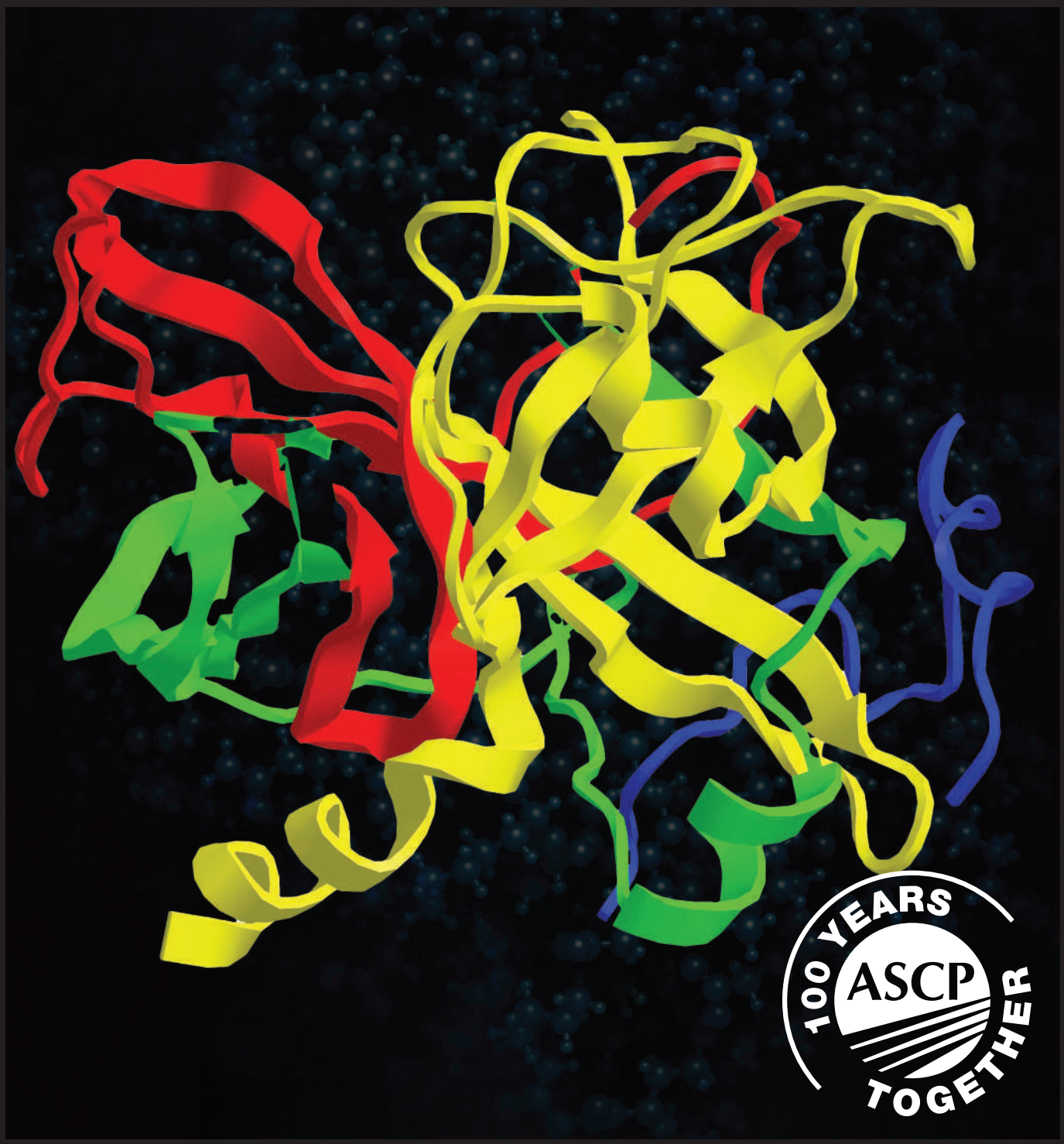
Laboratory Medicine

July 2022 Vol 53 No 4 Pgs 331–435

labmedicine.com



Downloaded from https://academic.oup.com/labmed/issue/53/4 by guest on 28 February 2025

BOARD OF EDITORS

Editor in Chief
Roger L. Bertholf, PhD
Houston Methodist Hospital and Weill Cornell Medicine

Reviews
ASSOCIATE EDITOR
Deniz Peker, MD
Emory University School of Medicine
ASSISTANT EDITOR
Rahul Matnani, MD, PhD
Rutgers Robert Wood Johnson Medical School

Clinical Chemistry
ASSOCIATE EDITOR
Uttam Garg, PhD
University of Missouri Kansas City School of Medicine
ASSISTANT EDITORS
David Alter, MD
Emory University School of Medicine
Veronica Luzzi, PhD
Providence Regional Core Laboratory
Alejandro R. Molinelli, PhD
St Jude Children's Research Hospital

Cytology
ASSOCIATE EDITOR
Antonio Cajigas, MD
Montefiore Medical Center

Hematology
ASSOCIATE EDITOR
Shiyong Li, MD, PhD
Emory University School of Medicine
ASSISTANT EDITORS
Elizabeth Courville, MD
University of Virginia School of Medicine
Alexandra E. Kovach, MD
Children's Hospital Los Angeles
Sanam Loghavi, MD
MD Anderson Cancer Center

Histology
ASSOCIATE EDITOR
Carol A. Gomes, MT(ASCP)HTL
Stony Brook University Hospital

Immunohematology
ASSOCIATE EDITOR
Richard Gammon, MD
OneBlood
ASSISTANT EDITORS
Phillip J. DeChristopher, MD, PhD
Loyola University Health System
Gregory Denomme, PhD
Grifols Laboratory Solutions

Immunology
ASSOCIATE EDITOR
Thomas S. Alexander, PhD
FlowMetric Life Sciences

Laboratory Management and Administration
ASSOCIATE EDITOR
Lauren Pearson, DO, MPH
University of Utah Health
ASSISTANT EDITORS
Daniel D. Bankson, PhD, MBA
University of Washington
Joseph Rudolf, MD
University of Utah

Microbiology
ASSOCIATE EDITOR
Yvette S. McCarter, PhD
University of Florida College of Medicine
ASSISTANT EDITORS
Kevin Alby, PhD
University of North Carolina School of Medicine
Allison R. McMullen, PhD
Augusta University–Medical College of Georgia
Elitza S. Theel, PhD
Mayo Clinic

Molecular Pathology
ASSOCIATE EDITOR
Gerald A. Capraro, PhD
bioMérieux
ASSISTANT EDITORS
Holli M. Drendel, PhD
Atrium Health Core Laboratory
Rongjun Guo, MD, PhD
ProMedica Health System
Shuko Harada, MD
University of Alabama at Birmingham

Pathologists' Assistant
ASSOCIATE EDITOR
Anne Walsh-Feeks, MS, PA(ASCP)
Stony Brook Medicine

STAFF

EXECUTIVE EDITOR FOR JOURNALS
Kelly Swails, MT(ASCP)
DIRECTOR OF SCIENTIFIC PUBLICATIONS
Joshua Weikersheimer, PhD
SENIOR EDITOR, JOURNALS
Philip Rogers

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, $173 (electronic) or $225 (electronic and print); single issues for individuals are $29 and for institutions $64. Annually for Rest of World, £112/€159 (electronic) or £144/€206 (electronic and print); single issues for individuals are £19/€27 and for institutions £40/€57. 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, 2001 Evans Road, Cary, NC 27513. 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.

CLAIMS: Publisher must be notified of claims within four months of dispatch/ order date (whichever is later). Subscriptions in the EEC may be subject to European VAT. Claims should be made to Laboratory Medicine, Journals Customer Service Department, Oxford University Press, 2001 Evans Road, Cary, NC 27513, Tel: 800-852-7323 (toll-free in USA/Canada) or 919-677-0977, e-mail: jnlorders@oup.com.

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

Laboratory Medicine is a registered trademark. Authorization to photocopy items for internal and personal use, or the internal and personal use of specific clients, is granted by ASCP Press for libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the base fee of USD 15.00 per copy is paid directly to the CCC, 222 Rosewood Drive, Danvers, MA 01923, 978.750.8400. In the United States prior to photocopying items for educational classroom use, please also contact the CCC at the address above.

Printed in the USA

© 2022 American Society for Clinical Pathology (ASCP)

Downloaded from https://academic.oup.com/labmed/issue/53/4 by guest on 28 February 2025

Advertising Sales Office Classified and Display Advertising

CORPORATE ADVERTISING
Jane Liss
732-890-9812
jliss@americanmedicalcomm.com

RECRUITMENT ADVERTISING
Lauren Morgan
267-980-6087
lmorgan@americanmedicalcomm.com

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

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

LaboratoryMedicine

July 2022
Vol 53, No 4
p. 331-435

ON THE COVER: Thrombin [activated factor II (IIa)] is a 37 kDa serine protease cleaved from prothrombin (factor II) by the action of activated factor X and its cofactor, factor V, that form a "prothrombinase." Thrombin plays a central role in thrombus formation by activating factors XI, VIII, and V, which regulate its production, and thrombin cleaves fibrinopeptides A and B from fibrinogen (factor I) to form the fibrin (factor Ia) monomers that polymerize, and then cross-link via the action of fibrin-stabilizing factor (factor XIII; which also is activated by thrombin), stabilizing the platelet plug that contributes to the thrombus. In addition, thrombin activates platelets. Later in the coagulation cascade, thrombin will inhibit coagulation through its binding to thrombomodulin, thereby inhibiting the procoagulant effects of thrombin, and inactivating factors Va and VIIIa. In this issue of *Laboratory Medicine* pp 349–359, Marin and colleagues provide a rational approach to coagulation testing.

Downloaded from https://academic.oup.com/labmed/issue/53/4 by guest on 28 February 2025

EDITORIAL

A Century of Progress

Roger L. Bertholf, PhD,[1] *Steven H. Kroft, MD*[2]

[1]Editor in Chief, *Laboratory Medicine*; and [2]Editor in Chief, *American Journal of Clinical Pathology*

Laboratory Medicine 2022;53:333–335; https://doi.org/10.1093/labmed/lmac070

Anniversaries are natural moments to reflect on the passage of time—where we started, where we have been, where we are, and even where we are going. A 100th, or centurial, anniversary feels particularly momentous. Thus, as ASCP turns 100, it seemed appropriate to provide a retrospective on the enormous progress we have seen over a century of time in the field of pathology and laboratory medicine. To accomplish this, we asked members of our editorial boards to look back to the founding of the ASCP in 1922 and comment on the changes that have taken place in their specialties since then. Their outstanding essays are published on the Critical Values web site, and we highly recommend visiting them.

Geoffrey Wool, MD, PhD, is Coagulation Associate Editor for *American Journal of Clinical Pathology (AJCP)*. He describes how our understanding of hemostasis and thrombosis has evolved over the past 100 years. In 1922, Dr Wool points out, only 3 coagulation factors were known: calcium, thrombin, and fibrinogen. The traditional Roman numerology for the factors didn't begin to be developed until 1943, and wasn't formally adopted until 1959. The coagulation cascade with which we are all familiar was only completed in the 1960s. The prothrombin time test was first described in 1935 and the activated partial thromboplastin time assay was developed in 1953. Dr Wool concludes his essay with a comment on how unique features of COVID-19, including the limitations of conventional coagulation assays to predict thrombosis and the emergence of the novel entity of vaccine-induced immune thrombotic thrombocytopenia, highlighted some limitations of our current understanding of the coagulation system. He ends with this observation: "As we look forward to another successful 100 years in the hemostasis field, we must remember that dedicated, knowledgeable, and skilled laboratory professionals are the foundation of our work."

Yvette McCarter, PhD, is Microbiology Associate Editor for *Laboratory Medicine* (*LM*). She notes the sobering coincidence that in 1922 the world was just emerging from the Great Flu Pandemic, which claimed an estimated 50 million lives worldwide, with 675,000 of those in the US. The current COVID-19 pandemic is estimated to have caused over 5 million deaths worldwide, with about 1 million of those in the US. Thus, remarkably, the first 100-years of ASCP history are bookended by global public health catastrophes. And while it took over a decade for the causative agent of the 1918 pandemic to be discovered, the remarkable advances in virology allowed the causative agent of COVID-19 to be fully characterized in a matter of months. This, in turn, allowed clinical laboratories to rapidly develop and deploy accurate tests to detect the virus, and in so doing profoundly impact the course of the present pandemic in a way that was impossible 100 years ago. Regarding this dramatic evolution, Dr McCarter cites 2 developments that have had particularly profound influence on clinical microbiology: automation and molecular techniques (without both of which, the clinical laboratory response to COVID-19 would have been impossible). She opines: "While automation has had a significant impact on the ability of clinical microbiologists to contribute to patient care, the evolution of molecular techniques has changed the clinical microbiology paradigm from conventional laboratory methods that rely on phenotypic expression of antigens or biochemical products, to molecular methods for the rapid identification of infectious agents."

Andrew Fletcher, MD, is Medical Director of Consultative Services at ARUP Laboratories in Salt Lake City, UT. Dr Fletcher offers a fascinating perspective on the evolving role of the clinical laboratory in medical care, centering his comparison of 1922 and the present day on a single laboratory, the Finley Hospital Laboratory in Dubuque, IA, which has been in continuous operation for just over 100 years. He quotes the late Dr F.P. McNamara, a pathologist who joined the Finley Hospital medical staff in 1921, as saying that clinical laboratories were "having a hard time" proving their worth. It is astonishing to anyone practicing laboratory medicine in the 21st century that 100 years ago many doctors, according to McNamara, were critical of the laboratory, complaining that it had "assumed too great an importance" in medical practice. He went on to say, prophetically, that laboratory "sciences are developing so rapidly that it is practically impossible for the practitioner to keep in touch with all the advances." In contrast, the 2022 version of the Finley Hospital clinical laboratory is a modern, vibrant facility that performs nearly 200,000 tests per year. Dr Fletcher observes that even after 100 years of evolution, clinical laboratories still face the same challenges of under- and overutilization, and of educating their clinical colleagues in the appropriate application of laboratory techniques to diagnosis and clinical management.

Many laboratory procedures that are commonplace today were not available (or even conceivable) 100 years ago. Uttam Garg, PhD, Clinical Chemistry Associate Editor for *LM*, cites newborn screening as an example. Although the term "inborn errors of metabolism" was coined in 1902 by British physician Sir Archibald Garrod, no tests were available for these genetic disorders until the ferric chloride test for phenylpyruvic acid in urine was introduced in 1934 to detect phenylketonuria, caused by a mutation in the *PAH* gene coding the enzyme phenylalanine hydroxylase. The ferric chloride test was somewhat crude and insensitive, though, and was replaced in the 1960s by the Guthrie test,

Downloaded from https://academic.oup.com/labmed/article/53/4/333/6627554 by guest on 28 February 2025

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

which could be performed on dried blood spots. Dr Garg points out that newborn screening is one of the most successful public health programs in medical history. Contemporary newborn screening programs typically utilize liquid chromatography and tandem mass spectrometry to screen for abnormal concentrations of metabolic products in dried blood spots, improving both sensitivity and throughput.

Commenting on advances in breast cancer diagnosis and treatment over the past 100 years is Marilin Rosa, MD, Breast Pathology Associate Editor for *AJCP*. Dr Rosa reminds us that, at the time the ASCP was founded in 1922, the standard treatment for breast cancer was radical mastectomy, a surgical procedure introduced by William Halsted in 1882 at Roosevelt Hospital in New York City. However, in the 1920s, the necessity of the profoundly disfiguring procedure was being questioned, as surgeons experimented with more conservative surgery combined with radiation to treat breast cancer. Radical mastectomies are rarely performed today, since less invasive mastectomies or "lumpectomies" combined with radiotherapy and/or chemotherapy are preferred approaches to treating breast cancer. Dr Rosa further makes the very interesting observation that oncologic pathology has evolved from a strictly diagnostic service to a sophisticated combination of staging, molecular characterization, and classification of tumors, all of which directly guide therapy. As such, pathologists have greatly expanded their role in medical care by not only providing a diagnosis but also in advising clinicians on the most appropriate and effective treatment on an individualized basis for each and every patient. The expanding role of pathologists has undoubtedly contributed to the improvement in breast cancer survival from 5% to over 90% in the past 100 years.

The development of modern blood banking and transfusion medicine practice certainly deserves a place among the most important advances in the history of medical practice. Although blood groups, compatibility testing, and storage with anticoagulants were all known prior to 1922, widespread blood transfusion remained a rare practice until 1937, when the first blood bank was established. Prior to blood banking, transfusions involved end-to-end vascular anastomosis, a procedure fraught with risks to both patient and donor. Modern transfusion medicine services include testing of blood products for various infectious agents (first done in the 1950s), isolation of specific components of the collected blood, such as platelets or coagulation factors and, most recently, transfusion of plasma collected from recovered COVID-19 patients for the therapeutic benefit derived from the presence of antibodies to SARS-CoV-2. But global disparities exist in the availability and use of blood for transfusion, and in the safety of the blood supply. Dr Richard Gammon, Transfusion Medicine Associate Editor for *LM*, predicts that stem cell technology, biotherapies, and cord blood banking will be major areas of future focus in transfusion medicine, along with the possibility that blood donation may be replaced by synthetically produced substitutes.

Prior to the eponymous cervicovaginal cytology test pioneered by Dr George Papanicolaou in the 1930s, cervical cancer was a very common cause of cancer-related death in women. Currently, it is not among the top 10 causes of cancer-related death in women. *AJCP* Cytopathology Associate Editor Charles D. Sturgis, MD, traces the history of the "Pap" test, noting that it "has saved more lives than any other clinically utilized cancer prevention test." Cytology laboratories, Dr Sturgis notes, are among the most highly regulated clinical laboratory services. High error rates in Pap smear interpretations observed during the 1960s led to passage of the Clinical Laboratory Improvement Act in 1967 (CLIA '67), subsequently revised through the Clinical Laboratory Improvement Amendments in 1988 (CLIA '88), familiar to all clinical laboratory professionals as the statutory framework for all clinical laboratory practice in the US. Modern strategies for detecting cervical cancer include liquid-based cytology, a variety of immunohistochemical studies, methods to detect human papillomavirus (HPV; a primary cause of cervical cancer), and molecular analyses.

LM Hematology Assistant Editor Alexandra E. Kovach, MD, tells the story of pathologist Sidney Farber's pioneering work in the treatment of childhood acute lymphoblastic leukemia (ALL) with the dihydrofolate reductase inhibitor aminopterin, a therapy she characterizes as "one of the greatest medical achievements during the last century." Dr Kovach describes the central role that laboratory professionals play in staging and monitoring the disease by analysis of cerebrospinal fluid (CSF) for nucleated cells and blasts. She offers a critique of alternative methods that exist for detecting and characterizing the abnormal cells found in the CSF—namely, flow cytometry and molecular analysis—concluding that technical obstacles preclude their mainstream use at this time. However, Dr Kovach speculates that the more sophisticated laboratory methods may become prominent in monitoring ALL in the future.

Sanjay Mukhopadhyay, MD, *AJCP* Associate Editor for Pulmonary/Thoracic Pathology, reviews the most important advancements in thoracic pathology over the past 100 years. Among the discoveries cited by Dr Mukhopadhyay as having significantly influenced the field of thoracic pathology are immunohistochemistry, computed tomography (CT), molecular testing, and advanced diagnostic techniques such as bronchoscopy and endobronchial ultrasound-guided transbronchial needle aspiration. He also mentions new treatments, such as lung transplantation, molecular targeted therapies, and immunotherapy, which have improved the prognosis of many lung diseases, including cancer. Finally, Dr Mukhopadhyay notes that, in 1922 when the ASCP was founded, the link between smoking and lung cancer had yet to be established, and over the past several decades the steady decline in tobacco use has had a significant effect on the prevalence of smoking-related disorders, including lung cancer.

Perhaps due to some combination of a politically polarized populace, the 24-hour news cycle, pervasive consumption of social media, a somewhat disorganized governmental response to the pandemic, or the terrifying prospect of a rapidly mutating, highly infectious respiratory virus, the COVID-19 pandemic caused many to distrust government officials and medical experts. Thus, when a vaccine was developed for the virus, many refused to take it, suspicious of ulterior motives or inadequate attention to safety. It is easy to attribute vaccine resistance to the current political climate, but *LM* Reviews Editor Deniz Peker, MD, reveals that resistance to vaccination has existed for as long as vaccines themselves. In 1922, Dr. Peker recalls, San Antonio, TX, required all school children to receive the smallpox vaccine, and the legality of the mandate was challenged all the way to the US Supreme Court, where it was upheld. Vaccine acceptance peaked in the 1950s, when Jonas Salk developed a vaccine for polio, a dreaded disease at the time. However, the anti-vaccination movement was partly revived by fraudulent research suggesting a link between vaccination and autism (with one

Downloaded from https://academic.oup.com/labmed/article/53/4/333/6627554 by guest on 28 February 2025

paper describing the fraudulent studies published in *LM,* since retracted by the Editor). Regarding the vaccines for COVID-19, Dr Peker warns that "skepticism and efforts to challenge vaccination mandates, including many lawsuits across the United States, remain an obstacle to efforts to curb the virus by vaccinations."

We are sincerely grateful to our editors for these excellent essays, all of which highlight how the medical landscape has changed since ASCP was founded in 1922. We heartily congratulate ASCP on this remarkable milestone, and join in the celebration marking 100 years of progress in pathology and laboratory medicine.

Downloaded from https://academic.oup.com/labmed/article/53/4/333/6627554 by guest on 28 February 2025

OVERVIEW

Methods to Correct Drug-Induced Coagulopathy in Bleeding Emergencies: A Comparative Review

*Shaughn Nalezinski, MS, MLS(ASCP)BB, MT(AMT)**

The George Washington University School of Medicine and Health Sciences, Washington, DC, USA; *To whom correspondence should be addressed. snalezinski@live.com

Keywords: viscoelastic, coagulopathy, coagulation, frozen plasma, warfarin, prothrombin time

Abbreviations: FDA, US Food and Drug Administration; EMA, European Medicines Agency; DTI, direct thrombin inhibitor; ADP, adenosine diphosphate; FII, factor II; FVII, factor VII; NSAID, nonsteroidal anti-inflammatory drug; ROTEM, rotational thromboelastometry; TEG, thromboelastography; FFP, fresh frozen plasma; PF24, plasma frozen after 24 hours; PF24RT24, plasma frozen within 24 hours after phlebotomy held at room temperature for up to 24 hours; INR, international normalized ratio; AABB, Association for the Advancement of Blood and Biotherapies; PCC, prothrombin complex concentrate; TACO, transfusion-associated circulatory overload; TRALI, transfusion-related acute lung injury; HLA, human leukocyte antigen; PEO-CO, polyoxyethylated castor oil.

Laboratory Medicine 2022;53:336–343; https://doi.org/10.1093/labmed/lmab115

ABSTRACT

Objective: Anticoagulant and antiplatelet therapy have become increasingly popular. The goal of therapy is to prevent venous thromboembolism and platelet aggregation, respectively. Traditional anticoagulant and antiplatelet drugs are quickly being replaced with novel medications with more predictable pharmacokinetics. Unfortunately, these drugs carry the risk of uncontrolled hemorrhage because of drug-induced coagulopathy. Uncontrolled hemorrhage continues to be a major cause of preventable death: hemorrhage accounts for approximately 30% of trauma-related deaths, second to brain injury. Controlling hemorrhage while dealing with comorbidities remains a challenge to clinicians. There are many gaps in care and knowledge that contribute to the struggle of treating this patient population.

Methods: This literature review is focused on the most effective ways to achieve hemostasis in a patient with drug-induced coagulopathy. The antiplatelet therapies aspirin, clopidogrel, ticlopidine, pasugrel, and ticagrelor are analyzed. Anticoagulant therapies are also reviewed, including warfarin, rivaroxaban, apixaban, edoxaban, and dabigatran. In addition, viscoelastic testing and platelet function assays are reviewed for their ability to monitor drug effectiveness and to accurately depict the patient's ability to clot. This review focuses on articles from the past 10 years. However, there are limitations to the 10-year restriction, including no new research posted within the 10-year timeline on particular subjects. The most recent article was then used where current literature did not exist (within 10 years).

Results: Traditional anticoagulants have unpredictable pharmacokinetics and can be difficult to correct in bleeding emergencies. Vitamin K has been proven to reliably and effectively reverse the effect of vitamin K antagonists (VKAs) while having a lower anaphylactoid risk than frozen plasma. Prothrombin complex concentrates should be used when there is risk of loss of life or limb. Frozen plasma is not recommended as a first-line treatment for the reversal of VKAs. Novel anticoagulants have specific reversal agents such as idarucizumab for dabigatran and andexxa alfa for factor Xa (FXa) inhibitors. Although reliable, these drugs carry a large price tag. As with traditional anticoagulants, cheaper alternative therapies are available such as prothrombin complex concentrates. Finally, static coagulation testing works well for routine therapeutic drug monitoring but may not be appropriate during bleeding emergencies. Viscoelastic testing such as thromboelastography and rotational thromboelastometry depict in vivo hemostatic properties more accurately than static coagulation assays. Adding viscoelastic testing into resuscitation protocols may guide blood product usage more efficiently.

Conclusion: This review is intended to be used as a guide. The topics covered in this review should be used as a reference for treating the conditions described. This review article also covers laboratory testing and is meant as a guide for physicians on best practices. These findings illustrate recommended testing and reversal techniques based off evidence-based medicine and literature.

The aging populations (>65 years) of the United States and Europe present many challenges compared to younger patients because they have more comorbidities, disabilities, and fragility. Among these challenges are anticoagulation and antithrombotic agents. Direct oral anticoagulants have increased in use in this demographic along with traditional vitamin K antagonist (VKA) therapies. In addition, 40% to 66% of the aging population are taking at least 1 antithrombotic agent.[1]

This trend is cause for concern because 7.2 out of 100 patients taking anticoagulants require management for drug-induced coagulopathy or complications associated with antithrombotic prophylaxis or treatment.[1] Many of these bleeds are gastrointestinal, although intracranial

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

hemorrhage is also of concern for those taking both VKAs and direct oral anticoagulants. The risk of fatal bleeding from anticoagulation is 1.31 people out of 100.[2,3]

Uncontrolled hemorrhage continues to be a major cause of preventable mortality in trauma patients, only second to brain injuries. In total, uncontrolled hemorrhage accounts for approximately 40% of deaths.[4] Managing hemorrhage during a bleeding emergency has difficulties of its own; adding anticoagulants and antiplatelet drugs further complicates treatment and resuscitation. In addition, novel direct oral anticoagulants are becoming increasingly popular and lack assays or assay availability to measure their degree of efficacy.

As a result, many providers have historically been uncomfortable with the thought of treating coagulopathy during a bleeding emergency, mainly because of the number of drugs and assays available and that there is no "one size fits all" treatment. Correcting coagulopathy and achieving hemostasis is vital in managing these patients. Improper treatment can lead to overtransfusion of blood products and, ultimately, death. Massive transfusion, although beneficial and lifesaving when indicated, still carries risks such as hypocalcemia, acidosis, shock, and multisystem failure.[5,6]

Traditional anticoagulants, direct oral anticoagulants, thrombin inhibitors, and antiplatelet drugs are explored in this article. Traditional anticoagulants include warfarin, which is a VKA that inhibits the activation of multiple coagulation factors such as II, VII, IX, and X along with proteins S, C, and Z.[7] In a cohort study by Yu and colleagues,[8] it was found that patients on warfarin therapy had a significant decrease in death resulting from stroke. However, there was a notable increased risk of hemorrhagic stroke (hazard ratio 1.01) and an association with subdural and gastrointestinal hemorrhage.[9]

Novel anticoagulants, such as rivaroxaban, apixaban, and edoxaban, are part of a drug group called direct oral anticoagulants. These drugs directly inhibit factor Xa (FXa). Direct oral anticoagulants act more reliably than traditional anticoagulants. Unfortunately, the U.S. Food & Drug Administration (FDA) and the European Medicines Agency (EMA) do not recommend them for patients with mechanical heart valves and severe mitral stenosis.[10]

Direct thrombin inhibitors (DTIs) inhibit free and clot-bound thrombin along with thrombin platelet aggregation. Dabigatran, lepirudin, desirudin, bivalirudin, and argatroban are also DTIs with similar mechanisms of action. Dabigatran is the only oral DTI in this group. Because it can be taken orally, more patients are on dabigatran than the other DTI drugs.

The antiplatelet agents aspirin and clopidogrel affect platelet aggregation and activation, respectively. Aspirin inhibits platelet aggregation via thromboxane-a2, and clopidogrel irreversibly blocks the P2Y12 receptor, inhibiting the adenosine diphosphate (ADP) pathway for platelet aggregation. Ticlopidine, ticagrelor, prasugrel, and cangrelor are antiplatelet drugs that belong to the same group as clopidogrel.

The purpose of this article is to highlight the challenges faced when thrombus formation is inhibited by drugs in the setting of coagulopathy associated with trauma (ie, dilutional and consumption coagulopathy). In addition, assays that are used to measure hemostasis/drug efficacy are explored. Finally, recommendations on how to treat these patients are identified using novel research. The drugs' mechanism of action is explored and considered with these recommendations.

Anticoagulants and Antiplatelet Drugs

Novel direct oral anticoagulants are becoming increasingly popular in the medical industry because of their predictable pharmacokinetics. Although these drugs are efficacious, their use presents unique challenges in situations requiring emergency drug reversal. In addition to novel anticoagulants, novel antiplatelet therapies are becoming increasingly popular. These also contribute to challenges in emergency medicine where ensuring hemostatic equilibrium is crucial.

Anticoagulants

Traditional anticoagulants refer to warfarin, other coumarins, and heparin. Because heparin is intravenous and not prescribed for routine prophylactic use, warfarin is the main focus in this group. Warfarin and coumarins act as a competitive antagonist to vitamin K–dependent coagulation factors and some coagulation inhibitors. These factors include II (FII; prothrombin), VII (FVII), IX (Christmas factor), and X (Stuart-Prower factor). Proteins S and C are also inhibited by this drug. Warfarin acts on both intrinsic and extrinsic pathways. The inhibition of FIX and FX leads to inhibiting the intrinsic pathway. FVII inhibition prevents activation of the extrinsic pathway. In addition, because warfarin acts competitively, factors can be added to overload the drug effect causing a reversal. This information is discussed in greater detail later in the article.

Research has shown that VKAs have a reputation for being unreliable because they act on numerous different and diverse factors, each with their own respective half-lives. The half-lives of these factors are as follows: FII, 60 hours; FVII, 4 to 6 hours; FIX, 24 hours; and FX, 48 to 72 hours.[11] Proteins C and S have half-lives of 8 and 30 hours, respectively. Therefore, routine drug monitoring is needed while a patient is on warfarin therapy. This monitoring includes the prothrombin time and the international normalized ratio, which the World Health Organization introduced to standardize results for anticoagulant therapy management. Therapeutic ranges differ for the disease states being treated. Patients must also modify their diets while on this therapy because vitamin K–rich foods can adversely affect their drug levels. Foods rich in vitamin K include dark leafy greens such as spinach, kale, collards, and romaine lettuce.

Studies have shown that VKAs pose a greater risk for intracranial hemorrhage when compared to novel anticoagulants.[12] Both drugs carry the risk for bleeding and hemorrhage but warfarin carries a higher risk for hemorrhage and intracranial hemorrhage, in part because of its unpredictable pharmacokinetics and its action on far more coagulation factors than direct oral anticoagulants.

Warfarin is primarily prescribed for patients who have undergone mechanical valve replacements and for those with severe mitral stenosis, primarily because the FDA and EMA do not recommend other anticoagulants. Physicians have been trending toward using more reliable anticoagulants when possible because of better patient management and outcomes.[13]

Direct oral anticoagulants refer to both FXa inhibitors and DTIs. These drug classes directly inhibit their target (FXa and thrombin) and do not interfere with other coagulation factors. Because these drugs more reliably act on 1 factor and have predictable pharmacokinetics and limited drug interactions, there is no need for drug monitoring once a patient starts a regimen. In addition, no dietary or lifestyle changes are needed when these drugs are started. Factor Xa inhibitors are commonly prescribed for acute coronary syndrome and nonvalvular atrial fibrillation.

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

Factor Xa inhibitors include rivaroxaban, apixaban, and edoxaban. These drugs act by selectively and competitively inhibiting free and bound FXa along with prothrombinase activity,[14] deterring thrombin-induced platelet aggregation and the common coagulation pathway. Edoxaban is the only exception because it selectively and reversibly blocks the active Xa site. All of these drugs can block FXa activity without requiring a cofactor.

The only oral DTI that is commonly used in the United States is dabigatran, which acts as a competitive thrombin inhibitor, inhibiting free and bound thrombin from acting on fibrinogen. As with the FXa inhibitors, dabigatran also inhibits thrombin-induced platelet aggregation. Dabigatran is reversible and has a half-life of approximately 12 hours.[15]

Dabigatran is typically prescribed to prevent stroke in patients with atrial fibrillation, deep venous thromboembolism, and pulmonary embolism. When compared to warfarin, dabigatran has been equally effective in preventing ischemic stroke and carries the same risk of hemorrhage and mortality.[16]

There is a shift to using novel anticoagulants in place of warfarin for the prevention of venous thromboembolism. They are more predictable pharmacokinetically, have fewer drug interactions, and the overall cost is less than that of warfarin.[17] Warfarin requires routine drug monitoring at anticoagulation clinicals, can cause serious bleeding events, and requires lifestyle changes that are required to begin a regimen. Unfortunately, direct oral anticoagulants are not approved for as many conditions as warfarin. Therefore, warfarin is still needed and is prescribed.

Antiplatelet Agents

One of the most commonly used antiplatelet drugs is acetylsalicylic acid, or aspirin. Aspirin is a nonsteroidal anti-inflammatory drug (NSAID) that is available over the counter. Aspirin was first discovered in 1904 and was mainly used for its analgesic and antipyretic properties.[18] Later studies showed that the drug has antiplatelet properties and that low doses can prevent myocardial infarction and ischemic stroke.

