Laboratory Medicine

May 2023 Vol 54 No 3 Pgs 225–336

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 $349; fellow membership plus 1-year unlimited online CE is $519; 2-year fellow membership is $675; and 2-year fellow membership plus 2-year unlimited online CE is $1,015. The ASCP membership fees for laboratory professionals are as follows: newly certified membership is $49; annual membership is $99; annual membership plus 1-year unlimited online CE is $129; 3-year membership is $349. 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.

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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

LaboratoryMedicine

May 2023
Vol 54, No 3
Pgs 225-336

OVERVIEW

SPECIAL REPORT

SCIENCE

CASE STUDY

The following are online-only papers that are available as part of Issue 54(3) online.

ON THE COVER: Phlebotomists may be the most underappreciated of all medical laboratory professionals. Most of the preanalytical factors that can influence or invalidate results of a laboratory test are under the control of the phlebotomist. In addition to possessing the skills to perform venipuncture, phlebotomists must follow strict protocols for patient identification, understand the various types of blood collection tubes and the order in which they should be used, be familiar with any special instructions for patient preparation, and observe meticulous aseptic practices. As the only medical laboratory professionals who regularly interact with patients, phlebotomists are the face of the laboratory and must have the ability to explain the procedures for collecting blood and calm the fears of apprehensive patients. In this issue of *Laboratory Medicine*, Atias-Etzion and Azuri explore the physical demands on phlebotomists and suggest ergonomic approaches to reduce their susceptibility to musculoskeletal injuries.

OVERVIEW

The Clinical Significance of *Staphylococcus aureus* Small Colony Variants

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Keywords: *Staphylococcus aureus*, *Staphylococcus aureus* small colony variants, chronic infections, antimicrobial susceptibility, antimicrobial resistance, diagnostics

Abbreviations: SA, *Staphylococcus aureus*; SA-SCVs, small colony variants; SSTIs, skin and soft-tissue infections; ETC, electron transport chain; WT, wild-type; CF, cystic fibrosis; MIC, minimum inhibitory concentration; PA, *Pseudomonas aeruginosa*; AST, antimicrobial susceptibility testing; MRSA, methicillin-resistant SA; PJIs, prosthetic joint infections; HQNOs, alkyl-hydroxyquinoline N-oxides; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; PBP2', penicillin-binding protein 2'

Laboratory Medicine 2023;54:227–234; https://doi.org/10.1093/labmed/lmac101

ABSTRACT

A burdensome, atypical phenotype of *Staphylococcus aureus* (SA) called *S aureus* small colony variant (SA-SCV) has been identified, which is induced as a result of a combination of environmental stressors, including polymicrobial interactions. The SA-SCVs exhibit altered phenotypes as a result of metabolic dormancy caused by electron transport deficiency, leading to increased biofilm production and alterations to antimicrobial susceptibility. The SA-SCVs typically exhibit altered colony morphology and biochemical reactions compared with wild-type SA, making them difficult to detect via routine diagnostics. The SA-SCVs have been found to contribute to chronic or recurrent infections, including skin and soft-tissue infections, foreign-body associated infection, cystic fibrosis, and sepsis. There is evidence that SA-SCVs contribute to patient morbidity and mortality as a result of diagnostic difficulties and limited treatment options. New detection methods may need to be developed that can be incorporated into routine diagnostics, which would allow for better assessment of specimens and introduce new considerations for treatment.

Staphylococcus aureus (SA) is a bacterial species often found as a transient colonizer on the human body, particularly as part of the nasal microflora, in approximately 30% of the population.[1–3] Despite this, SA will oftentimes act as an opportunistic pathogen, secreting multiple virulence factors within its host and interacting with other pathogens, ultimately contributing to a wide range of human infections, both acute and chronic.[2,4–6] SA infections are important contributors to chronic infections, which are difficult to diagnose and treat due to antimicrobial resistance, including multidrug resistance, and the polymicrobial nature of many chronic infections.[7–10]

A burdensome, atypical phenotype of SA called small colony variants (SA-SCVs) has been identified as largely associated with chronic and recurrent infections such as cystic fibrosis, foreign-body associated infections, skin and soft-tissue infections (SSTIs), sepsis, etc.[6,7,11–23] This phenotype contributes to recurrent infection via increased biofilm formation, altered antimicrobial susceptibility, and intracellular persistence to evade the host's defense mechanisms.[5,8,9,11,22,24–26] Specific nutritional requirements, called auxotrophy, of the chemicals hemin, menadione, and thymidine cause SA-SCVs to be unable to produce key components for a functional electron transport chain (ETC), leading to a deficiency in electron transport and characterizes the phenotype as metabolically dormant.[5,6,24,25,27,28] Consequentially, anoxic conditions increase SA biofilm production and alter antimicrobial susceptibility, rendering the infection difficult to treat.[5,8,14,22,24,28,29]

The SA-SCVs are distinguished from wild-type (WT) SA by their unusual morphological characteristics and biochemical reactions.[5,11,28] The SA-SCVs are characterized by small pinpoint colony sizes that are almost 10 times smaller than WT-SA colonies[5,6,11,22,28,30] (**FIGURE 1**). The SA-SCVs exhibit a slow growth rate, decreased pigmentation, and decreased hemolysis.[5,6,14,22,31] Due to slower growth rates, SA-SCVs require a longer incubation time (approximately 48 to 72 hours for optimal growth) and can be easily overgrown by WT-SA and other organisms of interest in coculture, which makes coculture detection of SA-SCVs difficult.[5,11,14] This phenotype is spontaneously induced by environmental stressors, such as harsh conditions or polymicrobial interactions.[25,26,32–34] This phenotype may be unstable and can revert back to normal WT-SA growth, both colony morphology and biochemical responses.[5,9,22,25,28,30] Ultimately, these morphological characteristics can lead to misidentification or diagnostic failure[5,11,25,26] (**TABLE 1**).

The major clinical implication of the SA-SCV phenotype is chronic or recurrent infections, which can contribute to patient mortality because clinicians are often unable to detect, identify, and effectively treat SA-SCV infection.[5,8,24,28,31] Currently, there is no routine diagnostic procedure in the clinical laboratory for the detection of the SA-SCV phenotype.[24,31,37,38] Many clinical laboratories are unfamiliar with this

FIGURE 1. **Morphological differences exhibited by wild-type *Staphylococcus aureus* (WT-SA) and SA small colony variant (SA-SCV). Blood agar plates after 48 hours of incubation at 37°C, showing a comparison of the colony morphology of WT-SA (A) to SA-SCV phenotype (B). WT-SA with the normal phenotype, characterized by yellow pigmentation and hemolysis (A, *S aureus* Newman parental strain[35]). SA-SCV characterized by pinpoint colonies that are nonpigmented and nonhemolytic (B, *S aureus* NewmanΔmenB[36]). Images taken by Klara Keim of the TTUHSC Department of Surgery and TTU Honors College; strains provided by Catherine Wakeman, TTU Department of Biological Sciences.**

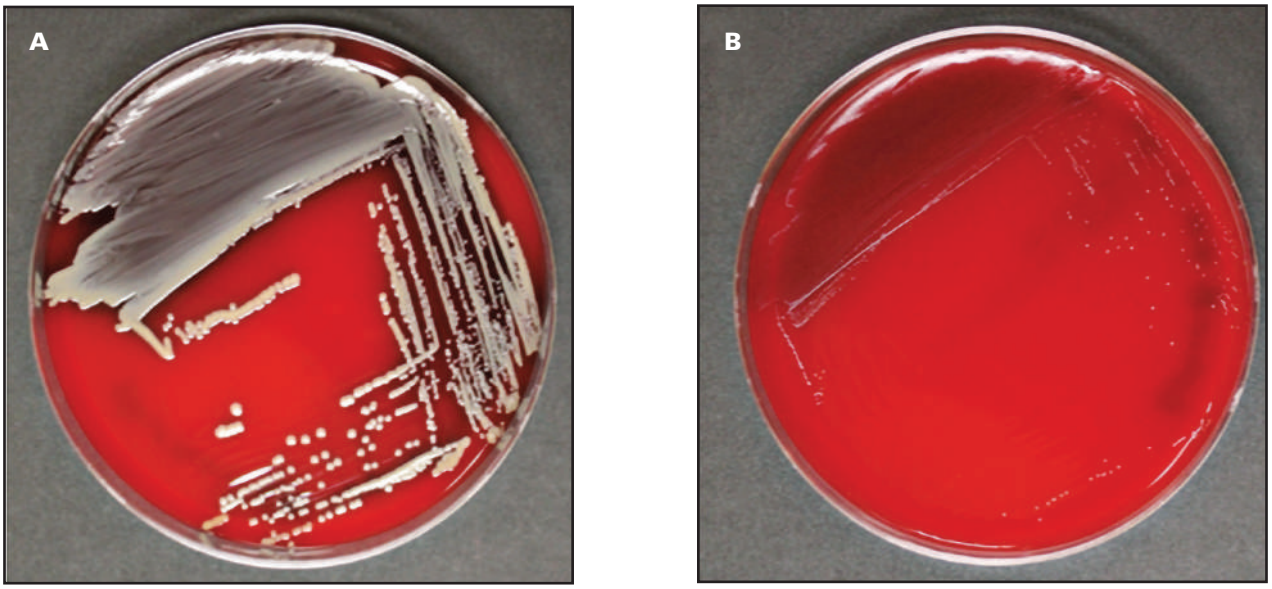


TABLE 1. **Morphological and Biochemical Characteristics Distinguishing WT-SA From SA-SCVs**

WT-SA	SA-SCVs
Large colonies (~10 mm)	Small, pinpoint colonies
Yellow pigmented	Nonpigmented
Hemolytic	Nonhemolytic
Catalase positive	Catalase negative
Coagulase positive	Coagulase negative
Agglutination positive	Agglutination negative
Mannitol fermenter	Mannitol negative

SA-SCV, SA small colony variant; WT-SA, wild-type Staphylococcus aureus.

phenotype, which can lead to oversight or misidentification of most small colonies as commensal organisms.[11,21,25,39] Altered antimicrobial susceptibility, abnormal biochemical test results, and altered morphological appearance collectively leave SA-SCVs frequently misidentified or undetected, limiting treatment options and affecting treatment success.[5,11,31,37,38] This stresses the importance of familiarizing the clinical laboratory with the morphological and biochemical characteristics, mechanisms of induction, and implications of SA-SCVs in clinical practice. Additionally, translating and increasing understanding of SA-SCVs in the clinical laboratory as well as research to incorporate new detection methods into routine procedure should be considered a priority. Ultimately, this will lead to better SA-SCV specimen assessment within an infection and introduction of new treatment options.

Infectious Processes

The SA-SCV isolates within patient specimens are important causative agents of recurring and persistent infections, leading to clinical implications of infection progression due to detection difficulties and poor antimicrobial responsiveness.[5,6,14,28] The SA-SCVs optimize their capacity to act as a reservoir for chronic infection by expressing altered patterns of virulence factors, increased biofilm formation, and intracellular persistence in host cells.[9,19,28,37] The SA-SCVs have been isolated in chronic diseases and infections, such as cystic fibrosis (CF), osteomyelitis, persistent SSTIs, chronic wounds, foreign body–associated infections, sepsis, and stomatitis.[5,6,11,13,22,40] Increased hospital intensive care unit mortality rates, prior exposure to anti-staphylococcal agents, long-term antimicrobial therapy, minimum inhibitory concentration (MIC) alterations, and treatment failure have been reported for infections harboring SA-SCVs compared to WT-SA.[11] Some SA-SCV infections may persist asymptomatically for years posttreatment and can exhibit recurrent relapse.[9] The SA-SCVs are likely common within the infection environment but are difficult to recover in the clinical laboratory.[11] Studies concerning the prevalence of SA-SCVs in the scope of wound infections could not be identified, and the dearth of data looking at prevalence of SA-SCVs in various clinical scenarios highlights the need to conduct such studies.[40] The rates of SA-SCVs could potentially be high and underestimated due to misdetection and misdiagnosis, but there is currently little sufficient clinical evidence to support this claim.

Cystic Fibrosis

The most frequently studied infectious process associated with SA-SCVs is in the CF lung. Clinical studies have found that approximately two-thirds of CF patients exhibit co-infection of SA, one-half being SA-SCVs and *Pseudomonas aeruginosa* (PA).[5,11,13] Co-infection with PA can induce SA-SCVs within 6 hours from production of pseudomonal exotoxins and cause SA persistence in persons with CF. The SA-SCV induction may be a cumulative and specific response to the environment of the CF airways

and aminoglycoside treatment.[30,41–43] Both SA and PA contribute independently and additively to the severity of infection in persons with CF by leading to variable SA-SCV phenotypic antimicrobial resistances, potential nonidentification of SA during antimicrobial susceptibility testing (AST), and increased lung inflammation and damage.[10,13,30,34,44] In addition to significantly higher SA-SCV antibiotic resistance rates compared to WT-SA, higher proportions of methicillin-resistant SA (MRSA) have been reported in SA-SCV isolates than WT-SA isolates.[10,44]

Persons with CF require frequent CF-related intravenous antibiotic therapy and have often received prior long-term antibiotic treatment[44]; prior and long-term antibiotic use are risk factors for developing SA-SCV infections. This results in higher MICs due to positive resistance selection by antimicrobials, specifically interventional aminoglycosides and trimethoprim-sulfamethoxazole therapy even after extended therapy discontinuation.[10,44] People with CF harboring SA infections were found to have significant resistance to sulfamethoxazole, trimethoprim, gentamicin, fosfomycin, ciprofloxacin, erythromycin, and clindamycin.[13] Antibiotic resistance acquired by SA-SCVs can allow them to persist and infect the host cells of persons with CF; these altered drug-resistance profiles often lead to difficulty in detection.[38]

A SA-SCV infection is directly related to poor clinical outcomes in those with CF by contributing to chronic inflammation of lung tissue, ultimately leading to collateral tissue damage caused by increased concentrations of host neutrophils.[38,44] The combined frequency of SA and PA indicates that clinical laboratories should be encouraged to improve active efforts of detection and monitoring of SA-SCV prevalence.[38]

Prosthetic Joint and Device-Related Infections

Implant-related infections harboring SA-SCVs—specifically prosthetic joint infections (PJIs), medical device–related infections, and prosthetic valve endocarditis—have poor clinical response to prolonged antimicrobial therapy, increased antimicrobial resistance upon implant adherence, and are identified as causative agents in recurrent infection.[5,6,22] The WT-SA readily attaches to medical devices and prostheses during implantation as a result of contamination by patient skin, medical staff, or even airborne particles.[5] Host material, such as matrix proteins like fibrinogen and fibronectin, associate with foreign surfaces in tissue. As a result, SA-SCVs upregulate extracellular adherence protein (Eap) and adhesion-fibronectin binding protein and downregulate alpha toxin, proteases, and other virulence factors to enhance bacterial adherence to the implant and invasion of host cells, often forming a biofilm.[11,29] The SA-SCV foreign-body infections can quickly lead to sepsis and bacteremia prior to isolation of the SA-SCV phenotype. Treatment of recurrent infections often requires removal of all infected prosthesis, tissue, and foreign material followed by antibiotic treatment continued through reimplantation to avoid treatment failure and persistent infection.[22] Therefore, clinical detection and treatment of PJI harboring SA-SCVs must be rigorous and thorough on initial infection to avoid treatment failure and reimplantation.[22]

Osteomyelitis

Osteomyelitis is a common comorbidity of device-related infections, PJIs, chronic wounds, and SSTIs caused by the spread of bacteria, making locating bacterial load and treating chronic infection difficult.[39,45] SA infection is a significant causative agent of osteomyelitis, and cases harboring SA-SCVs contribute to chronic infection via osteoblast intracellular persistence.[5,39,45] Bone and vascular tissue damage resulting from chronic bone infection prolongs SA-SCV persistence in poor perfusion areas because SA-SCV reservoirs cannot be reached by effective antibiotic concentrations.[39,45] SA-SCV biofilm formation is more likely in osteomyelitis infections than other SA-SCV-related chronic infections due to increased polysaccharide intracellular adhesin production for bone matrix adhesion.[24,39]

Immediate treatment with a milieu of antibiotics has been shown to reduce intracellular loads of SA-SCVs; findings suggest antimicrobial administration after 12 hours of intracellular persistence increases susceptibility with MICs up to 32-fold higher whereas after 7 days of persistence, antibiotic therapy became altogether ineffective.[24,39,46] Rifampicin was the only treatment method to have an effect on established SA-SCV persistence within bone tissue, indicating a sum of SA-SCV evasion mechanisms and environmental characteristics that impede the effect of other antibiotics.[45] Therefore, treatment of persistent SA-SCV infection must be a combination of interventional methods to clear infection, including surgical intervention and serial debridement of infected areas with long-term antimicrobial therapy.[24,39,45]

Chronic Wounds and Infections of the Skin and Soft Tissue

Most current literature has sought to identify SA-SCVs harbored in chronic infections: specifically CF, osteomyelitis, PJI, and device-related infections. *SA*-SCV occurrence is prevalent in other cases of chronic infection as well, such as SSTIs, chronic wound infections, and cases of sepsis. SA is the most prevalent pathogen causing SSTIs and chronic wound infections; however, these cases have not been studied thoroughly despite the similar observed mechanisms of SA-SCV pathogenesis and induction. The WT-SA commonly acts as an opportunistic pathogen alongside other pathogens, and treatment could ultimately select for SA-SCVs in chronic wounds and present other patient burdens, specifically manifestation of diabetic foot ulcer infection in persons with diabetes.[15,16,18,47,48]

SA-SCVs have been isolated from SSTI patients harboring chronic staphylococcal and polymicrobial infections previously administered long-term antibiotic treatment. Interestingly in the case of SSTIs, WT-SA and SA-SCVs have been isolated within the respiratory mucosa of patients in addition to the site of infection, sometimes without exhibiting a clinical presence until an opportunity arises for pathogenesis.[15] Notably, it has been found that SA-SCVs invade endothelial and epithelial cells of the integument and persist intracellularly, affecting the structure and integrity of the integument, and can exhibit antibiotic resistance (methicillin, vancomycin, etc), and cause recurrent purulent infection, limited treatment options, and infiltration of the circulation to infect other organs and cause septicemia.[15,37,49–52] The SA-SCVs notably persist within keratinocytes exhibiting low virulence to evade the host immune system.[49] This highlights the mechanism by which SA-SCVs can persist, demonstrate decreased antimicrobial susceptibility, and allow SSTIs to recur even after the infection is thought to be eradicated. Accurate identification and effective SA-SCV treatment application are also notably important to prevent further resistance and limit morbidity and mortality in SSTI cases.[15]

Mechanisms of Induction

When establishing chronic infection, SA faces harsh conditions and environmental stressors including aminoglycoside treatment and co-infection with PA that induce an anoxic state interfering

with ETC function and create selective pressure for the SA-SCV phenotype.[11,24,25,28,30,32,33,41–43,53] Therefore, reduced metabolic activity stimulates biofilm formation and decreases aminoglycoside sensitivity.[32,34,37,41–43]

Prior exposure or long-term treatment with subinhibitory concentrations of antimicrobials select for SA-SCVs and decrease antimicrobial susceptibility; most notably, aminoglycosides were found to induce SA-SCV emergence and exhibit the greatest susceptibility reduction.[13,25,28,29,42,43] Aminoglycosides cause mutations in genes involved in the ETC and ATP synthesis in addition to biosynthesis of thymidine, menadione, and hemin.[5,11,14,25,26,31] Electron transport interference by aminoglycosides limits nutrient availability, suppresses aerobic metabolism, and causes electron deficiency that ultimately causes spontaneous mutations that respond to selective environmental pressures. Additionally, ETC deficiency and upregulation of adhesin increases resistance and biofilm production.[5,24,25,33,41,42,53] People can initially harbor both WT-SA and SA-SCV strains but following antimicrobial treatment that successfully clears WT-SA, harbor predominantly SA-SCVs.[13,14,25,28] Ultimately, this leads to misidentification of SA-SCVs and misleads clinicians to suspend treatment. Improper treatment consequently produces a difficult-to-treat SA-SCV infection with increasing resistance to aminoglycosides and other antimicrobials[26,37] (**FIGURE 2**).

In conjunction with antibiotic-associated environmental stress, polymicrobial interactions also contribute to emergence of the SA-SCV phenotype.[30,32,34,41,43,53] Both SA and PA consistently co-infect diseased tissue, leading to mechanisms of microbial competition.[30,34,41] PA is usually the dominant pathogen and expresses exotoxins such as alkyl-hydroxyquinoline N-oxides (HQNOs), hydrogen cyanide, and pyocyanin to act against commensal organisms.[28,30,32–34,41,43,53] SA is sensitive to PA exotoxins, particularly those targeting ETC and aerobic metabolism. This metabolic switch from WT-SA to electron deficient SA-SCVs provides additional protection and toleration of aminoglycosides and vancomycin due to slowed metabolic activity[30,32,33,43,53] (**FIGURE 3**). By suppressing aerobic metabolism through interference of the SA ETC, PA selects for SA-SCVs and increased biofilm formation of this phenotype as a mechanism to tolerate the compounds produced by PA.[28,30,41,53] Therefore, the PA exotoxins drive SA to grow in a metabolically dormant and fermentative state as a survival strategy.[28,32–34] This indicates that aggressive and prolonged combination therapy may be required to eradicate SA-SCVs in co-infection.[5,24] Additionally, it may be important for the clinical laboratory to actively screen for SA-SCVs when SA/PA co-infection is present.[22,37,54]

Methods of Detection

The discussed characteristics of SA-SCV infections demonstrate the medical importance of this phenotype. The severe clinical implications suggest they must be actively investigated and considered distinct from WT-SA in the clinical laboratory.[22] SA-SCVs often remain undetected or misdiagnosed because they are a condition-, media-, and time-dependent phenotype with low metabolism and slow growth, only to be detected usually after 48 to 72 hours of incubation; simply prolonging the culture period can double detection and increase WT-SA overgrowth likelihood.[5,8,25,28]

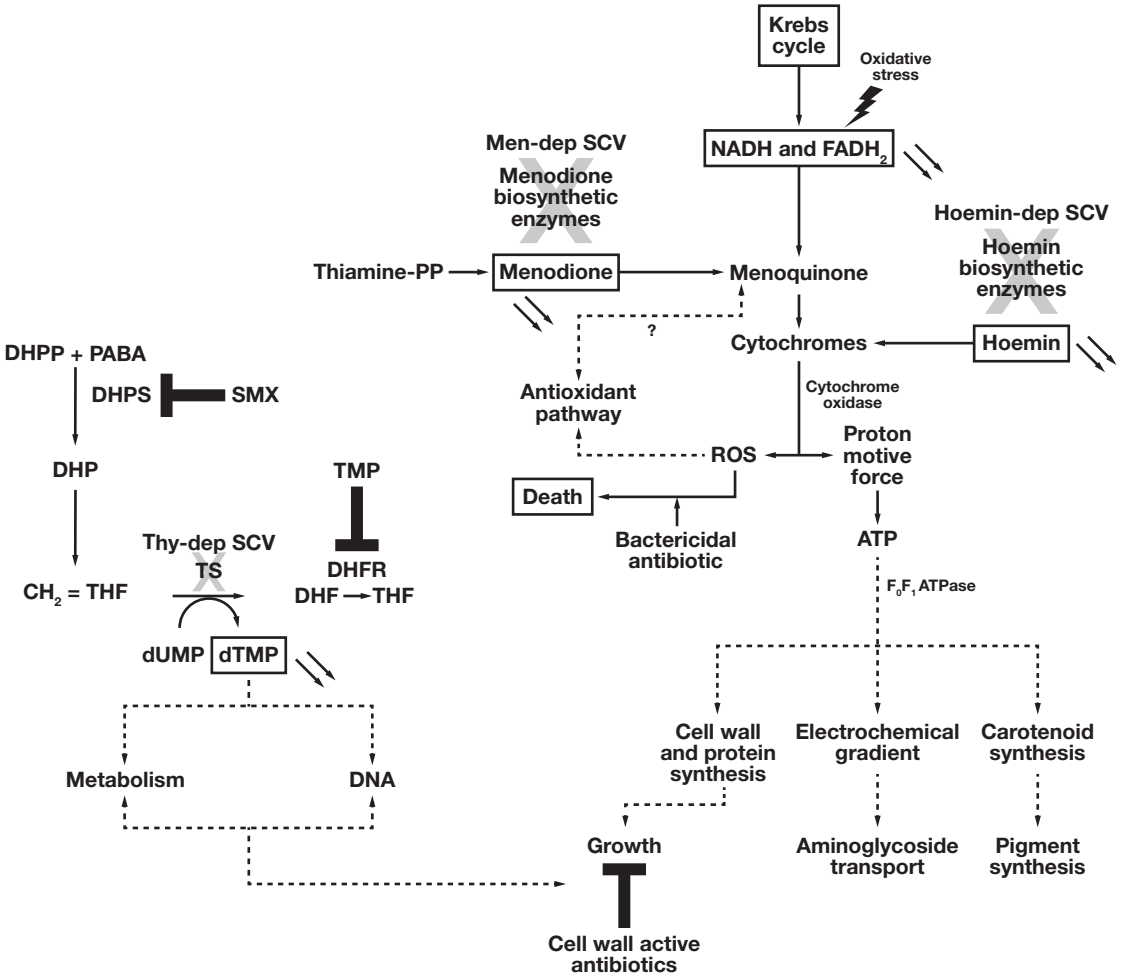
There are detection methods found to successfully culture and isolate SA-SCVs from clinical samples, distinguish SA-SCVs from WT-SA and other commensal organisms, and further identify antimicrobial susceptibility profiles.[8,11,13,22,30,31,38] Traditional methods of identification are based on analyzing the unusual morphological appearances and biochemical reactions observed in SA-SCV isolates.[5,11] Clinical SA-SCV isolates generally exhibit slow growth and 10× smaller, nonpigmented, and nonhemolytic colonies on mannitol salt agar, tryptic soy agar, sheep blood agar, rose bengal agar, clindamycin blood agar, and chocolate agar.[5,8,11,22] CHROMID SA Elite agar showed more rapid and reliable species identification for SA than chocolate agar, where 92.5% of SA was identified as green growth on SA Elite agar without dependence on auxotrophisms to hemin, menadione, or thymidine.[8] Additionally, SA-SCV isolates generally exhibit reduced coagulase production and increased resistance in AST to aminoglycosides and cell wall-active antibiotics.[5,8,11] Additional methods such as the ID color catalase test (Slidex Staph Plus) can further help confirm isolation of SA strains rather than commensal organisms.[11,22] The SA-SCVs also have a recognizable fingerprint observed on Fourier-transform infrared spectroscopy that can lead to further detection and confirmation of SA-SCVs.[11]

Aside from traditional diagnostics, the implementation of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) may be successful to identify SA-SCVs by comparing the spectra of the bio-analytes in an SA-SCV isolate with the established SA-SCV mass spectrometry profile in the identification software.[39,55,56] Previous studies describe the use of MALDI-TOF MS to identify SA and *Enterococcus faecium* SCVs.[38,56,57] The use of high performance liquid chromatography–mass spectrometry can also provide a clear distinction between WT-SA and SA-SCVs based on changes in their metabolic profiles during the phenotypic switch and the unique metabolic SA-SCV signature.[58] Moreover, proteomic analysis of WT-SA and SA-SCVs results in distinct protein profiles, such as SA-SCVs possessing larger amounts of induced proteins that are involved in glycolytic and fermentation pathways compared to their parent cells.[59,60]

SA-specific PCR assays can be used to verify that isolates suspected to be SA-SCVs phenotypically are indeed affiliated with the SA species.[11,39] Multiple gene targets have proven useful for SA identification via PCR, including the genes *nuc, coa, femA, eap,* and *sodM*, which are not associated with mutation in SA-SCV and should be conserved between WT-SA and SA-SCVs.[22,61–64] Polymerase chain reaction for the aforementioned genes may provide additional discrimination of WT-SA from coagulase-negative staphylococci, the latter tending to be characteristic of SA-SCVs.[65] Detection of penicillin-binding protein 2' (PBP2') in SA can potentially lead to the diagnosis of coagulase-negative staphylococci.[66,67] The PBP2 antigen test could also be a proxy test for the *mecA* gene resulting in the diagnosis of MRSA phenotypes.[66,67] Pulsed-field gel electrophoresis can serve as another way to discriminate SA-SCVs from WT-SA and determine antimicrobial susceptibility.[39,67] However, the unique growth and metabolic characteristics of SA-SCVs compared to WT-SA makes obtaining full antimicrobial susceptibility information difficult. These tests are not routinely used in the clinical laboratory and are not a replacement for full AST. The sensitive and specific identification of SA can permit reliable diagnosis of the species association of SCV isolates.[39,64]

A diverse range of detection methods may be used to determine the presence of SA-SCVs as a component of chronic, recurrent, and persistent infections. Therefore, the clinical laboratory should actively examine specimens for SA-SCVs and include detection methods in routine diagnostics. Clinicians should also consider patient history and any underlying comorbidities in arriving at a holistic diagnosis and treatment

FIGURE 2. Illustration of the mechanisms leading to the small colony variant (SCV) phenotype in *Staphylococcus aureus* and of their link to reduction in susceptibility to specific antibiotic classes. Electron-transport-deficient SCVs show alterations in the pathways leading to the synthesis of menadione or hemin (subsequent to mutations in biosynthetic enzymes), which causes a reduction in the amount of ATP produced. This leads to a reduced growth rate, which may affect the efficacy of antibiotics active against dividing bacteria, such as cell-wall-active agents, and to a reduction in transmembrane potential, which impairs aminoglycoside uptake. Menadione-dependent SCVs are hypersusceptible to oxidant species, possibly because of reduced electron transport and alteration of the induction of antioxidant pathways. They also show a reduced growth rate. Globally, antibiotics may also be less bactericidal towards electron-transport-deficient SCVs due to a reduced production of reactive oxygen species. DHF, dihydrofolate; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; DHP, dihydropteroate; DHPP, dihydropteridine pyrophosphate; Hemin-def, hemin dependent; Men-dep, menadione dependent; PABA, *para*-aminobenzoic acid; SMX, sulfamethoxazole; THF, tetrahydrofolate reductase; TMP, trimethoprim; TS, thymidylate synthaseThy-dep, thymidine dependent. From Garcia et al 2013.[24]



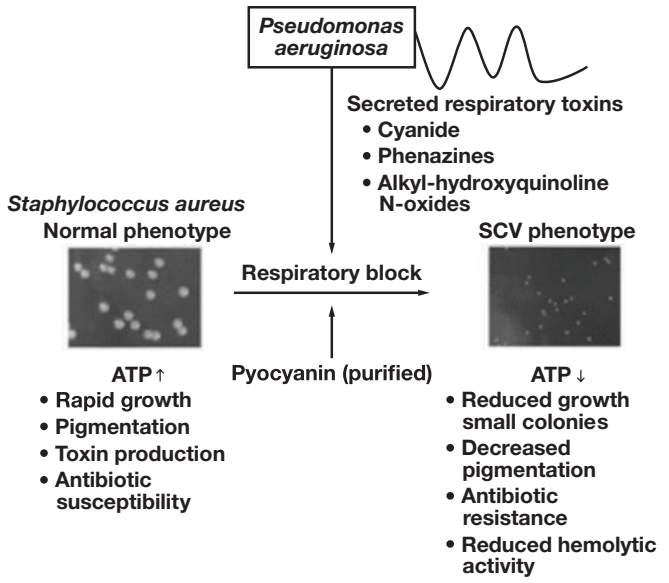
plan; otherwise, it is likely that undetected or misdiagnosed SA-SCVs will continue contributing to worsening infection and unresponsiveness to antimicrobial therapy.[5,11,38,54] Further studies should be conducted to determine which diagnostic methodology would be optimal to identify SA-SCVs from patient specimens.

Impact on Antimicrobial Susceptibility

The SA-SCVs experience reduced susceptibility to antimicrobials and antiseptics.[5,10,11,14,24,29,37] Altered AST results from reduced bacterial energy generation, intracellular transport, and downregulation of cell wall synthesis and toxin production caused by environmental stress.[11,14,29,32] Drug-resistance profiles show that SA-SCVs have increased resistance to aminoglycosides, sulfa drugs, cationic peptides, and cell-wall-active antibiotics.[8,10,14,22,24,28,38] Aminoglycoside MICs were found to be 8- to 32- times higher for SA-SCVs when compared to MICs of the WT-SA phenotype; gentamicin MIC was notably 32-times higher for the SA-SCV phenotype.[5,11,29,30,49]

Gene mutations involved in the biosynthesis of thymidine, thiamin, menadione, and hemin lead to reduced functions of metabolic pathways, specifically ETC or the tricarboxylic-acid cycle.[6,14,25,26,28] Impaired ETC inhibits antimicrobial uptake and mechanisms of aminoglycoside and antifolate activity.[11,13,14,24] Mutations that inhibit aerobic respiration and metabolism reduce ATP production that is required in WT-SA for rapid growth[14]; The SA-SCVs exhibit longer generation times and slower growth because cell wall synthesis requires large quantities of ATP.[5,6,11,25]

FIGURE 3. Illustration of *Pseudomonas aeruginosa*-induced *Staphylococcus aureus* small colony variant (SCV) selection. Left, Colony size of WT-SA under normal conditions. Right, In the presence of respiratory toxins, like alkyl-hydroxyquinoline N-oxides or the *Pseudomonas* quinolone signal, pyocyanin or cyanide produced by *P aeruginosa* leads to selection of the electron transport-deficient SCV phenotype in *S aureus*. Adapted from Biswas et al.[53]



Slowed growth and metabolism then decrease cross-membrane potential and uptake of cationic antimicrobial compounds, ultimately reducing aminoglycoside susceptibility.[14,24] Aminoglycoside resistance is a direct result of ETC inhibition because uptake is dependent on membrane potential created by electron transport.[11,14] Therefore, SA-SCV persistence and resistance to aminoglycosides is a major trade-off for reduction in growth rate.[14]

The SA-SCVs also exhibit increased biofilm formation and reduced virulence to optimize their ability to persist.[28,42,43] In polymicrobial infections, PA was shown to protect SA with antistaphylococcal compounds in the presence of vancomycin and aminoglycosides, such as tobramycin and streptomycin.[30,32,34,42,43] PA produces HQNO, pyocyanin, and siderophores that interfere with SA ETC, causing them to switch from respiration to grow fermentatively.[5,32,34,43] Anoxic conditions and fermentative lifestyle inhibits WT-SA growth without killing it, slowing metabolic activity and selecting for SA-SCVs.[6,14,30,32] Ultimately, PA induction of SA-SCV phenotype promotes viability and biofilm production in coculture, contributing to decreased aminoglycoside sensitivity.[30,34,43] Different doubling times between SA-SCVs, WT-SA, commensal organisms, and other wound pathogens, even in small concentrations, cause the SA-SCVs to become rapidly overgrown in liquid culture.[11,14,38] Therefore, AST becomes a major challenge because SA-SCVs are overgrown, leaving them undetected.[5,11,14,29,30]

The SA-SCVs can enter and persist within host cells, which contributes to reduced antimicrobial susceptibility.[5,8,15,22,26] SA-SCVs efficiently invade nonphagocytic cells due to high expression of adhesion-fibronectin binding protein and downregulation of alpha-toxin and proteinase.[9,11,22,26,28,38,42] This is a mechanism to evade immune system mechanisms such as antibodies and complement.[9,26,38] Ultimately, residing within host cells for long periods of time confers protection against antimicrobials as well, whereas continued antimicrobial use causes mutations that select for the SA-SCV phenotype and increases their occurrence, persistence, and genetic variation.[9,24,26,38] Therefore, intracellular persistence and slow growth ultimately lead to prolonged antimicrobial therapy, causing altered drug resistance profiles and increased resistance to aminoglycosides. Importantly, the SA-SCV phenotype is unstable and can revert to a highly virulent and rapidly growing WT-SA phenotype under the right conditions.[9,22,25] The SA-SCVs can therefore combine the ability to survive persistently with the propensity to generate fast-growing offspring when the right conditions are met to reestablish major infection.[14]

Treatment Guidelines and Recommendations

The SA-SCVs are a burdensome, fastidious organism that requires active consideration in clinical diagnosis and treatment. Treatment recommendations for SA-SCV infections vary based on the infection presented, further highlighting the importance of accurate diagnostics.[22] In cases of prosthesis or medical device infections, SA-SCVs readily attach to the devices post contamination and are more resistant upon adherence.[5,22] The SA-SCVs respond poorly to prolonged antimicrobial therapy.[11] Therefore, the current recommended treatment requires removal of all infected prosthesis, tissue, and foreign material followed by antibiotics prior to reintroduction of the prosthetic device.[22]

When treating SA-SCV infections with antimicrobial therapy, it is recommended to avoid aminoglycosides due to the phenotype's altered antimicrobial susceptibility profiles. Treatment with aminoglycosides can favor the emergence of SA-SCVs and increase persistence.[42] It has also been found that treatment with trimethoprim-sulfamethoxazole induces the formation of SA-SCVs in diabetic foot ulcers and persons with CF.[15] People treated with antifolates or antibiotics for over 1 month exhibit SA-SCV selection; therefore, prior long-term antimicrobial treatment also favors induction and increased persistence.[13,15] The SA-SCVs require immediate combination antibiotic therapy, which has been shown to successfully treat SA-SCV infections via rifampin with a fluoroquinolone rather than prolonged antimicrobial treatment.[5,11] The SA-SCVs have also shown response to flucloxacillin and rifampin combination therapy.[37]

Decreased SA-SCV antimicrobial susceptibility causes chronic and recurrent infection due to poor response to limited treatment options, which highlights the importance of accurate initial detection[31,68] To provide accurate diagnostics of SA-SCVs, clinicians must consider the sum of clinical signs and symptoms, blood tests, and radiography in addition to the microbiological workup.[5] The slow growth and reduced metabolism of SA-SCVs causes them to be detected only after 48 to 72 hours of culture; therefore, prolonging the culture time is recommended to increase detection likelihood.[5,28] Also, due to SA-SCV and PA co-infection frequency, it is recommended that when PA is detected in chronic or recurrent infections, SA-SCVs should be actively investigated.[54]

Conclusions

The SA-SCVs are a metabolically deficient phenotype of the common opportunistic pathogen SA that are widely associated with recurrent and persistent infections. This phenotype is difficult to treat due to decreased antimicrobial susceptibility, intracellular persistence, and increased biofilm formation. The phenotype is metabolically dormant

due to auxotrophism of hemin, menadione, or thymidine required for ETC component biosynthesis. The SA-SCV phenotype is induced by environmental stressors such as anoxic conditions, polymicrobial infection with PA, and pressure from certain classes of antibiotics. The SA-SCVs exhibit unusual phenotypic and biochemical characteristics that distinguish them from WT-SA. This can lead to misidentification or nonidentification in routine diagnostics. As a result of misdiagnosis, serious clinical implications arise from SA-SCV infections. This phenotype has been described in clinical cases of chronic infections, such as CF, SSTIs, osteomyelitis, foreign body-associated infections, and device-related infections, and others. Once induced by environmental stressors, SA-SCVs will often persist until the stressors are diminished, allowing them to revert to WT-SA and reinfect the host indefinitely until accurately detected and treated. Currently, there is not sufficient research on the mechanisms of pathogenesis or proposed treatment methods for these chronic infections. This highlights the importance of developing novel routine diagnostic methods to identify SA-SCVs in recurrent and persistent infections. Through active examination of the role of SA-SCV in infectious processes, elucidating new detection methods, and exploring novel treatment methods, the clinical implications of mortality and morbidity resulting from SA-SCV infection could be diminished.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Work-up of Patients with Decreased Hemoglobin A2 Identified by Capillary Zone Electrophoresis: A North American Institutional Experience

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Keywords: hemoglobin, capillary, electrophoresis, hemoglobinA2, iron, anemia

Abbreviations: HbA2, hemoglobin A2; CBC, complete blood count; ACOG, American College of Obstetricians and Gynecologists; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cell count; RDW, red cell distribution width; WHO, World Health Organization

Laboratory Medicine 2023;54:235–240; https://doi.org/10.1093/labmed/lmac114

ABSTRACT

Objective: Isolated low hemoglobin A2 (HbA2) is rarely encountered in our clinical practice using capillary zone electrophoresis. The study goal was to characterize the work-up at our institution of patients with low HbA2.

Methods: Patients with low HbA2 and a control cohort with normal capillary zone electrophoresis were identified and relevant information extracted from the medical record.

Results: Of 44 patients with isolated decreased HbA2, 28 (64%) had corresponding complete blood count/ferritin values. Compared to control patients, patients with low HbA2 were more likely to have iron deficiency and demonstrated a more microcytic, hypochromic blood picture. However, 46% (13/28) of patients with low HbA2 and ferritin for evaluation did not have iron deficiency. Only 2 patients had genetic testing.

Conclusion: This study redemonstrates the association between low HbA2 and iron deficiency and reinforces the need for iron indices to interpret capillary zone electrophoresis results. Our study population showed incomplete or absent iron studies in most cases.

An abnormal hemoglobin A2 (HbA2) fraction can be an indicator of thalassemia[1,2]; however, accurate evaluation of HbA2 may be challenging because both inherited and environmental factors influence the HbA2 fraction. In addition to alpha-thalassemia, delta-thalassemia, and other rare hemoglobin variants, decreased HbA2 has been associated with iron deficiency, sideroblastic anemia, anemia of chronic disease, lead poisoning, hematologic malignancies, aplastic anemia, hypothyroidism, and medications and treatment interventions.[1–4] Parsing out the different influences on the HbA2 fraction in a particular case may be difficult or impossible, as complete clinical and laboratory data are often not available to the laboratorian, both iron deficiency and thalassemia may present with anemia and microcytosis, and a subset of patients have multiple contributing factors.[5–8]

The association between iron deficiency and a decreased HbA2 fraction was first reported in the 1960s.[3,9–12] Importantly, iron deficiency may result in a false-negative interpretation for a patient with beta-thalassemia trait, as the HbA2 fraction may be lowered enough to fall within the normal range.[12–14] There has been less examination of the clinical relevance and diagnostic interpretation of hemoglobin electrophoresis patterns in which the HbA2 fraction is below the reference range. Prior studies evaluating the association between iron deficiency and a decreased HbA2 fraction used quantitation methods that are no longer commonly used in clinical practice, such as cellulose acetate electrophoresis.[3,9,15] Capillary zone electrophoresis is a relatively new and robust methodology for hemoglobin analysis that shows subtle quantitative variations when compared to other methods.[16–20] There is limited literature examining the relationship of iron status and HbA2 using quantification by capillary zone electrophoresis.[13]

Flowcharts for low HbA2 as suggested by Giambona et al[2] rely on MCV and iron studies to guide further evaluation. For carrier screening, the American College of Obstetricians and Gynecologists (ACOG) recommends DNA-based testing for alpha-thalassemia if the mean corpuscular volume is below normal, iron deficiency anemia has been excluded, and the hemoglobin electrophoresis is not consistent with

β-thalassemia. The scenario of a low HbA2 is not addressed.[21] In the course of our clinical sign-out of hemoglobin electrophoresis, we noted variable work-up of patients with low HbA2 and sought to formally characterize the extent of their evaluation.

Materials and Methods

This retrospective study was approved by the University of Virginia School of Medicine and University of Virginia Health System institutional review board (IRB# 13310).

Hemoglobin Electrophoresis

Peripheral blood samples were analyzed by capillary zone hemoglobin electrophoresis using the CAPILLARYS 2 FLEX-PIERCING instrument according to the manufacturer-specified CAPILLARY HEMOGLOBIN(E) Assay procedure (Sebia, **FIGURE 1**). Hemoglobin fraction reference intervals were as follows: hemoglobin A >94%, HbA2 2.0% to 3.8%, and hemoglobin F <2.0%. These reference intervals were implemented at our institution following internal institutional verification of reference intervals publicly available from Mayo Clinic Laboratories.

Standard procedure at our institution includes a review of all electropherograms by a laboratory technologist. Abnormal studies (including electropherograms with hemoglobin values outside the reference range) are then reviewed by a pathologist who adds an interpretative comment.

Case Selection and Data Collection

Capillary electrophoresis results from January 2019 to October 2020 were screened for patients with an isolated decrease in HbA2 (<2.0%) and no other detectable abnormalities by capillary electrophoresis. A subset of patients with a normal electropherogram result were selected from January 2020 to April 2020 to serve as controls (**FIGURE 2**); a limited time period was chosen for the control group for ease of data extraction.

Demographic information and additional laboratory indices were abstracted from the medical record. Patient ethnicity was self-reported and characterized as Hispanic or non-Hispanic. Race was self-reported and included the following categories: White or Caucasian, African American, Asian, Other, and Unknown/Not Recorded. Pregnancy status was determined by either clinical documentation of pregnancy or a positive human chorionic gonadotropin test. The presence or absence of hematologic malignancy was noted for all patients, as hemoglobin electrophoresis was routinely ordered at our institution during evaluation for autologous stem cell transplantation.[22,23]

Complete blood count (CBC) indices (hemoglobin, mean corpuscular volume [MCV], mean corpuscular hemoglobin concentration [MCHC], red blood cell count [RBC], and red cell distribution width [RDW]) were extracted. Mentzer (MCV/RBC) and Jayabose-RDW (MCVxRDW/RBC) indices, common discriminatory formulas used to distinguish between iron deficiency anemia and thalassemia trait, were calculated for each case.[24] A Mentzer index less than 13 and Jayabose-RDW calculation less than 220 were used as clinical cutoffs suggestive of thalassemia.[25,26] Anemia was defined according to age and sex-specific cutoffs from the 2011 World Health Organization (WHO) guidelines.[27] Laboratory markers of iron status were reviewed as available, including serum iron, transferrin, serum ferritin, and percent transferrin saturation. Serum ferritin was selected as the sole marker to evaluate iron status, as it was concurrently performed on the greatest number of patients. Ferritin values less than 15 ng/mL and 30 ng/mL were used as 2 alternative cutoffs suggestive of iron deficiency.[28,29] The CBC and ferritin values were required to be within 90 days of hemoglobin electrophoresis sample collection to be included for evaluation. The average time between collection of electrophoresis and ferritin was 6.8 days (range of 0–78 days), with 88 of the patients (81%) having the labs collected on the same day.

When available, results of alpha-globin gene analysis performed at a reference laboratory (Mayo Clinic Laboratories) were recorded.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM, version 28.0.0, 2021). Categorical variables were analyzed using Pearson's χ^2 analysis or Fisher's exact test (for analyses with expected frequencies less than 5). Continuous variables were analyzed using the independent samples *t*-test.

Results

Patient Characteristics

Out of 3273 hemoglobin electrophoresis results screened, 44 patients (1%) with decreased HbA2 were identified, of whom 28 (64%) had corresponding CBC and ferritin values available for review. Only 16 (36%)

FIGURE 1. Representative electropherogram results. Examples of normal electropherogram pattern (HbA, 97.5%; HbA2, 2.5%) (A) and electropherogram with decreased HbA2 (HbA, 98.4%; HbA2, 1.6%) (B).

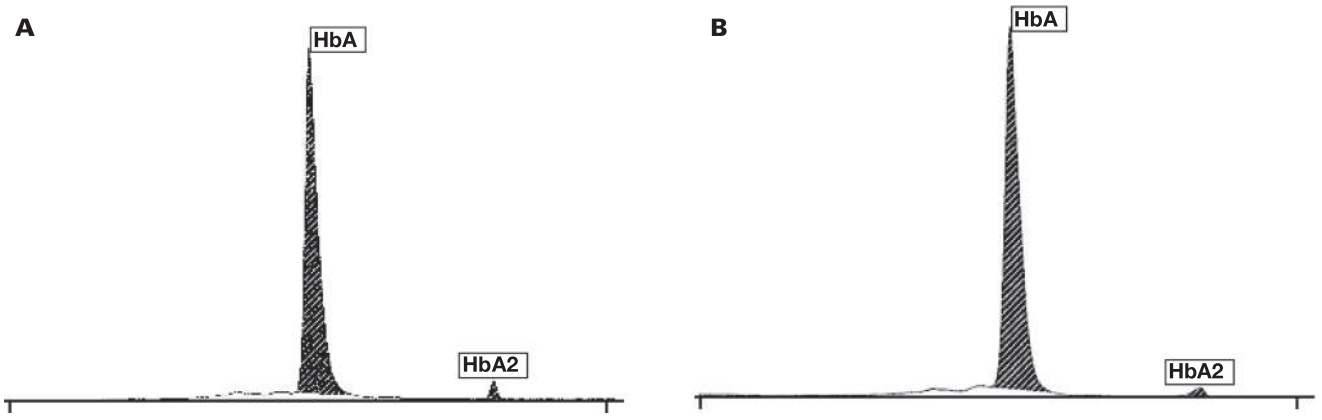
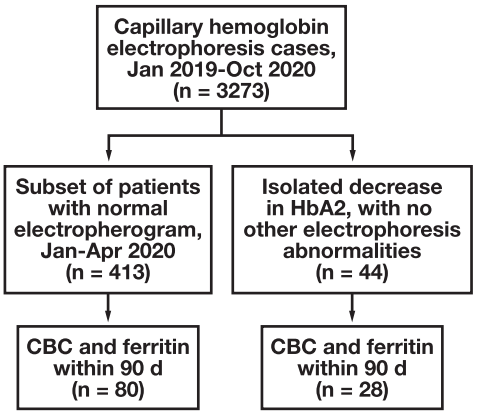


FIGURE 2. Patient selection criteria. HbA2, hemoglobin A2; CBC, complete blood count.



of the 44 patients with low HbA2 had complete iron studies (serum iron and % transferrin saturation in addition to ferritin), as recommended by Camaschella et al.[29] Four (29%) of the 14 pregnant patients in our cohort had complete iron studies performed. Eighty patients with normal electropherograms were selected as controls.

Comparing low HbA2 and normal HbA2 groups, there was no significant difference in self-identified ethnicity or race, age, or sex (**TABLE 1**). Among women, the proportion of pregnant patients was similar between the 2 groups. The control group was significantly more likely to have a diagnosed hematologic malignancy (61.3% vs 35.7%, P = .019).

CBC Indices

Although the presence of anemia by WHO criteria was not significantly different, the mean hemoglobin value was significantly lower in the decreased HbA2 group (9.8 g/dL) compared to controls (11.1 g/dL), suggesting a greater severity of anemia (P = .011, **FIGURE 3**). The MCV and MCHC values were significantly lower and the mean RDW value was significantly higher in the low HbA2 cohort. The RBC and platelet count did not significantly differ between HbA2 groups. The average Mentzer index was significantly lower in the low HbA2 group; however, there was no significant difference in the presence of a Mentzer value less than 13 between the 2 groups (P = .11), with only 2 patients in the normal HbA2 group (2.5%) and 3 patients in the decreased HbA2 group (10.7%) showing a Mentzer index less than 13. There was no significant difference in Jayabose-RDW value between the 2 groups. Five patients in the normal HbA2 group (6.3%) demonstrated a Jayabose-RDW value less than 220, compared to 1 patient in the low HbA2 group (3.6%, P = 1.0).

Iron Status

The average HbA2 was significantly lower in patients with a ferritin value less than 15 ng/mL compared to the iron-replete group (2.1% vs 2.3%, P = .015). A similar, nonsignificant trend was identified when HbA2 levels were compared using a ferritin cutoff of 30 ng/mL (P = .070).

After excluding the 6 patients (5 from the control group, and 1 from the low HbA2 group) with a ferritin value >2000 ng/mL, suggestive of acute phase response, the mean ferritin value was higher in the normal HbA2 group (242 ng/mL) than in the low HbA2 group (105 ng/mL, P = .006). Using ferritin values of both 15 and 30 ng/mL as alternative criteria for iron deficiency, patients in the decreased HbA2 group were significantly more likely to be iron-deficient than controls. However, a large percentage were *not* iron deficient (46.4% using ferritin <30 ng/mL as cut-off, 61.7% using ferritin <15 ng/mL as cut-off).

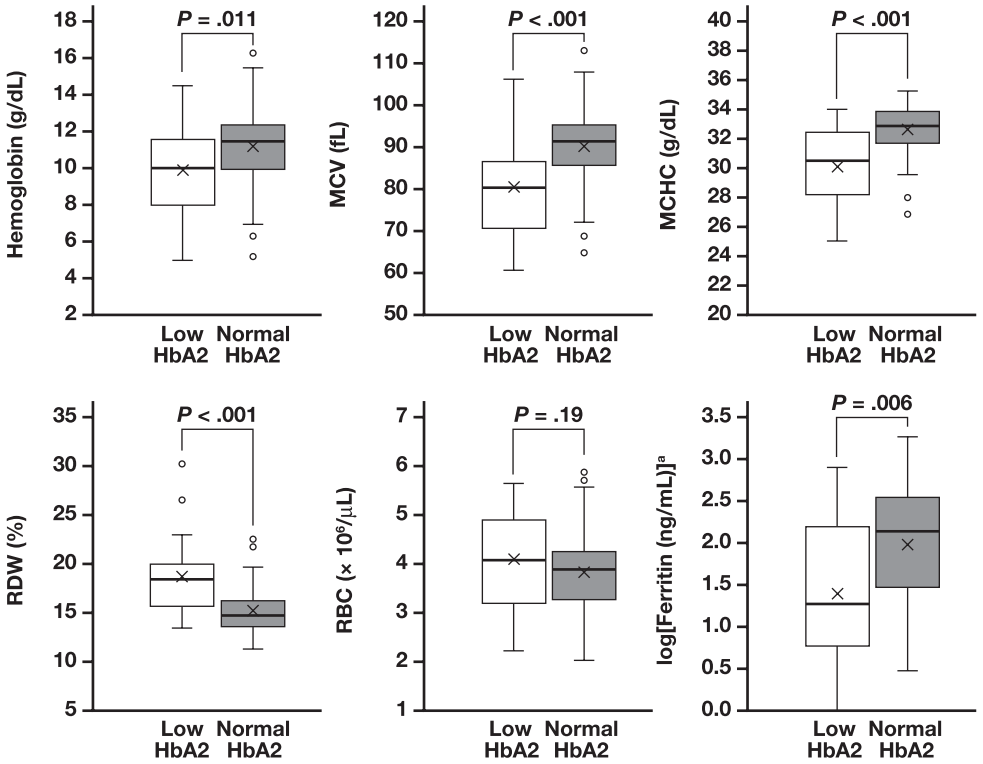
Among the 28 patients with low HbA2, the 11 patients with a ferritin value less than 15 ng/mL demonstrated lower mean MCV and MCHC values (71.7 fL and 28.5 g/dL) compared to those patients with a ferritin greater than 15 ng/mL (85.8 fL and 31.1 g/dL, P < .001 and P = .008). Only 1 of the 11 patients had an MCV > 80 fL and none of the 11 had a MCHC > 32 g/dL. The low ferritin group demonstrated a lower mean Mentzer index (16.8) than those with normal

TABLE 1. Summary of Patient Characteristics

	Normal Hb EP (n = 80)	Decreased HbA2 (n = 28)	*P* Value
Ethnicity, No. (%)			
Non-Hispanic	70 (87.5)	24 (85.7)	.88
Hispanic	7 (8.8)	3 (10.7)	
Unavailable	3 (3.8)	1 (3.6)	
Race, No. (%)			
African American	23 (28.8)	8 (28.6)	.70
Asian	1 (1.3)	0 (0)	
Other	7 (8.8)	5 (17.9)	
White or Caucasian	46 (57.5)	14 (50)	
Unavailable	3 (3.8)	1 (3.6)	
Age, mean (range), y	51.5 (3–73)	44.9 (2–73)	.13
Sex			
Male	37 (46.3)	11 (39.3)	.52
Female	43 (53.8)	17 (60.7)	
Pregnant			
No	33 (76.7)	12 (70.6)	.74
Yes	10 (23.3)	5 (29.4)	
History of hematologic malignancy, No. (%)	49 (61.3)	10 (35.7)	.019
Performance of alpha-globin gene analysis	1 (1.3)	1(3.6)	.45
Hemoglobin, mean, g/dL	11.1	9.8	.011
Patients with anemia, No. (%)	61 (76.3)	22 (78.6)	.80
RBC, mean, × 10^6/μL	3.8	4.1	.19
MCV, mean, fL	90.0	80.3	<.001
MCHC, mean, g/dL	32.6	30.0	<.001
RDW, mean, %	15.2	18.7	<.001
Mentzer index, mean	25.0	21.3	.024
Mentzer index <13, No. (%)	2 (2.5)	3 (10.7)	.11
Jayabose-RDW, mean	380	393	.66
Jayabose-RDW <220, No. (%)	5 (6.3)	1 (3.6)	1.0
Platelet count, mean, × 10^3/μL	214	271	.094
Ferritin, mean, ng/mL[a]	242	105	.006
Ferritin <30 ng/mL, No. (%)	19 (23.8)	15 (53.6)	.003
Ferritin <15 ng/mL, No. (%)	10 (12.5)	11 (39.3)	.002

EP, electrophoresis; HbA2, hemoglobin A2; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; Mentzer index, MCV/RBC; Jayabose-RDW calculation, (MCVxRDW)/RBC; RBC, red blood cell count; RDW, red cell distribution width.
[a]Six patients with ferritin values >2000 ng/mL were excluded from analysis.

FIGURE 3. Comparison of CBC indices in patients with low HbA2 and normal electropherograms. [a]Six patients with ferritin values > 2000 ng/mL were excluded from analysis. HbA2, hemoglobin A2; CBC, complete blood count; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cell count; RDW, red cell distribution width.



ferritin (24.1, $P = .012$), and all 3 patients in the low HbA2 group with a Mentzer index <13 had iron deficiency. Similar differences in MCV, MCHC, and Mentzer index were identified using a ferritin cutoff of 30 ng/mL.

Genetic Testing

Of the 108 patients with detailed analysis, 2 had genetic testing for alpha-globin gene variants (**TABLE 2**). One patient had an HbA2 fraction of 2.4% (within reference range) and a 2-gene deletion consistent with alpha-thalassemia trait and the other had a low HbA2 fraction of 1.1% and a negative study. Using ACOG guidelines, none of the pregnant women in either cohort had MCV and ferritin values that would suggest the need for DNA-based testing for alpha-thalassemia.

Discussion

In this study, we examined characteristics and work-up of a cohort of patients with HbA2 below the reference range and compared characteristics to a reference cohort of patients with HbA2 within the reference range. As noted in prior studies using other methodologies,[3,9–12] our results showed an association between decreased HbA2 and iron deficiency, with the low HbA2 cohort showing enrichment for patients with iron deficiency as measured by ferritin.

Although iron deficiency was more common in the low HbA2 group, many of the patients with a normal HbA2 were iron deficient and many of the patients with a low HbA2 were iron replete. This emphasizes the importance of evaluating a patient's iron status in conjunction with, or prior to, hemoglobin analysis. Of the 44 patients initially identified as having a decreased HbA2, only 28 had complete CBC indices and a ferritin value available for review around the time of hemoglobin analysis and additional laboratory markers of iron status (transferrin, serum iron, percent transferrin saturation) were ordered for only 16 patients. Although commonly recommended as a screening test for iron deficiency, ferritin is an imperfect laboratory value in the assessment of iron deficiency anemia due to its status as an acute phase reactant.[30] This incomplete evaluation is both a limitation of this study and reflects the unfortunate reality faced by many pathologists and laboratorians tasked with interpreting hemoglobin electrophoresis.

When the cohort of 28 patients with low HbA2 was evaluated, those with iron deficiency had more microcytic, hypochromic indices than patients who were iron replete. Nearly all patients with low HbA2 and iron deficiency had an MCV <80 fL and MCHC <32 g/dL. Of the 3 patients with a low Jayabose and/or Mentzer calculation and low HbA2, all had ferritin values <15 ng/mL, indicating limited diagnostic utility of these formulas in discriminating between thalassemia and iron deficiency in this clinical context.

Another potential question faced by pathologists and laboratorians is when to recommend additional genetic work-up for a low HbA2. Guidelines for such work-up may become more important in the United States as thalassemia is becoming increasingly prevalent.[31,32] Our study findings emphasize that work-up to exclude iron deficiency is an appropriate first step. Villegas et al[33] demonstrated that low HbA2 in the absence of iron deficiency is commonly associated with thalassemia, with 112 of 209 patients in a Spanish cohort with low HbA2 demonstrating thalassemia upon confirmatory genetic testing and many additional patients demonstrating a structural hemoglobin variant. As genetic

TABLE 2. Characteristics of Two Patients with Alpha Globin Genetic Testing

Clinical History	Age, y	Sex	Ethnicity	Race	HbA2 Fraction (%)	Hemoglobin, (g/dL)	RBC ($\times 10^6$/µL)	MCV (fL)	MCHC (g/dL)	RDW (%)	Platelet Count ($\times 10^3$/µL)	Ferritin (ng/mL)	Mentzer Index	Jayabose Calculation	Alpha Globin Genetic Testing Result
12 year history of anemia	51	M	Non-Hispanic	African American	2.4	12.1	5.11	77.3	30.6	13.4	181	155	15.1	203	2-gene deletion consistent with alpha-thalassemia trait
G4P1021, Anemia identified at initial prenatal visit	29	F	Non-Hispanic	African American	1.1	12.7	4.89	83.4	31.1	14.3	299	8	17.1	244	Negative, no duplications or deletions

HbA2, hemoglobin A2; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell count; RDW, red cell distribution width; Mentzer index, MCV/RBC; Jayabose-RDW calculation, (MCVxRDW)/RBC.

testing was performed in only 2 patients in our study (1 patient with low HbA2 and 1 patient with normal HbA2), our results cannot provide data regarding the prevalence of thalassemia in our cohort.

Although the association between low HbA2 and iron deficiency has been known for decades, our study shows that laboratory evaluation of patients with low HbA2 remains highly variable, with complete iron studies being performed in only a subset of patients. The results of our study provide the laboratorian, pathologist, and clinician with additional data to further evaluate a low HbA2 value and confirms the necessity of iron studies as an initial first step in the work-up.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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SPECIAL REPORT

Musculoskeletal Pains among Phlebotomists in Outpatient Clinics: Prevalence and Association with Personal Factors and Ergonomic Analysis of the Workstation

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Keywords: phlebotomist, clinical laboratory technicians, ergonomics, musculoskeletal disorders, workstation, blood drawing

Abbreviations: MSDs, musculoskeletal disorders; MHCS, Maccabi Health Care Services; OSS, one-stop shop; MOccH, Master of Occupational Health

Laboratory Medicine 2023;54:241–247; https://doi.org/10.1093/labmed/lmac106

ABSTRACT

Objective: To investigate the prevalence of musculoskeletal problems reported by phlebotomists, and the association of these problems with workstation characteristics and personal factors.

Methods: Self-administered questionnaires were distributed to phlebotomists (n = 115; 65.2% response rate). In addition to the Nordic Musculoskeletal Questionnaire, we asked respondents to provide sociodemographic data. An ergonomic score was used for the characterization of workstations.

Results: Symptoms in at least 1 body area within a year were reported by 80.7% of the participants. The lower back, neck, and shoulders were the most common areas in which symptoms occurred (72.7%, 60.9%, and 59%, respectively); these were also the areas with the highest incidence of symptoms disrupting work. A logistic regression model for predicting pain within a year found adequate leg space as a significant component of the ergonomic score (P = .045, OR = 0.222), which was also a protective factor for neck pain disrupting work (P = .047, OR = 0.385).

Conclusions: Musculoskeletal problems among phlebotomists were high. Therefore, the positive effects of simple ergonomic measures should be confirmed in further research.

Musculoskeletal disorders (MSDs) are a significant and multifactorial health problem that includes pain and soreness and can be work related.[1,2] Work-related MSDs are primarily caused or worsened by the work environment and task demands, and some are considered occupational diseases.[2–5] Demographic, personal, and psychosocial factors correlate with MSDs.[1,4,5] Among the ergonomics risk factors for MSDs are repetition, prolonged static postures, awkward postures, and excessive force.[1–6] The World Health Organization has described MSDs as a cause of concern for worker disability, workforce loss, and increased health care and public expenditures.[7] Simple measures—even as little as awareness campaigns, modification of working tools, and enhanced administrative approaches—can frequently reduce MSDs. However, employers need to be aware of the existence of work-related MSDs.[8,9]

Phlebotomists are a group of health care workers who specialize in taking venous blood specimens.[10] This practice requires repetitive hand movement and the adoption of static and awkward postures, including repetitive or continuous reaching, twisting, bending (especially neck and back), and prolonged static positions. These movements and positions can result in discomfort and/or pain, including in the hands, arms, shoulders, neck, back, and knees. However, to our knowledge, the literature does not refer to the duration of static postures among phlebotomists. Other stressor components are the demand for accuracy, time pressure, stab hazards, and unexpected reactions from patients.[11,12] Clinical work by laboratory technicians is relatively similar to the work of phlebotomists; however, other tasks, such as microscope use, are irrelevant to phlebotomists. López-González et al[13] investigated the work of laboratory technicians and emphasized the need to examine each workforce sector and its unique characteristics separately.

The literature regarding MSDs has rarely dealt with workers whose main task is collecting blood specimens from patients. A case report[14] demonstrated epicondylitis in a phlebotomist due to forceful gripping

and repetitive twisting. Another study report[11] showed a correlation between prolonged standing, posture (bending/twisting of the back), and the risk of developing lower-back disorders. Cong et al[15] described an ergonomic procedure among phlebotomists to reduce their discomfort during blood drawing from neonates. Further, a negative correlation between the number of MSDs and the workstation evaluation score was also found.[16] However, all of those studies were conducted in a hospital setting.

This study aimed to investigate the prevalence of MSDs among phlebotomists in outpatient clinics and the association of MSDs with workstation characteristics and personal factors. Although this study deals with workers who specialize in phlebotomy, this procedure is also performed by other health care workers, including nurses, making the findings generalizable beyond the target population.

Methods

Maccabi Health Care Services (MHCS) is a health maintenance organization in Israel that provides medical services to 26.5% of the population nationwide.[17] Phlebotomists perform blood specimen collection in MHCS community clinics. This cross-sectional study was conducted among phlebotomists in MHCS nationwide.

Study Design

Direct observations of 48 participants were made by a physiotherapist, to analyze the workstation. We looked for the physical characteristics of the workstation and the sequence, duration, and frequency of the tasks performed by the phlebotomists. The observations identified ergonomic risk factors for MSDs that are typical for computer users and laboratory workers. Most of the specimen collection work takes place in a standard, "one-stop shop" (OSS) workstation, used by the phlebotomist for the administrative registration process in a computerized system and then for collecting the blood specimen (**FIGURE 1**). The task is performed in a sitting position, whereas the phlebotomist uses a chair with an adjustable height and backrest; however, not all chairs have armrests. The desk space is divided into equipment drawers, with a computer station provided for administrative tasks. Some workstations contain an additional surface and a stand on which the arm of the patient is placed during blood collection.

FIGURE 1. Typical "one-stop shop" workstation. 1 signifies adjustable chair; 2, desk; 3, equipment drawers; 4, additional surface area; 5, stand on which arm of patient is placed during blood drawing.

The total duration of patient admission ranges from 5 to 10 minutes. We recorded that approximately 60% of the time spent by phlebotomists was devoted to blood-drawing procedures and the rest was for administrative actions. Each phlebotomist serves an average of 30 individuals per work shift. The blood-drawing procedure includes collecting the required equipment from the drawers through repetitive reaching postures, including twisting and bending the back. Blood drawing is performed in a static position of the body that lasts for at least 1 minute, including static holding with the wrist and fingers of 1 hand, with repetitive movements, such as pronation-supination, of the other hand. Removing the needle and cleaning also requires repetitive reaching and hand movements while twisting and bending the back.

FIGURE 2 illustrates examples of static and awkward positions during the phlebotomy procedure. Workstations differ by the proximity to the equipment drawers and whether the dominant hand could be close to the desk. Sitting close to the equipment drawers was defined as an arm reach distance to the drawers, thus requiring less repetitive, less awkward reaching. Sitting with the dominant hand close to the desk resulted in dominant arm support during work tasks. The dominant hand was defined as the hand that one usually uses to activate the computer mouse. Accordingly, we defined 4 types of workstation according to the phlebotomist position with the station, as follows: type 1: proximity to equipment and desk; type 2: proximity to equipment, distance from desk; type 3: distance from equipment, closeness to desk; and type 4: distance from equipment and desk (**FIGURE 3**).

Following the observations and workstation analysis, we developed an ergonomic score based on previous study reports[16,18] and the Laboratory Ergonomics Checklist (Environment Health and Safety, University of California Santa Cruz).[19] The score included 5 items describing the station proximity, dominant hand position, leg space, additional surface area, and use of a chair with armrests. Because all chairs had backrests and adjustable height, these characteristics were not included in the final score. Adequate leg space was defined as enough space under the desk for both knees and legs. The score was calculated by summing the number of positive answers for each of the 5 items, resulting in a final score of 0–5. The higher the score, the more ergonomic the workstation.

Study Setting

Data were collected using an anonymous, self-administered questionnaire. Inclusion criteria included working at least 15 hours per week in phlebotomy and using the same workstation for a minimum of 6 months. Because most phlebotomists in MHCS are female, male phlebotomists were excluded from the study. Pregnant women were also excluded.

The questionnaire included sociodemographic data, such as age, seniority, educational attainment, and religiosity. Weight and height, level of physical activity, smoking status, and background diseases (diabetes, rheumatoid disease, and/or other chronic illness) were also recorded as health variables. In addition, participants were asked about the number of children they had, number of their children who are younger than 6 years, number of working hours per week, additional occupation(s), size of the local phlebotomist team, and the number of patients they serve per shift, with all of these as load variables. We also included questions regarding the form

FIGURE 2. **Types of postures used during phlebotomy procedures. A, Static. B, Twisting. C, Bending posture. D, Awkward reaching.**

of sitting in the workstation (ie, whether the sitting is close to the drawers and the dominant hand is close to the desk) and the ergonomic components of the workstation. The detailed questionnaire (personal data and information regarding the phlebotomy workstation) is presented in the Supplementary Material. The Standardized Nordic Questionnaire for musculoskeletal complaints in different body regions (neck, shoulders, elbows, carpal bones, fingers, lower back, and knees) was used to assess MSDs in the past 12 months and the past week.[20]

The questionnaires were sent by mail, and 2 reminders were sent within 2 weeks. Finally, the questionnaires were returned by mail directly to the researcher. Outcome variables included the reported musculoskeletal problems as a categorical variable, the number of body areas in which problems were reported and the frequency of those problems within 1 week and within 1 year, and whether the pain had disturbed work within the past year.

Data Analysis

Data were presented first by using descriptive statistics. Descriptive statistics were performed by averaging, median, SD, and range of the quantitative variables; categorical variables were described using frequency and percentages. Subsequently, we performed a comparison between the background variables of the 4 types of workstations, using χ^2 testing for categorical variables and ANOVA for sequential variables, to verify their homogeneity. Further, univariate analysis was performed by comparing exposure groups using *t*-testing for independent sequential variables and with χ^2 or Fisher testing for categorical variables. All variables found related to the reported musculoskeletal problems in the univariate analysis were examined with multivariate analysis. We used a logistic regression model (backward selection method) to investigate musculoskeletal complaints and exposure variables in the workstation. Results were defined as statistically significant for *P* value of <.05. These analyses were performed using SPSS, version 23 (IBM). The study and the attached questionnaire were approved by the MHCS research committee and the MHCS local ethics committee (IRB).

