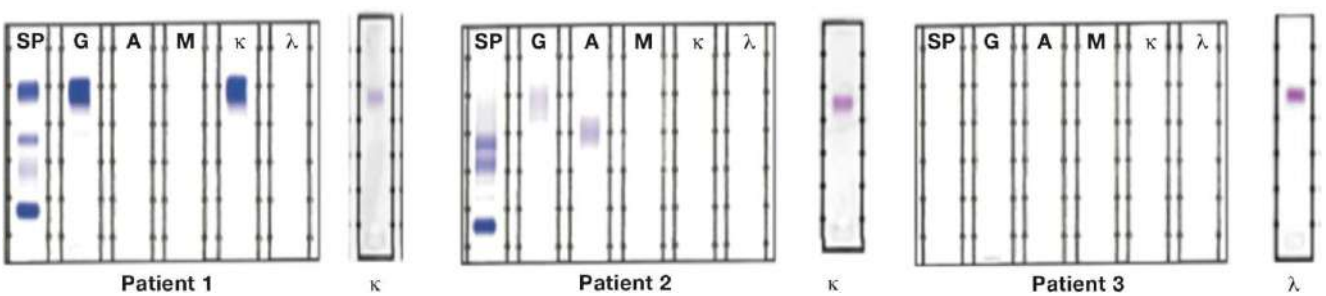
FIGURE 1. Urine from a patient with IgM kappa monoclonal immunoglobulin in serum was tested via conventional urine immunofixation electrophoresis reagents. Staining with antikappa antibody is presented in lane A and shows a low-intensity kappa monoclonal light chain (LC). No kappa LC was detected with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



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

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.

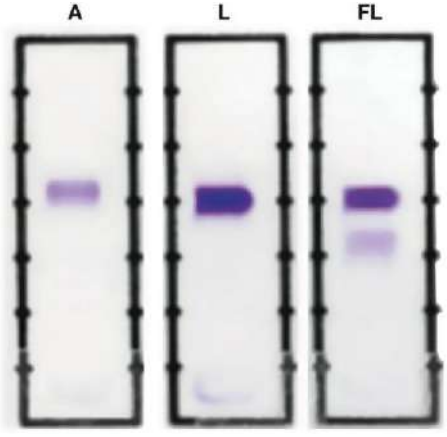


TABLE 5. Performance of FLC-UIFE with Respect to K/L Ratio

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.

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 Site[18] 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 Electrophoesis" (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

1. 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
2. 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
3. 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
4. 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
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.
6. 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.
7. 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
8. 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
9. 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
10. 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
11. 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
12. 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
13. 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
14. 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
15. 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
16. 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
17. 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
18. Badwell AR. *Serum Free Light Chain Analysis Plus Hevylite*. 7th ed. Birmingham, UK: The Binding Site; 2015.
19. 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.
20. 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.
21. 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
22. 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
23. 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
24. 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
25. Leung N, Bridoux F, Nasr SH. Monoclonal gammopathy of renal significance. *N Engl J Med.* 2021;384(20):1931–1941. doi:10.1056/NEJMra1810907

Verifying the nonreporting hemolysis index for potassium, phosphate, magnesium, AST, LDH, iron, CA 19-9, and vitamin D, using Beckman Coulter AU5800 and DxI800 automated analyzers

Samuel Sheerin [1]

[1]Department of Clinical Laboratory, Wuhan University, Renmin Hospital, Wuhan, China. *To whom correspondence should be addressed: lejuan0323@whu.edu.cn.

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

Laboratory Medicine 2024;54:534-542; https://doi.org/10.1093/labmed/lmad005

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)D_3). 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 1000*g* 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 $25(OH)D_2$ and $25(OH)D_3$; 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 (KaALB = 6×10^5 L/mol) and the affinity constant of VDBP for 25(OH)D (KaVDBP = 6×10^8 L/mol) using the following equation[18]:

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

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/m^2).[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 $25(OH)D_2$ and $25(OH)D_3$. The level of $25(OH)D_2$ and epi-$25(OH)D_3$ showed no significant differences between the 2 groups. We detected $25(OH)D_2$ in 31.2% (68/218) SP and 33.5% (79/236) TP, and epi-$25(OH)D_3$ in 67.4% (147/218) SP and 74.6% (176/236) TP. Epi-$25(OH)D_3$ concentration can make up 0% to 37.56% of the total 25(OH)D concentration. Furthermore, the Pearson correlation coefficient between epi-$25(OH)D_3$ and 25(OH)D was 0.602 ($P < .001$) in SP and 0.638 ($P < .001$) in TP. $25(OH)D_3$ could be detected in every participant, and significant differences were found in $25(OH)D_3$ (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