Aspirin works by acetylating platelet cyclooxygenase; acetylation then irreversibly inactivates cyclooxygenase. Cyclooxygenase is needed for prostaglandin H2 synthesis, which includes prostaglandins I2, E2, D2, F2a, and thromboxane A2. Thromboxane A2 is necessary for hemostasis; it acts by increasing platelet aggregation and vasoconstriction. By blocking prostaglandin formation, platelets cannot properly aggregate.[19,20] Other drugs fall under the class of cyclooxygenase inhibitors under the drug class of NSAIDs but do not effectively inactivate platelet aggregation as does aspirin and are thus not a focus in this review.

P2Y12 inhibitors are antiplatelet drugs that block the P2Y12 receptor on platelets. The P2Y12 receptor is an ADP-dependent receptor on the surface of the platelet. In the presence of ADP, the platelet will change its shape, aggregate, and generate thromboxane A2. There are 2 main groups of P2Y12 inhibitors: thienopyridines and the drug ticagrelor. Thienopyridines make up a group of prodrugs that selectively and irreversibly inactivate the P2Y12 receptor on the platelet. After the liver metabolizes the prodrug into its active metabolite, the ADP receptor is enzymatically changed so that it will no longer function in the presence of ADP.[21] Ticlopidine, clopidogrel, and prasugrel belong to this group of prodrugs. Ticagrelor is in the other group of antiplatelet drugs that make up P2Y12 inhibitors. This drug differs from the thienopyridines because it is reversible and does not require the liver to metabolize it into an active metabolite. Ticagrelor does not alter the P2Y12 receptor, making the block reversible. That being said, there is no antidote to immediately reverse the effects of ticagrelor.

Low-dose "baby" aspirin continues to be prescribed or recommended more than novel antiplatelet drugs. Aspirin is relatively inexpensive and is available over the counter. Many physicians will start patients on routine low-dose aspirin regimens for routine prevention of platelet aggregation. Patients who have a higher risk for platelet aggregation/activation may be started on a novel antiplatelet therapy rather than aspirin.

Viscoelastic Testing in Trauma

Anticoagulation and antiplatelet assays are a necessity to quantify the effects, impacts, and dynamics of coagulation for each drug. It is important that the assay accurately and representatively depicts the drug activity and its effect on the coagulation system. In addition, it is important to have assays that can depict the patient's hemostatic profile in vitro. In vivo and in vitro coagulation differ greatly in that in vivo coagulation has multiple factors that contribute to thrombi formation. Static in vitro coagulation assays typically measure 1 factor or 1 pathway. Assay results do not always accurately predict hemostasis. Different coagulation assays have different targets, and not all assays will detect drug effects.

Viscoelastic Testing

Rotational thromboelastometry (ROTEM) and thromboelastography (TEG) work in different ways but are functionally the same. The TEG assay has a stationary pin on a tension wire. The pin is placed in the middle of a cup with the patient specimen and reagent. The cup then moves slowly and the pin measures tension when the clot is forming. The ROTEM assay works in the opposite manner: the cup is stationary and the pin rotates. The principle of both tests is measuring the tension during clot formation to provide parameters such as clot time, maximum clot firmness, and clot lysis time. These values have been proven to accurately guide massive transfusion to selective therapy to cut down on blood product usage while achieving hemostasis more reliably than traditional transfusion algorithms.

Static coagulation assays such as the prothrombin time and activated partial thromboplastin time have significant limitations compared to viscoelastic testing. Unlike viscoelastic testing, the static coagulation tests measure the function of coagulation factors in plasma. They do not give a full picture of a patient's hemostatic capabilities. In viscoelastic testing, whole blood is used, which provides a better understanding of hemostasis by also measuring platelet clot contribution, endothelial tissue factor, blood flow, and other factors that contribute to clotting in vivo.[22,23] The common parameters measured with viscoelastic testing are clotting time, clot formation time, maximum clot formation, alpha angle, clot diameter after 10 minutes, and clot lysis.

Both viscoelastic testing assays described here are considered waived point-of-care tests. They can be done in the emergency department, the intensive care unit, and the operating room. This flexibility can be beneficial and can also cause issues with obtaining accurate and usable results. The TEG 5000 series and ROTEM platform both require pipetting reagent into the cups for the reaction to take place. Inadequate reagent preparation and addition can greatly affect the assays. Instrumentation Laboratories has added an automated pipette to mitigate this issue and provides animated, onscreen prompts for reagent preparation and addition.

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

Haemonetics Corporation has come out with a new platform, the TEG 6s. This device is designed to be used as a point-of-care assay. It is cartridge-based, so there is less variability from reagent preparation and addition, which allows the assay to be used more reliably at the bedside. The specimen is added into the cartridge wells and then resonance frequency analysis is used to detect viscoelastic properties and provide testing parameters. There is also a rapid TEG that can provide results in as little as 10 minutes. Every minute counts when managing a hemorrhaging patient.

Platelet Function Assays

Certain platelet assays are approved for measuring the effectiveness of antiplatelet therapy. Instrumentation Laboratories, Haemonetics, and Siemens all make commercially available platelet tests that require whole blood. They are not readily available and therefore are seldom used in bleeding emergencies.

Instrumentation Laboratories, a diagnostics company focusing on acute care and hemostasis, has a VerifyNow aspirin and P2Y12 assay that measures aspirin reaction units and P2Y12 reaction units, respectively. The test takes approximately 1 hour to run and measure platelet aggregation in the presence of activators that target thromboxane A2 and ADP receptors. This test can be used in conjunction with the ROTEM assay, which is also manufactured by Instrumentation Laboratories.

Haemonetics has a Platelet Mapping assay that runs on 4 channels on the TEG analyzer. The first test measures thrombin response to activate platelets and cleave all available fibrinogen, which ultimately shows the maximum clot strength. The second test blocks thrombin activity and uses a special activator to measure the clot strength from fibrin. The third and fourth assays use reagent specifically designed to activate platelets using their thromboxane A2 receptor (arachidonic acid), and the ADP receptor. These 4 parameters create a personalized platelet function profile that indicates the percentage of inhibition. Lam et al[24] concluded that the platelet mapping assay is not effective in measuring platelet inhibition in patients with spontaneous intracranial hemorrhage because of its lack of specificity.

Finally, Siemens, a multinational conglomerate, has a platelet function assay (PFA-100) that is approved for detecting drug-induced platelet dysfunction and acquired platelet disorders. The assay uses 2 cartridges that contain collagen and epinephrine or collagen and ADP. Citrated whole blood moves through the impregnated nitrocellulose membrane, activating platelets. The end point is measured in closure time, when the membrane is blocked, preventing blood from flowing through. Aspirin prolongs the closure time 94% of the time (collagen/epinephrine membrane). Because the collagen and nitrocellulose membrane alone can activate platelets, these tests are not considered reliable for the measurement of drug-induced platelet dysfunction.

Emergency Reversal Methods

Correcting drug-induced coagulopathy and managing a hemorrhaging patient has proven to be a difficult, multifaceted challenge. In addition to the coagulopathy caused by antiplatelet/anticoagulant drugs, there are often other coexisting coagulopathic events including disseminated intravascular coagulation, trauma-induced coagulopathy, secondary fibrinolysis, and dilutional coagulopathy. Maintaining hemostatic equilibrium needs to be done fast when a patient is losing blood. At the same time, there is a fine line between bleeding and thrombosis, both of which include their own challenges.

Traditional Anticoagulants

Frozen Plasma

Fresh frozen plasma (FFP), plasma frozen after 24 hours (PF24), and plasma frozen within 24 hours after phlebotomy held at room temperature for up to 24 hours (PF24RT24) are frequently used for the reversal of vitamin K agonists (warfarin and coumarins) and direct oral anticoagulants (apixaban). These products all contain plasma coagulation factors, the former product containing the highest concentration because of its preparation. Studies have shown that PF24 and PF24RT24 contain decreased levels of factors V and VIII.

The main benefit of plasma is that it contains all of the coagulation factors and half-lives. An average unit of plasma has an international normalized ratio (INR) of approximately 1.4, but this varies with each unit of plasma collected. It is recommended to administer 20 to 25 mL/kg plasma for urgent anticoagulation reversal.[25,26] The Association for the Advancement of Blood and Biotherapies (AABB) does not recommend plasma for this use and does not provide guidelines for transfusion.[27] American and British guidelines for anticoagulation reversal only recommend frozen plasma when prothrombin complex concentrate (PCC) is unavailable.[28]

One of the largest issues with administering plasma is that it is often underdosed. Plasma is typically ordered in quantities of 1 to 2 units for the correction of an elevated INR. This dosing is inadequate and does not provide benefit to the patient.[26] Current recommendations are to dose off of the patient's weight and provide rapidly, only when PCC is unavailable. An average unit of plasma has approximately 225 mL plasma; for a 115 kg adult, 1725 mL (1.7L) or 7 to 8 units of plasma would need to be infused, which does not guarantee anticoagulation reversal.

Another adverse effect of plasma administration is related to pulmonary complications. Marshall et al[25] found a correlation between doses of plasma at >3 units and transfusion-associated circulatory overload (TACO). Furthermore, plasma administration puts the recipient at risk for transfusion-related acute lung injury (TRALI), which is an immune response involving human leukocyte antigen (HLA) antibodies in the donor unit attacking the recipient cells. This process causes white blood cell infiltrates in the lungs and leads to mortality if not treated immediately. HLA antibodies can occur in both men and women without transfusion or transplantation. Women who have been pregnant had an increased number of HLA antibodies. HLA antibodies increased in women with multiple pregnancies as well. Due to the increased HLA antibody formation, female donors were deferred for plasma donations to mitigate the risk of TRALI. Even with the deferral of female donors and modern HLA antibody screening, TRALI continues to be one of the leading causes of transfusion-associated death in the United States.[29] From 2005 to 2016, the FDA compiled transfusion-related fatalities and found that TACO was the leading cause of death, followed by TRALI.[30] This finding is partially because TRALI is markedly underdiagnosed.

Vitamin K (Phytonadione)

Warfarin acts on vitamin K–dependent coagulation factors, blocking y-carboxylation by hepatocytes. Vitamin K1 administration returns the hepatic synthesis of K-dependent coagulation factors back to baseline, correcting coagulopathy.[31] Vitamin K1 provides a rapid and reliable way to reverse overcoagulation by VKAs.

Vitamin K administration is most effective when it is intravenous. This type of administration has the fastest onset, approximately 4 to

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

FIGURE 1. Joined dot-plot of the pre– and post–vitamin K administration international normalized ratios (INRs). Adapted from *Transfusion* 2017;57(8):1885–1890. doi: 10.1111/trf.14166.

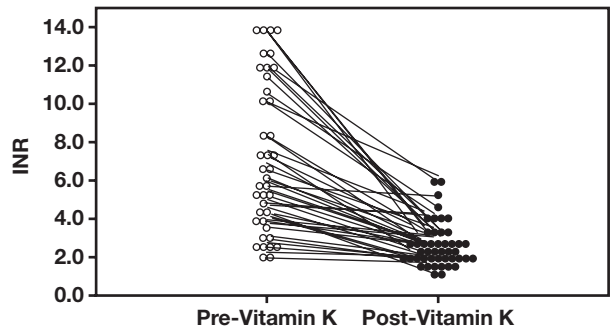
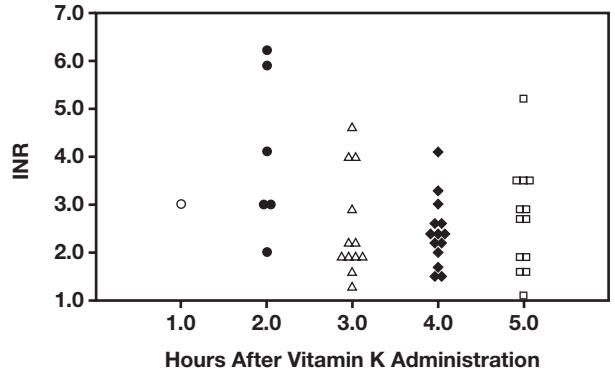


FIGURE 2. Dot-plot of the post–vitamin K administration international normalized ratios (INRs), stratified by hour after vitamin K infusion. Adapted from *Transfusion* 2017;57(8):1885–1890. doi: 10.1111/trf.14166.



6 hours (**FIGURES 1** and **2**). The next-effective route of administration is oral. These methods both provide reliable absorption of the drug. The subcutaneous and intramuscular administration routes should be avoided because of erratic absorption of the drug and unreliable reversal of supratherapeutic INR.

There has been concern regarding anaphylactic/anaphylactoid reactions to intravenous vitamin K1 administration, but rates of reaction have dropped.[32] Burbury et al[33] showed in their study that vitamin K1 can be administered safely at low doses for those awaiting surgery. New micelle preparations and slower infusion rates have dropped reactions from <0.1% to <0.03%.[34] Britt and Brown[35] showed that the rate of reactions correlated with the solubilizer in the medication. Vitamin K is fat-soluble and requires an emulsifier for intravenous, intramuscular, and subcutaneous administration. Polyoxyethylated castor oil (PEO-CO) has accounted for the majority of anaphylactoid reactions associated with intravenous vitamin K administration. Reactions were seen with this solubilizer at infusion rates as low at 0.5 mg over 1 hour and were seen with the micelle solubilizer, but not as much as with the PEO-CO.[35] Meehan et al[36] also reported a decrease in reactions with intravenous vitamin K solubilized in polyoxyethylated fatty acid derivatives compared to PEO-CO. They also highlighted the risk of anaphylactic/anaphylactoid reaction with plasma as being 1:500 to 1:100,000, considering the dose needed for the average patient (15–20 mg/kg or 4–6 units for the average adult); the risk with intravenous vitamin K is approximately 1:1000 to 1:250,000.[36]

3- and 4-Factor PCC

Studies have shown that PCC exists as both 3 factors (II, IX, and X) and 4 factors (FII, FVII, IX, and X). The 4-factor PCC is split into 2 different products: non-active (K-centra, octaplex), and active factor eight inhibitor bypassing activity (FEIBA). The difference between the 2 products is that FVII is active in FEIBA and not in K-centra or octaplex. Both the American and British guidelines consider PCC to be first-line therapy for anticoagulation reversal.[37-39]

When compared to frozen plasma, PCC more rapidly and reliably provides hemostasis. On average, it takes 15 to 20 minutes for the full effect of PCC, whereas plasma can take up to 24 hours for full effect after an adequate dose.[40] Compared to frozen plasma, PCC contain 25 times more factors.

Furthermore, PCC does not pose a risk for TACO or TRALI. The amount needed is markedly less than that for frozen plasma. The main disadvantage to using PCC is the prothrombotic risk from administration. Ideally, PCC should only be used when trying to save life or limb.

A major drawback for PCC is its price tag. On average, it costs approximately $6000 to $14,500 for anticoagulation reversal.[41] It is difficult for hospitals to be reimbursed for the product unless the patient is a private payor or has Medicare/Medicaid. Therefore, many hospitals will not allow PCCs to be given unless there is a life or limb threat to the patient. Many providers are forced to use frozen plasma, which costs approximately $40 a unit.

Direct Oral Anticoagulants

There are no recommended blood products for the reversal of direct oral anticoagulants. In healthy human volunteers, both 3- and 4-factor prothrombin complexes have been able to correct the drug effects and normalize coagulation assays.[40] In life or limb situations, PCCs should be trialed on patients taking direct factor IIa or Xa inhibitors.

Idarucizumab

Idarucizumab is a humanized monoclonal antibody fragment that binds to both dabigatran and its acylglucuronide metabolites. The antibody fragment works by binding with the drug and neutralizing its effect on thrombin. Idarucizumab has been shown to normalize coagulation assays within minutes of infusion and is the recommended reversal agent for dabigatran.

Andexxa Alfa

Andexxa alfa is a recombinant, inactivated FXa that is used for the emergent reversal of FXa inhibitors, including low-molecular-weight heparin. Andexxa alfa works by binding to the circulating FXa inhibitor and removing it. The drug can also inhibit the tissue factor pathway inhibitor, which increases the amount of tissue factor–activated thrombin. The main throwback with the drug is cost and availability, removing it from first-line therapy choices. An average dose of andexxa alfa costs between $29,700 and $59,400.[41]

Ciraparantag

Ciraparantag, also known as aripazine, is a small-molecule drug that is given by infusion. It binds directly to FXa and FIIa inhibitors, along with unfractionated and low-molecular-weight heparins. It rapidly

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

neutralizes the drug and restores hemostasis. Clinical studies have shown the effectiveness of this drug using enoxaparin. Healthy volunteers were given enoxaparin and placebo; their clotting times were measured. The volunteers then received ciraparantag. Clotting times were corrected by 20 minutes when using 100 mg. Using 200 mg corrected their clotting time in 5 minutes, a 75% reduction in time.[42]

Antiplatelet Reversal Agents

Unlike for anticoagulants, there are no specific reversal agents for antiplatelet drugs, partially because of the mechanism of action and the complexity of platelet development and action. Certain drugs can help patients who are on antiplatelet drugs so that they can clot. It has been recommended that 1 to 2 adult doses of donor platelets be given to reverse antiplatelet effects, despite the lack of evidence to support this recommendation. In fact, there is more evidence against this recommendation.

Viscoelastic testing should be used in these instances to see how much platelets are contributing to clot strength and whether the patient would benefit from medication or platelet transfusion. Patients can also receive cryoprecipitate to help boost fibrinogen and von Willebrand factor, both of which act on platelets in different ways to activate platelets. In addition, increasing a patient's hematocrit can help with platelet activation, possibly because of the blood pushing the existing platelets to the endothelial wall, where it can activate and participate in hemostasis.

Baharoglu et al[43] published the trial on platelet transfusion versus standard care after acute stroke because of spontaneous cerebral hemorrhage associated with antiplatelet therapy (the PATCH trial). This study split patients on antiplatelet drug therapy who developed acute intracerebral hemorrhage into a standard care group and a platelet transfusion group. This study showed that patients who received platelet transfusion had an increased risk of mortality and complication compared to those who received standard care (P = .0114).

Desmopressin

Desmopressin is a synthetic analogue of vasopressin. It has a more specific antidiuretic action on patients. It also increases von Willebrand factor, factor VIII, tissue plasminogen levels, intracellular platelet calcium/sodium ion concentrations, and the formation of procoagulant platelets in a dose-dependent manner.[44,45] It has been shown to help shorten activated thromboplastin time and bleeding time.

Although the benefit of desmopressin has not been fully evaluated, Desborough et al[45] showed that patients on antiplatelet therapy did have a change in hemostasis after desmopressin administration. They concluded that patients who received desmopressin needed fewer units of red cells transfused, had less blood loss, and had a lower risk of returning to the operating room because of surgical bleeding.

Conclusion

Managing bleeding/hemodynamically unstable patients is a challenge on its own. Adding anticoagulant and antiplatelet drugs presents an additional layer for the treating provider. There are no set guidelines for anticoagulation reversal because not every person bleeds in the same manner (eg, a gastrointestinal bleed vs a postpartum hemorrhage). It is important to consider the patient's condition when treating hemorrhage and to develop an individualized plan.

There is a lack of evidence suggesting that a mild to moderate supratherapeutic INR is indicative of bleeding risk, nor are there sufficient studies that outline when surgery becomes unsafe. Therefore, clinicians cannot rely solely on the prothrombin time and INR as a justification for anticoagulation reversal. Extreme supratherapeutic INR should be promptly reversed, especially in the presence of bleeding.

For VKAs such as warfarin, FFP is not recommended for anticoagulation reversal. Intravenous vitamin K should be used, time permitting. Intravenous vitamin K has the quickest absorption rate, followed by oral vitamin K. Intramuscular and subcutaneous administrations should be avoided because of erratic drug absorption. In a life or limb emergency, PCC should be utilized for rapid anticoagulation reversal. If PCC is not available, then frozen plasma should be used at a dosage of 20 to 25 mL/kg and should be rapidly infused.

When plasma is dosed correctly, it carries the same risk for hypersensitivity reactions as intravenous vitamin K. Vitamin K is now being emulsified with polyoxyethylated fatty acid derivatives, which have been shown to decrease the amount of anaphylactic/anaphylactoid reactions. The risk for intravenous vitamin K is approximately 1:1000 to 1:250,000, whereas plasma carries a 1:500 to 1:100,000 risk of a hypersensitivity reaction.

Factor Xa inhibitors do not currently have many FDA-approved reversal agents. Frozen plasma is not recommended for this drug class. The drug andexxa alfa, which is a recombinant FXa, has been shown to rapidly correctly coagulopathy in patients on FXa inhibitors. Unfortunately, the price of the medication is a limiting factor and most hospitals do not routinely stock it. Both 3- and 4-factor PCCs have been successfully used off-label to correct coagulopathy in these patients. There are more drugs currently under development for the reversal of FXa inhibitors. In addition, FXa assays calibrated for low-molecular-weight heparin can be used in an emergency situation to monitor other FXa inhibitors such as apixaban.

Idarucizumab is approved by the FDA for the reversal of dabigatran, a direct thrombin (FIIa) inhibitor. This monoclonal antibody has the ability to correct coagulation assays within minutes of infusion. The drug is not recommended for other anticoagulants, because it was designed specifically for dabrigatran.

For antiplatelet drugs, there are no good recommendations or guidelines for the correction of platelet dysfunction. Some studies have recommended 2 to 3 adult platelet apheresis doses for transfusion in emergency, despite the lack of evidence to support this dosage. The PATCH trial[43] showed that in the setting of acute intracerebral hemorrhage, platelet transfusion may contribute to morbidity and mortality. The AABB does not recommend for or against platelet transfusion in spontaneous and traumatic intracerebral hemorrhage in the presence of antiplatelet drug therapy because of the lack of supporting evidence.

During bleeding emergencies with platelet dysfunction, other factors can help supplement for dysfunctional platelets. Von Willebrand factor and fibrinogen both act directly on platelets. Transfusion of cryoprecipitate can help strengthen clotting and attempt to activate the inhibited platelets. Desmopressin causes an increase of von Willebrand factor and factor VIII, intracellular platelet

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

calcium/sodium ion concentrations, and the formation of procoagulant platelets. When clinically appropriate, this drug has the potential to correct platelet dysfunction.

Acknowledgments

The author confirms sole responsibility for the following: review conception and design, data collection, analysis and interpretation of results, and manuscript preparation. Figure rights were purchased from *Transfusion* magazine.

REFERENCES

1. Wong H, Lovett N, Curry N, Shah K, Stanworth SJ. Antithrombotics in trauma: management strategies in the older patients. *J Blood Med.* 2017;8:165–174.
2. Bradshaw PJ, Hung J, Knuiman M, et al. Warfarin use and mortality, stroke, and bleeding outcomes in a cohort of elderly patients with non-valvular atrial fibrillation. *J Atr Fibrillation.* 2019;12(1):2155.
3. Gage BF, Birman-Deych E, Kerzner R, Radford MJ, Nilasena DS, Rich MW. Incidence of intracranial hemorrhage in patients with atrial fibrillation who are prone to fall. *Am J Med.* 2005;118(6):612–617.
4. Curry N, Hopewell S, Dorée C, Hyde C, Brohi K, Stanworth S. The acute management of trauma hemorrhage: a systematic review of randomized controlled trials. *Crit Care.* 2011;15(2):R92.
5. Schöchl H, Grassetto A, Schlimp CJ. Management of hemorrhage in trauma. *J Cardiothorac Vasc Anesth.* 2013;27(4 suppl):S35–S43.
6. Wirtz MR, Jurgens J, Zuurbier CJ, et al. Washing or filtering of blood products does not improve outcome in a rat model of trauma and multiple transfusion. *Transfusion.* 2019;59(1):134–145.
7. Edardes JP. *Coumarin Anticoagulant Research Progress*. New York, NY: Nova Science Publishers; 2008.
8. Yu AY, Malo S, Wilton S, Parkash R, Svenson LW, Hill MD. Anticoagulation and population risk of stroke and death in incident atrial fibrillation: a population-based cohort study. *CMAJ Open.* 2016;4(1):E1–E6.
9. Witt DM, Delate T, Hylek EM, et al. Effect of warfarin on intracranial hemorrhage incidence and fatal outcomes. *Thromb Res.* 2013;132(6):770–775.
10. Anderson S, Marrs J. Direct oral anticoagulant use in valvular heart disease. *Clin Med Insights Ther.* 2018;10:1–6.
11. Kerins GM, MacNicoll AD. Comparison of the half-lives and regeneration rates of blood clotting factors II, VII, and X in anticoagulant-resistant and susceptible Norway rats (Rattus norvegicus Berk.). *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.* 1999;122(3):307–316.
12. Lopes RD, Guimarães PO, Kolls BJ, et al. Intracranial hemorrhage in patients with atrial fibrillation receiving anticoagulation therapy. *Blood.* 2017;129(22):2980–2987.
13. Fosbøl EL, Vinding NE, Lamberts M, et al. Shifting to a non-vitamin K antagonist oral anticoagulation agent from vitamin K antagonist in atrial fibrillation. *Europace.* 2018;20(6):e78–e86.
14. Wong PC, Pinto DJ, Zhang D. Preclinical discovery of apixaban, a direct and orally bioavailable factor Xa inhibitor. *J Thromb Thrombolysis.* 2011;31(4):478–492.
15. Lee CJ, Ansell JE. Direct thrombin inhibitors. *Br J Clin Pharmacol.* 2011;72(4):581–592.
16. Baetz BE, Spinler SA. Dabigatran etexilate: an oral direct thrombin inhibitor for prophylaxis and treatment of thromboembolic diseases. *Pharmacotherapy.* 2008;28(11):1354–1373.
17. Julia S, James U. Direct oral anticoagulants: a quick guide. *Eur Cardiol.* 2017;12(1):40–45.
18. Demir D. *Aspirin Therapeutic Uses, Adverse Effects and Pharmacokinetics.* New York, NY: Nova Science Publishers; 2011.
19. Patrono C, Ciabattoni G, Patrignani P, et al. Clinical pharmacology of platelet cyclooxygenase inhibition. *Circulation.* 1985;72(6):1177–1184.
20. Miner J, Hoffhines A. The discovery of aspirin's antithrombotic effects. *Tex Heart Inst J.* 2007;34(2):179–186.
21. Kam PC, Nethery CM. The thienopyridine derivatives (platelet adenosine diphosphate receptor antagonists), pharmacology and clinical developments. *Anaesthesia.* 2003;58(1):28–35.
22. Davis JPE, Northup PG, Caldwell SH, Intagliata NM. Viscoelastic testing in liver disease. *Ann Hepatol.* 2018;17(2):205–213.
23. McGlasson DL. Laboratory variables that may affect test results in prothrombin times (PT)/international normalized ratios (INR). *Lab Med.* 2003;34(2):124–129.
24. Lam H, Katyal N, Parker C, et al. Thromboelastography with platelet mapping is not an effective measure of platelet inhibition in patients with spontaneous intracerebral hemorrhage on antiplatelet therapy. *Cureus.* 2018;10(4):e2515.
25. Marshall AL, Levine M, Howell ML, et al. Dose-associated pulmonary complication rates after fresh frozen plasma administration for warfarin reversal. *J Thromb Haemost.* 2016;14(2):324–330.
26. Nixon CP, Tavares MF, Sweeney JD. How do we reduce plasma transfusion in Rhode Island? *Transfusion.* 2017;57:1863–1873.
27. Cohn CS, Delaney M, Johnson ST, Katz LM. *Technical Manual.* Bethesda, MD: AABB; 2020.
28. Zareh M, Davis A, Henderson S. Reversal of warfarin-induced hemorrhage in the emergency department. *West J Emerg Med.* 2011;12(4):386–392.
29. Weber LL, Roberts LD, Sweeney JD. Residual plasma in red blood cells and transfusion-related acute lung injury. *Transfusion.* 2014;54(10):2425–2430.
30. Triulzi DJ, Kleinman S, Kakaiya RM, et al. The effect of previous pregnancy and transfusion on HLA alloimmunization in blood donors: implications for a transfusion-related acute lung injury risk reduction strategy. *Transfusion*. 2009;49(9):1825–1835. doi:10.1111/j.1537-2995.2009.02206.x.
31. Hanley JP. Warfarin reversal. *J Clin Pathol.* 2004;57(11):1132–1139.
32. Riegert-Johnson DL, Volcheck GW. The incidence of anaphylaxis following intravenous phytonadione (vitamin K1): a 5-year retrospective review. *Ann Allergy Asthma Immunol.* 2002;89(4):400–406.
33. Burbury KL, Milner A, Snooks B, Jupe D, Westerman DA. Short-term warfarin reversal for elective surgery—using low-dose intravenous vitamin K: safe, reliable and convenient. *Br J Haematol.* 2011;154:626–634.
34. Navarro JL, Cesar JM, Fernández MA, Fontcuberta J, Reverter JC, Gol-Freixa J. Morbidity and mortality in patients treated with oral anticoagulants. Article in Spanish. *Rev Esp Cardiol.* 2007;60(12):1226–1232.
35. Britt RB, Brown JN. Characterizing the severe reactions of parenteral vitamin K1. *Clin Appl Thromb Hemost.* 2018;24(1):5–12.
36. Meehan R, Tavares M, Sweeney J. Clinical experience with oral versus intravenous vitamin K for warfarin reversal. *Transfusion.* 2013;53(3):491–498.
37. Keeling D, Baglin T, Tait C, et al. Guidelines on oral anticoagulation with warfarin--fourth edition. *Br J Haematol.* 2011;154(3):311–324. https://doi.org/10.1111/j.1365-2141.2011.08753.x.
38. Tomaselli GF, Mahaffey KW, Cuker A, et al. 2020 ACC expert consensus decision pathway on management of bleeding in patients on oral anticoagulants: a report of the American College of Cardiology Solution Set Oversight Committee. *J Am Coll Cardiol.* 2020;76(5):594–622. doi:10.1016/j.jacc.2020.04.053.
39. Cohn CS, Delaney M, Johnson ST, Katz LM. *Technical Manual.* Bethesda, MD: AABB; 2020.

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

40. Christos S, Naples R. Anticoagulation reversal and treatment strategies in major bleeding: update 2016. *West J Emerg Med.* 2016;17(3):264–270.

41. Momin JH, Candidate P, Hughes GJ. Andexanet Alfa (Andexxa) for the reversal of direct oral anticoagulants. *P T.* 2019;44(9):530–549.

42. Milling TJ Jr, Kaatz S. Preclinical and clinical data for factor Xa and "universal" reversal agents. *Am J Med.* 2016;129(11S):S80–S88.

43. Baharoglu MI, Cordonnier C, Al-Shahi Salman R, et al.; PATCH Investigators. Platelet transfusion versus standard care after acute stroke due to spontaneous cerebral haemorrhage associated with antiplatelet therapy (PATCH): a randomised, open-label, phase 3 trial. *Lancet.* 2016;387(10038):2605–2613.

44. Li C, Hirsh J, Xie C, Johnston MA, Eikelboom JW. Reversal of the anti-platelet effects of aspirin and clopidogrel. *J Thromb Haemost.* 2012;10(4):521–528.

45. Desborough MJ, Oakland KA, Landoni G, et al. Desmopressin for treatment of platelet dysfunction and reversal of antiplatelet agents: a systematic review and meta-analysis of randomized controlled trials. *J Thromb Haemost.* 2017;15(2):263–272.

Downloaded from https://academic.oup.com/labmed/article/53/4/336/6514454 by guest on 28 February 2025

REVIEW

Oncology Patients Who Develop Transfusion-Associated Circulatory Overload: An Observational Study

Marisol Maldonado, MSN, Colleen E. Villamin, MSN, Leah E. Murphy, MSN, Amitava Dasgupta, MD, Roland L. Bassett, MS, Mayrin Correa Medina, MD, Tonita S. Bates, DNP, Fernando Martinez, MD, Adriana M. Knopfelmacher Couchonal, MD, Kimberly Klein, MD, James M. Kelley, MD

Hemovigilance Unit, Department of Laboratory Medicine, Division of Pathology and Laboratory Medicine; The University of Texas MD Anderson Cancer Center, Houston, Texas; *To whom correspondence should be addressed. KKlein@mdanderson.org

Keywords: oncology patients, transfusion-associated circulatory overload, risk factors, cardio-toxic chemotherapy, outcomes, blood transfusion

Abbreviations: TACO, transfusion-associated circulatory overload; CDC, Centers for Disease Control and Prevention; PRBCs, packed red blood cells; EHRs, electronic health records; CHF, congestive heart failure; SDP, single-donor platelets; RPDs, random-donor platelets; LVEF, left ventricular ejection fraction; GFR, glomerular filtration rate; GVHD, graft-versus-host disease; ACE, angiotensin-converting enzyme; RR, respiratory rate; SBP, systolic blood pressure

Laboratory Medicine 2022;53:344–348; https://doi.org/10.1093/labmed/lmab119

ABSTRACT

Background: Transfusion-associated circulatory overload (TACO) is a largely preventable transfusion complication that results in significant morbidity and mortality. Cancers, related treatments, and comorbidities are among the factors that can predispose patients to TACO, but currently there are limited data on this topic in the literature.

Methods: We collected data retrospectively from the electronic health records of 93 adult patients with cancer who met Centers for Disease Control and Prevention (CDC) criteria for TACO from July 1, 2019, through October 31, 2020. The parameters we studied included demographics, comorbidities, treatment modalities, transfusion practices, and outcomes. We summarized data by means and ranges for continuous variables, and proportions for categorical variables.

Results: During the study period, the incidence of TACO among oncology patients was 0.84 per 1000 transfusions (95% CI, 0.68–1.02), representing 6.6% of all reactions. This percentage is high, compared with 1%–6% among other populations. Unique characteristics such as hematology malignancy (75.3%), receipt of cardiotoxic chemotherapy (87.1%), pneumonia (57.0%), preexisting oxygen use (59.1%), dyspnea (62.4%), hypertension (55.9%), renal insufficiency (46.2%), daily use of corticosteroids (43.0%), daily use of diuretics (40.9%), daily use of beta-blockers (36.6%), and elevated NT-proBNP (33.3%) were frequently observed in these group of oncology patients.

Conclusions: Our study indicates that oncology patients have unique factors that may lead to diagnosis of TACO. Developing appropriate guidelines that apply to oncology patients, in addition to those set forth by the CDC, should be considered. Implementation by ordering healthcare providers of a tools that can predict TACO can help in early recognition and mitigation of TACO.