Results

Questionnaires were distributed to 333 phlebotomists using OSS in MHCS nationwide, and 217 were returned (65.2% response rate). After

FIGURE 3. The 4 types of "one-stop shop" workstation. A, Phlebotomist close to equipment and desk. B, Phlebotomist close to equipment, at a distance from desk. C, Phlebotomist at a distance from equipment, close to desk. D, Phlebotomist at a distance from equipment and desk.

applying the inclusion and exclusion criteria, we analyzed 115 questionnaires. We excluded 102 questionnaires because of using the same workstation less than 6 months (n = 61), working hours <15 hours per week in phlebotomy (n = 38), sex (n = 6), and pregnancy (n = 10). The background demographic data from the questionnaires were compared and found to be similar before and after the exclusion, thus demonstrating that the cohort represented the whole responded phlebotomist population. Sociodemographic, personal characteristics, load variables (at work and in daily life), and ergonomic factors are shown in **TABLE 1**. Nearly half of the participants (49%) used a workstation with a low ergonomic score (0.1 or 2), whereas only 1.9% used a workstation that contained all the items in the ergonomic checklist (score of 5).

Although 80.7% of participants reported at least 1 symptomatic body area in the past year, 61.6% reported at least 1 symptomatic body area in the past week. The 3 most common body areas in which symptoms were reported were the lower back, neck, and shoulders, for the past year (72.7%, 60.9%, and 59%, respectively) and the past week (43%, 34.8%, and 36.1%, respectively). The problems in these areas were also reported as interfering with the ability to work within 1 year (54.7%, 51.6%, and 43.4%, respectively). The carpal bones were the fourth most common body area in which problems occurred, with 45.9% in the past year, 30.4% in the past week, and 37.7% for pains interfering with the work ability within the past year.

We found no significant correlations between the workstation type and the country of origin, educational attainment, age, work seniority, and any additional work (including additional work in another laboratory). Also, no significant correlations were found between these variables, except age, to the outcome variables.

Age, seniority, body mass index, having children younger than 6 years, and background diseases correlated with the outcome variables. In the single-variable analysis, space for legs, additional surface area, and presence of chair armrests significantly correlated with pain in the past year ($P = .02$, $P = .01$, and $P < .001$, respectively). All of these variables were included in a multivariate logistic regression model. Physical activity showed no significant association with any outcome variables but it was also included in the multivariate model due to its theoretical importance. **TABLE 2** presents multivariate analyses for pain prediction.

TABLE 1. Sociodemographic Data, Personal Characteristics, and Ergonomic Factors of the Female Phlebotomists in Our Cohort[a]

Variable	Frequency (Valid Percentage)	Mean (SD)
Age, y		46.6 (10.1)
Education, y		14.4 (2.6)
Seniority in phlebotomy, y		10.1 (7.6)
Body mass index, kg/m^2		26.5 (5.7)
Physical activity	60 (54.1)	
Current smoker	15 (13.3)	
Background diseases	18 (15.8)	
Diabetes	4 (3.5)	
No. of children		2.5 (1.3)
Children aged <6 y		0.3 (0.7)
Working hours/wk		20 (4.7)
Patients/d		36.1 (14.1)
Phlebotomy team size[b]		
Small	37 (32.5)	
Medium	35 (30.7)	
Big	42 (38.8)	
Type of workstation		
1	43 (38.7)	
2	20 (18.0)	
3	32 (28.8)	
4	16 (13.9)	
Ergonomic score[c]		
0	8 (7.4)	
1	13 (12.0)	
2	32 (29.6)	
3	26 (24.1)	
4	27 (25.0)	
5	2 (1.9)	
Ergonomic factors		
Close to equipment	66 (57.9)	
Close to workstation	76 (67.9)	
Adequate space for legs	69 (60.5)	
Additional surface	70 (61.4)	
Armrests	11 (9.6)	
Additional job	46 (43.4)	
Additional lab job	13 (11.6)	

[a]*n = 115. Male phlebotomists were excluded from the participant pool because there were very few of them.*
[b]*Laboratory size: small, 1–2 workers; medium, 3–5 workers; big, ≥5 workers.*
[c]*The higher the ergonomics score, the more ergonomic the workstation.*

Having children younger than 6 years and adequate leg space were associated with reported problems in at least 1 body area within a year (P = .01 and P = .045, respectively). Also, sufficient leg space was associated with neck pain interfering with work (P = .047), and age was associated with shoulder pain interfering with work (P = .03).

Discussion

Phlebotomists are a unique group of medical workers at high risk of developing MSDs who remain underdiagnosed. Herein, we describe the prevalence of MSDs and the influencing factors among phlebotomists. Musculoskeletal problems in at least 1 body area were reported by 80.7% of participants during the past year. No identical group of employees was found in the literature for reference. However, this finding is high compared with the findings from other types of health care workers engaged in tasks with similar requirements.[3,21–24]

Similar work characteristics to phlebotomists in outpatient clinics were found in other laboratory workers and the dentistry professions. Maulik et al[21] investigated laboratory technicians who also carried out tasks involving a computer. A prevalence of 73.5% for pain in the past year and 53.1% in the past week was found, and the most common body area in which problems were reported was the lower back (30.6%), followed by the upper back, knees, and neck. The prevalence of carpal bone pain in that group was as low as 2%. The authors could not determine whether this occurred because of computer use or due to the nature of the job. Sadeghian et al[22] investigated clinical laboratory workers and found a similarly higher prevalence of MSDs (72.4%) in the past year. AlNekhilan et al,[3] who also surveyed clinical laboratory workers, showed a high prevalence (82%) for pain in at least 1 body area during the past year. However, when evaluating specific body areas of pain, the prevalence was lower: 61% reported in the lower back, 46% in the neck, and 45% in the shoulders. These differences can be explained by the inclusion of men in the analysis and the different nature of work tasks, including posture (sitting vs standing position) in the study.

Ratzon et al[23] investigated dentists and reported similar prevalent body areas of pain (lower back, neck, and shoulders) in the past year and the past week. Still, the reporting rate in our study was higher than that of the dentists. Alghadir et al[24] also investigated dental professionals, including dentists, dental assistants, and dental hygienists. The researchers reported that these workers are required to use overstrained, awkward postures and repetitive joint movements and are at risk for MSDs, thus resembling phlebotomists. This finding may explain the same common body areas of pain reported in those studies. The reported gap in pain prevalence between the studies can be explained by the fact that the cohorts of dental professionals included men and heterogeneous professional status, compared with the homogenous population of phlebotomists in the current study. The current study excluded men, given that MHCS phlebotomists are predominantly women. In many study reports, women report MSDs more frequently than men do. Therefore, this factor might contribute to the high prevalence of symptoms in this study.[2,5,11,13,20–22,24–26]

Age and having children younger than 6 years were significantly associated with reported musculoskeletal problems. Previous study reports[2,12,13,18,22] have supported the association between worker age and MSDs. Having children younger than 6 years was included in this study as a reflection of the daily load. Previous study reports[18,22] did not find any association between daily load variables and musculoskeletal pain among laboratory workers. However, in 2015, Bevan[5] claimed that caring responsibilities, such as lifting or bathing, could increase the risk for MSDs. In the current study, having children younger than 6 years was a protective factor for any pain in the past year. This finding may be due to the confounder of young respondent age. Further studies are required to assess the influence of young children on MSDs and as a daily load marker.

TABLE 2. Multivariate Analyses for Pain Prediction

Outcome Variable	Risk Factor	OR	95% CI	*P* Value
Problem reported during past y				
	Children aged <6 y	0.199	0.055–0.713	.01
	Physical activity	0.288	0.077–1.077	.06
	Adequate leg space	0.222	0.051–0.965	.045
	Armrests	0.227	0.046–1.126]	.07
Neck pain at work				
	Age	1.045	0.998–1.094	.06
	Adequate leg space	0.385	0.150–0.987	.047
Shoulder pain at work				
	Age	1.056	1.005–1.109	.03
	Body mass index	2.793	0.968–8.055	.06
	Additional surface	0.427	0.166–1.098	.08
Lower back pain at work				
	Body mass index	3.152	0.989–10.041	<.05
	Additional surface	0.389	0.150–1.009]	<.05

[a]*Lower odds ratio (OR) indicates decreased risk of adverse outcomes.*

There was no correlation between phlebotomist position and workstation type as a significant exposure factor. However, items in the ergonomic score reflecting different exposure situations, especially adequate leg space, were important in the context of symptoms overall, especially neck pain interfering with work. This finding is supported by that of Agrawal et al,[25] who reported that the lack of leg space resulted in a 1.2-fold increased risk of work-associated musculoskeletal pain. Mukhtad et al[27] also suggested expanding the knee and foot clearance to prevent ergonomic risk factors from laboratory technicians. Haile et al[18] also showed that poor workstation ergonomics was the main factor for reporting work-related MSDs.

This study found an insignificant association between symptoms and the lack of a chair with armrests. Those insignificant findings are supported by Andersen,[28] who noted that one of the ergonomic problems among laboratory workers is the lack of arm support, and by Alghadir et al,[24] who found that dental professionals who used chairs with armrests suffered less than those who used chairs without armrests. The low number of participants who had chairs with armrests n = 23, valid percentage, 9.6%) may have affected the results in this study. There were also insignificant associations between symptoms and the existence of additional surface in the workstation. Additional surface enabled the placement of equipment closer to the phlebotomist, thus reducing awkward reaching. All of those findings, although partially statistically insignificant, are supported by Arora and Uparkar,[29] who gave recommendations to decrease work-related MSDs among pathology-laboratory technicians. They advised certain modifications at the workstation so that the workers are within close reach of their equipment, increased knee and foot clearance, and use of adjustable armrests to support the shoulders in a natural position.

Lima-Oliveira et al[30] reported a shorter duration of drawing blood in private laboratories, compared with public laboratories. They explained this finding in the presence of more ergonomic furniture in the private laboratories. However, this study did not address workload issues or MSD reduction rates, although the findings may indicate an additional benefit for phlebotomist workstations with ergonomic properties.

This study has several limitations. First, psychosocial factors may also play an important role in forming MSDs.[31] However, this factor was outside the scope of the current study and should be addressed in further studies. Other limitations are the response rate (65.16%), which could suggest a nonresponder bias. Still, the study represents a nationwide sample of phlebotomists. The phlebotomists filled in the questionnaires, which may lead to further bias in reporting symptoms. Even so, the questionnaires were distributed and returned anonymously to the researchers and not through employers, thus decreasing reporting bias. Finally, due to sample size restriction, we could not adjust to confounding factors such as age and number of children younger than 6 years. Future research should address psychosocial risk factors and examine the impact of appropriate intervention programs.

Conclusions

Phlebotomists are health care employees with unique characteristics. This study expands the existing knowledge and understanding of phlebotomists as a unique workforce and their individual and ergonomic risk factors for MSDs. Also, it highlights the necessity to adopt relatively simple and well-known ergonomic principles, such as increasing the knee clearance, to decrease musculoskeletal problems, especially neck pain that interferes with work.

Acknowledgments

This study was submitted as partial of the academic requirements for the degree of Master of Occupational Health (MOccH) in the School of Public Health, Sackler Faculty of Medicine, Tel Aviv University, Israel.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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The Dismal History of Cancer Treatment and Ongoing Racial Disparities

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Laboratory Medicine 2023;54:248–249; https://doi.org/10.1093/labmed/lmad031

One of the many horror stories stemming from World War II was the use of nitrogen mustard, a chemical weapon, which would later inspire a common treatment for cancer. On December 2, 1943, the German military conducted an air attack on Bari, a port city in Italy, dropping more than 200 bombs loaded with deadly nitrogen mustard gas, taking more than 1000 lives in the process. Survivors of the attack presented to nearby hospitals with swollen bodies, blistered skin, and temporary blindness. The treating medical staff noted a significant drop in the white blood cell (WBC) counts of the victims of the bombing who were exposed to nitrogen mustard gas. Medical experts studying the symptoms experienced by these patients later determined their significant drop in WBC count to be caused by bone marrow toxicity.[1]

Meanwhile, in early 1940s, the United States Army was studying mustard gas as an effective weapon for war, as well as determining the necessary measures for protection from the effects of the chemical compound. Alfred Zack Gilman, PhD, a pharmacologist at Yale University, became the section chief for pharmacology at the US Army Edgewood Arsenal. In 1942, Gilman, his colleague Louis Goodman, PhD, and Yale surgeon Gustaf Elmer Linskog, MD, experimented with nitrogen mustard in a patient who was terminally ill with lymphosarcoma (now known as lymphoma), who was in the care of Dr Linskog. Although the patient did not survive, nitrogen mustard was found to work effectively against the growth of cancer cells. Nitrogen mustard, initially used as a deadly weapon that devastated thousands of lives, the production and use of which is now strongly restricted by the Hague Conventions governing laws of war, served as the origin for alkylating agents that are still being widely used today in treating cancer. Nitrogen mustard was developed as a drug treatment for cancer shortly thereafter.

Shortly after this discovery, Sidney Farber, MD, a pediatric oncologist in Boston, MA, developed a new treatment for childhood leukemia using folic acid antagonists, which led to the development of other chemotherapy agents to combat other types of cancer. Treatments against metastatic cancer followed; methotrexate was successfully used in treatment of metastatic choriocarcinoma in 1956. As many agree, chemotherapy continues to be a source of hope for millions of patients with cancer, many of whom benefit significantly from it.

However, there is always a "but" in our imperfect world. Racial/ethnic disparities in cancer treatments still exist in the United States, the motherland of chemotherapy. A recent comprehensive review by Zavala and colleagues[2] outlined these disparities and contributors for multiple types of cancer. Despite the provisions of the Affordable Care Act, which was implemented in the United States in 2010, millions of Americans continue to lack health insurance, of which Hispanics/Latinos are the most affected groups. Also, with most insurance plans having the patients identify their health care providers and coordinate their own care, notable lapses occur, with many people in low socioeconomic status groups having limited access to proper (or sometimes any) health care. These patients typically have low screening rates, delayed treatment after diagnosis, and a substantially lower rate of essential follow-ups.

Racial disparity in health care is a multidimensional issue that cannot be explained solely by unequal access to health care. According to the National Cancer Institute,[3] White women are more likely to be diagnosed with breast cancer, but Black women are more likely to die from it.

Breast cancer can be effectively managed, especially when diagnosed early, and screening is an essential tool for preventing deaths from this type of cancer. However, unequal access to health care is an ongoing problem that results in significant disparities (although the unequal access is not the only problem). According to an analysis conducted by Field et al,[4] which included more than 20,000 patients with invasive breast cancer, Black patients still had worse prognoses despite equal access to care. The data from a similar study by Guan et al[5] showed similar results.

The authors of both study reports concluded that socioeconomic status has the biggest impact in mortality rates regarding disparities. This finding could be attributed to differences in disease biology and differences in quality of care, such as inadequate molecular testing due to unclassified molecular markers and inadequate dosages of chemotherapy (ie, disproportionate to the body surface areas of patients). Another researcher, Shariff-Marco and colleagues[6] found that racism is a stressor that may contribute to behaviors that increase cancer risk, including smoking, drinking, and being overweight, which eventually deepens disparities in health care. Also, persons who experience racism in health care are less likely to regularly obtain annual screenings (ie, prostate-specific antigen screening and annual imaging).

The American Association for Cancer Research recently published its 2022 Cancer Disparities Progress Report (https://cancerprogressreport.aacr.org/disparities/). Although a significant

decline was reported in overall cancer incidence and mortality rates for racial and ethnic minorities, the economic burden of cancer health disparities is still enormous, due to disparities in premature cancer deaths between Black and White patients. Also, the report emphasized that the substantial lack of sociodemographic diversity among clinical trial participants represents a major barrier to advancing cancer care for the entire patient population. Improved access to clinical trials for a variety of racial and ethnic groups will certainly enable scientific observation of biologic and behavioral differences among these groups and will assist in the development of more effective and tailored treatments.

The tragic history of nitrogen mustard that led to the discovery of chemotherapy has enabled us to save many lives. Still, we must continue to identify and dismantle ongoing structural racism and to mitigate the resulting adverse health effects on patients from racial minority groups.

Conflict of Interest Disclosure

The author has nothing to disclose.

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SCIENCE

Association of Interleukin-6 Polymorphisms with Schizophrenia and Depression: A Case-Control Study

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Keywords: schizophrenia, interleukin-6, polymorphism, depression, genotype, cytokine

Abbreviations: *IL-6*, interleukin-6; SNV, single nucleotide variant; CIs, confidence intervals; OR, odds ratio

Laboratory Medicine 2023;54:250–255; https://doi.org/10.1093/labmed/lmac099

ABSTRACT

Objective: Growing evidence suggests a crossover in genetic susceptibility to schizophrenia and depression. We aimed to investigate the association of the rs1800795 and rs1800796 polymorphisms of the *IL-6* gene with schizophrenia and depression in the Han Chinese population, combined with *IL-6* serum levels.

Methods: Gene sequencing and enzyme-linked immunosorbent assay were performed on 113 subjects with schizophrenia, 114 subjects with depression, and 110 healthy controls.

Results: Our findings showed that *IL-6* concentrations in schizophrenia and depression groups were significantly higher than in the control group. The rs1800796 CC genotype and C allele were significantly associated with depression ($P = .012$ and $P < .05$, respectively). The rs1800796 CC and CG genotype was significantly associated with chronic schizophrenia ($P = .020$ and $P = .009$, respectively). Regarding the rs1800795 polymorphism, only one case of CG genotype was detected. The remainder were of the GG genotype.

Conclusion: The *IL-6* rs1800796 might serve as a protective factor for depression and schizophrenia in the Han Chinese population.

Depression is a common and serious mental disorder, manifested by persistent and significant low mood, loss of interest and pleasure, and decreased energy. It can lead to self-harm through suicidal ideations and attempts. In 2017, the World Health Organization reported that depression affects approximately 322 million people worldwide, which corresponds to a prevalence rate of 4.4%.[1] In 2019, a report on the epidemiology of mental disorders in China published by *The Lancet Psychiatry* showed that the lifetime weighted prevalence of the various types of depression has reached 6.8%.[2]

In recent years, research studies have questioned the efficacy of traditional antidepressant therapy. This has resulted in interest in the cytokine theory, as well as other mechanisms, which have brought new opportunities for the diagnosis and treatment of depression.[3] Recent studies point to the inflammatory response as a potential cause of depression. Among these, the increase in pro-inflammatory cytokines, such as interleukin-6 (*IL-6*), is one of the most reliable biological markers.[4]

Inflammation in the central nervous system is closely related to neurodegeneration in patients with psychiatric diseases.[5] The neurodevelopmental model postulates that schizophrenia is the result of a behavioral disorder that begins long before the individual presents with clinical symptoms.[6] Schizophrenia is a heterogeneous disorder with a complex etiological background involving the environment and numerous genetic/epigenetic factors, estimated to affect approximately 1% of the world's population.[7] Numerous previous studies have proposed a role for genetic polymorphisms in schizophrenia risk, including single nucleotide variants (SNVs) in the promoters of microRNAs and intronic SNVs.[8–13] Studies have shown that blood protein expression in patients with schizophrenia is influenced by corresponding gene polymorphisms, and the effect of certain SNVs on expression of the respective proteins may vary by diagnosis. The combination of patient-specific genetic information and blood biomarker data thus opens a novel approach to investigate disease mechanisms in schizophrenia and other psychiatric disorders.[14]

Immune dysfunction has been demonstrated in schizophrenia and depression,[15] suggesting that there may be common etiological risk factors for both disorders. Cytokines are important for communication among the nervous, endocrine, and immune systems. They regulate neurotransmitters and neuropeptides, as well as participate in the pathophysiological process of nervous system diseases. *IL-6* is a multifunctional cytokine with proinflammatory, immunomodulatory, and neuroprotective functions that plays an important role in the pathophysiology and antidepressant response of depression. *IL-6*, a

pro-inflammatory cytokine, has been reported to be a key mediator of maternal immune activation disrupting fetal brain development.[16] The gene for *IL-6* is located on chromosome 7 and is organized into 5 exons and 4 introns. Its expression might be modulated by polymorphisms such as rs1800795 and rs1800796 in the promoter region of the gene.[17]

Changes in *IL-6* levels and gene polymorphism were found to be associated with psychopathology, disease severity, cognition, and brain morphometry.[18] Genetic variations of *IL-6* have been reported to modulate chronic stress exposure in the development of depressive symptoms and increase the risk of interferon-induced depression.[19,20] Few studies have assessed the effect of *IL-6* gene polymorphisms on depression.[21]

In this study, we aimed to investigate the association of the rs1800795 and rs1800796 polymorphisms of the *IL-6* gene with schizophrenia and depression in the Chinese Han population, combined with *IL-6* serum levels. This study provides a basis for taxonomic and etiological studies.

Materials and Methods

Participants

This study included 113 participants with chronic schizophrenia, 114 with depressive disorders, and 110 healthy controls. All subjects were recruited from the Fifth People's Hospital of Nanning between August 2019 and July 2020. All participants were diagnosed with schizophrenia or depression by at least 2 psychiatrists based on the International Statistical Classification of Diseases and Related Health Problems 10th Revision. The criteria were of the chronic type and an illness duration of at least 6 months. Individuals with head injuries, substance-induced psychotic disorders, alcoholic psychosis, and other symptomatic psychoses were excluded from the study. All participants had received stable doses of oral antipsychotic drugs for at least 6 months prior to inclusion into the study and were treated using several different types of atypical antipsychotics or antidepressants.

A random selection of 110 age- and sex-matched subjects, who visited the general health check-up centers of the Fifth People's Hospital of Nanning for a physical examination, were randomly selected among the healthy controls. None of them had a personal or familial history of mental disorders as assessed by 2 experienced independent psychiatrists and through electronic medical record review. None of the participants included in this study had a substance abuse disorder, and all of them were Han Chinese from the Guangxi district. Written informed consent was obtained from all participants. This study was approved by the Ethics Committee of the Fifth People's Hospital of Nanning.

Serum *IL-6* Level

For those eligible for inclusion, 3 mL elbow venous blood was collected on an empty stomach from 8:00 am to 10:00 am on the day of inclusion or the next day. The blood samples were centrifuged at 3500 rpm for 10 min after standing until coagulated, and the serum was subpacked and stored at −20°C for examination. The concentration of *IL-6* in the serum of all subjects was detected by enzyme-linked immunosorbent assay. The optical density of the sample to be tested was measured at 450 nm with a microplate meter. The standard curve was calculated according to the optical density of the standard substance and the corresponding concentration gradient, and then the *IL-6* concentration of the sample to be tested was further calculated according to the standard curve.

SNV Genotyping

To evaluate the SNVs, a sample of approximately 2 mL of ethylenediaminetetraacetic acid anticoagulated venous blood was collected from each of the participants and stored at −80°C until DNA extraction. Genomic DNA was extracted using a QIAamp DNA blood mini kit (QIAGEN). All extracted samples were sent to Nanning Guotuo Biotechnology Science and Technology, and SNV genotype analysis was performed by Shotgun and Sanger sequencing methods.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation (normal distribution) or median (quartile; skewed distribution). Continuous and categorical variables were compared using Student's *t*-test and χ^2 test, respectively. The Hardy–Weinberg equilibrium was assessed via a goodness-of-fit χ^2 test. To investigate the potential association of SNVs with schizophrenia and depression, χ^2 tests were performed to compare the difference in genotype and allele frequency in patients with schizophrenia, depression, and the controls. The odds ratios and corresponding 95% confidence intervals (CIs) were calculated using a binary logistic regression model adjusted for age and sex to examine whether there were significant differences between the patients and controls after controlling for confounders. The reference category used in the regression analysis was the homozygote of the ancestral allele based on the National Center for Biotechnology Information dbSNV database. Haplotypes with frequencies of >1% were included in the association analysis. The haplotype construction was conducted using SHEsis software. All statistical analyses were performed using the Statistical Package for Social Sciences, version 22.0 (SPSS); 2-sided *P* values of <.05 were considered statistically significant. Statistical power calculations were performed using power and sample size calculation.

Results

Characteristics of Study Participants

The demographic characteristics of the study participants are shown in **TABLE 1**. There were no significant differences based on age or sex between the case and control groups (P>.05), suggesting that the 3 sets of data are comparable.

Association of *IL-6* Gene Polymorphism and Serum *IL-6* Levels with the Risk of Schizophrenia and Depression

The genotype distribution of these 2 SNVs in case and control groups were in accordance with the HWE (P > .05). The genotype and allele frequencies of the candidate SNVs for the patient and control groups are shown in **TABLE 2**. As shown in **TABLE 2**, *IL-6* concentrations in schizophrenia and depression groups were significantly higher than

TABLE 1. Baseline Characteristics of Study Participants

Variables	Schizophrenia (n = 113)	Depression (n = 114)	Controls (n = 110)	*P* Value
Age (mean ± SD)	32.23 ± 9.26	31.21 ± 11.95	32.35 ± 6.29	NS
Sex, No. (%)				
Male	54 (47.8)	55 (48.2)	54 (49.1)	NS
Female	59 (52.2)	59 (51.8)	56 (50.9)	

NS, not significant.

TABLE 2. Genotype and Allele Frequencies of *IL-6* Gene Polymorphisms in Patients with Schizophrenia, Depression, and Healthy Controls

Polymorphisms	Cases (n = 113/114)[a]	Controls (n = 110)	χ^{2}[b]	*P* Value[b]	OR (95% CI)[c]	P_{OR}[c]
Schizophrenia (*IL-6* [pg/mL])	38.22 (43.51)	2.58 (2.70)	130.729	<.05[d]		
Rs1800796[e]						
CC	59 (52.2)	56 (50.9)	7.77	.020[d]	0.252 (0.079–0.807)	.020[d]
CG	50 (44.3)	39 (35.5)			0.206 (0.063–0.672)	.009[d]
GG	4 (3.5)	15 (13.6)			1[ref]	
C allele	168 (74.3)	151 (68.6)	1.780	.182	0.756 (0.500–1.142)	.184
G allele	58 (25.7)	69 (31.4)			1[ref]	
Rs1800795[e]						
CC	0 (0.00)	0 (0.00)		NS		NS
CG	1 (0.9)	0 (0.00)		NS		NS
GG	112 (99.1)	110 (100)				
C allele	1 (0.4)	0 (0.0)		NS		NS
G allele	225 (99.6)	220 (100)				
Depression (*IL-6* [pg/mL])	15.68 (22.82)	2.58 (2.70)	98.910	<.05[d]		
Rs1800796[e]						
CC	82 (71.9)	56 (50.9)	14.30	<.05[d]	0.071 (0.009–0.557)	.012[d]
CG	29 (25.5)	39 (35.5)			0.152 (0.019–1.251)	.080
GG	3 (2.6)	15 (13.6)			1[ref]	
C allele	193 (84.6)	151 (68.6)	16.11	<.05[d]	0.393 (0.248–0.622)	<.05[d]
G allele	35 (15.6)	69 (31.4)			1[ref]	
Rs1800795[e]						
CC	0 (0.00)	0 (0.00)		NS		NS
CG	0 (0.00)	0 (0.00)		NS		NS
GG	114 (100)	110 (100)				
C allele	0 (0.00)	0 (0.0)		NS		NS
G allele	228 (100)	220 (100)				

CI, confidence interval; NS, not significant; OR, odds ratio; ref, reference genotype.
[a]*n = 113 patients with chronic schizophrenia and n= 114 patients with depressive disorders.*
[b]*Values regarding genotype distribution.*
[c]*Adjusted for sex and age by logistic regression model.*
[d]*P < .05 was considered statistically significant.*
[e]*Values are given as No. (%) for Case and Control columns.*

in the control group (both $P < .05$). The genotype distribution of the rs1800796 polymorphism was statistically significantly different between the subjects with schizophrenia or depression and healthy controls ($P = .020$ and $P < .05$, respectively). The distributions of the rs1800796 alleles were then compared across the groups, and a significant difference was found between the depression and control groups (adjusted odds ratio [OR] = 0.393, 95% CI 0.248–0.622, $P < .05$). Further, logistic regression analyses adjusted for age and sex revealed that the rs1800796 C and G alleles were not associated with a reduced risk of schizophrenia compared with the C allele (adjusted OR = 0.756, 95% CI 0.500–0.142, $P = .184$). However, rs1800795 was not statistically significant between the case and the control groups in this study.

Stratified Effects of rs1800796 Polymorphisms on the Risk of Schizophrenia/Depression Estimated by Sex

The genotype and allele frequencies by sex of the candidate SNVs are described in **TABLE 3**. The CC genotype distribution of the rs1800796 polymorphisms was statistically significant in female depression groups(adjusted OR = 0.080, 95% CI 0.010–0.666, $P = .020$). The C allele distribution of the rs1800796 polymorphisms were statistically significant in both male and female depression groups(adjusted OR = 0.339, 95% CI 0.169–0.680, $P = .002$; OR = 0.439, 95% CI 0.237–0.811, $P = .009$). Furthermore, the C allele of schizophrenia distribution of the rs1800796 polymorphisms was not significant in both male and female groups. However, the CC genotype of schizophrenia distribution of the rs1800796 polymorphisms was significant in the female groups (adjusted OR = 0.173, 95% CI 0.035–0.865, $P = .033$).

Haplotype Analysis

The haplotype distribution in the schizophrenia/depression patients and healthy control subjects is shown in **TABLE 4**. The GG haplotype was associated with a significantly increased risk of depression (OR = 2.546, 95% CI 1.609–4.028) whereas the GC haplotype was associated with a significantly decreased risk of depression (OR = 0.393, 95% CI 0.248–0.622). The GC and GG haplotypes in the schizophrenia patients were not significantly different from those in healthy controls (both $P > .05$).

TABLE 3. Stratified Effects of rs1800796 Polymorphisms on the Risk of Schizophrenia/Depression Estimated by Sex[a]

Model	Genotypes/Alleles	Male					Female				
		Cases (n = 54)	Controls (n = 54)	*P* Value[b]	OR (95% CI)[c]	P_{OR}[c]	Cases (n = 59)	Controls (n = 56)	*P* Value[b]	OR (95% CI)[c]	P_{OR}[c]
Schizophrenia	Rs1800796										
Co-dominant	CC	23 (42.6)	28 (51.9)	.254	0.406 (0.075-2.205)	.296	36 (61.0)	28 (50.0)	.065	0.173 (0.035-0.865)	.033[d]
	CG	29 (53.7)	20 (37.0)		0.230 (0.042-1.257)	.090	21 (35.6)	19 (33.9)		0.201 (0.038-1.050)	.057
	GG	2 (3.7)	6 (11.1)		1[ref]		2 (3.4)	9 (16.1)		1[ref]	
Allele	C	75 (69.4)	76 (70.4)	.886	1.018 (0.570-1.819)	.952	93 (78.8)	75 (67.0)	.043[d]	0.557 (0.308–1.007)	.053
	G	33 (30.6)	32 (29.6)		1[ref]		25 (21.2)	37 (33.0)		1[ref]	
Depression		n = 55	n = 54								
	CC	43 (78.2)	28 (51.9)	.038[d]	0.212 (0.040–1.126)	.069	39 (66.1)	28 (50.0)	.043[d]	0.080 (0.010–0.666)	.020[d]
	CG	10 (18.2)	20 (37.0)		0.667 (0.113–3.919)	.654	19 (32.2)	19 (33.9)		0.111 (0.013–0.965)	.046[d]
	GG	2 (3.6)	6 (11.1)		1[ref]		1 (1.7)	9 (16.1)		1[ref]	
	C	96 (87.3)	76 (70.4)	.002[d]	0.339 (0.169–0.680)	.002[d]	97 (82.2)	75 (67.0)	.008[d]	0.439 (0.237–0.811)	.009[b]
	G	14 (12.7)	32 (29.6)		1[ref]		21 (17.8)	37 (33.0)		1[ref]	

CI, confidence interval; OR, odds ratio; ref, reference genotype.
[a]*Data are given as No. (%) for Case and Control columns.*
[b]χ^2 *test.*
[c]*Adjusted for age by logistic regression model.*
[d]P < *.05 was considered statistically significant.*

TABLE 4. Haplotype Analysis of *IL-6* Gene Polymorphisms Between Cases and Healthy Controls

rs1800795	rs1800796	Schizophrenia, No. (%)	Controls, No. (%)	OR (95% CI)	*P* Value[a]
G	C	168 (74.3)	151 (68.6)	1.347 (0.890–2.037)	.158
G	G	57 (25.7)	69 (31.4)	0.734 (0.491–1.123)	.158
C	C	0 (0.00)	0 (0.00)	—	—
C	G	1 (0.004)	0 (0.00)	—	—
		Depression, No. (%)			
G	C	193 (84.8)	151 (68.6)	0.393 (0.248–0.622)	<.05
G	G	35 (15.2)	69 (31.4)	2.546 (1.609–4.028)	<.05

CI, confidence interval; OR, odds ratio.
[a]P < *.05 was considered statistically significant.*

Discussion

Our study revealed several novel findings. First, the *IL-6* rs1800796 CC and CG genotypes were associated with significantly decreased schizophrenia risk. In other words, the association of rs1800796 polymorphism with schizophrenia was present among CC and CG heterozygotes, but the rs1800796 C allele was not significantly correlated with schizophrenia. Second, *IL-6* rs1800796 CC genotype was associated with a lower risk for depression. Similarly, the rs1800796 C allele was inversely associated with depression as well. Thirdly, *IL-6* concentrations in schizophrenia and depression groups were significantly higher than those in the control group. In addition, haplotype analysis of the 2 candidate SNVs revealed that the haplotype GC was significantly associated with a decreased risk for depression, which was consistent with the association analysis between rs1800796 and depression risk. Therefore, this study found that acute stage immune activation was prevalent in patients with schizophrenia and depression, which may have the same pathological mechanisms.

Depressive symptoms are one of the common clinical features of schizophrenia. Studies have shown that 30% to 70% of persons with schizophrenia have depressive symptoms,[22] which likely worsen the prognosis and social functioning of these individuals. Previous studies have shown that cytokines such as *IL-6* and tumor necrosis factor-α are involved in the incidence and development of schizophrenia and depression.[23,24] This literature suggests that *IL-6* may be a common factor in the occurrence of these 2 diseases.

According to the literature, the functional rs1800795 polymorphism is located in the promoter region of the *IL-6* gene and has been previously associated with an increased risk of interferon-induced depression.[20] High transcriptional activity was found in rs1800795 GG homozygotes and GC heterozygotes.[25] According to 1 study, the rs1800795 C allele is more frequently found in schizophrenia patients than in controls,[26] and previous reports have found that that the presence of allele C at position -174 of the *IL-6* promoter sequence (rs1800795) may be correlated with an increased risk of paranoid schizophrenia in the Polish population; however, this was only found in the female subgroup.[27] In our study, we observed that all participants had the GG gene polymorphism in *IL-6* rs1800795 except for 1 case of the CG genotype in the schizophrenia group. This indicates that the frequency of gene polymorphisms is low in this study population. This can be due to obvious ethnic and regional differences in gene

polymorphisms, leading to different phenotypic effects of the same mutation in different populations, as well as inconsistent results of different studies. Moreover, in the *IL-6* promoter region, there may be other polymorphic sites involved in the complex expression regulation. Nevertheless, the results are in line with that of Zhong et al[28] and Fu et al,[29] where no genetic polymorphism was found at the rs1800795 loci, and all the individuals were GG homozygous. Our study population is similar to that of those conducted in South Korea and Japan, where there are no such polymorphisms or the frequency of the C allele is low. The *IL-6* gene rs1800795 polymorphism that supports our findings is indeed different from that of European and American populations, since the C allele is less widely distributed in East Asian populations than in Western ones. In the study of the correlation between the gene polymorphism and schizophrenia/depression in the Han Chinese population, it can be concluded that the gene polymorphism of *IL-6* rs1800795 is not associated with schizophrenia and depression.

It has been widely established that inflammation is implicated in schizophrenia and depression, although the exact nature of that association is unclear. There are multiple polymorphic sites in the promoter region of the *IL-6* gene, including -174G/C, -373An/Tn, -572C/G, and -597G/A, which may affect transcription.[30] Previous studies have shown that the level of plasma *IL-6* in the G allele of rs1800796 locus is higher than in the C allele.[31,32] Therefore, we selected rs1800795 and rs1800796 to explore the role of gene polymorphisms of the *IL-6* promoter region in the incidence and development of schizophrenia and depression at the genetic level. In this study population, there was an *IL-6* rs1800796 gene polymorphism. Our results revealed that the rs1800796 CC genotype and C allele were significantly negatively correlated with depressive disorders. Furthermore, the rs1800796 CC and CG genotype were significantly negatively correlated with chronic schizophrenia. The genotype distribution of the rs1800796 polymorphism was statistically significant in female groups. This is inconsistent with a study showing no association between the *IL-6* gene rs1800796 polymorphism and schizophrenia.[30]

Inflammation has also been implicated in depression, although the extent of that association is unclear as well. Inflammation is thought to play a role in the pathogenesis of depression, but depression itself may result in increased inflammation. They may also have similar etiological causes, such as chronic stress.[33] Pro-inflammatory cytokines, such as *IL-6*, have been reported to play a fundamental role in the pathogenesis of depression in some individuals.[34] Increased levels of *IL-6* have also been associated with reduced grey matter in the brain, which is commonly seen in depression.[35] It appears to be one of the most powerful markers of inflammation associated with depression.[36] Irrespective of the presence of chronic disease that may influence *IL-6* production, the effect of long-term medications cannot be excluded. Therefore, more data are needed to support the relationship between *IL-6* inflammatory factor and depression.

Several potential limitations of our study must be acknowledged when interpreting our results. First, our sample size was relatively limited, which could have led to false-positive results related to the lack of statistical power. A replication study that includes a larger sample size is thus needed. Second, we did not control for other potential confounders, such as schizophrenia subtype, body mass index, smoking, or drinking status, and this might have caused bias in the statistical analysis. Third, only 2 SNVs were studied, which may not have allowed us to fully understand the effects of *IL-6* genes. Identification of more functional SNVs and investigation of their influence on *IL-6* expression and protein production is needed. Fourth, this study did not assess the relationship between *IL-6* and clinical symptoms in patients with schizophrenia and depression. Ideally, a larger sample might have allowed for an assessment of variations in the gene and environment interaction between other ethnic groups. This is particularly interesting when we consider the importance of culture in personalized medicine.[37,38] The SNVs could serve as valuable biomarkers relevant to broad clinical applications of personalized medicine.[39] Several recent developments in nanotechnology-based SNV sensing methods enable specific and ultrasensitive detection of low-concentration SNVs and rare mutations, which will allow rapid and precise disease diagnosis and prognosis.[40]

In summary, our study suggests that the *IL-6* rs1800796 gene polymorphism might serve as protective factor in depression and schizophrenia. Although these 2 diseases may have a common pathological basis, the exact association of *IL-6* with them remains unclear. Additional studies are necessary to detect the interrelationship between *IL-6* SNVs and clinical symptoms in schizophrenia and depression.

Acknowledgments

We would like to thank all the participants of this study. We also thank Scribendi.com for linguistic assistance during the preparation of the manuscript.

This work was supported by the Health Commission of Guangxi Zhuang Autonomous Region (No. Z20190713).

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Platelet MicroRNA-484 as a Novel Diagnostic Biomarker for Acute Coronary Syndrome

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Keywords: miR-484, miR-223-5p, miR-126-5p, miR-130a-3p, platelet, acute coronary syndrome

Abbreviations: miR, microRNA; ACS, acute coronary syndrome; CAD, coronary artery disease; AUC, area under the curve; CI, confidence interval; UA, unstable angina; AMI, acute myocardial infarction; STEMI, AMI with ST-segment elevation; NSTEMI, AMI without ST elevation; SA, stable angina; PRP, platelet-rich plasma; ROC, receiver operating characteristic; SCAD, severe CAD

Laboratory Medicine 2023;54:256–261; https://doi.org/10.1093/labmed/lmac102

ABSTRACT

Objective: Platelet microRNAs (miRs) have been indicated as a diagnostic biomarker in various diseases, including acute coronary syndrome (ACS). This study aimed to investigate the expression of miR-223-5p, miR-126-5p, miR-484, and miR-130a-3p in individuals with coronary artery disease (CAD).

Methods: Forty subjects with CAD and 13 healthy individuals were under study. The expression of miR-223-5p, miR-126-5p, miR-484, and miR-130a-3p was measured in platelets by quantitative reverse transcription–polymerase chain reaction. The relationship between miRNA expression and various parameters of the subjects was analyzed using analysis of variance and Spearman and *t*-tests.

Results: The miR-484 expression was significantly upregulated in the ACS subjects ($P = .0097$). Moreover, miR-484 had diagnostic value for screening subjects with unstable angina vs controls (area under the curve [AUC] = 0.978, 95% confidence interval [CI] 0.92–1, $P = .0006$) and NSTEMI patients versus controls (AUC = 0.910, 95% CI 0.74–1, $P = .005$).

Conclusion: The results of this study indicate that the upregulated expression of miR-484 in ACS patients might be used as a diagnostic biomarker in ACS.

Acute coronary syndrome (ACS) is a major severe medical emergency that imposes a high burden on health care costs, mortality, and morbidity in many countries. It can be classified into unstable angina (UA), acute myocardial infarction (AMI) with ST-segment elevation (STEMI), and AMI without ST elevation (NSTEMI).[1] Several pathological studies have indicated that overcrowding and platelet hyperactivity are associated with the incidence and progression of cardiovascular disease. Platelets are the main agent in hemostasis and acute atherothrombosis in the incidence of cardiovascular disease, especially ACS.[2] Atherosclerosis plaque as an inflammatory situation activates platelets and releases their vesicle content, which leads to plaque rupture, thrombosis, and ACS progression.[3,4] Interestingly, although platelets lack a nucleus and transcript gene mechanism, they have RNA, including microRNA (miR). At present, several studies have pointed out that some platelet miRs, such as miR-146a-5p, miR-21-5p,[5,6] miR-361-5p,[7] miR-19b-1-5p,[8] and miR-4286,[9] are associated with the occurrence and development of ACS. Currently, the understanding of pathological mechanisms of ACS is limited, and those biomarkers that have been discovered and used to diagnose MI are not considered sufficiently effective.[10] Therefore, further investigation of platelet miRs in ACS disease might add potential biomarkers and novel therapeutic targets for individuals with ACS. In addition, platelet miRs are related to platelet function and related diseases; their physiological role, especially as a potential diagnostic biomarker, remains unknown. The diagnostic value of platelet miR-223-5p, miR-126-5p, miR-484, miR-130a-3p in ACS diseases is not clear. This study aimed to evaluate whether platelet miR-484 can be used as a diagnostic biomarker in ACS.

Materials and Methods

Study Population

This case-control study was conducted in 2021 with 40 individuals, including 23 diagnosed with ACS and 17 with stable angina (SA) who were visited at the Taleqani Hospital (Tehran, Iran) and 13 healthy

volunteers as controls. Definitive diagnosis of ACS and classification into STEMI, NSTEMI, and UA was done using international ACS diagnostic criteria.[11,12] All persons with heparin therapy, platelet count <100,000/μL or >450,000/μL, active malignancy, or end-stage renal disease, and pregnant and lactating women were excluded from the study.

Ethical Considerations

The Ethical Committee of Shahid Beheshti University of Medical Sciences approved this study (IR.SBMU.RETECH.REC.1399.325), and all the participants provided informed consent following the Declaration of Helsinki.

Sample Collection and PRP Preparation

A total of 9 mL anticoagulant (3.2% sodium citrate containing and indomethacin 10 μmol/L) peripheral blood was obtained (9 parts of blood, 1 part of anticoagulant). To obtain platelet-rich plasma (PRP), whole blood was centrifuged at 100*g* for 10 min at room temperature, and PRP was isolated by successive centrifugation of sterile conical tubes at 1000*g* for 15 min at room temperature. Next, the platelets were suspended in Tyrode HEPES buffer (10 mmol/L N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid [HEPES], 137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2[6H_2O]$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4, 5.5 mmol/L glucose, 0.35% bovine serum albumin [pH 7.4]) and stored at −80°C.

TABLE 1. Primer Sequences of Platelet miRNAs for Real-Time PCR

miRNA	Sequence 5′–3′
hsa-miR-223-5p	CGUGUAUUUGACAAGCUGAGUU
hsa-miR-126-5p	CAUUAUUACUUUUGGUACGCG
hsa-miR-484	UCAGGCUCAGUCCCCUCCCGAU
hsa-miR-130a-3p	CAGUGCAAUGUUAAAAGGGCAU
U6	CTCGCTTCGGCAGCACA

Platelet miRNA Isolation and RT-PCR

Extraction of miRNA was prepared using Trizol (Thermo Fisher Scientific) according to the manufacturer's instructions. The concentration of platelet miRNA was assessed using Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific). The RNA integrity was evaluated by agarose gel electrophoresis.

The cDNA was synthesized using a reverse transcription kit (SMOBIO) according to standard protocol. The expression of platelet miR-223-5p, miR-126-5p, miR-484, and miR-130a-3p was calculated using quantitative reverse transcription–polymerase chain reaction (RT-PCR) on Rotor-gene Q (Qiagen) using Taq DNA Polymerase Master Mix Green 2 mmol/L $MgCl_2$ (Ampliqon). Relative platelet miRs were measured by the $2^{-\Delta\Delta Ct}$ method after U6 normalization. All platelet miRs primer sequences are shown in **TABLE 1**.

Statistical Analysis

For data analysis, GraphPad Prism version 8.4.3 (686) (GraphPad Software) was used. Comparison of fold-change miRs in subject and control groups was done by unpaired *t*-test with Welch's correction or 1-way ANOVA followed by Tukey's post hoc test. Receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic potential of miR-484. The correlation between the expression of platelet miRs and variables was analyzed by Spearman correctional analysis. The statistical significance was considered $P < .05$.

Results

Subject Characteristics

The characteristics of subjects with STEMI (n = 10), NSTEMI (n = 6), UA (n = 7), and SA (n = 17) and the 13 controls are shown in **TABLE 2**.

TABLE 2. Characteristics of the Subject Populations[a]

	Subjects with ACS (n = 40)				Control (n = 13)	P_1 Value	P_2 Value	P_3 Value	P_4 Value
	STEMI (n = 10)	NSTEMI (n = 6)	UA (n = 7)	SA (n = 17)					
Age, y	53.6 ± 11.53	60 ± 6.164	59.71 ± 6.39	52.94 ± 12.01	53 ± 19.99	.9289	.2673	.2835	.9926
BP, mm Hg	138.4 ± 22.41	137.6 ± 12.27	154.8 ± 25.76	133 ± 22.37	120.4 ± 13.61	.0416	.0194	.0113	.0665
Troponin, μg/L	4.225 ± 12.57	11.26 ± 21.69	0.06514 ± 0.04	0.1216 ± 0.14	—	.3157	.2596	.0078	.0038
Hypertension, %	30	33.33	71.42	17.64	0	.0811	.1747	.0082	.0826
Diabetes mellitus, %	40	66.66	28.57	52.94	0	.0368	.025	.1723	.0006
Family history, %	40	33.33	14.28	23.52	0	.0368	.0756	.3559	.0413
Cigarette smoking, %	30	16.66	14.28	47.05	0	.0811	.3632	.3559	.0017
ASA, %	40	50	28.57	41.17	0	.0368	.0756	.1723	.0041
Clopidogrel, %	20	16.66	14.28	23.52	0	.1679	.3632	.3559	.0413
Statin, %	37.5	60	50	54.54	0	.0796	.0705	.0756	.0061
β-blocker, %	50	50	33.33	23.52	0	.0331	.1817	.1747	.0162
PLT, $\times 10^9$/L	209.4 ± 58.97	221.2 ± 74.12	206.1 ± 14.52	238.3 ± 77.64	253.4 ± 63.15	.1009	.3822	.0219	.5621
PDW, fL	12.96 ± 2.21	13.3 ± 2.10	13.03 ± 1.18	12.52 ± 1.62	13.92 ± 2.66	.358	.5965	.3181	.1117
MPV, fL	10.26 ± 1.09	10.32 ± 1.16	10.03 ± 0.85	10.04 ± 0.92	10.12 ± 1.05	.7527	.7273	.8446	.8294

ACS, acute coronary syndrome; BP, blood pressure; MPV, mean platelet volume; NSTEM, non-ST segment elevation acute myocardial infarction; PDW, platelet distribution width; PLT, platelet; SA, stable angina; STEMI, ST segment elevation acute myocardial infarction; UA unstable angina.
[a]P_1, *STEMI vs control;* P_2, *NSTEMI vs control;* P_3, *UA vs control;* P_4, *SA vs control.*

FIGURE 1. Expression of platelet miR-223-5p, miR-126-5p, miR-484, and miR-130a-3p in acute coronary syndrome (ACS) subjects. A, Expression of miR-223-5p in controls, ACS subjects, and stable angina (SA) subjects. B, Expression of miR-126-5p in controls, ACS subjects, and SA subjects. C, Expression of miR-484 in controls, ACS subjects, and SA subjects. D, Expression of miR-130a-3p in controls, ACS subjects, and SA subjects. Data are mean ± SEM. *P < .01.

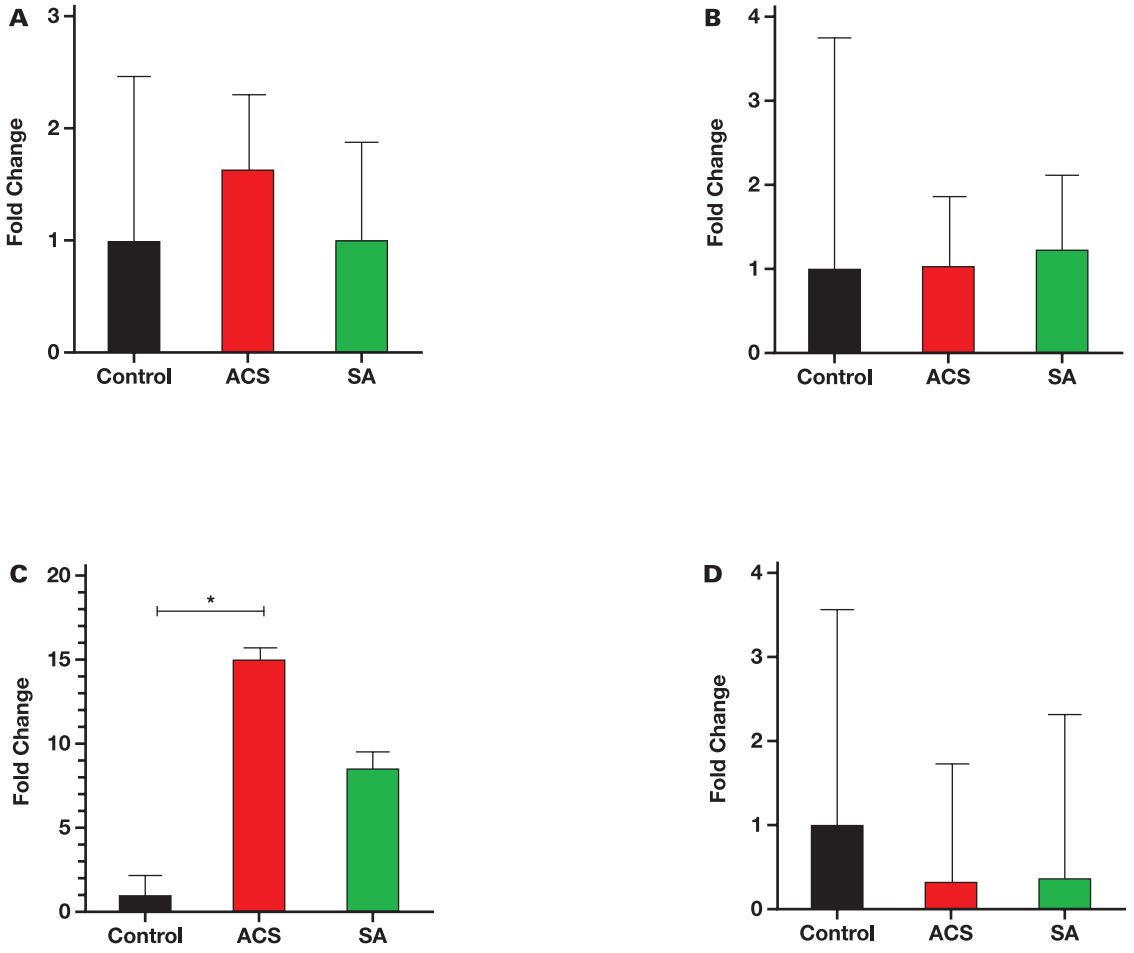


FIGURE 2. Fold change platelet miR-223-5p, miR-126-5p, miR-484 and miR-130a-3p in ST segment elevation acute myocardial infarction (STEMI), non-STEMI (NSTEMI), unstable angina (UA), and stable angina (SA) subjects. Data are mean ± SEM. *P < .05, **P < .01, ***P < .001.

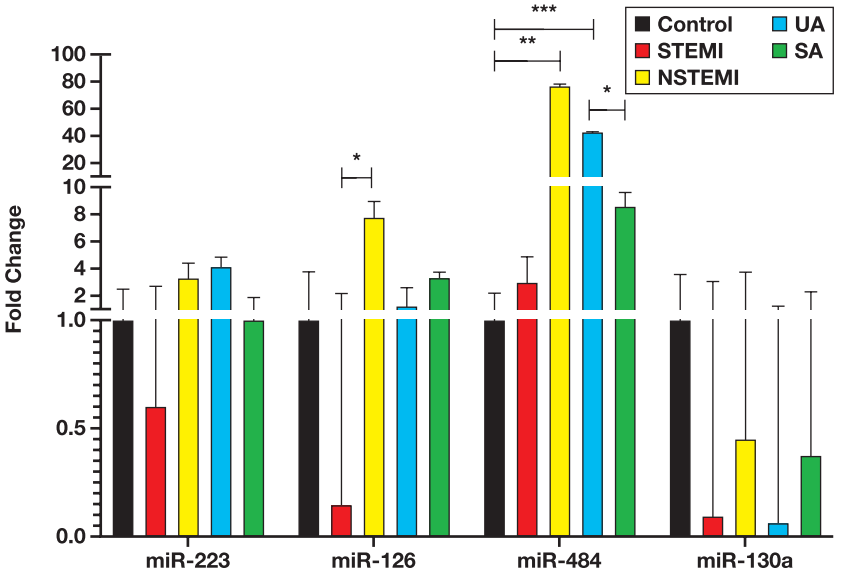
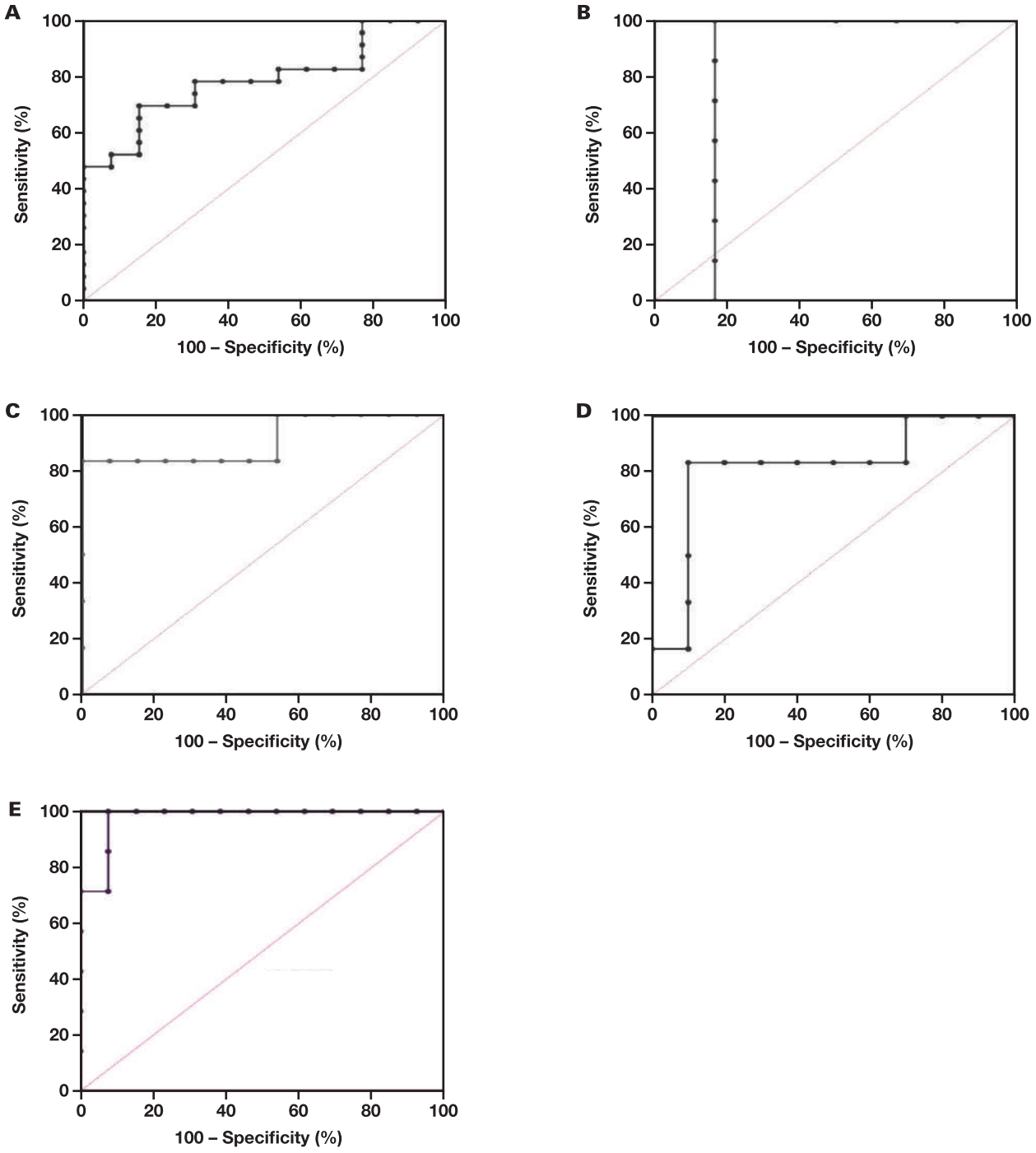


FIGURE 3. **Diagnostic value of platelet miR-484. A, The receiver operating characteristic (ROC) curve miR-484 expression in distinguishing acute coronary syndrome (ACS) from healthy controls. *P* = .0049; area under the curve (AUC) = 0.786; sensitivity = 69.57%; specificity = 84.62%. B, A ROC analysis based on miR-484 expression discriminates between non-ST segment elevation acute myocardial infarction (NSTEMI) and unstable angina (UA). *P* = .0455; AUC = 0.833; sensitivity = 100%; specificity = 83.33%. C, The ROC curve based on the miR-484 expression for separation NSTEMI from healthy subjects. *P* = .005; AUC = 0.910; sensitivity = 83.33%; specificity = 100%. D, A ROC curve corresponding to miR-484 expression levels from STEMI and NSTEMI patients. *P* = .0393; AUC = 0.816; sensitivity = 83.33%; specificity = 90%. E, A ROC curve miR-484 expression for screening UA healthy from controls. *P* = .006; AUC = 0.978; sensitivity = 100%; specificity = 92.31%.**



Expression of Platelet miRNAs

Platelet miRNAs expression levels in subjects with ACS were compared with controls (**FIGURE 1**). The results showed that expression of miR-484 upregulated in ACS patients in comparison with controls (*P* = .0097) whereas there was no statistical difference between subjects with SA vs controls (*P* = .0571) and those with ACS vs SA subjects

(P = .5112). In addition, the data show that miR-223-5p, miR-126-5p, and miR-130a-3p expression in subjects with ACS was not significantly different compared with controls.

We further assessed the expression of platelet miRNAs in ACS subtypes (STEMI, NSTEMI, and UA) and subjects with SA in comparison with healthy volunteers. The results revealed that miR-126-5p was higher in those with NSTEMI than those with STEMI (≈53-fold; P = .0272) whereas no statistical differences were observed in other groups. As shown in **FIGURE 2**, miR-484 levels increased in NSTEMI vs healthy individuals (≈77-fold; P = .0023) and UA vs controls (≈43-fold; P = .0006). Overexpression of miR-484 in UA vs SA was found (≈5-fold; P = .0425). In addition, the data demonstrated that miR-223-5p and miR-130a-3p expression levels were not statistically significant between ACS subtypes in comparison to each other or the control group.

Diagnostic Value of Platelet miR-484

The ROC was applied to investigate the diagnostic value of platelet miR-484 (**FIGURE 3A**), which found the AUC of miR-484 was 0.786, suggesting that platelet miR-484 has a diagnostic potential in distinguishing ACS subjects from healthy subjects. As shown in **FIGURE 3B**, the AUC of miR-484 was 0.833, with a sensitivity of 100% and specificity of 83.33%, indicating that miR-484 has good diagnostic value for screening NSTEMI from UA. **FIGURE 3C** shows the AUC of miR-484 was 0.910, suggesting that platelet miR-484 has some diagnostic value for the screening of NSTEMI.

The miR-484 expression showed an AUC of 0.816 with a sensitivity of 83.33% and a specificity of 90% in distinguishing NSTEMI from STEMI (**FIGURE 3D**). In addition, the AUC of miR-484 was 0.978, which may be a diagnostic biomarker useful to distinguish UA from healthy persons (**FIGURE 3E**)

Discussion

ACS is the most common cause of death in the world.[13] This syndrome is subdivided into UA, NSTEMI, and STEMI.[14,15] Although several guidelines have been established for the proper diagnosis of the disease, the number of individuals who succumb to the disease annually increases at an astonishing pace.[14] Moreover, as the mortality rate of ACS is 7 times higher than other severe CAD (SCAD),[16] and due to the low validity of cardiac markers at the primary stages of the disease,[17] the rapid, easy, and accessible diagnosis of ACS is of particular importance for physicians. In this study, we investigated the expression of platelet miR-223-5p, miR-126-5p, miR-484, and miR-130a-3p in PRP of subjects with ACS and SA against those of healthy controls and identified miRs that are differentially regulated in those with ACS. Our results indicate that miR-484 expression was significantly upregulated in ACS, NSTEMI, and UA subjects when compared with healthy controls, and the expression of miR-484 was upregulated in subjects with UA in comparison with subjects with SA. In addition, miR-126-5p expression was downregulated in subjects with STEMI in comparison to subjects with NSTEMI. The differences were not significant in miR-223-5p, miR-130a-3p, and miR-126 for ACS vs control, SA vs control, or ACS vs SA, which may be due to the relatively small sample size in our study. Wang et al[18] found that miR-484 was higher in CAD patients than in healthy individuals. In line with our results, Su et al[19] also found a significant increase of circulating miR-484 in subjects with acute heart failure compared with controls. Moreover, previous studies show that miR-126 expression was profoundly elevated in ACS compared with control[20] and SCAD.[3] Additionally, the aberrant expression of miR-484 was reported in different diseases. For example, Zare et al[21] indicated a significant downregulation of miR-484 in patients with gastric cancer. Moreover, Hu et al[22] documented that downregulation of miR-484 contributes to cervical cancer metastasis. It has also been claimed that miR-484 could also increase the risk of apoptosis in myocardia cells via targeting with Yap1 anti-apoptotic protein.[23] Moreover, it has also been claimed that miR-484 is a reason why doxorubicin could induce cardiomyopathy.[24,25] In the our study, we found that the expression of miR-484 elevated in the platelet of ACS patients suggested that their transport to the cardiomyocytes could induce apoptotic cell death and thereby result in MI. In contrast, Wang et al[26] have shown that circulating miR-126 was lower in subjects with CAD than in healthy individuals. Importantly, a positive correlation between platelet miR-223-5p and hematocrit was found in ACS patients, since hematocrit is related to the incidence of MI; therefore, miR-223-5p might be considered in the development of ACS. Moreover, a positive correlation was found between platelet miR-130a-3p and miR-223-5p, miR-126-5p, and miR-484. Several studies document that microRNAs could be a diagnostic biomarker in different diseases, such as miR-143-3p, miR-195-5p, miR-20b-5p, miR-204-5p, miR-423-3p, and miR-484 in endometrial cancer[27]; miR-125b in Alzheimer disease[28]; miR-155 and miR-21 in asthma[29]; miR-34a-5p in Parkinson disease[30]; miR-16-5p and miR-21-5p in inflammatory bowel disease[31]; miR-101-3p, miR-195-5p, and miR-223-3p in syphilis[32]; miR-3135b and miR-107 in severe hypertension[33]; and miR-21, miR-23a, and miR-27a in colorectal cancer.[34] Interestingly, ROC analysis showed that miR-484 has a potential diagnostic value for distinguishing subjects with ACS and healthy controls, subjects with UA and healthy controls, subjects with NSTEMI and healthy individuals, and subjects with STEMI and those with NSTEMI, NSTEMI, and UA. Many studies have also reported the diagnostic value of miR-484 in various cancers such as endometrial cancer, lung adenocarcinoma, and colorectal cancer. However, more studies with a large sample size are required.[27,34]

Conclusion

Our findings show that miR-484 could be helpful as a diagnostic biomarker for distinguishing ACS, UA, and NSTEMI from healthy individuals and differentiating NSEMI from UA and STEMI from NSTEMI. Further investigations are needed to shed light on the role of platelet miR-484 in the pathogenesis of ACS.

Conflict of Interest Disclosure

The authors have nothing to disclose.

Acknowledgment

The authors thank the Deputy Researcher of Shahid Beheshti University of Medical Sciences.

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Circulating Levels of C1q/TNF-Related Protein 3 (CTRP3) and CTRP9 in Gestational Diabetes and Their Association with Insulin Resistance and Inflammatory Cytokines

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Keywords: adipokine, CTRP3, CTRP9, inflammation, insulin resistance, gestational diabetes

Abbreviations: GDM, gestational diabetes mellitus; CTRP3, C1q/TNF-related protein 3; TNF, tumor necrosis factor; IL, interleukin; HOMA-IR, homeostasis model assessment of insulin resistance; BMI, body mass index; CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ELISA, enzyme-linked immunosorbent assay; CV, coefficient of variation; CI, confidence interval; AMPK, 5' adenosine monophosphate-activated protein kinase; AMPK, 5'-adenosine monophosphate-activated protein kinase

Laboratory Medicine 2023;54:262–269; https://doi.org/10.1093/labmed/lmac096

ABSTRACT

Objective: Gestational diabetes mellitus (GDM) is closely related to obesity, adipose tissue, and adipokines. Adiponectin-homologous adipokines with anti-inflammatory properties, including C1q/TNF-related protein 3 (CTRP3) and CTRP9, regulate glucose and lipid metabolism, which was measured in pregnant women with GDM with the aim to assess their circulating levels and their relation with inflammatory cytokines and other biochemical data.

Methods: Serum levels of CTRP3, CTRP9, adiponectin, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 were measured in 43 subjects with GDM and 42 healthy controls by enzyme-linked immunosorbent assay.

Results: Serum levels of adiponectin and CTRP3 were lower in GDM subjects than in controls, whereas CTRP9, TNF-α, and IL-6 showed higher concentrations in subjects with GDM than in controls. In the subjects with GDM, there was a significant association of CTRP3 with homeostasis model assessment of insulin resistance (HOMA-IR), body mass index, and triglycerides, whereas CTRP9 is associated with TNF-α and HOMA-IR.

Conclusion: The differences in the assessed levels of CTRP3 and CTRP9 suggest a possible relation with the pathogenesis of GDM, in particular insulin resistance, which showed significant association with both adipokines.

Gestational diabetes mellitus (GDM) is a metabolic disease that exerts a serious threat to maternal and neonatal health during pregnancy and might lead to preterm delivery or even death.[1] According to studies,[1,2] women with GDM can have serious consequences during the perinatal period, such as premature delivery, polyhydramnios, stillbirth, fetal macrosomia, pulmonary immaturity, and neonatal jaundice. Glucose levels of some women with GDM return to the normal range; however, they are also reported to have a 17% to 63% chance of getting type 2 diabetes within 5 to 16 years after childbirth.

Insulin resistance (IR) as the main underlying mechanism for GDM is caused by elevation in some hormone levels, including estrogen, human placental lactogen, growth hormone, and cortisol, in women during pregnancy.[1,3] Furthermore, it has been proposed that obesity and adipokines could have a substantial role in insulin resistance. In line with this concept, recent reports show that some adipokines affect β cell function in pregnant women.[3]

Two newly discovered adiponectin-homologous adipokines, C1q/TNF-related protein 9 (CTRP9) and CTRP3 (CORS26 or cartonectin), with anti-inflammatory properties are considered to regulate glucose and lipid metabolism.[4–7] Moreover, CTRP3 shows a property that suppresses the inflammatory responses caused by lipopolysaccharide, Toll-like protein 4, and fatty acids, and ameliorates the release of adiponectin and resistin from mouse adipocytes.[8] The results of an animal study showed that CTRP3 inhibits the hepatic output of glucose and decreases plasma glucose levels.[5] Furthermore, human studies have illustrated the relation of CTRP3 with diseases such as coronary artery disease (CAD), diabetes mellitus, and nonalcoholic fatty liver disease.[9,10] It has been shown that CTRP9 with a 45% shared sequence with adiponectin plays a key role in glucose metabolism.[4] The CTRP9 administration to mice remarkably reduced plasma glucose and insulin concentration.[11] Moreover, clinical studies have revealed the association of CTRP9 with the pathogenesis of several diseases, such as diabetes mellitus and CAD.[12]

There are limited studies and data supporting alteration in CTRP3 in GDM,[13] and no study has specifically assessed the association of CTRP9 circulating levels with GDM. This study sought to assess circulating levels of CTRP3 and CTRP9 in subjects with GDM and their relation with inflammatory cytokines and biochemical data.

TABLE 1. Comparison of Demographic Characteristics of the Studied Group

Variables	Control (n = 42)	GDM (n = 43)	*P* Value
Age, y	58.62 ± 7.97	58.44 ± 8.51	.921
BMI, kg/m^2	26.09 ± 3.55	26.74 ± 3.8	.421
SBP, mm Hg	136.02 ± 18.62	137.44 ± 16.69	.713
DBP, mm Hg	84.17 ± 12.45	85.60 ± 13.02	.604
FBS, mg/dL	92.04 ± 6.12	160.95 ± 20.75	<.001
Insulin, uU/mL	6.14 ± 4.13	11.39 ± 5.43	<.001
HOMA-IR	1.41 ± 0.97	4.61 ± 2.42	<.001
TG, mg/dL	130.7 ± 41.9	156.50 ± 57.69	.021
TC, mg/dL	191.48 ± 41.07	186.58 ± 47.11	.610
LDL-C, mg/dL	114.31 ± 31.5	114.72 ± 34.58	.954
HDL-C, mg/dL	43.84 ± 8.62	40.87 ± 5.72	.066

BMI, body mass index; DBP, diastolic blood pressure; FBS, fasting blood sugar; GDM, gestational diabetes mellitus; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

Materials and Methods

Study Population

This case-control study included 43 subjects with GDM and 42 healthy controls. Diagnosis of GDM was made according to American Diabetes Association criteria.[14] Persons with liver and kidney diseases, cancer,

FIGURE 1. Comparison of adiponectin (A), CTRP3 (B), CTRP9 (C), TNF-α (D), and IL-6 (E) levels between controls and subjects with GDM. **P* < .01. CTRP, C1q/TNF-related protein; GDM, gestational diabetes mellitus; IL, interleukin; TNF, tumor necrosis factor.