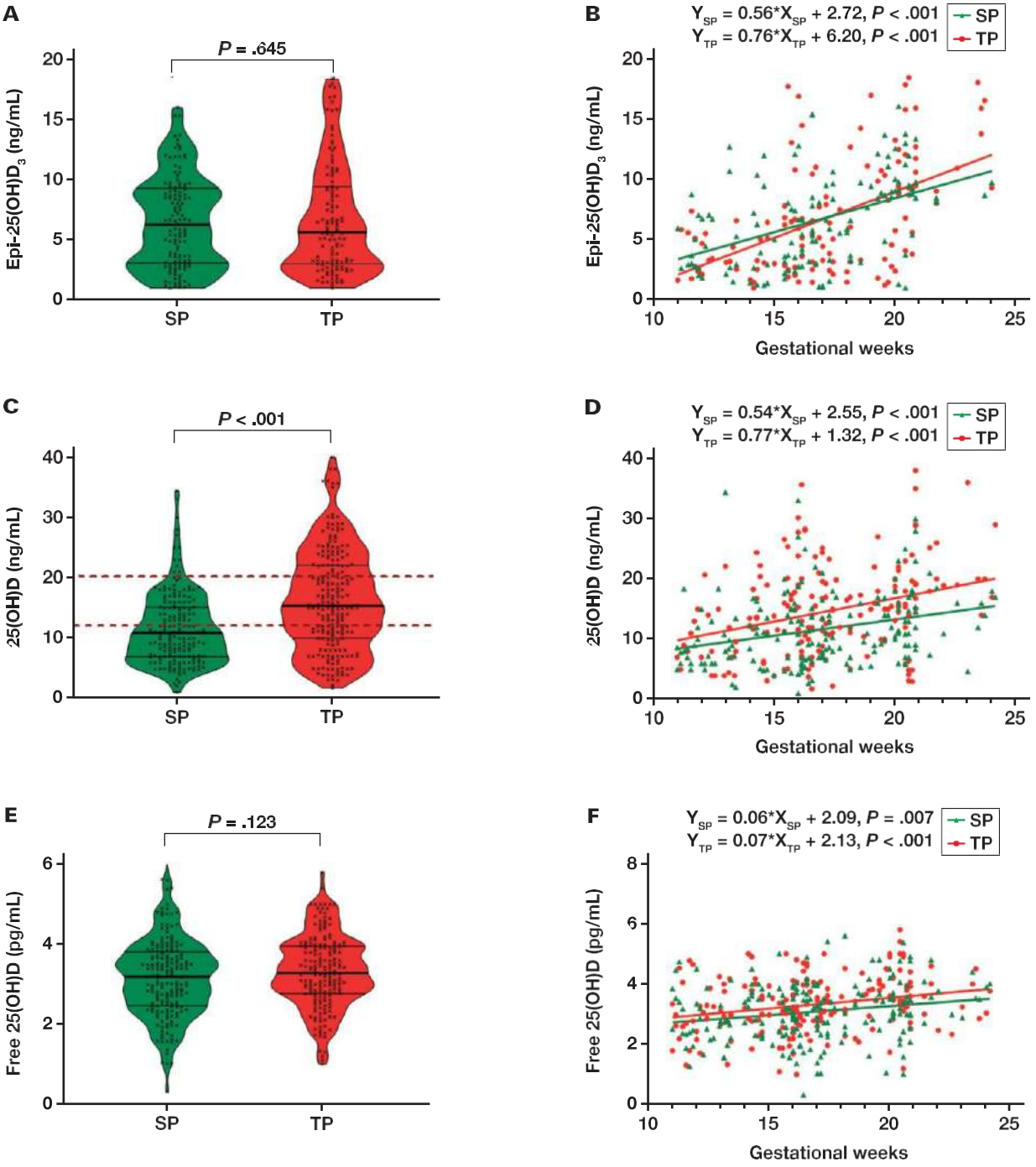
TABLE 1. Maternal Clinical Characteristics of Twin and Singleton Pregnancies[a]

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/m²)	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.
[a]Age, 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)D_3 (**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)D_3 (**FIGURE 1B**), 25(OH)D (**FIGURE 1D**), and VDBP (**FIGURE 1H**) increased with gestational progress in the second trimes-