Transfusion-associated circulatory overload (TACO) is the leading cause of transfusion-related death and transfusion-related sequelae, such as increased length of hospital stay[2] (including longer ICU stay) and incidence of mechanical ventilation.[1-2] The Centers for Disease Control and Prevention (CDC) defines TACO as any 3 of the following conditions occurring within 6 hours of transfusion: acute respiratory distress, elevated NT-proBNP, elevated central venous pressure, evidence of left-sided heart failure, evidence of positive fluid balance, and radiographic evidence of pulmonary edema.[3] According to the US Food and Drug Administration, between 2014 and 2018, TACO accounted for 32% of reported transfusion-related deaths.[2]

Despite its high mortality rate, TACO remains one of the most underreported and possibly preventable transfusion-related reactions.[4] Barriers in diagnosing TACO include delay in the appearance of specific symptoms, the multifactorial nature of circulatory overload, and the broadness of the guidelines set forth by the CDC.[1,4] Clinicians are more likely to diagnose TACO in patients with contributing comorbidities, such as renal and cardiac dysfunction, and transfusion of multiple units of blood products. In contrast, clinicians are less likely to diagnose TACO based on vital-sign changes such as hypertension and tachycardia, which may indicate early signs of fluid overload.

Oncology patients possess unique factors that may increase their risk for TACO. Many chemotherapies and immunotherapies are associated with cardiotoxicity and/or nephrotoxicity.[5] The multiple blood-component transfusions used to correct pancytopenia due to cytotoxic

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/344/6548260 by guest on 28 February 2025

chemotherapies, combined with the use of other intravenous therapies (eg, anti-infective agents), increase the risk of fluid overload. In this descriptive study, our aims are to characterize TACO occurrence among oncology patients, to identify variables unique to this patient population, and to reevaluate transfusion practices.

Materials and Methods

This descriptive study is based on retrospective auditing of all inpatient and ambulatory clinical encounters for patients aged 18 years and older who received a blood component transfusion (packed red blood cells [PRBCs], platelets, or thawed plasma) and developed TACO from July 1, 2019 to October 31, 2020 at the M.D. Anderson Cancer Center in Houston, TX. The institutional review board of that institution approved the study. Data were summarized by means and ranges for continuous variables and proportions for categorical variables. We used R software, version 3.6.1 (http://www.r-project.org) for data analysis. There was no control group for summary statistics. We did not compare TACO cases with non-TACO cases due to the large database of transfusions. Also, a noncancer, non-TACO control group would have been infeasible, given that we conducted this study in a cancer care institution.

We monitored each transfusion using a digital dashboard that alerts a hemovigilance nurse that the patient may be experiencing a transfusion reaction. When the hemovigilance nurse receives such alerts, that person deploys an advanced practice healthcare provider to the bedside for assessment and examination of the patient in real time. Supportive care is provided and laboratory tests are ordered if a diagnosis of TACO (and or other transfusion reaction) is suspected. The transfusion medicine physician then performs a medical records review and documents a diagnosis in those records.

A transfusion medicine board-certified physician, using the CDC guidelines, diagnosed TACO among our study population. The threshold for diagnosis was defined as new onset or exacerbation of 3 or more of the following within 6 hours of cessation of transfusion: acute respiratory distress (dyspnea, orthopnea, and/or cough), elevated NT-proBNP, elevated central venous pressure, evidence of left-sided heart failure, evidence of positive fluid balance, and radiographic evidence of pulmonary edema.[3]

Inclusion criteria included adult patients aged 18 years and older with a diagnosis of TACO that involved an imputability rating of definite, probable, or possible. Exclusion criteria included clinical encounters involving blood component transfusions without an ordered transfusion reaction panel and without a diagnosis of TACO. Encounters with an imputability classification of doubtful (n = 6) were excluded, given that cases were volume-overloaded, which led to adverse events (these events, however, were unlikely to be transfusion-related). Encounters involving massive bleeding requiring multiple transfusions simultaneously (n = 3) were excluded, to account for outliers who had been given resuscitation processes, several blood products, and other fluids. We also excluded encounters for patients younger than 18 years (n = 2; small group, age outlier) and multiple TACO events from a single patient (n = 6; to negate duplicate demographics).

Data on patients with TACO were manually collected by hemovigilance nurses (L.E.M.) from patient electronic health records (EHRs) using paper forms and were validated for completeness and accuracy by a transfusion specialist nurse practitioner (M.M.). Data collected included patient demographics of age, sex, race, ethnicity, cancer diagnosis, weight at hospital admission, weight at time of transfusion, and inpatient or outpatient. Additional variables abstracted included comorbidities and cancer treatment modalities, to help identify oncology-specific associations that contribute to the development of TACO. Comorbidities included history of congestive heart failure (CHF), diastolic/systolic dysfunction, coronary-artery disease, acute myocardial infarction, valvular heart disease, atrial fibrillation, type 2 diabetes, hypertension, and obesity (BMI ≥30). We also noted daily use of certain medications such as corticosteroids, diuretics, and antihypertensives.

We also collected data regarding history of treatment with cardiotoxic chemotherapy or radiation to the breast or chest. In addition, we collected the results of laboratory and diagnostic tests, including pre-/post-transfusion high-sensitivity troponin T higher than the normal range using the institutional laboratory range (≤18 ng/L) as the standard, along with pre-/post-transfusion echocardiogram with left ventricular ejection fraction ≤50%, and NT-proBNP higher than the normal range between 24 and 72 hours after transfusion using the institutional laboratory range (≤125 pg/mL) as the standard.

We reviewed clinical variables to determine whether they were risk factors for TACO, included renal insufficiency, hemodialysis, and preexisting positive fluid balance ≥1000 mL since admission and 24 hours before transfusion. We also reviewed pre-/post-transfusion creatinine level (0.67–1.17 mg/dL), glomerular filtration rate (≥60 mL/min/1.73 m^2), and blood urea nitrogen (6–23 mg/dL) levels out of normal range (using the institution laboratory range as the standard).

Additional clinical variables we reviewed included pneumonia, chronic obstructive pulmonary disease, graft-versus-host disease of the lung, diffused alveolar hemorrhage, preexisting supplemental oxygen use, preexisting dyspnea, and preexisting evidence of pulmonary edema with an indication on chest radiography of opacities attributed to pulmonary edema, enlarged cardiac silhouette, and/or pleural effusions related to oncotic pressure. We retrieved data on the transfusion practices used during each clinical encounter to help examine the impact on the occurrence of TACO, on clinical characteristics of the reaction, and on patient outcomes.

Results

During the study period, the incidence of TACO among oncology patients was 0.84 per 1000 transfusions (95% CI, 0.68–1.02), representing 6.6% of all reactions. There were 111,285 transfusions, defined as the total number of units issued. Blood products were issued from a single blood bank in our cancer hospital. There were 1401 transfusion reactions diagnosed, including 240 allergic (17.1%), 1032 febrile nonhemolytic (73.7%), 10 hypotensive (0.7%), 9 transfusion-associated dyspnea (0.6%), and 110 TACO (7.8%) cases. Of these, 17 were excluded (see the Materials and Methods section), and 93 TACO cases were used for this study. The transfusion medicine physician (M.C.M., F. M., A.M.K.C., K.K., and J.M.K.) determined the imputability category of TACO diagnoses to be "possible" in 48.4% of these clinical encounters, "probable" in 37.6%, and "definite" in the remaining 14.0%.

The demographics of the study patients who developed TACO are listed in **TABLE 1**. Although we did not compare TACO cases with non-TACO cases, **TABLES 1–5** allow for observation of variables that are typical in TACO cases. Comorbidities present in oncology patients before diagnosis with TACO are listed in **TABLE 2**. The treatment modalities used for oncologic patients before they developed TACO are listed in **TABLE 3**.

Downloaded from https://academic.oup.com/labmed/article/53/4/344/6548260 by guest on 28 February 2025

TABLE 1. Demographics of the 93 Study Patients Who Developed TACO[a]

Variable	Category	
Age, y, mean (range)	62.8 (18–86)	
Sex, No. (%)	Female	46 (49.5%)
	Male	47 (50.5%)
Race, No. (%)	White	64 (68.8%)
	Black	11 (11.8%)
	Asian	6 (6.4%)
	Other	9 (9.7%)
	Not reported	3 (3.2%)
Ethnic group, No. (%)	Hispanic/Latino	16 (17.2%)
	Not Hispanic/Latino	70 (75.3%)
	Declined to answer	7 (7.5%)
Cancer diagnosis, No. (%)	Hematologic malignancy	70 (75.3%)
	Solid tumor	23 (24.7%)
Location, No. (%)	Inpatient	85 (91.4%)
	Outpatient	8 (8.6%)

Abbreviation: TACO, transfusion-associated circulatory overload.
[a]Percentages may not total 100% because of rounding.

TABLE 2. Comorbidities Present in the 93 Studied Patients Before They Developed TACO

Category	Variable	No. (%)
	Pretransfusion NT-proBNP	31 (33.3%)
	Hypertension	52 (55.9%)
	BMI ≥30	30 (32.3%)
	Congestive heart failure	26 (28.0%)
	Type 2 diabetes mellitus	23 (24.7%)
	Coronary artery disease	22 (23.7%)
	Atrial fibrillation	14 (15.0%)
	Acute myocardial infarction	4 (4.3%)
	Valvular heart disease	3 (3.2%)
	Pretransfusion LVEF ≤50	5 (5.4%)
Renal[a]	Renal insufficiency	43 (46.2%)
	Pretransfusion creatinine higher than normal range	40 (43.0%)
	Pretransfusion GFR lower than normal range	37 (39.8%)
	Hemodialysis	8 (8.6%)
	Positive fluid balance ≥1000 mL (since hospital admission)	70 (75.2%)
	Positive fluid balance ≥1000 mL (24 h pre-TACO)	43 (46.2%)
Pulmonary	Pneumonia	53 (57.0%)
	Chronic obstructive pulmonary disease	7 (7.5%)
	GVHD of the lungs	0
	Diffused alveolar hemorrhage	4 (4.3%)
	Preexisting supplemental oxygen	55 (59.1%)
	Preexisting dyspnea	58 (62.4%)
	Preexisting pulmonary edema via chest x-ray	11 (11.8%)
	History of radiation of chest/breast	10 (10.8%)

Abbreviations: TACO, transfusion-associated circulatory overload; LVEF, left ventricular ejection fraction; GFR, glomerular filtration rate; GVHD, graft-versus-host disease.
[a]Admission weight (mean average), 76.9 kg; weight at time of transfusion, kg (mean average), 79.3 kg.

TABLE 3. Treatment Modalities Used Before Development of TACO in the 93 Study Patients

Drug Class	No. (%)
Cardiotoxic chemotherapy	81 (87.1%)
Antimetabolites	57 (61.3%)
Alkylating agents	47 (50.5%)
Anthracyclines	37 (39.8%)
Kinase inhibitors	13 (14.0%)
Antimicrotubule agents	12 (13.0%)
Proteasome inhibitors	7 (7.5%)
Monoclonal antibodies	6 (6.4%)
Corticosteroids	40 (43.0%)
Diuretics	38 (40.9%)
Beta-blockers	34 (36.6%)
Calcium-channel blockers	25 (26.9%)
ACE inhibitors	13 (14.0%)
Antiarrhythmic	9 (9.7%)

Abbreviations: TACO, transfusion-associated circulatory overload; ACE, angiotensin-converting enzyme.

Clinical Characteristics

TACO was more commonly observed in inpatients (n = 85; representing 91.4% of all TACO cases), compared with ambulatory patients (n = 8; representing 8.6% of all TACO cases). The primary cancer diagnoses of patients with TACO were hematologic malignancy (70 [75.3%]) and solid tumor (23 [24.7%]). The average length of stay for patients who developed TACO was 28.8 days (total hospital stay) and 11.7 days before TACO.

The mean hemoglobin before PRBC transfusion was 7.7 g/L, compared with the mean hemoglobin count of 8.7 g/L after PRBC transfusion. The median platelet count before apheresis platelet transfusion (single-donor platelets [SDP]) or pooled platelet (random-donor platelets [RDPs]) transfusion was 20,000 × 10^3/μL, compared with the median platelet count of 26,000 × 10^3/μL after platelet transfusion.

The mean laboratory values before the administration of thawed plasma were prothrombin time of 27.3 seconds, partial thromboplastin time of 48.7 seconds, and international normalized ratio of 2.6, compared with 19.9 seconds, 43.3 seconds, and 1.74 seconds, respectively, after thawed plasma administration. The mean admission weight was 76.9 kg, and the mean weight at the time of transfusion was 79.3 kg.

Transfusion Practices

The clinical service assigned to patients at the time of TACO diagnosis included leukemia (48 [51.6%]), stem cell transplant (14 [15.0%]), general internal medicine (13 [14.0%]), lymphoma (7 [7.5%]), emergency medicine (2 [2.2%]), gynecologic oncology (2 [2.2%]), sarcoma (2 [2.2%]), urology (1 [1.1%]), and colorectal surgery (1 [1.1%]). The type of blood component ordered and administered that resulted in the highest incidence of TACO was PRBCs, leading to 60 TACO cases (64.5%). The most common indications for ordering PRBCs, platelets, and plasma, respectively, were low hemoglobin levels, low platelet counts, and high international normalized ratio.

Blood administration orders with the indication "Other" require the ordering provider to discuss the clinical indication with the transfusion

Downloaded from https://academic.oup.com/labmed/article/53/4/344/6548260 by guest on 28 February 2025

TABLE 4. Characteristic Symptoms of TACO in the 93 Study Patients

Variable	No. (%)
Shortness of breath	82 (88.2%)
Hypoxemia (increase of 5% SpO_2 from baseline and/or increase in oxygen requirements)	66 (71.0%)
Tachypnea (RR change >25%)	66 (71.0%)
Edema	63 (67.7%)
Tachycardia (pulse change >25%)	52 (55.9%)
Crackles/rales	50 (53.8%)
Hypertension (SBP >30 from baseline)	44 (47.3%)
Cough	37 (39.8%)
Wheezing	22 (23.7%)
Orthopnea	13 (14.0%)
Chest tightness	12 (12.9%)
Jugular venous distention	4 (4.3%)
S3 Gallop	3 (3.2%)
TACO occurring during transfusion	36 (38.7%)
TACO occurring after transfusion (as long as 6 h)	56 (60.2%)
TACO severity	
Nonsevere	53 (57.0%)
Severe	39 (41.9%)
Not determined	1 (1.1%)
Confidence level of diagnosis	
Possible	45 (48.4%)
Probable	35 (37.6%)
Definitive	13 (14.0%)

Abbreviations: TACO, transfusion-associated circulatory overload; RR, respiratory rate; SBP, systolic blood pressure.

medicine physician before approval and blood product release. Premedications had been ordered in 40 of clinical encounters (43.0%) with patients in whom transfusions resulted in a TACO diagnosis.

The mean volume of blood components transfused in the 24 hours before the TACO event was 629.6 mL. Those who developed TACO had a mean of 2.5 units of blood products transfused in the 24 hours before the event. Repeat laboratory values after transfusion were ordered for 45 patients (48.4%). The types of repeat laboratory tests ordered were hemoglobin and hematocrit (n = 4), platelet count (n = 6), complete blood count (n = 34), and coagulation factors (n = 4). See **TABLE 4** for the characteristic symptoms of TACO in our study population.

Outcomes

Patient outcomes after development of TACO are described in **TABLE 5**. Of the 93 patients diagnosed with TACO, 14 patients (15.0%) were admitted to the ICU because of TACO, and 34 patients (36.6%) died within 30 days of TACO diagnosis due to other causes, none of which were deemed to be a result of TACO.

Discussion

A descriptive study of oncology patients who developed TACO revealed characteristics unique to this patient population. Developing appropriate

TABLE 5. Outcomes After Development of TACO in the 93 Studied Patients

Variable	No. (%)
Length of hospital stay (total admission), d	28.8 (7.5%)
Length of hospital stay before TACO, d	11.7 (15.1%)
Ambulatory TACO needing higher level of care	7 (7.5%)
Admission to ICU related to TACO	14 (15.0%)
Death 30 d after TACO (other causes not related to TACO)	34 (36.6%)

Abbreviation: TACO, transfusion-associated circulatory overload.

guidelines that apply to oncology patients, in addition to those set forth by the CDC, should be considered. Also, implementation by ordering providers of a predictive TACO tool can help in early recognition and mitigation of TACO.

The incidence of TACO among oncology patients is high, compared with 1%–6% among other populations.[6-9] The higher proportion of transfusion reactions diagnosed as TACO is likely due to real-time hemovigilance monitoring in combination with a higher-acuity population. We observed that 91.4% of cases of TACO occurred in the inpatient setting; a hematology malignancy diagnosis was made in 75.3% of all patients with TACO and a solid tumor diagnosis in the remaining 24.7%. This finding may suggest that inpatients are more susceptible to TACO events, possibly related to baseline clinical status. Also, identifying medical history, comorbidities, and unique factors common among oncology patients who develop TACO is critical information that healthcare providers can use to help prevent reactions and mitigate the severity of those reactions.

The oncology patients in our study who were diagnosed with TACO had similar incidences of CHF, hypertension, diabetes mellitus, coronary artery disease, and kidney dysfunction as cohort patients in previous works of research.[10-13] However, unique characteristics such as preexisting hematologic malignancy, receipt of cardiotoxic chemotherapy, pneumonia, preexisting oxygen use, preexisting dyspnea, daily use of corticosteroids, daily use of beta-blockers, daily use of diuretics, and elevated NT-proBNP levels were observed in our study, which may lead to a diagnosis of TACO.

Most patients who developed TACO in our study had received a PRBC transfusion. Most of the blood products were ordered 1 unit at a time (53.8%), with indication of hemoglobin ≤8 g/L (91.4%). A study report by Daurat et al[13] also stated that 53% of patients diagnosed with TACO developed symptoms after receiving 1 unit of RBC, indicating that fluid status can change quickly.

In our study, intravenous furosemide was the most common diuretic administered on the day of the adverse event; it was administered after the completion of transfusion to help manage TACO reactions. This observation indicates a need for further research on preemptive use of diuretics. Implementation of more-thorough examination of susceptible patients and review of their EHRs by the ordering clinicians would be beneficial.

In a case-control study, Li et al[6] observed that patients diagnosed with TACO were more likely to have a larger volume transfused at a faster rate than patients with similar characteristics receiving their transfusions in the ICU. Standardizing orders to transfuse 1 unit at a time, along with required repeat laboratory tests before ordering more products for susceptible patients, would allow for the reassessment of volume status and the need for additional blood products in such patients. Addressing gaps in transfusion practices is critical in reducing and mitigating a preventable reaction in an already-vulnerable population.

Downloaded from https://academic.oup.com/labmed/article/53/4/344/6548260 by guest on 28 February 2025

Our study was limited by its retrospective nature and the lack of a control group. However, assembling a noncancer, non-TACO control group was infeasible, given that we conducted our study in a cancer institution. Nevertheless, we believe that our results provide useful preliminary data. We hope that future researchers can conduct further studies of this topic, involving larger sample sizes, to validate our findings.

Acknowledgments

This work was supported in part by the NIH/NCI Cancer Center support grant (award No. P30 CA016672) and involved the work of the Biostatistics Resource Group. J.M.K. received funding from the Amon G. Carter Foundation. We acknowledge the contribution of Anecita P. Fadol, PhD, APRN, FNP, FAANP, FAAN, for her cardiology expertise and input with cardiology risk factors in development of our data-abstraction tool. Also, we thank Cory Wongsa, BSN, and Nancy Tomczak, BSN, for their assistance with data collection and data entry. We thank Yimin Geng, MS, in the Research Medical Library at MD Anderson Cancer Center, for her assistance with literature review. We thank Bryan Tutt, MA, in the Research Medical Library at MD Anderson Cancer Center for his editorial support. Lastly, we would like to thank Luisa Gallardo, MSN.

REFERENCES

1. Bosboom JJ, Klanderman RB, Migdady Y, et al. Transfusion-associated circulatory overload: a clinical perspective. *Transfus Med Rev.* 2019;33(2):69–77.
2. United States Food and Drug Administration. Fatalities reported to FDA following blood collection and transfusion: annual summary for fiscal year 2017. https://www.fda.gov/media/124796/download. Accessed January 6, 2022.
3. The National Healthcare Safety Network Biovigilance Component Hemovigilance Module Surveillance Protocol (version 2.5). Division of Healthcare Quality Promoting, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention; 2021. https://www.cdc.gov/nhsn/pdfs/biovigilance/bv-hv-protocol-current.pdf.
4. Raval JS, Mazepa MA, Russell SL, Immel CC, Whinna HC, Park YA. Passive reporting greatly underestimates the rate of transfusion-associated circulatory overload after platelet transfusion. *Vox Sang.* 2015;108(4):387–392.
5. Gutierrez C, McEvoy C, Munshi L, et al. Critical care management of toxicities associated with targeted agents and immunotherapies for cancer. *Crit Care Med.* 2020;48(1):10–21.
6. Li G, Rachmale S, Kojicic M, et al. Incidence and transfusion risk factors for transfusion-associated circulatory overload among medical intensive care unit patients. *Transfusion.* 2011;51(2):338–343.
7. Menis M, Anderson SA, Forshee RA, et al. Transfusion-associated circulatory overload (TACO) and potential risk factors among the inpatient US elderly as recorded in Medicare administrative databases during 2011. *Vox Sang.* 2014;106(2):144–152.
8. Popovsky MA, Audet AM, Andrzejewski C Jr. Transfusion-associated circulatory overload in orthopedic surgery patients: a multi-institutional study. *Immunohematology.* 1996;12(2):87–89.
9. Bierbaum BE, Callaghan JJ, Galante JO, Rubash HE, Tooms RE, Welch RB. An analysis of blood management in patients having a total hip or knee arthroplasty. *J Bone Joint Surg Am.* 1999;81(1):2–10.
10. Lieberman L, Maskens C, Cserti-Gazdewich C, et al. A retrospective review of patient factors, transfusion practices, and outcomes in patients with transfusion-associated circulatory overload. *Transfus Med Rev.* 2013;27(4):206–212.
11. Roubinian NH, Hendrickson JE, Triulzi DJ, et al. Contemporary risk factors and outcomes of transfusion-associated circulatory overload. *Crit Care Med*. 2018;46(4):577–585.
12. Bosboom JJ, Klanderman RB, Zijp M, et al. Incidence, risk factors, and outcome of transfusion-associated circulatory overload in a mixed intensive care unit population: a nested case-control study. *Transfusion.* 2018;58(2):498–506.
13. Daurat A, Grenie J, Roger C, et al. Outcomes and risk factors of transfusion-associated circulatory overload: a case control study. *Transfusion.* 2019;59(1):191–195.

Downloaded from https://academic.oup.com/labmed/article/53/4/344/6548260 by guest on 28 February 2025

SCIENCE

A Rational Approach to Coagulation Testing

Maximo James Marin,[1,] Neil Harris,[1] William Winter,[1,*] Marc Stuart Zumberg[1]*

[1]Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL, USA; *To whom correspondence should be addressed. winter@pathology.ufl.edu

Keywords: coagulation, prothrombin time, activated partial thomboplastin time, fibrinogen, platelet count, viscoelastic testing, test selection

Abbreviations: FDA, U.S. Food & Drug Administration; EUA, emergency use authorization; LMWH, low-molecular-weight heparin; LIS, Laboratory Information System; PT, prothrombin time; aPTT, activated partial thromboplastin time; INR, international normalized ratio; BAT, Bleeding Assessment Tool; ISTH, International Society on Thrombosis and Haemostasis; VWF, von Willebrand factor; VWF:AG, von Willebrand factor evaluation: antigen; TT, thrombin time; VWF:RCo, von Willebrand factor evaluation: ristocetin cofactor assay; ACT, activated clotting time; UFH, unfractionated heparin; CAC, COVID-19-associated coagulopathy; DIC, disseminated intravascular coagulation; DOAC, direct-acting oral anticoagulant; ADP, adenosine diphosphate; VWD, von Willebrand disease; PED, pipeline embolization device.

Laboratory Medicine 2022;53:349–359; https://doi.org/10.1093/labmed/lmac005

ABSTRACT

Quality patient care requires the appropriate selection of laboratory tests. Irrelevant testing must be avoided, whereas pertinent testing is indispensable. The goals of this review are 3-fold: (1) to describe appropriate coagulation test selection for medical and surgical patients, (2) to describe appropriate coagulation testing specifically in individuals infected with SARS-CoV-2 causing COVID-19, and (3) to define the rational use of anticoagulant monitoring.

Quality patient care requires the appropriate selection of laboratory tests. Irrelevant testing must be avoided, whereas pertinent testing is indispensable. The goals of this review are 3-fold[1]: to describe appropriate coagulation test selection for medical and surgical patients,[2] to describe appropriate coagulation testing specifically in individuals infected with SARS-CoV-2 causing COVID-19 disease, and[3] to define the rational use of anticoagulant monitoring. The urgency of coherent and judicious test selection has been triggered by the nationwide shortage of light blue-top sodium citrate tubes that are used for essentially all functional coagulation studies. To this end, by reducing unnecessary coagulation testing, sodium citrate tubes will be available for those patients who truly require hemostatic assessment. Therefore, an important goal of this discussion is the proper stewardship of sodium citrate tubes because of their limited availability.[4]

Overview

In spring 2021, US laboratory professionals received the surprising news that light blue tubes (3.2% sodium citrate) were in short supply.[1] The shortage of sodium citrate tubes reportedly resulted from increased demand and reduced production of sodium citrate tubes because of "recent vendor supply challenges." Among other factors, the worldwide pandemic of SARS-CoV-2 infections had markedly increased the demand for coagulation testing.[2]

The US Food & Drug Administration (FDA) subsequently warned laboratories that sodium citrate tubes from different manufacturers may not be interchangeable. The agency recommended that when using tubes from a different manufacturer, laboratory staff should perform validation studies. Furthermore, the FDA wrote that "Sodium citrate tubes that are not FDA-cleared or authorized under an emergency use authorization [EUA]…are not legally marketed devices and should not be used."[1] In addition, according to the FDA, outdated tubes should not be used. How then could laboratories respond to this looming crisis?

In July 2021, the FDA did approve an EUA to Becton Dickinson for the use of BD Vacutainer Plus Citrate Plasma Tubes (UK manufacturing site).[3] The FDA and many health care organizations advised "conservation strategies" to reduce the utilization of sodium citrate tubes.[4,5] Such conservation strategies can be grouped into 3 approaches: (1) ordering practices, (2) phlebotomy practices, and (3) laboratory practices (including laboratory information system [LIS] modifications).

Ordering Practices

Regarding ordering practices, providers should carefully consider the necessity for coagulation testing in each patient. Only medically necessary coagulation testing should be ordered.[6] When medically necessary coagulation testing is performed, physicians should consider monitoring coagulation less frequently if such practices do not jeopardize patient care. Decreased coagulation testing can also be achieved by eliminating routine preoperative coagulation testing in patients at low risk for bleeding who lack a previous history of hemorrhage and who lack a family history of excessive bleeding. Patients treated with certain anticoagulants (eg, low-molecular-weight heparins [LMWHs], direct thrombin inhibitors, and direct factor X inhibitors) do not require coagulation monitoring in the absence of hemorrhage or thrombosis. Physicians should be

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

encouraged to discontinue the practice of placing standing orders for coagulation testing when not clinically indicated.[7] Likewise, there are clinical circumstances when measuring certain coagulation factors or functions is contraindicated (eg, protein S, protein C, and antithrombin should not be measured in search of hypercoagulability during an acute thrombotic episode). These points are summarized in **TABLE 1**.

Phlebotomy Practices

Concerning phlebotomy practices, sodium citrate tubes for coagulation studies should not be used unless a specific order is placed for coagulation testing (eg, do not include sodium citrate tubes in "rainbow draws"). The use of such practices, in which a predefined set of tubes is drawn in the emergency department, even before a patient has been clinically evaluated, has been shown to be wasteful.[8-10] Sodium citrate tubes should not be used as discard (ie, blank) tubes. When coagulation testing is appropriate, the proper volume of tube should be used (eg, 1.8 mL tubes may be necessary for smaller patients). Consolidation of testing is important, such as drawing the minimum number of tubes (and minimum volumes) necessary to complete the ordered tests. For example, prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen can be drawn in 1 tube vs 3 tubes. These points are summarized in **TABLE 2**.

Laboratory Practices

With respect to laboratory practices, if alternate sources of sodium citrate tubes are used, then such tubes must be validated. The issue of tube validation by reference laboratories is intriguing because reference laboratories recommend specific sources of tubes only for a limited number of specialty tests. Upon examining the tube requirements for PT testing for the 4 major reference laboratories, none provide specifications other than "3.2% sodium citrate." Recommendations for collection tube validation and verification were provided more than 10 years ago by the Clinical and Laboratory Standards Institute.[11,12]

TABLE 1. A Rational Approach to Coagulation Testing: Ordering Practices

Perform coagulation tests only when medically necessary.
Monitor coagulation only when clinically indicated.
Eliminate routine preoperative coagulation testing.
Limit anticoagulant monitoring to medications whose dosages are adjusted via such monitoring (eg, PT/INR testing for persons treated with vitamin K antagonists and anti-Xa testing in persons treated with high-dose IV UFH).
Discontinue standing orders for coagulation testing unless clinically indicated.
Assess the timing of coagulation testing: acute hemorrhage or thrombosis can temporally affect coagulation testing results.

INR, international normalized ratio; IV, intravenous; PT, prothrombin time; UFH, unfractionated heparin.

TABLE 2. A Rational Approach to Coagulation Testing: Phlebotomy Practices

Sodium citrate tubes should not be used unless a specific order is placed for coagulation testing.
Sodium citrate tubes should not be used as discard ("blank") tubes.
Tubes with the proper volumes should be used.
Combining orders when possible can reduce tube utilization (and possibly limit the development of phlebotomy-induced anemia).

If they are available and clinically appropriate, then point-of-care testing devices can be used that do not require a sodium citrate tube (eg, PT/international normalized ratio [INR] testing of patients treated with vitamin K antagonists such as warfarin). Although the FDA has warned against the use of outdated tubes, the College of American Pathologists advised, "Only during the public health emergency declaration, CMS [the Centers for Medicare & Medicaid Services] will allow laboratories to use expired test kits and reagents if they pass quality control tests with each assay run."[13] These points are summarized in **TABLE 3**. Smart LIS ordering systems can be enabled to assist in ordering the proper coagulation testing and in reducing duplicate orders.[14,15]

Laboratory Response to Sodium Citrate Tube Shortage

Immediately after the FDA issued its warning, laboratories began to contact their suppliers and to review the indications for coagulation testing requiring sodium citrate tubes.[16,17] Sodium citrate tubes once thought abandoned or hidden in closets and cupboards were collected and cataloged. Of the 3 components of conservation strategies, we next sought to address the sentinel question: "What coagulation testing is 'really' necessary for the medical care of patients?"

When Is Coagulation Testing Clinically Indicated?

Excessive coagulation testing adds to wasted medical testing that costs the United States more than $200 billion per year.[18,19] As part of the routine history and physical examination, patients should be questioned about any tendency to excess bleeding or thrombosis.[20] A history of blood in saliva, vomitus, urine, or stool or a history of excess bleeding with menses should be sought. Especially important is the patient's bleeding tendency (if any) and timing of bleeding after the extraction of teeth, after surgery or trauma, with menses, and after childbirth. Clinicians must ask whether the patient has ever had a blood transfusion. In addition, the family history should be queried for evidence of hemostatic defects. During the physical examination the skin, nail beds, mucosal membranes, soft tissues, and joints should be studied for evidence of petechiae, ecchymosis, hematomas, hemarthroses, hemangiomas, and telangiectasias.

Standardized questionnaires and bleeding scores can be included in the evaluation of patients with possible bleeding disorders. The Bleeding Assessment Tool (BAT) developed by the International Society on Thrombosis and Haemostasis (ISTH)/Scientific and Standardization Committee is designed to standardize a patient's bleeding risk. The BAT is a standardized questionnaire comprised of 14 categories for assessing bleeding symptoms (see https://cdn.ymaws.com/www.isth.org/resource/resmgr/ssc/isth-ssc_bleeding_assessment.pdf). Such an approach has been shown to help identify patients who should be evaluated for type 1 von Willebrand disease.[21] Bleeding assessment tools have also been used in the practice of gynecology.[22]

Certainly, traumatic causes of bleeding must always be sought in the history and may not reflect any coagulopathy (at least initially).[23]

TABLE 3. A Rational Approach to Coagulation Testing: Laboratory Practices

Consider point-of-care testing that does not require sodium citrate tubes.
If a public health emergency has been declared, then consider the use of outdated tubes if no other sodium citrate tubes are available.
Validate alternatively sourced sodium citrate tubes.

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

Bleeding may result internally or externally from blunt-force trauma, lacerations, or bleeding from the upper or lower gastrointestinal tract (potentially massive), genitourinary tract (eg, placenta previa or retained products of conception), or even the lung. In surgery, most bleeding results from insufficient mechanical hemostasis.[24,25]

Indications for Coagulation Testing

There are several clinical scenarios in which coagulation testing may be medically indicated.[26-32] Those scenarios are outlined in **TABLE 4**. Without a clear clinical indication that coagulation testing is required, coagulation testing should not be performed. Of course, this argument can be made for any laboratory test.[33] The types of testing that may be required for personal or family histories of thromboses are very complex and go beyond the scope of this article. Certainly, there is controversy about the value and need for specific testing seeking a genetic cause for hypercoagulability that could contribute to thromboses.[34-36]

Definition of Excessive Bleeding or Thrombosis

A significant variable in the above directives is what defines "excessive" bleeding or thrombosis. It is very difficult to find a standard medical definition of excessive bleeding. Excessive bleeding can be defined as the development of anemia or the requirement for a blood transfusion in the setting of acute or chronic hemorrhage. Palmer wrote, "The clinician should be alert that a hemostatic problem possibly exists when undue bleeding is observed that is inconsistent with antecedent trauma, occurs from two or more anatomic sites, or lasts for over 24 hours."[37] Unprovoked thrombosis is generally considered to be a thrombosis (often repeated) in the absence of recognized risk factors (eg, older age, obesity, post surgery [especially orthopedic], stasis/immobility, inflammation/trauma, pregnancy, estrogen use) and/or a thrombosis that occurs in an unusual location (eg, central nervous system, venous system, upper extremity, or splanchnic circulation).[38]

Selection of Coagulation Tests

The spectrum of the various coagulation tests is broad, and such tests can be highly specialized. According to a collection of health care centers in the United Kingdom, most inherited bleeding disorders involve clotting factors (87%), platelets (8%), or fibrinolytic defects,[3] whereas 2% are unclassified.[39] Some elements of the hemostatic system can be measured in mass units by immunoassay, such as that for the von Willebrand factor (VWF) antigen (VWF:Ag), but many more tests are defined functionally, often by the detection of fibrinogen formation via mechanical, photo-optical, or viscoelastic methods (eg, PT, PTT, thrombin time [TT], fibrinogen concentration, and clotting factor concentrations). Some tests use chromatographic substrates in the analysis (eg, chromatographic measurement of fibrinogen or heparin measurements).