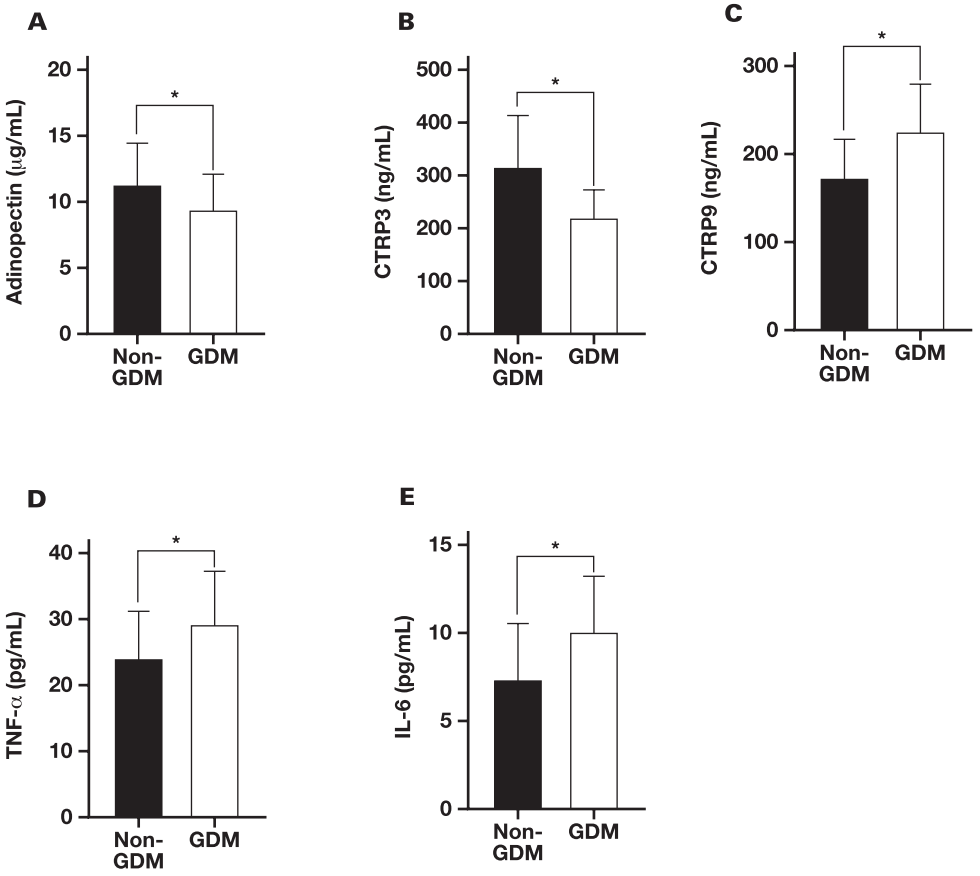


TABLE 2. Odds Ratios of Disease Status According to Serum Levels of CTRP3 and CTRP9

Models		B	Standard Error	Wald	Odds Ratio (B)	95% CI for Odds Ratio (B)		*P* Value
						Lower	Upper	
CTRP3	Crude	–0.018	0.005	15.988	0.982	0.973	0.991	<.001
	Adjusted[a]	–0.020	0.005	16.277	0.980	0.970	0.990	<.001
CTRP9	Crude	0.021	0.005	15.889	1.021	1.011	1.032	<.001
	Adjusted[a]	0.022	.006	15.872	1.022	1.011	1.034	<.001

[a]*Adjustment was performed for age and body mass index.*

TABLE 3. Pearson Correlation of CTRP3 and CTRP9 with Anthropometric and Biochemical Data in Control and Patient Groups

	CTRP3		CTRP9	
	Controls	Subjects with GDM	Controls	Subjects with GDM
CTRP9	–0.262	–0.031	1	1
Age	0.202	–0.204	0.056	–0.109
BMI	–0.447[a]	–0.319[b]	0.391[b]	0.289
SBP	0.019	0.070	0.048	–0.110
DBP	–0.119	–0.095	–0.143	–0.104
HOMA-IR	0.065	–0.421[a]	–0.064	0.396[a]
FBS	–0.118	–0.150	–0.005	0.374[b]
Insulin	0.075	–0.411[a]	–0.056	0.338[b]
TG	–0.002	0.332[b]	0.237	0.275
TC	–0.012	0.182	–0.074	0.078
LDL-C	0.056	0.202	–0.073	0.121
HDL-C	–0.026	–0.119	–0.181	–0.071
Adiponectin	0.436[a]	0.094	–0.368[b]	–0.202
TNF-α	0.060	0.256	0.227	0.414[a]
IL-6	–0.223	0.085	0.321[b]	0.336[b]

BMI, body mass index; CTRP, C1q/TNF-related protein; DBP, diastolic blood pressure; FBS, fasting blood sugar; GDM, gestational diabetes mellitus; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; IL, interleukin; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; TNF, tumor necrosis factor.
[a]P < *.01.*
[b]P < *05.*

and inflammatory diseases were excluded from the study. Informed written consent was provided by all study participants and the study was conducted under the Declaration of Helsinki and approved by the Ethics Committee of Iran University of Medical Sciences (code: IR.IUMS.RETECH.REC.1398.527).

The body mass index (BMI) was calculated by the standard equation, weight (kg)/height (m^2). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) values were estimated by a standard sphygmomanometer.

Sampling

Five mL of venous blood was collected from each participant after overnight fasting. Concentrations of fasting blood sugar (FBS), and lipid profile including triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), were measured by the commercially available kits (Pars Azmoon). Fasting insulin was determined by an enzyme-linked immunosorbent assay (ELISA) kit (Monobind). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by a standard equation: insulin uIU/mL × glucose (mg/dL)/405.

Measuring Serum Levels of Adipokines and Cytokines

Serum levels of adiponectin were measured using an ELISA kit (Adipogen) with intra- and interassay coefficients of variation (CVs) of 3.4% and 4.3%. The CTRP9 levels were measured using an ELISA kit (USCN Life Science) with an intra-assay CV of 4.4% and an interassay CV of 4.5%. The serum concentration of CTRP3 using intra-assay was 7.3% and with interassay was 5.8%. The CVs were investigated using the ELISA method (Adipogen). Serum levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were determined by ELISA kits (R & D Systems). The minimum detectable range for IL-6 and TNF-α was 0.11 pg/mL and 0.5 pg/mL, respectively. Inter- and intra-assay CVs for IL-6 and TNF-α were <10%.

Statistical Analysis

Categorical data were evaluated by the χ^2 test and were presented via frequency and percentage. Categorical data was shown by the mean and standard deviation (SD) and analyzed by Student's *t*-test. Spearman correlation test was used for correlation analysis, and variables that had a significant correlation with the target variable were included in multiple linear regression. All analysis was performed using SPSS version 16, and a *P* value less than .05 was considered a significant threshold.

Results

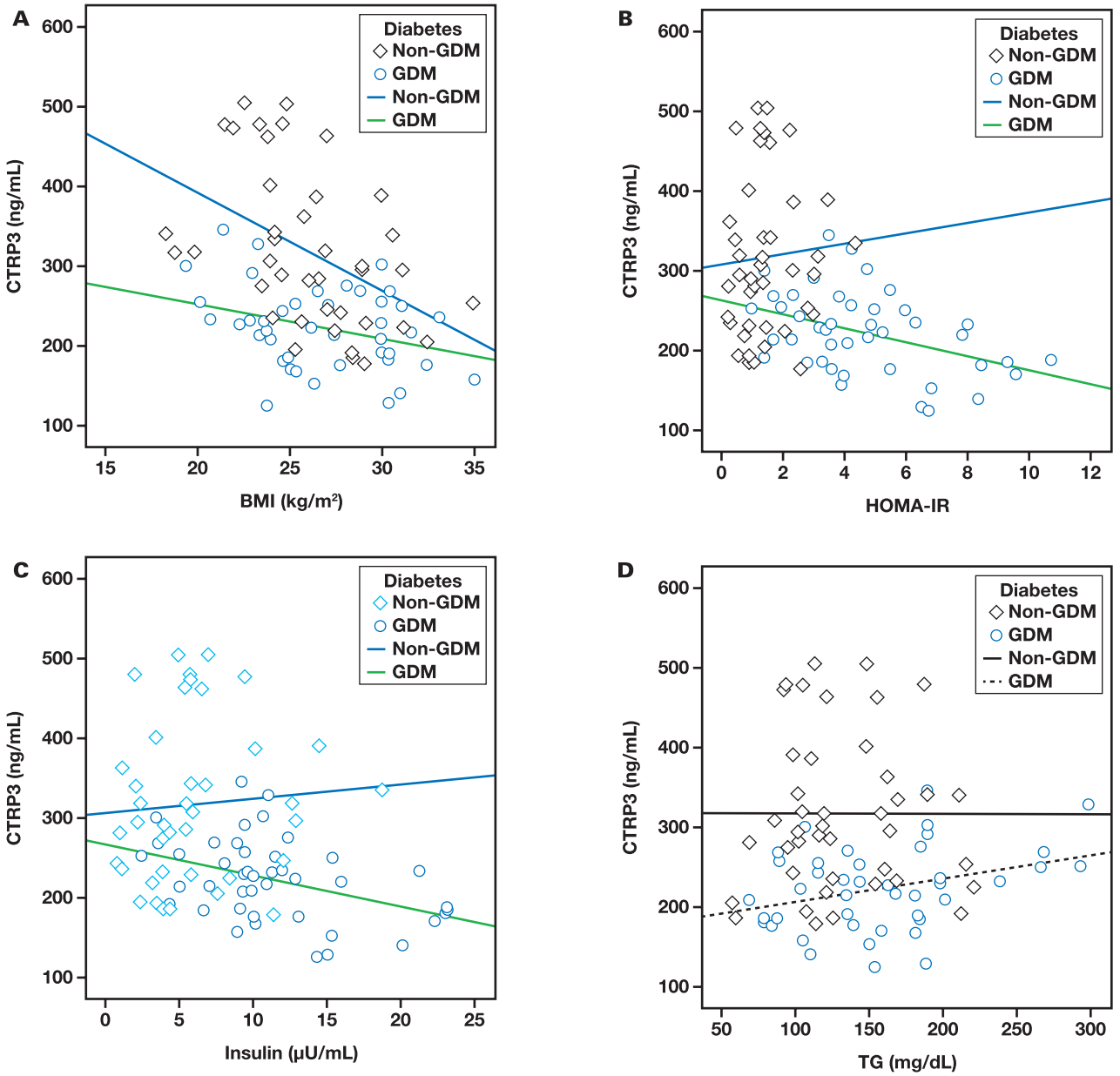
Anthropometric and Laboratory Data

Details of demographic characteristics studied are shown in **TABLE 1**. As shown, there was no significant difference between the GDM group and controls in terms of age, BMI, SBP, and DBP. As expected, FBS, insulin, and HOMA-IR were elevated in the GDM group in comparison to the control group. The TG levels increased in the GDM group whereas TC, LDL-C, and HDL-C indicated no significant change between the groups.

Serum Levels of Adipokines and Cytokines

Serum levels of adiponectin in subjects with GDM (9.39 ± 2.51 μg/mL) were significantly lower than controls (11.17 ± 3.12 μg/mL), *P* = .005) (**FIGURE 1A**). Serum levels of CTRP3 were found to be lower in subjects with GDM (222.3 ± 51.31 ng/mL) than controls (317.11 ± 98.19 ng/mL, *P* < .001) (**FIGURE 1B**), whereas CTRP9

FIGURE 2. Pearson analysis of the correlation between CTRP3 levels and variables of BMI (A), HOMA-IR (B), insulin (C), TG (D), and adiponectin levels.



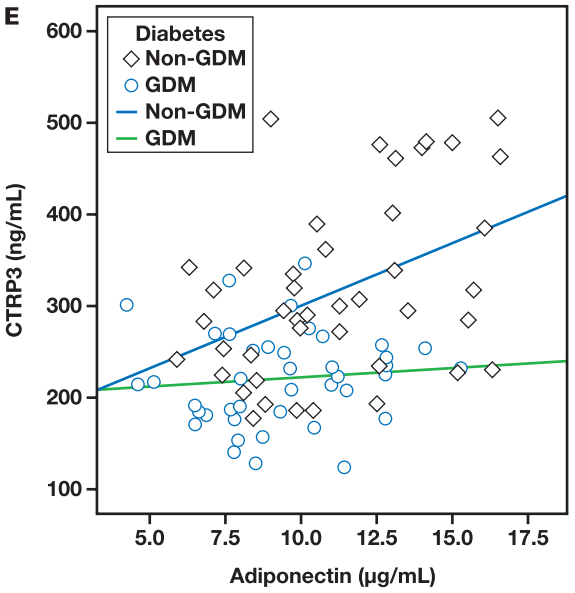
levels of the GDM group (225.77 ± 53.82 ng/mL) were higher than controls (173.36 ± 43.16 ng/mL, $P < .001$) (**FIGURE 1C**). In addition, TNF-α (29.34 ± 7.78 vs 24.23 ± 7.04 pg/mL, $P = .002$) and IL-6 (10.07 ± 3.17 vs 7.34 ± 3.23 pg/mL, $P < .001$) levels of subjects with GDM were significantly higher than controls (**FIGURE 1D** and **1E**, respectively).

The possible impact of covariates (age and BMI) was adjusted on the serum levels of CTRP3 and CTRP9 using analysis of covariance. The results show that the difference between the groups in terms of CTRP3 and CTRP9 remained significant. Also, the relation of CTRP3 and CTRP9 with the risk of GDM was determined using binary logistic regression; the results are given in **TABLE 2**. As shown, when the association of CTRP3 and CTRP9 with the risk of GDM was adjusted for age and BMI, the results remained significant.

Association of CTRP3 and CTRP9 with Other Variables

Pearson correlation was performed to determine the correlation of CTRP3 and CTRP9 with other variables in controls and subjects with GDM. The results of the correlation analysis are given in **TABLE 3** and the significant correlation of CTRP3 and CTRP9 with other variables are illustrated in **FIGURES 2** and **3**, respectively. In the control group, CTRP3 was found to have a significant inverse correlation with BMI and a positive correlation with adiponectin; multiple linear regression indicated the independent association of CTRP3

FIGURE 2. (cont) (E) in GDM patients and controls. BMI, body mass index; CTRP, C1q/TNF-related protein; GDM, gestational diabetes mellitus; HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglycerides.



with BMI (B [95% confidence interval (CI)]: −11.26 [−18.49, −4.04], P = .003) and adiponectin (B [95% CI]: 12.43 [4.21, 20.65], P = .004). The CTRP9 had a positive correlation with BMI and IL-6 and an inverse correlation with adiponectin. Multiple linear regression indicated an independent association of CTRP9 with BMI (B [95% CI]: 4.45 [1.11, 7.79], P = .010).

In the GDM group, CTRP3 had an inverse correlation with BMI, insulin, HOMA-IR, and TG. Moreover, multiple linear regression indicated an independent association of CTRP3 with BMI (B [95% CI]: −3.93 [−7.49, −0.36], P = .032), HOMA-IR (B [95% CI]: −7.66 [−13.253, −2.062], P = .009) and TG (B [95% CI]: 0.297 [0.065, 0.530], P = .014). Furthermore, there were significant positive correlations of CTRP9 with FBS, insulin, HOMA-IR, IL-6, and TNF-α. It was shown that CTRP9 independently associated with HOMA-IR (B [95% CI]: 11.22 [−17.32, −5.17], P = .001) and TNF-α (B [95% CI]: 1.89 [0.064, 3.71], P = .043).

Discussion

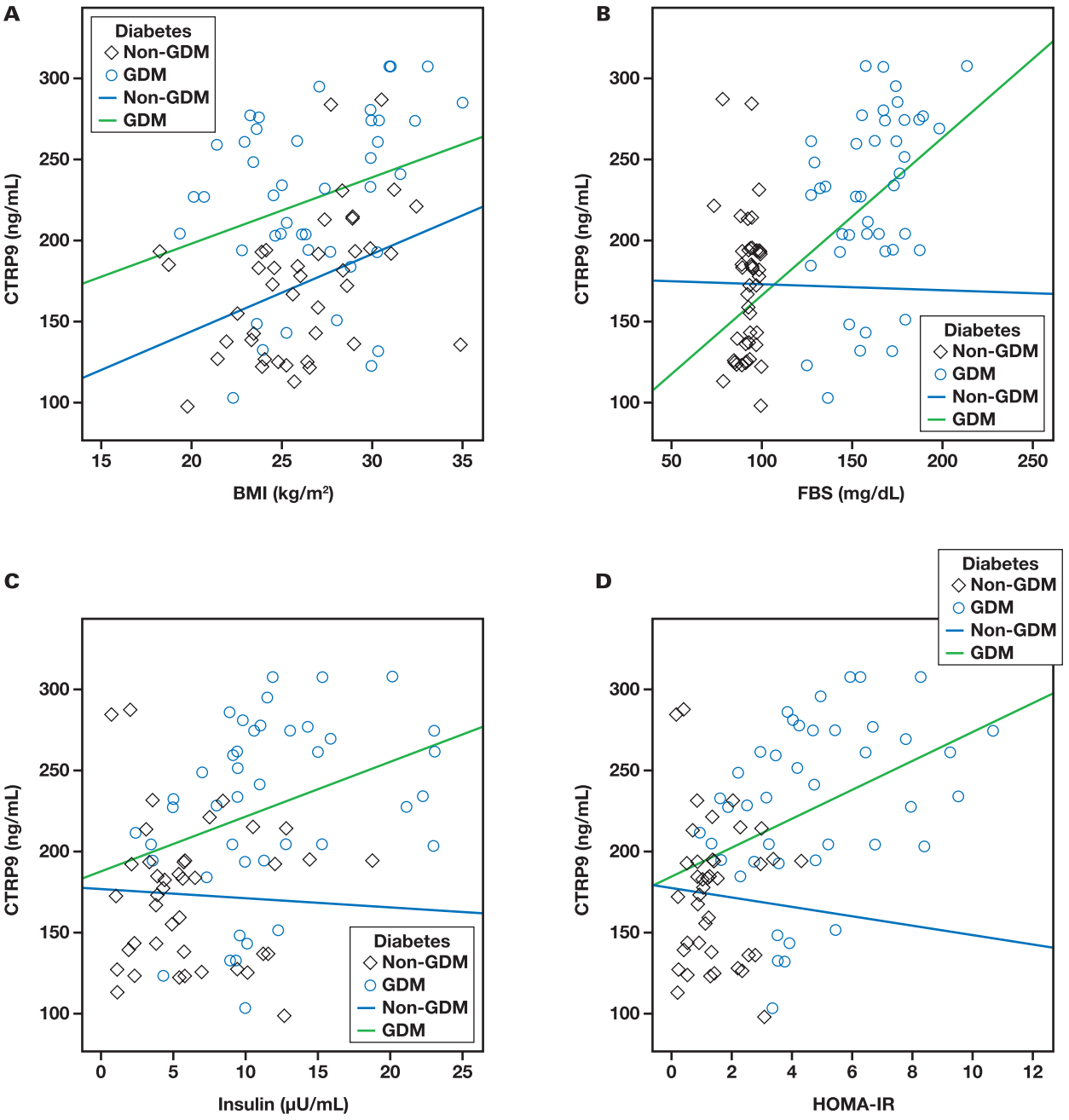
Gestational diabetes is the most common metabolic disease during pregnancy that could lead to serious clinical maternal and neonatal outcomes that threaten the health of mother and child.[1,3] Our previous studies identified a remarkable perturbation in CTRP family levels in diabetes and CAD.[10,12,15] This study is the first study that has evaluated CTRP3 and CTRP9 levels simultaneously in pregnant women with gestational diabetes. The main findings of the study were a significant increase in CTRP9 levels in subjects with GDM compared with controls and decreased CTRP3 levels in subjects with GDM.

In agreement with our results, a previous study by Li et al[13] showed that CTRP3 level in fasting serum in subjects with GDM was lower than in controls. Decreased CTRP3 levels in GDM subjects was suggested to have a metabolic role in GDM pathogenesis.[5] In addition, studies in subjects with type 2 diabetes showed lower CTRP3 than controls.[10,15] Previous studies reported the impact of CTRP3 on insulin sensitivity and glucose metabolism.[5] In line with this concept, in our study CTRP3 indicated an inverse relation with the insulin resistance index. Moreover, clinical studies confirmed the relation of CTRP3 with parameters of insulin and glucose metabolism.[3,10,16] In vitro studies have dissected the mechanism of the impact of CTRP3 on these pathways.[5,17] Peterson et al[11] demonstrated that CTRP3 inhibits glucose release from the liver. Moreover, it has been found that CTRP3 increases the rate of 5'-adenosine monophosphate-activated protein kinase (AMPK) phosphorylation that improves insulin sensitivity.[6,18] Our study also found an inverse association between CTRP3 and TG levels. Peterson et al[17] reported TG synthesis gene reduction in mice after administration of CTRP3. Collectively, it seems likely that CTRP3 reduction in persons with GDM might be a factor related to metabolic complications, especially insulin resistance and hypertriglyceridemia.

To our knowledge, there are no data on circulating levels of CTRP9 in people with GDM; however, there are studies that measured this adipokine in subjects with type 2 diabetes.[12,19] In line with the results of our study, several studies have reported a higher concentration of CTRP9 in subjects with T2DM than controls.[12,19] CTRP9 also has a favorable effect on glucose and insulin metabolism. Experimental studies have shown that CTRP9 improves insulin sensitivity.[11] CTRP9 overexpression in mice resulted in a significant decline in fasting glucose and insulin. On the other hand, CTRP9 deletion leads to higher insulin resistance.[20] Moreover, it has been reported that CTRP9 increases the activities of protein kinase B, AMPK, and p42/44 mitogen-activated protein kinase promoting glucose uptake.[20]

There was a positive association between CTRP9 and TNF-α, which suggests a possible relation between these adipokines and inflammation. Previous studies have reported similar results in subjects with diabetes mellitus and CAD.[12] In addition, CTRP9 was found to ameliorate inflammation in macrophages and increase plaque stability.[21] These results suggest that increased levels of CTRP9 might be a compensatory response to insulin resistance, and

FIGURE 3. Pearson analysis of the correlation between CTRP9 levels and BMI (A), FBS (B), insulin (C), HOMA-IR (D), TNF-α (E), and IL6 (F) levels in GDM patients and controls. BMI, body mass index; CTRP, C1q/TNF-related protein; FBS, fasting blood sugar; GDM, gestational diabetes mellitus; HOMA-IR, homeostasis model assessment of insulin resistance; IL, interleukin; TNF, tumor necrosis factor.



an inflammatory milieu in subjects with GDM; as in the present study, CTRP9 had an independent association with HOMA-IR and TNF-α.

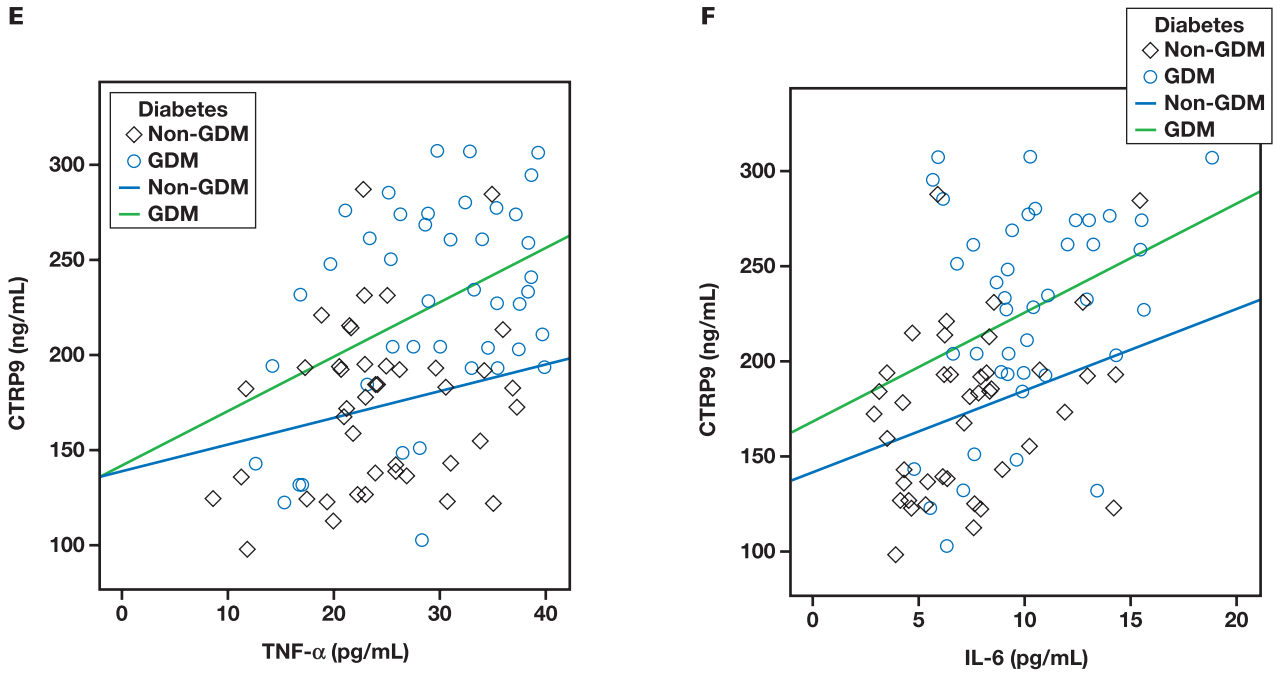
This study has several limitations. First, it was based on a cross-sectional analysis, and thus it was not possible to determine causal relationships. Second, the sample size was might be too small to draw a definite conclusion.

In conclusion, this study showed an independent association of CTRP3 and CTRP9 with GDM. The CTRP3 showed a relation with the pathological mechanisms of GDM (eg, insulin resistance, obesity, and high TG levels) and CTRP9 was associated with inflammatory marker and insulin resistance. These findings suggested a relation of these adipokines with the pathogenesis of GDM.

Acknowledgments

The authors are thankful to Iran University of Medical Sciences for financial support (grant No. 90527).

FIGURE 3 (cont)



Conflict of Interest Disclosure

The authors have nothing to disclose.

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Evaluation of Serum FGL1 as Diagnostic Markers for HBV-Related Hepatocellular Carcinoma

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Keywords: hepatocellular carcinoma, fibrinogen-like protein 1, alpha-fetoprotein, hepatitis B virus, diagnosis, molecular diagnostics

Abbreviations: HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; HBV-HCC, HBV-related hepatocellular carcinoma; FGL1, fibrinogen-like protein 1; EMT, epithelial to mesenchymal transition; LC, liver cirrhosis; CHBV, chronic HBV; HC, healthy control; GGT, gamma-glutamine transferase; ALB, albumin; TBIL, total bilirubin; hs-CRP, hypersensitive C-reactive protein; DBIL, direct bilirubin; FIB, fibrinogen; AUROC, area under the ROC curve; +LR, positive likelihood ratio; −LR, negative likelihood ratio; NA, nonapplicable

Laboratory Medicine 2023;54:270–281; https://doi.org/10.1093/labmed/lmac094

ABSTRACT

Objective: Based on the current difficulties in early diagnosis of HBV-related hepatocellular carcinoma (HBV-HCC), we assessed the values of preoperative serum fibrinogen-like protein 1 (FGL1) by itself and in combination with alpha-fetoprotein (AFP) for the diagnosis of HBV-HCC.

Methods: We used ELISA and chemiluminescence assays to detect the serum levels of FGL1 and AFP, respectively.

Results: Serum FGL1 level in the HBV-HCC group was significantly higher than in the chronic HBV (CHBV) group, the liver cirrhosis (LC) group, and the healthy control (HC) group. Serum FGL1 had an outstanding performance in distinguishing AFP-negative HBV-HCC from different control conditions. In the patients with AFP-negative HBV-HCC, the sensitivity of serum FGL1 was high. Moreover, serum FGL1 had a stronger performance than AFP in distinguishing early-stage HBV-HCC.

Conclusions: Serum FGL1 is significantly elevated among patients with HBV-HCC, including those with negative AFP and with disease at an early stage. Hence, serum FGL1 may serve as a potential diagnostic marker in the early diagnosis of HBV-HCC.

Hepatocellular carcinoma (HCC) was the sixth most common cancer and the third leading cause of cancer death in 2020, with approximately 906,000 new cases and 830,000 deaths worldwide.[1] HBV accounts for half of all liver cancer deaths, especially in China.[2,3] One of the reasons for the high mortality in HCC was that most of the patients with HCC were diagnosed at an advanced disease stage, when curative resection was no longer feasible because of intrahepatic and extrahepatic metastases. Consequently, early detection and diagnosis of HCC still present the best chance for successful treatments and improving the 5-year survival rate of patients.[4]

Alpha-fetoprotein (AFP) has been widely used as a serologic diagnostic tumor marker in the laboratory for HBV-related hepatocellular carcinoma (HBV-HCC). However, the sensitivity was unsatisfactory at the cutoff of 20 ng/mL, especially in the detection of early-stage HCC.[5] Besides, the results of a large nationwide study[6] recently demonstrated a downtrend of AFP levels at HCC diagnosis, with the strongest trend seen in early-stage HCC. These findings highlight the urgent need for novel surveillance strategies, such as novel biomarkers and biomarker panels, that can detect HBV-HCC at an early stage and/or with negative AFP results.

Fibrinogen-like protein 1 (FGL1), a 68-kDa protein comprised of a disulfide bond-linked homodimer, was secreted mainly by hepatocytes in the liver and involved in hepatocyte mitosis and liver energy utilization.[7–10] In a previous circulating mRNA transcriptome study report,[11] FGL1 mRNA was identified as a potential novel biomarker for cancer detection. Also, substantial evidence[12–18] has indicated that FGL1 played a significant role in response to injury stimulation, tumor epithelial to mesenchymal transition (EMT), tumor proliferation, apoptosis, and radiation, as well as drug sensitivity in many types of solid tumors, including HCC. In this study, we focused on the diagnostic value of serum FGL1 for HBV-HCC, particularly for patients with negative AFP results or at the early disease stage, which, to our knowledge, has not yet been investigated comprehensively.

TABLE 1. Clinical Characteristics for the Study Population

Characteristics	Group				*P* Value
	HC (n = 68)	CHBV (n = 63)	LC (n = 61)	HBV-HCC (n = 142)	
Sex (male/female)	44/24	45/18	43/18	112/30	>.05
Age, y, mean (SD)	51.49 (11.58)	52.49 (9.55)	55.08 (11.79)	53.08 (12.10)	.13
ALT, U/L (range)	14.00 (19.00–27.00)	82.00 (26.00–277.50)[a]	39.00 (24.00–64.00)[a]	32.00 (24.25–48.75)[a,b]	<.001
AST, U/L (range)	20.00 (17.00–23.00)	63.00 (24.50–166.00)[a]	47.00 (32.00–85.00)[a]	44.50 (28.25–87.50)	
ALP, U/L (range)	66.00 (59.53–76.00)	96.00 (76.10–122.25)[a]	98.60 (74.00–54.00)[a]	112.90 (80.03–195.40)[a]	
GGT, U/L (range)	17.50 (14.00–27.25)	52.00 (23.00–150.00)[a]	50.00 (25.00–100.00)[a]	80.00 (39.25–205.00)[a,c]	
ALB, g/L (range)	43.65 (42.65–4.85)	40.80 (35.75–43.40)[a]	31.90 (27.90–38.10)[a,b]	38.50 (33.78–41.75)[a,c]	
TBIL, μmol/L (range)	12.20 (9.20–15.00)	21.80 (14.95–47.55)[a]	25.90 (16.70–40.90)[a]	19.60 (14.28–32.10)[a]	
DBIL, μmol/L (range)	3.85 (3.18–4.70)	7.40 (4.75–20.90)[a]	12.70 (6.60–43.00)[a]	7.05 (4.63–12.65)[a,c]	
D-dimer, mg/L (range)	0.29 (0.13–0.49)	0.33 (0.20–0.69)	1.24 (0.29–4.61)[a,b]	7.05 (4.63–12.65)[a,b]	
FIB, g/L (range)	2.70 (2.30–3.12)	2.09 (1.86–2.49)[a]	1.85 (1.52–2.14)[a]	2.73 (2.21–3.46)[b,c]	
hs-CRP, mg/dL (range)	2.22 (1.23–3.81)	2.31 (0.50–7.78)	3.95 (0.66–10.96)	4.70 (1.25–15.81)[a,b]	
HBVDNA, IU/mL (range)	NA	4250.00 (126.00–385,500.00)	336.50 (100.00–10,000.00)[b]	100.00 (100.00–779.00)[b]	
FGL1, ng/mL	0.63 (0.33–0.87)	19.67 (9.78–26.73)	16.55 (4.89–24.90)	39.04 (19.43–99.77)	
AFP, ng/mL (range)	2.18 (2.90–4.05)	2.20 (4.27–35.55)	5.20 (2.67–18.04)	30.94 (5.98–1761.72)	
Early-stage cases	NA	NA	NA	26	NA
Middle-stage cases	NA	NA	NA	37	NA
Advanced-stage cases	NA	NA	NA	79	NA

AFP, alpha-fetoprotein; ALB, albumin; CHBV, chronic HBV; DBIL, direct bilirubin; FGL1, fibrinogen-like protein 1; FIB, fibrinogen; GGT, gamma-glutamine transferase; HC, healthy control; HCC, hepatocellular carcinoma; hs-CRP, hypersensitive C-reactive protein; LC, liver cirrhosis; NA, nonapplicable; TBIL, total bilirubin.

[a] $P < .05$, compared with the HC group.

[b] $P < .05$, compared with the CHBV group.

[c] $P < .05$, compared with the LC group.

Materials and Methods

Research Object Selection

All participants were recruited to a test cohort from April 2021 through November 2021 in the Department of Oncology and the Physical Examination Center at Renmin Hospital of Wuhan University (main hospital area), Wuhan, China. Our research was performed according to the guidelines of the Chinese Society of Hepatology, the Chinese Society of Infectious Diseases, and the Chinese Medical Association.[19] A validation cohort comprising patients with HBV-HCC, chronic hepatitis infection, and liver cirrhosis (LC), along with healthy control (HC) individuals, was recruited from Renmin Hospital of Wuhan University (east hospital area), China, from January 2022, through April 2022. The 334 participants were divided into the following groups: HBV-HCC (102 cases [test cohort], 40 cases [validation cohort]), liver cirrhosis LC (41 cases, 20 cases), chronic HBV (CHBV; 43 cases, 20 cases), and healthy control HC (48 cases, 20 cases). All patients with HBV-HCC had their disease confirmed by liver pathological examination, X-ray examination, or MRI examination. Exclusion criteria were as follows: malignant tumors other than primary liver cancer; hepatitis virus infections other than HBV; diseases such as severe diabetes, hyperthyroidism, and cardiovascular disease; and/or pregnancy. All healthy control populations tested negative for HBV, HCV, syphilis, and HIV; also, the results of all biochemical tests were normal.

This study was reviewed and approved by the Medical Ethics Review Committee of Renmin Hospital of Wuhan University. All patients approved and signed a written informed-consent form in accordance with the policies of the Renmin Hospital of Wuhan University Ethics Committee. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Ethics Review Committee of Renmin Hospital, Wuhan University (No. WDRY2021-K112).

Specimen Collection

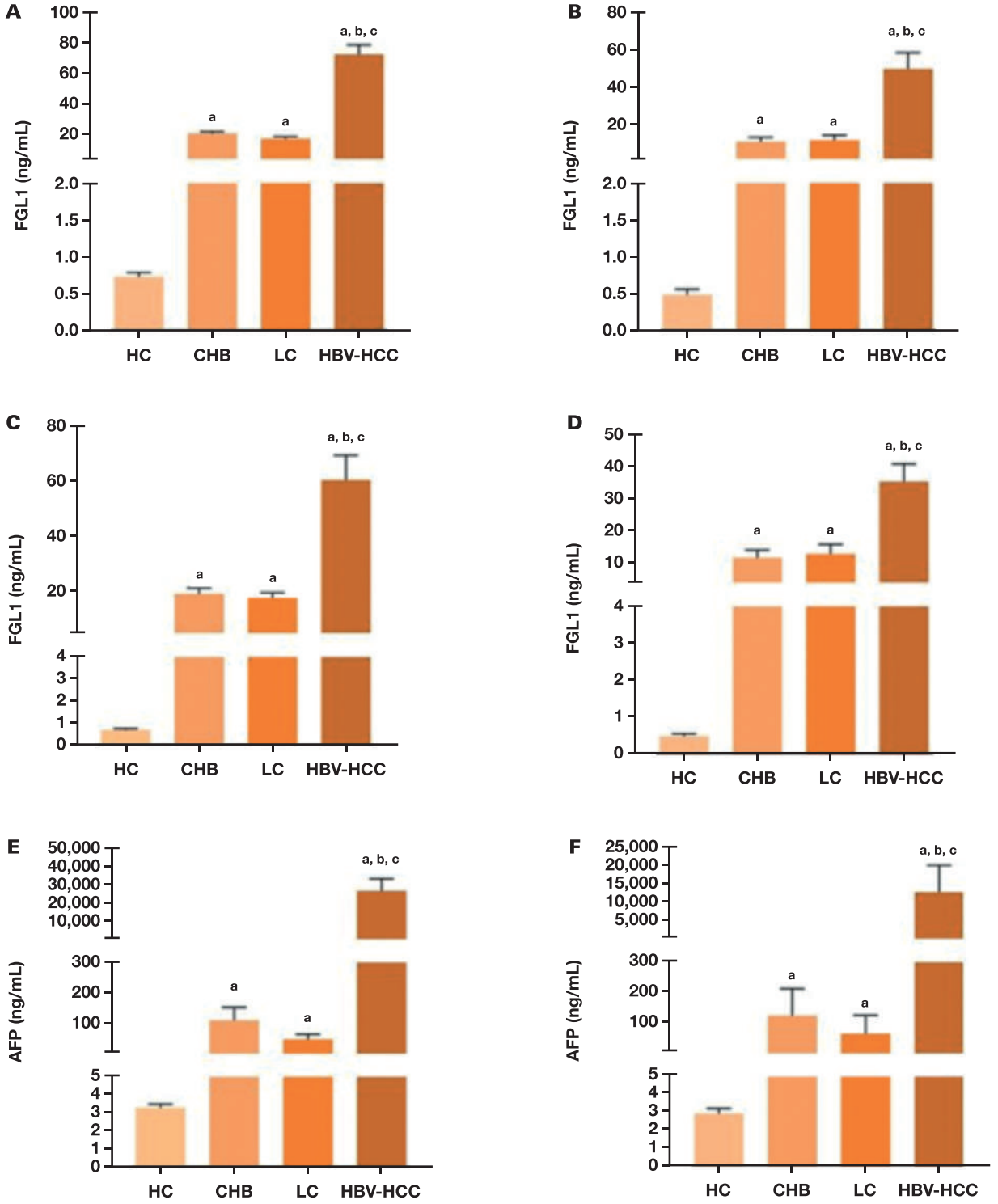
Venous blood was collected in the morning from participants who had fasted for more than 8 hours immediately before collection; it was then centrifuged at 2603*g* for 15 minutes at room temperature. Next, we transferred the serum from the collection tube with separating gel to the cryotube and stored it at −80°C for further use.

Laboratory Analysis

The levels of ALT, AST, ALP, gamma-glutamine transferase (GGT), albumin (ALB), total bilirubin (TBIL), hypersensitive C-reactive protein (hs-CRP), and direct bilirubin (DBIL) were analyzed via the enzymatic method using the fully automatic biochemical analyzer ADVIA 2400 (Siemens). The Sysmex CA-7000 (Sysmex) was used to detect the levels of fibrinogen (FIB) and D-dimer. We used the ABI ViiA7 real-time fluorescent quantitative polymerase chain reaction system (Thermo Fisher Scientific) to measure the HBV DNA level. The concentration of AFP was detected using the Siemens ADVIA Centaur CP (Siemens).

We used a commercial ELISA kit (product number JL47875) produced by the J&I Biological Company to detect FGL1. The kit used a double-antibody 1-step sandwich method for ELISA. The detection range is 0.047 ng/mL–1.500 ng/mL, the specimens were diluted 100 times for testing, and the auxiliary hole was set to ensure the detection accuracy.

FIGURE 1. Serum concentrations of fibrinogen-like protein 1 (FGL1) and alpha-fetoprotein (AFP) and correlation analysis. A, FGL1 for the test cohort. B, FGL1 for the validation cohort. C, FGL1 for the test cohort when testing AFP negative. D, FGL1 for the validation cohort when testing AFP negative. E, AFP for the test cohort. F, AFP for the validation cohort.



Data Analysis

The experimental data were analyzed using SPSS software, version 20.0 (IBM); MedCal 15.2.2 (MedCalc Software); and GraphPad Prism 6.0 (GraphPad Software). The measurement data used the single-sample Kolmogorov-Smirnov method to test whether the data from each group conform to normality; the normal distribution data were represented by $\bar{\chi} \pm s$, the comparison between multiple groups was performed by ANOVA, and the further pairwise comparison was performed by LSD-*t* testing. The nonnormal distribution data were represented by M (P_{25}–P_{75}), the comparison between multiple groups used the Kruskal-Wallis *H* test, the comparison between the 2 groups used the Mann-Whitney *U* test, and the pairwise comparison used the Bonferroni adjustment test

FIGURE 1. (cont) G, Correlation of FGL1 with AFP in the test cohort (r = 0.155; P = .02). H, Correlation of FGL1 with AFP in the validation cohort (r = 0.166; P = .002). [a]Other groups compared with the healthy control (HC) group; P <.001. [b]Other groups compared with the chronic HBV (CHBV) group; P <.001. [c]Other groups compared with the liver cirrhosis (LC) group; P <.001.

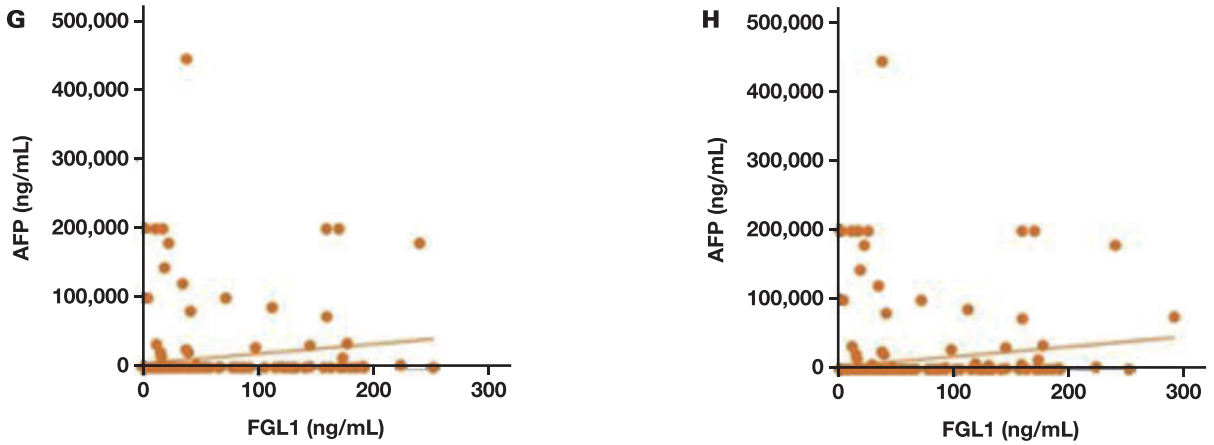


TABLE 2. Correlation Analysis of Serum FGL1 and Hepatic Biochemical Index

Index	r	P Value
ALT	0.291	<.001
AST	0.157	.004
ALP	0.042	.45
GGT	0.057	.30
ALB	−0.051	.35
TBIL	0.053	.34
DBIL	0.057	.30
D-dimer	0.048	.39
FIB	−0.009	.86

ALB, albumin; DBIL, direct bilirubin; FGL1, fibrinogen-like protein 1; FIB, fibrinogen; GGT, gamma-glutamine transferase; TBIL, total bilirubin.

level method. Pearson correlation was used to analyze the correlation of indicators. A stepwise logistic regression model was used to calculate the diagnostic value. The Hanley-McNeil nonparametric method was used to compare ROC curves. P <.05 indicates statistically significant difference.

Results

Characteristics of the Study Population

The demographic characteristics of the HBV-HCC group are displayed in **TABLE 1**. There was no significant difference in age and sex between the groups (P > .05). Through the analysis of liver biochemical indicators, the differences of the levels of ALT, AST, ALP, GGT, ALB, TBIL, DBIL, and hs-CRP in the HC, CHBV, LC, and HCC groups were found to be statistically significant (P <.001). The differences in the expression of blood coagulation indexes D-dimer and FIB in each group were also statistically significant (P <.001). The HBV DNA content in the CHBV group was significantly higher than the other 3 groups (P <.001). The disparity in the expression of AFP and FGL1 in each group was statistically significant (P <.001). Comparisons between groups are shown using the corner scale. The level of FGL1 in the HBV-HCC group was the highest (39.04 ng/mL; range, 19.43–99.77), and FGL1 level was not significantly different in the LC group and the CHBV group (P> .05).

Serum FGL1 and AFP Levels in Different Groups

As shown in **TABLE 1**, in all controls, the median serum FGL1 level in the HBV-HCC group (39.04 ng/mL; range, 19.43–99.77) was significantly elevated compared with that in the HC group (0.63 ng/mL; 0.33–0.87; P <.001), LC group (16.55 ng/mL; 4.89–24.90; P <.001), and CHBV group (19.67 ng/mL; 9.78–26.73; P<.001). AFP expression was higher in the HBV-HCC group (30.94 ng/mL; 5.98–1761.72) than in the other groups [(2.18 ng/mL; 2.90–4.05), (2.20 ng/mL; 4.27–35.55), and (5.20 ng/mL; 2.67–18.04)] and was statistically significant (P <.001), More importantly, the serum FGL1 level in the AFP-negative HBV-HCC group (31.59 ng/mL; 19.43–64.21) was significantly higher than that in the HC group (0.63 ng/mL; 0.33–0.87; P <.001), the AFP-negative CHBV group (18.18 ng/mL; 9.58–26.64; P <.001), and the AFP-negative LC group (17.07 ng/mL; 4.34–24.65; P <.001).

In addition, we discovered a significant positive correlation between serum FGL1 and AFP levels (r = 0.167, P = .002). The expression of FGL1 in the test and validation cohorts was consistent with that of AFP and in all subjects, as shown in **FIGURE 1**.

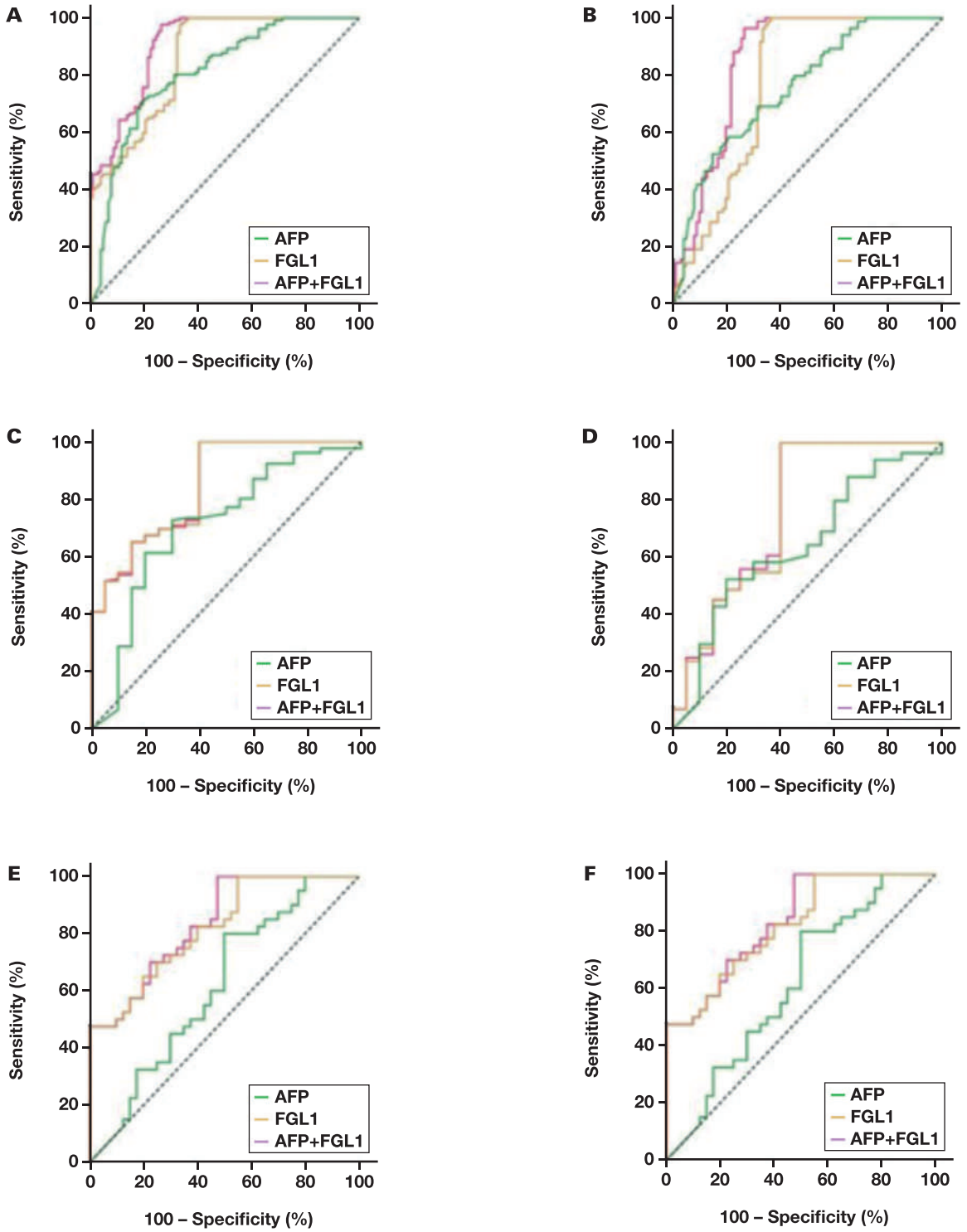
Correlation Analysis of Serum FGL1 and Hepatic Biochemical Index

As shown in **TABLE 2**, we conducted Pearson correlation analysis and found that serum FGL1 revealed a significant correlation with serum ALT and AST. The correlation coefficients were 0.291 and 0.157 (P <.001 and P = .004), respectively.

Diagnostic Performance of Serum FGL1 Compared with AFP in HBV-HCC

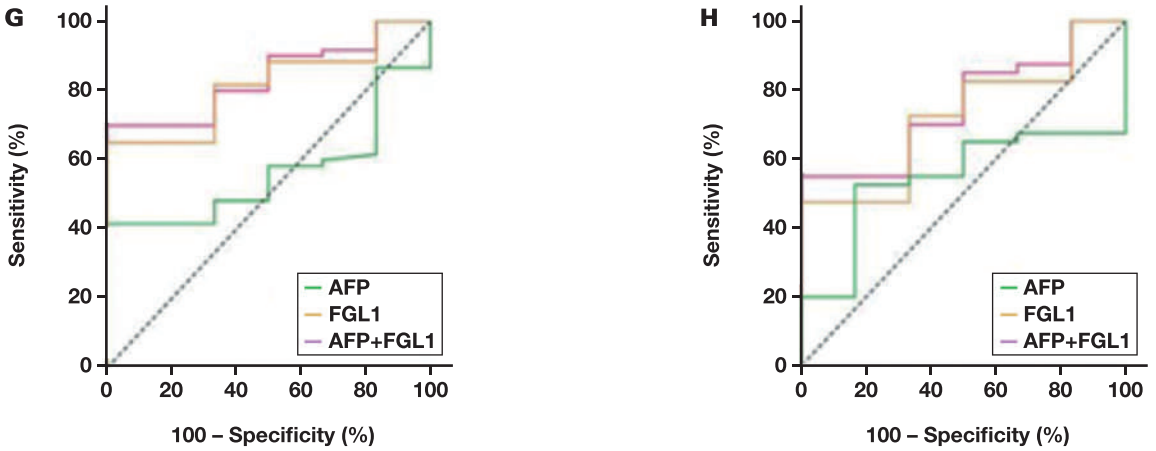
Next, we analyzed the ROC curves to evaluate the sensitivity and specificity of serum FGL1 for the HBV-HCC group (**FIGURE 2**). In the test cohort, the area under the ROC curve (AUROC) of FGL1

FIGURE 2. Diagnostic outcomes for serum fibrinogen-like protein 1 (FGL1) in the diagnosis of HBV-related hepatocellular carcinoma (HBV-HCC). A, ROC curve for FGL1, alpha-fetoprotein (AFP), or both for all patients with HBV-HCC vs all control individuals in the test cohort. B, ROC curve of FGL1, AFP, or both, for all patients with HBV-HCC vs controls at risk of HBV-HCC in the test cohort. C, ROC curve for FGL1, AFP, or both for patients with early-stage HBV-HCC vs all controls in the test cohort. D, ROC curve of FGL1, AFP, or both for patients with early-stage HBV-HCC vs controls at risk of HBV-HCC in the test cohort. E, ROC curve for FGL1, AFP, or both for all patients with HBV-HCC vs all controls in the validation cohort. F, ROC curve of FGL1, AFP, or both, for all patients with HBV-HCC vs controls at risk of HBV-HCC in the validation cohort.



(0.758; 95% CI, 0.664–0.836) was found to be much larger than that of serum AFP (0.645; 0.545–0.736; P <.001), especially in the early-stage HBV-HCC group and in other controls. Similarly, the ROC of FGL1 (0.721; 0.569–0.843) was significantly higher than that of AFP (0.546; 0.392–0.693) in the validation cohort. The sensitivity, specificity, PPV, NPV, positive likelihood ratio (+LR) and negative

FIGURE 2. (cont) G, ROC curve for FGL1, AFP, or both for patients with early-stage HBV-HCC vs all controls in the validation cohort. H, ROC curve of FGL1, AFP, or both for patients with early-stage HBV-HCC vs controls at risk of HBV-HCC in the validation cohort.



likelihood ratio (–LR) at various cutoff values of FGL1 and AFP, according to their ROC curves, were calculated and are shown in **TABLE 3**. Moreover, binary logistic regression modeling indicated that the combination of FGL1 and AFP could improve the diagnostic efficiency significantly.

In the test cohort, the AUROC of the combined index was 0.852 (0.792–0.899) in the HBV-HCC group vs the LC and CHBV groups, and 0.762 (0.669–0.840) in the early-stage HBV-HCC group vs the LC and CHBV groups. In the validation cohort, the AUROC of the combined index was 0.836 (0.736–0.909) in the HBV-HCC group vs the LC and CHBV groups, and 0.754 (0.605–0.869) in the early-stage HBV-HCC group vs the LC and CHBV groups. In the stepwise regression model, FGL1 showed good diagnostic value (P <.001, Wald = 31.753). We compared the ROC curves of FGL1 by itself and combined index diagnosis, and found that the diagnostic efficiency of the combined index was significantly better than that of FGL1 by itself (P = .0052; Z = 2.796).

For further research, we calculated the optimum cutoff value of FGL1 to be 34.05 ng/mL in the test cohort, and was 7.57 ng/mL in the validation cohort. Also, 20 ng/mL is currently accepted as the cutoff value for AFP. We analyzed the positive ratio of FGL1 and AFP in patients based on the cutoff values. As shown in **FIGURE 3**, a greater proportion of patients with HBV-HCC tested positive for FGL1 than for AFP (61.76% vs 59.80% in the test cohort; 100.00% vs 37.50% in the validation cohort). Moreover, 60.98% patients who tested AFP negative and had HBV-HCC had positive FGL1 results in the test cohort; 100.00% of patients who tested AFP negative with HBV-HCC had positive FGL1 results in the validation cohort. The combination of FGL1 and AFP had a higher positive rate (81.37%, in the test cohort; 100.00% in the validation cohort) in the HBV-HCC cohort.

In addition, no patients showed FGL1 positivity in the CHBV group and the LC group, whereas 27.91% and 29.27% of patients in the CHBV group and LC groups, respectively, exhibited AFP positivity in the test cohort. However, there is a difference in this case in the validation cohort. The FGL1 positivity rate (62.20%) of patients with advanced-stage HBV-HCC (BCLC B/C/D) was higher than that of early-stage HBV-HCC (BCLC 0/A) (60.00%). In the validation cohort, the positivity rate of FGL1 in early-stage and advanced HBV-HCC was 100%. However, there was no association between serum FGL1 levels and BCLC stages.

Performance of Serum FGL1 for the Diagnosis of HBV-HCC with Negative AFP Results

To further evaluate the diagnostic performance of FGL1, we focused on a subset of patients with negative AFP results. In the assessment of differential diagnostic accuracy, FGL1 has good AUC and sensitivity values to distinguish the HBV-HCC group from the HC, LC, and CHBV groups (0.892; 95% CI, 0.826–0.940; 97.56% in the test cohort; 0.864; 0.767–0.931; 100.00% in the validation cohort). We noticed that when distinguishing the early-HCC group from the CHBV and LC groups, serum FGL1 showed an obviously high specificity of 100% in the test cohort. Serum FGL1 had good performance in distinguishing early-stage AFP-negative HBV-HCC from other control conditions (0.862, 0.780–0.922, in the test cohort; 0.814, 0.690–0.904, in the validation cohort; **FIGURE 4**; **TABLE 4**). Because none of the patients with early-stage HBV-HCC in the validation cohort had serum AFP levels above the threshold, the relevant uncountable data were expressed using NA (nonapplicable).

Discussion

HCC is a primary tumor with extremely high morbidity and mortality worldwide.[20] To reduce those outcomes in hepatitis-related cancers, it is necessary to focus on prevention and conduct large-scale screening and treatment.[21] The cumulative incidence rate of developing HCC in the LC groups was 53.1% at 10 years, so cirrhosis has become an important risk factor for HCC.[22] Liver biopsy is usually used to confirm the diagnosis of HBV-HCC; however, this method is traumatic and not easily tolerated by patients.[23] A trial performed that using abdominal ultrasound every 6 months in more than 18,000 Chinese patients resulted in mortality risk lowered by 37% in screened patients.[24]

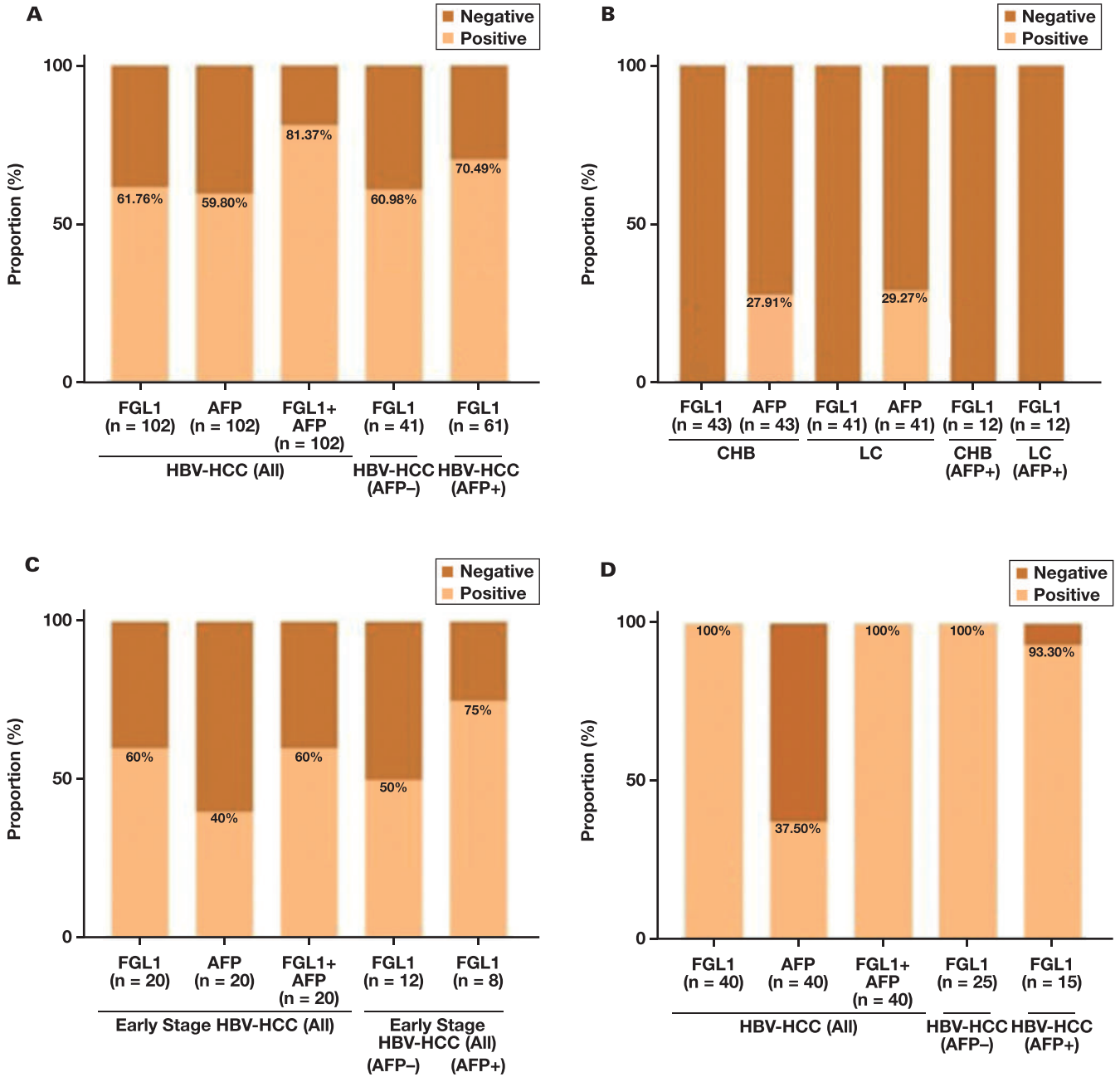
Although abdominal ultrasound was recommended as the primary surveillance test by the AASLD, EASL, and APAS, its sensitivity for detection of early-stage HCC was only 47% (95% CI, 0.33–0.61).[25] The

TABLE 3. Performance of FGL1 and AFP for Detecting Early-Stage HBV-HCC

Variable	Test							Validation						
	AUC (95%CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	−LR	AUC (95%CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	−LR
HBV-HCC vs CHBV, LC, and HC														
FGL1	0.858 (0.806–0.900)	66.67	97.73	100	77.20	NA	0.30	0.880 (0.799–0.936)	100	65.00	65.57	100	1.90	0
AFP	0.808 (0.752–0.856)	79.41	71.97	81.00	76.30	4.25	0.38	0.675 (0.574–0.765)	50.00	86.67	65.22	67.53	1.88	0.48
FGL1 + AFP	0.905 (0.860–0.939)	73.53	97.73	66.90	82.70	2.02	0.21	0.890 (0.812–0.944)	100	65.00	64.52	100	1.82	0
HBV-HCC vs CHBV and LC														
FGL1	0.777 (0.710–0.834)	63.73	100	100	68.30	NA	0.46	0.819 (0.717–0.896)	100	47.50	0.65	100	1.90	0
AFP	0.756 (0.688–0.816)	79.41	58.33	81.00	72.50	4.25	0.68	0.615 (0.500–0.722)	50.00	80.00	0.65	0.56	1.88	0.78
FGL1 + AFP	0.852 (0.792–0.899)	73.53	96.43	64.10	75.00	1.78	0.33	0.836 (0.736–0.909)	52.50	100	0.65	100	1.82	0
Early-stage HBV-HCC vs CHBV, LC, and HC														
FGL1	0.846 (0.778–0.899	60.00	100	100	94.30	NA	0.06	0.814 (0.699–0.899)	100	65.00	0.22	100	0.29	0
AFP	0.709 (0.630–0.780)	70.00	72.73	25.00	16.70	0.33	0.11	0.563 (0.435–0.684)	100	41.67	0.00	0.90	0	0.12
FGL1 + AFP	0.846 (0.778–0.899	60.00	100	100	94.30	NA	0.06	0.836 (0.435–0.684)	100	70.00	0.27	0.95	0.38	0.06
Early-stage HBV-HCC vs CHBV and LC														
FGL1	0.758 (0.664–0.836)	60.00	100	100	91.30	NA	0.10	0.721 (0.569–0.843)	100	47.50	0.22	0	0.29	0
AFP	0.645 (0.545–0.736)	80.00	52.38	25.00	16.70	0.33	0.20	0.546 (0.392–0.693)	88.33	52.50	0	0.16	0	0.19
FGL1 + AFP	0.762 (0.669–0.840)	60.00	100	100	91.30	NA	0.10	0.754 (0.605–0.869)	100	55.00	0.27	0.91	0.38	0.09

AFP, alpha-fetoprotein; CHBV, chronic HBV; HC, healthy control; FLG1, fibrinogen-like protein 1; HBV-HCC, HBV-related hepatocellular carcinoma; LC, liver cirrhosis; +LR, positive likelihood ratio; –LR, negative likelihood ratio; NA, nonapplicable.

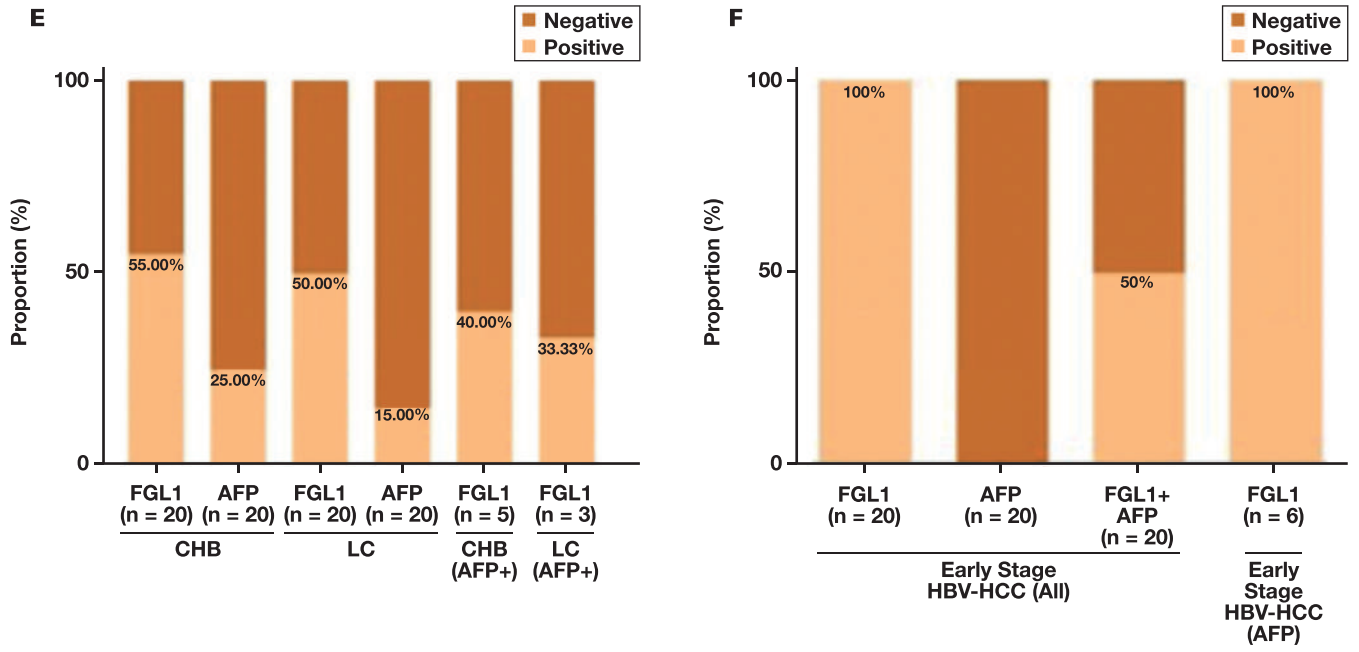
FIGURE 3. The rate of positive results for alpha-fetoprotein (AFP), fibrinogen-like protein 1 (FGL1), and both in different groups. A, The rate of positive results for AFP, FGL1, and both in all patients with HBV-related hepatocellular carcinoma (HBV-HCC), and for FGL1 by AFP status, in the test cohort. B, The rate of positive results for AFP and FGL1 for patients with chronic HBV infection or liver cirrhosis (LC), and for FGL1 by AFP-positive status, in the test cohort. C, Rate of positive results for AFP, FGL1, or both in patients with early-stage HBV-HCC, and for FGL1 by AFP status, in the test cohort. D, The rate of positive results for AFP, FGL1, and both in all patients with HBV-HCC, and for FGL1 by AFP status, in the validation cohort.



combination of abdominal ultrasound and AFP has better performance for the diagnosis in early-stage HCC. The diagnosis of HBV-HCC is easy when substantially evaluated serum AFP levels and definitive imaging features are present; however, the proportion of patients with AFP negative is high. Therefore, it is urgent to find an effective biomarker to improve the diagnostic efficiency of HBV-HCC, especially in patients with early-stage disease and AFP-negative results. An ideal marker for screening HBV-HCC requires several characteristics, namely, the capacity to detect a secreted protein or molecule in blood or other excreta, having strong performance for the diagnosis of HBV-HCC, and having stable physical and chemical properties to ensure reliable and stable test results.

FGL1 is a secreted protein specifically expressed in the liver. It has been verified that FGL1 is an inhibitory ligand for LAG3, and it has been

FIGURE 3. (cont) E, The rate of positive results for AFP and FGL1 for patients with chronic HBV infection or cirrhosis, and for FGL1 by AFP-positive status, in the validation cohort. F, Rate of positive results for AFP, FGL1, or both in patients with early-stage HBV-HCC, and for FGL1 by AFP status, in the validation cohort.



shown that FGL1 expression is upregulated in the serum of patients with non–small-cell lung carcinomas. Liu et al[26] reported that FGL1 is a novel biomarker for predicting disease activity and prognosis of rheumatoid arthritis. A study report by Guo et al[27] demonstrated that FGL1 expression was upregulated in tissues with HCC from patients with the disease. However, reports in the literature have evaluated the levels of FGL1 in the serum of patients with HBV-HCC. Therefore, we studied the serum levels of FGL1 and assessed their diagnostic value. We hope that the combination of FGL1 and AFP as a classical marker can improve the diagnostic efficiency of HBV-HCC.

Until now, AFP has been widely used in the laboratory for early screening of HBV-HCC, but its diagnostic value still needs to be improved.[28] As a result, in this work of research, we address the potential of a novel blood biomarker, FGL1, as a diagnostic factor in patients with HBV-HCC. In the test cohort, we found that serum FGL1 was significantly increased in patients with HBV-HCC compared with healthy controls and patients with CHBV and LC. At the cutoff value of 34.05 ng/mL, none of the patients with CHBV and LC exceeded the threshold, and 61.67% patients with HBV-HCC tested positive for FGL1.