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)

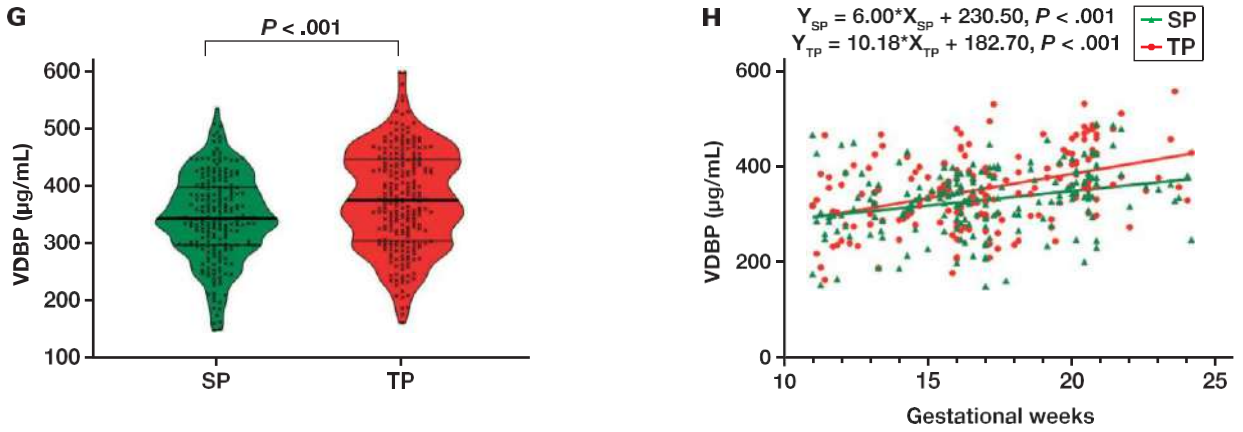


TABLE 2. Adjusted and Unadjusted Vitamin D Levels in Singleton and Twin Mothers[1]

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

[a]*Covariate in adjusted model include age, body mass index, and hemoglobin.*
[b]*In 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)D_3 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.
[a]Crude odds and 95% CI in parentheses (all such values) of univariate logistic regression. P < *.05 indicates significance.*
[b]Adjusted 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)D_3 in over 70% of the enrolled subjects, accounting for 0% to 37% of 25(OH)D concentration. In other research, Bailey and Di Mao[28] found epi-25(OH)D_3 in 77% of Korean and 94.5% of Hong Kong pregnant women,[29] and in Ireland, O'Callaghan et al[30] found that epi-25(OH)D_3 was detectable in all maternal samples. We found epi-25(OH)D_3 was not only proportional to 25(OH)D_3, but also increased as gestational progress (**FIGURE 1B**). This result indicated the effect of pregnancy on the production of epi-25(OH)D_3. However, many immunological and mass spectrometry methods cannot exclude the interference and influence of epi-25(OH)D_3 in the detection of the 25(OH)D level, which often leads to overestimation of VD levels.[14] Therefore, identifying epi-25(OH)D_3 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 $25(OH)D_3$ using its precursor, cholecalciferol,[35] and placental fragments incubated ex utero could convert vitamin D_2 to $25(OH)D_2$.[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, free25(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 Society[47]) 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 D_3) level decreases with age,[48] thus weakening capacity to produce vitamin D_3. 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-$25(OH)D_3$ 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, Robsahm 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. Kominiarek 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, Souz 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

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
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
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
37. Dovnik A, Mujezinović F. The association of vitamin D levels with common pregnancy complications. *Nutrients*. 2018;10(7):867. doi:10.3390/nu10070867
38. Filipecka-Tyczka D, Jakiel G, Kajdy A, Rabijewski 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
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
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
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
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
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
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
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
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
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
48. Wyskida M, Wieczorowska-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
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
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
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

CORRECTION

Correction to: DNA variants detected in primary and metastatic lung adenocarcinoma: a case report and review of the literature

This is a correction to: Christina Kelly, Caitlin Raymond, Song Han, Youmin Lin, Linyijia Chen, Gengming Huang, Jianli Dong, DNA variants detected in primary and metastatic lung adenocarcinoma: a case report and review of the literature, *Laboratory Medicine*, 2024;, lmae019, https://doi.org/10.1093/labmed/lmae019.

In the originally published version of this manuscript, the credentials of author Caitlin Raymond were erroneously omitted. This error has been corrected.