TABLE 4. Indications for Coagulation Testing

Personal history of unexplained excess bleeding or thrombosis.
Family history of unexplained excess bleeding or thrombosis.
Acute presentation with unexplained excess bleeding or thrombosis.
Treatment with an anticoagulant where coagulation monitoring is used to adjust dosages (eg, vitamin K antagonists or UFH), or bleeding or thrombosis occur in a patient being treated with an anticoagulant regardless of whether the patient's anticoagulation is routinely monitored.[119-121]
Diagnosis with a condition known to be associated with a high risk of bleeding or thrombosis (eg, COVID-19 infection, sepsis with DIC, heparin-induced thrombocytopenia, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura). In such patients in whom the risk of bleeding or thrombosis is substantial, despite the patient not yet experiencing bleeding or thrombosis, anticipatory coagulation testing may be indicated.[122-124]

DIC, disseminated intravascular coagulation; UFH, unfractionated heparin.

Although there are many tests to assess the procoagulant pathways of primary (platelet-plug formation) and secondary (non-cross-linked fibrin formation) hemostasis, there are fewer tests of early tertiary hemostasis (ie, factor XIII assays involving fibrin strand cross-linking) and late tertiary hemostasis (D-dimers, plasminogen activity) and even fewer tests to assess anticoagulant pathways (eg, antithrombin, protein S, protein C, and alpha-2 plasmin inhibitor). Viscoelastic testing (eg, thromboelastogram (TEG) [Haemonetics, Boston, MA], rotational thromboelastometry (ROTEM) [Werfen, Bedford, MA], and Quantra (HemoSonics, Charlottesville, VA]) can evaluate both thrombosis formation and dissolution. However, viscoelastic testing is only available in limited centers and is typically restricted to central laboratories or surgical suites.

Bleeding can result from defects in platelets, plasma proteins, or the endothelium/blood vessel wall. Blood vessel disease with normal platelet function and plasma proteins can be of intrinsic origin (eg, Osler-Weber-Rendu disease, Ehlers-Danlos syndrome) or extrinsic origin (eg, vitamin C deficiency or trauma).[40-42] There are no laboratory plasma tests that can directly assess blood vessel integrity. Trauma may be recognized or hidden (even intentionally, such as in child or elder abuse[43]). Bleeding times are no longer included in most laboratory directories because of their lack of reproducibility, poor sensitivity and specificity for a vascular etiology of bleeding, and relatively high cost.[30,44-46]

The selection of tests used to initially evaluate bleeding is controversial. Some experts recommend 3 basic tests: PT, PTT, and platelet count.[32,47] This recommendation is because these tests (1) investigate primary hemostasis (the platelet count) and secondary hemostasis (PT and PTT), (2) are broadly available from routine laboratories, (3) can be rapidly performed, and (4) are of relatively modest individual cost. Other experts would add to this list the functional (Clauss) measurement of fibrinogen, the TT, and the VWF evaluation.[48] This evaluation includes the antigenic mass of VWF (ie, VWF:Ag) and the VWF functional activity. The latter activity can currently be determined by 4 different assays: the ristocetin cofactor assay (VWF:RCo), VWF:GPIbR, VWF:GPIbM, and VWF:Ab.[49,50] The ristocetin cofactor assay (VWF:RCo) is the traditional assay that forms the bulk of the existing literature. Recently, an international panel consisting of the American Society of Hematology, ISTH, the National Hemophilia Foundation, and the World Federation of Hemophilia[1] recommended the VWF:GPIbR and the VWF:GPIbM assays above all others. However, these 2 latter assays are not commercially available in the United States and are not FDA-approved. Only the VWF:RCo and VWF:Ab are FDA-approved. The VWF collagen-binding assay is a fifth functional assay that is distinct from the assays described above. The VWF:RCo, VWF:GPIbR, VWF:GPIbM, and VWF:Ab measure platelet binding while the collagen-binding assay assesses the interaction of vWF with collagen. Platelet aggregometry is used to assess platelet function and to determine the nature of a defect should it exist. The activated clotting time (ACT) is a rapid global test of hemostasis performed in a point-of-care setting. Platelet-dense granule electron microscopy can determine whether the platelets have an adequate complement of granules containing ATP, ADP, and serotonin.[9,31,51-53] Although there is no

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

consensus on the answer to this question, the current authors limit their consideration of "basic" procoagulant testing to the following tests: PT, PTT, functional fibrinogen (if the PT or PTT are prolonged), and platelet count (with microscopic morphology evaluated as part of the routine complete blood count).[54,55]

Reasonable Guidelines for Ordering Coagulation Testing

It is intuitive that the choice of specific coagulation tests should be guided by the clinical presentation of the bleeding patient.[56] When patients present with mucosal bleeding and/or bleeding that is observed in association with petechiae or bleeding immediately after trauma or surgery, primary hemostasis would be the initial focus of coagulation testing (eg, platelet count and microscopic morphology).[57] Further testing would examine VWF concentration and function and platelet function via aggregation testing.

When patients present with ecchymoses, soft-tissue hematoma, hemarthrosis, and/or bleeding that is delayed by hours after trauma or surgery, secondary hemostasis would be the initial focus of coagulation testing (eg, PT, PTT, and fibrinogen).[58] Abnormalities of fibrin cross-linking (eg, factor XIII deficiency) causing early defective tertiary hemostasis would display a normal PT and PTT and require specialized testing such as the factor XIII activity and antigen assay, the euglobulin lysis test, the 5M urea solubility test, a 1% monochloroacetic acid dissolution, or an analysis of clot stability by viscoelastic testing.

Viscoelastic testing (TEG, ROTEM, or Quantra) displays an integrated analysis of the total coagulant cascade (platelets, VWF, and plasma protein clotting factors) and thrombolysis.[59] The disadvantage of viscoelastic testing is that in our experience it is not as sensitive as the PT or PTT for detecting the presence of therapeutic anticoagulants, moderate factor deficiencies, or lupus anticoagulants or certain classes of antiplatelet drugs. However, the advantage of this testing modality is its ability to assess intrinsic platelet dysfunction and fibrinolysis. Viscoelastic testing seems to be best used in trauma and other emergency settings. Furthermore, it is not usually available on a "stat" basis outside of certain critical care operating rooms (eg, a cardiovascular, orthopedic, or neurosurgical operating room) and therefore should not be considered as a "routine" coagulation test.

Thrombophilia Testing

We have no validated tests that can predict thrombotic susceptibility that would be performed in the absence of a personal or family history of repeated or unusual location thromboses.[60] The measurement of antithrombin, protein C, protein S, antiphospholipid autoantibodies (against beta-2 glycoprotein I and cardiolipin), and lupus anticoagulant testing, and the genetic detection of the factor V Leiden mutation and the prothrombin 20210 polymorphism are usually performed *after* the patient presents with a history of unexplained, multiple, and/or unusual location thromboses. Lupus anticoagulant testing can be performed using a variety of methods, the chief of which is the dilute Russell viper venom time, which activates coagulation via the common pathway. Other commonly used methods use a PTT-based format where the intrinsic pathway is activated with silica. It is always important to recognize that anticoagulants and vitamin K antagonists can significantly affect the outcome and interpretation of lupus anticoagulant testing. Furthermore, in the timing of any coagulation test request, it is critical to recognize that bleeding and thromboses themselves can alter coagulation test results, as will anticoagulant medications. Specifically, nongenetic and nonautoimmune tests seeking evidence of a prothrombotic predisposition (eg, antithrombin, protein S, and protein C) should not be performed in the presence of an acute thrombosis where such factors can be consumed.[61] Testing for antithrombin, protein S, and protein C should be delayed for many weeks after the patient has recovered. This timing can be further complicated if the patient was placed on prolonged anticoagulation after the acute thrombotic event.[62] Anticoagulants can affect hemostasis in complex ways. For example, intravenous unfractionated heparin (UFH) can deplete antithrombin levels.

Summary

The appropriate use of coagulation testing is based on the premise that testing is indicated when there is a valid clinical concern about actual or potential excess bleeding or thrombosis.

The COVID-19 Pandemic—A Major Cause of Increased Coagulation Testing

Because of increased coagulation testing in the management of COVID-19 infection, it is especially important to define the appropriate use of coagulation testing in affected persons.

Although lung injury is the primary feature of COVID-19, many studies have shown that a hypercoagulable state is often present.[63] This section focuses on the practical indications and selection of coagulation testing for hospitalized patients with COVID-19.

Features of COVID-19–Associated Coagulopathy

In patients with severe COVID-19, the SARS-CoV-2 infection promotes an acute systemic inflammatory state that involves endothelial injury and dysfunction, the release of prothrombotic molecules, platelet activation, and reduced antithrombotic activity that precedes a hypercoagulable condition.[64-66] The hypercoagulable condition leads to thrombotic complications that impact the morbidity and mortality of patients with COVID-19.[67,68]

Although the pathophysiology of COVID-19–associated coagulopathy (CAC) continues to be investigated, it does share similarities with other known coagulopathic conditions such as sepsis-induced coagulation, thrombotic microangiopathies, and disseminated intravascular coagulation (DIC). Nevertheless, there are unique features that distinguish CAC from these other entities.[69,70] Although this review does not discuss or focus on the pathophysiological similarities and differences between the other coagulopathic conditions and CAC, it highlights the clinical laboratory findings that are both helpful and practical in monitoring for CAC.

Important Laboratory Testing in the Progression of CAC

Although the pathophysiology of CAC seems to evolve in parallel with the increasing severity of disease, there is evidence that some individuals with asymptomatic to mild COVID-19 may be at an increased risk for thrombosis including stroke.[71,72] Still, most of the evidence and discussion in the CAC literature revolves around hospitalized patients. Thus, we derive our discussion from the evidence surrounding coagulation testing at admission and in hospitalized patients.

To discuss the evaluation and practicality of coagulation testing, it is important to identify a conceptual framework in the evolution of CAC within the context of coagulation laboratory test results. From

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

TABLE 5. CAC Stages

Stage	Coagulation Events	Degree of Illness	D-dimer	Platelet Count	PT	Fibrinogen
1	Localized activation	Not severe	2- to 3-fold increase	Normal	Normal	Normal to increased
2	Systemic activation	Moderate to severe	3- to 6-fold increase	Mild decrease	Mild increase	Increase
3	Consumptive coagulation	Severe to critical	>6-fold increase	Decrease	Increase	Decrease

CAC, COVID-19–associated coagulopathy; PT, prothrombin time.

the literature, we have found essentially 3 stages of CAC,[70,73-77] which are described in **TABLE 5**.

Medically Relevant and Nonrelevant Testing in CAC

Coagulation experts (including the ISTH recommended guidelines[48]) overwhelmingly favor testing for CAC using (1) the platelet count (complete blood count, EDTA/purple-top tube), (2) D-dimers, (3) PT, and (4) fibrinogen levels as shown above in the CAC staging.[74,77] Platelet counts <100,000/μL are more commonly observed in nonsurvivors. The PT remains relatively normal or slightly prolonged in stages 1 and 2 but may progressively prolong as patients trend toward a DIC-like profile in stage 3. Fibrinogen levels are generally elevated, >200 mg/dL, but levels dropping below 200 mg/dL after being elevated are associated with a high mortality rate, which likely demarcates the beginning of the consumptive coagulation phase (stage 3 CAC). Regarding D-dimers, this important marker coincides with disease progression and prognosis advancing from CAC stage 1 through stage 3. The significance of the D-dimer is supported by the ISTH guidelines, among other studies.[70,73-77] However, D-dimers and other biomarkers should not be the only criteria for making clinical decisions. Clinical decisions should be made in the context of a comprehensive clinical assessment of the patient.

In a routine clinical setting (as opposed to research), for patients with CAC, the value of other coagulation markers and/or alternative instrument methods such as VWF, soluble P-selectin, soluble thrombomodulin, antithrombin complexes, tissue plasminogen activator, plasminogen activator inhibitor-1, factor VIII, ADAMTS-13, and viscoelasticity remain unproven and are not recommended at this time.[73,75,78-82]

Utilization and Practicality in CAC Testing

From a utilization and practicality perspective, the importance of using the recommended tests is significant for 2 fundamental reasons. First, these tests are currently the same ones used for the diagnosis and monitoring of DIC, which in stage 3 of CAC trends toward a DIC-like profile in critically ill patients.[75,83]

First, the ISTH diagnostic criteria for overt DIC use the platelet count, PT, fibrinogen, and a fibrin-related marker such as D-dimers.[84] The ISTH DIC scoring system incorporates the coagulation laboratory test results by assigning points (0–3) based upon the results meeting particular designated cutoff values. The overall score then categorizes patients as either compatible with overt DIC or suggestive for nonovert DIC.[84] Note that the ISTH DIC score is a snapshot in time and may guide treatment in acute situations.

Second, in our experience, many medical centers caring for severely ill patients with COVID-19 have these tests readily available in their respective in-house laboratories whereas many of the other biomarkers and methods evaluated in the literature are not. For example, although VWF and ADAMTS-13 may show some level of evidence for diagnostic and prognostic value, tests assessing their presence are not as likely to be found in many medical center clinical laboratories. In addition, these markers are not superior to tests included in the recommended guidelines (ie, D-dimer, fibrinogen, PT, and platelet count).

Baseline, Frequency, and Anticoagulation Testing in CAC

When reviewing the literature recommendations for adult baseline testing, we found that the recommended frequency of anticoagulation monitoring varies.[77,85,86] However, an increasing consensus suggests that coagulation baseline testing at the time of hospital presentation should include a platelet count, D-dimers, PT, and fibrinogen. Once a patient is hospitalized, in general coagulation testing should be done at least every 48 hours with the caveat that the patient is stable; otherwise, the testing frequency should be increased as needed. Specific guidance regarding testing frequency should be established by each institution with consideration of the respective patient population, laboratory resources, and input from leadership in hematology and clinical pathology. A general guide to increase the frequency in testing may include changes from baseline oxygen requirements or mechanical ventilation, blood pressure, worsening coagulation markers, changes in anticoagulation treatment, and other risk factors for potential thromboembolic risk such as a suspicion of a new deep vein thrombosis. However, once a new baseline is established and the patient stabilizes, there should be a de-escalation of coagulation testing frequency.[85,86]

With regard to anticoagulation therapy, in the absence of contraindications such as increased bleeding risk and medications, a simple algorithm includes placing all patients with COVID-19 on a standard dose of LWMH at the time of hospitalization and making changes based on a thromboembolic risk assessment, which may include a combination of the patient's oxygen requirements, body mass index, and medical history such as cancer and/or recent thromboembolic events.[77,85-87] Increased oxygen requirements, suspicion of a new venous thromboembolism, and/or renal failure may justify an increased LMWH dose or a switch to ultrafractionated heparin. However, note that this topic remains complex because some studies have shown that escalated dosing in moderately ill patients may be beneficial, but in critically ill patients it may not be.[88] Nevertheless, the frequency of monitoring with an anti-Xa heparin assay (or PTT, if an anti-Xa heparin assay is not available) will depend on a number of variables that include the patient's clinical status (eg, renal insufficiency, risk for a thromboembolic event), type of anticoagulation being given, dosage, and available laboratory resources.[58,86,89,90]

Summary

The unexpected rise of CAC in patients with COVID-19, especially early in the pandemic, led to a major shift in coagulation testing. Although our understanding of the pathophysiology of CAC is still evolving, not unexpectedly, several routine and specialized coagulation laboratory findings seen in CAC do share some similarities with other known coagulopathic conditions.[69,81,91-93] In this context,

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

TABLE 6. Recommended Conservation and CAC Coagulation Testing Strategies

A comprehensive clinical evaluation for bleeding and thrombotic risk is essential because it will guide appropriate testing.
CAC should be evaluated primarily using PT, fibrinogen, platelet count, and D-dimer measurement at baseline and for subsequent monitoring.
Coagulation testing outside of PT, fibrinogen, platelet count, and D-dimer measurement should rarely be performed, but if there are concerns, consultations with hematology and clinical pathology departments are recommended before potentially ordering unnecessary tests.
Laboratory should consider a discussion with the primary clinical team and hematology department to cancel orders of other coagulation biomarkers outside of the developed guidelines.
The frequency of coagulation testing should be determined by clinical status, changes in treatment, type of anticoagulation, and available laboratory resources.
The inpatient hematology team should be consulted to discuss specific guidance if the patient's coagulopathy appears to be worsening or treatment changes are needed.
Standardized guidance protocols/algorithms for common situations for patients with CAC should be developed by each institution with input from hematology and clinical laboratory leadership.

CAC, COVID-19–associated coagulopathy; PT, prothrombin time.

it is not surprising that the various features of CAC may have led to confusion, overuse, and misuse of coagulation testing beyond the recommended ISTH guidelines. Thus, based on the current evidence and recommended guidelines, conservation strategies for coagulation testing in CAC summarized in **TABLE 6** should be valid into the foreseeable future.

Monitoring Drugs that Inhibit Hemostasis

Monitoring drugs that inhibit hemostasis represents a major demand for coagulation testing. These drugs can be divided into 2 broad classes: anticoagulants and antiplatelet agents. Closely tied in with antiplatelet agents is the process of monitoring platelet function.

Anticoagulant Monitoring

Anticoagulants, defined here as drugs that inhibit the coagulation pathways (ie, secondary hemostasis), are used in the treatment or prevention of arterial and venous thrombosis. Their mode of action is to inhibit thrombin generation. The anticoagulants covered in this section include the various heparins, the direct thrombin inhibitors, and the oral direct anti-Xa inhibitors. Vitamin K antagonists (eg, warfarin) differ from the other medications because they are not inhibitors: They block the vitamin K–dependent carboxylation of specific coagulation factors, and their use requires regular monitoring via the INR.

Research has shown that UFH[94,95] has a variable clearance and, when used intravenously, requires regular monitoring. The pharmacokinetics of UFH are unpredictable, and the half-life of UFH varies between 30 and 120 minutes depending on the heparin dose.[94] Studies have indicated that UFH binds to a number of plasma proteins, which creates variability in its pharmacokinetics, and UFH is mainly cleared by binding to various cell surface receptors. It was traditionally monitored by evaluating the PTT, but in many health care centers the use of this method has been replaced by the enzymatic chromogenic anti-Xa assay. The problem with the PTT monitoring of UFH is that the PTT can be significantly influenced by many coagulation abnormalities, including defects in the intrinsic coagulation pathway, the presence of antiphospholipid antibodies, and the acute phase response with elevations in various clotting factors such as factor VIII and fibrinogen.[96]

The LMWHs display reduced binding to proteins and cells and exhibit a more constant rate of elimination with a half-life (after subcutaneous injection) of approximately 4 hours.[94] The ultra-low-molecular-weight synthetic heparin fondaparinux has an extended half-life of approximately 17 hours after subcutaneous injection.[94] Both LMWH and fondaparinux are renally excreted and, because of their stable half-lives, their effects or concentrations do not need to be monitored in most patients. However, monitoring these heparins may well be necessary in the intensive care unit setting, particularly if the patient has renal impairment or there are extremes of age or weight. Neither LMWH nor fondaparinux significantly prolong the PTT, so the measurement of plasma concentrations requires an anti-Xa assay calibrated for the specific drug.

The anti-Xa assay plays no role in the measurement of direct thrombin inhibitors such as argatroban, bivalirudin, and the oral agent dabigatran. Argatroban and bivalirudin are both used to control thrombosis including patients in whom UFH is contraindicated such as those with heparin-induced thrombocytopenia. Bivalirudin is also used for short-term[97] procedures such as percutaneous coronary intervention. Both of these medications require monitoring especially for intermediate and long-term usage; typically, the PTT or ACT perform this function; the latter is a point-of-care whole blood assay usually used in the operating room or in the interventional cardiology suite.[98] The ACT does not require a sodium citrate tube.

Caution should be applied in interpreting PTT results in patients treated with argatroban in the presence of a lupus anticoagulant, critical illness, and coagulopathy, and in patients with greatly elevated factor VIII concentrations.[99] For argatroban, the ecarin clotting time is the optimal assay but is not widely available in clinical laboratories.[100] In monitoring direct thrombin inhibitors, the PTT may correlate poorly with the ecarin chromogenic assay.[101] In recent years, a modification of the standard thrombin clotting time, termed the dilute (or diluted) TT, has been developed for monitoring direct thrombin inhibitors. In this method, the patient plasma is diluted in normal plasma.[102,103] The dilute TT is more responsive to the plasma concentrations of direct thrombin inhibitors and shows a better correlation with liquid chromatography-tandem mass spectrometry measurements compared with the PTT.[104,105]

Monitoring the direct-acting oral anticoagulants (DOACs) has raised some interesting questions.[97,106,107] These medications include the direct factor Xa inhibitors (eg, rivaroxaban, apixaban) and the direct (oral) thrombin inhibitors (eg, dabigatran). All these anticoagulants were FDA-approved with the understanding that they did *not* require regular monitoring by laboratory tests. However, it is apparent that in certain urgent situations, monitoring is necessary. Patients in the emergency department with trauma (especially head trauma) who may or may not have taken DOACS are a prime example. Patients recently admitted to critical care units with a history of DOAC administration may also require a plasma DOAC determination. The preferred assay for the anti-Xa inhibitors is an anti-Xa chromogenic assay calibrated with the DOAC under investigation. Dabigatran can be monitored with the dilute TT. The ecarin clotting time is another possibility for dabigatran monitoring. The PT and PTT are not recommended for DOAC monitoring. **TABLE 7** summarizes these recommendations.

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

TABLE 7. Recommended Monitoring (if Indicated) of Anticoagulants

Class of Drug	Requires Regular Monitoring	Recommended Routine Monitoring Test	Nonroutine Monitoring Test
UFH, IV	Yes	Anti-Xa assay	N/A
LMWH, subcutaneous	No	N/A	In acute care settings—anti-Xa assay calibrated for LMWH
Fondaparinux, subcutaneous	No	N/A	In acute care settings—anti-Xa assay calibrated for fondaparinux
Direct thrombin inhibitors (IV) Argatroban and bivalirudun	Yes	PTT	Dilute thrombin time, ecarin clotting time
Dabigatran (PO)	No	N/A	Dilute thrombin time
Direct Xa inhibitor (PO)	No	N/A	In acute care settings—anti-Xa assay calibrated for specific direct Xa inhibitor

IV, intravenous; LMWH, low-molecular-weight heparin; N/A, not applicable; PO, per os (by mouth); PTT, partial thromboplastin time; UFH, unfractionated heparin.

Platelet Function Monitoring

The goal of primary hemostasis is platelet aggregation in response to an injured blood vessel. Excluding the blood vessel wall, the effectiveness of primary hemostasis is dependent upon the platelet count, platelet function, and the presence of active VWF.

There are multiple methods of testing platelet function, including PFA-100 (Siemens Medical Solutions, Malvern, PA), platelet aggregometry (light transmission aggregation or whole blood aggregometry), VerifyNow (Werfen, Bedford, MA), and viscoelastic testing (TEG, ROTEM, or Quantra).[108] Some methods are aimed at testing intrinsic or baseline platelet function in the absence of medication; others focus on the response of platelets to inhibitory drugs. With respect to the latter, there are 2 main groups of antiplatelet agents: aspirin and the adenosine diphosphate (ADP)–receptor antagonists (P_2Y_{12} antagonists). Antiplatelet therapy is used in the management of arterial thrombosis including strokes, interventional neuroradiology (especially flow diversion with a pipeline embolization device [PED]) for the treatment of aneurysms, cardiology patients undergoing treatment for acute coronary syndrome, and patients undergoing percutaneous coronary intervention.

The use of these devices for hemostasis assessment is not always appropriate. Many of these methods aimed at assessing platelet function are negatively affected or even invalidated by thrombocytopenia. If platelet dysfunction (inherited or acquired) is suspected, then it is essential to first exclude von Willebrand disease (VWD), which may itself be inherited or acquired. The correlation between these different platelet function methods is not consistent and may vary significantly with different modalities. If a defect in primary hemostasis is suspected, then it is vital to obtain a good hemostatic history, including the use of the BAT questionnaire.

Platelet aggregometry aims to elucidate minor or major defects in platelet response. Its use, as noted herein, should always be preceded by testing for VWD. Individual agonists such as ADP, arachidonic acid, collagen, U46619 (a thromboxane A_2 analog), and ristocetin are added to a fresh preparation of platelets, and the aggregation response is monitored together with the release of adenosine triphosphate from the platelet dense granules. Responses are assessed by determining the degree of platelet aggregation compared with a normal control specimen run concurrently, along with a reference range previously established by the testing laboratory.

The PFA-100 platelet function analyzer (Siemens Medical Solution, Malvern, PA) performs a rapid analysis of platelet adhesion to a collagen-coated cartridge.[109] Specifically, fresh citrated whole blood is drawn through a very small aperture in the center of a cartridge. Two types of cartridges are available. Both cartridges are coated with type I collagen and with an additional agonist, either epinephrine or ADP. The PFA-100 is particularly susceptible to mild/moderate thrombocytopenia and anemia, for which it may indicate platelet dysfunction where there is none. Furthermore, the use of a P_2Y_{12} inhibitor does not always display a prolonged closure time, giving the incorrect impression that the medication is ineffective.[108]

The VerifyNow (Werfen/Instrumentation Laboratories, Bedford, MA) uses whole blood and is a relatively small self-contained cartridge-based optical aggregometer designed to test platelet responses to arachidonic acid and ADP.[110,111] It is not intended as a tool for assessing platelet function; rather, it tests the effectiveness of antiplatelet drugs such as aspirin and clopidogrel. The intended use of this device is to assess these drug effects; testing is carried out with the assumption that baseline platelet function is within normal limits and that the patient does not have VWD.

Viscoelastic hemostasis testing (TEG and ROTEM) provides valuable information about platelet function, mainly reflected by the TEG maximum amplitude or the ROTEM maximum clot firmness, both of which reflect clot strength, which is a function of platelet action (predominantly) and active fibrinogen concentration.[59,112] Platelet dysfunction and or thrombocytopenia can significantly reduce clot strength. What is often not well appreciated is that TEG or ROTEM clot strength is unaffected by the use of aspirin or P_2Y_{12} inhibitors, because the latter medications block either the synthesis of thromboxane A_2 or the function of the P_2Y_{12} receptor. Studies have shown that TEG and ROTEM are performed on venous blood activated by kaolin or kaolin and thromboplastin together; in this scenario, platelet activation occurs via thrombin that acts via a distinct set of protease-activated receptors. If viscoelastic testing is to be used to monitor antiplatelet drugs, then TEG platelet mapping is required. This process is a modification of the standard TEG protocol; briefly, high concentrations of heparin are used to inhibit all thrombin activity, thereby preventing thrombin-induced platelet aggregation. To form a clot, fibrinogen is polymerized by a combination of reptilase (a thrombin-resistant snake venom) and factor XIIIa. Then platelets can be activated by either arachidonic acid or by ADP in the absence of thrombin.

The Quantra hemostasis analyzer is a novel viscoelastic point-of-care testing coagulation monitor that uses sonography. Quantra testing is not considered a tool in coagulation testing outside of the operating room, and its intended use is in monitoring coagulation in real time while assisting in the choice of therapy for intraoperative coagulopathies.

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

TABLE 8. Occurrence of Inappropriate Use of Testing for Defects in Primary Hemostasis

Bleeding history is incomplete.
Other causes of bleeding (not related to problems with platelets/VWF) are not fully investigated, particularly with epistaxis and menorrhagia. For epistaxis, common causes in children include mucosal dryness, trauma, and rhinitis. Noncoagulopathic causes of menorrhagia are many and include fibroids, endometriosis, endocrine disturbances, and malignant neoplasms.
The medication history is incomplete. There are many prescription medicines and over-the-counter supplements that impair normal platelet function.
The patient is an inpatient in a critical care unit and is receiving IV fluids and various medications.
Overuse of testing for antiplatelet medication can occur because (1) many testing modalities are not universally supported by the literature—they are either refuted entirely by some groups or the available studies are equivocal; and (2) different laboratory methods of testing platelet responses to antiplatelet medication may yield contrasting information.[125,126]

IV, intravenous; VWF, von Willebrand factor.

Platelet function testing to monitor antiplatelet therapy has proved controversial. In the setting of interventional neuroradiology and the use of the PED (see above), Texakalidis et al concluded, "There was no statistically demonstrable difference in regard to thrombotic events between centers that conducted at least one PFT [platelet function test] and centers that did not test their patients with a PFT."[113] Another study by Neyens et al showed no benefit in a situation where the VerifyNow was utilized to monitor antiplatelet therapy in the setting of PED management of aneurysms.[114] Conversely, Li et al suggested that TEG platelet mapping might be of benefit in this situation.[115] Corliss et al concluded that platelet mapping had no value in monitoring antiplatelet therapy in interventional neuroradiology.[116]

Monitoring for suspected platelet dysfunction is also used in trauma situations. However, Stettler et al found no advantage in the use of TEG platelet mapping. "No advantage" means that this test did not predict mortality, massive transfusion, or platelet transfusion.[117]

Summary

The evaluation of anticoagulants can be complex and situational: Routine testing for DOACs is not required, but testing for DOACs may be required in persons so treated who suffer from bleeding or thrombosis. **TABLE 8** provides testing guidance for the assessment of primary hemostasis (platelet function).

Closing Remarks

One of the most difficult challenges in laboratory medicine is restricting the utilization of testing.[118] Nevertheless, when funds, staff, and/or supplies are limited, this is what we must do. Our discussion has provided referenced and rational guidelines to better manage coagulation testing.

REFERENCES

1. Sodium citrate blood specimen collection (light blue top) tubes: frequently asked questions. 2021. https://www.fda.gov/medical-devices/coronavirus-covid-19-and-medical-devices/sodium-citrate-blood-specimen-collection-light-blue-top-tubes-frequently-asked-questions.
2. Asakura H, Ogawa H. COVID-19-associated coagulopathy and disseminated intravascular coagulation. *Int J Hematol.* 2021;113(1):45–57.
3. U.S. Food & Drug Administration. Sodium citrate blood specimen collection tube conservation strategies: letter to health care and laboratory personnel. https://www.fda.gov/medical-devices/letters-health-care-providers/sodium-citrate-blood-specimen-collection-tube-conservation-strategies-letter-health-care-and. Published June 10, 2021. Accessed January 25, 2022.
4. College of American Pathologists. CAP publishes strategies to mitigate national shortage of "blue top" test tubes. https://newsroom.cap.org/latest-news/cap-publishes-strategies-to-mitigate-national-shortage-of--blue-top--test-tubes/s/96bd2447-418a-4c78-b914-604842575c11. Published June 16, 2021. Accessed January 25, 2022.
5. College of American Pathologists. COVID-19 information. https://www.cap.org/news/2020/latest-on-the-novel-coronavirus-covid-19. Accessed January 25, 2022.
6. Choosing Wisely, An Initiative of the American Board of Internal Medicine Foundation. American Society for Clinical Pathology: Thirty five things physicians and patients should question. https://www.choosingwisely.org/societies/american-society-for-clinical-pathology/. Updated July 2021. Accessed January 25, 2022.
7. Spectrum Health. Coagulation blue top tube shortage. https://lab.spectrumhealth.org/2021/06/10/coagulation-blue-top-shortage/. Updated September 1, 2021. Accessed January 25, 2022.
8. Claytor JD, Redberg RF. Product hopping—an expensive and wasteful practice. *JAMA Intern Med.* 2020;180(9):1154–1155.
9. Humble RM, Hounkponou HG, Krasowski MD. The "rainbow" of extra blood tubes—useful or wasteful practice? *JAMA Intern Med.* 2017;177(1):128–129.
10. Snozek CLH, Hernandez JS, Traub SJ. "Rainbow draws" in the emergency department: clinical utility and staff perceptions. *J Appl Lab Med.* 2019;4(2):229–234.
11. Dubrowny N, Armstrong E, Berube J, et al. Validation and verification of tubes for venous and capillary blood specimen collection; approved guideline. Clinical and Laboratory Standards Institute. https://clsi.org/media/1376/gp34a_sample.pdf. Published December 2010. Accessed January 25, 2022.
12. Dubrowny N, Armstrong E, Berube J, et al. Tubes and additives for venous and capillary blood specimen collection; approved standard—sixth edition. Clinical and Laboratory Standards Institute. https://clsi.org/media/2464/gp39a6e_sample.pdf. Published December 2010. Accessed January 25, 2022.
13. College of American Pathologists. Blue top tube recommendations. https://documents.cap.org/documents/cap-blue-top-tubes-recommendations.pdf. Published June 15, 2021. Accessed January 25, 2022.
14. Blennerhassett R, Favaloro E, Pasalic L. Coagulation studies: achieving the right mix in a large laboratory network. *Pathology.* 2019;51(7):718–722.
15. Rudolf JW, Baron JM, Dighe AS. Order indication solicitation to assess clinical laboratory test utilization: D-dimer order patterns as an illustrative case. *J Pathol Inform.* 2019;10:36.
16. Cleveland Clinic Laboratories. Light blue coagulation tubes: national supply shortage—update. https://clevelandcliniclabs.com/2021/05/20/light-blue-coagulation-tubes-national-supply-shortage-update/. Published May 20, 2021. Updated December 29, 2021. Accessed January 25, 2022.
17. Rouse J. Supply shortage of sodium citrate collection tubes. https://www.salemhealth.org/for-healthcare-professionals/common-ground/common-ground-2021/june-20-2021/supply-shortage-of-sodium-citrate-collection-tubes. Published June 20, 2021. Accessed January 25, 2022.
18. The Ophthalmologist. The high price of unnecessary tests. https://theophthalmologist.com/subspecialties/the-high-price-of-unnecessary-tests. Published May 11, 2015. Accessed January 25, 2022.
19. Patrick Malone & Associates P.C. With costly medical over-testing, causes can be blurry, solutions complex. https://www.jdsupra.com/legalnews/with-costly-medical-over-testing-causes-39353/. Published April 9, 2019. Accessed January 25, 2022.