Also, ROC curves showed a higher specificity of FGL1 than AFP when distinguishing HBV-HCC from other control conditions. We obtained a positive correlation between FGL1 and AFP. In addition, serum FGL1 levels were also positively correlated with AST and ALT. AFP often does not show abnormalities in liver cancer, which decreases the detection rate of liver cancer. Consequently, we further analyzed the diagnostic value of FGL1 for HBV-HCC in the presence of negative AFP. The results of this analysis showed that FGL1 exhibited good diagnostic value in the presence of negative AFP, with high AUROC and specificity. These findings indicate that FGL1 is a novel marker with a higher true-positive rate in diagnosing and differentiating HBV-HCC from other control conditions. Especially after it is combined with AFP, AUROC and sensitivity were improved. In distinguishing patients with HBV-HCC from all patients, the AUROC and specificity of the joint index were higher than the AFP value.

In the other groups, the diagnostic values of the combined index also improved. The validation cohort had the same trend of FGL1 at each group level as the test cohort. The AUC of FGL1 for the diagnosis of HBV-HCC from other control conditions in the validation cohort (0.880; 95% CI, 0.799–0.936) was similar to that in the test cohort (0.858; 0.806–0.900); the AUC of FGL1 for the diagnosis of early-stage HBV-HCC from other controls in the validation cohort (0.814; 0.699–0.899) was similar to that in the test cohort (0.846; 0.778–0.899), which demonstrates that FGL1 has good clinical value in HBV-HCC.

Once diagnosed, patients are usually in advanced stages of HCC because early HCC symptoms are not significantly distinguishable from liver disease, especially cirrhosis and hepatitis. In China, HBV infection accounted for 63% of all deaths due to LC and other chronic liver diseases and for 53% of all deaths due to HCC.[3] Therefore, the biggest obstacle to the diagnosis of early-stage liver cancer is the current shortage of tumor markers.[29]

In this study, we statistically analyzed the diagnostic value of FGL1 in early-stage HCC. We were surprised to discover that FGL1 had better AUROC and specificity than AFP in the diagnosis of early-stage HBV-HCC, and the diagnostic value of the combined index has been further improved. A large nationwide study report mentioned a downtrend of AFP levels at HCC diagnosis, with the most significant decline observed among those with early-stage HCC.[6] Therefore, we further analyzed the diagnostic value of FGL1 in AFP-negative early-stage HBV-HCC; FGL1 exhibited good AUROC. Those results suggested that FGL1 has excellent diagnostic value in the diagnosis of early-stage HBV-HCC and is equally capable of distinguishing early-stage HBV-HCC when AFP results are negative.

FIGURE 4. Diagnostic outcomes for serum fibrinogen-like protein 1 (FGL1) in the diagnosis of alpha-fetoprotein (AFP)–negative HBV-related hepatocellular carcinoma (HBV-HCC). A, ROC curve for FGL1 for all patients with AFP-negative HBV-HCC vs all controls in the test cohort. B, ROC curve of FGL1 for all patients with AFP-negative HBV-HCC vs control individuals at risk of HBV-HCC, in the test cohort. C, ROC curve for FGL1 for patients with AFP-negative early-stage HBV-HCC vs all controls in the test cohort. D, ROC curve of FGL1 for patients with AFP-negative early-stage HBV-HCC vs controls at risk of HBV-HCC, in the test cohort. E, ROC curve for FGL1 for all patients with AFP-negative HBV-HCC vs all controls in the validation cohort. F, ROC curve of FGL1 for all patients with AFP-negative HBV-HCC vs controls at risk of HBV-HCC, in the validation cohort.

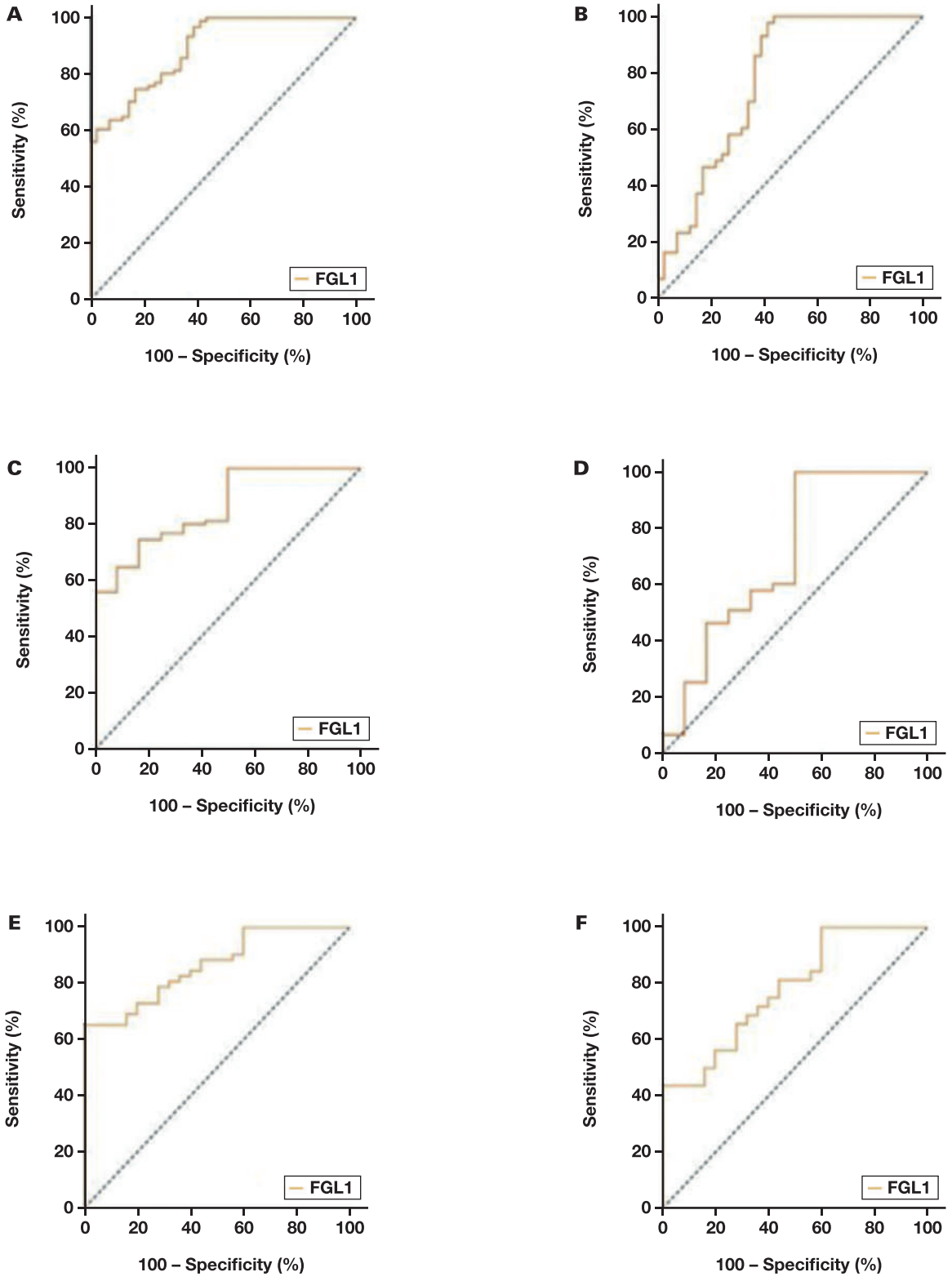


FIGURE 4. (cont) G, ROC curve for FGL1 for patients with AFP-negative early-stage HBV-HCC vs all controls in the validation cohort. H, ROC curve of FGL1 for patients with AFP-negative early-stage HBV-HCC vs controls at risk of HBV-HCC, in the validation cohort.

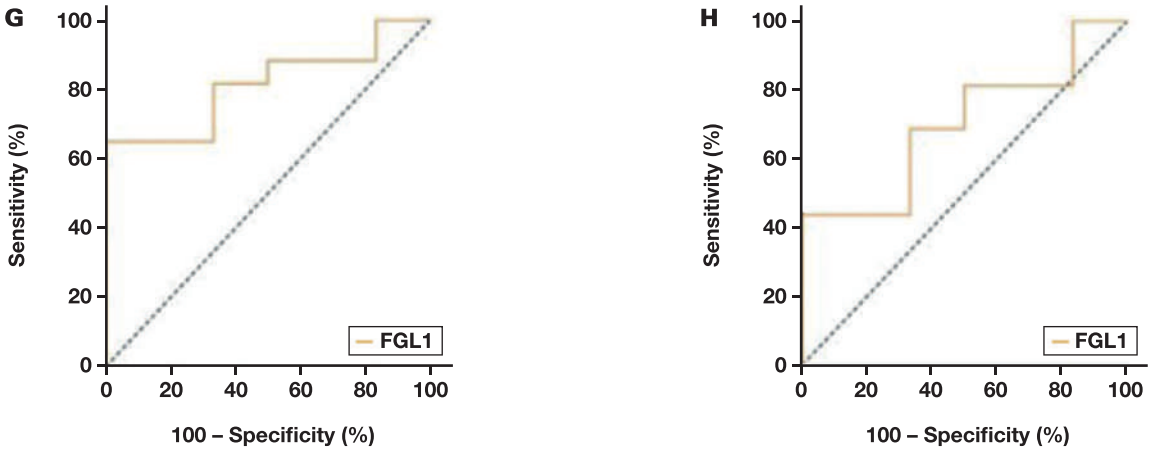


TABLE 4. Performance of Serum FGL1 for the Diagnosis of HBV-HCC with Negative AFP

Variable	Test							Validation						
	AUC (95%CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	–LR	AUC (95%CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	+LR	–LR
AFP negative														
HBV-HCC vs CHBV, LC, and HC	0.892 (0.826–0.940)	97.56	60.44	92.31	83.96	12.00	0.19	0.864 (0.767–0.931)	100	65.38	0.57	100	1.32	0
HBV-HCC vs CHBV and LC	0.772 (0.668–0.856)	58.54	97.67	92.31	70.69	12.00	0.41	0.779 (0.649–0.878)	100	43.75	0.57	100	1.32	0
Early-stage HBV-HCC vs CHBV, LC, and HC	0.862 (0.780–0.922)	83.33	74.73	75.00	93.68	3.00	0.07	0.814 (0.690–0.904)	100	65.38	0.21	100	0.32	0
Early-stage HBV-HCC vs CHBV and LC	0.707 (0.569–0.822)	50.00	100	75.00	87.23	3.00	0.15	0.698 (0.528–0.836)	100	43.75	0.21	100	0.32	0
AFP positive														
HBV-HCC vs CHBV, LC, and HC	0.767 (0.663–0.852)	70.49	100	100	57.10	NA	0.75	0.908 (0.713–0.988)	93.33	75.00	0.82	0.83	4.67	0.20
HBV-HCC vs CHBV and LC	0.767 (0.663–0.852)	70.49	100	100	57.10	NA	0.75	0.908 (0.713–0.988)	93.33	75.00	0.82	0.83	4.67	0.20
Early-stage HBV-HCC vs CHBV, LC, and HC	0.833 (0.660–0.941)	75.00	100	100	92.30	NA	0.08	NA	NA	NA	NA	NA	NA	NA
Early-stage HBV-HCC vs CHBV and LC	0.767 (0.663–0.852)	70.49	100	100	92.30	NA	0.08	NA	NA	NA	NA	NA	NA	NA

AFP, alpha-fetoprotein; CHBV, chronic HBV; FLG1, fibrinogen-like protein 1; HBV-HCC, HBV-related hepatocellular carcinoma; HC, healthy control; LC, liver cirrhosis; NA, nonapplicable.

Conclusions

In this article, we show that serum FGL1 could potentially be used to diagnose HBV-HCC, especially early-stage HBV-HCC, and will help to resolve the deficiencies of AFP in the diagnosis of patients with HBV-HCC who test AFP-negative. Further, the combination of AFP and FGL1 can improve the diagnostic efficiency of HBV-HCC. However, the number of specimens included in this study is small, and we suggest that scholars can expand the sample size for multicenter studies to provide more evidence to validate the fitness of FGL1 in testing for HBV-HCC.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (81773444). The datasets generated during 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.

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Intraday Changes and Clinical Applications of Thyroid Function Biomarkers in Healthy Subjects

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Keywords: thyroid-stimulating hormone, triiodothyronine, thyroxine, free T3, free T4, intraday changes

Abbreviations: TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxine; FT3, free T3; FT4, free T4; ANOVA, analysis of variance; CV_I, within-subject biological variation; BMI, body mass index

Laboratory Medicine 2023;54:282–286; https://doi.org/10.1093/labmed/lmac105

ABSTRACT

Objective: We evaluated the intraday changes of thyroid function biomarkers in healthy subjects to help clinicians diagnose thyroid diseases in appropriate timing.

Methods: Blood samples were collected from 31 subjects at 0:00, 4:00, 8:00, 12:00, 16:00 and 20:00 on the sampling day and analyzed for thyroid-stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4), free T3 (FT3), and free T4 (FT4). The intraday concentration changes were analyzed using Friedman's 2-way analysis of variance by ranks.

Results: The concentrations of TSH, T3, T4, FT3, and FT4 in males were significantly higher than those in females ($P < .01$). The obvious peak circadian rhythm of TSH was observed at 0:00 AM with gradual decline thereafter, whereas other biomarkers showed no rhythmic changes.

Conclusion: Sex differences should be considered in interpreting thyroid function tests. It is important to select the sampling time according to the clinician's diagnostic needs, especially at night when TSH secretion peaks.

Thyroid is an important endocrine organ in human body. Thyroid hormones regulate growth and development and metabolic activities of human body, and its physiological mechanism of feedback regulation by the hypothalamus-pituitary-thyroid axis has been extensively reviewed.[1,2] Thyroid disease is a common endocrine disorder, and its diagnosis must be combined with medical history, clinical manifestations, and laboratory tests, among which thyroid hormone test results from the laboratory are particularly important. Using laboratory test results to diagnose thyroid disease may seem simple, but unexpected or unusual results often confuse clinicians.[3] Thyroid-stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T4), free T3 (FT3), and free T4 (FT4) are common indicators to evaluate thyroid function. Patients can come to the laboratory for testing at any time and the time of sample collection is not considered. If there are significant intraday changes in hormone levels, test results from samples taken at different times may allow the wrong judgment to be made about thyroid disease. In one study, it was reported that 50% of 19 subjects with subclinical hypothyroidism were diagnosed in the morning as having hypothyroidism but were not so diagnosed in the afternoon.[4] On the other hand, clinicians often compare a patient's test results to previous results in addition to considering intraindividual and interindividual differences in thyroid biomarkers. Different sampling time points of samples will also lead to incorrect analysis of differences in one individual's results over time, which may lead to a misdiagnosis of thyroid disease. Our objective was to evaluate the intraday changes of serum thyroid biomarkers in healthy adults and to ascertain whether the time of sampling is critical or not.

Materials and Methods

Subjects

This prospective study was approved by the ethics review committee of the Traditional Chinese Medicine Hospital of the Pidu District and was conducted following the Helsinki Declaration of 1975 (1983 revision). All volunteers provided signed informed consent before participation. A total of 31 healthy subjects were enrolled, including 17 males (21 to 54 years, median age 30 years) and 14 females (18 to 48 years, median age 33 years). The inclusion criteria were as

follows: no known thyroid disease, obvious goiter, or other diseases; no abnormalities in liver function, kidney function, blood lipid, blood sugar, thyroid function or related antibodies, or ultrasonography in recent physical examination; no recent history of medication; no smoking, alcohol consumption, or other health-related habits. All participants maintained their daily lifestyle before and on the day of sample collection.

Sampling and Preservation

On the day of sampling, blood samples were collected every 4 h from 00:00 to 20:00 h (a total of 6 times, considered t1 through t6, successively) using silicon vacuum tubes. After collection, all blood samples were left at room temperature (22°C to 28°C) for 30 min for complete coagulation, centrifuged at 2500*g* for 10 min to separate the serum, and stored at −70°C before analysis.

Test Methods

We assessed 2/3 levels of quality control materials produced by Bio-Rad (Bio-Rad Laboratories) each day and participated in external quality assessment to monitor the quality performance of the methods used. To reduce preanalysis error, the sample pretreatment was standardized. All samples were thawed and centrifuged again on the same day, and serum TSH, T3, T4, FT3, and FT4 were measured for each sample using the Abbott automatic immune system I2000 and Abbott original reagents (Abbott).

Statistical Analysis

All data were statistically analyzed by SSPS22.0 (IBM). GraphPad Prism v 9.0 (Northside) was used to draw line charts. The Shapiro-Wilk test was used to analyze whether the data were normally distributed, and the median, 25th percentile, and 75th percentile were used as descriptive statistics for nonnormally distributed data. The Mann-Whitney *U* test was used for statistical analysis of gender differences in study variables. The intraday concentration changes were analyzed using Friedman's 2-way analysis of variance (ANOVA) by ranks. The median of hormone concentrations at each time point and time points (t1-t6) were used as variables to make a curve tracing the circadian rhythm. Within-subject biological variation (CV_I) data were obtained from the European Federation of Clinical Chemistry and Laboratory Medicine Biological Variation Database.[5]

Results

Population Characteristics and Thyroid Function Biomarker Concentrations

All variables were nonnormally distributed and were described using the median, 25th percentile, and 75th percentile. The demographic characteristics and thyroid function biomarker concentrations of the subjects are shown in **TABLE 1**. There was no significant difference in age between males and females ($P > .05$). The differences in body mass index (BMI), TSH, T3, T4, FT3, and FT4 concentrations were statistically significant ($P < .01$).

Concentration Distribution of Thyroid Function Biomarkers at Each Time Point and Circadian Rhythm

TABLE 2 and **FIGURE 1** show the concentrations and distribution of thyroid function biomarkers measured at different time points. There was significant difference in TSH concentration among the 6 time points ($P < .01$), whereas no significant differences are found in concentrations of other markers. The obvious peak in circadian rhythm of TSH appeared to be at 0:00, with gradual decline thereafter, as shown in **FIGURE 2**, and the concentration was higher in the morning (from 8:00 to 12:00) than in the afternoon (from 12:00 to 16:00). The TSH secretion rhythm is almost the same in both males and females. There were no rhythmic changes in other biomarkers. The differences between males and females in T3 at t1 to t6, T4 at t2, FT3 at t1 to t6, and FT4 at t4 were statistically significant, and there were no statistically significant differences at other time points. There was no significant difference between males and females at all time points for TSH ($P > .05$).

Intraday Amplitudes of Thyroid Function Biomarkers Compared with CV_I

The intraday amplitudes of thyroid hormones and their ratio to CV_I are shown in **TABLE 3**. The amplitudes of TSH, T3, T4, FT3, and FT4 are narrower in males than in females and their ratio to CV_I also smaller. The changes in TSH, FT3, and FT4 were wider than those of CV_I, whereas the changes in T3 and T4 were within the range of CV_I.

Discussion

The incidence of thyroid disease has been increasing in recent years, and clinicians often judge its condition and adjust the treatment plan according to changes in results of tests taken over weeks or months. The

TABLE 1. Characteristics and TSH, T3, T4, FT3, and FT4 Concentrations of the Subjects[a]

	Males (n = 17)	Females (n = 14)	All Subjects (n = 31)	*P* Value[b]
Age (years)	30 (25–44)	33 (22–38)	33 (24–40)	.465
BMI (kg/m^2)	23.3 (22.9–25.6)	21.1 (18.7–23.2)	23.1 (21.0–24.3)	.004
TSH (ulU/mL)	2.481 (1.622–3.514)	2.036 (1.489–2.976)	2.236 (1.512–3.199)	.002
T3 (ng/mL)	1.12 (1.03–1.19)	0.96 (0.87–1.07)	1.06 (0.93–1.16)	<.001
T4 (ug/dL)	8.50 (7.61–9.43)	7.76 (7.07–8.46)	8.17 (7.26–9.06)	<.001
FT3 (pg/mL)	3.56 (3.32–3.80)	3.12 (2.84–3.40)	3.38 (3.03–3.68)	<.001
FT4 (ng/dL)	1.08 (1.01–1.15)	1.03 (0.96–1.08)	1.05 (0.99–1.12)	<.001

BMI, body mass index; FT3, free T3; FT4, free T4; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxine.
[a]The analyzed data were nonnormally distributed and the median, 25th percentile, and 75th percentile were used as descriptive statistics.
[b]The P value is the comparison between males and females.

changes in test results is not only related to the course of disease but also related to biological and analytical variation.[3,4,6] It can also be affected by the time of day of sample collection when there are obvious rhythm changes in the analyte. When clinicians judge changes in test results over time of the same individual, the results may not be comparable, either because of biological variation[7] or the influence of rhythm changes within the day, and different clinical decisions may be made due to differences between different sampling time points. In general, circadian rhythm and biological variation characteristics represent 2 basic issues in the clinical application of biomarkers.[8] It is important to evaluate the changes of thyroid function biomarkers within 1 day, whether using reference intervals or biological variations to interpret the results.

TABLE 1 shows that the BMIs of all study participants were in the healthy range and the difference between males and females was statistically significant. The age difference was not statistically significant, so the analysis was not affected by age. Analysis of all data showed that the overall concentrations of TSH, T3, T4, FT3, and FT4 in males were higher than those in females, and the differences were statistically significant. This finding is contrary to Qiu et al's[9] report that TSH concentrations in females are significantly higher than in males; Shatynska-Mytsyk et al[10] in the US found similar results, that TSH and T4 concentrations were lower in males than in females. This may be due to the relatively small number of subjects, different sampling times (within 24 h), and the different geographical environments. Zhang[11] proposed that serum TSH concentration was significantly related to BMI, and its average concentration increased with the increase of BMI. This may be the reason why the overall level of all the biomarkers shown in this study is higher in males than in females.

Concentrations and distributions of thyroid biomarkers at the different time points are reported in **TABLE 2** and **FIGURE 1**. The concentration difference at each time point of TSH was statistically significant, although there was no significant difference in the concentrations of other markers. As shown in **FIGURE 2**, the secretion of TSH has an obvious circadian rhythm and the concentration varies greatly; the concentration is higher at night than during the day and higher in the morning than in the afternoon, which conforms to the circadian secretion change law of TSH.[12] So the sampling time points should be relatively the same when analyzing the changes of monitoring results over time.

In addition, we can see that the median concentration of TSH is higher in females than in males only at 00:00 and then declines gradually. After that, the concentration of TSH in males was higher than females, but there was no significant difference between genders at each time point, and the circadian rhythm was consistent. Pan et al[13] reported that there was no significant difference in TSH concentration between genders, which may be because the samples were all collected in the morning at the same time. There was no significant difference in the concentrations of T3, T4, FT3, and FT4 at each time point, and there was no circadian change. The differences of T3 at t1-t6, T4 at t2, and FT3 at t1-t6 and FT4 at t4 were statistically significant between males and females. Most clinical monitoring samples are collected during the daytime. However, the concentrations of T3, T4, FT3, and FT4 are significantly different between males and females during daytime, so the gender differences in hormones, especially for T3 and FT3, should be considered when interpreting results. **TABLE 3** shows that the intraday average variation ranges of TSH, T3, T4, FT3, and FT4 concentrations in males are narrower than those in females and their ratio to CV_I is also smaller. This is inconsistent with the widths of T3, FT3, and FT4 reference intervals for males and females given in the Haikou Healthy Population survey.[13] The amplitudes of all hormones differed from their CV_I, which may be due to the fact that the samples in this study were collected within 24 h and the data used to calculate CV_I are mostly sample test data collected on different days.

In this study, the TSH concentration of 5 subjects exceeded the reference range from 0:00 to 8:00. However, the thyroid function examination of these subjects was done after 8:00, and no abnormality was found. Thyroid ultrasonography during the same period of sampling also showed no abnormality. During the follow-up investigation, 4 out of 5 subjects with abnormal results had thyroid imaging abnormalities of varying degrees during subsequent physical examination within 1 to 2 years, although the results of thyroid function biomarkers taken after 8:00 remained normal. In fact, nocturnal TSH results in these subjects had been suggestive of subclinical hypothyroidism more than a year earlier, so subclinical hypothyroidism may be detected earlier when samples are taken between 0:00 and 8:00 when TSH secretion peaks. The results of Sviridonova et al's[4] study also showed that the diagnostic efficacy of thyroid disease was different at different sampling times.

TABLE 2. Concentrations of TSH, T3, T4, FT3, and FT4 at Different Times

	Sex	t1 (0:00)	t2 (4:00)	t3 (8:00)	t4 (12:00)	t5 (16:00)	t6 (20:00)	*P* Value[a]
TSH (ulU/mL)	Males	3.116	3.031	2.357	2.146	1.658	1.946	<.001
	Females	3.377	2.608	2.127	1.394	1.584	1.724	<.001
T3 (ng/mL)	Males	1.16	1.12	1.11	1.08	1.13	1.10	.074
	Females	0.97[b]	0.92[b]	0.98[b]	0.98[b]	0.91[b]	0.92[b]	.167
T4 (ug/dL)	Males	8.43	8.70	8.77	8.59	8.73	8.52	.619
	Females	7.69	7.51[b]	7.99	7.76	7.87	8.21	.076
FT3 (pg/mL)	Males	3.68	3.65	3.55	3.54	3.57	3.47	.324
	Females	3.10[b]	3.05[b]	3.12[b]	3.13[b]	3.01[b]	2.95[b]	.107
FT4 (ng/dL)	Males	1.04	1.07	1.08	1.10	1.10	1.08	.552
	Females	1.02	1.00	1.03	1.01[b]	1.04	1.05	.077

FT3, free T3; FT4, free T4; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxine.
[a]*P values are the results of statistical analyses comparing analyte concentrations at different time points based on Friedman's 2-way ANOVA by ranks.*
[b]*Means comparison between female and male results at the same time point,* $P < .05$.

FIGURE 1. A, The median and 25th and 75th percentiles of thyroid-stimulating hormone (TSH) concentrations in males and females. B, The median and 25th and 75th percentiles of triiodothyronine (T3) concentrations in males and females. C, The median and 25th and 75th percentiles of thyroxine (T4) concentrations in males and females. D, The median and 25th and 75th percentiles of free T3 (FT3) concentrations in males and females. E, The median and 25th and 75th percentiles of free T4 (FT4) concentrations in in males and females.

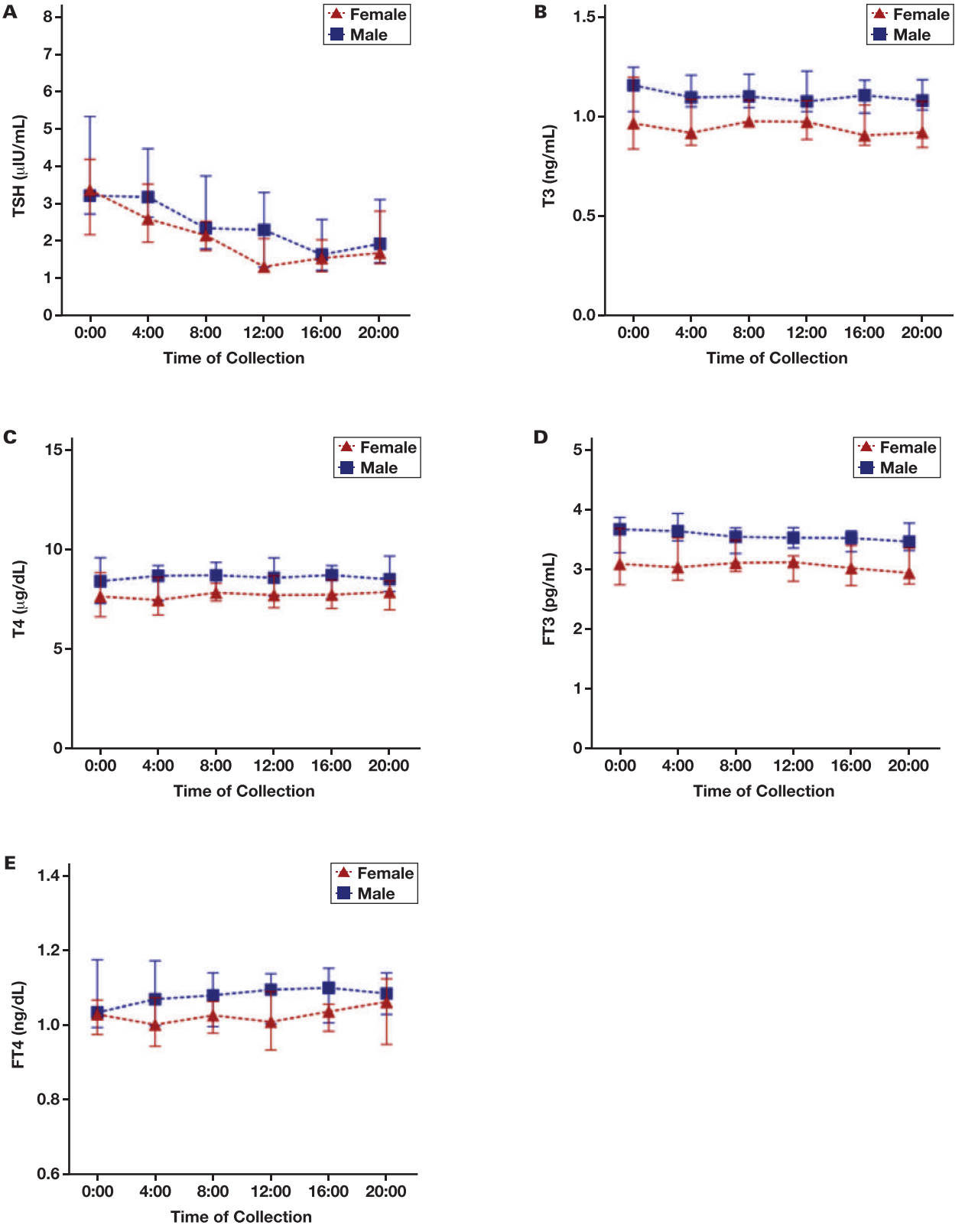

FIGURE 2. The circadian changes of thyroid-stimulating hormone (TSH) concentrations in males and females.

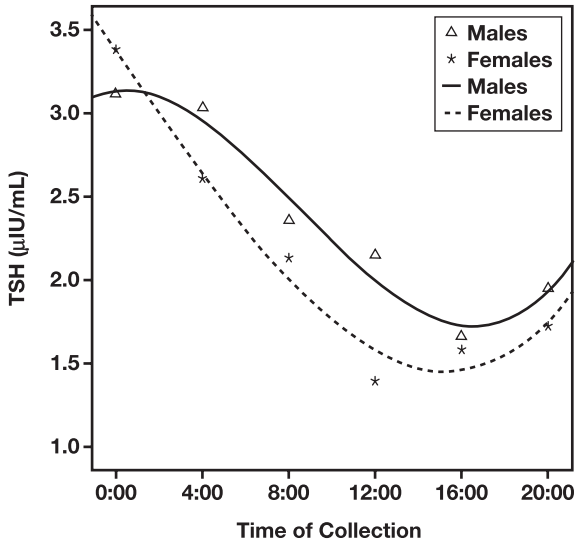

TABLE 3. Intraday Average Amplitude of Tsh, T3, T4, Ft3 and Ft4 Concentrations and Their Ratio to Cv_I

		TSH (%)	T3 (%)	T4 (%)	FT3 (%)	FT4 (%)
Amplitude	Males	54.47	6.67	6.06	6.24	4.96
	Females	74.81	9.11	6.29	8.21	5.04
Ratio to CV_I	Males	307.71	70.92	94.61	124.87	101.19
	Females	422.68	96.90	98.30	164.24	102.91

CV_I, within-subject biological variation; FT3, free T3; FT4, free T4; TSH, thyroid-stimulating hormone; T3, triiodothyronine; T4, thyroxine.

We aimed to evaluate the changes in thyroid function biomarkers concentration at different time points during the day and found the concentrations of all biomarkers were significantly different between males and females. Due to the rhythmical changes of TSH, the time of sample collection is very important for the diagnosis of thyroid disease, and the results of samples at different time intervals will enable clinicians to make different decisions. In addition, when comparing the changes in results over time, the collection time point of samples from the same individual should be set at the same time within the 24-h day, which is more conducive to the accurate interpretation of the results.

The deficiency of this study is that relatively few healthy people were recruited, and we will include more samples to confirm these results in later studies.

Conclusions

This study confirms that the concentrations of TSH, T3, T4, FT3, and FT4 in males are higher than those in females, and sex differences should be considered when interpreting thyroid function tests. Because of the circadian rhythm of TSH, clinicians may make different decisions about thyroid disease depending on the results from samples taken at different points in the 24 h period, especially at late-night hours. Therefore, it is important to select the sampling time accordingly.

Acknowledgments

This study was supported by the Department of Laboratory Medicine, Traditional Chinese Medicine Hospital of Pidu District.

Funding

This study was supported by Chengdu Municipal Health Commission (2021178) and "Xinglin Scholar" Hospital Special Fund of Chengdu University of Traditional Chinese Medicine (YYZX2021142).

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Improvement in Platelet Product Wastage and Reduction of Costs through Implementation of the Pan Genera Detection Test

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Abbreviations: RBC, red blood cell; PGD, Pan Genera Detection; IRB, institutional review board

Laboratory Medicine 2023;54:287–290; https://doi.org/10.1093/labmed/lmac111

ABSTRACT

Objective: The aim of this study was to evaluate the effects of Pan Genera Detection (PGD) testing on reducing platelet product wastage and transfusion service costs.

Methods: We conducted a retrospective cross-sectional study comparing the number of platelet apheresis units wasted before (March 2017 to February 2019) and after (March 2019 to February 2021) PGD implementation. The PGD testing was performed before transfusion on days 6 and 7. Cost analysis considered the costs of platelet units wasted ($500.00/unit) and PGD test supplies and performance (estimated $26.50 per test). Paired samples *t*-test was used to compare platelet wastage pre- and post-PGD implementation.

Results: The number of wasted platelet units decreased from pre-PGD (419) to post-PGD (195), representing a significant decrease in platelet wastage from 17.5% to 9.2% ($P < .0001$). During the post-PGD period, 366 and 133 units were tested on days 6 and 7, with 28 and 36 units discarded each day, allowing transfusion of an additional 302 platelet units. Costs from platelet wastage decreased from $209,500.00 pre-PGD to $97,500.00 post-PGD.

Conclusion: Our results showed that PGD testing effectively reduced platelet wastage, extended platelet availability, and reduced transfusion service costs.

Whole blood and blood components are considered drugs because of their crucial role in medical care, with component therapy defined as the provision of the specific blood component required by a patient.[1] Platelet products are the second most frequently transfused blood component after red blood cells (RBCs).[2] Apheresis platelets, which are obtained from a single donor, are prepared in many blood centers because of their high platelet yield and in-process reduction of leukocytes.[1] As the majority of the platelet product supply in the United States relies on altruistic donors who donate voluntarily, wasting platelets results in undesirable ethical issues.[3]

Managing a robust platelet product inventory remains a key issue for blood centers and transfusion services. Whereas it is crucial to have an adequate supply of platelets for patients requiring transfusion, outdating of platelet products wastes a valuable resource and imposes a substantial financial burden.[4] Platelet products have a short shelf life of 5 to 7 days, which limits their availability. Effective platelet product inventory management should minimize platelet wastage and reduce costs without compromising patient safety. As per the Standards for Blood Banks and Transfusion Services from the Association for the Advancement of Blood and Biotherapies,[5] platelets must be tested using a United States Food and Drug Administration–approved method to detect bacterial contamination before being released for transfusion. Currently, several methods are used in the clinical setting to minimize platelet wastage, reduce costs, and improve inventory management, including immunoassays to detect bacterial contamination in apheresis platelets.

The Pan Genera Detection (PGD) test by Verax Biomedical is a rapid qualitative immunoassay for detecting aerobic and anaerobic bacteria in apheresis platelets near the time of their release, providing a method to extend the expiration of apheresis platelets from 5 to 7 days.

Collins et al[6] showed that simple inexpensive interventions can result in marked reductions in blood component wastage, as well as the costs associated with wasted units. Implementing PGD testing to detect bacterial contamination and extend platelet shelf life is a potential strategy for reducing platelet wastage and blood bank costs. The purpose of our study was to evaluate the effectiveness of PGD testing for reducing platelet wastage and decreasing costs at a tertiary care hospital. We hypothesized that implementing PGD testing for identifying bacterial contamination before issuing platelet units for transfusion would reduce platelet wastage and costs. We compared the number of platelet components received

and wasted before and after implementing PGD testing at our institution and determined the number of platelet units transfused on days 6 or 7 after implementation of this test. Potential cost savings to the transfusion service were also calculated based on the number of platelet products discarded in the pre-PGD and post-PGD periods.

Materials and Methods

This cross-sectional retrospective study was conducted at a 529-bed private, nonprofit, tertiary care teaching hospital in which approximately 12,000 to 13,000 blood products are transfused per year. An average of 1,200 to 1,300 apheresis platelet transfusions are provided annually to patients on the medical and surgical wards, in outpatient clinics, during surgery, and in intensive care units. The hospital's transfusion service began using the PGD test in March 2019 to reduce platelet product wastage and costs within the transfusion service. A review of existing transfusion service operational data was conducted using Softbank (Electronic Medical Records) to determine the number of platelet components received, transfused, extended, and wasted from March 2017 through February 2021. The step-by-step data collection process is summarized in **FIGURE 1**.

Data were stratified into 2 24-month periods: March 2017 through February 2019 (pre-PGD implementation) and March 2019 through February 2021 (post-PGD implementation). Disposition of apheresis platelets was compared between the pre-PGD period and post-PGD period. Platelet wastage in the pre-PGD study period was compared to platelet wastage in the post-PGD period using the paired samples *t*-test, with *P* values < .05 indicating statistical significance. We also performed a cost analysis, considering the cost of PGD supplies and test performance and the cost of platelet units wasted.

The institutional review board (IRB) of the study facility determined that this study did not require IRB approval or oversight because the project focused on quality assessment/quality improvement.

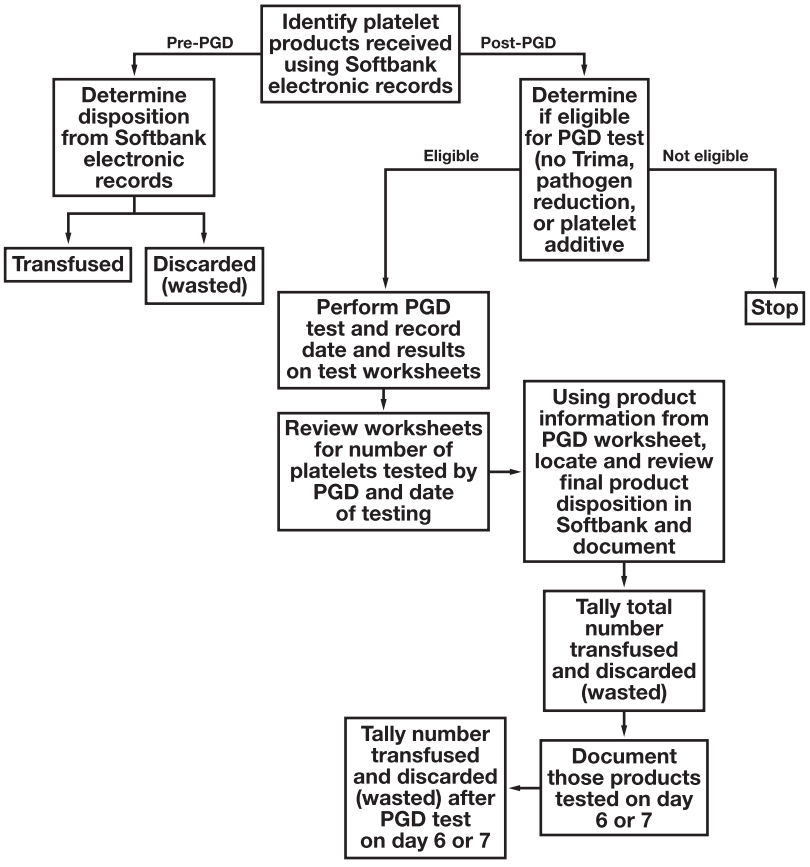
Results

In total, 4516 units of apheresis platelets were received by the transfusion service during the study: 2391 in the pre-PGD period and 2125 in the post-PGD period (**TABLE 1**). Despite the receipt of 266 fewer platelet units in the post-PGD period, platelet usage decreased by only 42 units after PGD implementation (from 1972 to 1930 units). A total of 419 units were wasted in the pre-PGD study period and 195 units were wasted in the post-PGD study period. Platelet wastage was thus reduced from 17.5% to 9.2% after PGD implementation, a statistically significant decrease ($P < .0001$).

TABLE 2 shows the number of platelet units tested and wasted on day 6 and day 7 during the post-PGD period. The number of units tested on day 6 was 366, with 28 units wasted; 133 units were tested again on day 7, with 36 units wasted. Thus, use of PGD testing allowed an additional 302 platelets to be transfused that would have been otherwise discarded.

The results of our cost analysis comparing pre-PGD and post-PGD periods are shown in **TABLE 3**. We used $500.00 as the estimated cost

FIGURE 1. Data collection process in the pre-Pan Genera Detection (PGD) and post-PGD study periods.



of 1 unit of apheresis platelets, as that was the average price from the blood supplier over the study period. The total cost for all platelets received in the post-PGD period was somewhat lower than in the pre-PGD period, as fewer units were received in the second time period. The total cost attributed to wasted units was much lower after PGD testing began, decreasing from $209,500.00 before PGD implementation to $97,500.00 after PGD implementation.

Discussion

The goal of this study was to determine the effectiveness of PGD testing in reducing platelet product wastage and increasing platelet availability while reducing transfusion service costs. We found that PGD testing was associated with a significant reduction in platelet product wastage, from 17.5% before implementation of PGD testing to 9.2% after implementation of PGD testing. Although the number of platelet units received by the transfusion service fell substantially between these time periods, this was accompanied by a much smaller decrease in platelet usage.

Our results were similar to those of previous reports showing reduced platelet wastage after implementation of PGD testing. Dunbar et al[7] reported a decrease in outdated platelets units from 5% to 1% during their 46-month experience with PGD. Harm et al[8] evaluated PGD testing in 2 hospitals and found that PGD implementation was associated with decreases in outdated rates from 5% to 2% in 1 hospital ($P < .0001$) and from 28% to 14% in the other hospital ($P < .001$). In another study, Shahshahani and Taghvai[9] showed that among different types of blood components, platelets had the highest rate of wastage, and platelet wastage decreased from 18.5% to 10.5% after implementing a number of strategies (although not PGD testing) to reduce blood component wastage. Expiration was the most common reason for discarding platelets and RBCs, and even after implementing strategies to reduce wastage, platelets continued to have the highest wastage rate of all blood components because of their short shelf life.

The PGD test allows extension of the platelet shelf life from 5 days to 7 days. When the shelf life is extended an additional 2 days, platelet product availability is increased, and wastage is decreased. Li et al[10] reported that the 2 additional storage days afforded by PGD testing had a marked effect on rates of wasted platelets and increased platelet availability during critical situations. They noted that in 1 facility, the mean platelet discard rate decreased from 24% to 12% after 3 months of implementing a 7-day platelet outdating protocol, and in another facility, the discard rate decreased from 8.7% to 1.7% after 4 months of implementing 7-day outdating. In both facilities, the change in platelet wastage occurred shortly after implementing PGD testing. In our study, 366 platelet apheresis units were tested on day 6 and of those, 133 were tested on day 7, with 28 and 36 units discarded on days 6 and 7, respectively. Thus, PGD testing allowed transfusion of an additional 302 platelet units beyond the traditional 5-day shelf life, and a total of 64 PGD tested platelet products were discarded. Platelets tested on day 6 that were considered for transfusion on day 7 were tested again, according to the facility's procedures.

TABLE 1. Comparison of Platelet Product Disposition before and after Implementation of PGD Testing[a]

Platelet Disposition	Pre-PGD	Post-PGD
Units received	2391	2125
Units transfused	1972	1930
Units wasted	419	195
Percentage wasted	17.5%	9.2%
P value	—	<.0001

PGD, Pan Genera Detection.
[a]Pre-PGD implementation: March 2017 through February 2019. Post-PGD implementation: March 2019 through February 2021.

TABLE 2. Platelet PGD Testing on Days 6 and 7 during the Post-PGD Period

Time Period	Units Tested on Day 6	Units Tested on Day 7	Units Wasted on Day 6	Units Wasted on Day 7
Post-PGD 2019 (Mar–Dec 2019)	157	41	16	12
Post-PGD 2020	198	88	11	24
Post-PGD 2021 (Jan–Feb 2021)	11	4	1	0
Total	366	133	28	36

PGD, Pan Genera Detection.

TABLE 3. Comparison of Costs for Wasted Platelets Between Pre-PGD and Post-PGD Periods

Pre-PGD		Post-PGD	
Variable	**Units or Cost**	**Variable**	**Units or Cost**
Platelet units received	2391	Platelet units received	2125
Cost of 1 apheresis unit	$500.00	Cost of 1 apheresis unit	$500.00
Total cost of received platelets[a]	$1,195,500.00	Total cost of received platelets[a]	$1,062,500.00
Platelet units wasted		Platelet units wasted	
2017 (Mar–Dec 2017)	186	2019 (Mar–Dec 2019)	116
2018	200	2020	71
2019 (Jan–Feb 2019)	33	2021 (Jan–Feb 2021)	8
Total	419	Total	195
Total cost of wasted platelets[a]	$209,500.00	Total cost of wasted platelets[a]	$97,500.00
Estimated hospital cost for Verax PGD test	N/A	Estimated hospital cost for Verax PGD test[b]	$56,312.50

N/A, not applicable; PGD, Pan Genera Detection.
[a]Based on $500.00 per apheresis unit.
[b]Total cost including PGD supplies and technologist time, $26.50/test.[10] Number of test performed in Post-PGD totaled 2125, assuming all platelets received were PGD-tested (total cost × No. of tests performed).

As noted by Stanger et al,[11] inventory management is a trade-off between shortage and wastage. Both Li et al[9] and Stanger et al[10,11] emphasized the use of PGD testing to meet the challenge of maintaining an optimal inventory of platelets while keeping platelet outdates at a minimum.

Economic considerations are increasingly important for most blood banks and transfusion services, and wastage of precious products such as platelets has substantial financial impact. Our cost analysis showed that implementation of PGD testing reduced costs for the transfusion service. By reducing the total number of wasted platelet units, PGD testing decreased the total cost of wasted units from $209,500.00 before PGD implementation to $97,500.00 after implementation. Even if one considers the cost of PGD supplies and test performance, estimated at $56,000, there remains a considerable cost savings. In their recent study, Jacobs et al[12] reported the results of a survey of 66 hospitals using PGD testing. The median reduction in platelet unit outdating was 74% (range, 17% to 100%), and the mean cost savings was $176,803 (range, $30,000 to $1,200,000), which was similar to our results.

This study has some limitations. The demand and supply of platelets was affected by the COVID-19 pandemic, which was the major reason for the reduction in platelets received by the transfusion service in the post-PGD period. Additionally, units that did not meet the volume requirement for PGD testing were not included in this study. Platelets obtained using a Trima apheresis system, processed using a pathogen reduction technique, or stored in platelet additive solution cannot be PGD tested and were also excluded from the study and from all calculations and statistical analysis.

Conclusion

In conclusion, our study provided evidence of reduced platelet product wastage through implementation of PGD testing in the blood bank. The ability to transfuse platelets up to 7 days increases platelet product availability and simplifies platelet inventory management. Outdating platelets affects blood bank costs, as well as human lives. Detecting bacterial contamination by PGD testing on the day of transfusion enhances safety of platelet products and reduces the risk of adverse reactions in transfusion recipients. As platelet products are an expensive, perishable, and limited resource, effectively managing a platelet inventory is challenging. The PGD testing is a useful strategy to reduce platelet product wastage and improve platelet product availability.

Acknowledgments

The authors are grateful for the support of Dr Gutman, the medical director and IRB chairperson at Holy Cross Hospital, Silver Spring, MD, as well as the blood bank manager and all other staff at the Holy Cross Hospital.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Validation of Two Revised, Simplified Criteria for Assessing Sepsis-Associated Disseminated Intravascular Coagulation in ICU Patients with Sepsis-3: A Retrospective Study

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Keywords: sepsis, diagnostic criteria, disseminated intravascular coagulation, sepsis-induced coagulopathy, early-stage DIC, ICU

Abbreviations: ICU, intensive care unit; m-JAAM, modified Japanese Association for Acute Medicine; s-JSTH, simplified Japanese Society on Thrombosis and Hemostasis; SIC, sepsis-induced coagulopathy; ISTH, International Society on Thrombosis and Hemostasis; ROC, receiver operating characteristic; AUC, area under the curve; Sepsis-3, Third Edition of the International Consensus on the Definition of Sepsis; DIC, disseminated intravascular coagulation; SSC, Scientific and Standardization Committee; SIC, sepsis-induced coagulopathy; SIRS, systemic inflammatory response syndrome; TAT, thrombin-antithrombin; SF, soluble fibrin; PF, prothrombin fragment; PT-INR, prothrombin-international normalized ratio; FDP, fibrinogen degradation product; AT, antithrombin; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; PPV, positive predictive value; NPV, negative predictive value; OR, odds ratio; CI, confidence interval; J-SSCG, Japanese Clinical Practice Guidelines for the Management of Sepsis and Septic Shock

Laboratory Medicine 2023;54:291–298; https://doi.org/10.1093/labmed/lmac112

ABSTRACT

Objective: This study aimed to validate the performance of modified Japanese Association for Acute Medicine (m-JAAM) and simplified Japanese Society on Thrombosis and Hemostasis (s-JSTH) criteria in diagnosing coagulation disorders in sepsis and examine their prognostic value compared with sepsis-induced coagulopathy (SIC) and International Society on Thrombosis and Hemostasis (ISTH) criteria.

Methods: This retrospective study included subjects diagnosed with sepsis (August 2020 to February 2021, n = 296). The m-JAAM, s-JSTH, SIC, and ISTH criteria were evaluated and compared using receiver operating characteristic (ROC) curves and areas under the curve (AUCs).

Results: There was no significant difference in AUC for predicting in-hospital 28-day mortality by m-JAAM, s-JSTH, SCI, and ISTH criteria (0.745, 0.763, 0.760, and 0.730, respectively). The proportion of patients fulfilling the m-JAAM and SIC criteria was higher than that of the s-JSTH and ISTH criteria (43.2%, 56.1% vs. 25.0%, 22.6%, $P < .05$).

Conclusion: The m-JAAM criteria might be more suitable for early-stage disseminated intravascular coagulation of sepsis than s-JSTH criteria.

The Third Edition of the International Consensus on the Definition of Sepsis (Sepsis-3) was published in 2016 and defines sepsis as a dysregulated host response to infection that produces life-threatening impairment of organ function.[1,2] Sepsis is associated with in-hospital mortality rates reaching 11.7% to 19.5%[3–5] and late mortality.[6,7]

Coagulation dysfunction plays a very important role in the process of impaired organ function in sepsis.[1] Unlike disseminated intravascular coagulation (DIC) caused by other diseases, a prominent feature of sepsis-associated DIC is the overexpression of plasminogen activator inhibitor-1, resulting in an over suppression of fibrinolysis.[8] In addition, the "immune thrombus" that forms in the early stages of coagulopathy helps the host trap and remove pathogens from the circulation.[9] Anticoagulation at this time is not conducive to patient survival.[10,11] On the other hand, patients with advanced coagulopathy, including many who meet the criteria of overt DIC from the International Society on Thrombosis and Hemostasis (ISTH), can no longer benefit from anticoagulant therapy.[12] Therefore, targeting the right moment to initiate anticoagulant therapy in patients with sepsis has always been a difficult problem for clinicians.

An important signal for anticoagulation in sepsis is the onset of DIC, but the prognosis can be affected if the signal appears

too early or too late. In 2017, the Scientific and Standardization Committee (SSC) on DIC of the ISTH first introduced a new concept, sepsis-induced coagulopathy (SIC), and the corresponding diagnostic criteria.[8,11] The SIC criteria aim to identify the early stages of DIC and find the optimal timing of anticoagulation for sepsis treatment.

Coagulation disorders in sepsis have 2 phases: SIC and sepsis-associated DIC. Currently, there are 3 diagnostic criteria designed for coagulation disorders in sepsis. In addition to the SIC criteria, there are the Japanese Association for Acute Medicine (JAAM) DIC criteria of 2006[12] and the Japanese Society on Thrombosis and Hemostasis (JSTH) DIC criteria of 2016.[13] The diagnostic value of the JAAM criteria for sepsis-associated DIC has been challenged by the introduction of the Sepsis-3 definition. Therefore, in 2016, Iba et al[14] proposed the modified JAAM (m-JAAM) criteria, which replaced the systemic inflammatory response syndrome (SIRS) with antithrombin activity. Because some molecular markers (thrombin-antithrombin [TAT] complex, soluble fibrin [SF], and prothrombin fragment$_{1+2}$ [PF$_{1+2}$]) are difficult to detect routinely, the JSTH criteria are not appropriate for use in emergency critical care units. Hence, the Society revised this scoring system in 2017 to remove molecular marker testing and propose the simplified JSTH (s-JSTH) criteria.[15]

Few studies have examined these 2 improved diagnostic criteria, especially in the Chinese population. Therefore, this study aimed to validate their performance in diagnosing coagulation disorders in sepsis and examine the criteria's prognostic value compared with the SIC and ISTH criteria. The results could help improve the management of patients with sepsis.

Materials and Methods

Study Design and Patients

This retrospective study included persons treated in the intensive care unit (ICU) of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, from August 2020 to February 2021 and diagnosed with sepsis at admission. Sepsis diagnosis met the Sepsis-3 criteria.[1] The exclusion criteria were (1) <18 years of age, (2) hospital stay <24 h, (3) major bleeding, (4) pregnancy, (5) hematologic malignancy, (6) cirrhotic Child-Pugh grade C, (7) admission to hospital for cardiopulmonary resuscitation, anticoagulation, chemotherapy, or radiotherapy, or (8) incomplete clinical information or laboratory data related to this study.

Data Collection and Definitions

The data were fully anonymized during the data collection process. Through the electronic medical record system, we collected clinical information such as subjects' sex, age, comorbidities, infection site, and the results of the biological indicators within 6 h of admission to the ICU (platelet count, prothrombin-international normalized ratio [PT-INR], fibrinogen, fibrin/fibrinogen degradation product [FDP], D-dimer and antithrombin [AT] activity). The Acute Physiology and Chronic Health Evaluation (APACHE) II score[16] and sequential organ failure assessment (SOFA) score[17] were recorded within 24 h of ICU admission. These 2 scores were used to assess disease severity.

The primary outcome measure was all-cause in-hospital 28-day mortality. The SIC,[8] s-JSTH,[13,15] m-JAAM,[12,14] and ISTH criteria[8,18] are listed in Supplementary Table S1.

Statistical Analysis

Statistical analyses were performed using SPSS 22.0 (IBM). The continuous variables with a skewed distribution according to the Shapiro-Wilk normality test were presented as medians and interquartile range and compared between 2 or more than 2 groups using the Mann-Whitney *U* test or the Kruskal-Wallis *H* test, respectively. The categorical data were presented as n (%) and analyzed using the χ^2 test, Fisher's exact test, McNemar test, or kappa test, as appropriate. The relationships between in-hospital 28-day mortality and the prognostic scores were examined using univariable and multivariable logistic regression analyses. Cochran's Q test was used to compare the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Dunn's test (corrected by the Bonferroni method) was used for pairwise comparison after events. Receiver operating characteristic (ROC) curves were used to analyze the predictive value of the 4 criteria for prognosis. The Delong test was used to compare the areas under the curve (AUCs). Two-sided *P* values < .05 were considered statistically significant.

Results

Characteristics of the Subjects

The data of 296 subjects (19–92 years of age, median of 55 years; 189 males and 107 females) with sepsis are shown in **TABLE 1**. The all-cause in-hospital 28-day mortality rate was 30.4% (90/296) in subjects with sepsis. The most common comorbidity was hypertension (37.9%). The major sites of infection were respiratory (64.2%) and abdominal cavity (23.0%).

The subjects were divided into survivors and nonsurvivors according to their in-hospital outcomes. There were no statistically significant differences in sex, age, comorbidities, and site of infection between the 2 groups ($P > .05$). Compared with survivors, the nonsurvivors showed lower platelets (90 vs 152×10^9, $P < .001$), higher PT-INR (1.45 vs 1.23, $P < .001$), longer PT (3 vs 0.8 s, $P < .001$), higher FDP (21.7 vs 11.2 μg/mL, $P < .001$), higher D-dimer (5.7 vs 3.5 μg/mL, $P < .001$), and lower AT activity (64% vs 75%, $P < .001$) (**TABLE 1**).

Univariable and Multivariable Analyses of In-Hospital 28-Day Mortality

The univariable logistic regression analyses showed that the scores of the 6 scoring systems, SOFA (odds ratio [OR] = 1.236, 95% confidence interval [CI]: 1.143–1.337, $P < .001$), APACHE II (OR = 1.134, 95% CI: 1.083–1.187, $P < .001$), SIC (OR = 2.277, 95% CI: 1.797–2.884, $P < .001$), s-JSTH (OR = 1.681, 95% CI: 1.441–1.960, $P < .001$), m-JAAM (OR = 1.496, 95% CI: 1.318–1.698, $P < .001$), and ISTH (OR = 1.843, 95% CI: 1.511–2.248, $P < .001$) were significantly associated with in-hospital 28-day mortality in subjects with sepsis (all $P < .001$) (**TABLE 2**). The multivariable analyses showed that the SIC (OR = 2.053, 95% CI: 1.600–2.633, $P < .001$), s-JSTH (OR = 1.538, 95% CI: 1.309–1.807, $P < .001$), m-JAAM (OR = 1.404, 95% CI: 1.231–1.601, $P < .001$), and ISTH score (OR = 1.652, 95% CI: 1.349–2.221, $P < .001$) were independently associated with in-hospital 28-day mortality.

TABLE 1. Baseline Characteristics of Subjects with Sepsis

Characteristics	Total (n = 296)	Survivors (n = 206)	Nonsurvivors (n = 90)	*P*
Sex (male/female)	189/107	137/69	52/38	.151
Mean age (range), y	55 (42–67)	55 (42–67)	53 (41–67)	.433
Comorbidities, No. (%)				
Hypertension	102 (37.9)	76 (36.9)	26 (28.9)	.183
Diabetes	56 (18.9)	43 (20.9)	13 (14.4)	.194
Heart failure	29 (9.8)	23 (11.2)	6 (6.7)	.231
Chronic lung disease	29 (9.8)	17 (8.3)	12 (13.3)	.176
Chronic liver disease	22 (7.4)	14 (6.8)	8 (8.9)	.528
Chronic kidney disease	18 (6.1)	12 (5.8)	6 (6.7)	.781
Malignant tumor	13 (4.4)	9 (4.4)	4 (4.4)	>.999[a]
Others	44 (14.9)	31 (15.0)	13 (14.4)	.893
Main site of infection, No. (%)				
Respiratory tract	190 (64.2)	139 (67.5)	51 (56.7)	.074
Abdominal cavity	68 (23.0)	47 (22.8)	21 (23.3)	.922
Urinary tract	36 (12.2)	21 (10.2)	15 (16.7)	.117
Others	38 (12.8)	28 (13.6)	10 (11.1)	.557
Biomarkers				
Platelet count, $\times 10^9$/L (range)	134 (77–204)	152 (103–211)	90 (48–180)	<.001
PT-INR (range)	1.27 (1.14–1.53)	1.23 (1.12–1.37)	1.45 (1.26–1.75)	<.001
Prolonged PT, s (range)	1.3 (0.1–3.8)	0.8 (0–2.4)	3 (1.1–5.4)	<.001
Fibrinogen, g/L (range)	4.4 (3.2–5.9)	4.4 (3.3–5.7)	4.4 (3.2–6.2)	.610
FDP, μg/mL (range)	13.4 (5.7–38.5)	11.2 (5.3–27.0)	21.7 (8.8–57.6)	<.001
D-dimer, μg/mL (range)	3.8 (1.9–9.9)	3.5 (1.6–7.7)	5.7 (2.6–13.6)	<.001
AT activity, % (range)	72 (58–85)	75 (61–89)	64 (51–74)	<.001
Severity of illness				
SOFA score	7 (4–9)	6 (3–8)	9 (6–11)	<.001
APACHE II score	13 (10–18)	12 (9–16)	18 (12–21)	<.001
Diagnostic criteria for coagulation disorders				
SIC score	4 (3–5)	3 (2–4)	5 (4–6)	<.001
Simplified JSTH score	2 (1–3.75)	1 (1–3)	3 (3–5)	<.001
Modified JAAM score	3 (1–5)	2 (1–4)	5 (3–6)	<.001
ISTH score	3 (2–4)	3 (2–4)	4 (3–5)	<.001

APACHE, Acute Physiology and Chronic Health Evaluation; AT, antithrombin; DIC, disseminated intravascular coagulation; FDP, fibrin/fibrinogen degradation product; INR, international normalized ratio; ISTH, International Society on Thrombosis and Hemostasis; JAAM, Japanese Association for Acute Medicine; JSTH, Japanese Society on Thrombosis and Hemostasis; PT, prothrombin time; SIC, sepsis-induced coagulopathy; SOFA, sequential organ failure assessment.

[a]*Fisher exact test.*

Value of Indicators in Predicting In-Hospital 28-Day Mortality

TABLE 3 shows the proportion and in-hospital 28-day mortality of subjects fulfilling each of the 4 diagnostic criteria. The proportion of subjects fulfilling the SIC criteria was the highest (56.1%), followed by the m-JAAM (43.2%), s-JSTH (25.0%), and the lowest proportion of subjects fulfilling the ISTH criteria (22.6%) ($P < .001$), but the proportion for the SIC and m-JAAM criteria were similar (56.1% vs 43.2%, adjusted $P > .05$). The positive rates for the s-JSTH and ISTH criteria were similar (25.0% vs 22.6%, adjusted $P > .05$). Subjects with coagulation disorders had significantly higher in-hospital 28-day mortality rates than those without ($P < .001$). Although the risk of death was the highest in subjects with sepsis who met the SIC criteria (OR = 5.218), the difference in in-hospital 28-day mortality between subjects with coagulation disorders fulfilling the diagnostic criteria was not statistically significant ($P > .05$).

TABLE 4 and **FIGURE 1** show the performance parameters for predicting in-hospital 28-day mortality for the 4 diagnostic criteria of coagulation disorders. The AUC of s-JSTH (0.763) was higher than that of ISTH (0.730) and SCI score (0.760). The AUC of m-JAAM (0.745) was higher than that of ISTH (0.730) but lower than for SIC (0.760). There were no significant differences in AUC among the 4 diagnostic criteria (**FIGURE 1**). The SIC criteria had the highest predictive sensitivity (81.1%), yet a post hoc pairwise comparison revealed that there were no significant differences between the SIC and m-JAAM criteria (81.1% vs 67.8%, adjusted $P = .105$, **TABLE 4**), whereas the sensitivity of the ISTH criteria was close to that of the s-JSTH criteria (41.1% vs 42.2%,

TABLE 2. The Univariable and Multivariable Analyses of In-Hospital 28-Day Mortality in ICU

Characteristics	Univariable Analysis, OR (95% CI), *P*	Multivariable Analysis, OR (95% CI), *P*			
		Model 1	Model 2	Model 3	Model 4
Age[a]	0.994 (0.980–1.009), .457				
Sex (male vs female)	0.689 (0.414–1.146), .151	0.759 (0.424–1.356), .351	0.752 (0.424–1.334), .330	0.753 (0.425–1.337), .333	0.697 (0.394–1.230), .213
SOFA score[b]	1.236 (1.143–1.337), <.001				
APACHE II score	1.134 (1.083–1.187), <.001	1.092 (1.040–1.147), <.001	1.089 (1.037–1.143), .001	1.101 (1.050–1.155), <.001	1.101 (1.049–1.156), <.001
SIC score	2.277 (1.797–2.884), <.001	2.053 (1.600–2.633), <.001			
Simplified JSTH score	1.681 (1.441–1.960), <.001		1.538 (1.309–1.807), <.001		
Modified JAAM score	1.496 (1.318–1.698), <.001			1.404 (1.231–1.601), <.001	
ISTH score	1.843 (1.511–2.248), <.001				1.652 (1.349–2.221), <.001

95% CI, 95% confidence interval; APACHE, Acute Physiology and Chronic Health Evaluation; ISTH, International Society on Thrombosis and Hemostasis; JAAM, Japanese Association for Acute Medicine; JSTH, Japanese Society on Thrombosis and Hemostasis; OR, odds ratio; SIC, sepsis-induced coagulopathy; SOFA, sequential organ failure assessment.
[a]Age is included in APACHEII score and does not participate in multivariable analysis.
[b]Some SOFA score items (respiratory SOFA, cardiovascular SOFA, hepatic SOFA, renal SOFA) are included in SIC score system, so SOFA score does not participate in multivariable analysis.

TABLE 3. In-Hospital 28-Day Mortality of Subjects Diagnosed by Each Coagulation Disorder Criteria

Diagnostic Criteria	Cut-off Value	No. (%)	28-Day Mortality, No. (%)	Univariable Analysis		
				OR	95% CI	*P*
SIC	≥4	166 (56.1)	73 (44.0)	5.218	2.878–9.459	<.001
	<4	130 (43.9)	17 (13.1)			
Simplified JSTH	≥4	74 (25.0)[a]	38 (51.4)	3.451	1.988–5.991	<.001
	<4	222 (75.0)[b]	52 (23.4)			
Modified JAAM	≥4	128 (43.2)[c]	61 (47.7)	4.364	2.570–7.410	<.001
	<4	168 (56.8)[d]	29 (17.3)			
ISTH	≥5	67 (22.6)[a,e]	37 (55.2)	4.096	2.314–7.250	<.001
	<5	229 (77.4)[b,f]	53 (23.1)			

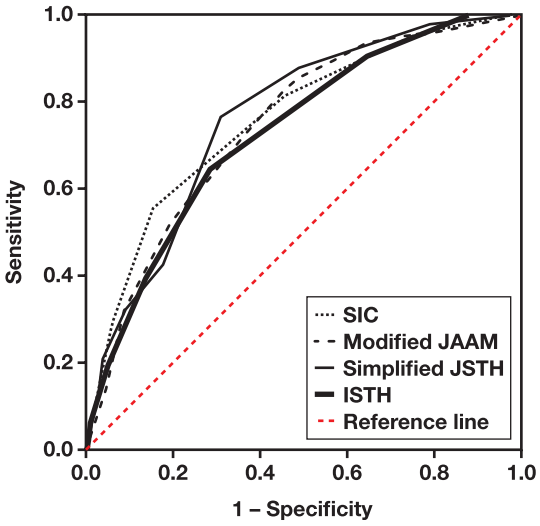
95% CI, 95% confidence interval; ISTH, International Society on Thrombosis and Haemostasis; JAAM, Japanese Association for Acute Medicine; JSTH, Japanese Society on Thrombosis and Hemostasis; OR, odds ratio; SIC, sepsis-induced coagulopathy.
[a]Compared with SIC ≥4, P < *.05.*
[b]Compared with SIC <4, P < *.05.*
[c]Compared with simplified JSTH ≥4, P < *.05.*
[d]Compared simplified JSTH <4, P < *.05.*
[e]Compared with modified JAAM ≥4, P < *.05.*
[f]Compared with modified JAAM <4, P < *.05.*

TABLE 4. Performance for Predicting In-Hospital 28-day Mortality of Diagnostic Criteria for Coagulation Disorders

Diagnostic Criteria	Cut-off	AUC (95% CI)	*P*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SIC	4	0.760 (0.701–0.819)	<.001	81.1	54.9	44.0	86.9
Simplified JSTH	4	0.763 (0.706–0.819)	<.001	42.2[a]	82.5[a]	51.4	76.6
Modified JAAM	4	0.745 (0.686–0.804)	<.001	67.8[b]	67.5[a,b]	47.7	82.7
ISTH	5	0.730 (0.670–0.790)	<.001	41.1[a,c]	85.4[a,c]	55.2	76.9
P				<.001	<.001	.152	.053

95% CI, 95% confidence interval; AUC, area under the curve; ISTH, International Society on Thrombosis and Hemostasis; JAAM, Japanese Association for Acute Medicine; JSTH, Japanese Society on Thrombosis and Hemostasis; NPV, negative predictive value; PPV, positive predictive value; SIC, sepsis-induced coagulopathy.
[a]Compared with SIC, P < *.05.*
[b]Compared with simplified JSTH, P < *.05.*
[c]Compared with modified JAAM, P < *.05.*

FIGURE 1. Receiver operating characteristics (ROC) curves for predicting prognosis of patients with different diagnostic criteria. ISTH, International Society on Thrombosis and Hemostasis; JAAM, Japanese Association for Acute Medicine; JSTH, Japanese Society on Thrombosis and Hemostasis; SIC, sepsis-induced coagulopathy.



Diagnostic Criteria	z Statistic	*P*
SIC vs simplified JSTH	0.143	.886
SIC vs modified JAAM	0.615	.539
SIC vs ISTH	1.365	.172
Simplified JSTH vs modified JAAM	1.170	.242
Simplified JSTH vs ISTH	1.822	.068
Modified JAAM vs ISTH	0.669	.503

adjusted $P > .999$). The ISTH criteria had the highest predictive specificity (85.4%), but a post hoc pairwise comparison showed the s-JSTH criteria had similar predictive specificity (82.5% vs 85.4%, adjusted $P > .999$).

Relationships Between Diagnostic Criteria of Coagulation Disorders

The overlaps of the 4 diagnostic criteria for coagulation disorders are shown in **FIGURE 2**. To identify the risk of DIC in ICU patients with sepsis, the s-JSTH criteria showed a high degree of concordance with the ISTH criteria (kappa = 0.712, $P < .001$), and there were no significant differences between them ($P = .281$) (**FIGURE 2A**). In addition, 1.4% (1/74) of the subjects with DIC diagnosed by the s-JSTH criteria did not fulfill the m-JAAM criteria (**FIGURE 2B**); 10.4% (7/67) of the subjects with DIC fulfilling the ISTH criteria did not meet the m-JAAM criteria (**FIGURE 2C**); 17.2% (22/128) of the subjects with DIC meeting the m-JAAM criteria did not meet the SIC criteria (**FIGURE 2D**). All subjects with DIC meeting the ISTH or the s-JSTH DIC criteria met the SIC criteria (**FIGURE 2E**). The population distributions of coagulation disorders identified by the 4 diagnostic criteria were not completely consistent (**FIGURE 2F**).