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

20. Girolami A, Luzzatto G, Varvarikis C, Pellati D, Sartori R, Girolami B. Main clinical manifestations of a bleeding diathesis: an often disregarded aspect of medical and surgical history taking. *Haemophilia.* 2005;11(3):193–202.
21. Rodeghiero F, Castaman G, Tosetto A, et al. The discriminant power of bleeding history for the diagnosis of type 1 von Willebrand disease: an international, multicenter study. *J Thromb Haemost.* 2005;3(12):2619–2626.
22. Kadir RA, Davies J. Hemostatic disorders in women. *J Thromb Haemost*. 2013;11(suppl 1):170–179.
23. Caspers M, Maegele M, Fröhlich M. Current strategies for hemostatic control in acute trauma hemorrhage and trauma-induced coagulopathy. *Expert Rev Hematol.* 2018;11(12):987–995.
24. Curnow J, Pasalic L, Favaloro EJ. Why do patients bleed? *Surg J (N Y).* 2016;2(1):e29–e43.
25. Ghadimi K, Levy JH, Welsby IJ. Perioperative management of the bleeding patient. *Br J Anaesth*. 2016;117(suppl 3):iii18–iii30.
26. de Moerloose P. Laboratory evaluation of hemostasis before cardiac operations. *Ann Thorac Surg.* 1996;62(6):1921–1925.
27. Eckman MH, Erban JK, Singh SK, Kao GS. Screening for the risk for bleeding or thrombosis. *Ann Intern Med.* 2003;138(3):W15–24.
28. Nicolson D, Nadra A, Hodzovic I. Preoperative coagulation screening—take a bleeding history. *Eur J Anaesthesiol*. 2008;25:84.
29. Roberts JC. Evaluation of abnormal bleeding in children. *Pediatr Ann.* 2020;49(1):e36–e42.
30. Rohrer MJ, Michelotti MC, Nahrwold DL. A prospective evaluation of the efficacy of preoperative coagulation testing. *Ann Surg.* 1988;208(5):554–557.
31. Samková A, Blatný J, Fiamoli V, Dulíček P, Pařízková E. Significance and causes of abnormal preoperative coagulation test results in children. *Haemophilia.* 2012;18(3):e297–301.
32. Leung LLK. Preoperative assessment of hemostasis. https://www.uptodate.com/contents/preoperative-assessment-of-hemostasis?topicRef=1368&source=see_link. Accessed January 25, 2022.
33. Mayer M, Wilkinson I, Heikkinen R, Orntoft T, Magid E. Improved laboratory test selection and enhanced perception of test results as tools for cost-effective medicine. *Clin Chem Lab Med.* 1998;36(9):683–690.
34. Long S, Goldblatt J. MTHFR genetic testing: controversy and clinical implications. *Aust Fam Physician.* 2016;45(4):237–240.
35. Turrini M, Bourgain C. Genomic susceptibility in practice: the regulatory trajectory of non-rare thrombophilia (NRT) genetic tests in the clinical management of venous thrombo-embolism (VTE). *Soc Sci Med*. Published online March 3, 2020. doi: 10.1016/j.soscimed.2020.112903.
36. Turrini M, Bourgain C. Appraising screening, making risk in/visible. The medical debate over non-rare thrombophilia (NRT) testing before prescribing the pill. *Sociol Health Illn.* 2021;43(7):1627–1642.
37. Palmer RL. Excessive bleeding and bruising. In: Walker HK Hall WD, Hurst JW, eds. *Clinical Methods: The History, Physical, and Laboratory Examinations*. 3rd ed. Boston, MA: Butterworths; 1990.
38. Connors JM. Thrombophilia testing and venous thrombosis. *N Engl J Med.* 2017;377(23):2298.
39. Sivapalaratnam S, Collins J, Gomez K. Diagnosis of inherited bleeding disorders in the genomic era. *Br J Haematol.* 2017;179(3):363–376.
40. Abdullah M, Jamil RT, Attia FN. *Vitamin C (Ascorbic Acid)*. Treasure Island, FL: StatPearls; 2021.
41. Jesudas R, Chaudhury A, Laukaitis CM. An update on the new classification of Ehlers-Danlos syndrome and review of the causes of bleeding in this population. *Haemophilia.* 2019;25(4):558–566.
42. Macri A, Wilson AM, Shafaat O, Sharma S. *Osler-Weber-Rendu Disease*. Treasure Island, FL: StatPearls; 2021.
43. Knöfler R, Streif W, Watzer-Herberth I, Hahn G, Schmidt U. Child abuse or bleeding disorder—an interdisciplinary approach. *Hamostaseologie.* 2019;39(1):28–35.
44. De Caterina R, Lanza M, Manca G, Strata GB, Maffei S, Salvatore L. Bleeding time and bleeding: an analysis of the relationship of the bleeding time test with parameters of surgical bleeding. *Blood.* 1994;84(10):3363–3370.
45. Russeau AP, Vall H, Manna B. *Bleeding Time*. Treasure Island, FL: StatPearls; 2021.
46. Srichumpuang C, Sosothikul D. Comparison between bleeding time and PFA-200 to evaluate platelet function disorder in children. *J Pediatr Hematol Oncol.* 2021;43(5):e748–e749.
47. Neutze D, Roque J. Clinical evaluation of bleeding and bruising in primary care. *Am Fam Physician.* 2016;93(4):279–286.
48. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC Joint VWF and Perinatal/Pediatric Hemostasis Subcommittees Working Group. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost.* 2010;8(9):2063–2065.
49. Harris NS, Pelletier JP, Marin MJ, Winter WE. Von Willebrand factor and disease: a review for laboratory professionals. *Crit Rev Clin Lab Sci*. Published online December 28, 2021. doi: 10.1080/10408363.2021.2014781.
50. Higgins RA, Goodwin AJ. Automated assays for von Willebrand factor activity. *Am J Hematol.* 2019;94(4):496–503.
51. Meijer K, van Heerde W, Gomez K. Diagnosis of rare bleeding disorders. *Haemophilia*. 2021;27(suppl 3):60–65.
52. Thomas W, Downes K, Desborough MJR. Bleeding of unknown cause and unclassified bleeding disorders; diagnosis, pathophysiology and management. *Haemophilia.* 2020;26(6):946–957.
53. Warhadapande S, Dariushnia SR, Kokabi N, et al. Clinical approach to and work-up of bleeding patients. *Semin Intervent Radiol.* 2020;37(1):24–30.
54. Andreason CL, Pohlman TH. Damage control resuscitation for catastrophic bleeding. *Oral Maxillofac Surg Clin North Am.* 2016;28(4):553–568.
55. Lee A. Emergency management of patients with bleeding disorders: practical points for the emergency physician. *Transfus Apher Sci.* 2019;58(5):553–562.
56. Hrachovinová I. Diagnostic strategies in disorders of hemostasis. *Vnitr Lek.* 2018;64(5):537–544.
57. Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Platelets at work in primary hemostasis. *Blood Rev.* 2011;25(4):155–167.
58. Winter WE, Flax SD, Harris NS. Coagulation testing in the core laboratory. *Lab Med.* 2017;48(4):295–313.
59. Faraoni D, DiNardo JA. Viscoelastic hemostatic assays: update on technology and clinical applications. *Am J Hematol.* 2021;96(10):1331–1337.
60. Moll S. Thrombophilia: clinical-practical aspects. *J Thromb Thrombolysis.* 2015;39(3):367–378.
61. Owings JT, Bagley M, Gosselin R, Romac D, Disbrow E. Effect of critical injury on plasma antithrombin activity: low antithrombin levels are associated with thromboembolic complications. *J Trauma.* 1996;41(3):396–405.
62. Alameddine R, Nassabein R, Le Gal G, Sié P, Mullier F, Blais N. Diagnosis and management of congenital thrombophilia in the era of direct oral anticoagulants. *Thromb Res.* 2020;185:72–77.
63. Abou-Ismail MY, Diamond A, Kapoor S, Arafah Y, Nayak L. The hypercoagulable state in COVID-19: incidence, pathophysiology, and management. *Thromb Res.* 2020;194:101–115.
64. Gupta A, Madhavan MV, Sehgal K, et al. Extrapulmonary manifestations of COVID-19. *Nat Med.* 2020;26(7):1017–1032.
65. Hanff TC, Mohareb AM, Giri J, Cohen JB, Chirinos JA. Thrombosis in COVID-19. *Am J Hematol.* 2020;95(12):1578–1589.
66. Perico L, Benigni A, Casiraghi F, Ng LFP, Renia L, Remuzzi G. Immunity, endothelial injury and complement-induced coagulopathy in COVID-19. *Nat Rev Nephrol.* 2021;17(1):46–64.

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

67. Bilaloglu S, Aphinyanaphongs Y, Jones S, Iturrate E, Hochman J, Berger JS. Thrombosis in hospitalized patients with COVID-19 in a New York City health system. *JAMA.* 2020;324(8):799–801.
68. Malas MB, Naazie IN, Elsayed N, Mathlouthi A, Marmor R, Clary B. Thromboembolism risk of COVID-19 is high and associated with a higher risk of mortality: a systematic review and meta-analysis. *EClinicalMedicine.* 2020;29:100639.
69. Iba T, Levy JH, Connors JM, Warkentin TE, Thachil J, Levi M. The unique characteristics of COVID-19 coagulopathy. *Crit Care.* 2020;24(1):360.
70. Iba T, Warkentin TE, Thachil J, Levi M, Levy JH. Proposal of the definition for COVID-19-associated coagulopathy. *J Clin Med.* 2021;10(2):191.
71. Luo Y, Trevathan E, Qian Z, et al. Asymptomatic SARS-CoV-2 infection in household contacts of a healthcare provider, Wuhan, China. *Emerg Infect Dis.* 2020;26(8):1930–1933.
72. Tu TM, Seet CYH, Koh JS, et al. Acute ischemic stroke during the convalescent phase of asymptomatic COVID-2019 infection in men. *JAMA Netw Open*. 2021;4(4):e217498.
73. Lee AYY, Connors JM, Kreuziger LB, et al. COVID-19 and coagulopathy: frequently asked questions. https://www.hematology.org/covid-19/covid-19-and-coagulopathy. Updated January 29, 2021. Accessed January 25, 2022.
74. Liao D, Zhou F, Luo L, et al. Haematological characteristics and risk factors in the classification and prognosis evaluation of COVID-19: a retrospective cohort study. *Lancet Haematol.* 2020;7(9):e671–e678.
75. Tang N, Bai H, Xiong D, Sun Z. Specific coagulation markers may provide more therapeutic targets in COVID-19 patients receiving prophylactic anticoagulant. *J Thromb Haemost.* 2020;18(9):2428–2430.
76. Thachil J, Cushman M, Srivastava A. A proposal for staging COVID-19 coagulopathy. *Res Pract Thromb Haemost.* 2020;4(5):731–736.
77. Thachil J, Juffermans NP, Ranucci M, et al. ISTH DIC subcommittee communication on anticoagulation in COVID-19. *J Thromb Haemost.* 2020;18(9):2138–2144.
78. Bazzan M, Montaruli B, Sciascia S, Cosseddu D, Norbiato C, Roccatello D. Low ADAMTS 13 plasma levels are predictors of mortality in COVID-19 patients. *Intern Emerg Med.* 2020;15(5):861–863.
79. Goshua G, Pine AB, Meizlish ML, et al. Endotheliopathy in COVID-19-associated coagulopathy: evidence from a single-centre, cross-sectional study. *Lancet Haematol.* 2020;7(8):e575–e582.
80. Mancini I, Baronciani L, Artoni A, et al. The ADAMTS13-von Willebrand factor axis in COVID-19 patients. *J Thromb Haemost.* 2021;19(2):513–521.
81. Mei ZW, van Wijk XMR, Pham HP, Marin MJ. Role of von Willebrand factor in COVID-19 associated coagulopathy. *J Appl Lab Med.* 2021;6(5):1305–1315.
82. Zuo Y, Warnock M, Harbaugh A, et al. Plasma tissue plasminogen activator and plasminogen activator inhibitor-1 in hospitalized COVID-19 patients. *Sci Rep.* 2021;11(1):1580.
83. Taylor FB, Toh CH, Hoots WK, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. *Thromb Haemost*. 2001;86(5):1327–1330.
84. Toh CH, Hoots WK; SSC on Disseminated Intravascular Coagulation of the ISTH. The scoring system of the Scientific and Standardisation Committee on Disseminated Intravascular Coagulation of the International Society on Thrombosis and Haemostasis: a 5-year overview. *J Thromb Haemost.* 2007;5(3):604–606.
85. Cuker A, Tseng EK, Nieuwlaat R, et al. American Society of Hematology 2021 guidelines on the use of anticoagulation for thromboprophylaxis in patients with COVID-19. *Blood Adv*. 2021;5(3):872–888.
86. Susen S, Tacquard CA, Godon A, et al. Prevention of thrombotic risk in hospitalized patients with COVID-19 and hemostasis monitoring. *Crit Care.* 2020;24(1):364.
87. Leentjens J, van Haaps TF, Wessels PF, Schutgens REG, Middeldorp S. COVID-19-associated coagulopathy and antithrombotic agents—lessons after 1 year. *Lancet Haematol.* 2021;8(7):e524–e533.
88. Ten Cate H. Surviving Covid-19 with heparin? *N Engl J Med.* 2021;385(9):845–846.
89. Arachchillage DRJ, Kamani F, Deplano S, Banya W, Laffan M. Should we abandon the APTT for monitoring unfractionated heparin? *Thromb Res.* 2017;157:157–161.
90. Hardy M, Lecompte T, Douxfils J, et al. Management of the thrombotic risk associated with COVID-19: guidance for the hemostasis laboratory. *Thromb J.* 2020;18:17.
91. Iba T, Connors JM, Levy JH. The coagulopathy, endotheliopathy, and vasculitis of COVID-19. *Inflamm Res.* 2020;69(12):1181–1189.
92. Merrill JT, Erkan D, Winakur J, James JA. Emerging evidence of a COVID-19 thrombotic syndrome has treatment implications. *Nat Rev Rheumatol.* 2020;16(10):581–589.
93. Ortega-Paz L, Capodanno D, Montalescot G, Angiolillo DJ. Coronavirus disease 2019-associated thrombosis and coagulopathy: review of the pathophysiological characteristics and implications for antithrombotic management. *J Am Heart Assoc.* 2021;10(3):e019650.
94. Garcia DA, Baglin TP, Weitz JI, Samama MM. Parenteral anticoagulants: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest.* 2012;141(2 suppl):e24S–e43S.
95. Linhardt RJ. 2003 Claude S. Hudson Award address in carbohydrate chemistry. Heparin: structure and activity. *J Med Chem.* 2003;46(13):2551–2564.
96. Zehnder J, Price E, Jin J. Controversies in heparin monitoring. *Am J Hematol*. 2012;87(suppl 1):S137–S140.
97. Samuelson BT, Cuker A, Siegal DM, Crowther M, Garcia DA. Laboratory assessment of the anticoagulant activity of direct oral anticoagulants: a systematic review. *Chest.* 2017;151(1):127–138.
98. Van Cott EM, Roberts AJ, Dager WE. Response to: "More on the limitations of the aPTT for monitoring argatroban therapy." *Semin Thromb Hemost.* 2017;43(6):644.
99. Guy S, Kitchen S, Maclean R, Van Veen JJ. Limitation of the activated partial thromboplastin time as a monitoring method of the direct thrombin inhibitor argatroban. *Int J Lab Hematol.* 2015;37(6):834–843.
100. Nowak G. The ecarin clotting time, a universal method to quantify direct thrombin inhibitors. *Pathophysiol Haemost Thromb.* 2003;33(4):173–183.
101. Lind SE, Boyle ME, Fisher S, Ishimoto J, Trujillo TC, Kiser TH. Comparison of the aPTT with alternative tests for monitoring direct thrombin inhibitors in patient samples. *Am J Clin Pathol.* 2014;141(5):665–674.
102. Love JE, Ferrell C, Chandler WL. Monitoring direct thrombin inhibitors with a plasma diluted thrombin time. *Thromb Haemost.* 2007;98(1):234–242.
103. Wanat MA, Hart SR, Putney D, Liebl MG, Chandler W. Alternative monitoring of argatroban using plasma-diluted thrombin time. *Ann Pharmacother.* 2013;47(4):e18.
104. Beyer JT, Lind SE, Fisher S, Trujillo TC, Wempe MF, Kiser TH. Evaluation of intravenous direct thrombin inhibitor monitoring tests: correlation with plasma concentrations and clinical outcomes in hospitalized patients. *J Thromb Thrombolysis.* 2020;49(2):259–267.
105. Scalambrino E, Padovan L, Chantarangkul V, et al. Responsiveness of the activated partial thromboplastin time and dilute thrombin time to argatroban: Results of an in vitro study. *Int J Lab Hematol.* 2020;42(3):e128–e131.
106. Chen A, Stecker E, Warden BA. Direct oral anticoagulant use: a practical guide to common clinical challenges. *J Am Heart Assoc.* 2020;9(13):e017559.
107. Dunois C. Laboratory monitoring of direct oral anticoagulants (DOACs). *Biomedicines*. 2021;9(5):445.

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

108. Bolliger D, Lance MD, Siegemund M. Point-of-care platelet function monitoring: implications for patients with platelet inhibitors in cardiac surgery. *J Cardiothorac Vasc Anesth*. 2021;35(4):1049–1059.

109. Kundu SK, Heilmann EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer—PFA-100. *Semin Thromb Hemost*. 1995;21(suppl 2):106–112.

110. Bansal V, Fortlage D, Lee J, Doucet J, Potenza B, Coimbra R. A new clopidogrel (Plavix) point-of-care assay: rapid determination of antiplatelet activity in trauma patients. *J Trauma.* 2011;70(1):65–69.

111. Karon BS, Tolan NV, Koch CD, et al. Precision and reliability of 5 platelet function tests in healthy volunteers and donors on daily antiplatelet agent therapy. *Clin Chem.* 2014;60(12):1524–1531.

112. Selby R. "TEG talk": expanding clinical roles for thromboelastography and rotational thromboelastometry. *Hematology Am Soc Hematol Educ Program.* 2020;2020(1):67–75.

113. Texakalidis P, Bekelis K, Atallah E, Tjoumakaris S, Rosenwasser RH, Jabbour P. Flow diversion with the pipeline embolization device for patients with intracranial aneurysms and antiplatelet therapy: a systematic literature review. *Clin Neurol Neurosurg.* 2017;161:78–87.

114. Neyens R, Donaldson C, Andrews C, Kellogg R, Spiotta A. Platelet function testing with a VerifyNow-directed personalized antiplatelet strategy and associated rates of thromboembolic complications after pipeline embolization for complex cerebral aneurysms. *World Neurosurg.* 2020;138:e674–e682.

115. Li Y, Zhang X, Guo Z, et al. Standard vs. modified antiplatelet therapy based on thromboelastography with platelet mapping for preventing bleeding events in patients undergoing stent-assisted coil for a ruptured intracranial aneurysm. *Front Neurol*. 2020;11:615829.

116. Corliss BM, Freedman R, Brennan MM, et al. Laboratory assessments of therapeutic platelet inhibition in endovascular neurosurgery: complication prediction using the VerifyNow P2Y12 assay and thromboelastography with platelet mapping. *J Neurosurg.* 2020;134(3):884–892.

117. Stettler GR, Moore EE, Moore HB, et al. Platelet adenosine diphosphate receptor inhibition provides no advantage in predicting need for platelet transfusion or massive transfusion. *Surgery.* 2017;162(6):1286–1294.

118. Bindraban RS, Ten Berg MJ, Naaktgeboren CA, Kramer MHH, Van Solinge WW, Nanayakkara PWB. Reducing test utilization in hospital settings: a narrative review. *Ann Lab Med.* 2018;38(5):402–412.

119. Clark NP. Role of the anticoagulant monitoring service in 2018: beyond warfarin. *Hematology Am Soc Hematol Educ Program.* 2018;2018(1):348–352.

120. Funk DM. Coagulation assays and anticoagulant monitoring. *Hematology Am Soc Hematol Educ Program.* 2012;2012:460–465.

121. Onundarson PT, Flygenring B. Oral anticoagulant monitoring: are we on the right track? *Int J Lab Hematol*. 2019;41(Suppl 1):40–48.

122. Al-Samkari H, Karp Leaf RS, Dzik WH, et al. COVID-19 and coagulation: bleeding and thrombotic manifestations of SARS-CoV-2 infection. *Blood.* 2020;136(4):489–500.

123. Levi M, Thachil J, Iba T, Levy JH. Coagulation abnormalities and thrombosis in patients with COVID-19. *Lancet Haematol.* 2020;7(6):e438–e440.

124. Levi M, van der Poll T. Coagulation and sepsis. *Thromb Res.* 2017;149:38–44.

125. Lordkipanidzé M, Pharand C, Schampaert E, Turgeon J, Palisaitis DA, Diodati JG. A comparison of six major platelet function tests to determine the prevalence of aspirin resistance in patients with stable coronary artery disease. *Eur Heart J.* 2007;28(14):1702.

126. Lordkipanidzé M, Pharand C, Nguyen TA, Schampaert E, Palisaitis DA, Diodati JG. Comparison of four tests to assess inhibition of platelet function by clopidogrel in stable coronary artery disease patients. *Eur Heart J.* 2008;29(23):2877–2885.

Downloaded from https://academic.oup.com/labmed/article/53/4/349/6548261 by guest on 28 February 2025

Serum Metabolomics in Patients with Coexisting NAFLD and T2DM Using Liquid Chromatography-Mass Spectrometry

Cheng Hu, MS,[1,a,] Xiaoyu Zhuang, MD,[1,a] Jiaqi Zhang, MS,[1,2,a] Tao Wang, MS,[3] Shengnan Du, MS,[3] Jinping Wang, MS,[3] Xuelian Peng, MS,[3] Qin Cao, MS,[3,*] Mingcai Zhang, MD,[4,*] Yuanye Jiang, MD[1,3,*]*

[1]Experiment Center for Science and Technology, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [2]Shanghai TCM-Integrated Institute of Vascular Anomalies, Shanghai TCM-Integrated Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [3]Department of Gastroenterology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [4]Shuguang Hospital affiliated with Shanghai University of Traditional Chinese Medicine, Shanghai, China; *To whom correspondence should be addressed. zhangmc816@126.com. [a]These authors contributed equally to this work.

Keywords: metabolomics, NAFLD, T2DM, LC-MS, noninvasive biomarkers, OPLS-DA

Abbreviations: NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; TP, total protein; TG, triglycerides; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; ChE, cholinesterase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ApoA, apolipoprotein a; ApoB, apolipoprotein B; Glu, glucose in urine; GHb, gamma hydroxybutyric acid; LC-MS, liquid chromatography-mass spectrometry; QC, quality control; UPLC, ultraperformance liquid chromatography; PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; FA, fatty acid; HCC, hepatocellular carcinoma; HbA1c, hemoglobin A1c; CPT-1, carnitine palmitoyl transferase-1; S1-P, sphingosine 1-phosphate.

Laboratory Medicine 2022;53:360–368; https://doi.org/10.1093/labmed/lmab118

ABSTRACT

Objective: Nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) frequently coexist and can act synergistically to drive adverse outcomes of one another. This study aimed to unravel the metabolomic changes in patients with NAFLD and T2DM, to identify potential noninvasive biomarkers, and to provide insights for understanding the link between NAFLD and T2DM.

Methods: Three hundred participants aged 35 to 70 years who were diagnosed with NAFLD (n = 100), T2DM (n = 100), or a comorbidity of NAFLD and T2DM (n = 100) were included in this study. Anthropometrics and routine blood chemistry were assessed after overnight fast. The global serum metabolomic analysis was performed by ultra-performance liquid chromatography-Orbitrap mass spectrometry. Multivariate data analysis methods were utilized to identify the potential biomarkers.

Results: A set of serum biomarkers that could effectively separate NAFLD from NAFLD + T2DM and T2DM from NAFLD + T2DM were identified. We found that patients with coexisting NAFLD and T2DM had significantly higher levels of total protein (TP), triglycerides (TG), glucose in urine, and gamma-hydroxybutyric acid than those with NAFLD and had significant increased levels of TP, albumin, alanine aminotransferase, aspartate aminotransferase, total cholesterol, cholinesterase, TG, low-density lipoprotein, and apolipoprotein A when compared to patients with T2DM.

Conclusion: The metabolomics results provide evidence that the comorbidity of NAFLD and T2DM considerably altered patients' metabolomics patterns compared to those of patients with only NAFLD or T2DM.

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and affects approximately 20%–30% of individuals worldwide.[1,2] The pathogenesis of NAFLD is multifactorial. Studies have shown that the high prevalence rates of NAFLD have been paralleling the rapidly progressing epidemic of components of metabolic syndrome such as obesity, type 2 diabetes mellitus (T2DM), dyslipidemia, and hypertension.[3]

The relation between NAFLD and T2DM is considered bidirectional and complicated. Studies have shown that NAFLD predicts the development of diabetes and vice versa and that each condition serves as a progression factor for the other.[4] In addition, evidence has shown that insulin resistance promotes the progression of liver disease, and NAFLD may also increase the risk of diabetes complications. Although NAFLD affects a large proportion of patients with T2DM, the diagnosis may frequently be overlooked.[5]

Currently, liver biopsy remains the gold standard for NAFLD diagnosis, especially in differentiating between simple steatosis and

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

steatohepatitis and staging the degrees of fibrosis.[6] However, liver biopsy carries the limitations of sampling errors and interobserver variations, along with the risk of invasive procedure–related complications including bleeding, pain, and infection near the biopsy site.[7,8] Thus, liver biopsy is unlikely to be carried out widely as a routine examination method in clinical practice. The new guidelines released by the American Diabetes Association suggest that patients with T2DM or prediabetes and elevated liver enzymes or fatty liver on ultrasound should be evaluated for the presence of NAFLD.[9] However, there is a lack of sensitive noninvasive markers. Alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels, which physicians usually rely on, are normal in many patients with NAFLD.

Metabolomics has recently emerged as a powerful tool that allows the assessment of global metabolite profiles in easily accessible biofluids to evaluate the progress of disease, discover potential biomarkers, and provide insights into the underlying pathophysiology. The rapidly developing metabolomics technology holds the promise of significantly improving the diagnosis and prognosis of NAFLD and T2DM.[10] Thus, this work was designed to explore novel biomarkers in patients with NAFLD and T2DM and enable a more-precise prognosis of disease development.

Methods

Participants

Three hundred participants aged 35 to 70 years who were inpatients and outpatients in the digestive department of Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China) were recruited from 2019 to 2020. The protocol was approved by the medical ethics committee of the hospital. All participants signed informed consent.

According to the guidelines for the diagnosis and treatment of NFLD (2018) formulated by the National Workshop on Fatty Liver and Alcoholic Liver Disease and the Chinese Society on Hepatology, a diagnosis of NAFLD is based on the detection of steatosis by abdominal ultrasonography.[11] A diagnosis of T2DM is according to the criteria defined by the World Health Organization, including having diabetes symptoms with a random plasma glucose level ≥11.1 mmol/L, a fasting plasma glucose level ≥7.0 mmol/L, or a 2-hour plasma glucose level ≥11.1 mmol/L during an oral glucose-tolerance test conducted with a standard loading dose of 75 g.

Exclusion criteria were as follows: (1) a history of liver diseases other than NAFLD, including viral hepatitis, cirrhosis, liver cancer, autoimmune liver disease, alcoholic liver disease, or hereditary liver disease; (2) excessive alcohol consumption (≥210 g/week for men, ≥140 g/week for women); (3) type 1 diabetes or other specific diabetes; (4) acute complications of diabetes; (5) severe heart disease (myocardial infarction, heart failure, and/ or severe arrhythmia); (6) severe infections and severe trauma; and (7) pregnant or breastfeeding.

Clinical Measurement

In the morning after overnight fasting, all patients underwent anthropometric evaluation, ultrasound examination, a questionnaire survey, and venous blood sampling. Serum levels of total bilirubin, direct bilirubin, total protein (TP), albumin (ALB), ALT, AST, alkaline phosphatase, γ-glutamyltransferase, total cholesterol (TCHO), cholinesterase (ChE), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoprotein A (ApoA), apolipoprotein B (ApoB), glucose in urine (Glu), and γ-hydroxybutyric acid (GHb) were measured using standard clinical methods on an automated chemistry analyzer (Hitachi 7600d-210, Japan).

Specimen Preparation for Metabolomics

One hundred μL of each serum specimen was mixed with 400 μL cold methanol and then vortexed for 3 minutes to precipitate the protein. After centrifugation (12,000 rpm at 4°C for 15 minutes), the supernatant was transferred to autosampler vials for further liquid chromatography-mass spectrometry (LC-MS) analysis. A pooled quality control (QC) specimen was prepared by mixing equal amounts of each specimen. Aliquots of pooled QC were analyzed every 10 specimens to monitor the stability of the analytical platform.

LC-MS Analysis

Serum metabolic profiling was performed using an Ultimate 3000 ultraperformance liquid chromatography (UPLC) system coupled to an linear ion trap-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Liquid chromatographic separation was achieved at a flow rate of 0.3 mL/min on an high-strength silica T3 column (100 mm × 2.1 mm, 1.8 μm, Waters) using 0.1% formic acid (buffer A) and acetonitrile (buffer B). The gradient elution was as follows: 0 to 2 minutes, 95% A; 2 to 12 minutes, 5% A; 12 to 15 minutes, 5% A; 15 to 17 minutes, 95% A. Mass spectrometric data acquisition was completed in both positive and negative ionization modes. Compounds were detected by full-scan mass analysis from 50 to 1000 *m/z* at a resolution of 60,000. In positive electrospray ionization mode, the spray voltage was set at 3.8 kV; the heater temperature was 300°C and the capillary temperature was 350°C; the S-Lens F level was set at 30%; and flow rates of sheath gas, auxiliary gas, and sweep gas were 45, 10, and 0 arb (arbitrary units), respectively. In negative ionization mode, the following parameters were used: spray voltage, –3.2 kV; capillary temperature, 350°C; heater temperature, 300°C; S-Lens F level, 60%; sheath gas flow, 45 arb; auxiliary gas flow, 5 arb; sweep gas flow, 1 arb.

Data Processing and Metabolite Identification

The raw LC-MS data were preprocessed using Compound Discoverer 2.1 software (Thermo Fisher Scientific), including alignment of the chromatograms, normalization, de-isotoping, adduct deconvolution, peak picking, and peak annotation, and the resultant data matrices that consisted of mass, retention time, and peak intensity were exported to SMICA-P software (Umetrics, Umea, Sweden) for multivariate statistical analyses. Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed for both serum and cell specimens of mice in each group.

Metabolites of interest were identified according to their accurate masses and MS/MS spectra patterns by searching the HMDB, KEGG, and *mz*Cloud metabolite databases.

Statistical Analysis

The SPSS 21.0 software (Chicago, IL) was used for statistical analysis. If the measurement data were normally distributed, then they were expressed as the mean ± standard deviation, and if not then the median (quartile) was used. Analysis of variance was used when the measurement data met the normal distribution and the homogeneity of variance

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

test; otherwise, the H test was used. Normally distributed measurement data between the 2 groups (T2DM vs NAFLD) were compared using the independent-sample *t*-test; otherwise, the Wilcoxon nonparametric test was used. The values $P < .05$, $P < .01$, and $P < .001$ were considered as statistically significant.

Results

Biochemical Analysis

The clinical laboratory data are displayed in **TABLE 1**. Compared to patients with T2DM, significantly increased levels of TP, ALB, ALT, AST, TCHO, ChE, TG, LDL, and ApoA ($P < .01$) were observed in patients with T2DM and NAFLD. Patients with coexisting NAFLD and T2DM also had significantly higher levels of TP, TG, Glu, and GHb than those with NAFLD. Higher levels of TP, ALB, ALT, AST, TCHO, ChE, LDL, ApoA, ApoB, Glu, and GHb were observed in the NAFLD group compared to the T2DM group.

Repeatability and Stability for UPLC-Orbitrap-MS Platform

The QC specimens were employed to evaluate system repeatability and stability. In both positive and negative ion modes, a high degree of aggregation of all QCs that were clearly separated from other groups in the PCA score plot was observed, indicating the good stability of the UPLC-Orbitrap-MS platform throughout the entire experiment.

Metabolomics

Multivariate Analysis of Serum Metabolic Profile

We used PCA to determine the general interrelations between the groups. A PCA plot shows clusters of specimens based on their similarity. As shown in **FIGURES 1 to 3**, the PCA score plots showed a clear separation between the NAFLD and NAFLD + T2DM groups in both positive and negative scan modes. A similar trend was observed between the T2DM and NAFLD + T2DM groups. We performed OPLS-DA to highlight the differences between the groups and indicate the driving forces among the variables. As shown in **FIGURES 1 to 3**, the datasets were divided into 2 clusters in all patients with valid model fits. The relevant R^2Y and Q^2Y values were all above 0.7, indicating good predictive accuracy of the model.

Identification of Potential Biomarkers

In the OPLS-DA model, metabolites with a variable importance in projection value > 1 and $P < .05$ were selected as variables that were responsible for the separation between the groups. A total of 50 serum metabolites that significantly changed between the NAFLD group and the NAFLD + T2DM group were identified as potential biomarkers (**TABLE 2**), including phosphatidylcholine (PC), lysophosphatidylcholine (LPC), fatty acid (FA), and components of the glutamate and carnitine metabolism pathways. By comparing the serum metabolomic profiles of the T2DM group and the NAFLD + T2DM group, we also identified 41 potential serum biomarkers (**TABLE 3**), most of which were lipids, amino acids, and fatty acids.

Discussion

The pathogenesis of NAFLD, which used to be described as a "two-hit" process, is now regarded as a "multiple parallel hits model,"[12] which considers multiple "hits, strikes, blows" acting together on genetically predisposed patients to induce NAFLD.[13] Among the multiple factors contributing to the development of NAFLD, insulin resistance plays a key role and is critical for the establishment of lipotoxicity, oxidative

TABLE 1. Clinical Biochemistry Characteristics

Index	Group			*P* Value
	NAFLD + T2DM	T2DM	NAFLD	
TB	14.57 ± 0.65	13.98 ± 0.77	14.39 ± 0.61	.534
DBIL	2.63 ± 0.12	2.67 ± 0.12	2.67 ± 0.12	.924
TP	71.47 ± 0.6	65.97 ± 0.77**	69.35 ± 0.46*##	.001***
ALB	40.57 ± 0.43	37.83 ± 0.52**	39.63 ± 0.38##	.001***
ALT	40.87 ± 3.63	22.1 ± 1.49**	37.15 ± 3.01##	.001***
AST	33.17 ± 2.26	24.82 ± 1.92**	30.93 ± 1.86#	.001***
ALP	88.5 ± 2.7	89.63 ± 5.47	89.97 ± 2.8	.105
GGT	48.8 ± 5.35	37.74 ± 6.74	50.27 ± 4.65	.001***
TC	5.13 ± 0.12	4.34 ± 0.12**	5.06 ± 0.2##	.001***
ChE	8832.22 ± 204.47	7015.26 ± 222.42**	8902.01 ± 166.2##	.001***
TG	2.53 ± 0.21	1.54 ± 0.09**	1.9 ± 0.09**	.001***
HDL	1.19 ± 0.05	1.09 ± 0.03	1.1 ± 0.04	.331
LDL	3.27 ± 0.1	2.89 ± 0.09**	3.38 ± 0.11##	.001***
ApoA	1.22 ± 0.02	1.11 ± 0.02**	1.22 ± 0.03##	.002**
ApoB	0.91 ± 0.02	0.87 ± 0.03	0.97 ± 0.04#	.045*
FPG	8.2 ± 0.27	7.93 ± 0.35	5.45 ± 0.07**##	.001***
HbA1c	7.91 ± 0.14	8.35 ± 0.26	5.69 ± 0.1**##	.001***

ALB, albumin; ALP; alkaline phosphatase; ALT, alanine aminotransferase; ApoA, apolipoprotein A; ApoB, apolipoprotein B; AST, aspartate aminotransferase; ChE, cholinesterase; DBIL, direct bilirubin; FPG, fasting plasma glucose; GGT, γ-glutamyltransferase; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TB, total bilirubin; TC, total cholesterol; TG, triglycerides; TP, total protein.
*T2DM vs NAFLD vs NAFLD + T2DM: *P < .05,**P < .01;***P < .001; T2DM or NAFLD vs NAFLD + T2DM: *P < .05,**P < .01; NAFLD vs T2DM: #P < .05,##P < .01.*

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

FIGURE 1. Principal component analysis score plots for nonalcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM), and NAFLD + T2DM. A, Positive ionization mode. B, Negative ionization mode.

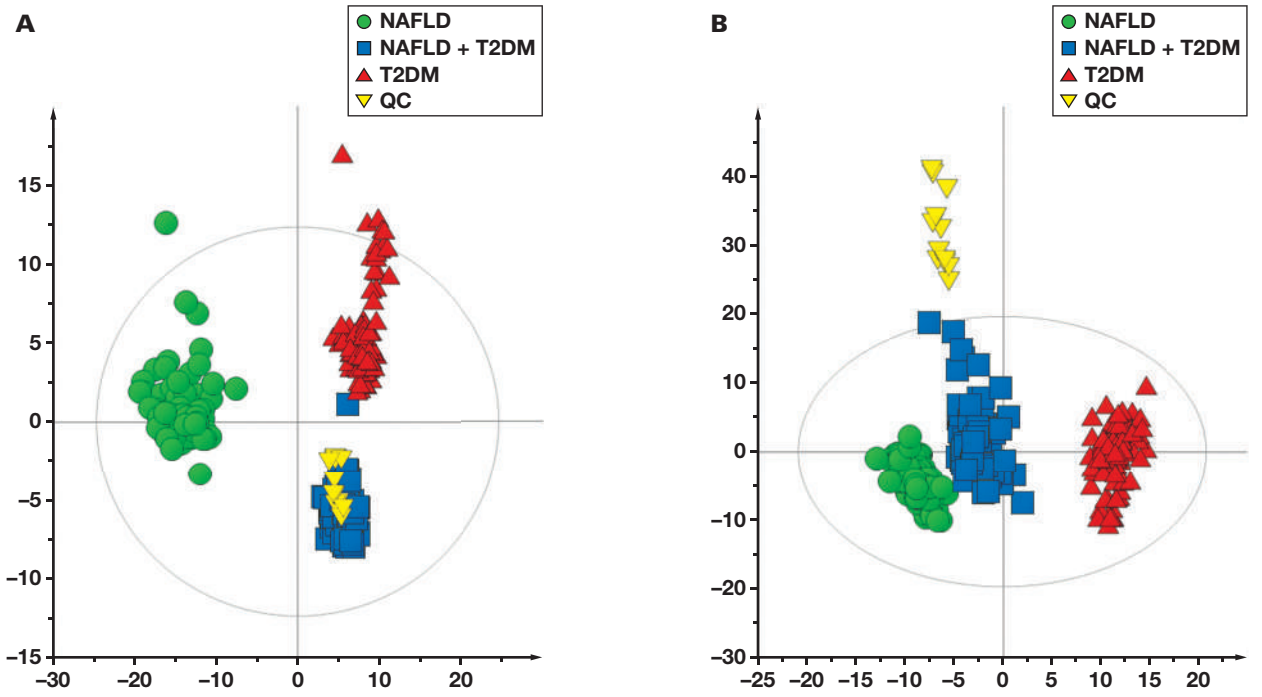
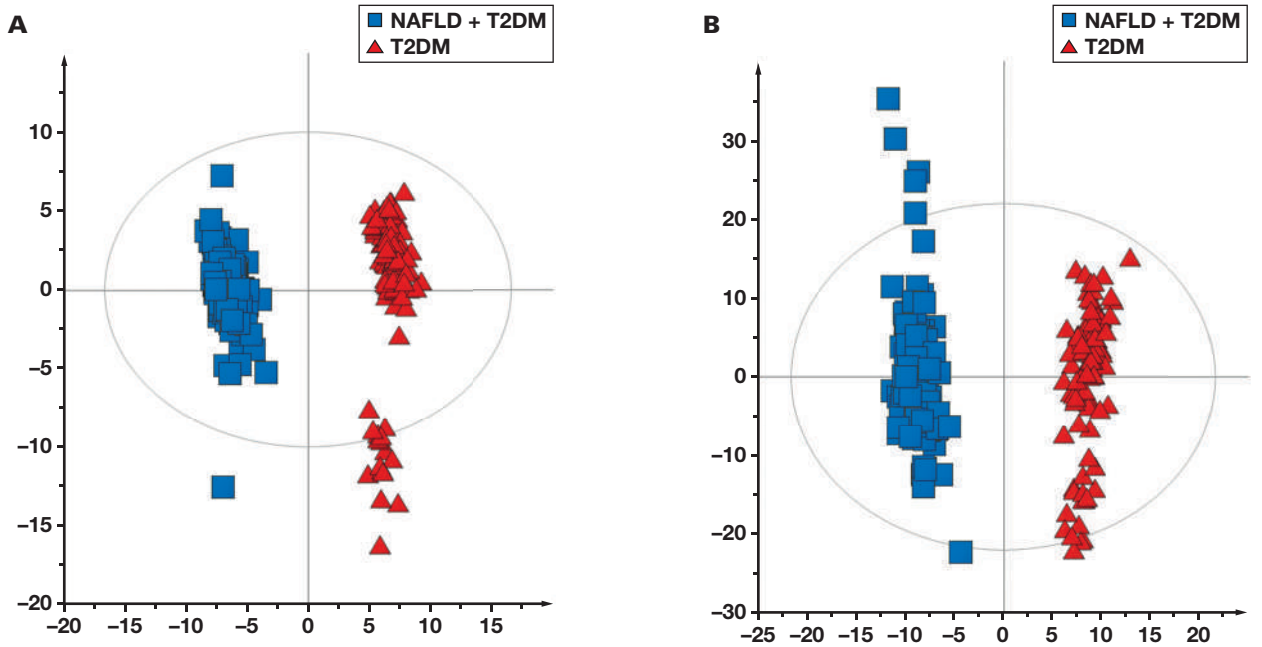


FIGURE 2. Orthogonal partial least-squares discriminant analysis plots for type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD) + T2DM. A, Positive ionization mode. B, Negative ionization mode.



stress, and inflammatory cascade activation. Insulin resistance is also related to T2DM and seems to be a risk factor for the progression of NAFLD in patients with T2DM.[13-15]

There is a more complex relationship between NAFLD and T2DM, with 2-way influence and correlation.[16] Accumulating evidence has implied that NAFLD, as one of the independent risk factors for T2DM, often appears before T2DM and is an early predictor and important influencing factor of T2DM.[17,18] The incidence of NAFLD in patients with T2DM is significantly increased. A cross-sectional study by Petersen and Shulman[19] reported that NAFLD occurs in up to 65% of patients with T2DM and that the incidence of liver fibrosis is as high as 7.1%. A meta-analysis of 296,439 patients showed that NAFLD can sig-

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

TABLE 2. Identities of Differential Metabolites Between NAFLD and NAFLD + T2DM

Number	Name	Molecular Weight	Fold Change (NAFLD + T2DM/NAFLD)	VIP	*P* Value
1	UDP-L-rhamnose	550.06207	1.11	1.302	6.46E-08
2	UDP-D-galacturonate	580.03679	0.46	1.826	1.57E-19
3	Taurine	125.01419	1.48	1.286	1.07E-15
4	Sphingosine 1-phosphate	301.2968	1.15	2.626	1.16E-04
5	sn-glycero-3-Phosphocholine	257.1022	0.72	1.606	1.50E-10
6	SM(d18:1/24:1(15Z))	812.67613	0.55	1.203	3.63E-08
7	SM(d18:0/22:3(10Z,13Z,16Z))	780.61113	0.55	1.195	3.13E-08
8	PC(18:2(9Z,12Z)/P-18:1(11Z))	767.58348	0.51	1.021	4.19E-06
9	PC(14:1(9Z)/22:2(13Z,16Z))	783.57685	1.76	1.260	1.89E-09
12	PC(14:0/20:1(11Z))	759.57433	1.73	1.089	1.49E-07
13	PC(14:0/18:2(9Z,12Z))	729.52812	0.44	1.485	1.25E-12
14	PC(14:0/18:1(11Z))	731.54517	1.96	1.002	4.16E-06
15	Malic acid	134.02097	2.62	1.360	2.87E-19
16	LysoPC(20:4(5Z,8Z,11Z,14Z))	543.33071	0.67	1.651	2.82E-14
17	LysoPC(18:0)	523.36246	0.44	2.128	5.25E-27
18	L-Threose	120.04174	2.28	1.539	7.46E-26
19	L-Threonicacid	136.03663	1.35	1.062	3.15E-11
20	L-glutamine	144.08942	1.57	1.161	1.64E-01
21	L-Glutamate	147.05255	1.61	1.177	2.44E-13
22	Leucyl-Proline	228.14688	1.47	1.143	6.84E-08
23	L-Asparagine	132.05321	1.21	1.163	4.51E-07
24	L-Arginine	174.11137	1.27	1.033	3.89E-06
25	FA oxo(12:0)	212.14057	1.23	1.289	1.85E-02
26	FA methyl(18:0)	294.25445	0.64	1.552	4.89E-11
27	FA hydroxy(20:4)	334.21346	1.09	1.107	1.00E-05
28	FA hydroxy(20:3)	338.24455	1.11	1.306	5.10E-08
29	FA hydroxy(18:0)	314.2456	0.17	1.726	1.44E-33
30	FA(20:3)	320.23502	0.08	1.335	7.85E-18
31	FA(18:1)	278.22349	1.40	1.063	4.67E-06
32	FA(16:2)	299.28129	0.27	2.876	1.54E-03
33	D-Serine	105.0423	1.35	1.738	9.32E-17
34	Dipalmitoylphosphatidylcholine	733.56062	0.22	2.116	1.25E-27
35	Dehydroepiandrosteronesulfate	368.16548	0.14	1.413	2.95E-20
36	alpha-L-Arabinose	150.05213	1.69	1.088	1.16E-10
37	Acetyl-L-carnitine	203.11531	0.77	2.088	1.33E-06
38	6 alpha-Hydroxy-castasterone	466.36593	0.27	1.161	3.56E-13
39	5-Valerolactone	100.05206	1.34	1.375	5.12E-05
40	5-Hydroxy-L-tryptophan	220.08433	0.77	1.474	6.55E-12
41	3-O-Sulfogalactosylceramide (d18:1/24:0)	891.64548	0.60	1.346	4.34E-11
42	2-Octenoylcarnitine	441.34381	1.54	1.015	2.62E-06
43	1-Phenylethylamine	121.08869	0.32	2.191	1.74E-31
44	1-Palmitoylglycerophosphocholine	495.3325	0.23	1.444	3.14E-21
45	1-Oleoylglycerophosphocholine	521.34637	0.43	1.063	1.17E-06
46	1-Linoleoylglycerophosphocholine	519.33072	0.68	1.243	7.71E-08
47	FA amino(5:0)	117.07847	1.59	1.129	4.15E-05
48	O-Palmitoyl-R-carnitine	399.33352	0.83	1.035	2.23E-06
49	FA(6:1)	98.07271	22.29	1.129	3.41E-07
50	(R)-10-Hydroxystearate	300.26616	0.57	1.111	3.56E-12

NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; VIP, variable important in the projection.

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

TABLE 3. Identities of Differential Metabolites Between T2DM and NAFLD + T2DM

Number	Name	Molecular Weight	Fold Change (NAFLD + T2DM/NAFLD)	VIP	*P* Value
1	sn-glycero-3-Phosphocholine	257.1022	0.32	2.381	1.20E-36
2	SM(d18:1/24:1(15Z))	812.6761	0.63	1.081	5.91E-06
3	SM(d18:1/18:1(11Z))	728.5804	0.42	1.402	3.32E-12
4	Phenylalanylphenylalanine	312.1466	1.59	1.216	1.77E-08
5	PC(o-20:1(11Z)/20:4(8Z,11Z,14Z,17Z))	821.6316	1.33	1.232	9.23E-08
6	PC(o-18:2(9Z,12Z)/22:0)	827.6751	1.65	1.051	7.86E-07
7	PC(o-16:0/22:0)	803.6749	4.67	2.026	1.86E-26
8	PC(o-14:0/16:1(9Z))	689.5387	2.34	1.340	3.67E-08
9	PC(18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	833.5919	1.75	2.128	2.91E-21
10	PC(16:0/22:5(4Z,7Z,10Z,13Z,16Z))	807.5759	3.42	1.973	2.17E-25
11	PC(14:1(9Z)/22:2(13Z,16Z))	783.5769	2.62	1.666	8.53E-17
12	PC(14:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	777.5288	1.60	1.121	5.41E-06
13	PC(14:0/22:5(4Z,7Z,10Z,13Z,16Z))	779.543	3.93	1.752	3.92E-19
14	PC(14:0/22:4(7Z,10Z,13Z,16Z))	781.5621	2.44	1.503	1.82E-13
15	PC(14:0/20:4(5Z,8Z,11Z,14Z))	753.5302	2.68	1.562	2.79E-14
16	PC(14:0/20:2(11Z,14Z))	757.5586	1.71	1.091	1.31E-07
17	PC(14:0/20:1(11Z))	759.5743	2.20	1.368	4.80E-12
18	PC(14:0/18:2(9Z,12Z))	729.5281	1.50	1.109	7.79E-07
19	N-Acetyl-L-histidine	197.0801	0.59	1.715	4.91E-12
20	Malic acid	134.021	0.43	1.195	1.06E-06
21	LysoPC(18:0)	523.3625	0.51	1.837	7.68E-18
22	L-Leucine	131.094	1.70	2.202	8.91E-22
23	L-Glutamate	147.0527	0.58	1.009	4.11E-05
24	L-Carnitine	161.1048	0.71	1.007	3.00E-06
25	L-Arginine	174.1114	1.37	1.051	1.70E-06
26	Lactic acid	90.03127	0.87	1.005	5.13E-05
27	L-Tryptophan	204.0893	1.47	2.236	4.44E-30
28	L-Phenylalanine	165.0785	1.27	1.418	9.35E-12
29	Dipalmitoylphosphatidylcholine	733.5606	1.71	1.039	1.53E-06
30	Sphingosine 1-phosphate	379.2478	1.18	1.112	5.69E-06
31	Cytidine	243.0854	0.52	1.787	3.85E-13
32	Acetyl-L-carnitine	203.1153	0.36	2.347	2.06E-32
33	2-Hydroxy-2_4-pentadienoate	114.0312	0.45	1.788	2.96E-19
34	2-coumarate	164.0469	1.17	1.017	9.02E-07
35	1-Pyrroline-4-hydroxy-2-carboxylate	129.0421	0.80	1.015	4.64E-05
36	1-phenylpropane-1_2-dione	148.052	1.27	1.363	5.82E-12
37	1-Linoleoylglycerophosphocholine	519.3307	0.67	1.250	1.61E-07
38	1-alpha-D-Galactosyl-myo-inositol	342.1167	0.13	1.541	3.87E-10
39	FA(18:2)	280.2406	3.48	1.746	1.56E-13
40	(S)-N-Methylcanadine	353.1618	1.85	1.153	9.38E-08
41	(E)-Glutaconate	130.0261	0.24	1.065	1.37E-05

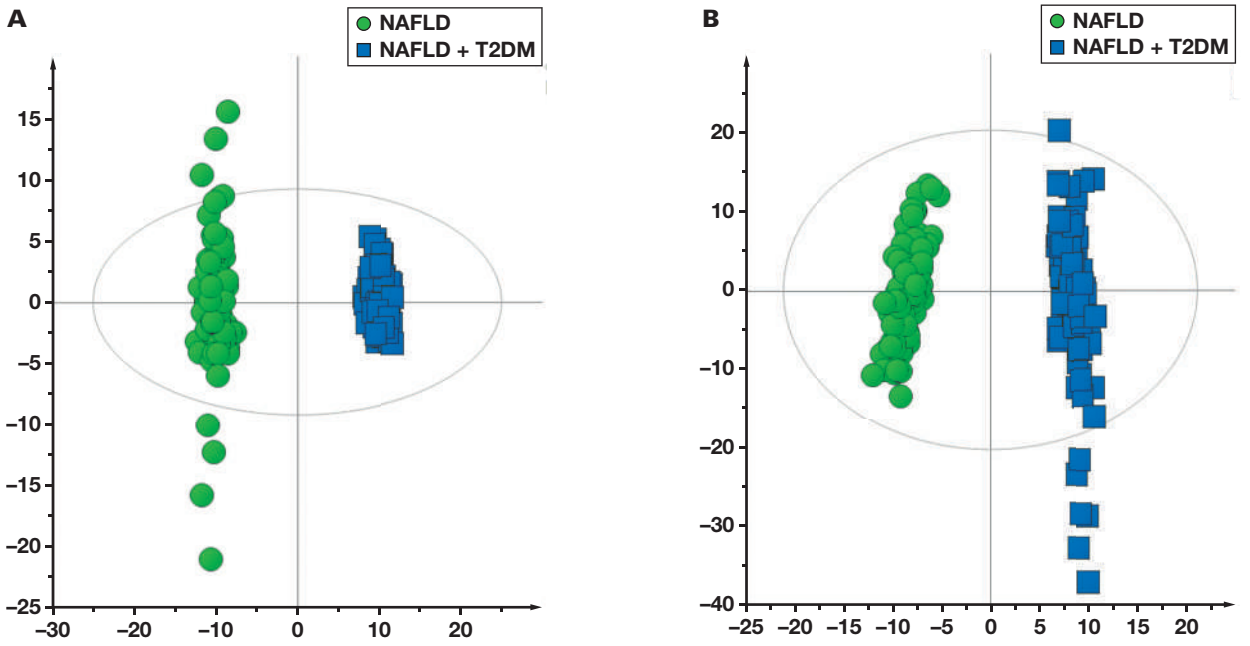
NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; VIP, variable important in the projection.

nificantly increase the risk of diabetes.[8] Another meta-analysis showed that patients with NAFLD diagnosed by ultrasound or liver enzymes have a significantly increased risk of diabetes in 5 years of follow-up.[7] In a regression model that predicted the risk of T2DM, patients with NAFLD were at a greater risk of T2DM than those with increased waist circumference, suggesting that for nonobese individuals, NAFLD is an important risk factor for T2DM.[20] Lipotoxin, mitochondrial function, cytokines, and adipocytokines have been proposed to play a major role in NAFLD and T2DM.[21]

Research has shown that T2DM is an important risk factor for NAFLD. In addition, T2DM seems to accelerate the progression of liver disease in NAFLD.[5] It is also well established that T2DM has adverse

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

FIGURE 3. Orthogonal partial least-squares discriminant analysis plots for nonalcoholic fatty liver disease (NAFLD) and NAFLD + type 2 diabetes mellitus (T2DM). A, Positive ionization mode. B, Negative ionization mode.



effects on NAFLD, including its promotion of the progression of NAFLD to more severe forms (nonalcoholic steatohepatitis, advanced fibrosis, and cirrhosis). Patients with T2DM are at a 2-fold higher risk of developing cirrhosis and hepatocellular carcinoma (HCC).[22] A large-scale epidemiology study in China reported that patients with T2DM are at a substantially increased risk for NAFLD, cirrhosis, and HCC compared to people without diabetes.[23] Epidemiological studies in Europe also have shown that T2DM is a risk factor for liver fibrosis.[24,25] In patients with prediabetes or T2DM, continuous liver biopsy has shown progressive fibrosis.[26] Another study showed that patients with T2DM are at a substantially increased risk of moderate to severe liver damage and are more likely to develop HCC.[27] In addition, T2DM seems to be a risk factor for nonalcoholic steatohepatitis progression to end-stage fibrosis, cirrhosis, and HCC.[25,28] At present, a deeper understanding of the relationship between NAFLD and T2DM is lacking, and there is still a need for much experimental data and research results for further confirmation.

Liver enzymes are the first laboratory tests that every clinician will consider worth evaluating the degree of liver damage. Elevated ALT and AST levels often indicate inflamed or injured liver cells.[29,30] As shown in the clinical laboratory data displayed in **TABLE 2**, patients in the NAFLD and NAFLD + T2DM groups had significantly more elevated ALT and AST levels than those in the T2DM group ($P < .01$), and the highest levels of ALT and AST were observed in patients with NAFLD and T2DM, suggesting that the coexistence of NAFLD and T2DM may increase the likelihood of developing more severe liver damage.

Our data show that patients in the NAFLD + T2DM group showed a significantly increased level of fasting glucose, in agreement with the evidence that patients with T2DM and NAFLD often have poorer glycemic control than those without NAFLD, suggesting that NAFLD may have adverse effects on glycemic control in T2DM. However, hemoglobin A1c (HbA1c) in T2DM was higher than in the other 2 groups, suggesting that there is an unusual relationship between HbA1c and NAFLD.[31]

With respect to lipid levels, patients with NAFLD and T2DM had significantly higher levels of TG, total cholesterol, and LDL ($P < .01$) than those who had only NAFLD or T2DM, and there was no statistical difference in HDL. The association between NAFLD and T2DM can be explained by insulin resistance, dyslipidemia, and TG accumulation.[32] It is suggested that insulin resistance can promote hepatic TG accumulation.[33] These data all indicate that patients with NAFLD who also have T2DM may suffer from more severe liver inflammation, dyslipidemia, abnormal blood glucose, and metabolic disorders.

Metabolomics results showed that 50 serum metabolites significantly changed in patients with NAFLD with T2DM compared to those without T2DM. Among these, the levels of PC, LPC, and FA were significantly increased, as were the metabolites involved in the phenylalanine, sphingolipid, and carnitine metabolic pathways.

Carnitine transports fatty acids into the mitochondria for oxidation and energy production, and carnitine palmitoyl transferase-1 (CPT-1) is an essential enzyme responsible for this process. In NAFLD and other hepatic injury models, whereas significant upregulated carnitine was observed, the expression of CPT-1 was decreased.[34,35] Studies have shown that CPT-1 activity promotes fatty acid oxidation and decreased steatosis, so CPT-1 is thus emerging as a new drug target for the treatment of liver diseases. Our results indicate that the serum concentrations of carnitine in patients with NAFLD and T2DM were clearly less than in those with only NAFLD or

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

T2DM. Compared to the patients with T2DM, the levels of carnitines and acyl-carnitines in patients with T2DM complicated by NAFLD decreased 34% and 64%, respectively ($P < .01$). However, no significant change in carnitines was observed between patients with NAFLD and NAFLD + T2DM ($P > .05$). These data suggest that patients with NAFLD may have reduced carnitines.

Phenylalanine and its related metabolites are mainly metabolized in the liver.[36-38] Evidence has shown that the increased levels of phenylalanine are highly correlated with obesity and liver steatosis. It has also been reported that phenylalanine levels in patients with T2DM patients are significantly increased.[39] Therefore, the deterioration of liver function in patients with NAFLD may cause a decline in phenylalanine metabolism and ultimately lead to the accumulation of phenylalanine and its related metabolites in the liver and serum.

Sphingosine 1-phosphate (S1-P) is an important circulating lipid mediator that is derived from ceramide. It plays a vital role in regulating cell growth, apoptosis, and inflammation. Studies have found that the S1P-mediated SphK1/S1P/S1PR signaling pathway is involved in the pathobiology of liver injury and liver fibrosis.[40,41] The S1-P levels of patients with coexisting T2DM and NAFLD were 1.18 and 1.15 times that of patients in the T2DM group and the NAFLD group, respectively. The results indicate that the comorbidity of NAFLD and T2DM will further increase the S1-P level.

Among the biomarkers listed in **TABLES 2** and **3**, there are 13 common metabolites that show similar changing trends. In addition to carnitine and S1-P, most of these biomarkers are phospholipids, including SM(d18:1/24:1(15Z)), PC(14:1(9Z)/22:2(13Z,16Z)), PC(14:0/20:1(11Z)), lysoPC(18:0), sn-glycero-3-phosphocholine, 1-linoleoyl-glycerophosphocholine, and PC(14:0/18:2 (9Z,12Z). Studies have reported that the levels of phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and lysophosphatidylethanolamine were increased in patients with NAFLD and patients with T2DM.[42-44] These accumulated lipid metabolites play important roles in the development of NAFLD and T2DM. In our study, these lipid biomarkers increased significantly when NAFLD and T2DM compared with patients with NAFLD or T2DM. This result also confirms the close correlation between NAFLD and T2DM.

Conclusion

We identified a set of serum metabolites and clinical variables that offer a potential application for diagnosing the coexistence of NAFLD and T2DM. In clinical practice, the potential biomarkers could be used as a first step to appropriately characterize each patient's cohort and enable a more precise prognosis of disease development. In addition, this study helps provide new molecular insights into the links between NAFLD and T2DM. In the future, we will conduct in-depth mechanism research on the signal pathways related to carnitine, phenylalanine, and S1-P and conduct lipidomics research to find more lipid markers and signal pathways related to lipid metabolism.

Acknowledgments

Study concept and design: CH and JZ. Acquisition of data: TW, DN, and YJ. Analysis and interpretation of data: JW, QC, and MZ. Drafting of the manuscript: XZ. Revision of the manuscript: all authors. Statistical analysis: YJ. This work was financially supported by the National Natural Science Foundation of China (21904022), the Shanghai Municipal Commission of Health and Family Planning general project for clinical research of the health industry (201840377, 201940449), and the Putuo District of Shanghai Science and Technology Commission Research Project (ptkwws201813). MZ was funded by a project within the budget of Shanghai University of Traditional Chinese Medicine (2021LK079). All data are included in this article.

REFERENCES

1. Li J, Zou B, Yeo YH, et al. Prevalence, incidence, and outcome of non-alcoholic fatty liver disease in Asia, 1999-2019: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol.* 2019;4(5):389–398.
2. Pimpin L, Cortez-Pinto H, Negro F, et al; EASL HEPAHEALTH Steering Committee. Burden of liver disease in Europe: epidemiology and analysis of risk factors to identify prevention policies. *J Hepatol.* 2018;69(3):718–735.
3. Williams R, Karuranga S, Malanda B, et al. Global and regional estimates and projections of diabetes-related health expenditure: results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes Res Clin Pract.* 2020;162:108072.
4. Targher G, Corey KE, Byrne CD, et al. The complex link between NAFLD and type 2 diabetes mellitus—mechanisms and treatments. *Nat Rev Gastroenterol Hepatol*. 2021;18(19):599–612.
5. Younossi ZM, Golabi P, de Avila L, et al. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: a systematic review and meta-analysis. *J Hepatol.* 2019;71(4):793–801.
6. European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO clinical practice guidelines for the management of non-alcoholic fatty liver disease. *Diabetologia*. 2016;59(6):1121–1140.
7. Ballestri S, Zona S, Targher G, et al. Nonalcoholic fatty liver disease is associated with an almost twofold increased risk of incident type 2 diabetes and metabolic syndrome: evidence from a systematic review and meta-analysis. *J Gastroenterol Hepatol.* 2016;31(5):936–944.
8. Mantovani A, Byrne CD, Bonora E, Targher G. Nonalcoholic fatty liver disease and risk of incident type 2 diabetes: a meta-analysis. *Diabetes Care.* 2018;41(2):372–382.
9. American Diabetes Association. 4. Comprehensive medical evaluation and assessment of comorbidities: *Standards of Medical Care in Diabetes-2020*. *Diabetes Care*. 2020;43(suppl 1):S37–S47.
10. Mato JM, Alonso C, Noureddin M, Lu SC. Biomarkers and subtypes of deranged lipid metabolism in non-alcoholic fatty liver disease. *World J Gastroenterol.* 2019;25(24):3009–3020.
11. National Workshop on Fatty Liver and Alcoholic Liver Disease, Chinese Society of Hepatology, Chinese Medical Association; Fatty Liver Expert Committee, Chinese Medical Doctor Association. Guidelines of prevention and treatment for nonalcoholic fatty liver disease: a 2018 update [in Chinese]. *Zhonghua Gan Zang Bing Za Zhi* 2018;26(3):195–203.
12. Takaki A, Kawai D, Yamamoto K. Multiple hits, including oxidative stress, as pathogenesis and treatment target in non-alcoholic steatohepatitis (NASH). *Int J Mol Sci.* 2013;14(10):20704–20728.
13. Gastaldelli A, Cusi K. From NASH to diabetes and from diabetes to NASH: mechanisms and treatment options. *JHEP Rep.* 2019;1(4):312–328.
14. Brar G, Tsukamoto H. Alcoholic and non-alcoholic steatohepatitis: global perspective and emerging science. *J Gastroenterol.* 2019;54(3):218–225.

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

15. Smith GI, Shankaran M, Yoshino M, et al. Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *J Clin Invest.* 2020;130(3):1453–1460.
16. Jarvis H, Craig D, Barker R, et al. Metabolic risk factors and incident advanced liver disease in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of population-based observational studies. *PLoS Med.* 2020;17(4):e1003100.
17. Lee YH, Cho Y, Lee BW, et al. Nonalcoholic fatty liver disease in diabetes. Part I: epidemiology and diagnosis. *Diabetes Metab J.* 2019;43(1):31–45.
18. Valenti L, Bugianesi E, Pajvani U, Targher G. Nonalcoholic fatty liver disease: cause or consequence of type 2 diabetes? *Liver Int.* 2016;36(11):1563–1579.
19. Petersen MC, Shulman GI. Roles of diacylglycerols and ceramides in hepatic insulin resistance. *Trends Pharmacol Sci.* 2017;38(7):649–665.
20. Kim SS, Cho HJ, Kim HJ, et al. Nonalcoholic fatty liver disease as a sentinel marker for the development of diabetes mellitus in non-obese subjects. *Dig Liver Dis.* 2018;50(4):370–377.
21. Tilg H, Moschen AR, Roden M. NAFLD and diabetes mellitus. *Nat Rev Gastroenterol Hepatol.* 2017;14(1):32–42.
22. Weiß J, Rau M, Geier A. Non-alcoholic fatty liver disease: epidemiology, clinical course, investigation, and treatment. *Dtsch Arztebl Int.* 2014;111(26):447–452.
23. Pang Y, Kartsonaki C, Turnbull I, et al. Diabetes, plasma glucose, and incidence of fatty liver, cirrhosis, and liver cancer: a prospective study of 0.5 million people. *Hepatology.* 2018;68(4):1308–1318.
24. Roulot D, Costes JL, Buyck JF, et al. Transient elastography as a screening tool for liver fibrosis and cirrhosis in a community-based population aged over 45 years. *Gut.* 2011;60(7):977–984.
25. Koehler EM, Plompen EP, Schouten JN, et al. Presence of diabetes mellitus and steatosis is associated with liver stiffness in a general population: the Rotterdam study. *Hepatology.* 2016;63(1):138–147.
26. Hazlehurst JM, Woods C, Marjot T, Cobbold JF, Tomlinson JW. Non-alcoholic fatty liver disease and diabetes. *Metabolism.* 2016;65(8):1096–1108.
27. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology.* 2018;67(1):328–357.
28. Alexander M, Loomis AK, van der Lei J, et al. Risks and clinical predictors of cirrhosis and hepatocellular carcinoma diagnoses in adults with diagnosed NAFLD: real-world study of 18 million patients in four European cohorts. *BMC Med.* 2019;17(1):95.
29. Gitter AH, Theis J. A low-cost voltage stepper device for use in patch-clamp experiments. *Pflugers Arch.* 1987;408(2):194–195.
30. Chen CH, Huang MH, Yang JC, et al. Prevalence and etiology of elevated serum alanine aminotransferase level in an adult population in Taiwan. *J Gastroenterol Hepatol.* 2007;22(9):1482–1489.
31. Amiri Dash Atan N, Koushki M, Motedayen M, et al. Type 2 diabetes mellitus and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *Gastroenterol Hepatol Bed Bench.* 2017;10(suppl 1):S1–S7.
32. Forlani G, Giorda C, Manti R, et al.; AMD-Annals Study Group. The burden of NAFLD and its characteristics in a nationwide population with type 2 diabetes. *J Diabetes Res.* 2016;2016:2931985.
33. Brown MS, Goldstein JL. Selective versus total insulin resistance: a pathogenic paradox. *Cell Metab.* 2008;7(2):95–96.
34. Zhang J, Liang X, Li J, et al. Apigenin attenuates acetaminophen-induced hepatotoxicity by activating AMP-activated protein kinase/carnitine palmitoyltransferase I pathway. *Front Pharmacol.* 2020;11:549057.
35. Dusabimana T, Park EJ, Je J, et al. P2Y2R deficiency ameliorates hepatic steatosis by reducing lipogenesis and enhancing fatty acid beta-oxidation through AMPK and PGC-1alpha induction in high-fat diet-fed mice. *Int J Mol Sci* 2021;22(11):5528.
36. Zello GA, Pencharz PB, Ball RO. Phenylalanine flux, oxidation, and conversion to tyrosine in humans studied with L-[1-13C]phenylalanine. *Am J Physiol.* 1990;259(6 Pt 1):E835–E843.
37. Burke PA, Stack JA, Wagner D, Lewis DW, Jenkins RL, Forse RA. L-[1-(13)C] Phenylalanine oxidation as a measure of hepatocyte functional capacity in end-stage liver disease. *Am J Surg.* 1997;173(4):270–273.
38. Ishii Y, Suzuki S, Kohno T, et al. Patients with severe liver cirrhosis followed up by L-[1-(13)C] phenylalanine breath test. *J Gastroenterol.* 2003;38(11):1086–1090.
39. Mook-Kanamori DO, de Mutsert R, Rensen PC, et al. Type 2 diabetes is associated with postprandial amino acid measures. *Arch Biochem Biophys.* 2016;589:138–144.
40. Lan T, Li C, Yang G, et al. Sphingosine kinase 1 promotes liver fibrosis by preventing miR-19b-3p-mediated inhibition of CCR2. *Hepatology.* 2018;68(3):1070–1086.
41. Wang Y, Aoki H, Yang J, et al. The role of sphingosine 1-phosphate receptor 2 in bile-acid-induced cholangiocyte proliferation and cholestasis-induced liver injury in mice. *Hepatology.* 2017;65(6):2005–2018.
42. Hu C, Wang T, Zhuang X, et al. Metabolic analysis of early nonalcoholic fatty liver disease in humans using liquid chromatography-mass spectrometry. *J Transl Med.* 2021;19(1):152.
43. Razquin C, Toledo E, Clish CB, et al. Plasma lipidomic profiling and risk of type 2 diabetes in the PREDIMED trial. *Diabetes Care.* 2018;41(12):2617–2624.
44. Hou B, He P, Ma P, et al. Comprehensive lipidome profiling of the kidney in early-stage diabetic nephropathy. *Front Endocrinol (Lausanne).* 2020;11:359.