Discussion

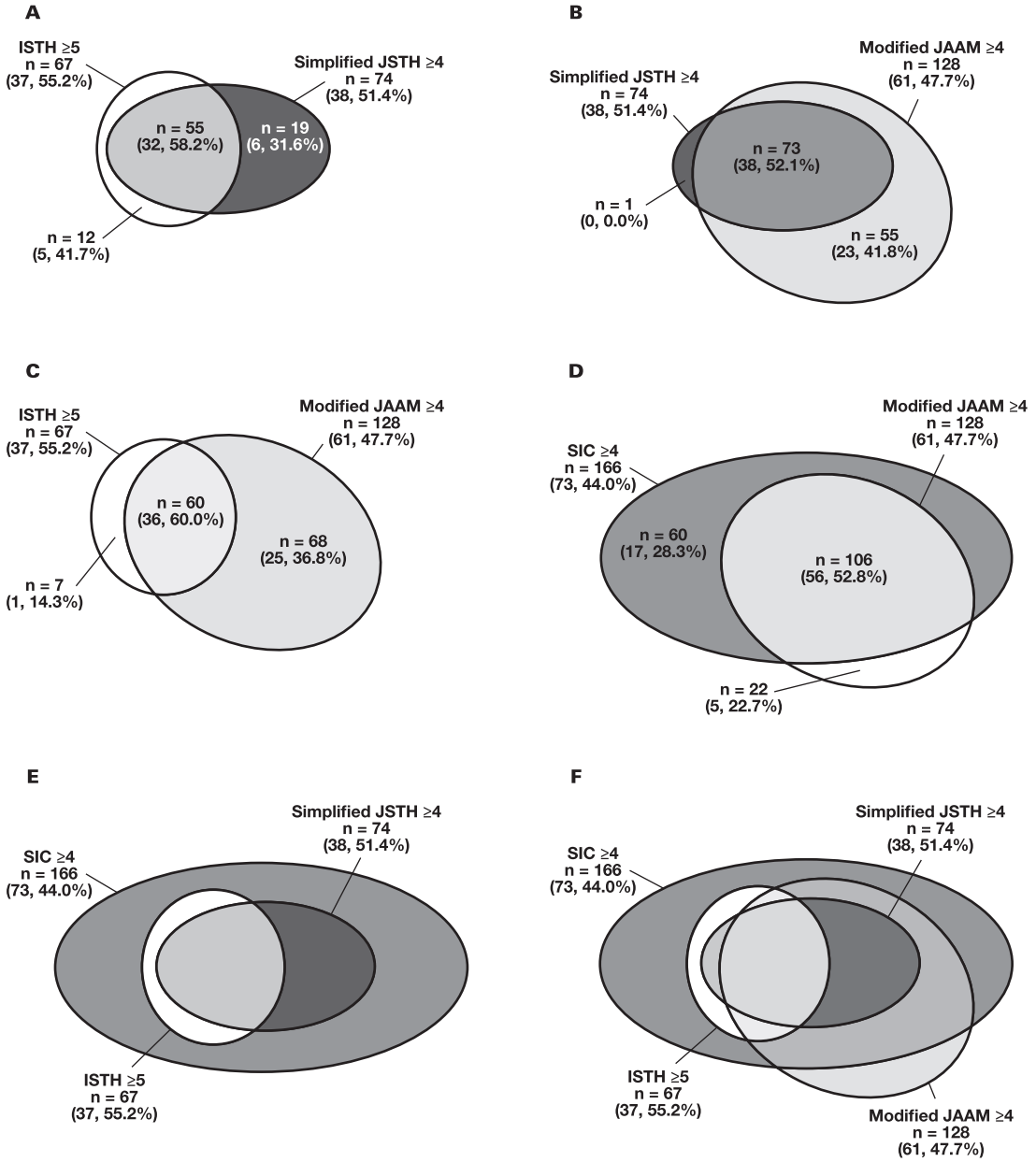
DIC is one of the common and most serious complications of sepsis. Once a patient develops DIC, the mortality rate increases significantly.[19–21] Unfortunately, there are no uniform and gold standard indicators or criteria for the identification of DIC. In 2019, the ISTH guidelines for the diagnosis and management of sepsis[8] indicated that SIC is identified before overt DIC and clarified the relationship between the SIC and ISTH overt DIC diagnostic criteria. Consequently, this study compared 4 diagnostic criteria for coagulation disorders in sepsis (the SIC, m-JAAM, s-JSTH, and ISTH criteria) from 2 main perspectives; namely, the diagnostic rate of coagulation disorders and the predictive value of in-hospital 28-day mortality. The results showed no significant difference in AUC for predicting in-hospital 28-day mortality by m-JAAM, s-JSTH, SCI, and ISTH criteria, and all subjects fulfilling the s-JSTH or ISTH criteria met the SIC criteria, and 82.8% of the subjects fulfilling the m-JAAM criteria met the SIC criteria. This study suggested that the m-JAAM criteria might be more suitable for early-stage DIC of sepsis than the s-JSTH criteria but is not a substitute for the SIC criteria. This study addressed some deficiencies of previous studies[14,15] by comparing the s-JSTH criteria and the m-JAAM criteria each with the ISTH criteria. Moreover, the relationships among the 3 simple diagnostic criteria currently used for sepsis-associated coagulation disorders were examined (the SIC, s-JSTH, and m-JAAM criteria).

The aim of diagnosing SIC and sepsis-associated DIC is to determine the timing of the initiation of anticoagulant therapy to ensure the immune role of the initial thrombus without delaying its management to the late stages of coagulopathy and to identify candidates who might benefit from anticoagulant therapy to improve their survival.[19,22] For anticoagulation in sepsis and septic shock, the anticoagulants that have been discussed more frequently are antithrombin, heparin, and thrombomodulin, but the evaluation of the efficacy of these anticoagulants is still controversial. For instance, the 2016 version of the international guideline[23] does not recommend antithrombin for all patients with sepsis and septic shock (including those with DIC) because antithrombin fails to significantly reduce patient mortality but instead increases the risk of bleeding. The anticoagulants commonly used in China are low-molecular-weight heparin followed by unfractionated heparin.[24] In contrast, the Japanese Clinical Practice Guidelines for the Management of Sepsis and Septic Shock (J-SSCG 2016),[25] the 2019 SSC on DIC, and the SSC on Perioperative and Critical Care of the ISTH[8] recommend antithrombin for DIC patients in sepsis with antithrombin activity <70%, considering that septic patients with severe coagulopathy could benefit from it.[15]

Activation of the coagulation system, downregulation of physiological anticoagulants, and inhibition of the fibrinolytic system are 3 major systemic dysfunctions that are present throughout the pathology of DIC in sepsis.[26–28] Platelet count and PT-INR reflect hemostasis/coagulation, FDP reflects fibrinolysis, and AT activity reflects anticoagulation. The first 3 biomarkers are traditional coagulation tests that are often adopted in the diagnostic criteria for DIC. The proportion of patients with sepsis with AT activity ≤70% in this study was 46.6% (138/296), corresponding to an in-hospital 28-day mortality rate of 42.0% (58/138). Reduced AT activity has been reported to be closely associated with sepsis mortality.[14] The major causes of reduced AT activity are increased consumption, slowed or impaired synthesis, and extravasation due to increased vascular permeability.[14]

The JAAM criteria are a diagnostic set of criteria designed not only for acute diseases (including sepsis and trauma) but also for determining the optimal timing of anticoagulant therapy.[19] The JAAM criteria were recommended by the J-SSCG 2016 and have been widely used in emergency and intensive care units in Japan.[29] As the SIRS score adopted in the criteria only assessed the systemic inflammatory status and did not directly reflect the presence of coagulation/fibrinolytic abnormalities, it was no longer

FIGURE 2. **Distribution diagram of patients according to the sepsis-induced coagulopathy (SIC), modified Japanese Association for Acute Medicine (JAAM), simplified Japanese Society on Thrombosis and Hemostasis (JSTH), and International Society on Thrombosis and Hemostasis (ISTH) diagnostic criteria (n = 296; n = 90 [30.4%] nonsurvivors). The numbers outside the parentheses indicate the number of eligible patients, and the numbers inside the parentheses indicate the number of nonsurvivors and the mortality rates for the patients who should be eligible. A, Overlaps of ISTH overt disseminated intravascular coagulation (DIC) and simplified JSTH DIC. κ = .712, *P* < .001; McNemar test: *P* = .281. B, Overlaps of simplified JSTH DIC and modified JAAM DIC. κ = .594, *P* < .001; McNemar test: *P* < .001. C, Overlaps of ISTH overt DIC and modified JAAM DIC. κ = .453, *P* < .001; McNemar test: *P* < .001. D, Overlaps of SIC and modified JAAM DIC. κ = .455, *P* < .001; McNemar test: *P* < .001. E, Overlaps of SIC, simplified JSTH DIC, and ISTH overt DIC. F, Overlaps of 4 diagnostic criteria for coagulation disorders in sepsis.**



adapted to the requirements of the latest Sepsis-3 definition and had to be adjusted accordingly to continue to take advantage of the JAAM criteria. Therefore, in 2016, Iba et al[14] proposed the m-JAAM criteria by replacing SIRS ≥3 with AT activity <70% and keeping the other biomarkers (platelet count, PT-INR, and FDP). The s-JSTH criteria were not only easy to use but specifically designed to diagnose the early stages of DIC in sepsis.[15] Compared with the original JSTH criteria, the s-JSTH criteria retained 4 routinely assessed biomarkers (platelet count, PT-INR, FDP, and AT activity) and discarded molecular markers (TAT complex, SF, and PF_{1+2}) that were not routinely performed. The 4 biomarkers (platelet count, PT-INR, FDP, and AT activity) used in the m-JAAM and s-JSTH criteria are in accordance with the understanding of DIC pathogenesis in sepsis.

The difference between the m-JAAM criteria and the s-JSTH criteria is that the former has relatively lenient scoring boundaries and the score

given (eg, FDP ≥10 μg/mL in the m-JAAM criteria scores 1 point and ≥25 μg/mL scores 3 points, while FDP ≥20 μg/mL in the s-JSTH criteria only scores 1 point) (see Supplementary Table S1), which inevitably results in more patients being included in the criteria and a wider range of inclusion. These 2 points have been confirmed by the present study. The predictive sensitivity of in-hospital 28-day mortality and predicted risk of death in patients with DIC were higher using the m-JAAM criteria than the s-JSTH criteria. As a result, the m-JAAM criteria are more suitable for screening in the early stages of DIC in sepsis than the s-JSTH criteria.

The SIC criteria appeared to have the highest diagnostic positivity and sensitivity for predicting in-hospital 28-day mortality, followed by the m-JAAM criteria, but the difference was not statistically significant between them. The relationship between the SIC criteria and the ISTH criteria has been repeatedly reported previously.[22,29,30] The present study reconfirmed that all patients with sepsis fulfilling the ISTH criteria were consistent with the SIC criteria.[22] Compared with the ISTH criteria, the SIC criteria facilitated the early recognition of overt DIC patients with sepsis,[29] enabling earlier screening of populations with sepsis who might benefit from anticoagulant therapy.[22] To this end, in 2019, ISTH proposed a simplified sequential scoring 2-step approach[8] for the early identification of DIC; that is, first screening with the SIC score and then calculating the overt DIC score for patients who met the SIC criteria, which greatly increased the likelihood of patients with sepsis who would benefit from anticoagulant therapy.

As the 3 diagnostic criteria to identify the early stages of DIC in sepsis, the m-JAAM criteria and the s-JSTH criteria require only 4 routine laboratory indicators. For the SIC criteria, although only 3 items are involved in the scoring system, the third item (the SOFA score) requires collecting the results of 4 other indicators, such as respiratory, cardiovascular, hepatic, and renal scores, in addition to platelet count and PT-INR for detection and acquisition of results. Therefore, its calculation is not as fast and convenient as the revised criteria. Nevertheless, in terms of the scientific value and preciseness of the diagnostic criteria design, the SIC criteria adopted the SOFA score, which is more in line with the Sepsis-3 definition. It is emphasized here that if the development of diagnostic criteria is simply to modify the cut-off values and/or scores of these biomarkers, all that will change is the sensitivity and specificity of predicted mortality, and ultimately, it will be difficult to make a major breakthrough.[19]

The s-JSTH criteria were the most similar to the ISTH criteria in DIC diagnostic rate, DIC population mapping, and predictive efficacy of in-hospital 28-day mortality. Although the diagnostic concordance between these 2 criteria was high and the difference in diagnosis was not statistically significant, the in-hospital 28-day mortality rate was found to be 10.1% higher in patients who met the ISTH criteria but not the s-JSTH criteria than in those who met the s-JSTH criteria but not the ISTH criteria. Therefore, whether the s-JSTH criteria have equivalent clinical utility as the ISTH criteria in diagnosing septic DIC should be considered with caution, and prospective studies are needed to confirm this finding.

This study had limitations. First, as a single-center retrospective study with a limited number of cases, data representativeness might be biased and needs to be further validated in a multicenter, large-sample prospective study. Second, anticoagulant efficacy could not be discussed due to the small number (13.9%) of patients treated with anticoagulants.

In conclusion, there was no significant difference in AUC for predicting in-hospital 28-day mortality by m-JAAM, s-JSTH, SCI, and ISTH criteria. The population distributions of coagulation disorders identified by the 4 diagnostic criteria were not completely consistent. All patients fulfilling the s-JSTH or ISTH criteria met the SIC criteria, and most patients (82.8%) fulfilling the m-JAAM criteria met the SIC criteria. This study suggests that the m-JAAM criteria might be more suitable for early-stage DIC of sepsis than the s-JSTH criteria but is not a substitute for the SIC criteria. However, the application of these 2 revised, simplified criteria in patients with sepsis needs further evaluation.

Supplementary Data

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

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Establishment and Evaluation of Recombinant Expression of HCV Transmembrane Protein (p7) and Detection of Anti-p7 Antibody in Serum of HCV-Infected Patients by Chemiluminescence

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Keywords: hepatitis C virus, transmembrane protein p7, antibody, detection, evaluation, chemiluminescence method

Abbreviations: HCV, hepatitis C virus; p7, transmembrane protein; PCR, polymerase chain reaction; GST, glutathione S-transferase; DTT, dithiothreitol; RLIV, relative luminescence intensity value; RLIR, relative luminescence intensity ratio; ELISA, enzyme-linked immunosorbent assay; HIS, histidine; Ni-NTA-agarose, nickel nitrilotriacetic acid agarose; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LOB, limit of blank; LOD, limit of detection

Laboratory Medicine 2023;54:299–307; https://doi.org/10.1093/labmed/lmac113

ABSTRACT

Objective: Our aim was to establish a chemiluminescence method for detecting anti-transmembrane protein (p7) antibody in the serum of patients with hepatitis C virus (HCV) infection.

Methods: The *p7* gene was amplified by polymerase chain reaction using the plasmid PUC-p7 containing the p7 nucleic acid sequence of the HCV 1b genotype as the template, and recombinant plasmid pGEX-KG-p7 was constructed. After p7 fusion, the protein was induced and expressed in the prokaryote, extracted, and purified; the anti-p7 antibody detection kit was prepared, and its efficacy was evaluated.

Results: The plasmid pGEX-KG-p7 was constructed correctly, and p7 fusion protein was obtained. The methodological indexes of the kit, the precision test, blank limit and detection limit, etc, met the requirements. The positive rate of serum anti-p7 antibody in 45 patients with HCV infection was 20%.

Conclusions: The kit can be used in screening diagnosis, condition monitoring, prognosis, and disease mechanism and epidemiological study of HCV infection. The p7 protein has immune response in HCV-infected patients.

Hepatitis C virus (HCV) is the pathogen causing hepatitis C, which can lead to chronic inflammation, necrosis, and fibrosis of the liver; some patients can develop liver cirrhosis and even hepatocellular carcinoma.[1] The nucleocapsid protein (Core), envelope proteins (E1, E2), and transmembrane protein (p7) of HCV are the main components of the HCV coat protein skeleton. The p7 is a small hydrophobic protein composed of 63 amino acid residues encoded by 2580 to 2768 nucleotides of the HCV genome.[2] Its coding gene is located between structural and nonstructural proteins and integrated on the endoplasmic reticulum membrane after expression in cells; the cationic channel of the hexamer is formed to facilitate the release of mature virus particles and promote the release of HCV virus particles, which is closely related to the synthesis of HCV virus particles and the mechanism of HCV infection.[3] The p7 can regulate the envelope of newborn virus particles and the pH value of some intracellular compartments, which may be of great importance to the protection and secretion of infectious particles.[4] Other studies have shown that one of the functions of p7 is to protect HCV glycoprotein from premature degradation during virus particle morphogenesis.[5] The development of HCV vaccines mostly focuses on targeting the HCV core, envelope, or unstructured NS3-NS5B protein; however, it is crucial to note that p7 is also targeted by T cells in HCV patients.[6] Several amino acids of p7 are conserved in HCV genotypes, and p7 ion channel activity can be specifically inhibited by different drugs, suggesting that p7 may represent a potential target for antiviral therapy and vaccine development.[7] However, there is no kit for detecting p7 single antibody at present, and the fragment of p7 protein is small, making it difficult to express and purify.[8] Therefore, we recombined and expressed p7 protein, established a method for detecting anti-p7 antibody in serum of HCV-infected persons, and evaluated its application. This research will provide technical support for further understanding the immune response of p7 protein in patients with hepatitis C virus infection, the correlation between the reaction products anti-p7 antibody and circulating immune complex and different hepatitis C genotypes, different clinical stages, and pre- and post-antiviral treatment.

Materials and Methods

Materials and Reagents

Relevant materials for recombinant expression of p7 protein included plasmid PUC-p7 (synthesized by Hangzhou Qingke Biotechnology) containing HCV 1b genotype p7 nucleic acid sequence (accession No. AJ238800), *Escherichia coli* competent cell BL21(DE3) (preserved in our laboratory), restriction endonucleases *Bam*HI and *Hind*III (NEB), KOD-PLUS series high fidelity polymerase (TOYOBO), T4 DNA ligase (Shanghai Sangon Bioengineering), glutathione S-transferases (GST protein) (100 μg, Beijing Yiqiao Shenzhou Technology), D2000 DNA marker (100-2000 bp, Mona Biotechnology), D5000 DNA marker (100-5000 bp, Mona Biotechnology), protein marker (14.4-116.0 kDa, Thermo Scientific), and a nickel nitrilotriacetic acid agarose (Ni-NTA-agarose) affinity chromatography column (QIAGEN).

Relevant materials for detecting anti-p7 antibody in serum included Microplate Nunc (Thermo Fisher), coating solution (0.02 mol/L phosphate buffer at pH 7.4), blocking solution (containing 5% bovine serum albumin, 1% sucrose, 4% gelatin, 0.2% Proclin 300 preservative, 0.01 mol/L phosphate buffered saline at pH 7.4), washing solution (containing 0.5% Tween-20, 0.2% Proclin 300 preservative, and 0.01 mol/L phosphate-buffered saline at pH 7.4), sample diluent (containing 20% bovine serum, 0.01 mol/L phosphate-buffered saline at pH 7.4), substrate solution B (containing 0.3 g/L carbamide peroxide, 0.2 mol/L phosphate buffer at pH 7.4), substrate solution A (containing 0.8 g/L luminol, 0.008 g/L o-phenylphenol, 0.025 g/L 4-imidazolophenol, 0.1 mol/L carbonate buffer solution at pH 9.0), enzyme-labeled antibody diluent solution (containing 5% bovine serum albumin, 0.5% enzyme stabilizer, 0.1% Tween-20, 5 μg/mL anti-mouse antibody blocker, 1 μg/L red pigment, 0.2% Proclin 300 preservative, 0.01 mol/L phosphate-buffered saline at pH 7.4), and enzyme marker working solution (obtained by diluting 1 mg/mL horseradish peroxidase labeled goat anti-human IgG marker stock solution with enzyme-labeled antibody dilution. The stock solution was purchased from Hangzhou Longji Biotechnology).

Negative and positive controls were heat inactivated non-HCV-infected (anti-p7 antibody–negative) and HCV-infected (anti-p7 antibody–positive) mixed serum or plasma (containing 0.2% Proclin 300 preservative), and the tests of human immunodeficiency virus antibody, *Treponema pallidum* antibody, and hepatitis B virus surface antigen were negative.

Serum Collection

Informed consent was obtained from all participants included in the study. Venous blood of 45 HCV-infected persons (Xiamen Xinchuang reagent screening anti-HCV antibody–positive, research group) and 50 healthy persons (anti-HCV antibody–negative, control group) were randomly used to collect serum, or plasma was collected by anticoagulation with disodium EDTA or potassium salt. The serum or plasma samples to be tested within 1 week can be stored at 2°C to 8°C. If more time is required, they should be placed at −20°C; repeated freezing and thawing and cross-contamination shall be avoided.

The main instruments used were the JY92-II ultrasonic cell pulverizer (Ningbo Xinzhi Biotechnology), Mini-Protean Tetra electrophoresis apparatus (Bole), Tanon 3500 fully automatic digital gel image analysis system (Shanghai Tianneng Technology), and the TZD-CL-200S chemiluminescence immunoassay analyzer (Xiamen Tianzhongda Biotechnology).

Experimental Method

In the polymerase chain reaction (PCR) amplification of full-length p7 of HCV, the primers of p7 gene were (p7f:5'-ATCGGA TCTGGTTCCGCGT*GGATCC*ACCCTAGAGAACCTGGTGGTCC-3', p7r:5'-TTAGTGGTGGTGGTGGTGGGCGTATGCTTGTGGTGGTAA-3',p7r1:5'-AGTCAGTCACGATGAATT *AAGCTT***TTAGTGGTGGTGGTG GTGGTG**-3'). *Bam*HI and *Hind*III digestion sites (italics) were introduced into the primers; 6 histidine (HIS) and stop codon TAA (bold part) were introduced into the downstream primer p7r1. The synthesized PUC-p7 plasmid was used as the template, p7f and p7r were used as primers for the first round of PCR amplification, and then the first round of PCR products were used as the template and p7f and p7r1 were used as primers for the second round of PCR amplification. The amplification parameters of the 2 rounds of PCR were pre-denatured at 94°C for 2 min, then 30 cycles at 94°C for 15 s, 53°C for 30 s and 68°C for 30 s, and finally extended at 72°C for 7 min.

Identification of PCR Products and Construction of Recombinant Plasmid pGEx-KG-p7

The PCR products and expression vector pGEx-KG were digested with restriction enzymes *Bam*HI and *Hind*III, and the enzyme digestion system was 50 μL. The enzyme digestion reaction system consisted of PCR product/pGEx-KG 43 μL, *Bam*HI 1 μL, *Hind*III 1 μL, enzyme digestion buffer NEBuffer 2.1 5 μL (0.5 mol/L Tris-HCl buffered saline solution containing 1 mol/L NaCl, 100 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol (DTT), and pH 7.9). After mixing, digestion was completed in a 37°C water bath for 3 to 4 h, and after digestion was completed, the desired target fragments and vector fragments were recovered by agarose gel. After recovery, the PCR tube was removed the loading buffer 6× Loading Buffer 9 μL (containing 0.05% bromophenol blue, 0.05% xylene nitrile blue FF, 36% glycerol, 30 mmol/L EDTA, adjust the pH to 7.0 with 2 mol/L NaOH) was added, mixed, and the sample was collected at mass concentration 1.0% agarose gel electrophoresis, voltage 150 V, time 8 min. When electrophoresis was finished, gel was placed in the gel imaging system to observe whether the size of PCR amplification fragment was about 189 bp. Then the gel containing the target fragments was cut and transferred to 2 new aseptic 1.5 mL centrifuge tubes, the gel pieces were chopped, and the AXYGEN gel recovery kit was used to recover DNA fragments. The target fragments digested by restriction enzymes *Bam*HI and *Hind*III and the expression vector pGEx-KG were connected with T4 DNA ligase at 16°C for 8 to 12 h. Finally, recombinant plasmid pGEx-KG-p7 was sent to Hangzhou Qingke Biotechnology for sequencing to check whether it was consistent with the sequence of p7 target fragment.

Induced Expression of p7 Protein

The plasmid P703 was transformed into *E coli* competent cell BL21 (DE3) and cultured in 100 mg/L ampicillin 2× yeast extract peptone agar medium at 37°C for 3 to 4 h. When the absorbance OD600 was between 1.0 and 2.0, 1 m mol/L isopropyl thiogalactoside was added to induce culture for 3 h.

Extraction and Purification of p7 Protein

The bacteria were collected by centrifugation and resuspended in pH 8.0 buffer A (50 mmol/L Tris-HCl buffered saline solution containing

0.2 mol/L NaCl at pH 8.0) for ultrasonic crushing; the precipitated inclusion bodies after centrifugation were washed once with pH 8.0 buffer B (50 mmol/L Tris-HCl buffered saline solution containing 0.15 mol/L NaCl, 1% Triton X-100 at pH 8.0); the precipitate after washing and centrifugation was resuspended and dissolved with pH 8.0 buffer solution C (containing 0.5 mol/L NaCl, 8 mol/L urea, 10 mmol/L imidazole, 50 mmol/L Tris-HCl buffered saline solution at pH 8.0), purified by Ni-NTA-agarose affinity chromatography column, and the target protein was collected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

p7 Protein Renaturation

The collected purified protein was treated and dialyzed for renaturation. The purified protein was calibrated as 1.0 mg/mL, 1 mmol/L DTT was added, and the reaction was performed at room temperature for 30 min; 0.1% SDS was added and reaction was performed at room temperature for 15 min. After the reaction, the sample was loaded into a dialysis bag containing dialysis buffer at 1:100 for dialysis, and the dialysis buffer (0.01 mol/L phosphate buffer at pH 7.5) was used for dialysis.

Preparation of Microplates for Detecting anti-p7 Protein Antibodies and GST Protein Antibodies

The microplates for detecting anti-p7 protein antibodies and GST protein antibodies were prepared based on the principle of indirect method. The optimal coating concentration of recombinant HCV transmembrane protein p7 antigen was selected by square matrix (chessboard) titration method, and then the recombinant HCV transmembrane protein p7 antigen was diluted to the optimal working concentration (the same concentration of GST protein) with coating solution, 100 μL of the diluted HCV transmembrane protein p7 antigen was added to the microplate, which was set at 2°C to 8°C for 20 to 24 h, and 300 μL washing solution was added to each well to wash away the protein not adsorbed on the plate. The wash was repeated 3 times and patted dry; 200 μL sealing liquid was added per hole and set at 2°C to 8°C for 20 to 24 h and patted dry; the sealing film was pasted and it was put into the sealing bag, desiccant added, and refrigerated at 2°C to 8°C.

Preparation of Optimal Enzyme Marker Working Solution

To prepare optimal enzyme marker working solution, enzyme-labeled antibody diluent was used to dilute 1 mg/mL horseradish peroxidase-labeled goat anti-human IgG marker stock solution, and the optimal enzyme marker working solution was selected by the square matrix (chessboard) titration method.

Detection of Anti-p7 Antibody and Anti-GST Protein Antibody in Serum

For sample loading, the coated microplate was fixed on the plate rack, 2 wells for negative control and 1 well for positive control for each test were set, and 100 μL of negative and positive controls were added. A blank control well without sample was added. Then 100 μL of sample diluent was added to the remaining wells and 10 μL of each sample to be tested in parallel to the anti-p7 antibody and anti-GST protein antibody microplates were added and the sealing plate was covered, shaken, and mixed well, incubated at 37°C ± 1°C for 30 min, and washed to remove unbound components. The same enzyme marker working solution (100 μL) was then added (except in blank control wells) to the other wells. The plates were covered with sealing film, shaken to mix, and incubated at 37°C±1°C for 30 min. The liquid in the wells was discarded, each well was filled with washing solution, and let stand for no more than 60 s. The washing solution in the wells was discarded, washing repeated 5 times, and patted dry. For color development, 50 μL of substrate solution B and 50 μL of substrate solution A were added to each well in turn, shaken and mixed at room temperature, and placed in the dark for 15 min. For detection, the relative luminescence intensity value (RLIV) of each well was measured with TZD-CL-200S chemiluminescence immunoassay analyzer, and the relative luminescence intensity ratio of each well (RLIR = sample RLIV/negative control RLIV mean) was calculated.

Result Judgment

When the ratio of sample RLIV in anti-p7 antibody microplate to the mean value of negative control RLIV was ≥2.1 and the ratio of sample RLIV in the anti-GST antibody microplate to the mean value of negative control RLIV was <2.1, the result was positive. When the ratio of the sample RLIV in the anti-p7 antibody microplate to the mean value of the negative control RLIV is ≥2.1 and the ratio of the sample RLIV in the anti-GST antibody microplate to the mean value of the negative control RLIV was ≥2.1, the result was negative (anti-GST antibody is present in the patient). When the ratio of sample RLIV in the anti-p7 antibody microplate to the mean value of the negative control RLIV was <2.1 and the ratio of the sample RLIV in the anti-GST antibody microplate to the mean value of the negative control RLIV was <2.1, the result was negative. In terms of quality control, the blank value should be <30,000 RLIV, each negative control value should be ≤115,000 RLIV, and the difference between the readings of 2 negative control wells and the average value of the negative control should be ≤10%; each positive control value should be >231,500 RLIV; otherwise, the test is invalid and must be repeated.

Methodological Index Evaluation of Anti-HCV Transmembrane Protein p7 Antibody Detection Kit

In the precision test,[9,10] for imprecision within the batch, the screened high and low concentration samples were used for detection that was repeated 20 times (well) for each, the average value of RLIR and SD were calculated and the CV% within the batch (<10% is qualified) was calculated. For interbatch imprecision, the high and low concentration samples that had been screened were tested once every morning and afternoon, 20 batches for 10 days, then the average value of RLIR and SD was calculated and the inter batch CV% (<15% is qualified) calculated.

For the limit of blank (LOB), 5 sera of uninfected HCV subjects were selected and 2 batches were tested every day for 6 days. The RLIR value was calculated and 60 results in total were obtained. By type I error ($\alpha = 0.05$), LOB is 5% more likely to contain the substance to be tested; the results of blank samples were sorted from small to large by nonparametric test, and the 95th percentile value is the blank limit LOB. For the limit of detection (LOD), 5 serum samples with expected concentration of 1 to 5 times LOB as low concentration samples were selected and tested twice a day for 6 consecutive days. The LOD was calculated by nonparametric analysis method; 5 low-concentration samples were determined 12 consecutive times, and the results showed nonnormal distribution. A nonparametric program was used to estimate LOD; that is, LOD = LOB + Ds.β, Ds, where β is the difference between the median (M) and the 5th percentile of the measured value of the low concentration sample.

The linear evaluation and analysis measurement range were designed according to the requirements of the EP6-A2 document[11]

The serum of subjects not infected with HCV (low value L, 1.252 RLIR) and the anti-p7 antibody serum of persons infected with HCV (high value H, 18.774 RLIR) were used to prepare 6 samples according to a ratio of 5L, 4L + 1H, 3L + 2H, 2L + 3H, 1L + 4H, and 5H, and each sample was tested twice and the mean value, the expected mean, and measured mean were used as abscissa and ordinates for regression analysis ($r^2 > 0.95$). To evaluate accuracy,[12] the anti-p7 antibody serum with a concentration of 37.5 RLIR (obtained by ultrafiltration concentration of U-Tube protein) was added to the anti-p7 negative serum in the proportion of 5% and 10%, respectively, parallel detection was conducted 3 times, and the average recovery (90%–110% is qualified) was calculated. In an interference experiment, the serum samples that were positive for rheumatoid factor, hepatitis B virus surface antibody, hepatitis B virus e antibody, *T pallidum* antibody, or human immunodeficiency virus type I and/or type II antibody were mixed with anti-p7 antibody positive (9.332RLIR), and the sample diluent was used as the control to observe whether there was cross-reaction. In an observation of stability, the same batch of anti HCV transmembrane protein p7 antibody detection kit (including independently packaged coated microplate, sample diluent, negative control, positive control, enzyme marker working solution, substrate solution a, substrate solution B, and washing solution) was stored at 2°C to 8°C, and the same positive control serum was detected at 0, 3, 6, 9, 12, 15, and 18 months, 3 repeats were made each time, the average value was taken, and stability was observed over time (compared with the first time, <10% was qualified). As an example application, the prepared anti-HCV transmembrane protein p7 antibody and anti-GST protein antibody detection kit were used to detect the anti-p7 antibody in the sera of 45 HCV-infected persons and 50 healthy persons, respectively. The positive rates of anti-p7 antibody in the 2 groups were counted and compared.

Statistical Analysis

The χ^2 test was used to compare the anti-p7 antibody test results between the study group and the control group. When $P < .05$, the difference was statistically significant.

Results

Amplification of the *p7* Gene and Construction of Expression Vector pGEX-KG

Using the synthesized PUC-P7 plasmid as the template, the full-length *p7* gene was amplified by PCR (**FIGURE 1**). After enzyme digestion, electrophoresis identification showed that the insertion size of p7 nucleic acid fragment was about 189 bp (excluding HIS), which was consistent with the expected size (**FIGURE 2**). The PCR product was connected to the pGEX-KG vector and sequenced correctly (**FIGURE 3**), and the plasmid pGEX-KG-p7 was obtained.

Expression, Purification, and Renaturation of *p7* Gene in *E coli* Competent Cells BL21(DE3)

Plasmid pGEX-KG-p7 was transformed into *E coli* competent cell BL21 (DE3) and induced to express GST fusion protein with a molecular weight of 34 kDa (**FIGURE 4**). After ultrasonic fragmentation,

FIGURE 1. Identification of the second round of polymerase chain reaction amplified p7 gene products by agarose gel electrophoresis. Lane 1, D2000 marker; lane 2, amplified products.

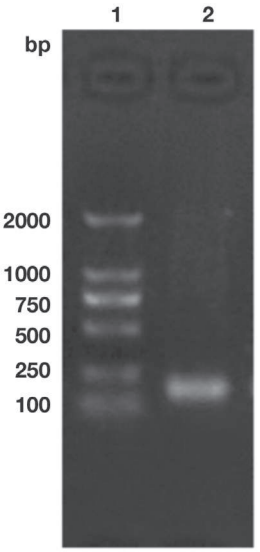
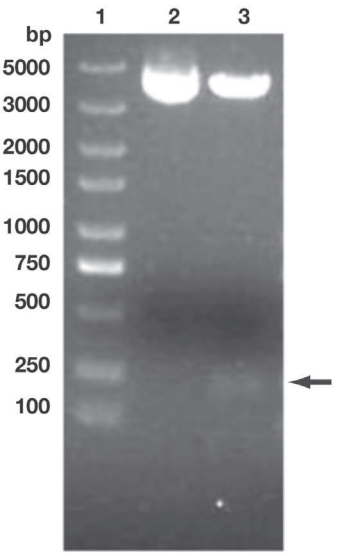


FIGURE 2. Identification of p7 recombinant protein expression plasmid by restriction enzyme digestion. Lane 1, D5000 marker; lane 2, pGEX-KG-p7 recombinant plasmid; lane 3. pGEX-KG-p7 recombinant plasmid digested by *Bam*HI and *Hind*III.

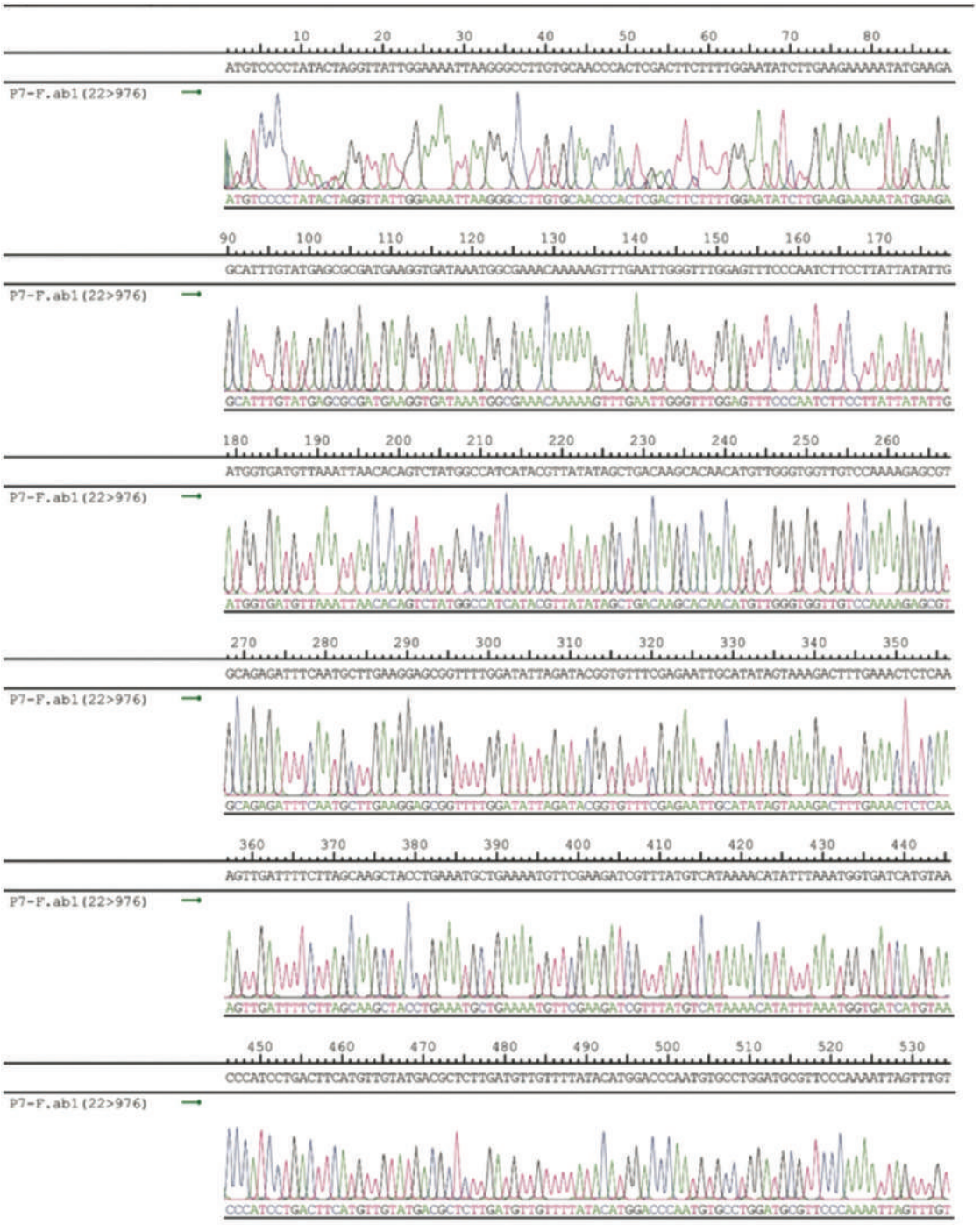


SDS-PAGE showed that the expressed protein formed inclusion bodies in the precipitation. The stable protein was obtained by inclusion body cleavage and dialysis renaturation after Ni-NTA-agarose affinity chromatography.

Optimal Coating Concentration of Recombinant HCV Transmembrane Protein p7 Antigen and Selection of Enzyme Marker Working Solution

The optimum coating concentration of recombinant HCV transmembrane protein p7 antigen and working solution of enzyme marker was

FIGURE 3. Sequencing map of p7 gene linked to pGEX-KG vector.



0.5 μg/mL and 0.05 μg/mL, respectively, and the optimum sample dosage was 100 μL (**TABLE 1**).

Evaluation of Kit Methodological Indicators

In the precision test, the intrabatch coefficient of variation was CV% <10% and interbatch coefficient of variation was CV% <15% (**TABLE 2**); the blank limit and detection limit were 1.56 RLIR and 5.99 RLIR, respectively. The linear regression equation is y = 1.0991x – 0.3412, the regression coefficient was between 0.97 and 1.03, the correlation coefficient was $r \geq 0.975$ ($r^2 \geq 0.95$), and the analytical measurement range was 1.252 to 18.774 RLIR. The accuracy evaluation found that the average recovery rate was 97.8%. The interference test showed serum samples positive for rheumatoid factor, hepatitis B virus surface antibody, hepatitis B virus e antibody, *T pallidum* antibody, human immunodeficiency virus type I and/or type II antibody had no cross-reaction to this test. The results of the kit

FIGURE 3. (cont)

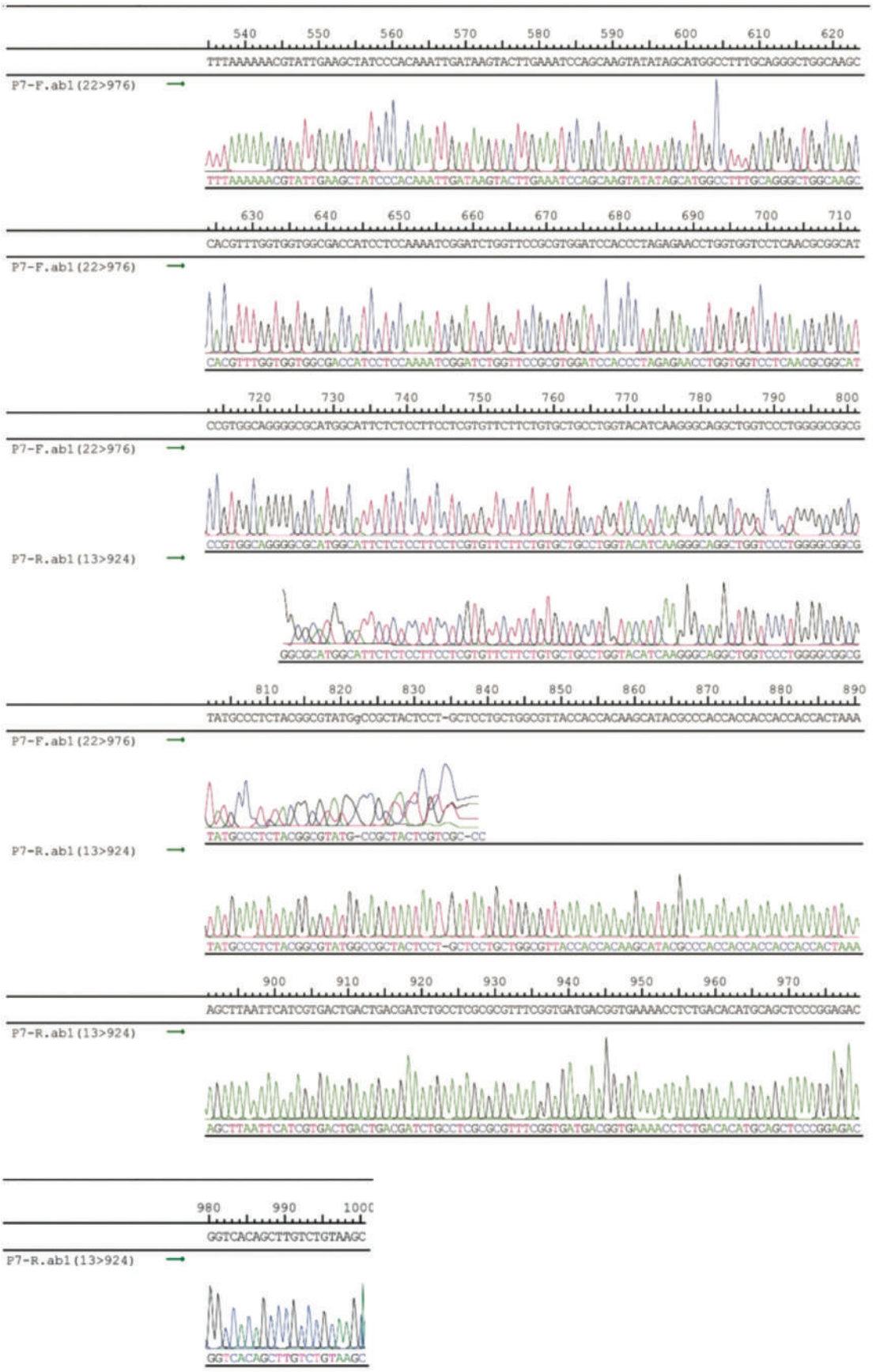


FIGURE 4. Electrophoretogram of induced expression p7 fusion protein (34 kDa).

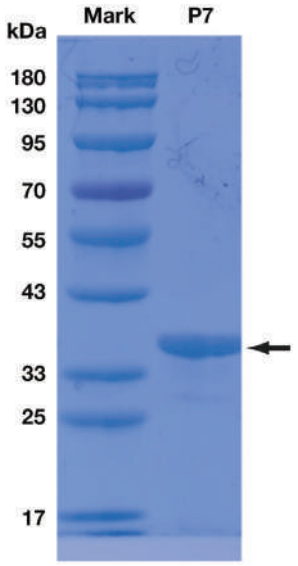


TABLE 1. Screening the Optimal Concentration of Antigen Coating and Enzyme Marker by Square Array Titration (RLIV)

Concentration of Enzyme Marker Working Solution		Concentration of Coating Antigen			
		2 μg/mL	1 μg/mL	0.5 μg/mL	0.25 μg/mL
Negative control	0.025 μg/mL	57,855	57,539	56,542	50,182
	0.05 μg/mL	96,612	97,288	96,723	89,972
	0.1 μg/mL	98,964	98,146	94,281	90,583
Positive control	0.025 μg/mL	579,286	578,892	573,853	439,566
	0.05 μg/mL	1,104,548	1,107,448	1,107,449	636,832
	0.1 μg/mL	1,218,351	1,205,387	1,196,894	695,854

were stable within 15 months. From the example application, the anti-transmembrane protein p7 antibody was detected in the sera of 45 HCV-infected patients and 50 healthy persons who were randomly collected. The positive rates of anti-p7 protein antibody were 20% (9/45) and 0, respectively, whereas the positive rate of anti-GST antibody was 0. The results are shown in **TABLE 3**.

Discussion

Hepatitis C is mainly transmitted by HCV through blood (blood transfusion, acupuncture, drug use), sex, from mother-to-child, and other ways, and it is a global epidemic. The mortality related to HCV infection (death caused by liver failure and hepatocellular carcinoma) will continue to increase in the next 10 years. The infection is very harmful to the health and life of those with infection and has become a serious social and public health problem.[1] In chronic hepatitis C infection, host immune factors and HCV protein components promote virus persistence and immune system dysregulation, which have an important impact on the immune pathogenesis of hepatitis C. Therefore, understanding these protein components and host-produced antibodies will contribute to the further study of the pathogenesis of hepatitis C.[13]

TABLE 2. Statistical Results of the Precision Experiment (n = 20)

Statistical Parameters		Mean Value	Standard Deviation	Coefficient of Variation (%)
Low level of anti-p7 antibody	In batch test	4.72	0.14	2.97
	Inter batch test	4.8	0.18	3.75
High level of anti-p7 antibody	In batch test	16.12	0.66	4.09
	Inter batch test	17.56	0.97	5.52

TABLE 3. Detection Results of Anti-p7 Antibody in HCV-Infected Patients[a]

Group	Research Group (n = 45)	Control Group (n = 50)
Anti-HCV antibody[b]	45	0
Anti-p7 antibody[c]	9	0

HCV, hepatitis C virus.
[a]The research group was compared with the control group (P < .05).
[b]Commercial kits were used to detect anti-HCV mixed antibodies (four recombinant proteins of NS3, NS4, NS5, and nucleocapsid).
[c]The recombinant transmembrane protein p7 was used to detect anti-p7 antibody.

At present, as a mature nonselective cation channel of HCV, p7 has been more studied with regard to its structure, immunological function, and interaction with other substances.[14,15] The p7 is a potential target of anti-HCV drugs and there are many relevant reports on this[16]; however, the research on chemiluminescence immunoassay kits and related technologies for the detection of anti-p7 antibody is still to be done. Some scholars have used recombinant Core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A, and NS5B proteins to establish an immunoblotting method to detect the corresponding protein antibodies in the serum of HCV-infected patients.[17] The results showed that there were differences in the positive rate of antibodies to different proteins in HCV-infected patients. We considered that this may be related to the different course of HCV in infected patients, different genotypes, pre- and post-direct-acting antiviral agents treatment, and the existence of circulating immune complexes in serum. Due to the low sensitivity of immunoblotting, the conventional kits used for clinical diagnosis of hepatitis C use HCV nucleocapsid region recombinant protein C-22, nonstructural protein NS3 region recombinant protein C-200 antigen, nonstructural protein NS4 region recombinant protein C-200, and NS5 as fourth-generation reagents for mixed-coated antigens to detect antibodies.[18] The methods for detecting antibodies mainly include enzyme-linked immunosorbent assay (ELISA), colloidal gold method, chemiluminescence method, etc. However, these kits can only detect mixed antibodies in the serum of HCV-infected patients and do not contain anti-p7 antibodies, nor can they distinguish which protein components are antibodies, and cannot fully reveal the clinical significance of a single HCV protein component in condition monitoring, prognosis evaluation, or epidemiological research of HCV-infected persons. Therefore, our laboratory recombinantly expressed p7 protein and established a method for detecting anti-p7 antibodies in the serum of HCV-infected patients and evaluated the application. The results

show that the indicators meet the methodological requirements, and the kit can be applied to screening, diagnosis, condition monitoring, prognostic assessment, disease mechanism, and epidemiological studies of HCV-infected patients. Furthermore, our kit for detecting anti-p7 antibody in serum of HCV-infected persons by chemiluminescence also has the following advantages.

The microplates in the kit developed in this research were coated with recombinant GST-fused p7 protein. Based on literature reports,[19,20] the p7 protein fragment was small, and direct recombinant expression cannot be achieved; only the fusion of p7 protein can be successfully expressed. In addition, GST has no effect on the spatial structure of p7 protein (also forming hexamer). Therefore, we chose GST and HIS double tag fusion to express p7 protein, which is conducive to the separation and purification of the target protein; it can stabilize and increase the expression of the protein. The serum of 45 patients with HCV infection was detected by serum anti-p7 antibody kit, and 9 patients were positive for anti-p7 antibody (negative for anti-GST antibody). The results showed that there was a certain proportion of anti-p7 antibody in HCV-infected persons (**TABLE 3**), suggesting that there was an immune response to transmembrane protein p7. These preliminary results provide a technical means to further reveal that transmembrane protein p7 stimulates the body to produce antibodies (anti-p7) and circulating immune complex and examine whether there are differences in different genotypes and subtypes, different clinical stages (chronic, liver cirrhosis, liver cancer), and before and after antiviral treatment of HCV-infected persons. Therefore, it is of great significance to establish a high-sensitivity detection method for the detection of anti-p7 antibodies in the serum of HCV-infected patients or the corresponding antibodies in p7 circulating immune complexes for the treatment, disease monitoring, prognosis evaluation and epidemiological research of HCV-infected patients. It also provides an objective verification index for evaluating the effect of p7 as a new type of vaccine targeting the induction of multifunctional $CD4^+$ and $CD8^+$ T cells in hepatocytes expressing viral antigens, or for the study of neutralizing epitope vaccines.[21,22]

Furthermore, the chemiluminescence immunoassay established in this study is based on the principle of indirect ELISA and uses 2 chemiluminescence enhancers (o-phenylphenol and 4-imidazolenol) to play a synergistic enhancement effect, significantly reduce the background signal, and keep the luminol luminescence signal stable for a long time, which indirectly amplifies the results of immune reaction; the determination method has high sensitivity and stability.

Finally, to obtain an effective concentration of p7 antibody positive control serum, we collected the mixed serum or plasma of HCV-infected patients (human immunodeficiency virus antibody, *T pallidum* antibody, and hepatitis B virus surface antigen test was negative), and heat inactivated them. The positive rate of anti-p7 antibody in HCV-infected patients was only about 20%; therefore, we used U-Tube protein ultrafiltration concentration (30KD) tubes (purchased from Merck; U-Tube 20-30) to concentrate IgG antibody (50 mL to 10 mL), and add 0.2% Proclin 300 preservative to the concentrated HCV-infected mixed serum or plasma to improve the detection rate of specific p7 antibody.

Acknowledgments

This work is supported by the major special fund for medical science and technology innovation of Nanjing Military Region (15DX025), the social development scientific research project of Hangzhou Science and Technology Bureau of Zhejiang Province (20160533B75), the medical and health scientific research project of Zhejiang Province (2016KYB250, 2021433791) and the public welfare technology research program of Zhejiang Natural Science Foundation (LGF21H200003). The product of the project has applied for national invention patent of China(202010946293.8).

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Differential Effects on HbA1c Detection by HPLC and Capillary Electrophoresis in Five Types of Hb Variants in China

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Keywords: HbA1c, hemoglobin, variants, sequencing, HPLC, capillary electrophoresis

Abbreviations: HbA1c, hemoglobin A1c; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; PCR, polymerase chain reaction; Glu, glucose; HC, healthy control; RBC, red blood cell

Laboratory Medicine 2023;54:308–316; https://doi.org/10.1093/labmed/lmac121

ABSTRACT

Objective: Hemoglobin A1c (HbA1c) can be used to evaluate blood glucose control. Its measurement will be affected by many factors, but Hb variation is the most critical factor. This study aimed to explore the types of variants found in routine work and their impact on test results.

Methods: Samples with abnormal HbA1c chromatograms found in routine testing were tested with high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) and then further tested with polymerase chain reaction (PCR) and sequencing.

Results: Five recessive heterozygous mutations were identified after PCR and sequencing. Hb Riccarton-II (a mutation in the *HBA2* gene), Hb E, Hb G-Coushatta, Hb G-Taipei, and Hb North Manchester (a mutation in the *HbB* gene) were identified. All HbA1c values of these variants detected by HLC-723 G8 (HPLC method) were lower than those of Sebia Capillarys 2 FP (C2FP, CE method) with $P < .0001$.

Conclusion: Five Hb mutations were identified in our routine HbA1c test, and their HPLC detection values were significantly lower than those obtained with the CE method.

Diabetes mellitus is a difficult health problem affecting the world population. According to the International Diabetes Federation, approximately 415 million adults aged 20 to 79 years had diabetes mellitus in 2015.[1] Diabetes mellitus is usually diagnosed by impaired fasting glucose (6.1 to 6.9 mmol/L), impaired glucose tolerance (with oral glucose tolerance test results of 7.8 to 11 mmol/L), or an elevated glycated hemoglobin A1c (HbA1c) level (6% to 6.4%).[2,3] Nevertheless, only HbA1c can be used to evaluate the level of glucose control because it represents the blood glucose (Glu) level over the previous 60 to 90 days.[4,5] The HbA1c measurement can be affected by Hb variants.[6] So far, more than 1350 Hb variants have been identified,[7] and we have previously reported a special case of an abnormally low HbA1c value of 0 due to Hb variation.[8] In the present study, based on abnormal peaks in patient HbA1c chromatograms, we identified another series of Hb variants and their impact on 2 different HbA1c assays.

Materials and Methods

Routine Laboratory Analysis

In Tianjin Medical University General Hospital, samples with abnormal peak patterns of HbA1c chromatograms were found from January 2021 to December 2021. The presenting reasons the persons with these patterns came to the hospital were type 2 diabetes, systemic lupus erythematosus (SLE), fever, or physical examination.

This study was approved by the Ethics Committee of Tianjin Medical University General Hospital (Ethical No. IRB2021-WZ-024), and all of our clinical findings and hematological indices were collected after written informed consent from the patient.

The hematological parameters were measured by an automated cell counter (Sysmex, XN-2000 + PA-990, Sysmex) and Hitachi 008AS Automated Biochemical Analyzer (Glu detection). The reasons for visiting the hospital and blood routine data of the patients are shown in **TABLE 1**. The HbA1c analysis was performed by an HLC-723 G8 analyzer in standard mode (Tosoh G8, Tosoh).

Screening of Hb Variants

Capillary electrophoresis (CE) was performed to screen for abnormal Hb by a Sebia Capillarys 2 FP instrument (Sebia).

DNA Sequence Analysis

Isolation of genomic DNA was performed with a TIANamp Blood DNA Kit (TIANGEN Biotech) according to the manufacturer-provided

TABLE 1. Hematological Parameters of 14 Patients[a]

Patient	Sex/Age, y	RBC (×10^{12}/L)	RDW (%)	Hb (g/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Glu (mmol/L)	Reason
1	M/62	5.16	13.1	142	83.7	27.5	329	5.5	Physical examination
2	F/55	4.91	14.2	116	71.3	23.6	331	5.6	Type 2 diabetes mellitus
3	F/65	4.43	12.5	122	82.2	27.5	335	5.1	Physical examination
4	F/44	5.12	12.9	132	77.5	25.8	332	4.6	Physical examination
5	F/94	4.74	13	145	88.6	30.6	345	8.0	Coronary disease
6	M/68	4.71	13.6	136	86.4	28.9	334	5.1	Physical examination
7	F/34	4.86	13.2	138	87	28.4	326	3.9	Physical examination
8	F/23	2.63↓	12.9	80↓	84.4	30.4	360↑	4.6	SLE
9	F/51	4.71	12.8	136	85.4	28.9	338	5.1	Physical examination
10	M/58	5.39	13.3	167	92	31	337	6	Physical examination
11	F/38	4.64	15.5	124	83.6	26.7	320	4.9	Physical examination
12	M/47	5.32	11.9	159	87.6	29.9	341	4.3	Physical examination
13	F/64	4.05	13.7	109	85.2	26.9	316	6.3	Fever
14	M/41	4.99	12.8	151	90.6	30.3	334	4.8	Physical examination

[a]*Normal ranges follow. RBC (red blood cell): M 4.3–5.8 × 10^{12}/L, F 3.8–5.1 × 10^{12}/L. RDW (red cell volume distribution width): 11%–15%. Hb (hemoglobin): M 130–175 g/L, F 115–150 g/L. MCV (erythrocyte mean corpuscular volume): 82–100 fL. MCH (mean corpuscular hemoglobin): 27–34 pg. MCHC (mean corpuscular hemoglobin concentration): 316–354 g/L. Glu (fasting blood glucose): 3.6–5.8 mmol/L.*

TABLE 2. Glu and HbA1c Values of the 14 Patients[a]

Patient	HLC-723 G8 (%)	C2FP (%)	Mutation Type	Reason for Visit
1	4.8	5.6	Hb E	Physical examination
2	4.6	5.4		Physical examination
3	4.6	5.5		Physical examination
4	6.7	8.0		Type 2 diabetes mellitus
5	5.6	7.8	Hb G-Coushatta	Coronary disease
6	4.2	5.4		Physical examination
7	3.9	5.9		Physical examination
8	4.8	5.3		SLE
9	4.4	5.4		Physical examination
10	5.3	6.4		Physical examination
11	4.6	5.8	Hb G-Taipei	Physical examination
12	4.1	4.8	Hb North Manchester	Physical examination
13	5.9	8.0		Fever
14	4.1	5.2	Hb Riccarton-II	Physical examination

HbA1c, hemoglobin A1c; C2FP, Sebia Capillarys 2 FP.
[a]*Normal ranges follow. HLC-723 G8 (HbA1c values of HLC-723 G8): 4%–6%. C2FP (HbA1c values of C2FP): 4%–6%.*

instructions. The α_1, α_2, $\beta_{1\text{-}2}$, and β_3 globin genes were amplified by polymerase chain reaction (PCR) using pairs of primers that were designed in our previous study.[8] The PCR was performed with Phusion DNA Polymerase (Thermo Fisher Scientific) with 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 20 minutes. Finally, the amplified fragments were tested by electrophoresis and sequenced by BGI Tech.

Statistical Analysis

Data were analyzed with SPSS 22.0 software. Paired *t*-test followed by Neuman-Keuls post hoc test were performed for data analysis, and $P < .05$ was considered statistically significant.

Results

Hb E Variants

Three patients (patients 1–3) came to the hospital for physical examination, and another patient (patient 4) with type 2 diabetes came to the hospital for treatment (**TABLE 1**). The HbA1c was tested by using a Tosoh G8 HPLC analyzer in our laboratory, and the results of patients 1 to 3 were 4.8%, 4.6%, 4.6%, respectively, and 6.7% was the result for patient 4 (reference range, 4%–6%) (**TABLE 2**). Normal peaks (**FIGURE 1A**) represent A1a (glycated hemoglobin A), A1b (glycated hemoglobin B), F (hemoglobin F, high in infants and young children and of great significance for the diagnosis of β-thalassemia in adults), LA1c+ (aldimine, labile glycated hemoglobin), s-a1c (glycated

FIGURE 1. Four Hb E variants. A, Hemoglobin A1c (HbA1c) high-performance liquid chromatography (left) and capillary electrophoresis (CE) (right) chromatogram results of healthy control. B–E, Patients 1 to 4, respectively, with the Hb E variant. The first row was a batch of HbA1c HPLC chromatograms. The second row shows the CE results and the third row is the polymerase chain reaction analysis of Hb variants.

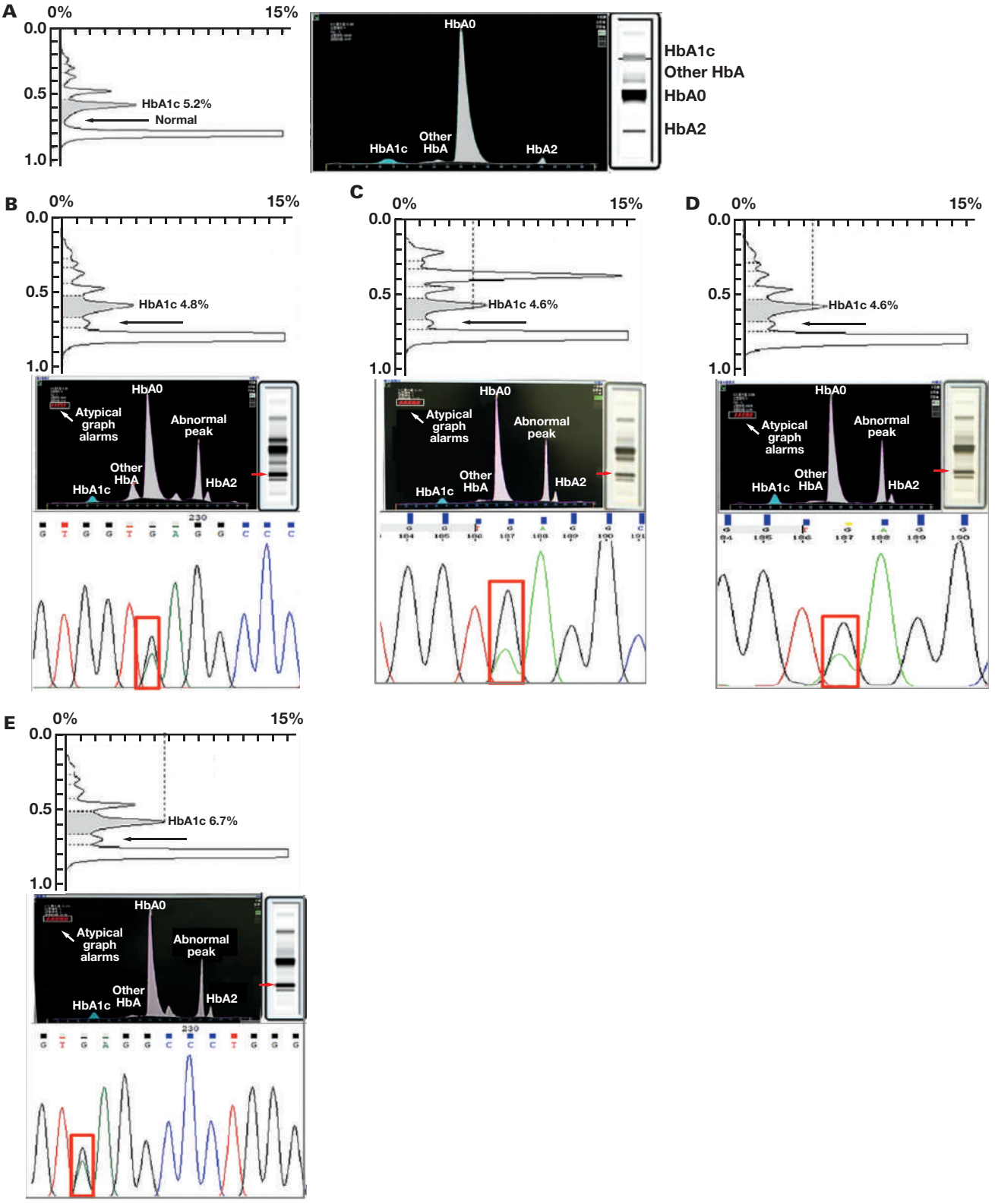
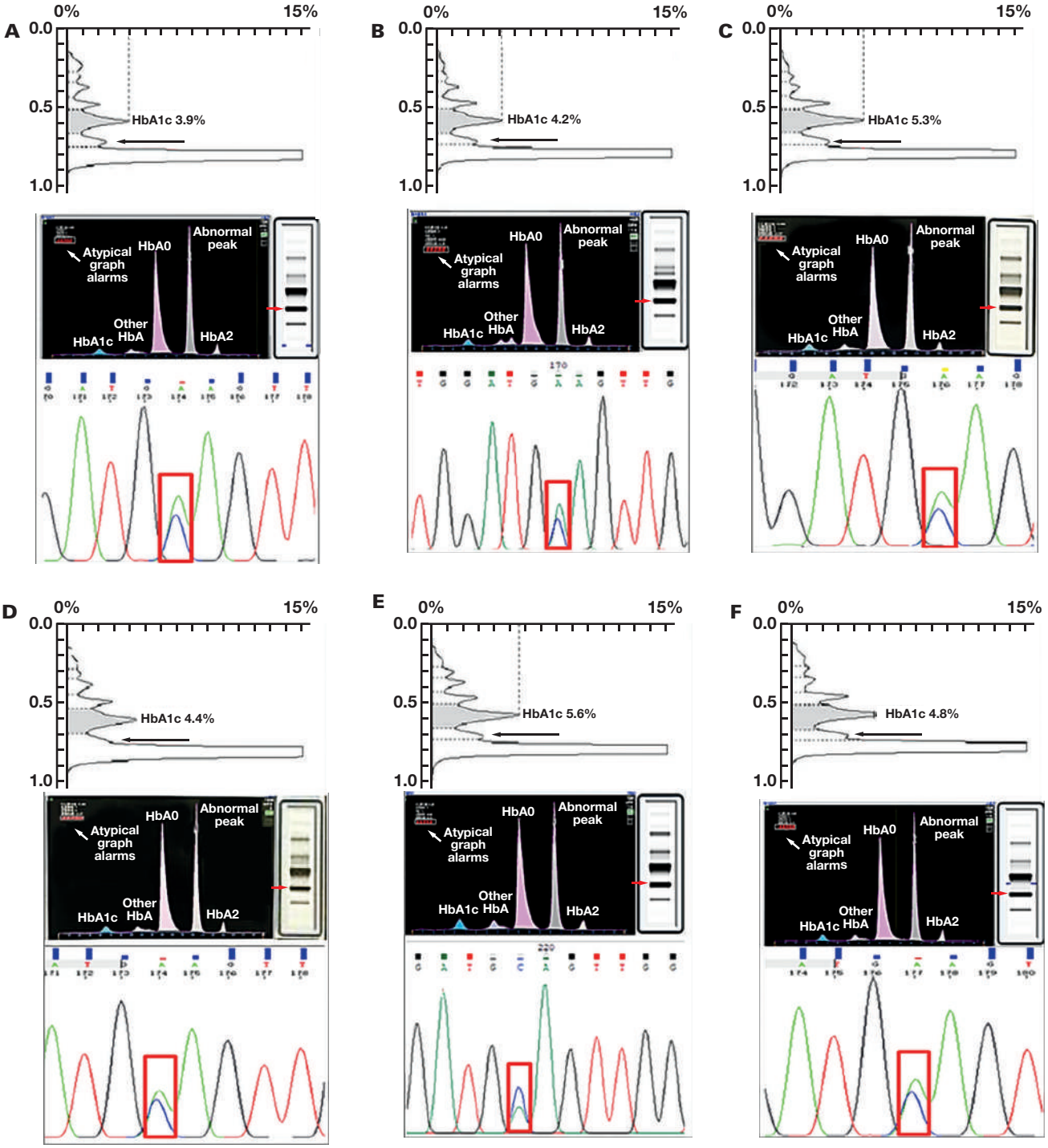


FIGURE 2. Six Hb G-Coushatta variants. A–F, patients 5 to 10, respectively, with Hb G-Coushatta variant. The first, second, and third rows are hemoglobin A1c (HbA1c) high performance liquid chromatography (HPLC) chromatograms, capillary electrophoresis (CE), and sequencing results for patients 5 to 7, respectively. The fourth to sixth rows were the HbA1c HPLC chromatograms, CE and sequencing results of patients 8 to 10 in order.



hemoglobin A1c, one of the indicators for the diagnosis of diabetes and used for treatment monitoring of diabetes) and A0 (unglycated normal hemoglobin), respectively. The shaded peak, s-a1c, is the HbA1c peak and the highest peak is the HbA0 peak. After comparing their peak spectra with that from a healthy control (HC) (**FIGURE 1A**), we discovered that the 4 patients' HbA1c peak spectra were abnormal: there was an extra peak between HbA1c and HbA0 (shown by red arrows in **FIGURE 1B** first line).

All routine blood parameters of the 4 patients showed no obvious abnormality (**TABLES 1** and **2**). Aiming to further analyze the

FIGURE 2. (cont) G, Polymerase chain reaction analysis of 6 Hb G-Coushatta patients.