Downloaded from https://academic.oup.com/labmed/article/53/4/360/6514475 by guest on 28 February 2025

Outcome of ABO-Incompatible Kidney Transplantation According to ABO Type of Transfused Plasma: Comparative Analysis Between "Universal" AB and Donor-Type Plasma

Han Joo Kim, MD, MSc,[1] Jin Seok Kim, MT, MD,[1] John Jeongseok Yang, MD, MSc,[1] Yousun Chung, MD, MSc,[2] Hyungsuk Kim, MD, MSc,[3] Sung Shin, MD, PhD,[4] Young Hoon Kim, MD, PhD,[4] Sang-Hyun Hwang, MD, PhD,[1] Heung-Bum Oh, MD, PhD,[1] Duck-Jong Han, MD, PhD,[4] Hyunwook Kwon, MD, PhD,[4] Dae-Hyun Ko, MD, PhD[1,]*

[1]Department of Laboratory Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea, [2]Department of Laboratory Medicine, Kangdong Sacred Heart Hospital, Seoul, Republic of Korea, [3]Department of Laboratory Medicine, Seoul National University Hospital, Seoul, Republic of Korea, [4]Division of Kidney and Pancreas Transplantation, Department of Surgery, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea; *To whom correspondence should be addressed. daehyuni1118@amc.seoul.kr

Keywords: ABO-incompatible, kidney transplantation, ABOi-KT, transfusion, plasma, universal blood type

Abbreviations: ABOi, ABO-incompatible; KT, kidney transplantation; ESRD, end-stage renal disease; TPE, therapeutic plasma exchange; FFP, fresh frozen plasma; DSA, donor-specific antibody; ABMR, antibody-mediated rejection; SD, standard deviation; TCMR, T-cell–mediated rejection; BSH, British Society for Haematology; ANZSBT, Australian and New Zealand Society of Blood Transfusion.

Laboratory Medicine 2022;53:369–375; https://doi.org/10.1093/labmed/lmab122

ABSTRACT

Objective: We compared the clinical outcomes of recipients of ABO-incompatible (ABOi) kidney transplantation (KT) according to the blood group of the plasma transfused.

Materials and Methods: We retrospectively analyzed the data of 60 recipients of ABOi-KT with blood type O and A or B donors. Demographic and clinical characteristics were compared between 2 groups of recipients: 1 group received AB plasma regardless of the donor's blood type (n = 30), and the other group received donor-type plasma (n = 30).

Results: There were no significant differences between the groups in terms of demographic characteristics. Transfusion of donor-type plasma was noninferior to transfusion of type AB plasma in terms of both rejection-free survival and rejection rate (P = .455, P = .335).

Conclusion: There was no significant prognostic difference between the 2 groups. In terms of blood supply and inventory management, we suggest that the blood group of the plasma should match the donor's type.

Kidney transplantation (KT) is the well-known treatment of choice for patients with end-stage renal disease (ESRD). Although the incidence and prevalence of ESRD are expected to increase, the availability of kidney donors remains limited.[1] Many methods to resolve the shortage of transplantable kidneys have been proposed, including those that have been contraindicated for decades, such as ABO-incompatible (ABOi) or HLA antigen–mismatched transplantations. There have been many attempts at ABOi-KT since the early 1950s, but most of them have been unsuccessful. In 1987, Alexandre et al[2] successfully demonstrated ABOi-KT with pretransplantation splenectomy and therapeutic plasma exchange (TPE) performed to overcome the immunologic barriers of ABOi transplantation. Since then, with several desensitization strategies including rituximab therapy, the long-term survival rate of recipients of ABOi-KT has greatly improved. According to the latest data from the Korean Network for Organ Sharing, 1301 KTs were performed in 2018, of which 342 (26%) were for patients who were ABOi.[3] At the Asan Medical Center (Seoul, Republic of Korea), the number of ABOi-KTs has been steadily increasing since 2009, when it was introduced, with up to 84 patients in 2018.

Transfusions are frequently performed for desensitization TPE and perioperative patient care in ABOi solid organ transplantation. The transfusion principle in ABOi hematopoietic stem cell transplantation is well established, but evidence-based guidelines have not been established for transfusion in solid organ transplantation.[4] Furthermore, some healthcare facilities conventionally provide AB-type plasma products or platelets without considering the blood type of the organ donor.[5] However, although AB is considered the universal blood type for plasma or platelet transfusions in emergencies or massive blood

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/369/6527640 by guest on 28 February 2025

transfusions, the AB blood type is rarer than other blood types. According to a 2012 survey in the United States, the distribution of plasma products decreased by 4% each year from 2008 to 2010, whereas the distribution of type AB plasma increased by 6% each year.[6] Moreover, as per this survey, 10 large blood centers in the United States had 27% more patients with AB plasma transfusion in 2011 than in 2006.[6] As per a recent report, plasma distributions decreased in the United States by 16.5% between 2017 and 2019.[7] According to these findings, the supply of AB plasma and platelets should be carefully managed. Moreover, data on the clinical efficacy and safety of universal AB plasma in ABOi solid-organ transplantation are insufficient.

We compared the clinical outcomes of recipients of ABOi-KT according to the blood group of the plasma that was transfused during the perioperative period (ie, AB vs donor-type plasma).

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Board of the Asan Medical Center (2020-0935). The requirement for written informed consent from patients was waived owing to the retrospective nature of this study.

Study Population

Since the first ABOi-KT at the Asan Medical Center, in August 2009, AB fresh frozen plasma (FFP) has been provided for recipients of ABOi-KT who require transfusion support. In December 2011, the blood type standard for FFP for recipients of ABOi-KT was changed to match the donor's blood type.

For this study, we selected patients who underwent ABOi-KT before December 2011 and those who underwent ABOi-KT after December 2011. A total of 162 patients who underwent ABOi-KT were recorded from August 2009 through June 2013. The donor and recipient blood groups were noted for each patient. We excluded 102 patients from our analysis because the donor blood type was AB or because there was a bidirectional mismatch. Finally, 60 patients with blood type O who had group A or B donors were enrolled and divided into 2 groups of 30 each (**FIGURE 1**). The first group included recipients of ABOi-KT with an AB FFP transfusion history, and the second group included recipients of ABOi-KT with a donor-type FFP transfusion history.

Study data were drawn from the electronic medical records of the Asan Medical Center. Parameters for analysis included recipients' age, sex, ABO blood group, cause of ESRD, underlying diseases, baseline and preoperative isoagglutinin titers, transfusion history of any blood product during the perioperative period, presence of donor-specific antibodies (DSAs), rituximab dose, results of kidney biopsy and renal imaging, serum creatinine level, and infection with cytomegalovirus and BK virus, along with the ABO blood group of the donor.

Perioperative Management

The immunosuppressive protocol and perioperative management for recipients of ABOi-KT have been described in previous reports.[8,9] In brief, each recipient was administered tacrolimus, mycophenolate mofetil, and methylprednisolone for immunosuppression during the perioperative period. In addition, 200 mg or 500 mg of single-dose rituximab was administered approximately 1 week before the first TPE for desensitization. The difference in dosage of rituximab was due to the process of establishing the kidney transplant protocol. Differences in rituximab doses are considered to have no effect on survival or prognosis.[10] An anti-CD25 monoclonal antibody, basiliximab (20 mg), was administered on the day of KT and on postoperative day 4, as induction therapy for immunosuppression.[10] Before transplantation, all recipients underwent TPE using COBE Spectra (CaridianBCT, Lakewood, CO) and a dual-lumen central venous catheter (Permcath; Quinton Instruments, Bothell, WA) with 1.0 plasma volume exchange until their isoagglutinin titer was reduced to <4. The replacement fluid was either 5% albumin or FFP. To minimize alloantigen exposure before transplantation, 5% albumin was the first choice of replacement fluid, provided that the recipient's coagulation tests were normal. To reduce the intraoperative bleeding risk, half of the replaced volume was replaced with FFP 2 days before transplantation, and the entire volume was replaced with FFP in the last TPE before transplantation. From August 2009 through November 2011, AB FFP was used (group 1). From December 2011 onward, the blood type of the FFP was dependent on the donor's ABO blood group (group 2). Some patients underwent TPE after KT because of rebound or isoagglutinin titer or a clinical status suggestive of antibody-mediated rejection (ABMR). Details of the protocols for isoagglutinin titer measurement and the plasma exchange strategy were explained in a previous publication.[11]

Renal scan was performed twice––on the day after KT and 2 days subsequently––to survey the function of the allograft. Serum creatinine level and the isoagglutinin titer were measured daily to monitor graft function and detect early acute rejection. If an abnormal result was observed, then renal scan or tissue biopsy was performed to confirm rejection.

Outcome Definitions

Graft survival was defined as the time from KT to graftectomy or return to dialysis. Overall survival was defined as the time from KT to death from any cause. Rejection-free graft survival time was defined as the time from transplantation to rejection. Rejection was defined according to the results of the kidney biopsy; we identified possible rejection when the renal scan results suggested rejection. If the recipient underwent both tests, then the result of the renal biopsy took precedence.

Statistical Analysis

Continuous variables are summarized as means ± standard deviations (SDs) and were compared using 2-sided Student's *t*-tests. Categorical data are presented as numbers and proportions and were compared using the chi-square test or Fisher's exact test. Graft survival and rejection-free graft survival were calculated using the Kaplan-Meier method and were compared using the log-rank test. Multivariable Cox proportional hazards models were used to estimate the relative risk of every clinical variant that is known to affect the outcome of ABOi-KT. All statistical analyses were conducted using PASW Statistics for Windows, version 18.0 (SPSS, Chicago, IL). *P* values ≤.05 were considered statistically significant.

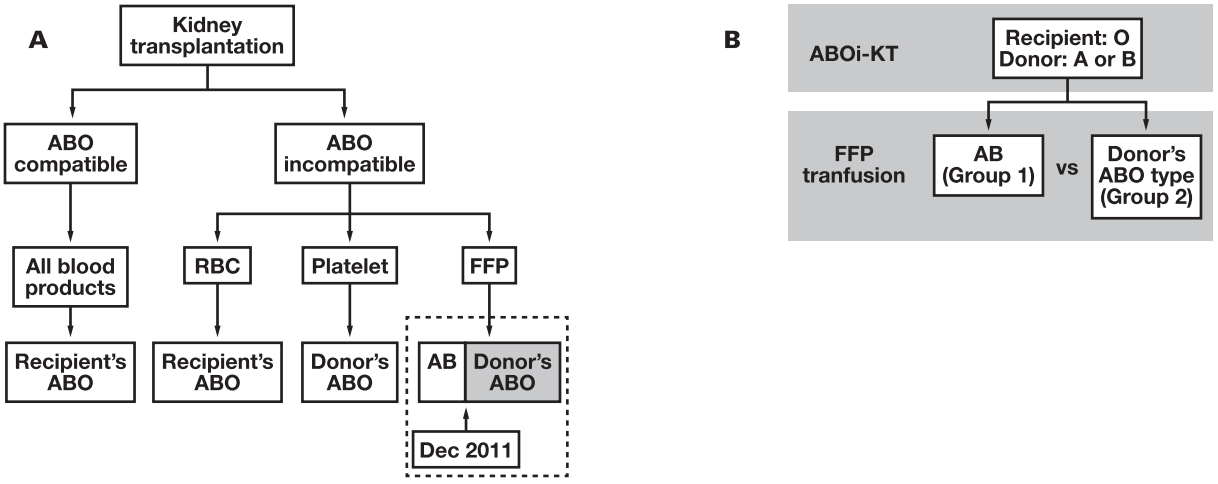
Results

Patient Demographics and Clinical Characteristics

Patient demographics and clinical characteristics are summarized in **TABLE 1**. From August 2009 through June 2013, of the 162 recipients of ABOi-KT, 60 were type O. The mean (SD) age of the recipients was 42.3 (24.2) years, and there was no significant difference in mean age between groups 1 and 2. Moreover, there were no significant intergroup

Downloaded from https://academic.oup.com/labmed/article/53/4/369/6527640 by guest on 28 February 2025

FIGURE 1. Study scheme. A, Transfusion strategy after transplantation at Asan Medical Center. The fresh frozen plasma (FFP) blood type for a recipient of ABO-incompatible (ABOi) kidney transplantation (KT) changed in December 2011. B, This study compared the group that received AB FFP (group 1) with the group that received FFP that matched the donor's blood type (group 2).



differences in terms of the causes of ESRD or underlying diseases. The isoagglutinin titer and transfusion history before and after the transplantation were not significantly different between the 2 groups. The group 1 patients underwent an average of 5.4 TPE sessions before transplantation, and group 2 patients underwent an average of 4.6 sessions of TPE before transplantation.

All recipients' creatinine levels were periodically assessed before and after the transplantation. No significant difference was noted in the postoperative creatinine levels between the 2 groups (**TABLE 2**).

Overall Survival, Rejection, Graft Loss, and Rejection-Free Survival

The survival rate, rejection-free survival, and rejection type are summarized in **TABLE 3**. Two patients, both in group 1, died after transplantation. One of these patients died from infective fungal endocarditis 457 days after KT, with no evidence of acute graft rejection. The other patient died from urosepsis 82 days after KT, with evidence of graft rejection upon renal scan. Although both patients were in group 1, there was no significant difference in the occurrence of death between groups 1 and 2 according to the Kaplan-Meier survival analysis with log-rank tests (P = .157; **FIGURE 2A**).

Three patients in group 1 and 1 patient in group 2 were diagnosed with ABMR (P = .612). Among the 3 patients with ABMR in group 1, the ABMR resulted from DSAs in 1 patient and from unknown causes in the other patients. The ABMR patient in group 2 also tested positive for DSAs. Four patients in group 1 and 2 patients in group 2 were diagnosed with T-cell–mediated rejection (TCMR) (P = .671) through kidney biopsy. Three of the 7 patients with rejection in group 1 and 2 of the 4 patients with rejection in group 2 involved both ABMR and TCMR according to kidney biopsy findings (P >.99). Three patients in group 1 and 2 patients in group 2 were neither ABMR nor TCMR (P >.99), but renal scan findings suggested the possibility of graft rejection. There were no significant intergroup differences in either type of rejection (7 in group 1 vs 4 in group 2; P = .506).

The graft survival graph from the Kaplan-Meier survival analysis with log-rank tests revealed no differences between the 2 groups (P = .455; **FIGURE 2B**). In addition, according to the Kaplan-Meier survival and log-rank analysis, the time from ABOi-KT to the first allograft rejection seemed more beneficial in group 2 (**FIGURE 2C**). However, this finding was not significant (P = .335). Multivariable Cox regression analysis did not converge, and we could not draw any statistical inferences from our dataset.

Discussion

The main finding of this study was that regardless of the donor's blood type, donor-type plasma transfusion trended toward improved overall survival, graft survival, and rejection-free survival compared with universal AB plasma for recipients of ABOi-KT. Only 1 recipient (from group 2) out of 60 patients had Rh-negative type O blood. Considering that Rh-negative blood types are rare among Korean patients, only 1 Rh-negative patient out of 60 was an expected finding.[12]

During the perioperative period, all recipients of ABOi-KT received various blood products, including plasma components for TPE. Previous papers have presented guidelines for transfusion blood type selection for recipients of ABOi transplantation; however, there is no evidence-based consensus. Some of the proposals are similar to ours. The British Society for Haematology (BSH) published guidelines on the spectrum of FFP and cryoprecipitate products.[13] Although the guidelines lack evidence-based references, the BSH recommends plasma components for transfusions based on the principle of ABO incompatibility and the time before which successful graft accommodation can be expected. After ABOi transplantation, plasma of the donor's ABO type should be used until accommodation is complete. The guidelines also suggest a 4-week duration for the completion of accommodation. The Australian and New Zealand Society of Blood Transfusion (ANZSBT) has also published guidelines for transfusion.[14] According to the ANZSBT guidelines, plasma should be "compatible" with the graft, but no comment is made concerning the specific blood type. Conversely, some authors have suggested that AB plasma should be the first choice for recipients of ABOi transplantation, without consideration of the donor's blood type.[5]

Downloaded from https://academic.oup.com/labmed/article/53/4/369/6527640 by guest on 28 February 2025

TABLE 1. Patient Demographics and Clinical Characteristics

Variable[a]	Group 1 (n = 30)	Group 2 (n = 30)
Age (y)[b]	20–63	20–63
Sex		
Male	17 (56.7%)	14 (46.7%)
Female	13 (43.3%)	16 (53.3%)
Donor blood type		
A	18 (60.0%)	13 (43.3%)
B	12 (40.0%)	17 (56.7%)
Cause of ESRD		
DM	4 (13.3%)	3 (10.0%)
HTN	4 (13.3%)	3 (10.0%)
IgAN	3 (10.0%)	7 (23.3%)
ADPKD	3 (10.0%)	1 (3.3%)
GN	1 (3.3%)	7 (23.3%)
Others	3 (10.0%)	3 (10.0%)
Unknown	12 (40.0%)	6 (20.0%)
Underlying disease		
DM	8 (26.7%)	2 (6.7%)
HTN	21 (70.0%)	22 (73.3%)
HBV	4 (13.3%)	4 (13.3%)
HCV	1 (3.3%)	0 (0.0%)
Others	3 (10.0%)	1 (3.3%)
None	6 (20.0%)	6 (20.0%)
Pre-TPE DSA		
Present	1 (3.3%)	2 (6.7%)
Absent	29 (96.7%)	28 (93.3%)
Pre-KT transfusion		
RBC	10 (33.3%)	4 (13.3%)
FFP	4 (13.3%)	8 (26.7%)
Post-KT transfusion		
RBC	10 (33.3%)	13 (43.3%)
FFP	11 (36.7%)	7 (23.3%)
PLT	1 (3.3%)	2 (6.7%)
Post-KT TPE		
Performed	8 (26.7%)	5 (16.7%)
Not performed	22 (73.3%)	25 (83.3%)
Isoagglutinin titer[c]		
Baseline	128 (16-512)	64 (32-1024)
Pre-KT	4 (1–8)	1 (1–4)
Viral infection		
BKV	8 (26.7%)	12 (40.0%)
CMV	2 (6.7%)	2 (6.7%)

ADPKD, autosomal dominant polycystic kidney disease; BKV, BK virus; CMV, cytomegalovirus; DM, diabetes mellitus; DSA, donor-specific antibody; ESRD, end-stage renal disease; FFP, fresh frozen plasma; GN, glomerulonephritis; HBV, hepatitis B virus; HCV, hepatitis C virus; HTN, hypertension; IgAN, immunoglobulin A nephropathy; KT, kidney transplantation; PLT, platelet; RBC, red blood cell; TPE, therapeutic plasma exchange.
[a]Data are presented as number (%) or range, unless otherwise specified. There were no significant intergroup differences in any of the investigated variables.
[b]Ages are presented as ranges.
[c]Isoagglutinin titers are presented as median (range).

TABLE 2. Baseline and Postoperative Serum Creatinine (mg/dL)[a]

Date	Group 1	Group 2
Baseline	6.88 ± 3.43	7.69 ± 2.71
POD 1	3.12 ± 2.28	2.73 ± 1.33
POD 2	1.61 ± 2.07	1.44 ± 0.97
POD 3	1.36 ± 1.87	1.37 ± 1.37
POD 4	1.42 ± 2.30	1.16 ± 0.84
POD 5	1.41 ± 2.43	1.19 ± 1.18
POD 6	1.29 ± 1.87	1.12 ± 0.84
POD 7	1.23 ± 1.51	1.09 ± 1.02
POD 8	1.28 ± 1.97	1.03 ± 0.63
POD 9	1.22 ± 1.53	1.05 ± 0.80
POD 10	0.93 ± 0.26	0.96 ± 0.50
POD 11	1.46 ± 2.82	0.94 ± 0.56
POD 12	1.39 ± 2.28	0.95 ± 0.58
POD 13	1.59 ± 3.04	0.88 ± 0.43
POD 14	0.91 ± 0.31	1.04 ± 0.58
POD 30	0.99 ± 0.29	0.98 ± 0.33
POD 60	1.48 ± 2.34	0.99 ± 0.29
POD 90	1.11 ± 0.26	1.05 ± 0.31
POD 180	1.18 ± 0.27	1.09 ± 0.36

POD, postoperative day.
**Data are presented as mean ± standard deviation. No significant intergroup differences were identified at any time point.*

TABLE 3. Survivals and Rejection Type Results[a]

Variable	Group 1 (n = 30)	Group 2 (n = 30)
Overall survival[b]	3316.5 (82–3893)	2610 (448–2995)
Graft survival[b]	3254 (6–3703)	2596.5 (448–2995)
Rejection-free survival[c]	3185.5 (4–3703)	2556.5 (5–2995)
Rejection type		
ABMR	3 (10.0%)	1 (3.3%)
TCMR	4 (13.3%)	2 (6.7%)
Both ABMR and TCMR	3 (10.0%)	1 (3.3%)
Unknown	3 (10.0%)	2 (6.7%)
Total	7 (23.3%)	4 (13.3%)

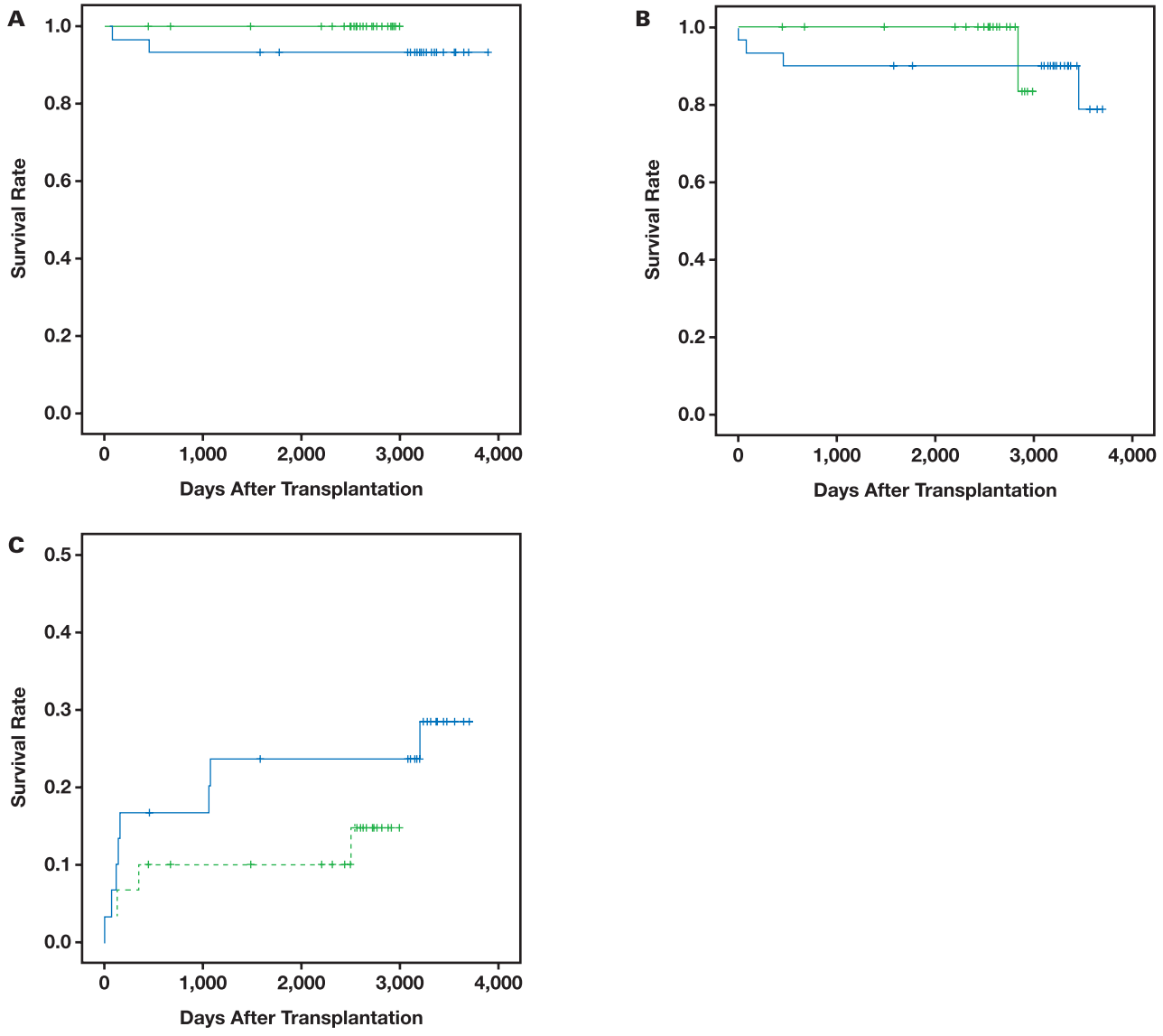
ABMR, antibody-mediated rejection; TCMR, T-cell–mediated rejection.
[a]There were no significant intergroup differences in any of the investigated variables.
[b]Survival data are presented as median and range of days.
[c]Rejection types are presented as number (%).

Of the 60 patients included in this study, only 2 patients died—on days 82 and 457 after transplantation, respectively. Based on these findings, the all-cause mortality after transplantation at 1 year was 1.7%. A meta-analysis conducted by Scurt et al[15] determined a 1-year all-cause mortality rate of approximately 3.1% associated with ABOi-KT, which is similar to the mortality rate in Korea, including that determined by our findings.

Recipients of KT in group 2 had similar or better prognosis than those in group 1; it is difficult to explain this finding based on the evidence, because both AB plasma and donor-type plasma were compatible

Downloaded from https://academic.oup.com/labmed/article/53/4/369/6527640 by guest on 28 February 2025

FIGURE 2. Survival curves for the study sample. Overall survival (A), graft survival (B), and rejection-free survival (C), comparing recipients with blood group O transfused with AB fresh frozen plasma (FFP) (group 1, blue line) vs patients transfused with FFP according to the donor's blood type (group 2, green line).



with the grafts. Other than ABO antigens on the surface of red cells and anti-A/B antibodies in plasma, blood components contain soluble A/B antigens, except in individuals with blood group O.[16] These soluble A/B antigens can bind to anti-A/B antibodies in the recipients' blood, forming immune complexes. These complexes have various effects on red cells or platelets, causing hemolysis or platelet activation, and they circulate for hours or days after ABO-mismatched transfusions.[17] For decades, AB plasma has been considered "universal," but it also harbors a significant amount of soluble A/B antigens. Therefore, there are some doubts about which blood type should be considered "universal."[16] We speculate that immune complex formation may have affected the prognosis of our patients; however, further study is necessary.

Perioperative management, including immunosuppressive therapy, was similar between the groups. Rituximab was administered in 2 different doses, 200 mg and 500 mg. Rituximab is a monoclonal antibody against the CD20 antigen and is frequently used for desensitization in recipients of ABOi or HLA antigen–mismatched organ transplantation.[18] Eleven recipients in group 1 and 4 recipients in group 2 received 500 mg of rituximab. There were no significant intergroup differences in terms of rejection incidence or rituximab doses. However, group 1 had more patients who received a high dose of rituximab, fewer patients with BK virus, and a higher rejection rate.

This study had some limitations. First, all patients were retrospectively reviewed through electronic medical records. Further studies with randomized controlled trial designs may provide stronger evidence to support our findings. Second, the sample size was insufficient. Although group 2 recipients seemed to have better survival curves, no significant differences were noted between the 2 groups. Considering the survival rate of our study, significant results would have been achieved with at least 125 patients per group.[19] Failure to converge on Cox regression may also have been because of the limited number of patients. It is quite challenging for a single-center study to obtain sufficient power for

Downloaded from https://academic.oup.com/labmed/article/53/4/369/6527640 by guest on 28 February 2025

statistical analyses. Further larger-scale, multicenter studies would be more robust and are warranted.

Determining how long ABO-mismatched transfusions should be maintained is another issue that lacks scientific and clinical evidence. Each institution has its own principles for the duration of ABO-mismatched transfusions after ABOi solid organ transplantation. At our center, once a patient is registered as an ABOi solid organ recipient, he or she is classified as a recipient for mismatched transfusion unless he or she undergoes another ABO-mismatched transplantation. After some time, the graft and recipient's immune systems will overcome the blood type inconsistency and reach a state called accommodation.[20] Although the BSH guidelines suggest that ABO-mismatched blood transfusions should be maintained until successful engraftment or accommodation is achieved (which usually takes 4 weeks),[13] the ANZSBT guidelines state that the mismatched transfusion protocol should be maintained indefinitely.[14] Further research on how long donor type plasma should be transfused must be conducted.

AQ11

Based on our data and previous studies, we propose a guide for transfusion blood type selection for patients with ABOi-KT (**TABLE 4**; modified from Kim and Ko[21] with permission). For every patient, the blood group of transfused red blood cells should match the recipient's blood group. For recipients with type O major mismatch, the first choice for the blood type of platelets and plasma should be that of the organ donor; AB plasma and platelets should be the second choice. For recipients with type A or B major mismatch, only AB plasma and platelets should be provided. For recipients with type A or B bidirectional mismatch, AB plasma and platelets should be the first choice; donor type plasma and platelets should be the second choice.

Conclusion

Transfusion of donor type plasma transfusion was noninferior to transfusion of universal type AB plasma in terms of both rejection-free survival and rejection rate. This blood type determination principle could be beneficial for the efficient operation of a blood bank. Therefore, for safe transfusion for recipients of ABOi-KT, each institution should readjust its current blood release standards based on the information in this article. This study is the first to show the clinical outcomes of ABOi-KT according to transfusion blood type.

TABLE 4. Suggestions for Blood Type Selection Criteria for ABOi-KT[a]

Recipient	Donor	RBC	Plasma and Platelets		Type of Mismatch
			First Choice	Second Choice	
O	A	O	A	AB	Major
O	B	O	B	AB	Major
O	AB	O	AB	NA	Major
A	B	A	AB	B	Bidirectional
A	AB	A	AB	NA	Major
B	A	B	AB	A	Bidirectional
B	AB	B	AB	NA	Major

ABOi, ABO-incompatible; KT, kidney transplantation; NA, not available.
[a]Modified with permission from Kim and Ko.[21]

Acknowledgments

The overall study was conceived and led by D.H. Ko and H. Kwon. H.J. Kim wrote the manuscript and performed statistical analyses. J.S. Kim, J.J. Yang, Y. Chung, H. Kim, S. Shin, Y.H. Kim, S.H. Hwang, H.B. Oh, and D.J. Han directly contributed to the acquisition of clinical data, their interpretation, or the revision of the article. The data used for the preparation of this article can be obtained from the corresponding author upon reasonable request.