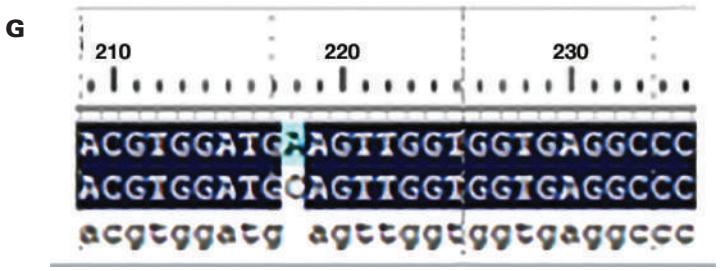
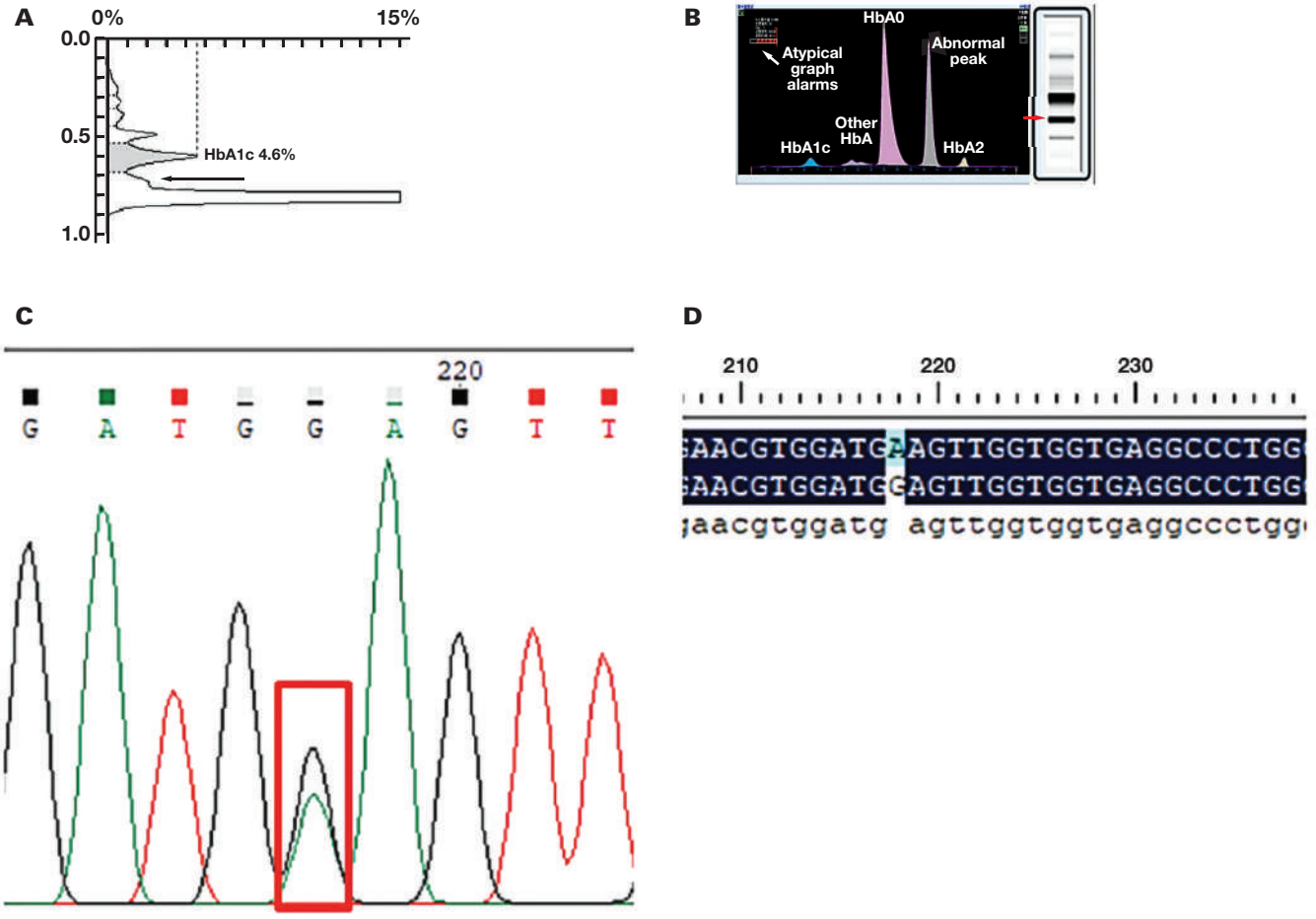


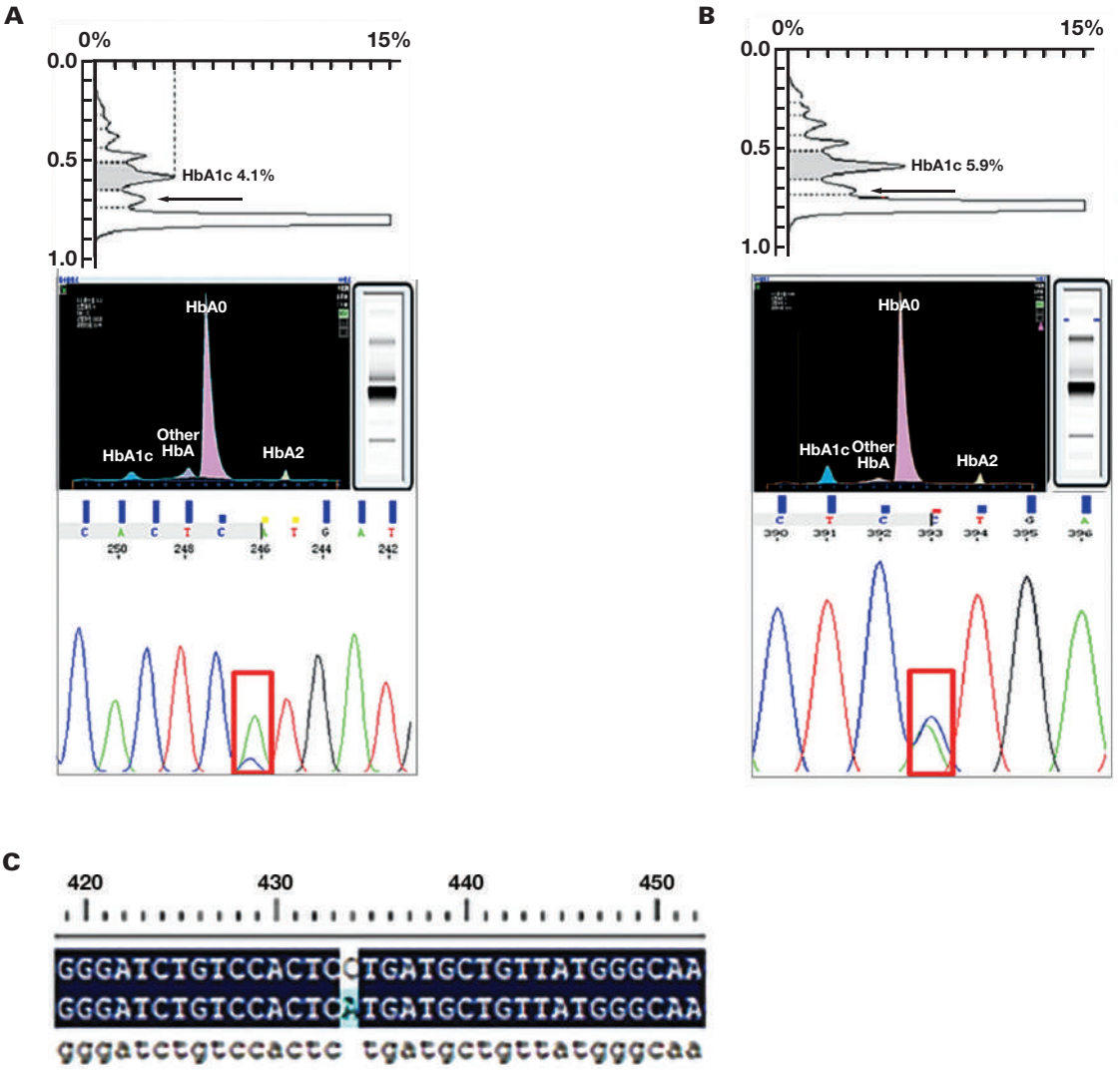
FIGURE 3. One Hb G-Taipei variant. A, Hemoglobin A1c (HbA1c) chromatogram of patient 11 with Hb G-Taipei variant. B, The capillary electrophoresis result. C, The sequencing results. D, Polymerase chain reaction analysis of Hb G-Taipei variant.



abnormal HbA1c peaks, we carried out CE. Compared with the HC electrophoretogram (**FIGURE 1A**), we found anomalous peaks and electrophoretic strips (illustrated in **FIGURE 1B**, second line). The abnormal peaks were regarded as abnormal hemoglobin with values of 18.4%, 18.5%, 21.3%, and 22.1% for patients 1 to 4, respectively, which were consistent with the electrophoretic strips (shown by red arrows).

The 4 PCR-amplified fragments (α_1, α_2, β_{1-2}, and β_3) of all patients were acquired for agarose gel electrophoresis. We confirmed that all electrophoretic bands met the predetermined length. To gather more information on the Hb-gene features, all amplified gene fragments were sequenced and compared with corresponding reference sequences (α_1: NM_000558.4, α_2: NM_000517.4, β: NM_000518.4). For these 4 patients, we found no mutation in the other genes but 1 heterozygous gene mutant in exon 1 of the β gene with HBB: c.79G > G/A (GAG to AAG) was found, which caused 1 amino acid change from Glu to Lys (**FIGURE 1B**, third line). After checking the HbVar database (http://globin.cse.psu.edu/), we confirmed that this hemoglobin mutant has been reported as Hb E.

FIGURE 4. Two Hb North Manchester variants. A and B, Patients 12 and 13, respectively, with the Hb North Manchester variant. The first row represents the hemoglobin A1c (HbA1c) high-performance liquid chromatography chromatogram. The second row is the capillary electrophoresis results and the third row is the sequencing results of these 2 Hb North Manchester variants. C, Polymerase chain reaction analysis in 2 Hb North Manchester variants.



Hb G-Coushatta Variants

A total of 6 patients developed Hb G-Coushatta mutation. Four of these patients (patients 6, 7, 9, 10) presented to the hospital for physical examination. Patient 5 had coronary disease and patient 8 had SLE (**TABLE 1**).

The HbA1c values of patients 6, 7, 9, and 10 were 4.2%, 3.9%, 4.4% and 5.3%, respectively, and those of patients 5 and 8 were 5.6% and 4.8%, respectively (**TABLE 2**). Compared with the HC (**FIGURE 1A**), these 6 patients had abnormal HbA1c peaks: 1 shoulder peak in the HbA0 peak (shown by red arrows in **FIGURE 2A**, first line and fourth line). **TABLE 1** shows the blood routine parameters. Patients 5 to 10 were the group with normal values with the exception of patient 8.

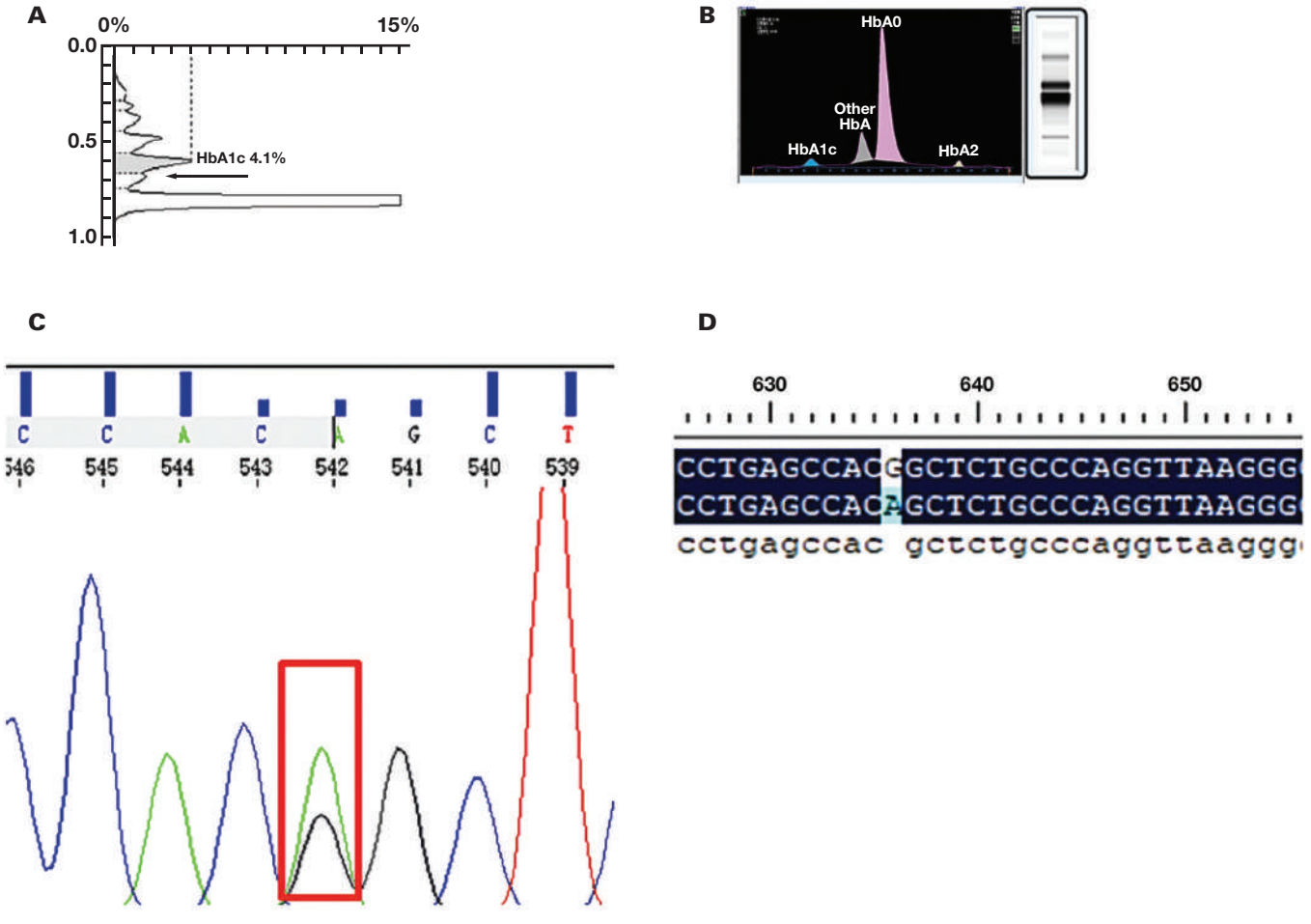
CE was carried out to further analyze the abnormalities. Not surprisingly, in comparison with the HC electrophoretogram (**FIGURE 1A**), there was an extra peak in the electrophoretograms of 6 patients (**FIGURE 2A**, second and fifth lines). The abnormal extra peaks on the electrophoretic peak maps were between the HbA0 and HbA2 peaks; the abnormal electrophoretic bands are indicated by red arrows. Contents of abnormal components displayed by the instrument were 40.5%, 38.3%, 39.3%, 39.2%, 38.0%, and 39.3%.

To confirm the occurrence of gene mutation that caused the abnormality in the electrophoresis and HbA1c analysis, the *HbA1*, *HbA2*, and *HbB* genes were amplified and sequenced. We indeed noticed a significant mutation [β22 (B4)Glu>Ala (GAA > GCA), HBB: c.68A > C] (**FIGURE 2A** third and sixth lines, and **2B**). However, there was no abnormality in the *HBA1* or *HBA2* genes. Referring to the HbVar database, we discovered that this mutation is known as Hb G-Coushatta.

Hb G-Taipei Variants

Patient 11, a 38-year-old woman, originally visited our hospital for physical examination and all blood routine and Glu results were normal (**TABLE 1**). However, on the HbA1c chromatogram, the HbA0 peak had an abnormal shoulder peak (**FIGURE 3A**, red arrow). Consistently, there was another abnormal peak between HbA0 and HbA2 in the CE (**FIGURE 3B**). The content of abnormal electrophoretic strip was measured as 32.9% by the apparatus (**FIGURE 3B**).

FIGURE 5. One Hb Riccarton-II variant. A and B, Hemoglobin A1c (HbA1c) high-performance liquid chromatography chromatogram and capillary electrophoresis results, respectively, of patient 11. C, The sequencing result of patient 11. D, Polymerase chain reaction analysis of Hb Riccarton-II variant.



Based on these results, we sequenced the patient's *HbA1*, *HbA2*, and *HbB* genes to confirm whether there was a mutation. Among these genes, we only found a heterozygous mutation [β22 (B4)Glu>Gly (GAA > GGA), HBB: c.68A > G] (**FIGURE 3C** and **3D**). In the HbVar database, this mutation is called Hb G-Taipei.

Hb North Manchester Variants

Patient 12 came to the hospital for physical examination and patient 13 for fever. Their blood routine results were presented in **TABLE 1**; their HbA1c values were 4.1% and 5.9%, respectively (**TABLE 2**). Both of these values are in the normal reference range. Nevertheless, HbA1c chromatograms showed an extra peak between HbA1c and HbA0 peak (shown by red arrows in **FIGURE 4A**, first line). The electrophoresis results (**FIGURE 4A**, second line) were the same as that of the HC (**FIGURE 1A**). To further analyze this anomaly, the *HbA1*, *HbA2*, and *HbB* genes of the 2 patients were amplified and sequenced. There was a single heterozygous nucleotide substitution (C > A) on the β gene at codon 51(CCT > CAT), leading to a transition (Pro > His) in β globin (**FIGURE 4A** third line, and **4B**). We confirmed that this mutation is known as Hb North Manchester.

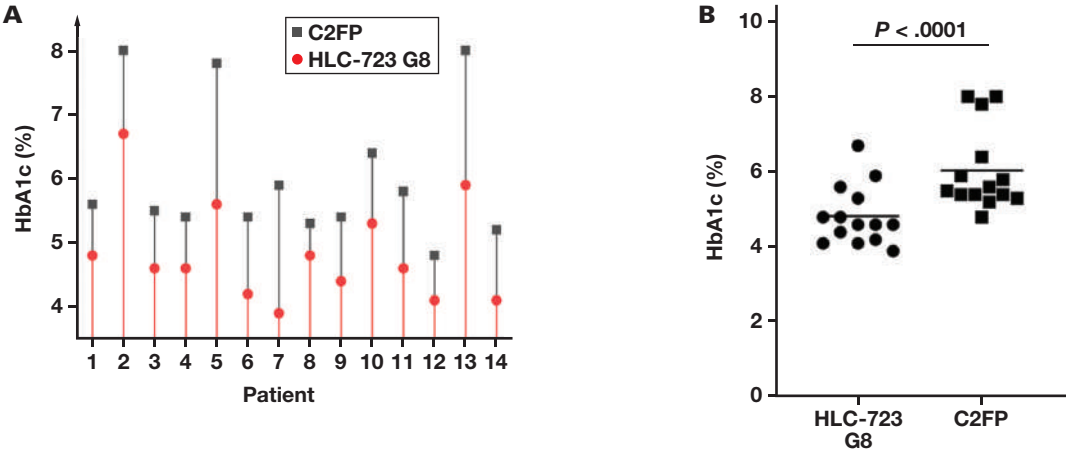
Hb Riccarton-II Variants

Patient 14, a 41-year-old man, visited our department for physical examination. His HbA1c was 4.1% and blood routine results were normal (**TABLE 1**). Compared with the results of the HC, this patient's HbA1c peak has an abnormal shoulder peak near the HbA0 peak (**FIGURE 5A**). Although the number of peaks did not change, we thought the composition of the different components might have changed, and there was an abnormal HbA0 band on the electropherogram (**FIGURE 5B**). After identifying the *HbA1*, *HbA2*, and *HbB* genes, the results revealed a heterozygous gene mutant in exon 2 of the α gene with HBB:c.154G > A (GGC to AGC), which resulted in 1 amino-acid change from glycine to serine (**FIGURE 5C** and **5D**). According to the HbVar database, this mutation is Hb Riccarton-II.

Hb Variant HbA1c Results in C2FP Were Higher than HLC-723 G8

The blood glucose results of patients 5, 10, and 13 were high according to the reference range (3.6–5.5 mmol/L), but the results of the other patients in the study were within the normal range. Interestingly, we found that for each patient, the HbA1c value given by the C2FP was higher than that by the HLC-723 G8 (**FIGURE 6A**). For all patients,

FIGURE 6. Comparison of hemoglobin A1c (HbA1c) results of 14 patients with HLC-723 G8 and Sebia Capillarys 2 FP (C2FP). A, All of the 14 patients had higher HbA1c test results than HLC-723 G8 test results. B, HbA1c result of C2FP was significantly higher than that of HLC-723 G8. Error bars represented the mean.



HbA1C assay results were significantly higher than HLC-723 G8 (**FIGURE 6B**).

Discussion

In this study, we discovered 5 different kinds of hemoglobin variants in 14 patients. These variants have been reported previously and are recessive heterozygous mutations. According to current reports, these 5 variants did not cause serious diseases like hemoglobin sickle.[9–13] Consistent with this, these patients did not report any obvious abnormal hematological symptoms. Blood routine results of most patients were in the normal reference intervals. Only patient 8 had developed anemia with normal red blood cell (RBC) size due to SLE. CE was used to analyze abnormal peaks on HbA1c chromatograms. With the possible exceptions of Hb North Manchester and Hb Riccarton-II (indicated on the electropherogram), we discovered the abnormal Hb readings on the electrophoretograms. Xu et al[14] reported that β-globin variants showed a single variant peak in Hb electrophoretograms because β globin only exists in HbA ($\alpha_2\beta_2$). However, α globin found in HbA ($\alpha_2\beta_2$), HbA2 ($\alpha_2\delta_2$), and HbF ($\alpha_2\gamma_2$) will lead to more types of mutation. Correspondingly, in our work, the Hb Riccarton-II (α-globin) variant electrophoretogram showed each peak's area and width were increased when compared to that of the HC (**FIGURES 5B** and **1A**). The electrophoretograms of Hb E (**FIGURE 1**), Hb G-Coushatta (**FIGURE 1**), and Hb G-Taipei (β-globin variants) (**FIGURE 3**) all had an anomalous peak. However, the electropherogram of the Hb North Manchester (β-globin) variant did not show a single abnormal peak. The Hb Riccarton-II mutation did not result in a significant change in amino-acid residue net-charge Gly > Ser, compared with other mutations. This explains the inability to detect it by clinically available electrophoresis. Moreover, Xu et al[14] observed that for HLC-723 G8, Hb E, Hb G-Coushatta, and Hb G-Taipei had statistically significant lower HbA1c values in comparison with the C2FP system (P = .000, .000, and .013, respectively) In our study, HbA1c results measured by C2FP were significantly higher than those measured by HLC-723 G8 in all 14 patients and variants, which was consistent with that study. Furthermore, our study found the same for Hb North Manchester and Hb Riccarton-II. The principle of HLC-723 G8 was HPLC whereas the C2FP was CE. Both HbA1c values (HPLC and CE) of patient 4 were higher than the normal reference interval (4%–6%) (**TABLE 2**), and those of patients 1, 2, 3, 6, 7, 8, 9, 11, 12, and 14 were all in the normal reference interval. For these patients, although the 2 results were different, there was no significant impact on the clinical diagnosis. The HLC-723 G8 results of patients 5, 10, and 13 were in the normal range but C2FP results were higher than the normal reference interval, which could lead to a different clinical diagnosis and treatment. In this case, the influence of variants on the test values will become particularly important. Unfortunately, because we were limited by the small sample size, more detailed statistical analysis could not be performed to provide an explanation for the differences in HbA1c values caused by Hb variants in the different detection methods.

Our study suggests that in addition to the numerical results of HbA1c testing, we need to be aware of tiny changes in HbA1c chromatograms that may represent unknown hemoglobin variants and their possible interference with diagnosis. Laboratories should be aware of the limitation of their methods with respect to Hb variants found commonly in their local population and suggest an alternative HbA1c quantification method (such as glycated albumin) to avoid misreading and misdiagnosis to the greatest extent.

Acknowledgments

This work was supported by New Century Talent Program Project of General Hospital of Tianjin Medical University (No.209060102501).

Conflict of Interest Disclosure

The authors have nothing to disclose.

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Frequencies of Anti-Troponin I vs Anti-Troponin T Autoantibodies and Degrees of Interference on Troponin Assays

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Keywords: cardiac troponin, autoantibody, interference, immunoassay, IgG-troponin, acute coronary syndrome

Abbreviations: cTn, cardiac troponin; CAD, coronary artery disease; CVDs, cardiovascular diseases; ACS, acute coronary syndrome; IgG, immunoglobulin G; hs-cTn, high-sensitivity cTn; LoD, limit of detection; HPLC, high-performance liquid chromatography; ROC, receiver operating characteristic; CI, confidence interval; eGFR, estimated glomerular filtration rate; Scr, serum creatinine; AUC, area under the curve; DCM, dilated cardiomyopathy; CKD, chronic kidney disease; ICM, ischemic cardiomyopathy

Laboratory Medicine 2023;54:317–323; https://doi.org/10.1093/labmed/lmac120

ABSTRACT

Objective: Presence of autoantibodies against troponin I (cTnI) or T (cTnT) has been reported to interfere with troponin assays. However, the extent of the interference with the measurement has not been explored sufficiently. The aims of this study were to examine the frequencies of autoantibodies against troponin I and troponin T and how much these antibodies would affect the measurement.

Methods: The study comprised 52 subjects who visited Hokkaido University Hospital with suspected ischemic heart diseases. To evaluate the presence of autoantibodies, we calculated the recoveries of cTnI or cTnT after immunoglobulin G depletion, and the distributions of peaks reactive with cTnI or cTnT by high-performance liquid chromatography were examined.

Results: Autoantibodies against cTnI and cTnT were identified in 8 subjects (15.4%) and 1 subject (1.9%), respectively. Although the greatest difference between cTnI and cTnT was 32-fold, the distributions of cTnI-to-cTnT ratios in groups with and without anti-cTnI were not statistically different.

Conclusion: Autoantibodies against cTnI were more frequent by several fold than those against cTnT. Their presence did not significantly expand the discrepancy between cTnI and cTnT assays.

Coronary artery disease (CAD), a generic term for diseases with coronary artery among cardiovascular diseases (CVDs), is the foremost single cause of mortality globally, accounting for 8.9 million deaths annually.[1] Within CAD, there is acute coronary syndrome (ACS), which is a syndrome with acute forms of coronary heart disease, including acute myocardial infarction and unstable angina. As described in the Fourth Universal Definition of Myocardial Infarction, elevation of cardiac troponin (cTn) is considered to be the indication of myocardial injury.[2] With the development of high-sensitivity cTn (hs-cTn) assays in recent years, the precise quantification of cardiomyocyte injury around the 99th percentile and thereby the detection of myocardial infarction with substantially increased accuracy were made possible.[3] From 21 troponin assays, Apple et al[4] designated 6 troponin assays as highly sensitive. The Abbott hs-cTnI and Roche hs-cTnT assays used in this study are included in these 6 assays.

Regarding the accuracy and specificity of the troponin measurement, however, several factors that could affect troponin levels have been reported. Welsh et al[5] reported that a significant increase in the percentage of the population above the 99th percentile was seen with cTnT in subgroups with advanced age in the evaluation of cTnI and cTnT levels in 19,501 members of the general population. Also, significant increases of cTnT levels in patients with reduced renal function have been reported from several sources.[6,7] In fact, Jaffe et al[8] reported the possibility that increases in cTnT in noncardiac diseases may occur and lead to a possible false-positive diagnosis of cardiac injury when skeletal muscle pathology is present.

In addition to these factors, research has shown the existence of autoantibodies against cTnI or cTnT,[9,10] which could be another factor affecting troponin measurement. In 1996, Bohner et al[11] identified autoantibodies against cTnI as one of the causes of false-negative cTnI

results. Eriksson et al[12] reported that circulating autoantibodies to cTnI could be a source of negative interference in cTnI assays, by isolating the immune complex with protein G. Akhtar et al[13] reported not only the false-negative interference by the autoantibodies mentioned above but that anti-cTnT IgG could cause false-positive cTnT elevation as well. Vylegzhanina et al[14] demonstrated by gel filtration that autoantibodies they identified bound to the cTn I-T-C ternary complex, indicating that both cTnI and cTnT could be affected by the presence of autoantibodies. Although autoantibodies to cTnI and cTnT have been reported to interfere with troponin assays, the extent of the interference on the measurement has not been explored sufficiently.

In this study, we examined the frequencies of anti-troponin I autoantibodies against those of anti-troponin T autoantibodies, whether these antibodies would affect measurement negatively or positively, and to what extent the measurement was affected.

Methods

Study Design

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Hokkaido University Hospital (approval No. 019-0352). Fifty-two subjects who visited Hokkaido University Hospital during February 2021 and August 2021 for suspected ischemic heart diseases were selected as consecutive samples and provided written informed consent for the participation to this study. Serum specimens collected from the subjects were used for the analyses. Clinical information and laboratory data of the subjects were obtained retrospectively from the medical records and subjected to analyses after anonymization.

hs-cTnI and hs-cTnT Assays

The cTnI level was measured by hs-cTnI Abbott assay on an Alinity I analyzer, the principle of which is based on a 2-step chemiluminescent microparticle immunoassay (Abbott Laboratories). The hs-cTnT level was measured by an Elecsys Troponin T-high sensitive test on a Cobas e801 analyzer, the principle of which is based on an electrochemiluminescence immunoassay (Roche Diagnostics). The 99th percentile cutoffs defined by the manufacturers were 26.2 pg/mL and 14.0 pg/mL for hs-cTnI and hs-cTnT, respectively. According to the report by Apple et al,[4] the limit of detection (LoD) of hs-cTnI was 1.2 pg/mL and the LoD for hs-cTnT was 5.0 pg/mL. Data below the LoD after depletion of IgG were used, as they were output for the calculation of recovery only to show that the recoveries were below certain levels.

Depletion of IgG with Protein G

To evaluate the presence of autoantibodies against cTnI or cTnT, IgG was depleted with protein G Sepharose 4 Fast Flow (Cytiva) from each specimen and the recovery of cTnI or cTnT after the protein G treatment was measured. According to the package insert of the protein G Sepharose 4 Fast Flow, the gel has a capacity to capture 18 mg of human IgG per mL gel suspension. The detailed procedure of the IgG depletion is the following: 100 μL protein G Sepharose suspension was diluted with 500 μL of 20 mmol/L phosphate buffer, pH 7.0 (binding buffer), followed by centrifugation at 500*g* for 2 min. After the supernatant was removed, the pellet was resuspended with 300 μL binding buffer, added with 100 μL serum specimen, and incubated at 4°C overnight using a rotator. After the incubation, the sample was centrifugated at 500*g* for 4 min and the supernatant was subjected to the measurement of cTnI and cTnT together with the original specimens before the protein G treatment.

Recovery of cTnI or cTnT was calculated by the following formula in which the dilution factor was four:

$$\text{Recovery of cTn } (\%) = \frac{\text{cTn level post} - \text{IgG depletion x dilution factor}}{\text{cTn level pre} - \text{IgG depletion}} \times 100$$

Size-Exclusion HPLC Analyses

Size-exclusion high-performance liquid chromatography (HPLC) was performed on a Superdex 200 Increase 10/300 GL column (10 × 300 mm, Cytiva). Five hundred microliters of serum specimen were injected for each run. The elution buffer consisted of 150 mmol/L NaCl and 10 mmol/L phosphate buffer (pH 7.4). The flow rate was 1.2 mL/min and the fraction volume was 0.5 mL. Protein levels were monitored by absorbance at 280 nm using the AKTA Explorer 100 System (GE Healthcare). The levels of cTnI and cTnT in the collected fractions were measured by hs-cTnI and hs-cTnT assays, respectively. The presence or absence of immune complex was determined by the molecular size of the peak of cTnI or cTnT.

Statistical Analyses

To determine upper limits of the recoveries of cTnI or cTnT for the presence of immune complex, we performed receiver operating characteristic (ROC) analyses between recoveries of cTnI or cTnT and the presence of the immune complex identified by HPLC analyses above. For the ROC analyses, we used JMP 16.0.0 (SAS Institute). The upper limits indicated by the ROC analyses were used to categorize the presence and absence of immune complex for specimens without HPLC.

For the calculation of upper and lower 95% confidence interval (CI) of linear regression between cTnT and cTnT, we performed linear regression analyses using the Microsoft 365 version 2102 of Excel (Microsoft). For subjects categorized as immune complex–absent by the analyses described above, we log-transformed the initial levels of cTnI and cTnT, performed the linear regression analyses between log (cTnT) and log (cTnI), and derived upper and lower 95% CIs.

For the assessment of statistical difference of distributions of cTnI-to-cTnT ratios in subject groups with and without macro-cTnI, we used JMP 16.0.0 to perform the Wilcoxon signed-rank test.

Results

Characteristics of the Study Subjects

Baseline characteristics of the study 52 subjects are presented in **TABLE 1**. Estimated glomerular filtration (eGFR) was calculated from serum creatine (Scr) values measured by the enzymatic method using the following formula: eGFR (male) = 194 × age−0.287 × Scr−1.094; eGFR (female) = 0.739 × 194 × age−0.287 × Scr−1.094. Briefly, medians of age, eGFR, cTnI, and cTnT of the total subjects were 76 years, 55.9 mL/min/1.73m^2, 310.2 pg/mL, and 118.5 pg/mL, respectively.

cTn Recoveries after IgG Depletion

The recoveries of cTnI after IgG depletion were plotted on the y-axis against the initial cTnI levels on the x-axis in **FIGURE 1A**. Similarly, the

recoveries of cTnT after IgG depletion were plotted on the y-axis against the initial cTnT levels on the x-axis in **FIGURE 1B**.

HPLC Analyses

We selected 15 specimens that included specimens of low troponin recoveries and subjected them to HPLC analyses. As shown in **FIGURE 2A–2D**, the peak patterns were categorized into 3 groups: group 1, without immune complex (**FIGURE 2A**); group 2, with macro-cTnI (**FIGURE 2B** and **2C**); and group 3, with both macro-cTnI and macro-cTnT (**FIGURE 2D**). Among the 15 specimens, 6, 8, and 1 specimen were categorized in group 1, 2, and 3, respectively. A specimen with macro-cTnT but without macro-cTnI was not observed. The recoveries and categorizations of immune complexes by HPLC analyses are summarized in **TABLE 2**.

Conformities between the Recovery and HPLC Methods

FIGURE 3A and **3B** were made in the same way as **FIGURE 1A** and **1B** but with the additional information of presence or absence of immune complexes by HPLC analyses. Using the data shown in **TABLE 2**, we performed ROC analyses to confirm the upper limit of cTnI recovery and that of cTnT for the presence of autoantibody. Regarding cTnI, area under the curve (AUC) was 0.981 and the upper limit was 41.2% (the recovery of subject 14). Regarding cTnT, the AUC was 1.000 and the upper limit was 49.8% (the recovery of subject 23).

TABLE 1. Background Characteristics of the Study Subjects (n = 52)[a]

Characteristic	Total
Age, y	76 (65–82)
eGFR, mL/min/1.73m^2	55.9 (44.0–67.4)
cTnI, pg/mL	310.2 (121.2–950.1)
cTnT, pg/mL	118.5 (79.3–259.8)

cTn, cardiac troponin; eGFR, estimated glomerular filtration.
[a]Data are given as No. (%) or median (interquartile range).

Comparison of Positive Rates of Autoantibodies against cTnI and cTnT

By using the upper limits of the recovery indicated above as the cutoffs, 8 specimens were categorized as with autoantibodies against cTnI (15.4%) whereas 1 specimen was categorized as with autoantibodies against cTnT (1.9%).

Linear Regression Analyses between cTnI and cTnT

To see the degree of interference by the autoantibodies on the measurement of cTnI or cTnT, we categorized the specimens into groups with and without autoantibodies using the cutoffs described above and overlaid the correlation plots between log (cTnT) and log (cTnI) of the 2 groups. As a result, all specimens were within 95% CIs except for 1 specimen (subject 27) as shown in **FIGURE 4A** and **4B**.

Distribution of cTnI-to-cTnT Ratios in Groups with and without Macro-cTnI

To assess whether the distributions of cTnI-to-cTnT ratios in groups with and without macro-cTnI would be statistically different, we performed the Wilcoxon signed-rank test and confirmed that the difference between the 2 groups was not significant (P = .446, **FIGURE 5**).

Discussion

In cardiomyocytes, the cTn complex consists of three subunits: TnI, TnT, and TnC. During myocardial damage, the cTn complex released into the circulation is degraded and found in various heterogeneous forms. The predominant forms of cTn in serum after ischemic heart disease are free cTnT and binary cTnI-C.[15,16] Moreover, free troponin T is easily degraded, whereas cTnI-C is less likely to be degraded because it forms a complex.

The protein peaks in **FIGURE 2A–2D** are considered to be albumin (on the right), IgG (in the center), and void (on the left). Bates et al[17] reported that cTnT was circulating as the free form (37 kDa) whereas cTnI was circulating as I-C complex (40 kDa) or I-T-C complex (77 kDa). The patterns of the HPLC analyses in this study showed that cTnI existed predominantly as I-C complex whereas cTnT existed as the free form when autoantibodies were absent (**FIGURE 2A**). The

FIGURE 1. Two-dimensional plot of recovery after IgG depletion (y-axis) against initial troponin level (x-axis) for cTnI (A) and cTnT (B).

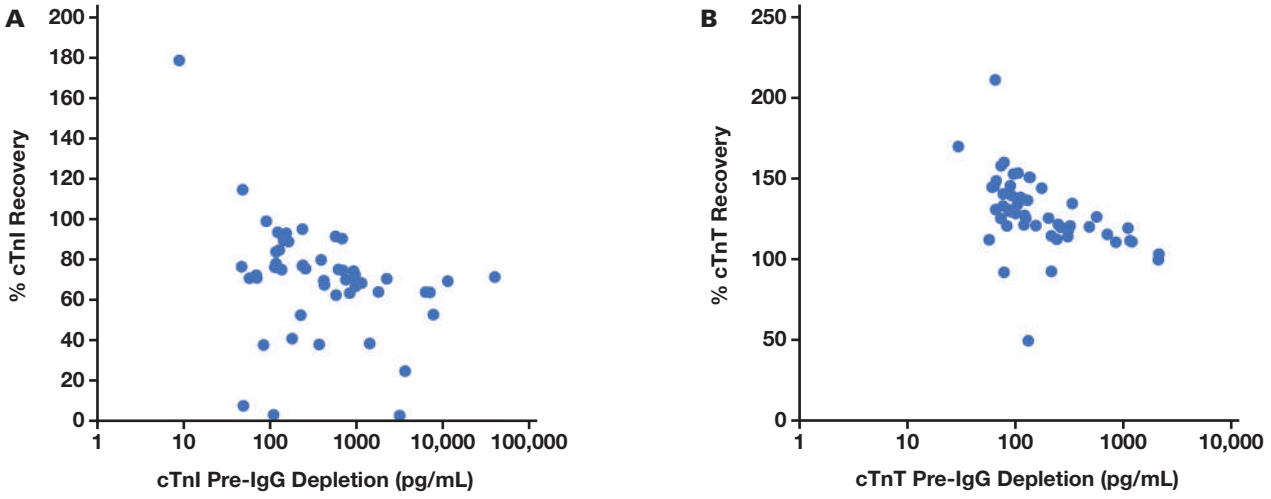
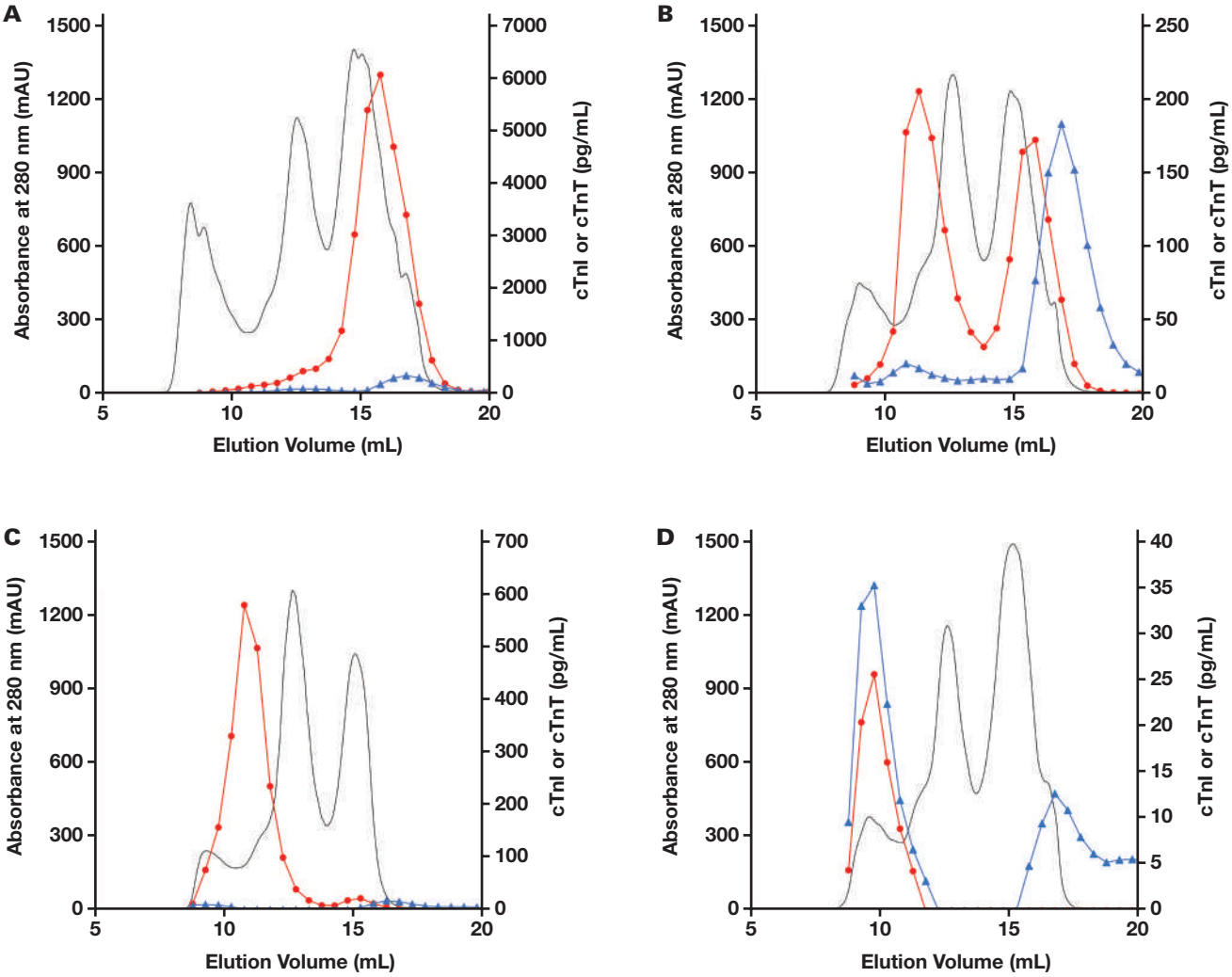


FIGURE 2. High-performance liquid chromatography chart patterns representing without immune complex (subject 24) (A), with macro cTnI (subject 51) (B), with macro cTnI (subject 27) (C), and with both macro cTnI and macro cTnT (subject 23) (D). Solid line, absorbance at 280 nm; red circle, cTnI level; blue triangle, cTnT level.



macromolecules in **FIGURE 2B** and **2C** are considered to contain I-C complex and the macro-molecule in **FIGURE 2D** is considered to contain I-T-C complex. By this interpretation, 9 specimens contained I-C complex whereas 1 specimen contained I-T-C among 15 specimens analyzed by HPLC (**TABLE 2**). Regarding cTn recoveries after IgG depletion, Lam et al[18] demonstrated low recovery even in specimens without immune complex. These low recovery results agree with those of our study. Additionally, the recovery of cTnT was higher than that of cTnI, with some specimens exceeding 100%, which may be due to the influence of the hydrophobic properties of I-C or the matrix of solutions. Using ROC analyses, we determined the upper limits of the recoveries to discover the levels of the autoantibody: 41.2% for anti-cTnI antibody and 49.8% for anti-cTnT antibody. By using these upper limits as cutoffs, we estimated the positive rate of anti-cTnI autoantibody presumably reacting with I-C to be 15.4% and that of anti-cTnT autoantibody presumably reacting with I-T-C to be 1.9%. This result, however, did not match that reported by Vylegzhanina et al[14] in which all the autoantibodies they examined bound to I-T-C ternary complex.

Shmilovich et al[19] reported that the positive rate of anti-cTnI antibody in patients with dilated cardiomyopathy (DCM) was 15.6% and that in patients with ischemic cardiomyopathy (ICM) was 18.2%. Similarly, Landsberger et al[20] reported that the positive rate of anti-cTnI antibody in patients with DCM was 20.4% and that in patients with ICM was 18.4%. The positive rate of anti-cTnI antibody obtained in our study (15.4%) could be considered comparable. Regarding the difference in the positive rate of anti-cTnI and that of anti-cTnT antibodies, Leuschner et al[21] reported that anti-cTnI antibodies with a titer of ≥1:160 were detected in 7.0% of patients with DCM and in 9.2% with ICM, whereas anti-cTnT antibodies with a titer of ≥1:160 were detected only in 1.7% of patients with DCM and in 0.5% with ICM. In a report by Lam et al,[18] the group of subjects with macro-cTnI and normal cTnT recovery was 40%, whereas the group of subjects with macro-cTnI and low cTnT recovery was 15%. Together with the positive rates obtained in this study, it may well be assumed that the occurrence of anti-cTnI autoantibodies is more frequent by several fold than that of anti-cTnT autoantibodies.

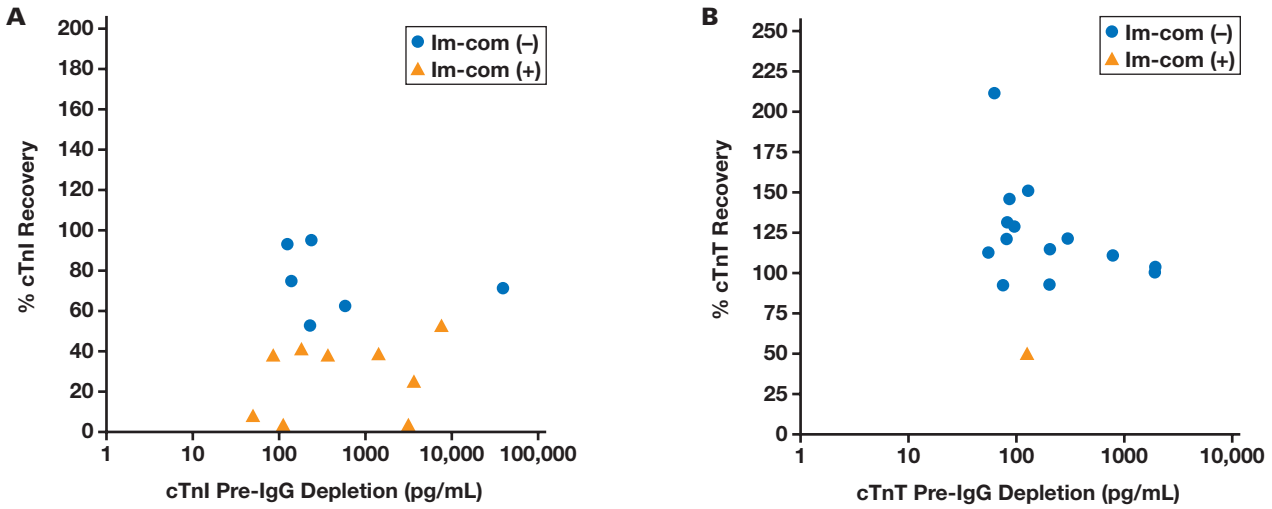
As shown in **FIGURE 4A** and **4B**, 1 specimen (subject 27) was outside of the 95% CI among the 52 specimens. The cTnI and cTnT levels

TABLE 2. Conformity between Recoveries after IgG Depletion and HPLC Analyses

Subject Number	Before IgG Depletion			After IgG Depletion		Recovery		Immune Complex Identified by HPLC	
	cTnI (pg/mL)	cTnT (pg/mL)	cTnI/cTnT	cTnI (pg/mL)	cTnT (pg/mL)	cTnI (%)	cTnT (%)	Macro-cTnI	Macro-cTnT
27	3096.0	96.6	32.0	25.1	31.0	3.2	128.4	Yes	No
42	109.3	86.6	1.3	1.0	31.5	3.7	145.5	Yes	No
23	49.2	126.0	0.4	1.0	15.7	8.1	49.8	Yes	Yes
51	3556.1	795.0	4.5	223.8	220.0	25.2	110.7	Yes	No
43	84.3	55.3	1.5	8.0	15.5	38.0	112.1	Yes	No
49	365.5	80.5	4.5	34.9	24.3	38.2	120.7	Yes	No
46	1393.7	304.0	4.6	135.0	91.8	38.7	120.8	Yes	No
14	178.8	62.8	2.8	18.4	33.1	41.2	210.8	Yes	No
9	224.7	83.2	2.7	29.6	27.2	52.7	130.8	No	No
31	7534.7	1967.0	3.8	995.5	508.0	52.8	103.3	Yes	No
8	573.1	129.0	4.4	89.5	48.6	62.5	150.7	No	No
24	38,250.0	1950.0	19.6	6823.3	487.0	71.4	99.9	No	No
32	135.5	205.0	0.7	25.4	58.7	75.0	114.5	No	No
5	122.6	75.6	1.6	28.6	17.4	93.3	92.1	No	No
35	234.0	205.0	1.1	55.5	47.5	94.9	92.7	No	No

cTn, cardiac troponin; HPLC, high-performance liquid chromatography.

FIGURE 3. Two-dimensional plot of recovery after IgG depletion (y-axis) against initial troponin level (x-axis) with the information of presence or absence of immune complex of selected specimens for cTnI (A) and cTnT (B). Im-com, immune complex.



of this specimen were 3096.0 pg/mL and 96.6 pg/mL, respectively, with a 32-fold difference (**TABLE 2**). As shown in **FIGURE 2C**, the height of the peak of the macro-cTnI is far greater than the peaks of presumed I-C complex or free T. The autoantibody in this specimen, therefore, may cause the hs-cTnI assay to overestimate the actual cTnI level or cause the hs-cTnT assay to underestimate the actual cTnT level. Regarding the specimen having the immune complex reacting both with cTnI and cTnT (subject 23), the deviations from the regression line were positive with cTnT and negative with cTnI, so that the effect of the autoantibody could be either positive on the hs-cTnT assay, negative on the hs-cTnI assay, or both. As shown in **FIGURE 5**, the difference in the distributions of the 2 groups with and without macro-cTnI was not significant (P = .446). Although previous studies have demonstrated that the presence of autoantibodies affects cTnI and cTnT assays,[22,23] this study shows for the first time, to our knowledge, that the presence of autoantibody may not necessarily interfere with the measurement of cTnI or cTnT in most cases.

Vilela et al[9] reported in a review article that the emergence of anti-cardiac troponin antibodies was seen in patients with ACS or chronic CVDs such as heart failure, DCM, ICM, peripartum cardiomyopathy, or ventricular hypertrabeculation. It is reasonable to assume that antibodies against cTn are elicited by long-term leakage of cTn from

FIGURE 4. Correlation between log (cTnT) and log (cTnI). A, Presence or absence of anti-cTnI by the recovery of cTnI. B, Presence or absence of anti-cTnT by the recovery of cTnT. Solid line, regression line; dotted line, upper and lower 95% confidence intervals; blue circle, specimen with more than the upper limit of the recovery for the presence of immune complex; orange triangle, equal to or less than the upper limit of recovery for the presence of immune complex.

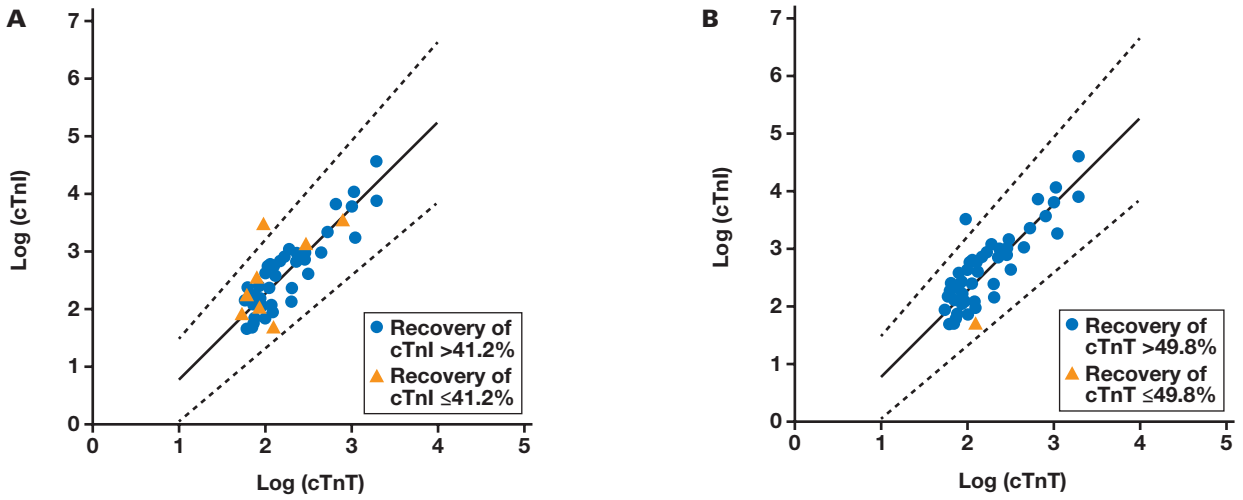
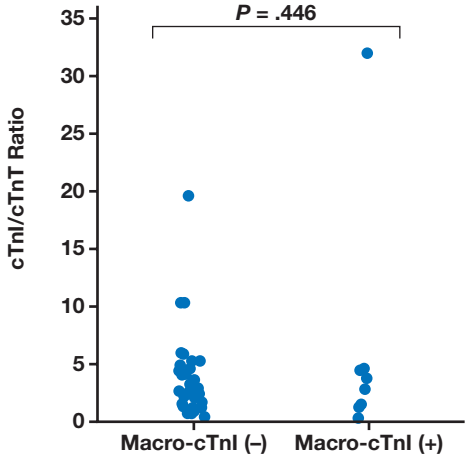


FIGURE 5. Distribution of cTnI-to-cTnT ratios in subjects with (right) and without (left) macro-cTnI.



the myocardium in such chronic CVDs. Regarding ACS, Pettersson et al[24] reported that ACS patients with cTn autoantibodies have higher cTnI release than patients without autoantibodies and that the release of cTnI also lasted longer, by at least months, suggesting that the amount and duration are determinants for the generation of cTn autoantibodies. It is also worth noting that cTnI levels were higher in patients with cTn autoantibodies than those without, because this suggests that the leakage of cTnI could have started long before the event of ACS in the former group compared with the latter group. Wu[25] suggested a possibility that autoantibodies to cTn function as a contributor to heart failure. Leuschner et al[21] reported that absence of autoantibodies against cTnI could be an indicator for improvement of left ventricular function after acute myocardial infarction. Warner et al[26] reported that macro troponin probably contributes to a difference in patient stratification in suspected ACS. From this viewpoint, the measurement of anti-troponin autoantibody could serve as an indicator for long-term myocardial injury and the prognosis of these diseases.

Conclusions

Autoantibodies against cTnI were more frequent by several fold than those against cTnT, but the presence of the autoantibodies did not significantly expand the discrepancy between cTnI and cTnT assays.

Acknowledgments

We thank the Open Facility, Global Facility Center, Creative Research Institution, Hokkaido University for allowing us to conduct the sizeexclusion HPLC analyses using the AKTA Explorer 100 System and for providing insight and expertise that greatly assisted the research. We are also grateful to Hikaru Okui of Abbott Japan for accommodating hs-cTnI reagents for this study.

Funding

This study was supported by the Division of Laboratory and Transfusion Medicine, Hokkaido University Hospital, Abbott Japan, and JSPS KAKENHI Grant Number 22H04358. This research was partially funded by Abbott Japan, where Satoshi Sugawa and Satoshi Murakami are employed. hs-cTnI reagents used in this study were provided by Abbott Japan.

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CASE STUDY

A Rare Case of Hemolytic Transfusion Reaction in a Premature Infant Caused by a Passive Anti-Jk^a Antibody

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Keywords: hemolytic transfusion reactions, anti-Jk^a, transfusion, passive antibody, donor

Abbreviations: HTR, hemolytic transfusion reaction; FFP, fresh frozen plasma; RBCs, red blood cells; SHOT, Serious Hazards of Transfusion

Laboratory Medicine 2023;54:324–326; https://doi.org/10.1093/labmed/lmac104

ABSTRACT

Hemolytic transfusion reaction (HTR) is an important type of transfusion-associated reaction and usually occurs after alloimmunization to red blood cell antigens. The HTRs caused by passively transferred Kidd blood group antibodies are not well documented. Here, we report about a premature infant who developed HTR owing to a passive anti-Jk^a antibody transfer following fresh frozen plasma transfusion. Anti-Jk^a antibody was detected in the infant's plasma and was also found in the donor plasma with a titer of 1:128. We reported this case to the local blood center, and they subsequently began testing for irregular antibodies of donor plasma, which is recommended but not mandated in China. This case reveals an unusual cause of HTR and emphasizes a possible need to screen donor plasma for antibodies to minimize risks to recipients.

Clinical History

A girl weighing 1390 g was vaginally delivered at 32 weeks of gestational age following premature membrane rupture. Her Apgar scores were 7, 9, and 9 points at 1, 5, and 10 min, respectively. She was transferred to the neonatology department immediately after birth and was diagnosed as having neonatal respiratory distress syndrome with gastrointestinal bleeding and diffuse intravascular coagulation. Immediately after admission, low molecular weight heparin and 20 mL of a split product of group A fresh frozen plasma (FFP) (both mother and baby were group

TABLE 1. Comparison of Laboratory Test Results of the Patient Pretransfusion and Posttransfusion

	Reference Interval	Pretransfusion	Day 3 Post FFP Transfusion
Hemoglobin (g/dL)	17–21	15.7	8.5
Reticulocyte count ($\times 10^{12}$/L)	0.024–0.084	0.025	0.269
Platelet count ($\times 10^9$/L)	100–300	67	110
PT (s)	9–15	18.3	14.3
APTT (s)	16.9–36.9	78	65
Fg (mg/dL)	200–400	150	220
D-dimer (mg/L)	<0.55	3.20	1.90
FDP (ug/mL)	<5	17.2	8.3
LDH (U/L)	120–246	186	427
Total bilirubin (mg/dL)	0.58–11.11	2.3	8.6
Direct antiglobulin test	Negative	ND	3+
Antibody screening	Negative	–	+
Antibody identification (in plasma)	Negative	ND	Anti-Jk^a
Antibody identification (in eluate)	Not applicable	ND	Anti-Jk^a

APTT, activated partial thromboplastin time; FDP, fibrinogen degradation products; Fg, fibrinogen; LDH, lactate dehydrogenase; ND, not done; PT, prothrombin time.

TABLE 2. Results of Screening Tests and Antibody Identification for Mother and Infant in Plasma and Eluate

Cells	Rh-Hr					Kell						Duffy		Kidd		Lewis		P	MNS				Luther		Xg	Experimental results		
	C	D	E	c	e	K	k	Kp^a	Kp^b	Js^a	Js^b	Fy^a	Fy^b	Jk^a	Jk^b	Le^a	Le^b	P1	M	N	S	s	Lu^a	Lu^b	Xg^a	Patient		Donor
																									Plasma	Eluate	Plasma
I	+	+	0	0	+	0	+	0	/	NT	/	+	0	+	0	0	+	+	0	+	0	+	0	/	+	2+	3+	3+
II	0	+	+	+	0	0	+	0	/	NT	/	+	+	+	0	0	+	+	+	0	+	0	0	/	+	2+	3+	3+
III	0	0	0	+	+	+	+	0	/	NT	/	0	+	0	+	+	0	0	0	+	+	+	0	/	+	0	0	0
1	+	+	0	0	+	0	+	0	+	/	+	+	0	+	0	0	+	+	+	0	+	0	0	+	0	2+	3+	3+
2	+	+	0	0	+	+	+	0	+	/	+	0	+	+	0	0	+	+	0	+	0	+	0	+	0	2+	3+	3+
3	0	+	+	+	0	0	+	0	+	/	+	0	+	+	+	0	0	+	0	+	+	0	0	+	+	2+	3+	3+
4	0	+	0	+	+	0	+	0	+	0	+	0	0	+	0	0	0	+	0	+	0	+	0	+	+	2+	3+	3+
5	+	0	0	0	+	+	+	0	+	/	+	+	0	+	0	+	0	+	+	0	+	+	0	+	+	2+	3+	3+
6	0	0	+	+	0	0	+	0	+	/	+	+	+	+	0	0	0	+	+	0	+	+	0	+	+	2+	3+	3+
7	0	0	0	+	+	+	0	0	+	0	+	0	+	+	+	0	+	0	+	0	+	+	0	+	+	2+	3+	3+
8	0	0	0	+	+	+	+	0	+	0	+	+	+	0	+	0	+	+	+	0	0	+	+	+	+	0	0	0
9	0	0	0	+	+	0	+	0	+	/	+	+	+	0	+	0	+	+	+	0	+	+	+	+	/	0	0	0
10	0	0	0	+	+	0	+	0	+	0	+	+	0	+	0	0	+	0	+	0	+	0	+	+	+	2+	3+	3+
11	+	+	+	0	+	0	+	0	+	/	+	+	0	0	+	0	+	+	0	+	0	+	+	+	0	0	0	0
12	W	+	+	+	0	0	+	0	+	/	+	0	+	+	+	+	0	+	0	+	+	+	0	+	+	2+	3+	3+
13	0	0	0	+	+	0	+	+	+	0	+	+	0	+	+	0	+	+	+	+	0	+	0	+	+	2+	3+	3+
14	+	+	0	+	+	0	+	0	+	/	+	+	+	+	+	0	+	+	+	0	0	+	0	+	/	2+	3+	3+
15	0	+	+	+	0	0	+	0	+	/	+	0	+	+	0	+	0	+	+	+	0	+	0	+	+	2+	3+	3+
16	+	0	0	0	+	+	+	0	+	0	+	+	+	+	0	0	+	+	+	+	0	+	0	+	0	2+	3+	3+
NC	+	+	0	0	+	/	/	/	/	/	/	/	/	+	0	/	/	/	/	/	/	/	/	/	/	2+	3+	3+

NC, neonatal cell; NT, not tested; W, weak.

A1) were administered to prevent severe bleeding and improve microcirculation and coagulation function. Because of visible yellowing of the skin, obvious symptoms of anemia, and brown urine on the third day posttransfusion, hemolysis was suspected, and transfusion reaction evaluation was immediately performed.

Laboratory Role in Diagnosis

The clerical recheck between the patient and component showed no evidence of identification errors. Phenotyping indicated that both the infant's and mother's blood types were A1, RhD(+), and Jk(a + b–). Posttransfusion, irregular antibody was detected in the baby's plasma, which was not present at pretransfusion. Other laboratory results of the infant are presented in **TABLE 1**.

Antibody identification of both the plasma and eluate showed that the culprit antibody was anti-Jk^a (**TABLE 2**), which could be completely adsorbed by Jk^a-positive cells but not by Jk^a-negative cells. The differential absorption with Jka-positive and -negative cells helped to determine alloantibody specificity. Though the autocontrol was positive, autoantibodies are usually nonspecific and can bind to all red blood cells (RBCs). The lack of pan-reactivity on the identification panel in our case is not congruent with autoantibody. Moreover, it is unlikely for a premature infant to produce autoantibodies in such a short time. To identify the source, both maternal plasma and the remainder of the donor FFP were tested. A negative antibody screening from the mother excluded the maternal source, and anti-Jk^a was detected in the donor plasma (**TABLE 2**) with a titer of 1:128. In summary, we confirmed that the anti-Jk^a antibody was passively transferred from the donor FFP to the infant. In other words, the infant developed HTR caused by the passive anti-Jk^a antibody.

Patient Follow-up

The patient's anemia was treated with Jk^a antigen-negative RBCs combined with intravenous immune globulin infusion to prevent the destruction of sensitized RBCs by the neonatal mononuclear phagocytic system. With a series of supportive treatments, such as keeping water and electrolytes balanced, maintaining temperature, and feeding properly, the patient's symptoms gradually improved. Her hemoglobin level increased to 13.5 g/dL on the third day after RBC transfusion and no further decline was observed.

Discussion

Blood transfusion is a critical lifesaving intervention and one of the most common procedures performed in hospitals; however, it can also result in adverse events.[1] The HTR is recognized as an important type of transfusion-associated reaction. Approximately 1% of transfusions result in serious adverse reactions,[2] of which 5% are HTR.[3] Excluding ABO-incompatibility, data from the Annual Serious Hazards of Transfusion (SHOT) Report provides evidence of antibodies that cause HTRs, including Rh and non-Rh systems, such as anti-E, -C, -S, -Fy^a, and -Jk^a.[4] According to SHOT, HTRs are largely preventable and adherence to established protocols for prompt identification and timely management, as well as reporting, remain the cornerstone of HTR management.[4]

There have been many reports of HTRs after RBC transfusion, which are presumed to occur after alloimmunization of RBC antigens. In reported cases where hemolysis is secondary to passively transferred blood group antibodies, the ABO system is most frequently involved, followed by the Rh system, and occasionally other blood groups.[5] The HTR caused by passive anti-Jk^a alloantibody in a hypovolemic premature infant has not been well documented. In our case, the root cause was the lack of antibody screening for irregular blood group antibodies in the donor plasma, which could have avoided the passive hemolytic transfusion reaction for this infant.

In China, antibody screening for donor plasma is recommended but not mandated by the Ministry of Health. If the recipient has the same ABO blood type as the donor, no other test must be performed before plasma transfusion.[6] In addition, the donor sample from our local blood center is often insufficient to perform additional tests. Therefore, most hospitals, including ours, did not previously conduct antibody screening of donor plasma. However, our case findings revealed that antibody screening of donor plasma is important, especially for patients with small blood volumes. We reported this case to the local blood center, which subsequently started testing for irregular antibodies in donor plasma.

Although donor antibody screening is routine in many developed countries, it is not universally conducted. In particular, in developing countries there is a risk that patients with hypovolemia will passively acquire irregular antibodies that may cause HTR. Our case findings show the importance of considering donor plasma antibody screening, especially for populations where the transfusion dose may comprise a considerable portion of the total blood volume, as with neonates.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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A Novel *INS* Mutation in the C-Peptide Region Causing Hyperproinsulinemic Maturity Onset Diabetes of Youth Type 10

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Keywords: insulin, diabetes mellitus, mody 10, proinsulin, C-peptide, genetics

Abbreviations: DM, diabetes mellitus; PNDM, permanent neonatal DM; MODY, maturity-onset diabetes of youth; DM2, type 2 DM; Arg, arginine; CPE, carboxypeptidase E; Gln, glutamine; Glu, glutamate; Gly, glycine; PC, prohormone convertase

Laboratory Medicine 2023;54:327–332; https://doi.org/10.1093/labmed/lmac115

ABSTRACT

Monogenetic diabetes mellitus (DM) describes a collection of single-gene diseases marked by hyperglycemia presenting in childhood or adulthood and the absence of immunological markers of type 1 DM. Mutations in the human insulin gene *INS* give rise to two separate clinical syndromes: permanent neonatal DM, type 4 (PNDM4), and maturity-onset diabetes of youth, type 10 (MODY10); the former presents shortly after birth and the latter presents in childhood and adulthood. We describe a 40-year-old man in a kindred with high prevalence of DM who presented with severe hyperglycemia but not ketoacidosis or hypertriglyceridemia. Twelve years after initial presentation, the patient had elevated proinsulin and normal plasma C-peptide when nearly euglycemic on treatment with insulin glargine. A novel *INS* mutation, Gln65Arg, within the C-peptide region was identified. The *INS* (p.Gln65Arg) mutation may cause MODY10 by disrupting proinsulin maturation.

Driven largely by the dramatic rise in the prevalence of type 2 diabetes mellitus (DM2),[1] DM has reached pandemic proportions. There has also been a rise in DM1 prevalence and survival of affected persons well beyond reproductive age in economically advanced societies.[2] Based on large-scale genomic efforts, single-gene mutations have been found in 4% of DM cases with the most commonly mutated genes in this subset carrying the numbered name maturity onset diabetes of youth (MODY). The assorted monongenetic DM conditions have different responses to therapies, indicating that greater precision in diagnosis can guide better treatment options. Likewise, refined understanding of the long-term complications of DM types can be realized with greater awareness and diagnosis of monogenetic DM, most practically in removing these persons from the polygenetic studies of DM2.[3]

The Neonatal Diabetes International Collaborative Group identified a collection of 10 heterozygous mutations in the insulin gene *INS* in (mostly) infants with permanent neonatal diabetes mellitus, type 4 (PNDM4, OMIM: 618858).[4] These workers and the Norwegian Childhood Diabetes Study Group reported subsequently that different mutations in *INS* give rise to MODY10 in children and adults (OMIM: 613370).[5] MODY10 is less prevalent than PNDM4, and much less common than other MODY types (particular MODY 3, 1, and 5). Causing confusing curation of the literature, MODY10 was reported several times prior to adoption of this name.[6] The *INS* mutations that give rise to PNDM4 and MODY10 do not overlap, generally, but both conditions can be caused by mutations in the signal sequence, B chain, C-peptide, and A chain regions. In short, there is little genotype-phenotype correlation between *INS* mutations and PNDM4 or MODY10.

We report a new *INS* mis-sense mutation within the region encoding the C-peptide in a man who presented with severe hyperglycemia without ketoacidosis or hypertriglyceridemia at age 40 years. He took metformin and various sulfonylureas for nearly 1 decade but then stopped taking any medications for DM. The patient then presented with severe hyperglycemia without ketoacidosis or hypertriglyceridemia. At this evaluation, autoimmune markers of DM1 were absent, and normal C-peptide concentration was found. He briefly began insulin glargine but discontinued insulin after 6 months. He presented to our attention approximately 3 years later with a similar pattern of severe hyperglycemia without ketoacidosis or hypertriglyceridemia. Insulin glargine was resumed. Genetic testing for monogenetic DM revealed a previously unreported *INS* variant causing a Gln65Arg mutation within the C-peptide region. After resuming insulin glargine and achieving near-normal blood glucose, he was found to have elevated proinsulin concentration. The unusual features of the patient's presentation, including the 12-year hiatus

from the initial diagnosis and brief treatment to a molecular diagnosis and resumption of tailored therapy, indicate that comprehensive laboratory phenotyping and a broad differential diagnosis are needed to provide optimal treatment of DM.

Case Report

A 52-year-old man presented for evaluation of uncontrolled DM. His hemoglobin A1c was 11.6% (103 mmol/mol; normal, 4.3%-5.6% or 23-38 mmol/mol) 1 month prior to evaluation in our facility, when he was prescribed insulin glargine (20 units daily). He was diagnosed with DM2 at age 40 years and had taken insulin glargine, metformin, and sulfonylurea medications intermittently until age 50 years. Three years prior to presentation he had severe hyperglycemia and was evaluated at another facility. At that evaluation, the hemoglobin A1c was 12.8% (116 mmol/mol), and nonfasting glucose was 413 mg/dL (22.9 mmol/L; normal 65 to 99 mg/dL or 3.6 to 5.5 mmol/L). He was given insulin glargine (20 units at bedtime) and metformin (1000 mg twice daily). Autoimmune markers of DM1 were absent. He took these medications for several months and had improvement in glycemic control but had stopped taking both for nearly 2 years (**TABLE 1**).

The patient's body mass index was 30.0 kg/m^2 and his mass had not increased nor decreased. There were no Cushingoid or acromegaloid features. He stated that he had never had a ketoacidotic episode. An extensive family history was identified at this encounter: both paternal grandparents, both parents, and all 9 siblings had DM or pre-DM, and several children had DM. None of these first-degree relatives had ketoacidosis or autoimmune endocrinopathies.

Nonfasting serum was collected at 5:16 PM showing severe hyperglycemia, again without acidosis or hypertriglyceridmeia (**TABLE 1**). Kidney function was normal, as were liver transaminases and thyroid-stimulating hormone. There was no evidence of iron overload. The C-peptide was normal, as it had been nearly 3 years prior. The DM1 autoantibodies were absent again. A monogenetic DM gene panel (Invitae) sequencing coding regions and intron-exon boundaries of 28 genes was obtained. A previously unreported coding variant in the *INS* gene was identified: c.194A > G(p.Gln65Arg). The mutated aminoacyl residue lies in the C-peptide region of insulin, suggesting the Gln65Arg mutation may cause disruption of proinsulin processing to generate any one of several proinsulin intermediates (**FIGURE 1**). The Gln65 residue is invariant among primates, but is less tightly conserved in other mammals, birds, or fish.

In addition to taking insulin glargine (20 units at bedtime), the patient resumed metformin (1000 mg twice daily). Within 2 months, the patient's fasting glucose values using a hand-held glucometer were 170 mg/dL (9.4 mmol/L). The hemoglobin A1c improved on this treatment to 10.6% (92 mmol/mol) after roughly 10 weeks of therapy. At 3:45 PM (approximately 18 hours after the last insulin glargine dose), the blood glucose was 151 mg/dL (8.4 mmol/L), the C-peptide was normal, and the proinsulin, as measured by an enzyme-linked immunosorbent assay with substantial cross-reactivity with proinsulin processing intermediates, was elevated (**TABLE 1**).

Insulin aspart at a fixed dose (5 units) with dinner was added to his treatment. A continuous glucose monitoring system (Dexcom G6) was initiated. Over the 14-day period of review, all days had data available (100% sensor usage). The glucose management index after 3 weeks of use was 7.7% (61 mmol/mol), reflecting a major (predicted) drop in A1c over this period. The average blood glucose was 184 ± 63 mg/dL (10.2 ± 3.5 mmol/L). The time spent in the normal range (glucose 70-180 mg/dL [3.9-10 mmol/L]) was 56%, the time spent in the high range (glucose 181-250 mg/dL [10.1-13.9 mmol/L]) was 30%, the time spent in the very high range (glucose >250 mg/dL [>13.9 mmol/L]) was 13%, the time spent in the low range (glucose 55-69 mg/dL [3.1-3.8 mmol/L]) was <1%, and the time spent in the very low range (glucose <54 mg/dL [<3.0 mmol/L]) was 0%. The high and very high range glucose values occurred most frequently after dinner. Additional education regarding counting carbohydrates at meals and treating with an insulin aspart dose that included a correction ("sensitivity factor") was provided.

Three months later, the continuous glucose monitor data was reviewed. Over a 14-day period of review, all days had data available (100% sensor usage). The glucose management index was 6.9% (52 mmol/mol), indicating a durable improvement in glycemic control. The average blood glucose was 151 ± 47 mg/dL (8.4 ± 2.6 mmol/L); the time spent in the normal range increased to 73%, the time spent in the high range decreased to 22%, the time spent in the very high range decreased to 2%, the time spent in the low range remained <1%, and the time spent in the very low range was <1%.

Discussion

Here we describe an unusual case of DM marked by diagnosis at age 40 years, lack of DM1 autoantibody markers on repeated testing, absence of ketoacidosis in the face of severe hyperglycemia on 2 occasions, and surprisingly normal triglycerides in the face of sustained severe hyperglycemia. Furthermore, there was rapid improvement in glycemic control with the implementation of basal insulin glargine and a single fixed dose of prandial insulin aspart. This pattern of discordant DM phenotypes (ie, not typical for DM1 nor DM2) prompted us to perform monogenetic DM testing. The comprehensive gene testing panel revealed a new *INS* variant. Additional biochemical testing disclosed hyperproinsulinemia, suggesting that mutated insulin fails to mature fully; there was elevated proinsulin in the face of nearly normal blood glucose (on treatment with insulin glargine), normal C-peptide, and normal insulin (again, 18 hours after the last dose of glargine).

Monogenetic DM, and MODYs in particular, are grossly underdiagnosed and their misdiagnosis affects long-term prognosis and management.[3] Since MODYs account for up to 4% of pediatric and young adult DM cases, there is an enormous gap in treatment across life stations such as puberty, pregnancy, intercurrent illnesses, and aging. Intensive efforts to increase awareness of MODY and to prompt referral for genetic testing, particularly in the United Kingdom, have shown success.[7]

Current American Diabetes Association guidelines recommend genetic testing of all persons diagnosed with DM as infants less than 6 months old and children and young adults who do not have typical features of DM1 or DM2 and have multigeneration family histories of DM. The individual genes to be tested and the methods to be used (eg, targeted exon sequencing or whole genome sequencing at a specified depth of coverage) are not specified in these recommendations; however, they are followed by the recommendation to refer all patients with identified mutations to "centers specializing in diabetes genetics" to "understand the significance of genetic mutations and how best to approach further evaluation, treatment, and genetic counseling.[8]"

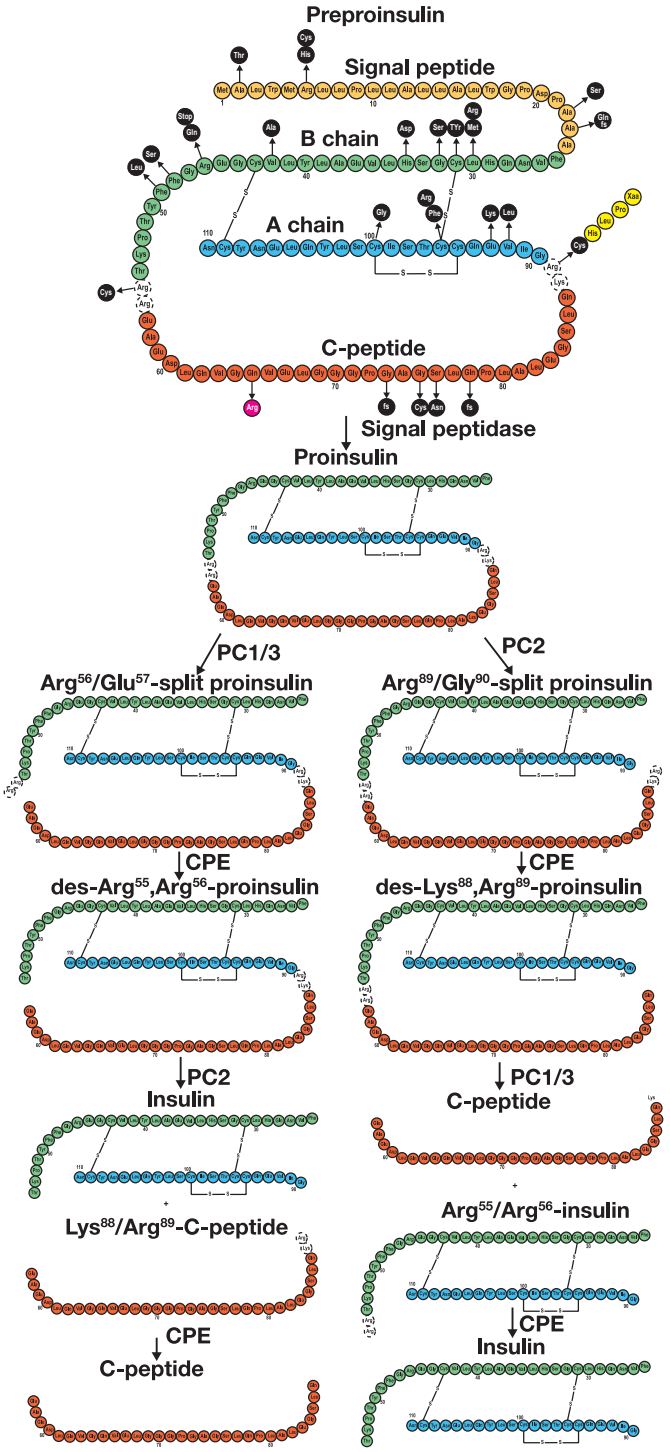
Our experience as a free-standing private practice, using a commercially available panel of exon sequencing tests, indicates these recommendations are too narrow in scale and require a potentially burdensome additional step.

TABLE 1. Select Laboratory Tests

Analyte	36 Months Prior to Evaluation	33 Months Prior to Evaluation	7 Months Prior to Evaluation	1 Months Prior to Evaluation	Evaluation 5:16 PM	2 Months after Evaluation 3:45 PM	Normal Range (SI Units if Not Identical to Conventional)
Hemoglobin A1c	12.8 (116)	7.3 (56)		11.6 (103)		10.6 (92.4)	4.3-5.6% (23.5–38 mmol/mol)
Glucose	413 (22.9)				425 (23.6)	151 (8.4)	65-99 mg/dL (3.6–5.5 mmol/L)
Estimated glomerular filtration rate					93 (1.55)	80 (1.34)	>59 mL/min/1.73 (>99 mL/sec/1.73)
Sodium	141				136	141	134–144 mmol/L
Potassium	4.3				4.3	4.4	3.5–5.2 mmol/L
Chloride	103				101	104	96–106 mmol/L
Bicarbonate	26				22	21	20–29 mmol/L
Albumin	4.8 (48)				4.5 (45)		3.8–4.9 g/dL (38-49 g/L)
Calcium	10.2 (2.6)				9.3 (2.3)	9.7 (2.4)	8.7–10.2 mg/dL (2.2–2.55 mmol/L)
AST	15 (0.25)				15 (0.25)		<40 U/L (<0.67 μkat/L)
ALT	24 (0.40)				18 (0.30)		<44 U/L (< 0.73 μkat/L)
Hemoglobin				15.8 (158)			12.0–18.0 g/dL (120-180 g/L)
Iron					94 (17)		38–169 μg/dL (6.8–30.3 μmol/L)
Ferritin					98 (98)		30–400 ng/mL (30–400 μg/L)
Transferrin					290 (35.7)		250–450 μg/dL (31–55 μmol/L)
Transferrin saturation					32		15%–55%
TSH					1.390		0.450–4.500 μIU/mL (0.450–4.500 mIU/L)
Total cholesterol			145 (3.8)	149 (3.9)			<200 mg/dL (<5.2 mmol/L)
HDL cholesterol			30 (0.8)	34 (0.9)			40–60 mg/dL (1.0–1.6 mmol/L)
Triglycerides			132 (1.5)	89 (1.0)			<200 mg/dL (<2.3 mmol/L)
Calculated LDL cholesterol			89 (2.3)[c]	97 (2.5)[c]			<160 mg/dL (<4.1 mmol/L)
Insulin						4.4 (31)[d]	2.6–24.9 μIU/mL (18-173 pmol/L)
C-peptide	1.5 (0.50)[a]				3.0 (0.99)[e]	1.4 (0.46)[e]	1.1–4.4 ng/mL (0.36–1.5 nmol/L)
Proinsulin						17.4[f]	0.0–10.0 pmol/L
Antipancreatic islet cells antibody	<1:4[b]				Negative		Negative dilution, <1:1
GAD-65 autoantibody	<5.8[b]				<5.0		<5.0 U/mL
Insulin antibodies					<5.0		<5.0 mIU/L
IA-2 autoantibodies	0.8[b]				<7.5		<7.5 U/mL
ZNT8 antibodies	<10[b]				<15		<15 U/mL
Urine albumin/creatinine	4 (0.45)[b]				<8 (<0.9)		<30 mg/g (<3.4 mg/mmol)

[a]*Normal range 0.8-3.5 ng/mL (0.26–1.2 nmol/L); measured by quantitative chemiluminescent immunoassay (ARUP).*
[b]*Different reference range; also negative or normal.*
[c]*Friedewald formula (in mg/dL): calculated LDL = total cholesterol – HDL – (triglycerides/5).*
[d]*Measured 20 hours after last insulin glargine injection by electrochemiluminescence immunoassay (LabCorp).*
[e]*Measured by electrochemiluminescence immunoassay (LabCorp).*
[f]*Measured by the Invitron Intact Proinsulin enzyme-linked immunoassay (LabCorp) with the following cross reactivities: intact proinsulin 100%, insulin 0.0%, C-peptide 0.0%, Arg^{56}/Glu^{57}-split proinsulin 5.6%, des-Arg^{55}, Arg^{56}-proinsulin 1.4%, Arg^{89}, Gly^{90}-split proinsulin 37%, des-Ly^{88}, Arg^{89}-proinslin 63%.*

FIGURE 1. Secondary structure of human preproinsulin and its derivatives culminating in mature insulin, with signal peptide (hazel), A chain (blue), B chain (green), C-peptide (orange) regions, disulfide bridges (S-S), and dibasic cleavage sites (open circles) marked. Black residues indicate previously described MODY10 mutations (the vast majority of which are cataloged in Rafique et al[13]), the yellow residues indicate the multiple hyperproinsulinemia variations identified previously (all at Arg 89). The Gln65Arg mutation found in this report is shown in magenta. The major pathway for proinsulin production is to the left, namely prohormone convertases 1 and 3 (PC1/3) first catalyze the generation of the intermediate Arg^{56}/Glu^{57}-split proinsulin (the most abundant proinsulin intermediate in blood[10]), then carboxypeptidase E (CPE) removes Arg^{55} and Arg^{56} from the B chain, and finally PC2 liberates mature insulin, leaving behind a C-peptide intermediate Arg89/Gly90-C-peptide that is matured by CPE. For clarity, unique permanent neonatal DM, type 4 mutations are not labeled in preproinsulin. fs, frame-shift; Xaa, stop codon.



We identified MODY10 due to a novel *INS* variant via a commercial genetic testing laboratory in a man previously given the incorrect diagnosis of DM2. Without any additional genetic counseling, we tailored his therapy to improve glycemic control. Wider-spread adoption of this approach will advance care for DM generally and allow for better research strategies to tackle the more prevalent DM forms; misdiagnosed monogenetic DM will be removed from the investigation of much more prevalent DM2 in particular.[3]

Consensus on this issue of wider-spread adoption of molecular diagnosis in new DM will emerge from greater appreciation for the spectrum of clinical presentation in monogenetic DM (eg, MODYs can present in middle-aged persons), and from greater recognition of the dropping costs of next-generation sequencing. Nevertheless, a major challenge is annotation of identified variants. Careful clinical and biochemical phenotyping (such as the case here) will improve this annotation. Indeed, previously identified insulin variants marked by elevated proinsulin concentrations are clustered at Arg89, part of the dibasic cleavage site between C-peptide and A chain. Interestingly, there is a MODY10 variant at this site as well: Arg89Cys.

Revealing the molecular mechanism accounting for the dominant nature of the *INS* Gln65Arg mutation will require in vitro studies. Comparisons not only with wild-type insulin but with other C-peptide mutations may reveal that more than 1 defect in insulin processing might be present.[9] For instance, measuring the half-life of the proinsulin should be assessed to differentiate overproduction from decreased degradation. The blood proinsulin was measured with a commercial immunoassay during near-normal glycemia achieved with treatment with insulin glargine. It would be dangerous to stop said treatment (ie, A1c was still very high) to perform an oral glucose tolerance test, where a maxium, stimulated proinsulin could be determined.

It will be interesting to determine what processing intermediates of insulin are in the blood of the patient by using liquid chromatography. Namely, commercial immunoassays for proinsulin vary in the degree to which they distinguish among Arg^{56}/Glu^{57}-split proinsulin, Arg^{89}/Gly^{90}-split proinsulin, des-Arg^{55},Arg^{56}-proinsulin, and des-Lys^{88},Arg^{89}-proinsulin, providing a "total" proinsulin, even when intact proinsulin is clearly distinguished from insulin and C-peptide concentration. The patient's proinsulin was measured with a commercial assay that detects intact proinsulin completely and assorted fragments to varying degrees. All of these intermediates are poorly processed to mature insulin in blood,[10] and proinsulin has nearly 90% lower affinity for insulin receptor than insulin.[11] It would be informative to test binding affinity of the prevalent proinsulin in the serum of an *INS* Gln65Arg mutation patient as well as any significant intermediates present. Likewise, assessment of activation of various cellular stress signaling pathways and apoptotic mechanism within the pancreatic β-cells that express *INS* Gln65Arg could be informative.[12]

In addition to the open biochemical and cell biological questions that the *INS* Gln65Arg mutation raises, there remain critical issues relating to phenotypic variation within the extended kindred and among other MODY10 and PNDM4 families. A unifying molecular mechanism to explain both the dominant molecular nature of the various *INS* mutations and how various mutations affect the age of onset of DM have not been elucidated to date; however, our patient's hyperproinsulinemia—even when made euglycemic by exogenous insulin—suggests that the sustained presence of proinsulin interferes with normal insulin action in peripheral tissues. Careful phenotyping of this proband and affected and seemingly unaffected relatives may yield insights into the pathogenesis of this form of MODY10. More generally, genetic study of individuals in large families, whether via genome-wide association for common variants implicated in DM2 or more sophisticated methods, holds the promise of providing not only better prognostic and therapeutic interventions for these rare monogenetic DMs but also for more common DM1 and DM2.

Conclusions

Monogenetic DM is present in 4% of all persons diagnosed as having DM1 or DM2. This 1-in-25 misdiagnosis exposes patients to potentially harmful therapies and delays care for their immediate relatives. Here we identified a novel *INS* variant that causes MODY10 in a man who was diagnosed with DM2 for over a decade. Glycemic control rapidly improved with relatively low dose basal-bolus insulin analogs. Broader adoption of molecular genetic testing of DM across life stations will improve diagnosis and care. Specialized diabetes genetic centers are not needed to affect this transformation.

Acknowledgments

We thank Sanjai Nagendra, MD, and Andre Valcour, PhD, of LabCorp for information regarding the proinsulin assay.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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A Case of Massive Fetal-Maternal Hemorrhage: Lessons Learned in Diagnosis and Treatment

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Keywords: massive fetal-maternal hemorrhage, Kleihauer-Betke test, Rhogam, Rh antigen, anti-D immunoglobulin

Abbreviations: IUFD, intrauterine fetal demise; IAT, indirect antiglobulin test; KB, Kleihauer-Betke; TMS, transfusion medicine service; HDFN, hemolytic disease of the fetus and newborn; AMIS, antibody-mediated immune suppression

Laboratory Medicine 2023;54:333–336; https://doi.org/10.1093/labmed/lmac116

ABSTRACT

The use of Rho(D) immune globulin in Rh-negative pregnant women has become standard of care, but many practicing clinicians do not know the dosing recommendations for this essential medication. In this article, we describe a case of a 15-year-old girl who presented with intrauterine fetal demise and was found to have massive fetomaternal hemorrhage. Kleihauer-Betke testing results indicated nearly 460 mL of fetal blood in the maternal circulation. The patient ultimately received 4800 μg of Rho(D) immune globulin, a dose that required close coordination with the obstetrical service and pharmacy. Although this is an unusual case of large-volume, potentially chronic, fetomaternal hemorrhage, it is also an excellent illustration of the principles for diagnosing this condition, as well as providing dosing guidelines for Rho(D) immunoglobulin to prevent alloimmunization.

Patient History

A 15-year-old girl, with medical history of one prior pregnancy that ended in miscarriage, presented at 36 weeks 0 days gestation to labor and delivery triage with concerns for loss of fetal movement in the previous 4 days. The patient had received regular prenatal care, and her medical history was further remarkable for Rh-negative status for which she was given a standard dose of Rho(D) RhIg (300 μg) at 28 weeks gestation. On the day of presentation at our hospital, ultrasound confirmed the absence of cardiac activity of the fetus, and a diagnosis of intrauterine fetal demise (IUFD) was made. The patient was admitted for induction of labor and delivered a dead infant girl vaginally the next day.

As part of her evaluation, the patient had a standard type and screen performed with results significant for type O, Rh-negative RBCs and a negative indirect antiglobulin test (IAT). A Kleihauer-Betke (KB) test was performed at the time of admission before the delivery of the dead infant; the results indicated 458 mL of fetal blood in the maternal circulation. A repeat test confirmed these results. The transfusion medicine service (TMS) was consulted to assist in managing the patient.

Considering the young age of the patient and her potential for future pregnancies, the team believed that treatment was preferential to watching and waiting. The TMS considered various treatment methods for this large-volume fetomaternal hemorrhage. Current guidelines from the American Society of Apheresis indicate that RBC exchange may be appropriate in cases of large-volume fetomaternal hemorrhage[1]; however, this is a Category III recommendation, indicating that the evidence supporting this recommendation is mixed and that an individualized clinical decision should be made.

After discussion among the team, we believed that the risks of proceeding with pharmacological treatment were no higher than those of RBC exchange, and ultimately the team decided to pursue pharmacological treatment. The TMS recommended that the patient be treated for the calculated fetal hemorrhage with 16 vials (4800 μg) of RhIg. The TMS, in close coordination with the pharmacy and obstetrical service, developed a plan to give the patient this massive dose during a 4-day period: 13 doses were delivered intramuscularly while the IV formulation was being obtained, and the final 3 doses were delivered intravenously (**TABLE 1**). It should be noted that the maximum dose of RhIg is 1000 μg every 12 hours, and that such high doses were an off-label use of the product based on the massive amount of fetomaternal hemorrhage. The patient remained in the hospital (nonemergency service) for close monitoring of potential adverse effects from this massive dose of RhIg, including hemolysis and allergic reaction.

Clinical and Laboratory Information

The KB test is used to calculate the volume of fetal cells in the maternal circulation. Created in 1957, it relies on differences between maternal and fetal hemoglobin.[2] Fetal hemoglobin contains 2 α and 2 γ subunits, whereas >95% of adult hemoglobin contains 2 α and 2 β subunits. The presence of γ subunits serves an important physiologic function: γ subunits have higher affinity for oxygen and allow fetal hemoglobin to

grab oxygen from maternal hemoglobin.[3] γ subunits are also more resistant to acid and alkali denaturation.[3,4] When exposed to acid, adult hemoglobin is removed from the cell, but γ subunits remain and can take up a stain.[4,5] Thus, after exposure to acid and subsequent staining, fetal RBCs appear darkly reddish-pink, whereas adult cells appear lightly pinkish-white (often referred to as ghost cells). Images of the KB test from the aforementioned case are provided in **FIGURE 1**.[4,5]

The laboratory technician counts 2000 RBCs under the microscope and calculates the percentage of fetal cells. This figure is then extrapolated to calculate the volume of fetal whole blood in maternal circulation and the RhIg dosage necessary to prevent alloimmunization of the mother. There are a number of published equations to convert the percentage of fetal RBCs into mLs of fetal whole blood.[4–6] At our institution, we use the formula presented in **TABLE 2**.[4–6] A single dose of RhIg (300 μg) is expected to neutralize as much as 30 mL of fetal blood in the maternal circulation (**TABLE 2**) and is expected to prevent the development of antibodies to the D antigen for as long as 12 weeks after immunization.[4,5]

Current recommendations are that all pregnant women who test negative for the D antigen receive a standard dose of 300 μg of RhIg at 28 weeks gestation, and within 72 hours of potential exposure to fetal blood.[7] We note that this prophylaxis is not necessary if the fetus tests D negative; however, the blood type of the fetus in most pregnancies is not known until birth. Since the introduction of RhIg prophylaxis, the incidence of anti-D alloimmunization has dropped from 14% to 1%–2%.[8] This regimen has been so successful that most current cases of hemolytic disease of the fetus and newborn (HDFN) are due to ABO incompatibility.[8,9]

In this case, a patient presented with third-trimester IUFD and known Rh-negative status. A KB test was performed with appropriate negative and positive controls to assess for the presence of fetal RBCs in the maternal circulation (**FIGURE 1**). The percentage of fetal RBCs was calculated to be 9.2%, and the volume of fetal blood in the maternal circulation was calculated to be 458 mL. Most healthy pregnancies will involve a few fetal RBCs cells entering the maternal circulation. Generally, a cutoff of >30 mL is accepted to define fetomaternal hemorrhage, and a cutoff of >150 mL is accepted to define massive fetomaternal hemorrhage.[10,11] To our knowledge, reports of cases of fetomaternal hemorrhage of >400 mL are rare, with the largest reported fetomaternal hemorrhage to date featuring a fetal RBC percentage of 27.6% via the KB test.[12]

Using a dose conversion of 30 mL of fetal blood per vial of RhIg, the recommended dose of RhIg was 16.3 vials. If the dose ends in a decimal, our current policy is to round down if the decimal value is <0.5 and to round up if it is ≥0.5. Thus, in this case, the calculated dose of 16.3 vials was rounded down, for a final dose of 16 vials. IV formulations were investigated but were not immediately available for administration, leading the patient to receive 13 separate intramuscular injections in the course of 3 days and causing her a great deal of discomfort. This case demonstrates the value of IV formulations of RhIg in delivering large doses in an efficient and comfortable manner.

Other than the KB test, there are a number of methods for identifying fetal RBCs in the maternal circulation. These include the rosette test, flow cytometry, gel agglutination, immunostaining, and the use of fluorochrome conjugated anti-D.[6] Of these methods, the KB is the most widely used[5]; however, this test has some drawbacks. Although highly specific, the KB test has poor sensitivity, and requires a minimum of 5 mL of fetal

TABLE 1. Dosing Route and Schedule for Rho(D) Immunoglobulin Administered to the 15-Year-Old Female Patient

Day of Tx	Dose in IU	Dose in μg	Dose in Vials, No.	Route
Day 1	7500	1500	5	IM
Day 2	4500	900	3	IM
Day 3	7500	1500	5	IM
Day 4	4500	900	3	IV
Total	24,000	4800	16	—

IM, intramuscular; Tx, transfusion.

FIGURE 1. Images from the Kleihauer-Betke test results for our patient, a pregnant 15-year-old girl. Specimens of maternal blood from the patient were treated according to the standard protocol at our institution. Images were collected using a ×100 objective and oil immersion. A and B, Fetal RBCs appear dark reddish-pink, in a background of light pinkish-white maternal "ghost" cells. Positive and negative control specimens appeared as expected (not shown).

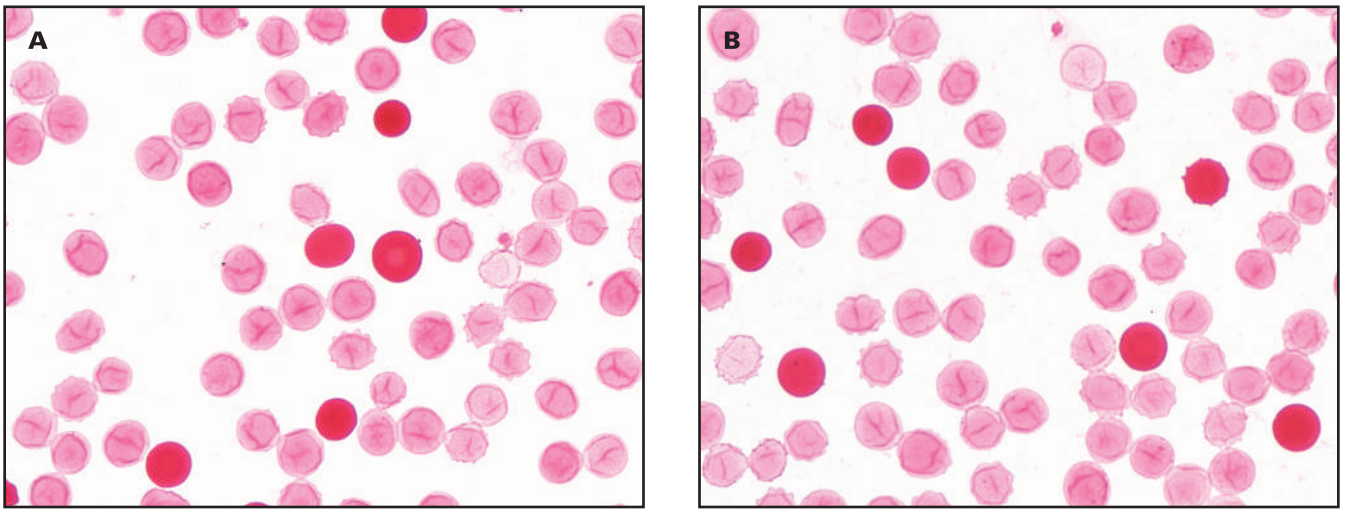

TABLE 2. Calculation of the Rho(D) Immune Globulin Dose After Kleihauer-Betke (Kb) Testing

Calculating the Anti-D Immunoglobulin Dose
Percentage of fetal cells on KB test = No. of fetal cells x 100/total No. of RBCs in field (at least 2000 RBCs must be counted)
Milliliters of fetal whole blood = % fetal RBCs x 50
30 mL of fetal whole blood requires administration of 1 vial of anti-D immunoglobulin + 1 additional vial
No. of vials needed = (% fetal RBCs x 50)/30 + 1
1 vial = 300 µg of anti-D immunoglobulin

blood in the maternal circulation for a positive result (studies indicate that 0.01–0.03 mL of fetal RBCs is enough for alloimmunization).[4] It is also widely known to over- and underestimate of the amount of fetal RBCs in circulation, compared to other methods, and has been documented to have increased interobserver variability.[5,6] Moreover, it can produce a false-positive result in the presence of a maternal hemoglobinopathy, such as sickle cell disease or hemoglobin C disease, in which there is maternal persistence of the fetal hemoglobin.[4] Despite all this, the KB test remains the most widely used in the United States due to its low cost, rapid results, and the minimal equipment needed.

In the case presented herein, we acknowledge the possibility that the KB stain overestimated the amount of fetal RBCs in the maternal circulation, and that the calculated dose of 16 vials of RhIg may have been more than necessary. Although confirmatory testing with flow cytometry could have been obtained at an outside laboratory, the potential for delay in treatment and the additional cost led the team to instead pursue treatment based on the results of the KB stain. In such cases, the cost and risks of treating with additional RhIg must be weighed against the potential of allowing alloimmunization to the D antigen and placing future pregnancies at risk of HDFN.

Discussion

The Rh blood group in humans has more than 50 defined antigens,[13] with the status of Rh(D) being designated in common nomenclature as positive (+) or negative (–) with the ABO status of the patient. The D antigen is an immunogenic antigen that is significant for pregnant women. Maternal immune cells in a mother negative for D-antigen can produce antibodies to D antigens expressed by a fetus. Antibodies to the D antigen are commonly IgG and can cross the placenta, making them one of the most common causes of HDFN. Before the 1970s, anti-D alloimmunization was the most common cause of HDFN.[7,14] This syndrome can present with symptoms ranging in severity from mild jaundice and anemia to severe brain injury or death of the fetus or newborn.[7]

Given the risk of severe outcomes with HDFN, a regimen to prevent anti-D antibodies was published in 1969.[14,15] This prophylaxis consists of injecting anti-D antibodies into the patient. The mechanism of action of this prophylaxis is not entirely understood. At the time of its introduction, 3 theories were posited: that RhIg masks D antigen from the maternal immune system, that RhIg facilitates the removal of D-antigen–positive cells to a sequestered space away from antibody-producing cells, and that there is central immune inhibition through an unknown mechanism.[14] To date, there is no confirmatory evidence for any of these models of action, although rodent models have provided support for antibody-mediated immune suppression (AMIS), in which the presence of free Ig antibodies in the circulation inhibits the development of antibodies to a wide number of antigens.[16]

The percentage of individuals testing D negative varies among ethnic groups and countries, and is highest among the White population, at 15%.[13,17] In the United States, access to prenatal care and identification of maternal D-negative status, followed by administration of RhIg, can differ between rural and urban settings.[18] Compounding this situation, many providers who deliver obstetric care may not be aware that a single dose only provides protection for a limited volume of fetal blood, or know how to calculate additional doses if necessary. This case of large volume fetal-maternal hemorrhage required a large dose of RhIg, far more than is standard, and provides an excellent example of how to calculate nonstandard RhIg doses.

The calculated volume of 458 mL in the maternal circulation of our patient is significant because it exceeds the estimated total volume of blood in a fetus of that gestational age. Blood volume of a fetus is calculated based on estimated weight and changes depending on gestational age. For 21 weeks or later, an estimation of 100 mL/kg has been suggested.[19] With an approximate weight of 3 kg, this infant might be expected to have as much as 300 mL of blood, which is only a fraction of the fetal blood calculated to be in the maternal circulation. There are 2 possible explanations for this discrepancy. One possibility is that the fetus had been chronically bleeding into the maternal circulation. In fact, the patient was examined at 28 weeks gestation for reduced fetal movement, although at that time, the viability of the fetus was confirmed by ultrasound. Another possibility is that the volume of fetal RBCs was overestimated by the KB test, which is a well-known weakness of this test.[4–6]

Patient Follow-Up

The patient experienced no immediate complications in the postpartum period. Six weeks after giving birth, she received an etonogestrel implant for contraception and at that time had resolution of vaginal bleeding and no symptoms of concern. She was scheduled for a laboratory visit at 12 weeks postpartum (after the RhIg would be catabolized) to assess her for alloimmunization that may complicate future pregnancies; however, the patient was lost to follow-up.

Acknowledgments

We acknowledge the contributions of Jack Alperin, MD, and Angela Novotny, MLT, SBB, to this manuscript.

Conflict of Interest Disclosure

The authors have nothing to disclose.

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SPECIAL REPORT

A historical perspective on diversity in Clinical Laboratory Sciences Programs

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Keywords: Clinical Laboratory Science program, Stony Brook University, diversity, lab medicine, NAACLS, medical laboratory

Abbreviations: CLS, Clinical Laboratory Sciences; LI, Long Island.

Laboratory Medicine 2023;54:e70–e76; https://doi.org/10.1093/labmed/lmac109

ABSTRACT

Objective: Diversity is integral for an effective learning environment and pedagogy. In this study, we aim to determine the student and faculty diversity in the Clinical Laboratory Sciences programs at Stony Brook University.

Methods: We retrospectively analyzed data over 20 years for the traditional program and 8 years for the hybrid program.

Results: Over 20 years, 502 students graduated from the traditional program, and 96 students graduated from the hybrid program. In the traditional program, an average of 25 students enrolled with 75% female and 25% male and in the hybrid program, an average of 12 students with 8.5 female and 3.5 male enrolled. The traditional program had the highest proportion of Asian students (50%), with White students making up 24.5%, whereas the highest proportions in the hybrid program were 35% White students and 24% Hispanic students. Among the 5 boroughs of New York City, the highest proportion of student representation was from Queens and Manhattan in both traditional (24.1%) and hybrid programs (16.7%). There were 30% male and 70% female faculty served in the traditional program whereas the current representation of fulltime faculty is 100% female.

Conclusion: Our data show the diversity of students and faculty in clinical laboratory sciences programs at Stony Brook University institution.

Since 1970, the Clinical Laboratory Sciences program at Stony Brook University has been committed to providing highly valued laboratory medicine–based education and clinical training to the next generation of medical laboratory scientists (MLS). The program was launched as a "Medical Technology Program", and in 2010 it was renamed as the "Clinical Laboratory Sciences (CLS) program". Since the inception of the program, hundreds of students have specialized in various laboratory medicine disciplines and are serving in laboratory medicine in different capacities. The program has trained a diverse population of national and international students; however, the extent of diversity in the program remains to be determined.

Diversity is a concept that differentiates a group of people from one another based on but not limited to age, ethnicity, gender, health care, mental health, and sexual orientation.[1] Contemporarily, diversity is uptrending in all disciplines of healthcare.[2–4] Since systemic inequalities have been reported in healthcare,[5,6] One way to end the differences is to prepare a diverse clinical team from early training so that deep-seated differences can be eliminated, which often merely stem from "cultural blankness" and lack of culturally diverse interaction. Diversity in education helps "furnish the minds" by providing profound acquaintance with other cultures, preventing the division that otherwise would continue.[7] In the current academic environment, racial, cultural, and religious differences are considered a building block for a prosperous and inclusive landscape for effective pedagogy. Expanding diversity in academic programs harnesses mutual ground for growth and interests, and it must be the touchstone to measure the quality of education. Diverse representation in a program is a direct measure of a welcoming and trustful learning environment; not only it is crucial for positive learning experiences for students, but nurturing the acceptance of diversity results in an effective workforce, which then drives innovation,[8] promotes socialization among groups,[9] and eliminates culture ignorance[10] which otherwise is difficult to achieve. Based on our experience, familiarity with others' cultures can be enhanced in a classroom by presenting a question among the circle of diverse groups to drive the discussion; this activity opens up participation and helps students understand others' unique perspective.

The goal of the CLS programs at our institution is focused on the "let us diversify" approaches to provide opportunities to inspire creativity, alleviate "cultural segregation," encourage students in civic engagement, and eventually promote advocacy of the profession. Over 50 years, the traditional CLS program has dedicated time and energy to meet professional

needs by training skillful laboratory scientists who will successfully engage in a career in laboratory medicine.

However, over time, the need for laboratory scientists has significantly increased for several reasons: (i) with advancement in technology, additional diagnostic tools are introduced that require a skilled and certified professional to perform the tests, (ii) the expansion of private laboratories[11] has opened up vacancies at the state and national level, and (iii) the lack of visibility of the profession[12] has undoubtedly hampered growth in numbers of certified laboratory scientists.[13] In an agreement with our clinical affiliates to address the shortage of highly trained and knowledgeable laboratory scientists in the field, we expanded from one program to two and introduced a hybrid program at our institution in 2014. Since the launch of the hybrid program, there has been a 19% increase in the number of certified laboratory students. Although we increased the number of Medical Laboratory Science programs to fill the gaps in the field, the level of diversity of trained students from our program remains unclear. Currently in the United States, we have 244 active bachelor-level Medical Laboratory Science programs[14] but the level of diversity in these programs remains elusive. Therefore, in this study, we aimed to determine the extent of diversity among the students trained in the laboratory sciences programs at our institution. Our accredited program remained committed to welcoming and training a diverse student body which is fundamental to producing a diverse workforce. Our study is the first to highlight the diversity in laboratory science programs over 20 years in the United States.

Material and Methods

In this study, we retrospectively analyzed the data of the traditional Clinical Laboratory Sciences program at Stony Brook University from 2002 to 2021 to better understand the level of diversity in our program over 20 years. We also examined the data of the hybrid CLS program from 2014-2021.

Results

The results herein represent a total of 598 students who graduated from either the traditional or hybrid CLS program. A total of 502 students graduated from the traditional program over 20 years, and since 2014, 96 students graduated from the hybrid program over 8 years. In the traditional program, the largest proportion (75%) was female, while males making up 25%. An average of 19 females and 6 males graduated from the program (**FIGURE 1A and B**). In the hybrid program, a total of 96 students graduated with an average of 12 students per class. Of the total, an average of 3.5 male and 8.5 female students (71% female, 29% male) represented the hybrid class (**FIGURE 1C and D**).

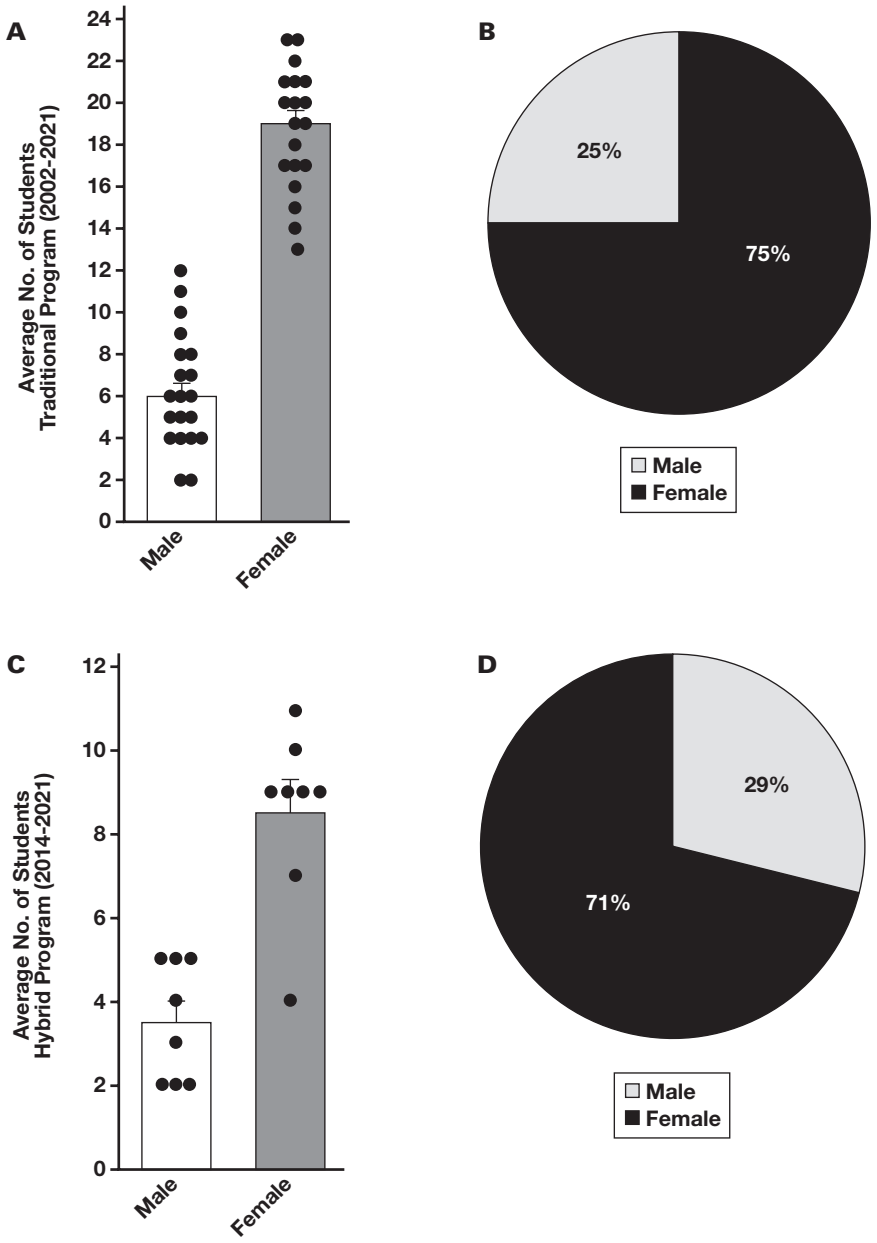
We sought to explore the diverse ethnic representation in the CLS programs. Of the total, the highest portion of those graduated from the traditional program were of Asian ethnicity (50%), followed by White (24.5%). African American (9.6%), Hispanic (8.6%), and students of unknown (7.2%) ethnicity made up one-quarter of those who graduated from the program, and in 2004, one student of American Indian/Alaskan Native descent (0.2%) graduated from our program as well (**FIGURE 2A**). **FIGURE 2C** shows the number of students per ethnicity from 2002 to 2021 in the traditional program. In the hybrid program, the majority (35%) of the representation was White, followed by Hispanic (24%), Asian (17%), African American (13%), and unknown (11%) ethnicity (**FIGURE 2B**). Students reported their age, gender, race, and ethnicity at admission. Reports and race and ethnicity include White, Hispanic (all racial groups combined), African American, Asian (all racial groups combined), and an unknown group where no race or ethnicity was available in the reported information. **FIGURE 2D** shows the number of students per ethnicity in the hybrid program from 2014 to 2021. We also sought to understand the geographic origin of the student population in both programs. Based on the data, the majority of the students in the traditional program were from New York State (91%), a small set (7%) of the student body was from other US states, and a smaller subset (2%) was international, mostly from the Southeast Asian region, including South Korea (44.44%), China (22.22%), India (22.22%), and Taiwan (11.11%) (**FIGURE 3A and D**). Since the largest percentage (91%) was from New York, next we categorized the student population within New York State and found that the majority (44%) of the students were from Long Island (Nassau and Suffolk Counties). Our institution is located on the Northshore of Long Island (LI) in southeastern New York and is approximately 60 miles east of New York City. Amongst the five boroughs of New York City, 24.1% of the students are from Queens and Manhattan, with the second-highest group (15.7%) from Brooklyn. A small portion (1.8%) was from Staten Island, located approximately 70 miles west of Long Island, and 2.8% of students were from the Bronx. We also had 3% representation from Upstate New York (**FIGURE 3B**). **FIGURE 3C** shows the breakdown of the 6.8 % of students from other states of the United States. A total of 2.6% represented the Northeastern states including New Jersey (1.6%), Pennsylvania (0.4%), Maryland (0.2%), Rhode Island (0.2%), and Massachusetts (0.2%). The southern states represented were Virginia (0.8%), Texas (0.6%), Tennessee (0.4%), South Carolina (0.2%), and Florida (0.2%). We also had representation from Washington (0.4%), Oregon (0.2%), California (0.4%), Ohio (0.2%), and Hawaii (0.2%), and the Midwest states representation included Kansas (0.2%), Wisconsin (0.2%), and Missouri (0.2%). In the hybrid program, 98% of the graduates were from New York, whereas only 2% enrolled in the program were from other US states: Kansas (1%) and Connecticut (1%) (**FIGURE 3E and G**). From New York, 54.2% of students were from Long Island, 16% from Queens/Manhattan, 10% from the Bronx, a small subset from Brooklyn (8%), 6% from Staten Island, and 2% from Upstate New York (**FIGURE 3F**).

Next, we sought to determine the diversity of the faculty serving in both traditional and hybrid programs. In the traditional program, 10 faculty members served full time, 90% of whom were White and 10% Hispanic, with 3 male (30%) and 7 female (70%) faculty members from 2002 to 2017 (**FIGURE 4A and B**). Currently, we have 5 full-time faculty serving in the program with 100% identifying themselves as female, 40% White, 20% East Asian, 20% Hispanic, and 20% South Asian (**FIGURE 4C and D**). In the hybrid program, the majority (90%) of the faculty are full-time clinicians and are teaching as adjunct faculty, whereas only 20% of the full-time faculty is teaching in the hybrid program.

Discussion

Diversity in academic institutions enhances creativity, builds a mutual ground for collaboration and interaction, provides an opportunity for intersubjectivity, and helps create a sense of community.[1–3] In a health-related academic program creating a safe learning environment and establishing relationships with the diverse body of students and providing

FIGURE 1 Average number and percentage of male and female students in the traditional and hybrid programs. A and C show the average number of 6 male and 19 female students in the traditional program (2002–2021) and 3.5 male and 8.5 female in the hybrid program (2014–2021). B and D show the percentage of female (75%) and male (25%) students in the traditional program and 71% female and 29% male in the hybrid program. Data are represented as mean ± SEM.

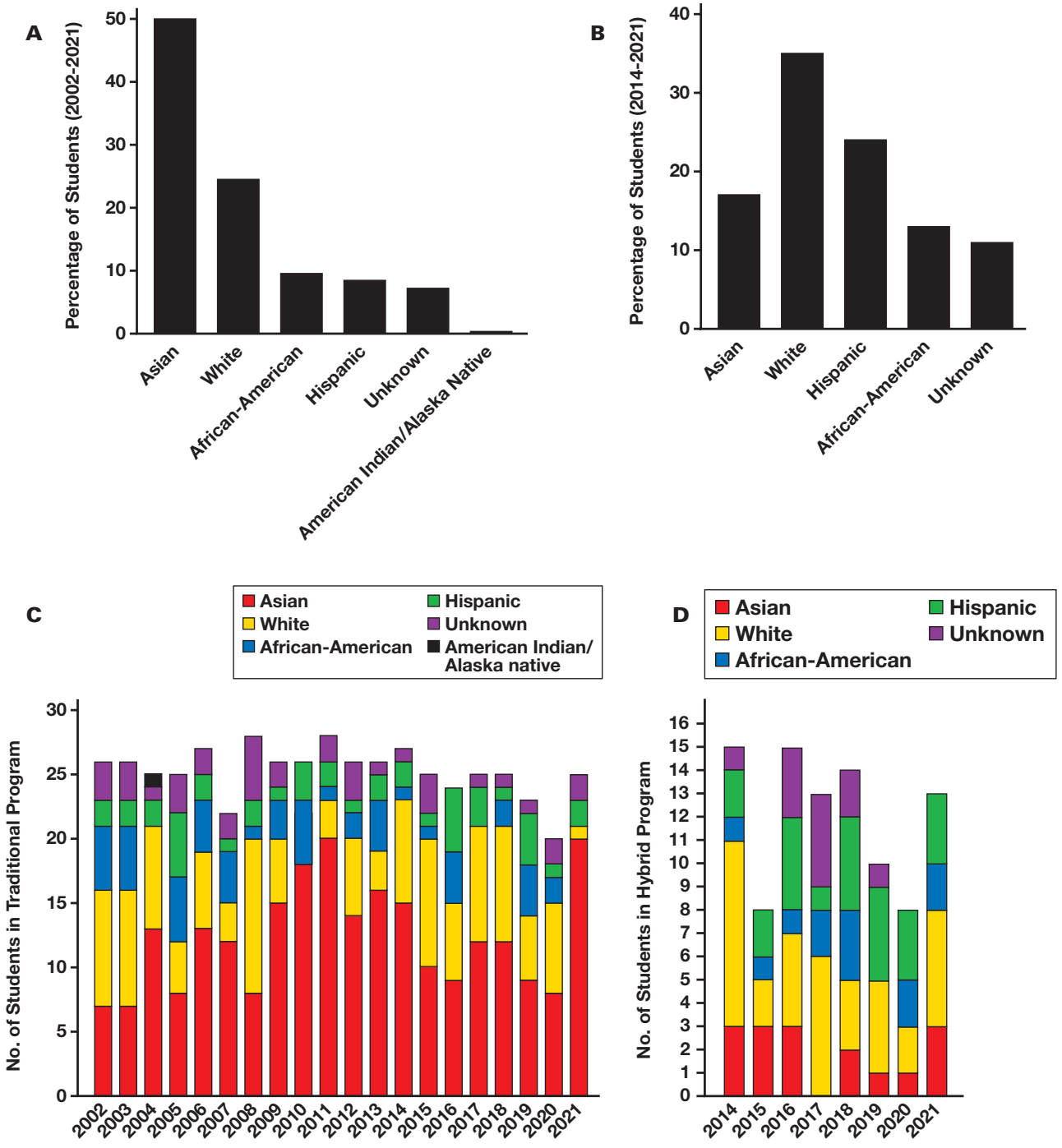


a welcoming environment, along with effective mentorship is a key to a successful healthcare force and leadership development.[4] Diversity among students helps enhance the inclusion of diverse perspectives not only in the classroom but also in the future workforce and can help add value to the overall team service.

The focus of the CLS program is educating and training students in developing skills in performing, evaluating, communicating, and troubleshooting diagnostic testing, moreover, to preparing students to become ambassadors of and leaders in the field of laboratory medicine. To better understand the level of effective pedagogy in the program, which can directly correlate to the level of diversity,[15] we measured the extent of diversity in both the traditional and hybrid programs. Over the last decade, diagnostic laboratories across the United States faced a significant shortage of certified laboratory personnel, and with the launch of a hybrid program, we increased the number of license-qualified laboratory students by 19%. Despite these efforts, the COVID-19 pandemic has further exacerbated the challenge of adequate staffing in diagnostic laboratories due to the many-fold increase in diagnostic testing,[16] in addition to all the aforementioned attributes.

Our data showed significant gender differences, with a higher percentage of females, 75% and 71% in the traditional and hybrid programs, respectively. The underlying reason why females gravitate more towards the lab medicine profession as opposed to males remains elusive. Also, it remains unclear if this difference in gender enrollment is merely based on the perception of the profession by males. Though equal opportunity is available for males to pursue a career in clinical laboratory medicine at our

FIGURE 2 Ethnicity of the students in the traditional and hybrid CLS programs. A and B show the percentage of students' ethnicity, Asian 50%, White 24.5%, African American 9.6%, Hispanic 8.6%, unknown 7.2%, and Alaskan Native American 0.2% graduated from the traditional program. In the hybrid CLS program 35% White, 25% Hispanic, 17% Asian, 13% African American, and 11 % of students of unknown ethnicity graduated from the hybrid program. C and D show the number of students from different ethnicities in the traditional program (2002–2021) and hybrid program (2014–2021), respectively.



institution, further investigation can help understand whether this difference can be alleviated via effective recruitment strategies and/or better professional development endorsement. While some studies showed that women are less likely to pursue careers in science, and minorities are less represented in the college.[17,18] Our data showed increased number of women graduates from CLS programs and that is an attestation to the welcoming and safe environment provided to CLS students at our institution. Also, our data is only limited to female and male gender percentages in our program; further analysis of gender such as transgender, queer, neutral, etc. was not possible due to the unavailability of the information.

Our data on ethnic diversity in the programs showed that the highest percentage of Asian ethnicity (50%) in the traditional program and

FIGURE 3 Percentage of students' origin from New York state, other US states, and international in the traditional and hybrid CLS program. A shows the percentage of students from New York (91%), other US states (7%), and international (2%) in the traditional program. B represents the percentage of students from Long Island (LI) (44%), Upstate NY (3%) and the five boroughs of New York, Queens, and Manhattan (24.1%), Brooklyn (15.7%), Bronx (2.8%), and Staten Island (1.8%). C and G represent the percentage of students from other US states who graduated from the traditional program (6.8%) from 2002 to 2021 and the hybrid program (2%) from 2014 to 2021. D shows the 2% international representation from South Korea (44.44%), China (22.22%), India (22.22%), and Taiwan (11.11%) in the traditional program, E shows the percentage of students in the hybrid program from NY (98%) and other U.S states (2%), F shows the students from NY in the hybrid program with 54.2% from LI, (16.7%) from Queens and Manhattan, Bronx (10.4 %), Brooklyn (8.3%), Staten Island (6.3%), and Upstate NY (2%).

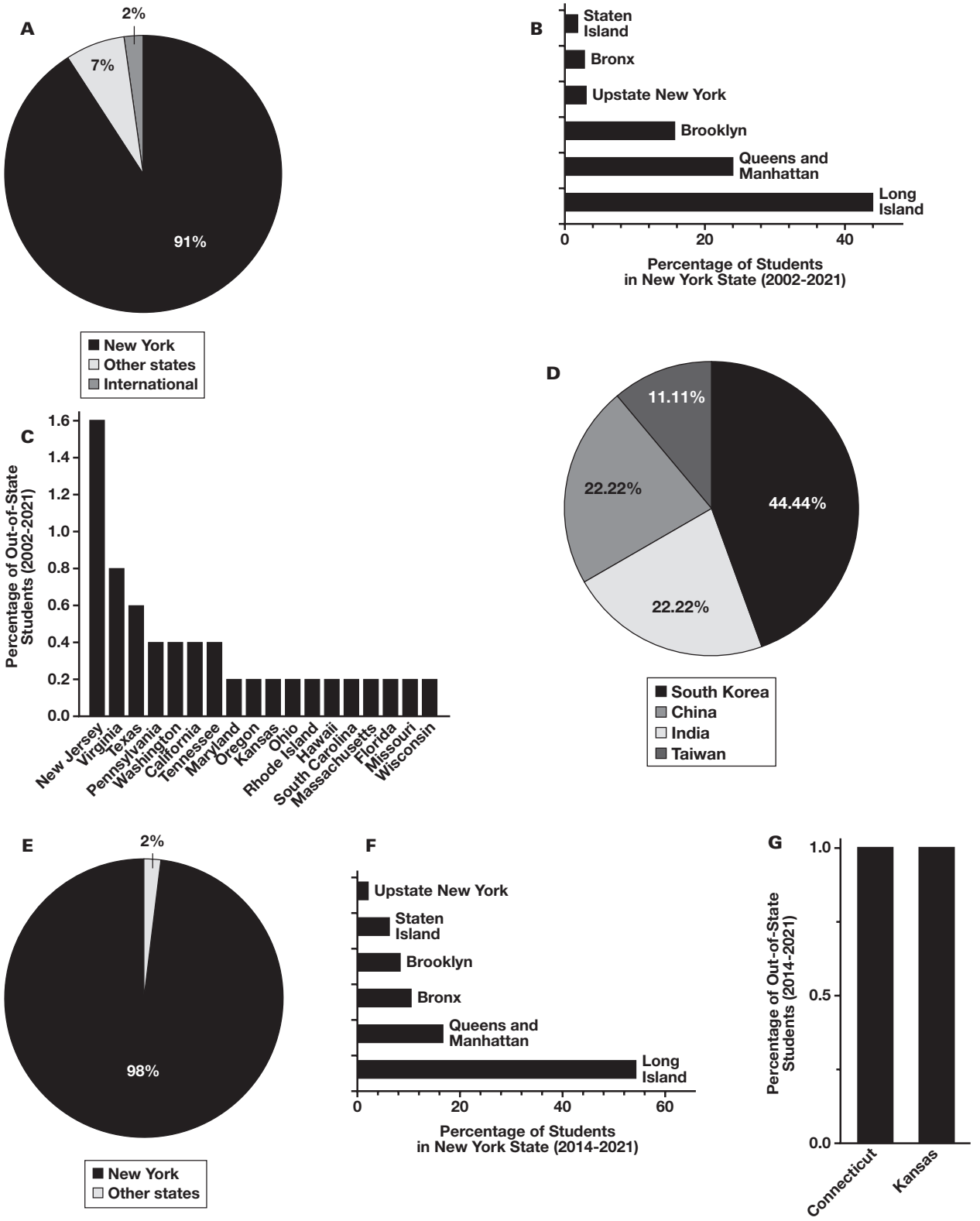
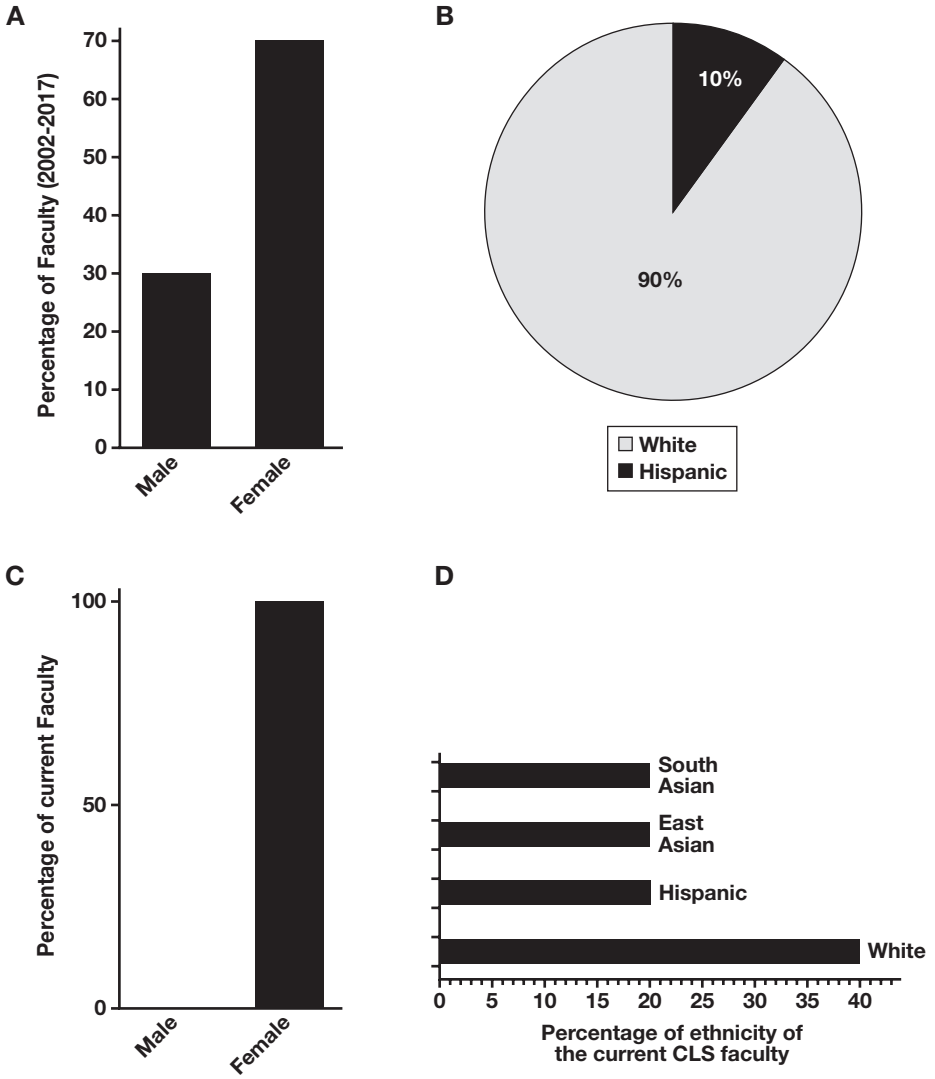


FIGURE 4 Diversity of the faculty in the CLS programs. A and C show the percentage of male and female faculty members in the traditional program from 2002 to 2017 and from 2017 to present, respectively. B shows the percentage of the faculty ethnicity in the traditional program, 90% White, 10% Hispanic (2002-2017). C shows the percentage of current faculty with 100% females and 0% males in the traditional program and D shows the ethnicity of the faculty members: 40% White, 20% Hispanic, 20% East Asian, and 20% South Asian.



17% in the hybrid program are from Manhattan and Queens. Based on the data (https://www.census.gov/library/stories/state-by-state/new-york-population-change-between-census-decade.html), the Asian population is higher in Manhattan and Queens as compared to Hispanic and African American, which are predominant in the Bronx and Brooklyn, and this is directly reflected in our data. In the traditional program, White ethnicity is 24.5 % as opposed to 52 % reported in the hybrid program, which resulted due to the origin of the majority of the enrolled students in the hybrid program being from Long Island.

Of note, the population in the United States is admixed, and based on the available information, one limitation of our study is that we could not dissect further whether the population was purely from one region or has African, European, or other ancestries mixed. Also, we could not establish that the distribution of students in the CLS program generally reflected population density across the regions. For example, 71.2% of the population in New York State is White, the percentage of White in Suffolk County on Long Island is 85.8% and in Nassau County on Long Island it is 77.3%. Asians make up 3.7% and 8.4% in Suffolk and Nassau Counties (8% of the population of New York), respectively, whereas the Hispanic or Latino population of any origin is 17.3% in Suffolk County and 15.3 % in Nassau County (8% of the population of New York) and the Black population is 8.2% in Suffolk County and 12.2% in Nassau County (17.5% of the population of New York).[19] Although the data showed a large portion of students enrolled in the program were from LI, the exact origin of the students cannot be determined, as some students could have reallocated to LI to attend the Stony Brook University CLS program.

Our data showed a portion of students of unknown ethnic origin, 7.2% and 11% in the traditional and hybrid program, respectively. Of note, different covariance affects the decision to identify as a specific race and ethnicity. Minorities are less likely to state their origin correctly and more likely to use a more generalized categorization of their ethnicity or prefer to state their ethnicity as "Unknown".[20–22] Societal stigma and "fear of racism" could also contribute and prevent students from identifying themselves with any particular race.[23,24]

Diverse faculty is integral for effective pedagogy as it enhances learning experiences for both mentor and mentee and helps bridge differences and promote cultural exchange by understanding self-efficacy. From 2002 to 2017, the CLS faculty was made up of 30% male and 70% female, with 90% White and 10% Hispanic representation. Currently, 100% of the faculty in the program are female. The current representation is 40% White, 20% East Asian, 20% South Asian, and 20% Hispanic. Our data show no implicit bias and displays a range of diverse representation, which is central to our educational goal of training the next generation of laboratory medicine leaders.

Conclusion

Overall, the objective of this study was to examine the spectrum of diversity in the CLS program at Stony Brook University. Diversity brings efflorescence of true creativity, expression, and collaboration and is integral for effective pedagogy in academic institutions. Both the traditional and hybrid CLS programs at our institution have a diverse representation of students and faculty. The student diversity in the CLS programs can directly correlate to future laboratory medicine leaders, educators, and laboratory scientists. Diversity in laboratory medicine is integral as it promotes effective professional advocacy, help establish connections with diverse communities, and largely contribute to diverse healthcare goals.

Acknowledgments

No financial support is available for the manuscript.

AA conceived the idea, designed the methodology, undertook the data curation and formal analysis, and wrote the original draft. AA and JG reviewed and edited the manuscript and agreed with the final version.

Conflict of interest

The authors declare no conflict of interest.

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SCIENCE

Establishment of Review Criteria Coordinating With the Automated Digital Cell Morphology Identification System in a Specialized Women's and Children's Hospital

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Keywords: blood cell count, review criteria, blood cell morphology, microscopic examination, automated image-analysis system, CellaVision DI-60

Abbreviations: WBC, white blood cell; RBC, red blood cell; IG, immature granulocytes; Micro R, microcytosis RBC; RET, reticulocyte Abn, abnormal; FRC, fragment cell; IRBC, infected RBC; HFLC, lymphocytes with high fluorescence intensity; ROC, receiver operating characteristic; nRBCs, nucleated red blood cells; ATL, atypical lymphocyte; PLT, platelet.

Laboratory Medicine 2023;54:e77–e84; https://doi.org/10.1093/labmed/lmac124

ABSTRACT

Objective: We aimed to establish appropriate review criteria for blood cell analysis in a specialized women's and children's hospital. Also, the CellaVision DI-60, was developed as one of the automated digital cell morphology analyzer, we evaluated if it was shown to be most effective under the certain review criteria.

Methods: A total of 2890 blood samples were detected to optimize the previously established review criteria for women and children with the Sysmex XE-2100. A total of 623 samples were used to validate the criteria.

Results: The microscopic-review rate based on the initial review criteria was 51.0%. After optimization, it was reduced to 17.3% and the false-negative rate was 3.85%. There was > 80% consistency between manual review results and CellaVision DI-60 preclassification when samples triggered the platelet- or red cell-related rules. The sensitivity for abnormalities (immature granulocytes, nucleated red blood cells) of reclassification was 90% to 100% and the false-negative rate was < 5%. However, direct microscopic review was required when the "Blasts/AbnLympho?" and "Atypical Lympho?" flags were triggered.

Conclusion: Specialized review criteria are needed for women and children. An automated morphology identification system might help to improve the review criteria.

Manual review of blood smears is usually regarded as the gold standard, but slide review is labor-intensive, time-consuming, and demanding, even for experienced examiners, thus affecting laboratory costs, productivity, and turnaround times. Analyzing blood samples with a high slide-review rate in clinical laboratories thus presents a challenge. The International Consensus Group for Hematology Review developed guidelines after the introduction of automated hematology analysis in 2005,[1] and the Chinese consensus group developed 23 guidelines for the Sysmex XE-2100 hematology analyzer (Sysmex, Kobe, Japan) in 2008.[2] Medical laboratories in China have accordingly achieved a consensus that each laboratory should have manual slide-review criteria for hematology analyzers.[3,4] However, research into the review criteria has mainly focused on adjusting the threshold for each parameter to establish optimized criteria with better efficiency,[5,6] and optimization of review criteria combining hematologic parameters obtained using the Sysmex XN-9000 hematology analyzer (Sysmex, Japan) and specific population characteristics has rarely been reported.

Standard criteria may not perform well in specific populations, and specialist women's and children's hospitals may thus need to develop their own review criteria. For example, more detailed criteria are needed to address the physiological crossover phenomenon between neutrophils and lymphocytes in white blood cell (WBC) classification in children and the phenomenon of hematopoiesis in pregnancy and newborns, and to focus on specific disease predilections in children, that is, childhood leukemia and infectious monocytosis.

The CellaVision DI-60 (CellaVision AB, Lund, Sweden) is an automated image-analysis system for peripheral blood smears, but its introduction into the clinical laboratory will necessitate a change in working habits, retraining of personnel, and increased spending on hardware and

software.[7,8] Two conditions need to be fulfilled to allow automated image analysis to replace direct microscopic review: the automated method must be shown to be at least as reliable clinically as direct microscopic slide review, and the new method must be associated with significant labor savings to justify the additional expenditure on the equipment.

In this study, we established appropriate review criteria for blood cell analysis in a specialist women's and children's hospital based on the specific characteristics of women and children,[9] the abnormalities indicated by the parameters, alarm information, and laser sensitivities of Sysmex XN-9000,[10] and on clinical information. We also evaluated the performance of the CellaVision DI-60 under certain triggering criteria.

Materials and Methods

Blood Samples

A total of 3513 blood samples were collected from patients admitted to West China Second University Hospital, including 103 newborns (median age 13 [range, 1–28] days, female:male, 2:3); 844 children (median age 6 [range, 1–16] years, female: male, 2:3), 1033 nonpregnant women, and 1533 pregnant women, including 404 early pregnancies (1–12 weeks), 634 midterm pregnancies (13–27 weeks), and 295 late pregnancies (28–42 weeks). The study was approved by the ethics committee of West China Second University Hospital, and signed informed consent was obtained from all subjects or their parents. Samples were analyzed using a Sysmex XN 9000 within 4 hours after collection. A slide of each sample was prepared by automated slide maker and stainer SP-10 (Sysmex, Kobe, Japan). Quality control, calibration, and management were carried out according to ISO15189/CAP requirements.

Groups

The establishment group included 2890 samples collected from outpatients or inpatients in different departments. To prevent interference from different sample origins over different time frames, the collected samples were uniformly distributed over a week for 3 different hours of each day (e.g., 8:00–11:00 on Monday and 12:00–15:00 on Tuesday). The validation group comprised an additional 623 samples collected in the same way after optimization of the review criteria. The CellaVision DI-60 group comprised 200 samples from the validation group that met the review criteria, which were chosen to evaluate the performance of the CellaVision DI-60.

Manual Differential Counting (Reference Method)

Seven certified medical laboratory technologists were asked to perform slide review in accordance with the reference leukocyte differential count methods.[11] All slides were reviewed twice by different technicians, and an additional review was carried out in the event of discrepancies between the initial results. Twelve criteria recommended by the International Society of Laboratory Hematology (**TABLE 1**)[1] were adapted to indicate positive smears. Abnormal blood cells were described according to standard recommendations.[12]

Analysis Using the CellaVision DI-60

The performance of the CellaVision DI-60 was evaluated by comparison with manual slide review (reclassification and manual review) and (preclassification and manual review) under certain triggered review criteria. Correlations between data from the CellaVision DI-60, technicians, and hematology analyzers were analyzed for WBCs including neutrophils, lymphocytes, monocytes, erythroblasts, immature granulocytes (including metamyelocytes, myelocytes, and promyelocytes), blasts, and atypical lymphocytes. For red blood cell (RBC) and platelet (PLT) parameters including polychromasia, hypochromia, anisocytosis, microcytosis, macrocytosis, poikilocytosis, giant platelets, smudge cells, artifacts, plasma cells, and thrombocyte aggregation, we also examined the consistency of morphological evaluation (qualitative) results between the CellaVision DI-60 and manual review.

Optimization of Review Criteria and Statistical Analysis

The overall goal of optimizing the criteria was to reduce the microscopic-review rate while maintaining a false-negative rate < 5%, without missing any serious hematological (leukemia or high percentage of atypical lymphocytes) cases. Laboman easy access 7.0s software was used to collect data and analyze the criteria. Cutoff values for items were determined using receiver operating characteristic (ROC) curves. The true-positive, false-positive, true-negative, false-negative, and microscopic-review rates were calculated taking microscopic examination as the gold standard. Quantitative comparisons were made by linear regression analysis followed by Pearson's correlation analysis. The sensitivity, specificity, and consistency of the CellaVision DI-60 system were defined as the ability to obtain qualitative results in concordance with manual review. All data were analyzed using SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, US).

Results

Review Rules

Evaluation of Primary Review Rules

A total of 2890 blood samples were used to evaluate the efficiency of the primary review criteria[13] (22-rule criteria on XE-2100 for children and women). The total review rate was 51.0%, with a high false-positive rate (53.8%, 1554 cases) (**TABLE 2**) and a low false-negative rate (0.7%, 21 cases).

Optimizing Primary Review Rules

The rule "IG (immature granulocytes) % >1.0% and WBC≥10 × 109/L" was mostly Triggered (**TABLE 2**). According to the population characteristics and research parameters of the Sysmex XN-9000, new cut-offs were chosen defined by the ROC curves, and this rule was modified to "IG%>2%, age ≤16 years or IG%>2.7%, age>16 years".[14]

The total percentage of RBC system-related rules (MCV<70 fl, RDW-CV > 22% and MCV > 105 fl) (**TABLE 2**) was 8.35%. We combined hematologic report, research, alarm parameters, Q-Flag (Schematic alarm diagram) from the Sysmex XN-9000, laser sensitivity, and the cutoff value with the best sensitivity and specificity and accordingly replaced former rules with the new combination rule "Micro R (Microcytosis RBC)>35%, RDW-SD>70 fl, RDW-CV>22%, Q-Flag (Fragment?) ≥100, RET Abn Scattergram (reticulocyte abnormal scattergram)>0, FRC (fragment cell) % >1%".[15] An "IRBC%? (infected RBC)" rule was added for Plasmodium screening. "Atypical Lympho?>150 and HFLC (Lymphocytes with high fluorescence intensity)>0.85" and "ABN(WBC)/WBC Abn Scattergram" (abnormal [WBC]/WBC abnormal scattergram) rules were added to enhance the ability to perform WBC analysis.

After optimization of the criteria, the total review rate was decreased to 17.96% (519 cases), with a false-positive rate of 13.94% (403 cases) and a false-negative rate of 3.18% (n = 92, 92/2890, without missing any cases of leukemia or high atypical lymphocytes). Cases triggering

IG,RBC, and atypical lymphocyte-related rules no longer accounted for the largest proportion in false-negative cases. The percentage of these rules in all false-negative cases was no higher than 1.5%. Instead, rules related to WBC, mostly relevant to left shift (metamyelocyte > 2%, toxic particles and promyelocyte/myelocyte ≥ 1%) and PLT (giant platelets/platelet aggregation) became the most triggered rules in false-negative cases (65.2%, 60/92) (**TABLE 3**). After optimization, there was an acceptable total review rate without increased risk. The review criteria for the Sysmex XN-9000 for a specialist women's and children's hospital are shown in **TABLE 4**.

TABLE 1. Criteria for a Positive Smear Recommended by the International Society of Laboratory Hematology

Morphology
RBCs
2+/moderate or greater; the only exception is malaria, where any finding will be considered a positive finding
Platelets
Giant platelets at either 2+/moderate or greater
Giant platelets at either 2+/moderate or greater
WBCs
Döhle bodies at either 2+/moderate or greater
Toxic granulation at either 2+/moderate or greater
Vacuoles at either 2+/moderate or greater
Abnormal cell types
Blasts ≥ 1%; myelocytes/promyelocytes ≥ 1%; metamyelocytes > 2%; Atypical lymphocytes > 5%; nucleated RBC ≥ 1%; plasma cells ≥ 1%

RBCs, red blood cells; WBCs, white blood cells. The International Society of Laboratory Hematology recommends that the use of band cell counts and left shift suspect flags (Left Shift?) should be in accordance with standard laboratory operating procedures. Thus, the Left Shift? Suspect flags were used as a screening criterion in this study, and the band count was considered a positive smear finding when it was > 8%.

Validation of Optimized Review Criteria

An additional 623 blood samples were analyzed to validate the above results. The total review rate was 17.3% (108/623), with a false-positive rate of 17.22% and a false-negative rate of 3.85% (<5.0%). No cases of leukemia or high atypical lymphocytes were missed.

CellaVision DI-60 Evaluation

Consistency between the CellaVision DI-60 and Manual Review for Case-Triggered RBC- or PLT-Related Rules

High consistency was found between manual review and preclassification of CellaVision DI-60 for cases that triggered RBC- and PLT-related rules (**TABLE 5**). The sensitivity of CellaVision DI-60 under each rule for RBCs and PLTs ranged from 92% to 94%, the consistency was approximately 88%, and the false-negative rate was approximately 7%. No cases of "RBC Fragment?" or "PLT Clump?" were missed. The results of CellaVision DI-60 preclassification could be reported directly when the rules for RBC or PLT (**TABLE 4**) (eg, "No. 10: RBC morphology related rules", "No. 12: RBC contents related rules", "No. 13: PLT Count and histogram abnormal related rules", or "No. 14: PLT aggregates related rules") were triggered.

Correlation Between Cellavision DI-60 and Manual Review for WBC Differential

The correlation between preclassification by CellaVision DI-60 and manual review (reclassification) for neutrophil (including segmented neutrophils and band forms), lymphocyte, and monocyte differentiation are shown in **FIGURE 1**. The correlation coefficients were high for band neutrophils (R^2 = 0.854) and segmented neutrophils (R^2 = 0.9575), but

TABLE 2. False-Positive Cases by Primary Review Criteria

Rule Number	Review Rules	Cases of Each Rule Triggered	Percentage of Total False-Positive Cases (%)
1	IG%>1.0% and WBC ≥ 10 × 10^9/L	1380	88.80
2	MCV < 70fl	95	6.11
3	Q-Flag Blasts/AbnLympho?	68	4.40
4	Lymphocyte%>50% Age>6 years	42	2.70
5	Q-Flag Atypical Lympho?	35	2.30
6	RDW-CV > 22%	22	1.40
7	Monocytes %>15%	22	1.40
8	PLT < 70 or PLT > 1000(10^{12}/L)	20	1.29
9	Neutrocyte # <0.5 or > 20(10^9/L)	19	1.20
10	MCV > 105fl	13	0.84

IG, immature granulocytes; MCV, mean corpuscular volume; PLT, platelet; RDW, red blood cell distribution width.

TABLE 3. Main Reason of Triggered Rules in False Negative Cases Triggered by Optimized Review Rules

Rule Number	Review Rules	Cases of Each Rule Triggered	Percentage of False-Negative Rules (%)
1	Metamyelocyte > 2%	22	23.9
2	Toxic particles	16	17.4
3	Promyelocyte/myelocyte ≥ 1%	13	14.1
4	Giant platelets/platelet aggregation	9	8.7

TABLE 4. Review Rules for the Sysmex XN-9000 in the Women's and Children's Specialized Hospital

Rule Number	Parameter	Review Rules	Review Requirements
1	Nucleated RBC?	Newborn: nRBCs > 2.0%, Non-newborn: nRBCs > 1.0%	Smear review
2	From the Pediatric Hematology-Oncology department	/	Smear review
3	Beyond linearity	WBC>440(10^9/L) or RBC>8(10^{12}/L) or Hb>250 (g/L) or PLT>5000(10^9/L) or RET%>23% or RET#>0.72(10^{12}/L)	Recheck sample, smear review
4	No results	No results of WBC, RBC, HGB, PLT, or no diff or incomplete diff	Recheck sample. Search for clots and rerun
5	WBC count abnormal	WBC<1.5(10^9/L)or WBC>30(10^9/L)	Smear review, leukocyte classification (abnormal cells)
6	Leukocyte classification abnormal	Neutrophil # <0.5(10^9/L)or>20(10^9/L)	Smear review, leukocyte classification (abnormal cells)
		Lymphocyte%>80%, age < 3 years	
		Lymphocyte%>70%, 6 years>age>3 years	
		Lymphocyte%>50%, age>6 years	
		Monocyte%>20%	
		Eosinophils#>2	
7	Immature granulocytes	IG%>2.7%, age > 16 years IG%>2.0%, age ≤ 16 years	Smear review, leukocyte classification (abnormal cells)
8	Blasts/AbnLympho?	Q-Flag (Blasts/AbnLympho?)>150 Ip ABN(WBC)/WBC Abn Scattergram	Smear review, leukocyte classification (abnormal cells)
9	Atypical Lympho?	Q-Flag (Atypical Lympho?)>150 and HFLC > 0.85	Smear review, leukocyte classification (abnormal cells)
10	RBC morphology	RDW-SD>70fl RDW-CV>22% Q-Flag (Fragment?) ≥100 Micro R > 35% RET Abn Scattergram > 0 FRC%>1%	Smear review, check for RBC morphology
11	MCHC	MCHC>380(g/L)	Check for lipemia, hemolysis, hyper leukocytosis, RBC agglutination, and blood anticoagulant relationship
12	RBC contents	Q-Flag IRBC%?	Smear review, check for RBC morphology
13	PLT Count and histogram abnormal	PLT<70(10^9/L) and platelet histogram abnormal or PLT>1000(10^9/L)	Search for clots, smear review, check for platelet aggregates and giant PLT
14	PLT aggregates	Q-Flag (PLT Clump?)>195	Search for clots, smear review, check for platelet aggregates

HGB, hemoglobin; HFLC, lymphocytes with high fluorescence intensity; RBC, red blood cell; Micro R, microcytosis red blood cell; WBC diff, WBC differential count; Hb, hemoglobin; nRBCs, nucleated RBC; IG, immature granulocytes; FRC, fragment cell; IRBC, infected RBC; ABN(WBC)/WBC Abn Scattergram, abnormal (WBC)/WBC abnormal scattergram.

lower for lymphocytes (R^2 = 0.7971) and monocytes (R^2 = 0.7693). These correlation coefficients indicated good ability of the CellaVision DI-60 system for preclassification of normal leukocytes. The results of CellaVision DI-60 preclassification could thus be reported directly when rules for abnormal leukocyte classification were triggered (**TABLE 4**) (eg, "No.6: Leukocyte classification abnormal related rules").

Ability of Cellavision DI-60 to Identify Important Clinical Abnormalities

CellaVision DI-60 preclassification was compared with manual review for some important clinical abnormalities, including immature granulocytes (promyelocytes, myelocytes, and/or > 2% metamyelocytes), nRBCs (nucleated red blood cells), atypical lymphocytes, and blasts (**TABLE 6**). Twenty-nine cases were identified as abnormal by CellaVision DI-60 but normal by manual review. In contrast, 34 cases were found to be abnormal by manual review but normal by CellaVision DI-60.

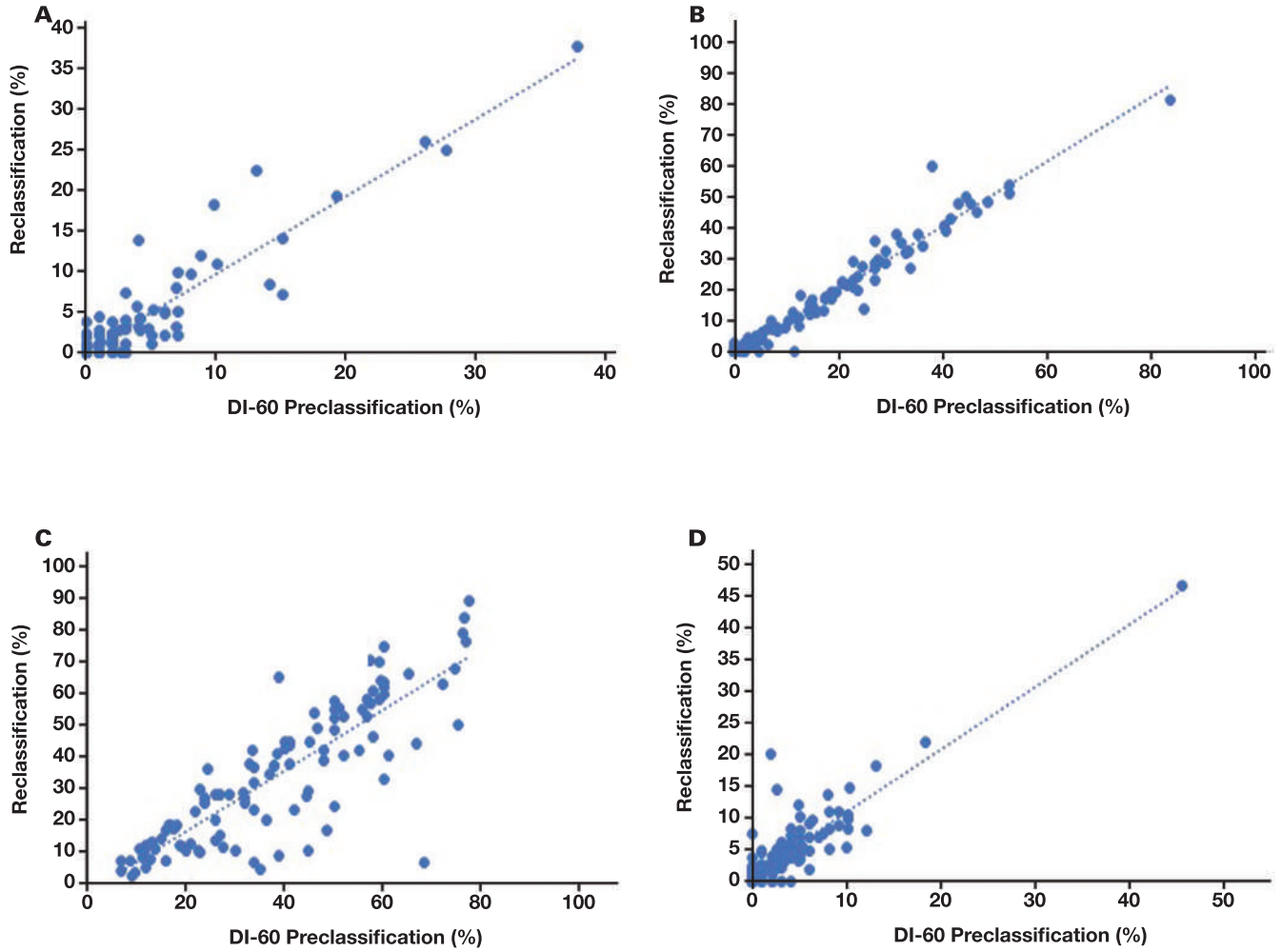
To further analyze these discrepant cases, the supervisors were asked to recheck the digital images and smears. Most of the discrepancies were attributed to insignificant statistical and small clinical variations, for example, deviation of 1% or 2% for nRBCs, metamyelocytes, or myelocytes between CellaVision DI-60 and manual review. An additional smear review resolved the discrepancies in another 15 cases. In the 3 most notable cases

TABLE 5. Consistency Between Manual Review and Preclassification of the DI-60 Image-Analysis System Under RBC- and PLT-Related Review Rules on 623 Validation Data.

Review Rule/Triggered Number RBC	Preclassification of DI-60	Manual Review		Consistency (%)
Morphology related/51	Positive	39	3	88.2%
including	Negative	3	6	
RDW-SD>70fl/13; RDW-CV>22%/14; Q-Flag (Fragment?)≥ 100/5; Micro R > 35%/18; RET Abn Scattergram > 0/1)				
PLT related/35	Positive	26	2	88.6%
including	Negative	2	5	
PLT < 70(10^9) and histogram abnormal; PLT>1000(10^9)/21				
Q-Flag (PLT Clump?)>195/14				

RDW, red blood cell distribution width; Micro R, microcytosis red blood cell; RET Abn, reticulocyte abnormal.

FIGURE 1. Correlation of band neutrophil (A), segmented neutrophil (B), lymphocyte (C), and monocyte (D) counts on preclassification and reclassification by reviewer of the CellaVision DI60(n = 108).



(CellaVision DI-60 found "promyelocytes" but manual review indicated normal), additional review of whole smears and 2 300-cell differentials finally identified a small group of immature myeloid cells.

Ignoring the above small variations, the sensitivities for abnormalities (promyelocytes, metamyelocytes, and/or myelocytes, nRBCs) of CellaVision DI-60 were as high as 90% to 100%, and the false-negative rate was < 5%, suggesting that a reclassification by CellaVision DI-60 could possibly be used to replace microscopic review when the rules "Nucleated RBC? related-" or "Immature granulocytes related-" (TABLE 4) were triggered.

The correlation for case-triggered rules of "Blasts/AbnLympho?" or "Atypical Lympho?" between the two methods is shown in **FIGURE 2**. The correlation coefficients were lower than the correlation coefficients for normal cells, although the correlation increased following manual reclassification of CellaVision DI-60. These data could not ensure that no cases of leukemia were missed, indicating that direct microscopic review was required when the rules "Blasts/AbnLympho? related-" or "Atypical Lympho? related-" (**TABLE 4**) were triggered.

Discussion

Although the patients remain the same, the technological changes from the Sysmex XE to the XN series, including routinely performed nRBCs, clearer distinction between lymphocyte and monocyte groups on the WBC scatter diagram, and cancellation of the IMI (immature granulocytes) channel (a specified channel of the XE series to indicate immature cells) means that the established primary review criteria for the XE-2100[13] are no longer adequate.

Atypical lymphocytes (ATL) is one of the most common morphologic abnormalities in pediatric patients. Using older hematology analyzers such as the XE-2100, ATL would often be identified incorrectly as monocytes (which is why the disease with high levels of ATL and clinical symptoms is named infectious mononucleosis), and the percentage of monocytes and Q-Flag related to monocytes and lymphocytes was therefore used as a review rule. However, the technological advances of the XN series have improved the distinction between the lymphocyte and monocyte groups on the WBC scatter diagram. Identification of ATL is thus unlikely to occur for the monocyte group when using the Sysmex XN-9000, and we accordingly adjusted the rule relating to the percentage of monocytes in our modified criteria. Because ATL frequently appears in smears from pediatric patients but only percentages > 5% or 10% have clinical significance, the threshold of Q-Flag "Atypical Lympho?" was modified from 100 to 150, together with a new research parameter HFLC > 0.85, to control the total review rate.

Acute lymphoblastic leukemia is the most common childhood cancer, and missing no leukemia cases is a key requirement of the review criteria for pediatric patients. Although the XN series no longer uses IMI channels to trigger Q-Flags, its ability to detect blast cells does not seem to be affected. Based on the improved WBC scatter diagram (Q-Flag "Blasts/AbnLympho?") and ABN(WBC)/WBC Abn Scattergram, the new review criteria could thus safely be used for leukemia screening with Sysmex XN-9000.

Identification of nRBCs is another improved aspect of the XN series. Compared with the Q-Flag of the XE series, the quantitative results for nRBCs from a specialized channel are more precise. Using a threshold of > 1% or 2% nRBCs could help to focus on cases with clinical significance.

Normal differential leukocyte count varies with age, which evaluation needs to take into account in specialist children's hospitals. In the first few days after birth, polymorphonuclear neutrophils are predominant but thereafter, lymphocytes account for the majority of leukocytes. This persists up to about 4 to 5 years of age, when polymorphonuclear leukocytes again become the predominant cell and remains so throughout the rest of childhood and adult life.[16] Therefore, we combined the population characteristics of our own laboratory and referred to the Reference Intervals of Blood Cell Analysis for Children, which was published by the Health Industry Standard of the People's Republic of China[17] and standardizes the age-related review criteria of differential leukocyte count abnormalities (**TABLE 4**).

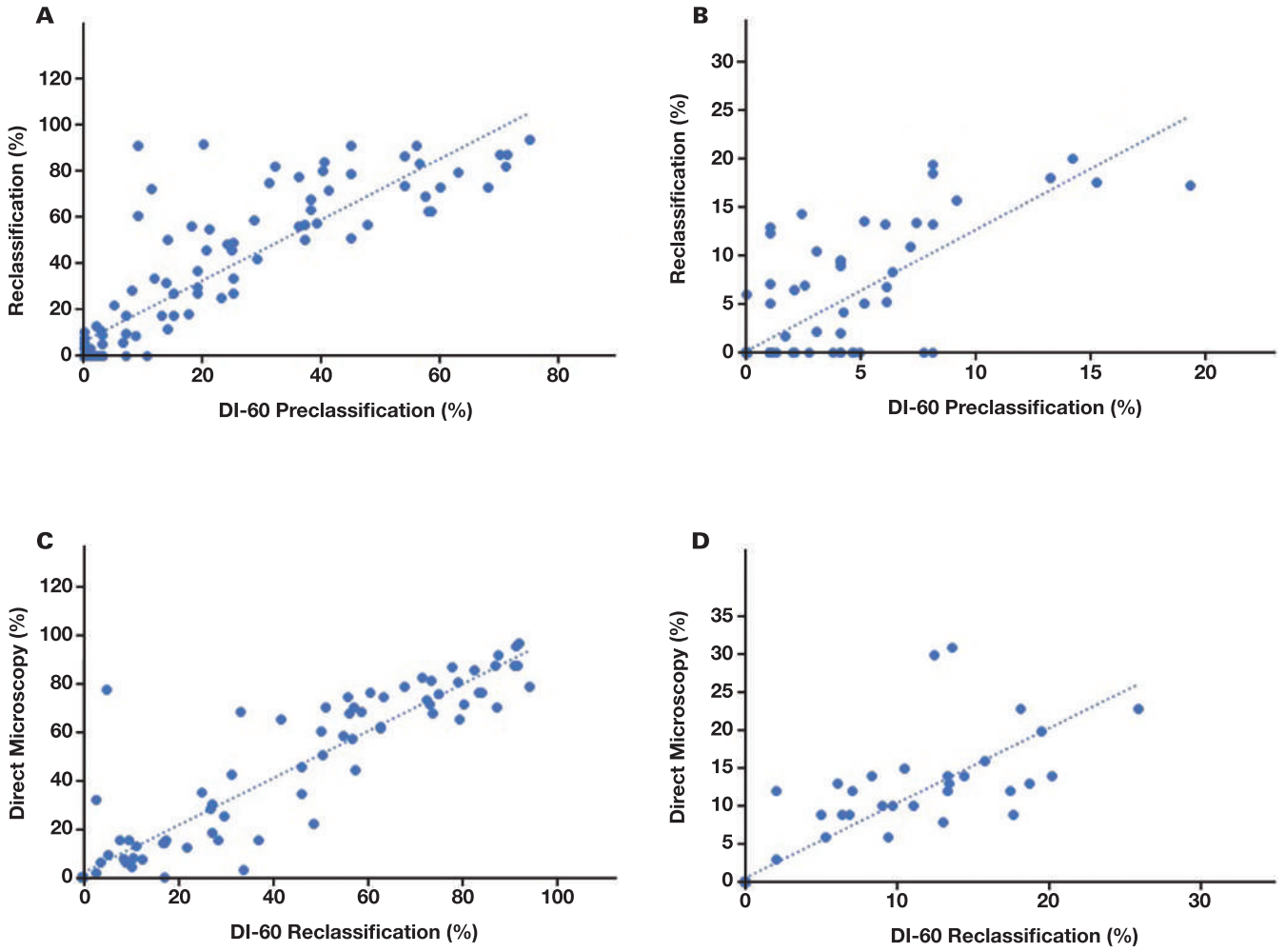
Also, the parameter IG is most affected by population characteristics. The IG increases significantly in the first trimester of pregnancy and in newborns, is associated with active hematopoiesis, and may remain higher in pregnant women and in children than in healthy, nonpregnant adults. Roehrl et al[18] found that IG concentrations for patients aged 10 years or younger and for those older than 10 years require different normal reference ranges. In addition, above the age of 10 years, pregnancy was a frequent benign cause of elevated IG counts in younger women. Roehrl et al[19] have seen another otherwise healthy pregnant woman with significantly elevated IG counts (4.2%/670 μL^{-1}) that normalized readily after delivery and show that even relatively high counts > 12%, that is, more than 4-fold greater than previous result in the early pregnancy stage, may hold no

TABLE 6. Identification of the Presence of Abnormal Findings by the CellaVision DI-60 Automated Image-Analysis System

Review Rule/Triggered Number	Preclassification of DI60	Manual Review		Consistency
IG ≥ 1% promyelocytes, myelocytes, and/or > 2% metamyelocytes				
IG%>2.7% >16Y/29	Positive	15	6	65.5%
	Negative	6	2	
IG%>2.0% ≤16Y/64	Positive	40	10	71.9%
	Negative	10	4	
nRBCs				
Newborn: nRBCs > 2.0%/34	Positive	19	9	61.8%
	Negative	4	2	
Non-newborn: nRBCs > 1.0%/4	Positive	2	2	50.0%
	Negative	0	0	
Blasts/AbnLympho? ≥1% blast				
Q-Flag (Blasts/AbnLympho?)>150/75	Positive	56	0	88.0%
	Negative	9	10	
Atypical Lympho? >5% Atypical Lympho?				
Q-Flag (Atypical Lympho?)>150 and HFLC > 0.85/47	Positive	34	2	85.1%
	Negative	5	6	

HFLC, lymphocytes with high fluorescence intensity; nRBCs, nucleated red blood cells; IG, immature granulocytes; Abn, abnormal.

FIGURE 2. Correlation of Blasts/AbnLympho? (A) and Atypical Lympho? (B) on preclassification and reclassification by the reviewer of the CellaVision DI60(n = 108). Correlation of Blasts/AbnLympho? (C) and Atypical Lympho? (D) on reclassification of DI-60 by the reviewer and on direct microscopy(n = 108).



pathological significance. Also, multicenter systems analysis[20] revealed that the expression levels of the immature-like neutrophil signature increased linearly with pregnancy. Therefore, new cutoffs for IG in children and women in our hospital were produced based on ROC curve analysis.

Although new cutoffs were modified to "IG%>2%, age ≤16 years or IG%>2.7%, age >16 years" by the ROC curves, "Metamyelocyte>2%" and "Promyelocyte/myelocyte≥1%" accounted for the largest proportion of false-negative cases (38.0%, 35/92 cases) (total percent: 1.2%, 35/2890 cases) (**TABLE 3**). The false-negative rate for detection of abnormal cells depends on the instrument and the detection limit desired (1%–5% abnormal cells).[16] Although new cutoffs were modified, the infrequent abnormal cells were still < 5%. Because of their low abundance under normal conditions, IGs are generally difficult to quantify precisely by manual smear and flow cytometry–based analyzers such as the Sysmex XN-9000. Also, IG is the problematic for specialist women's hospitals, because IG may remain higher in pregnant women and in children than in healthy, nonpregnant adults.

The CellaVision DI-60 system could supplement hematology analyzers and greatly reduce labor input. Under certain review criteria rules, especially for qualitative RBC or PLT morphological analysis, there was high consistency (>85%) between CellaVision DI-60 and direct microscopic review. For cases triggering these rules, direct reporting of CellaVision DI-60 results might be a reasonable option, without the need for microscopic review. In addition to qualitative analysis for PLTs or RBCs, CellaVision DI-60 still functions well for most mature WBC differentiation (eosinophils and basophils were not included because of a lack of high-level cases). The correlation coefficients between CellaVision DI-60 and manual review for normal WBC subtypes were all > 0.75 (lymphocytes, 0.80; monocytes, 0.77; and segmented neutrophils, 0.96). This result was similar to that for another automatic morphological analyzer, the DiffMaster Octavia.[21,22] Compared with the previously reported correlation coefficients of microscopic review between trained technicians (lymphocytes, 0.73; monocytes, 0.41; and segmented neutrophils, 0.87),[8,23] the performance of the CellaVision DI-60 was good enough to make it a possible choice for cases triggering rules of abnormal WBC percentage, but without abnormal morphological clues.

Most discrepancies between the CellaVision DI-60 and manual review results were case-triggered rules of IG, nRBCs, "Atypical Lympho?", or "Blasts/AbnLympho?", in line with the results of a previous study.[22] For these cases, the results from preclassification by CellaVision DI-60 were not suitable for direct reporting. In our study, most of the discrepancies were due to small variations (small cell populations < 5.0% or adjacent cell stages). Variations in items with different clinical importance could

be dealt with in different ways. Manual reanalysis using the CellaVision DI-60 resolved most of the discrepant cases under the IG and nRBCs rules, considering that a bias of 1% or 2% in differential or disagreement between metamyelocyte and myelocyte identification is usually unlikely to have any obvious clinical significance. For these cases, manual reclassification with the CellaVision DI-60 might thus be sufficient to save labor and time costs. However, manual reclassification with the CellaVision DI-60 was not adequate for cases flagged under the "Atypical Lympho?" and "Blasts/AbnLympho?" rules. As noted above, leukemia is a key childhood cancer of great clinical importance. Even small variations were therefore not allowed, to ensure that no leukemia diagnosis is missed, and microscopic review remains the only choice for cases under the rules related to leukemia, especially acute lymphoblastic leukemia.

In summary, appropriate review criteria should be established for specific populations such as women and children. Rules should be established to take account of particular age stages or age-related diseases (leukemia and infection monocytosis) in children and physiological stages in women (pregnancy). The CellaVision DI-60 could supplement the use of a hematology analyzer and was shown to be most effective for cases under the rules of RBC and PLT morphology. Manual reclassification using a CellaVision DI-60 could help to save labor and time costs for cases under the IG and nRBCs rules.

Acknowledgments

The authors would like to thank everyone who assisted in review and inputting data for this project.

Yuefang Wang and Lei Ye set up the concept, designed the experiments and the manuscript, wrote the manuscript, and equally contributed to this work. Ge Zhang supervised the project. Lan Chen, Qi Chen, Xia Zhang, Qingkai Dai, Luyun Peng, Chunqi Lai carried out the assays. All authors read and approved the final manuscript.

The study was approved by the ethical committee of West China Second University Hospital (an affiliated hospital of the Sichuan University in Chengdu, China), and signed informed consent forms were obtained from patients or parents.

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SCIENCE

An Adjusted Process to Improve Efficiency and Efficacy of Adsorption Procedures to Resolve Warm Autoantibody Cases

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Keywords: adsorption, warm autoantibody, warm auto, autoantibody, absorption, panreactivity, pan-reactivity

Abbreviations: PEG, polyethylene glycol; SOP, standard operating procedure.

Laboratory Medicine 2023;54:e85–e90; https://doi.org/10.1093/labmed/lmac131

ABSTRACT

Objective: The presence of warm autoantibodies in patient plasma typically causes extended delays in patient care due to panreactive antibody identification tests. Adsorption is the primary method for resolution. A modified adsorption procedure was devised with the intent to minimize delays, and the aim of the study was to evaluate its efficacy.

Methods: To evaluate the in-house developed (experimental) adsorption method, specimens were tested in parallel with the standard adsorption method described in the AABB Technical Manual, 20th edition. Specimens selected demonstrated panreactivity at the submitting facility and contained adequate volume for parallel adsorption studies.

Results: Ninety specimens were tested with both methods. Ninety specimens achieved complete adsorption with the experimental method and 88 specimens achieved complete adsorption with the standard method. Two underlying alloantibodies, which have been reported to cause hemolytic transfusion reactions and potential renal graft rejection, were detected using the experimental method that the standard method failed to detect.

Conclusion: The experimental method demonstrated a significant reduction in rounds of adsorption required to resolve warm autoantibody reactivity, enhanced antibody detection ability with adsorbed plasma, and more cost-effective outcomes compared to the standard method. A follow-up study is planned to assess whether the incubation time can be decreased with the experimental method to further improve the efficiency of the method without sacrificing efficacy.

Adsorption procedures are considered high-complexity pretransfusion laboratory tests in which antibodies causing broad spectrum interference in the plasma are selectively removed. This interference is often observed in the form of panreactivity caused by a warm autoantibody. In most cases, warm autoantibodies are unlikely to be clinically significant—defined as causing hemolytic transfusion reactions, reducing red cell survival in vivo, or causing hemolytic disease of the fetus and newborn. Since they tend to be polyclonal and react with all red cell antigens tested, there is an inherent risk of failing to detect the presence of underlying clinically significant alloantibodies to red cell antigens.[1]

In the presence of a warm autoantibody, autologous red cells are most appropriate for adsorption procedures if the patient has not been recently transfused and there is a reasonable degree of certainty that there are no coexisting donor cells in the specimen.[2] When there is a history of recent transfusion, or evidence thereof, such as observed mixed field reactivity when testing the patient's red blood cells, allogeneic red cells with known phenotypes are commonly used for adsorption procedures.[3] Allogeneic adsorption procedures can also be used when it is desired to remove or isolate a specific antibody, such as the presence of an antibody against a high incidence red cell antigen, for testing to detect underlying alloantibodies against more common red cell antigens.[4] Both autologous and allogeneic adsorption methods were used in this study; allogeneic adsorptions were performed with donor red cells with phenotypes known to match the patient for the common red cell antigens C, E, c, e, K, Fy^a, Fy^b, Jk^a, Jk^b, M, N, S, and s. Prior to use, autologous adsorbing cells were treated with Zzap and allogeneic adsorbing cells were treated with the proteolytic enzyme papain.

Research shows that approximately 30% to 40% of patients who present with panreactivity due to warm autoantibodies have also produced underlying alloantibodies.[5] Any failure to detect an underlying alloantibody should be considered critical as it has the potential to result in a hemolytic transfusion reaction.[6] Because of the combination of the probability of occurrence and the potential clinical significance of an underlying alloantibody, physicians are inclined to wait until a serological investigation is complete before transfusing, delaying patient care.

The purpose of this study is to improve the standard adsorption method without the disadvantages associated with techniques that use enhancement media, such as polyethylene glycol (PEG), which could result in the inability to detect clinically significant red cell antibodies due to dilution.[7]

Methods

The study was performed using 90 specimens collected in EDTA and submitted to an immunohematology reference laboratory for resolution of warm autoantibody interference that contained sufficient volume to allow both the standard adsorption method and the experimental adsorption method to be performed in parallel. Specimens were stored at 2°C to 8°C and tested within 72 hours of receipt. The testing facility's standard operating procedure (SOP) for warm autoantibody resolution via adsorption is based on the adsorption procedure detailed in the AABB Technical Manual, 20th edition, Methods and Appendices.[8] The SOP dictated that plasma was to be adsorbed using washed, packed red blood cells prepared with commercially prepared Zzap if autologous or commercially prepared papain if allogeneic, according to manufacturer's directions. Adsorptions were performed using a 1:1 cell-to-plasma ratio, incubated in a water bath at 37°C for 60 minutes, with intermittent mixing taking place approximately every 15 minutes. Following incubation, the plasma and adsorbing cells were separated by high-speed centrifugation and the plasma transferred to a clean test tube for subsequent antibody identification testing. A fresh aliquot of adsorbing cells was prepared as needed to continue until the adsorption process was complete. The testing facility's SOP limited the number of subsequent adsorptions to 6 without prior approval from the medical director. Once complete adsorption was achieved, adsorbed plasma was used to detect underlying antibodies to red cell antigens with the indirect anti-globulin test using commercially prepared reagent red cells with commercially prepared PEG enhancement, according to manufacturer's directions. In this study, complete adsorption is defined as adsorbed plasma exhibiting no extraneous reactivity with a panel of commercially prepared reagent red cells. After obtaining adsorbed plasma with the experimental procedure described below, testing to detect underlying antibodies was performed using identical methods to those used with adsorbed plasma obtained by the standard method:

Experimental Adsorbing Procedure

1. Treat the adsorbing cells with the appropriate enzyme or reagent according to the manufacturer's directions.
2. After treatment is complete, wash the treated cells 3 times or until the supernatant appears clear. After the last wash, centrifuge the red cells at 3500 rpm for 5 minutes, removing as much of the supernatant saline as possible with a transfer pipette. Insert a piece of narrowly trimmed filter paper to absorb any residual saline remaining. Allow the filter paper to rest in the tube for 60 seconds and then discard.
3. Combine 3 volumes of packed red cells and 1 volume of plasma in a 6 mL specimen tube that contains no additive, using a rubber stopper seal and/or parafilm if necessary to ensure specimen tube is watertight. Prepare each adsorbing tube with at least 1.0 mL but no more than 1.5 mL total volume, using as many tubes as necessary to achieve the desired volume of adsorbed plasma. Suggested volumes are 21 drops of red cells and 7 drops of plasma per 6 mL tube.
4. Mix well by gentle inversion and place adsorbing tubes horizontally in a specimen rack. Place the specimen rack in a 37°C water bath, ensuring that all tubes are completely submerged in water and do not become dislodged from the rack and rise to the surface.
5. Incubate at 37°C for 60 minutes. Periodic mixing is permitted, if desired.
6. After incubation, centrifuge the adsorbing tubes at 3500 rpm for 5 minutes to pack red cells tightly. If multiple tubes were used, use a transfer pipette to combine adsorbed plasma into a clean properly labeled 12 × 75 mm test tube prior to testing. If an eluate is to be prepared, save the adsorbing cells.
7. Test an aliquot of the adsorbed plasma, preferably against a reserved unused aliquot of the red cells used for adsorption or against a red cell of known phenotype if an unused aliquot is unavailable, to determine whether all reactivity has been removed. Alternatively, a direct antiglobulin test with anti-immunoglobulin G (anti-IgG) may be performed on the postadsorption cells to determine whether adsorption took place, if desired.
8. If adsorption is complete, proceed with antibody identification testing per appropriate SOP. If adsorption is incomplete, transfer adsorbed plasma to a fresh aliquot of packed red cells and repeat steps 3 to 7 until complete adsorption is achieved.
9. If complete adsorption is not achieved after 6 adsorptions, consult with the medical director.

Results

Using the standard method, an average of 2.93 rounds of adsorption were required to resolve warm autoantibody interference, and an average of 1.09 rounds were required using the experimental method (**TABLE 1**).

This study shows that the null hypothesis that warm autoantibody interference is resolved in the same number of rounds using either method has been rejected, with a P value of < .0001 from the Fisher's exact test (**TABLE 2**).

Discussion

The ratio of adsorbing cells to plasma in the experimental method was modified to 3:1, a 3-fold increase of adsorbing cells compared to the standard method. Since the adsorption process ceases when there are no antigen-binding sites available, any incubation time spent thereafter is of no value. The objective of this modification was to expose the warm autoantibody to the same or greater number of antigen binding sites in 1 incubation period that would require multiple rounds of adsorption with the standard method. The experimental method aims to improve on an inefficiency in the standard method of incubating beyond the point at which antigen-binding sites are at their maximum capacity.

By increasing the cells-to-serum ratio to 3:1, the potential for dilution of underlying alloantibodies in the patient plasma with residual saline from the washing process during adsorbing cell preparation is introduced. Filter paper was incorporated to counteract this risk. The experimental method used filter paper that was cut to uniform size with

TABLE 1. Testing Results

ID	Adsorptions Required (Standard)	Total Incubation Time (Standard, minutes)	Underlying Alloantibodies (Standard)	Adsorptions Required (Experimental)	Total Incubation Time (Experimental, minutes)	Underlying Alloantibodies (Experimental)
001	3	180	None	1	60	None
002	3	180	Anti-E	1	60	Anti-E
003	4	240	Anti-K	2	120	Anti-K, -Jka
004	3	180	None	1	60	None
005	3	180	None	1	60	None
006	2	120	None	1	60	None
007	4	240	None	1	60	None
008	3	180	Anti-c	1	60	Anti-c
009	2	120	None	1	60	None
010	3	180	None	1	60	None
011	5	300	Anti-C, -E	2	120	Anti-C, -E
012	3	180	None	1	60	None
013	2	120	None	1	60	None
014	3	180	None	1	60	None
015	3	180	None	1	60	None
016	4	240	Anti-e	2	120	Anti-e
017	6	360	Unsuccessful	2	120	Anti-U
018	3	180	None	1	60	None
019	2	120	None	1	60	None
020	2	120	Anti-K	1	60	Anti-K
021	3	180	None	1	60	None
022	3	180	None	1	60	None
023	3	180	Anti-E, -K	1	60	Anti-E, -K
024	3	180	None	1	60	None
025	3	180	None	1	60	None
026	3	180	None	1	60	None
027	3	180	None	1	60	None
028	3	180	Anti-S	1	60	Anti-S
029	2	120	None	1	60	None
030	2	120	None	1	60	None
031	3	180	Anti-D, -C	1	60	Anti-D, -C
032	3	180	Anti-c	1	60	Anti-c
033	3	180	None	1	60	None
034	3	180	None	1	60	None
035	3	180	None	1	60	None
036	4	240	None	2	120	Anti-Jkb
037	3	180	Anti-Fyb, -s	1	60	Anti-Fyb, -s
038	3	180	None	1	60	None
039	3	180	None	1	60	None
040	1	60	None	1	60	None
041	3	180	None	1	60	None
042	3	180	Anti-M	1	60	Anti-M
043	3	180	None	1	60	None
044	3	180	None	1	60	None
045	3	180	None	1	60	None
046	2	120	None	1	60	None
047	3	180	None	1	60	None
048	3	180	Anti-E, -K, -Fya	1	60	Anti-E, -K, -Fya

TABLE 1. Continued

ID	Adsorptions Required (Standard)	Total Incubation Time (Standard, minutes)	Underlying Alloantibodies (Standard)	Adsorptions Required (Experimental)	Total Incubation Time (Experimental, minutes)	Underlying Alloantibodies (Experimental)
049	3	180	None	1	60	None
050	3	180	None	1	60	None
051	3	180	Anti-e	1	60	Anti-e
052	2	120	None	1	60	None
053	3	180	None	1	60	None
054	3	180	Anti-K, -Jkb	1	60	Anti-K, -Jkb
055	2	120	None	1	60	None
056	2	120	None	1	60	None
057	3	180	None	1	60	None
058	3	180	Anti-Fya	1	60	Anti-Fya
059	3	180	None	1	60	None
060	4	240	Anti-C, -Cw	2	120	Anti-C, -Cw
061	3	180	None	1	60	None
062	3	180	None	1	60	None
063	2	120	None	1	60	None
064	3	180	Anti-Jsa	1	60	Anti-Jsa
065	3	180	None	1	60	None
066	3	180	None	1	60	None
067	2	120	None	1	60	None
068	3	180	Anti-K, -Kpa, -Jsa	1	60	Anti-K, -Kpa, -Jsa
069	3	180	None	1	60	None
070	3	180	None	1	60	None
071	2	120	None	1	60	None
072	3	180	Anti-c, -E, -K	1	60	Anti-c, -E, -K
073	3	180	None	1	60	None
074	3	180	None	1	60	None
075	3	180	None	1	60	None
076	2	120	None	1	60	None
077	3	180	Anti-Fyb, -Leb	1	60	Anti-Fyb, -Leb
078	3	180	None	1	60	None
079	6	360	Unsuccessful	2	120	Anti-K, -Jkb, -S
080	3	180	None	1	60	None
081	3	180	None	1	60	None
082	3	180	Anti-D, -C, -E	1	60	Anti-D, -C, -E
083	3	180	None	1	60	None
084	3	180	None	1	60	None
085	2	120	None	1	60	None
086	4	240	None	2	120	None
087	2	120	Anti-Jkb	1	60	Anti-Jkb
088	3	180	None	1	60	None
089	2	120	None	1	60	None
090	3	180	None	1	60	None

a guillotine trimmer and added to each aliquot of adsorbing cells after the final wash and before plasma was added. The filter paper was placed in the tubes for 60 seconds to absorb as much residual saline as possible and minimize its dilution effect. On average, 21 drops of red cells from a plastic transfer pipette (approximately 1 mL) were used in each experimental adsorbing tube, and on average, 3 to 4 drops of residual saline were removed with each application of filter paper. This was measured by using a plastic transfer pipette to drop saline onto clean, dry filter paper until a comparable amount was visibly pooled. Aliquots of standard method adsorbing cells did not use filter paper during preparation.

TABLE 2. Fisher's Exact Test

	1 Adsorption	>1 Adsorption	Total
Standard method	1	89	90
Experimental method	82	8	90
Total	83	97	180

Significance level = P < .0001.

Although far from definitive, it can be reasonably surmised that since the adsorbing cells were prepared in an identical fashion in both methods, approximately 3 to 4 drops of residual saline per 1 mL of adsorbing cells likely remained in each aliquot in the standard method.

Both adsorption methods were carried out in 6 mL specimen tubes with air-tight rubber stoppers containing no additives. As a result of the tube size and the lower volume limitation placed on each aliquot of adsorbing cells and plasma during incubation in the experimental method along with the horizontal placement of the tubes in the water bath, more optimal surface contact between antibodies in the plasma and antigens on the red blood cells was maintained throughout the incubation period. The standard method involved placing the tubes vertically in a conventional test tube holder, placing the test tube holder into the 37°C water bath, and mixing at approximately 15-minute intervals. The tubes were approximately half full to allow them to remain below the surface of the water while incubating; the total volume of the tubes was approximately 3 to 3.5 mL. The aliquot volume limitation and orientation of the tubes modified in the experimental method were designed to increase the likelihood of the formation of antigen-antibody complexes without the need for periodic mixing by minimizing gravity-induced separation of the antibodies in the plasma from the antigen-binding sites on the adsorbing cells.

All of the 90 specimens achieved complete adsorption with the experimental method, while 88 of the 90 specimens achieved complete adsorption with the standard method. A total of 25 of the 90 specimens, or approximately 28%, contained underlying alloantibodies, consistent with findings of previous studies.[5] In both cases where the standard method failed to achieve complete adsorption in 6 attempts, the experimental method was able to detect the presence of underlying alloantibodies in the adsorbed plasma. In 2 instances, the adsorbed plasma obtained from the experimental method demonstrated clinically significant underlying alloantibodies that were not detected by the standard method. In both cases where the standard method failed to detect an underlying clinically significant alloantibody, 4 adsorptions were required to achieve complete adsorption with the standard method, whereas 2 adsorptions were required with the experimental method. The additional underlying antibodies not detected by the standard method were in the Kidd blood group system, which produces antibodies that are known to rapidly and significantly drop in titer to levels that are difficult to detect with routine serological testing methods.[9] Although it is not possible to say for certain within the scope of this study, it seems logical to assume that, due to the characteristics of the aforementioned antibodies and the dilution potential of the standard method with multiple rounds of adsorption, the experimental method may have succeeded in detection because of its dilution-control modifications.

Conclusions

Compared to the standard method, the experimental method demonstrated improved efficiency through a reduction in rounds of adsorption required to resolve warm autoantibody interference and consequently shorter waiting periods for transfusions. A reduction in the amount of adsorbing rounds required to resolve interference in warm autoantibody cases could reduce the cost of staffing.

A further advantage to the experimental method was its greater sensitivity in detecting underlying alloantibodies, thereby reducing the risk of transfusion. Failure to detect clinically significant underlying alloantibodies in cases requiring warm autoantibody resolution can have potentially dire consequences for the recipient of transfused blood. The underlying alloantibodies detected by only the experimental method have been reported to cause both severe acute and delayed hemolytic transfusion reactions as well as possible renal graft rejection.[10–13]

There are plans to conduct a follow-up study using an incubation period of 30 minutes to determine whether the full 60 minutes are necessary, with the goal of further improving the efficiency of the experimental adsorption process without compromising its effectiveness.

Limitations

In the course of the study, it became apparent that the lack of a precision method to quantify the residual saline present in adsorbing cell aliquots following the washing process made it extremely difficult to determine whether the observed dilution potential in the standard method was in fact meaningful. Initially, this variable was not planned for in the study design, and a crude method to attempt to calculate how much residual saline remained was improvised. This improvised method permitted some insight into how much residual saline remained; however, no definitive conclusions could be drawn about its significance in the detection of underlying alloantibodies with the adsorbed plasma. As a result of this limitation, it can only be speculated that the failed detection of 2 underlying alloantibodies in the standard method may have been caused by the dilution of plasma by residual saline. Consequently, the effectiveness of the filter paper control is also uncertain. It would be beneficial in future research to develop a method for measuring this variable to determine whether methods of attempting to minimize residual saline are beneficial or necessary. Additionally, due to the complex nature of warm autoantibody cases, a comparison of total turnaround time between the standard and experimental methods could not be reliably performed.

Acknowledgements

The author thanks Laurie Gillard for general support by a departmental chair, Robert Harr for assistance with statistical analysis, and Chelsey Geurkink, Jules Zinni, and Vanessa Fortune, scientific advisers.

There are no conflicts of interest to disclose.

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SCIENCE

Stability of Hemoglobin Constant Spring Identified by Capillary Electrophoresis

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Keywords: hemoglobin Constant Spring, HbCS, capillary electrophoresis, alpha globin, specimen stability, pre-analytical error

Abbreviations: HbCS, hemoglobin Constant Spring; CE, capillary electrophoresis; IEF, isoelectric focusing; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; DMSO, dimethyl sulphoxide; SEA, Southeast Asian; HbHCS, hemoglobin H disease with hemoglobin Constant Spring.

Laboratory Medicine 2023;54:e93–e96; https://doi.org/10.1093/labmed/lmac130

ABSTRACT

Objective: Hemoglobin Constant Spring (HbCS) is often missed by routine hemoglobin analysis. The aim of this research was to study HbCS stability as identified by capillary electrophoresis (CE) to determine the specimen storage time limit.

Methods: The EDTA blood of 29 HbCS samples were kept at 4°C and analyzed every workday until CE could not detect HbCS or until 7 weeks after blood collection. The genotypes were confirmed by multiplex polymerase chain reaction.

Results: The median subject age was 27 years and 10 subjects were male. The HbCS levels were stable during the first 7 days but became undetectable in 5 cases (17.2%) after 1 week. All of them were heterozygous HbCS. Longer detection times were correlated with the higher baseline HbCS levels, with a correlation coefficient of 0.582 ($P \leq 0.001$)

Conclusion: Routine hemoglobin typing and quantitation should be performed within 1 week after blood collection to detect low HbCS levels, especially in heterozygous HbCS.

The α-globin protein is a major constituent of hemoglobin (Hb) and myoglobin and is produced by 2 homologous genes, *HBA1* and *HBA2*. Deletions in 1 (α^+ deletions that are $-\alpha^{3.7}$, $-\alpha^{4.2}$ or $-\alpha^{20.5}$) or both (α^0 deletions that are $--^{SEA}$, $--^{THAI}$, $--^{FIL}$ or $--^{MED}$) genes are found in 19.4% and 15.0% of Thai population, respectively.[1] Deletions of 3 of the 4 α-globin genes (α^+/α^0) cause Hb H disease, which typically shows mild-to-moderate anemia and needs only occasional transfusions, whereas deletions of all 4 genes (α^0/α^0) result in perinatal deaths from Hb Bart's hydrops fetalis.

Hemoglobin Constant Spring (HbCS) is the most common nondeletional α-thalassemia in Southeast Asia, with the gene frequency of 3% to 6%.[2] The HbCS is caused by a point mutation at the termination codon of the *HBA2* gene, resulting in 31 additional amino acids.[3–5] Due to the abnormal structure and unstable mRNA, the rate of α^{CS}-globin chain synthesis is markedly decreased. Notably, the HbCS homozygote or compound heterozygotes of HbCS and α^0 deletion (HbHCS) can have severe fetal anemia requiring life-saving intrauterine transfusions.[6,7] Interestingly, anemia in these cases is much improved postnatally, becoming thalassemia intermedia syndromes in adults. Therefore, detection of HbCS in parents is essential to identify and closely monitor the fetuses at risk. However, detectable HbCS levels are lower than 2% in heterozygotes[1] and often missed by routine laboratory testing.[3,4,8]

There are 3 major methods for detecting HbCS. Isoelectric focusing (IEF) electrophoresis is a manual method that was known to misdiagnose HbCS diseases.[9] Also, automated high-performance liquid chromatography (HPLC) can detect HbCS in only 26% to 86% of HbCS traits[4,5,10] without accurate quantification. Capillary electrophoresis (CE) is an automated method with the highest reported sensitivity for HbCS of 81% to 100%.[5,11]

Hemoglobin analysis is usually requested by general practitioners in community hospitals and sent to regional referral laboratories. The test is often delayed after specimen collection. This research aimed to study the stability of blood samples used for HbCS identification, providing data on the limit of specimen storage time.

Materials and Methods

This study was performed on leftover EDTA-blood specimens containing HbCS from the Thalassemia laboratory, Faculty of Medicine, Chulalongkorn University. All specimens were from Thai patients of all ages and both sexes. The HbCS was identified by Capillarys 2 flex piercing using the CAPILLARYS HEMOGLOBIN(E) kit including buffer, hemolyzing solution, wash solution, dilution segments, and

filters (Sebia, France) according to the manufacturer's instruction. The samples were analyzed within 24 hours and then every workday until the Capillarys 2 flex piercing could not detect HbCS or until 7 weeks after blood collection. The specimens were kept in a 2°C to 8°C refrigerator during the study.

The multiplex gap polymerase chain reaction (PCR) for $-^{SEA}$, $-^{THAI}$, $-^{FIL}$, $-^{MED}$ and $-\alpha^{20.5}$ α-globin gene deletions was performed in the presence of 2.5 mM $MgCl_2$, 0.75 M betaine, and 5% dimethyl sulphoxide (DMSO). The PCR cycles consisted of 95°C for 15 min (activation), followed by 35 cycles of 95°C for 1 min (denaturation), 62°C for 1 min (annealing), and 72°C for 2 min and 30 sec (extension), and the final extension of 72°C for 10 min. The primers have been previously described.[12] The multiplex gap PCR for $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions was performed in the presence of Q-solution using similar PCR cycles as above. The Q-solution is an additive that facilitates PCR amplification of templates with high Guanine/Cytosine contents and/or secondary structures by changing DNA melting behavior. The primers have been reported previously.[13]

The multiplex ARMS PCR for 6 nondeletional α-thalassemia genes that were initiation codon (-T), codon 30 (-GAG), codon 59 (G > A), HbCS, Hb Paksé, and Hb Quong Sze was done in the presence of 2.0 mM $MgCl_2$, 16.5 mM $(NH_4)_2SO_4$, 10% DMSO, and 0.01% (w/v) gelatin. The PCR cycles consisted of 94°C for 3 min (activation) followed by 30 cycles of 94°C for 1 min (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (extension), and the final extension of 72°C for 5 min. The primers were used as previously described.[14,15]

All PCRs were performed using HotStarTaq Plus DNA polymerase (QIAGEN, Germany) in the Biometra TRIO thermal cycler (Analytik Jena, Germany).

Results

There were 29 subjects with a median age of 27, ranging from 8 months to 52 years. Ten of them (34.5%) were male. By CE, there were 22 cases of heterozygous HbCS ($\alpha^{CS}\alpha/\alpha\alpha$), 6 cases of compound heterozygosity for 3.7 kb deletion and HbCS ($-\alpha^{3.7}/\alpha^{CS}\alpha$) and one compound heterozygosity for Southeast Asian (SEA) deletion and HbCS ($-^{SEA}/\alpha^{CS}\alpha$). The average HbCS levels at baseline were 0.46% (range 0.2–0.7), 0.68% (range 0.6–0.8) and 2.1%, for $\alpha^{CS}\alpha/\alpha\alpha$, $-\alpha^{3.7}/\alpha^{CS}\alpha$, and $-^{SEA}/\alpha^{CS}\alpha$, respectively. The HPLC and IEF could detect heterozygote HbCS in only 9 (40.9%) and 10 (45.5%) cases of HbCS heterozygotes, respectively. The respective numbers for $-\alpha^{3.7}/\alpha^{CS}\alpha$ were 4 (66.7%) for both HPLC and IEF. All 3 methods could detect HbCS in the $-^{SEA}/\alpha^{CS}\alpha$ case.

The longest and shortest storage times at which CE could identify HbCS were more than 7 weeks and 1 week after blood collection, respectively. The median detection times were 16 days and 28 days for heterozygous HbCS and $-\alpha^{3.7}/\alpha^{CS}\alpha$, respectively. Five of the 29 cases (17.2%) showed undetectable HbCS after 1 week of storage, and all of them were heterozygous HbCS. The cases of $-\alpha^{3.7}/\alpha^{CS}\alpha$ started to lose HbCS in week 3, but the $-^{SEA}/\alpha^{CS}\alpha$ still had detectable HbCS after 7 weeks of our study. The numbers of detectable cases by storage times are shown in **FIGURE 1**.

TABLE 1. The Correlation Between Durations of Detectable Hemoglobin Constant Spring (HbCS) after Storage and Baseline HbCS Levels

Last Detectable Times	Average Baseline %HbCS (Range)	Number of Cases
1 week	0.30 (0.2–0.5)	5
2 weeks	0.35 (0.2–0.5)	4
3 weeks	0.56 (0.3–0.7)	9
4 weeks	0.60 (0.4–0.8)	2
5 weeks	0.60	1
6 weeks	0.50	1
7 weeks	0.63 (0.6–0.7)	3
Over 7 weeks	1.08 (0.7–2.1)	4

FIGURE 1. The numbers of cases with detectable hemoglobin Constant Spring (HbCS), All blood specimens still had detectable HbCS in the first week but the numbers declined during the storage period. The median detectable times were 16 days in heterozygous HbCS and 28 days in compound heterozygous of HbCS and 3.7 kb deletion. HbCS/α, Heterozygous HbCS (left bar); HbCS/α^+, compound heterozygous of HbCS and 3.7 kb deletion (middle bar); HbCS/α^0, compound heterozygous of SEA deletion and HbCS (right bar). Number of patients.

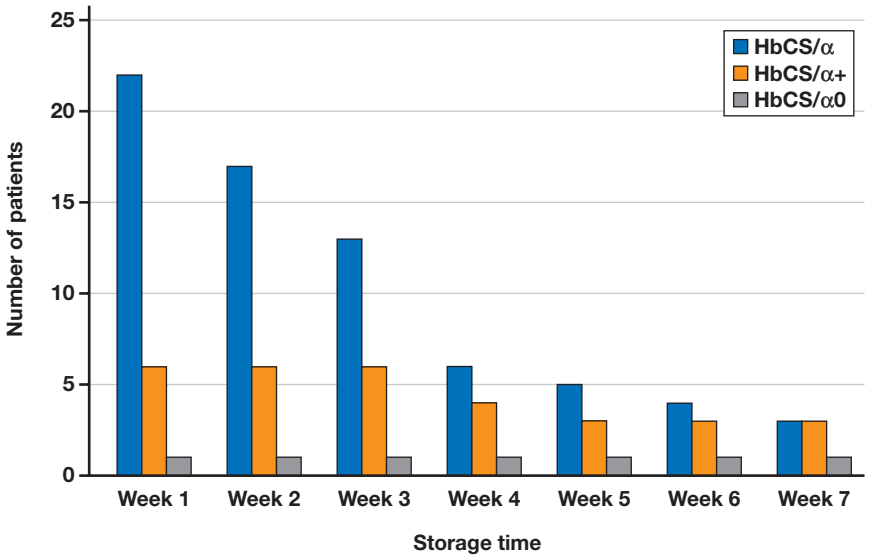
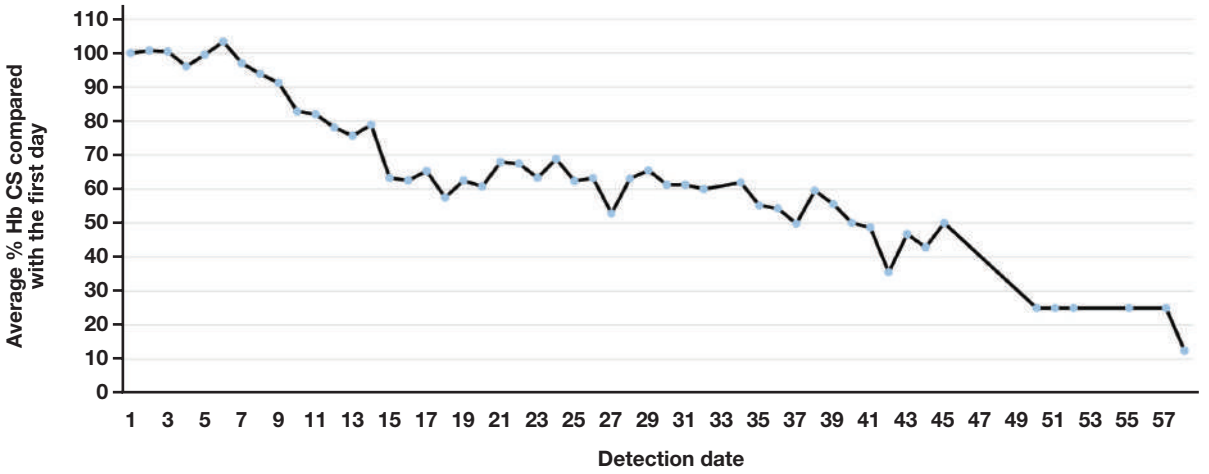


FIGURE 2. The percentage decrease in hemoglobin Constant Spring (HbCS) during storage compared with the baseline. This graph shows the average percentages of HbCS levels compared with their baselines over the storage time.



We observed that samples with higher baseline HbCS concentrations correlated with duration of HbCS detection postcollection with a correlation coefficient of 0.582 ($P < 0.001$), as shown in **TABLE 1**. In all 3 groups, the amounts of HbCS measured by CE progressively declined starting after 7 days. The percentage decreases in HbCS compared with baseline are shown in **FIGURE 2**. On average, HbCS levels declined by approximately 35% during the second week and subsequently decreased by 8% per week through week 7.

Discussion

In this study, we found that the optimal duration for HbCS detection in blood samples stored at 4°C was 1 week. Subsequently, the levels slowly declined. Although HbCS was still detectable in most specimens (82.8%) after 7 days, delaying analyses past 1 week is likely to lead to some patients being misdiagnosed, resulting in erroneous genetic counseling. In previous studies where CE performance was analyzed, it is possible that long storage durations contributed to the inability to detect HbCS, as storage durations before testing were not stated.[11,12] To the best of our knowledge, this is the first study on sample stability for detecting HbCS in HbCS heterozygous samples. Our study also confirms the higher sensitivity of HbCS detection using CE compared with HPLC and IEF.

Our study also demonstrates a good correlation between baseline concentrations of HbCS and duration of sample storage for its detection (**TABLE 1**). We thus hypothesize that lower HbCS concentrations in some samples that are heterozygote for HbCS contribute to their shorter sample stabilities. However, there were still some heterozygous HbCS specimens that showed persistent HbCS concentrations over 7 weeks. Although the reason is unclear, we suspect that those samples had higher concentrations of HbCS at baseline. The variations in baseline HbCS may be affected by other genetic polymorphisms that remain to be defined. Compound heterozygotes HbCS and deletional α-thalassemia genes had higher proportions of HbCS and thus longer detectable durations.

There are previous reports on the stability of HbCS during storage in specimens from 4 HbHCS cases. The HbCS levels were stable for 17 days in 1 case[16] and for 11 days in 3 cases.[11] Consistent with these data, we found that our HbHCS case showed stable levels of HbCS up to 27 days (data not shown). As far as we know, there has been no report of the HbCS stability in HbCS heterozygous samples.

In conclusion, the CE method can identify HbCS after several weeks of proper storage. However, routine hemoglobin typing and quantitation should be performed within 1 week after blood collection to detect low HbCS levels especially in heterozygous HbCS.

Acknowledgments

This study was supported by grants from H.H Princess Sudasiri Sobha's fund.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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CASE STUDY

Therapeutic Monoclonal Antibody Interference in Monoclonal Gammopathy Monitoring: a Denosumab Experience

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Keywords: denosumab, immunofixation electrophoresis, monoclonal antibody, multiple myeloma, serum protein electrophoresis, interference

Abbreviations: Ig, immunoglobulin; SIFE, serum immunofixation electrophoresis; TMA, therapeutic monoclonal antibody agent.

Laboratory Medicine 2023;54:e97–e99; https://doi.org/10.1093/labmed/lmac129

ABSTRACT

A 73-year-old woman was diagnosed with a lambda light chain myeloma. A follow-up immunofixation electrophoresis showed a monoclonal immunoglobulin (Ig)G kappa in addition to the regular lambda band. A monoclonal antibody therapy interference was suspected but her VRD (bortezomib, lenalidomide, dexamethasone) regimen did not include such a medication. Later it was learned that she was prescribed denosumab, a monoclonal human antibody agent to treat bone lesions. The IgG kappa band disappeared 7 months after the first and 4 months after the last dose of denosumab, confirming a case of interference. This case once again emphasizes the importance of delta check and close communication between clinicians to avoid a false result in electrophoresis. It also describes the migration pattern of denosumab. As therapeutic antibodies gain approval and enter into common clinical practice, drug interference will complicate electrophoresis testing in diagnosis and patient follow-up.

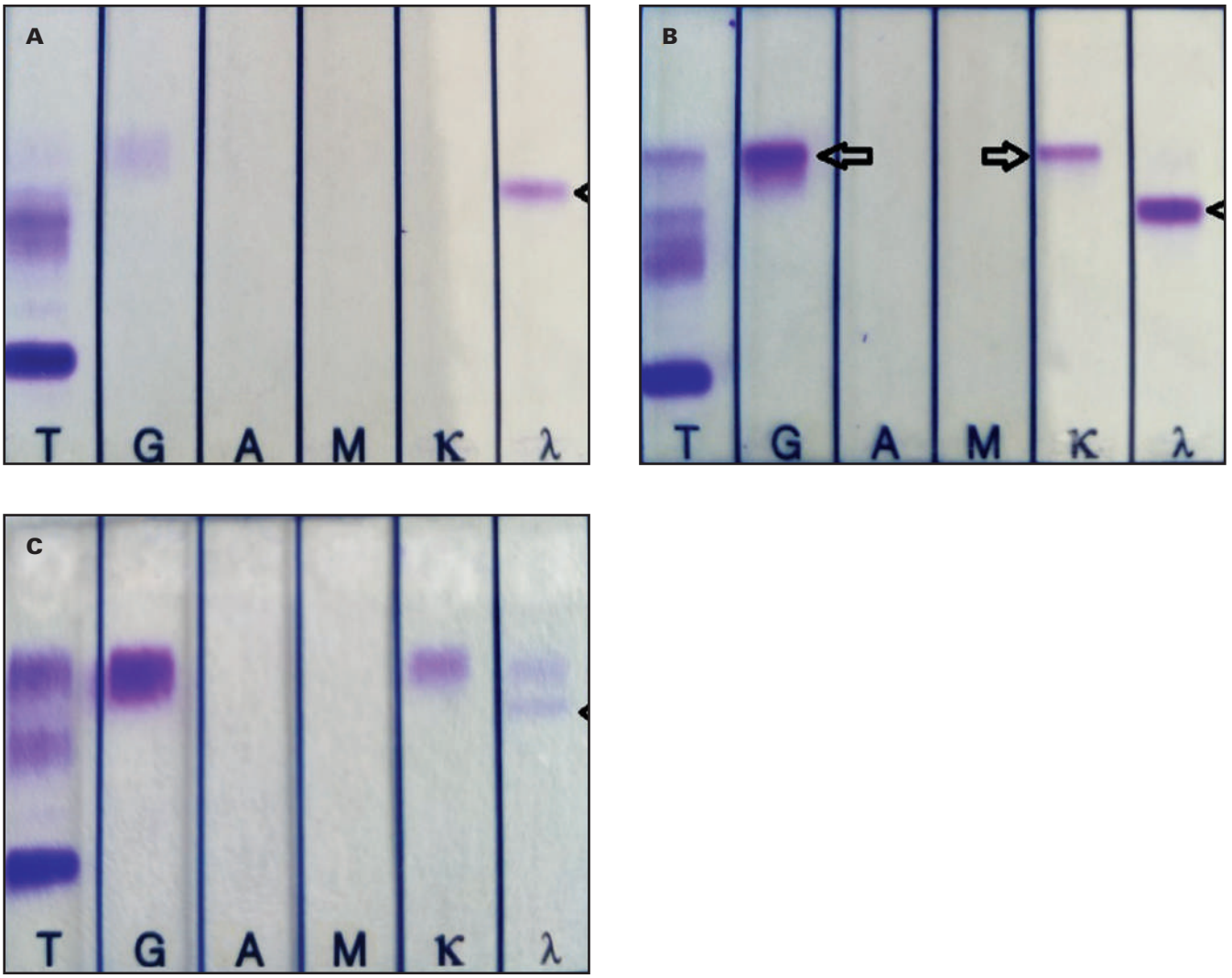
Clinical History

A 73-year-old woman had been followed with a diagnosis of free light chain multiple myeloma. She had chronic renal insufficiency and anemia at her first visit to the hospital in June 2021. Her initial serum immunofixation electrophoresis (SIFE) showed a clear monoclonal lambda band without a heavy chain component (**FIGURE 1A**). Serum free light chain ratio (kappa/lambda) was 0.005 (kappa: 19.3 mg/L, lambda: 3320 mg/L). She was treated with a VRD (bortezomib, lenalidomide, dexamethasone) regimen initially but lenalidomide was stopped because of complications. In July, a follow-up SIFE showed a weaker monoclonal lambda band. Her third SIFE in September 2021 showed the regular monoclonal lambda band and an extra monoclonal immunoglobulin (Ig)G kappa (**FIGURE 1B**). Therapeutic monoclonal antibody agent (TMA) interference was considered and the clinician was called for confirmation. It was learned that her primary therapy did not involve a TMA but she was using denosumab to treat bone lesions. She had her first dose of denosumab on July 8, 2021, and 2 more doses were planned every 4 weeks, 120 mg in each dose. Unfortunately, the therapy was interrupted because of a Covid-19 infection. Her blood sample for the third SIFE was gathered on September 2, 2021, before the second dose of denosumab, which was given on September 15, 2021, and the third dose was given on October 13, 2021. Meanwhile, the patient continued her routine chemotherapy, and she needed hemodialysis twice a week. On February 9, 2022, the fourth SIFE showed the primary monoclonal lambda band and the extra monoclonal IgG kappa band had disappeared (**FIGURE 1C**). Serum free light chain ratio (kappa/lambda) was 0.03 (kappa: 110 mg/L, lambda: 3800 mg/L) in February 2022 and 0.05 (kappa: 106 mg/L, lambda: 1960 mg/L) in March 2022.

Discussion

Interference is a major concern in clinical laboratories. Every test is prone to risk and electrophoresis is no exception. Traditional interference in electrophoresis are caused by the presence of fibrinogen, hemolysis, and the presence of radiologic dyes in the serum sample.[1] A high level of suspicion and some experience was previously sufficient to cope with this trio, as they show no reactivity in serum immunofixation electrophoresis that can be used as a rule-out check. Introduction of monoclonal agents as a therapeutic option in multiple myeloma is a break point in managing interference in electrophoresis.[2] The basic structure of these agents is very much like the protein end products of neoplastic myeloma cells: both are monoclonal Igs. The laboratory should be in close communication with clinicians and clinicians should be aware of their responsibility to inform the laboratory about medications in such a case.

FIGURE 1. **The patient's initial SIFE showed a monoclonal lambda band (arrow head) (A). On July 8, 2021, she received the first dose of denosumab; 56 days later, a follow-up SIFE showed a monoclonal IgG kappa band (two arrows) in addition to the expected monoclonal lambda band (arrow head) (B). She received 2 more doses of denosumab on September 15, 2021, and October 13, 2021. The next SIFE on February 9, 2022, showed the monoclonal lambda (arrow head) and the monoclonal IgG kappa had disappeared (C).**



Daratumumab is the most well-known therapeutic monoclonal antibody agent used in treatment of multiple myeloma, followed by elotuzumab.[3] Both are IgG kappa in nature and targeted against specific surface antigens of plasma cells. Shortly after their approval for use, they appeared to cause interference in follow-up electrophoresis of myeloma patients.[2] A band at the cathodal end of the gamma region with an IgG kappa immunophenotype is characteristic for daratumumab usage, whereas elotuzumab seems to migrate to the middle of the gamma region. The limited literature indicates that different agents show different migration patterns. Clinical confirmation is of ultimate importance in the diagnosis of therapeutic monoclonal antibody interference. We here describe a clear interference case with an unexpected agent as a cause.

A TMA interference complicated electrophoresis testing in this case. The incidence of interference is increasing as new drugs are introduced in a plethora of indications.[3] Laboratories that practice electrophoresis testing to diagnose or to follow monoclonal gammopathies must take extra care to consider TMA interference that may lead to unwanted consequences. This case emphasizes the importance of a delta check in reporting electrophoresis results. The reporters are advised to check patients' previous test results. The course of a monoclonal band should be traceable. Any variations, such as a shift in migration pattern or immunophenotype, should be investigated. In this case, it was fortunate that the original M-protein was a single monoclonal lambda band, so that an extra IgG kappa was a clear sign of TMA interference. However, most monoclonal gammopathies and most TMA agents share a common IgG phenotype, which is a major source of concern in differential diagnosis of TMA interference.

Denosumab is a monoclonal IgG antibody. It targets RANKL (receptor activator of NFjB ligand) protein, which is crucial for the differentiation of osteoclasts. Inhibition of osteoclasts results in reduced bone resorption. It has become a drug of choice as bone disease is a hallmark of myeloma.[4] Also, it is used efficiently in other solid tumors with bone

metastasis like breast carcinoma and prostate carcinoma.[5] And finally, the efficacy of denosumab on bone disease has been demonstrated in the treatment of osteoporosis.[6]

The MTAs have received exceptional interest. Their efficiency caused clinicians and manufacturers to design new drugs. They are already used in a wide range of indications.[7] Considering this case with denosumab, laboratories now face an interfering agent that is used for the treatment of bone disease in general, including osteoporosis. Myeloma, cancer, and osteoporosis share common diagnosis among the elderly. Most of the time, these patients are followed up in different clinics or even in different hospitals due to coexisting diseases. Lack of knowledge of medications may pose a dilemma in electrophoresis. Hopefully, hospital information systems and interactions between clinics and laboratories can prevent misdiagnosis.

This case is cautionary of the growing danger of misdiagnosis due to TMAs. We suggest that manufacturers include information about migration patterns of their drugs in electrophoresis. Hospital and laboratory information systems must adopt warning signals in the case of a patient with both an electrophoresis request and a TMA prescription. This case showed that the migration pattern of denosumab in electrophoresis mimics daratumumab's. Differential diagnosis needs a clinical correlation.

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