REFERENCES

1. McCullough KP, Morgenstern H, Saran R, Herman WH, Robinson BM. Projecting ESRD incidence and prevalence in the United States through 2030. *J Am Soc Nephrol.* 2019;30(1):127–135.
2. Alexandre GP, Squifflet JP, De Bruyère M, et al. Present experiences in a series of 26 ABO-incompatible living donor renal allografts. *Transplant Proc.* 1987;19(6):4538–4542.
3. Korean Network for Organ Sharing. 2018 Annual data report. https://www.konos.go.kr/konosis/common/bizlogic.jsp. Accessed January 4, 2022.
4. Kopko PM. Transfusion support for ABO-incompatible progenitor cell transplantation. *Transfus Med Hemother.* 2016;43(1):13–18.
5. Das J, Khanna S, Kumar S, Mehta Y. Blood transfusion practices in liver transplantation. *Indian J Anaesth.* 2015;59(4):266–267.
6. Yazer M, Eder AF, Land KJ. How we manage AB plasma inventory in the blood center and transfusion service. *Transfusion.* 2013;53(8):1627–1633.
7. Jones JM, Sapiano MRP, Mowla S, et al. Has the trend of declining blood transfusions in the United States ended? Findings of the 2019 National Blood Collection and Utilization Survey. *Transfusion*. 2021;61(suppl 2):S1–S10.
8. Shin E, Kwon SW, Yang WS, et al. Long-term outcomes of ABO-incompatible living donor kidney transplantation: a comparative analysis. *Transplant Proc.* 2015;47(6):1720–1726.
9. Won D, Choe W, Kim HJ, Kwon SW, Han DJ, Park SK. Significance of isoagglutinin titer in ABO-incompatible kidney transplantation. *J Clin Apher.* 2014;29(5):243–250.
10. Kwon H, Kim YH, Choi JY, et al. Analysis of 4000 kidney transplantations in a single center: across immunological barriers. *Medicine.* 2016;95(32):e4249.
11. Kim H, Choe W, Shin S, et al. ABO-incompatible kidney transplantation can be successfully conducted by monitoring IgM isoagglutinin titers during desensitization. *Transfusion.* 2020;60(3):598–606.
12. Lee SY. Further analysis of Korean blood types. *Yonsei Med J.* 1965;6:16–25.
13. Green L, Bolton-Maggs P, Beattie C, et al. British Society of Haematology Guidelines on the spectrum of fresh frozen plasma and cryoprecipitate products: their handling and use in various patient groups in the absence of major bleeding. *Br J Haematol.* 2018;181(1):54–67.
14. Australian & New Zealand Society of Blood Transfusion. *Guidelines for Transfusion and Immunohaematology Laboratory Practice*, 1st ed. Revised January 2020. https://anzsbt.org.au/wp-content/uploads/2020/03/Guideline_-for_Transfusion_and_Immunohaematology_Laboratory_Practice_20200326_FINAL_Published_SecurePW-1.pdf. Accessed December 22, 2021.
15. Scurt FG, Ewert L, Mertens PR, Haller H, Schmidt BMW, Chatzikyrkou C. Clinical outcomes after ABO-incompatible renal transplantation: a systematic review and meta-analysis. *Lancet.* 2019;393(10185):2059–2072.
16. Refaai MA, Cahill C, Masel D, et al. Is it time to reconsider the concepts of "Universal Donor" and "ABO Compatible" transfusions? *Anesth Analg.* 2018;126(6):2135–2138.

Downloaded from https://academic.oup.com/labmed/article/53/4/369/6527640 by guest on 28 February 2025

17. Zaffuto BJ, Conley GW, Connolly GC, et al. ABO-immune complex formation and impact on platelet function, red cell structural integrity and haemostasis: an in vitro model of ABO non-identical transfusion. *Vox Sang.* 2016;110(3):219–226.

18. Becker YT, Samaniego-Picota M, Sollinger HW. The emerging role of rituximab in organ transplantation. *Transpl Int.* 2006;19(8):621–628.

19. Machin D, Campbell MJ, Tan SB, Tan SH. *Sample Size Tables for Clinical Studies*. 3rd ed. Oxford, UK: Wiley-Blackwell; 2009.

20. Koch CA, Khalpey ZI, Platt JL. Accommodation: preventing injury in transplantation and disease. *J Immunol.* 2004;172(9):5143–5148.

21. Kim H, Ko DH. Transfusion in ABO-incompatible solid organ transplantation. *Korean J Blood Transfus.* 2020;31:70–72.

Downloaded from https://academic.oup.com/labmed/article/53/4/369/6527640 by guest on 28 February 2025

Validation of a Spectrophotometric Method for Urinary Iodine Determination on Microplate Based on Sandell-Kolthoff Reaction

Adrijana Oblak,[1,] Petra Arohonka,[2] Iris Erlund, PhD,[2] Sonja Kuzmanovska, PhD,[3] Katja Zaletel, PhD,[1,4] Simona Gaberšček, PhD[1,4]*

[1]Department of Nuclear Medicine, University Medical Centre Ljubljana, Ljubljana, Slovenia, [2]Finnish Institute for Health and Welfare, Department of Government Services, Helsinki, Finland, [3]Institute of Pathophysiology and Nuclear Medicine, Faculty of Medicine, Skopje, North Macedonia, [4]Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia;*To whom correspondence should be addressed. adrijana.oblak@kclj.si

Keywords: urinary iodine concentration, Sandell-Kolthoff reaction, thyroid, iodine deficiency, spectrophotometric detection

Abbreviations: UIC, urinary iodine concentration; APD, ammonium persulfate digestion; S-K, Sandell-Kolthoff; ICP-MS, inductively coupled plasma mass spectrometry; WHO, World Health Organization; EQUIP, Ensuring the Quality of Urinary Iodine Procedures; KIO_3, potassium iodate; $(NH4)_2S_2O_8$, ammonium peroxydisulfate; dH_2O, deionized water; NaOH, sodium hydroxide; As_2O_3, arsenic trioxide; NaCl, sodium chloride; H_2SO_4, sulfuric acid; $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$, ceric ammonium sulfate dihydrate; HNO_3, nitric acid; QC, quality control; CLSI, Clinical and Laboratory Standards Institute; LoB, limit of blank; LoD, limit of detection; THL, Finnish Institute for Health and Welfare; CV, coefficient of variation; CDC, Centers for Disease Control and Prevention; ICP-DRC-MS, inductively coupled plasma dynamic reaction cell mass spectrometry.

Laboratory Medicine 2022;53:376–380; https://doi.org/10.1093/labmed/lmab117

ABSTRACT

Objective: Iodine is an essential part of the thyroid hormones thyroxine and triiodothyronine. Therefore, it is essential to monitor iodine supply in a population. The biochemical marker for assessing and controlling iodine is urinary iodine concentration (UIC).

Materials and Methods: This cross-sectional study included 180 pregnant women and 308 women of reproductive age. Urine specimens from 185 of the 488 volunteers were used. The urine specimens were measured using 2 methods: (1) ammonium persulfate digestion (APD), followed by the Sandell-Kolthoff (S-K) reaction modified on microplate for spectrophotometric detection; and (2) the reference method, inductively coupled plasma mass spectrometry (ICP-MS).

Results: The regression equation between the methods was ICP-MS method = 1.137*(APD S-K)-5.57. A Passing-Bablok regression showed no deviation from linearity (P = .17). A Bland-Altman plot showed a negative mean bias of –2.7%.

Conclusion: The APD S-K reaction modified on microplate for spectrophotometric detection of UIC can be implemented into routine work. Its results are comparable to those of laboratories worldwide and to ICP-MS.

Iodine is an important trace element. It is used in the prevention of thyroid diseases and in radiation emergencies. Iodine is an essential component of the thyroid hormones thyroxine and triiodothyronine, which regulate a variety of important physiological processes. Iodine content in food varies geographically and is mostly low.[1]

Iodine deficiency leads to developmental and growth disorders. Adequate iodine supply is crucial for brain development from the 15th week of gestation to age 3 years. The consequences of iodine deficiency at that age are irreversible, leading to brain and central nervous system damage. Therefore, one important group affected by iodine deficiency is women of reproductive age.[2]

The World Health Organization (WHO) recommends iodine prophylaxis with universal salt iodization, which is an easy and successful way to overcome iodine deficiency disorders. The recommended daily intake of iodine is 150 µg for adults and 250 µg for pregnant and lactating women.[3]

The most important biochemical marker for assessing and controlling iodine supply in a population is the measurement of urinary iodine concentration (UIC), because in healthy patients with sufficient iodine approximately 90% of consumed iodine is excreted in urine.[4] The UIC can be measured using spot urine specimens or from 24-hour urine collection. For population-based studies, the WHO recommends the measurement of UIC using spot urine specimens. These data enable an estimation of the adequacy and effectiveness of salt iodization in a population and should be regularly acquired.[3]

Methods for UIC measurement include rapid semiquantitative methods, inductively coupled plasma mass spectrometry (ICP-MS), and spectrophotometric methods based on the Sandell-Kolthoff (S-K)

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/376/6514453 by guest on 28 February 2025

reaction with various modifications.[5] Rapid semiquantitative methods provide ranges rather than absolute concentrations of iodine. The ICP-MS method is a highly sensitive method with a low detection limit, a wide linear range, and a multielement technique that enables high specimen throughput and minimal specimen preparation. The disadvantage of the ICP-MS method is the high price. The spectrophotometric method enables usage of the equipment that is usually present in a laboratory. The disadvantage of this method is the need for specimen pretreatment before the S-K reaction to remove interfering substances. There are 2 variations of these pretreatment steps: chloric acid digestion, which requires safety precautions, and ammonium persulfate digestion (APD) in a heated environment, which is easier to work with.[5] The S-K reaction uses the catalytic activity of the iodide ion in the reaction of arsenic acid and ceric ammonium sulfate ($[NH_4]_4Ce[SO_4]_4$) in a sulfuric acid solution. Iodide's presence increases the reaction rate. In the absence of iodide ion, this reaction is very slow at room temperature and requires approximately 2 to 4 days for completion. The reaction rate is directly proportional to the iodide ion concentration in a solution[6]:

$$As^{3+} + 2Ce^{4+}\ (\text{yellow}) \xrightarrow{2I^-} As^{5+} + 2Ce^{3+}\ (\text{colorless})$$

The S-K reaction can be conducted in glass tubes or with modified quantities of specimens on microplates. Its main advantage over the ICP-MS method is cost-effectiveness. It also shows sustainability and good performance characteristics.[3,7]

The aim of our work was to implement and validate a spectrophotometric method using APD of urinary specimens in a heated environment followed by an S-K reaction modified on microplate to our laboratory for monitoring UIC in Slovenian women of reproductive age. Validation consisted of 3 main steps: implementation of a method in the laboratory using WHO and Ensuring the Quality of Urinary Iodine Procedures (EQUIP) guidelines,[3,8] method comparison with ICP-MS, and enrollment into an independent external quality control scheme.

Materials and Methods

Ethics Approval

Research involving human patients complied with all relevant national regulations and institutional policies and was in accordance with the tenets of the Helsinki Declaration (as revised in 2013). Ethics approval for this study was granted by the Republic of Slovenia National Medical Ethics Committee, number 0120-508/2017-2. The study was a part of European Horizon 2020 project EUthyroid (number 634453), entitled "Towards the Elimination of Iodine Deficiency and Preventable Thyroid-Related Diseases in Europe," the goal of which was harmonizing and sustainably improving iodine intake in Europe. Informed consent was obtained from all individuals included in this study.

Study Design

This cross-sectional study was conducted at the Department of Nuclear Medicine, University Medical Centre Ljubljana, Slovenia between October 2017 and July 2018. Two groups of volunteers, pregnant women and women of reproductive age from the local population, participated in the study. Only volunteers without known thyroid disease were included in the study. In all women, UIC was measured. Spot urine was collected between 8:00 AM and 2:00 PM into a urine container (120 mL, nonsterile, with screw cap, Golias, Slovenia). Specimens were then transported into the laboratory within 10 minutes of collection for immediate processing. Urine specimens were aliquoted and stored in a temperature-controlled freezer at –80°C before analysis. Measurements of urine specimens were taken in 2 batches, separately for each group.

Preparation of Reagents for APD S-K Reaction Method

Chemicals used included potassium iodate (KIO_3) (Sigma-Aldrich, Germany), ammonium peroxydisulfate ($[NH_4]_2S_2O_8$) (Sigma-Aldrich, Germany), deionized water (dH_2O) (Braun, Aqua B. Braun, Sterile, 1L, Ecotainer, Germany), sodium hydroxide (NaOH) (Merck, Germany), arsenic trioxide (As_2O_3) (Sigma-Aldrich, Germany), sodium chloride (NaCl) (Sigma-Aldrich, Germany), concentrated sulfuric acid (H_2SO_4) (Sigma-Aldrich, Germany), ceric ammonium sulfate dihydrate ($[NH_4]_4Ce[SO_4]_4 \times 2H_2O$) (Sigma-Aldrich, Germany), and 65% nitric acid (HNO_3) (Honeywell, USA).

All laboratory equipment was treated with HNO_3 before the preparation of the reagents to remove any additional iodine from the environment. The preparation of the reagents and the digestion processes and analysis performance took place in a ventilating fume hood in the laboratory. Reagents were prepared in volumetric flasks. A brief summary of preparation follows:

- 1 mol/L ammonium persulfate solution: dissolution of 228.2 g $(NH_4)_2S_2O_8$ in 1 L dH_2O.
- 0.875 mol/L NaOH solution: dissolution of 8.75 g NaOH in 0.25 L dH_2O.
- 0.05 mol/L arsenious acid solution: dissolution of 5 g As_2O_3 in 0.1 L of 0.875 mol/L NaOH on a magnetic stirrer. Afterward, the solution was put into an ice bath and 16 mL concentrated H_2SO_4 was added while stirring on the magnetic stirrer. After cooling, 12.5 g NaCl was added and diluted with dH_2O up to 0.5 L. The solution was then mixed for 90 minutes at 60°C. Afterward, the solution was filtrated.
- 1.75 mol/L H_2SO_4 solution: In an ice bath, 97 mL concentrated H_2SO_4 was slowly added to 0.5 L dH_2O and filled with dH_2O up to 1 L.
- 0.019 mol/L $(NH_4)_4Ce(SO_4)_4$ solution: dissolution of 6 g $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ in 0.5 L 1.75 mol/L H_2SO_4.

All reagents were stored in amber bottles in the dark at room temperature except for the ammonium persulfate solution, which was stored in a cool dark place at 2°C to 8°C.

Preparation of Standards and Quality Controls for Spectrophotometric Method

We dissolved 1.68 g KIO_3 in 1 L dH_2O. This solution was then used for the preparation of an intermediate standard with an iodine concentration of 1000 µg/L. From the intermediate standard, 6 working standards and 2 quality controls (QCs) were prepared. A certain volume of the intermediate standard was added to flasks and then diluted with dH_2O to prepare working standards with different concentrations of iodide: 0, 40, 80, 120, 200, and 400 µg/L. The preparation of the 2 QCs followed the same protocol, but the levels of concentration were 160 and 280 µg/L. Standards and controls were stored in a cool dark place at 2°C to 8°C.

Procedure for APD S-K Reaction Method

All reagents reached room temperature and were mixed thoroughly but gently before use. To 250 µL of standards, QC, and samples 1 mL of 1 mol/L ammonium persulfate solution was added into glass tubes. The solution

Downloaded from https://academic.oup.com/labmed/article/53/4/376/6514453 by guest on 28 February 2025

was then mixed and incubated for 1 hour at 95°C in a dry bath (Fisher Scientific Isotemp Digital Dry Bath/Block Heater, Fisher Scientific, UK). After cooling down to room temperature, 50 µL of standard, QC, and sample was transferred to microplate in duplicate. To each well, 100 µL arsenic acid solution was added. The microplate was sealed and incubated for 60 seconds on a microplate shaker. Afterward, 50 µL $(NH_4)_4Ce(SO_4)_4$ solution was added to each well within 40 seconds. The microplate was sealed and put on the microplate shaker for exactly 30 minutes. A measurement of an absorbance at 405 nm on a spectrophotometer (MRX Microplate Absorbance Reader, Dynatech Laboratories, USA) immediately followed. The results were calculated by plotting the optical density data on the *y* axis in linear model and the iodide ion concentration was plotted on the *x* axis in logarithmic mode. Concentration is inversely proportional to absorbance. Standard curves were developed for each microplate separately using a linear cubic spline fit with tails.

Limit of Blank, Limit of Detection, and Repeatability Estimation of Modified APD S-K Reaction Method

Following Clinical and Laboratory Standards Institute (CLSI) requirements, the limit of blank (LoB) and limit of detection (LoD) of the method were determined.[9] Sixty measurements of each were performed from 10 different specimens for LoB and LoD on 4 microplates using 4 different standard curves. Precision was evaluated in accordance with a modified protocol proposed by the CLSI.[10] For interassay precision, QCs at 2 concentration levels, 160 µg/L and 280 µg/L, were assayed on 18 microplates in duplicate and 9 single microplates. For intra-assay precision, 8 measurements on 1 microplate were used.

ICP-MS

For the method comparison, UIC was measured at the Finnish Institute for Health and Welfare (THL; Helsinki, Finland) using the ICP-MS method. The laboratory (number T077) has been accredited by the Finnish Accreditation Service and fulfills the requirement of the Finnish Standards Association/European Standards ISO/IEC standard 17025. The scope of accreditation covers the UIC method. In addition, the laboratory at THL participates in the EQUIP organized by the Centers for Disease Control and Prevention (CDC) 3 times per year. We determined the UIC using ICP-MS with an Agilent 7800 ICP-MS system (Agilent Technologies, Santa Clara, CA). We extracted 100 µL of urine specimens using an ammonium hydroxide solution. On the ICP-MS system, m/z = 127 was scanned for iodine determination. Tellurium was used as an internal standard. The limit of quantification of the method was 2 µg/L, and the linearity was $r = 0.9999$ up to 1500 µg/L). The National Institute of Standards and Technology reference standard materials SRM2670a (with certified mass concentration value) and SRM3668 level 1 and level 2 were used to validate the method. The coefficient of variation (CV) of the control specimens was 0.6% to 2.2%.

Method Comparison

The ICP-MS method, considered as a reference method, was used for method comparison. One hundred eighty-six (from 71 women of reproductive age and 115 pregnant women) specimens were sent to THL for UIC determination.

External QC Scheme

The evaluation of the APD S-K reaction method was also performed using EQUIP. Four rounds of the QC scheme were completed.

Statistical Analysis

The Kolmogorov-Smirnov test was used to assess the normality of distribution of the analyzed data. Data were not normally distributed. Differences were tested using the Wilcoxon test. A P value <.05 was considered statistically significant. Data were expressed as medians and ranges/percentiles. The Passing-Bablok regression was used for the method comparison of the analyzed data, and the Spearman coefficient was determined. A Bland-Altman plot was assessed to show bias between the methods. Statistical analysis was done using MedCalc Statistical Software version 18.6 (MedCalc Software, Ostend, Belgium).

Results

Four hundred and eighty-eight volunteers participated in the study, 308 women of reproductive age and 180 pregnant women. The median age was 31 (18–56) years. Eighteen women of reproductive age were excluded from the study because their UIC was below the LoD. The comparison of methods was done in two batches. The first batch represented 308 urine specimens from the women of reproductive age. All specimens were analyzed using the spectrophotometric method. Out of the 308 specimens, following the instructions of the EUthyroid project, 75 specimens representing a Gaussian curve were sent to THL for method comparison. Seventy-four specimens were analyzed, and 71 specimens were used for comparison. Three were excluded because the UIC was below the LoD. Urine specimens from the pregnant women were directly sent for UIC determination to THL, and 115 specimens were analyzed. Sixty-five specimens from the pregnant women were analyzed only using the APD S-K reaction method.

LoB and LoD Estimation, Analytical Range, and Repeatability of APD S-K Reaction

The LoD for UIC was 6.9 µg/L, which was determined to be consistent with the guidelines in the CLSI EP17-A protocol and with proportions of false positives (α) <5% and false negatives (β) <5%, was based on 120 determinations, where 60 blank and 60 low-level specimens were determined. LoB was set as 3.5 µg/L. Therefore, the analytical range was 6.9–400 µg/L. The calculated imprecisions expressed as CV (%) for 2 levels of control specimens, at 160 µg/L and 280 µg/L, respectively, are presented in **TABLE 1**.

TABLE 1. CV (%) for Intra-Assay, Interassay, and Total Imprecision for 2 Different QC Levels

Control Specimens	Target Value	Intra-Assay Imprecision (%)	Interassay Imprecision (%)	Total Imprecision (%)
1	160 µg/L	2.5	4.9	5.5
2	280 µg/L	2.3	6.3	7.2

CV, coefficient of variation; QC, quality control.

Downloaded from https://academic.oup.com/labmed/article/53/4/376/6514453 by guest on 28 February 2025

Method Comparison for UIC: ICP-MS and APD S-K Reaction

The 186 specimens selected covered all analytical ranges. One specimen with a very high UIC (>400 µg/L) was excluded from comparison, so the total size of specimens for comparison was 185. The median value of UIC measured using the APD S-K reaction method was 74.0 µg/L (range, 40.5–125.3 µg/L). The median value of UIC measured using the ICP-MS method was 67.3 µg/L (range, 34.9–136.7 µg/L). Both methods were not normally distributed, and the Spearman coefficient was 0.979 (95% confidence interval, 0.973–0.985). The *P* level of significance was <.0001. The Passing-Bablok regression analysis and Bland-Altman plot comparison are presented in **FIGURE 1** and **FIGURE 2**, respectively.

External QC Scheme

Enrolling in EQUIP enabled a comparison of measured UIC with other laboratories around the world. The target value in the QC scheme was set up with a dynamic reaction cell for inductively coupled plasma mass spectrometry (ICP-DRC-MS) by the CDC. It was derived by taking the mean of 9 individual results generated by the ICP-DRC-MS urine iodine method on at least 3 different days. The acceptable range was calculated by multiplying the target value by a fixed percentage as defined for the following concentration ranges: <50 µg/L, 30%; 50 to 100 µg/L, 25%; 100 to 200 µg/L, 20%; and >200 µg/L, 15%. This system considered the increase in analytical difficulty with the decreasing concentration of the analyte. Each round consisted of 4 specimens. In all 4 rounds of participation, all measured specimens were within the target range. Results are shown in **TABLE 2**.

FIGURE 1. Comparison of ammonium persulfate digestion Sandell-Kolthoff (APD S-K) reaction method with the inductively coupled plasma mass spectrometry (ICP-MS) method using Passing-Bablok regression. The solid line shows the regression line, dashed lines show 95% confidence interval (CI) for the regression line, and the dotted line shows the identity line Y = X. The sample size is n = 185. The regression equation is Y = 1.137*X-5.57. The slope is 1.137 (95% CI, 1.104–1.171), and the intercept is –5.57 (95% CI, –9.00 to –3.65). The Cusum test for linearity showed no significant deviation from linearity (*P* = .17). UIC, urinary iodine concentration.

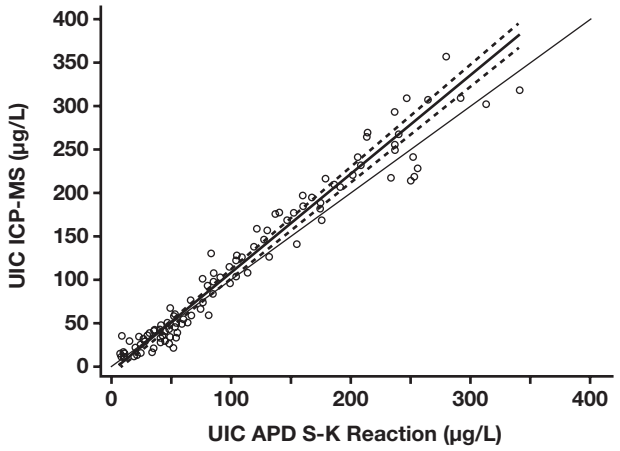
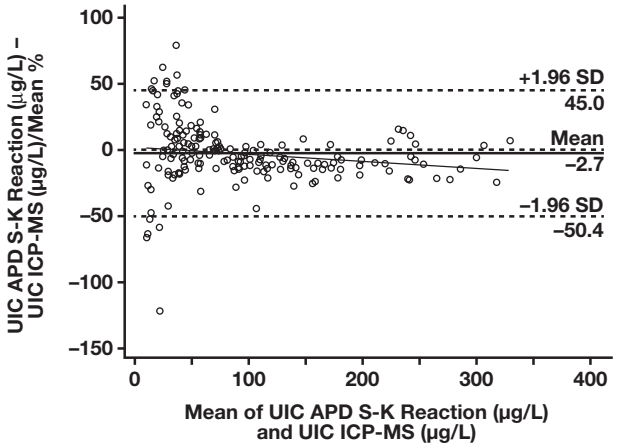


FIGURE 2. Bland-Altman plot of comparison of urinary iodine concentration (UIC) determination by ammonium persulfate digestion Sandell-Kolthoff (APD S-K) reaction method and the inductively coupled plasma mass spectrometry (ICP-MS) method. Differences between methods are plotted against the averages of both methods. The sample size is n = 185. Horizontal lines represent the mean of both methods, zero difference, and 95% confidence interval limits of agreement (confidence limits of bias), which are defined as the mean difference plus/minus 1.96 times the standard deviation (SD) of the differences. The mean shows a negative mean bias of 2.7%. Significant bias can be observed in values ≤50 µg/L. With higher values there is no significant bias.



Discussion

In 1999, the iodization of kitchen salt in Slovenia increased from 10 mg potassium iodide per kg salt to 25 mg (range, 20–30 mg). Consequent studies have shown an amelioration of the incidence and severity of several thyroid disorders and an adequate iodine supply in schoolchildren and in pregnant and lactating women.[11] Iodization programs should be monitored regularly. The recommended method for iodine level estimation is UIC measurement. Between 2017 and 2019, Slovenia participated in EUthyroid, which was intended to balance and sustainably improve iodine intake in Europe. As part of this project, a harmonization of laboratory UIC methods was performed. In line with the project objectives, to measure UIC we implemented and validated a spectrophotometric method using the APD of urine specimens in a heated environment followed by an S-K reaction modified on microplate. This method was compared with the reference ICP-MS method.

The WHO recommends various methods for UIC measurement. We chose spectrophotometric detection of the APD S-K reaction modified on microplate over other methods for a number of reasons. First, ICP-MS technology is very expensive, and the costs for using this technology only for monitoring population-based studies are too high. In addition, we wanted to avoid the potential hazards of the chloric acid digestion method, so we chose a nonexplosive, ammonium persulfate.[7] Laboratory equipment was already available. In comparison with the classic procedure of conducting an APD S-K reaction in glass tubes, our method modification of using microplates greatly increased specimen throughput and lowered the turnaround time, which is especially favorable in population-based studies. Another reason we chose this procedure is that fewer toxic products are formed compared to during an APD S-K reaction in glass tubes.

Downloaded from https://academic.oup.com/labmed/article/53/4/376/6514453 by guest on 28 February 2025

TABLE 2. Biases from Different Rounds of Participation in EQUIP[a]

EQUIP Round	CV (%) Deviation from CDC TV[b]				CV (%) Deviation from CDC TV[c]			
	1	2	3	4	1	2	3	4
51	–5.6	–10.9	–0.4	7.0	–2.5	–8.4	–4.9	–13.0
52	–6.8	–7.1	8.4	7.7	–6.0	13.3	1.9	1.7
53	23.8	6.2	5.9	–2.8	–11.1	–0.7	–9.4	–5.0
54	–10.2	–12.3	–5.0	–8.6	–7.3	–3.5	–2.6	–1.7

CV, coefficient of variation; EQUIP, Ensuring the Quality of Urinary Iodine Procedures; CDC TV, Centers for Disease Control and Prevention Target Value.
[a]Data shown separately for our laboratory and laboratories using the same method (in 4 rounds, 14–16 laboratories participated in evaluation).
[b]Data from our laboratory.
[c]Data from all laboratories using the same method.

One disadvantage of the method is that more care must be taken in preparing the reagents and analyzing the specimens. In addition, a number of substances affect the end result of the APD S-K reaction: inhibitors such as fluoride, mercury salts, cyanide, and silver; reducing substances such as nitrite, thiocyanate, and ferrous ion; AQ16 and substances that oxidize trivalent arsenic such as bromate and permanganate. Traces of iodide ion increase the speed of reaction, and interfering substances lower the amount of trivalent arsenic or tetravalent cerium to be available for reaction and therefore impact the end result of UIC. Special care is required in preparation and AQ17 pipetting the chemicals. Adequate preparation of equipment is necessary: It should be acid-washed to prevent interference from the surroundings to begin the reaction.[6]

The LoB and LoD were 3.5 μg/L and 6.9 μg/L, respectively. The method showed a good analytical sensitivity and enabled the detection of low UIC. The upper analytical range of the method was based on WHO recommendations on iodine intake that were reflected in the median UIC: adequate UIC was between 100 μg/L and 199 μg/L, above the recommended UIC was between 200 μg/L and 299 μg/L, and excessive UIC was >300 μg/L. Therefore, our highest standard was 400 μg/L.

The 2 methods that we compared agreed well, especially for specimens with UIC approximately ≥50 μg/L. However, values ≤50 μg/L were significantly different. These values are in the area of extremely inadequate iodine supplementation. Therefore, these significant differences did not influence any clinical decisions. The Spearman coefficient between both methods was 0.979 (95% confidence interval, 0.973–0.985). The overall bias was low, at –2.7%. The Bland-Altman graph showed a decreasing bias from lower to higher concentrations. Participation in the external QC scheme showed the comparability of our results to that of other laboratories worldwide.

Conclusion

Following WHO and EQUIP recommendations, the implementation of a spectrophotometric method with APD in a heated environment using a modified S-K reaction on microplate was successful. It can be implemented into routine work and does not require expensive equipment. The results are comparable to the ICP-MS method and to those of other laboratories worldwide.

Acknowledgments

We express our great appreciation to Darinka Levstek from the Department of Nuclear Medicine, University Medical Center Ljubljana (Ljubljana, Slovenia) for her technical support during the planning and implementing of a spectrophotometric method in our laboratory. Her willingness to give her time so generously is very much appreciated.

REFERENCES

1. Haldimann M, Alt A, Blanc A, Blondeau K. Iodine content of food groups. *J Food Drug Anal.* 2005;18:461–471.
2. Benoist de B, Andersson M, Egli I, Takkouche B, Allen H, eds. Iodine status worldwide: WHO global database on iodine deficiency. http://apps.who.int/iris/bitstream/handle/10665/43010/9241592001.pdf?sequence=1. Published 2004. Accessed December 7, 2021.
3. World Health Organization. Assessment of iodine deficiency disorders and monitoring their elimination: a guide for programme managers, 3rd ed. https://www.who.int/publications/i/item/9789241595827. Published September 1, 2007. Accessed December 7, 2021.
4. Zimmerman MB. Iodine deficiency. *Endocrine Reviews.* 2009;30:376–408.
5. Jooste PL, Strydom E. Methods for determination of iodine in urine and salt. *Best Pract Res Clin Endocrinol Metab.* 2010;24(1):77–88.
6. Sandell EB, Kolthoff IM. Micro determination of iodine by a catalytic method. *Microchim Acta.* 1937;1:9–25.
7. Pino S, Fang SL, Braverman LE. Ammonium persulfate: a new and safe method for measuring urinary iodine by ammonium persulfate oxidation. *Exp Clin Endocrinol Diabetes.* 1998;106(suppl 3):S22–S27.
8. Makhmudov AA, Caldwell KL. The challenge of iodine deficiency disorder: a decade of CDC's Ensuring the Quality of Urinary Iodine Procedures Program: EQUIP 10 year anniversary. https://stacks.cdc.gov/view/cdc/39393. Published September 2011. Accessed December 7, 2021.
9. Clinical and Laboratory Standards Institute. *Protocols for Determination of Limits of Detection and Limits of Quantitation, Approved Guideline. CLSI EP17-A*. Wayne, PA: Clinical and Laboratory Standards Institute; 2004.
10. Clinical and Laboratory Standards Institute. *Evaluation of Precision Performance of Quantitative Measurement Methods, Approved Guideline. CLSI EP05-A,* 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.
11. Zaletel K, Gaberšček S, Pirnat E, Krhin B, Hojker S. Ten-year follow-up of thyroid epidemiology in Slovenia after increase in salt iodization. *Croat Med J.* 2011;52:615–621.

Downloaded from https://academic.oup.com/labmed/article/53/4/376/6514453 by guest on 28 February 2025

Sialic Acid as a Suitable Marker of Clinical Disease Activity in Patients with Crohn's Disease

Yaoming Chen,MB,[1,#] Yuting He, MM,[1,#,] Xiaoxia Zhan, PhD[1,#] Dubo Chen, MB,[1] Pinning Feng, MB,[1] Yan Yan, MB,[1] Yichong Wang, PhD[1,*]*

[1]Department of Laboratory Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; *To whom correspondence should be addressed. yichong007@gmail.com; [#]These authors contributed equally to this work.

Keywords: sialic acid, Crohn's disease, diagnostic marker, disease activity, Harvey-Bradshaw Index, inflammatory markers

Abbreviations: SA, sialic acid; CD, Crohn's disease; HBI, Harvey-Bradshaw Index; CRP, C-reactive protein; IBD, inflammatory bowel disease; CDAI, Crohn's Disease Activity Index; ESR, erythrocyte sedimentation rate; ALB, albumin; WBC, white blood cell; PPV, positive predictive value; NPV, negative predictive value; ROC, receiver operating characteristic; AUC, area under the receiver operating characteristic curve.

Laboratory Medicine 2022;53:381–385; https://doi.org/10.1093/labmed/lmac006

ABSTRACT

Objective: Elevated serum levels of sialic acid (SA) have been verified in patients with various inflammatory conditions. The association between the Crohn's disease (CD) activity and serum SA has been insufficiently studied.

Materials and Methods: Serum SA concentrations were determined using an enzymatic colorimetric assay method, and the correlation of SA with the Harvey-Bradshaw Index (HBI) and other inflammation activity markers was evaluated using the Spearman correlation. The predictive value of SA in estimating CD disease activity was assessed using the receiver operating characteristic.

Results: The SA levels were positively correlated with HBI and C-reactive protein (CRP) levels. The correlation of SA with the HBI was superior to that of CRP with the HBI. The area under the curve for SA was higher than that for CRP, with an optimal cutoff value of 53.14 mg/dL for active CD.

Conclusion: Serum SA correlates with the HBI score better and has better predictive value in monitoring CD disease activity than CRP or other inflammatory markers.

Crohn's disease (CD) is 1 type of chronic nonspecific inflammatory bowel disease (IBD) whose etiologies remain unclear.[1] To date, there is no way to cure it completely. Consequently, medical treatment aims to maintain CD disease activity within remission.[2,3] The determination of disease activity is a crucial factor in the diagnosis and treatment of CD. In current clinical practice, a combination of methods is used when identifying CD disease activity, including symptom assessment, colonoscopy, histological and radiographic evaluation, and multiple laboratory markers.[4,5]

Endoscopic examination with biopsies is considered the gold standard for evaluating CD activity, but it is limited because of its invasiveness and the risk of multiple complications.[6,7] The Harvey-Bradshaw Index (HBI) is a simplified version of the Crohn's disease activity index (CDAI) that was developed in 1980[8,9] and is used to monitor the clinical disease activity of patients with CD. However, the HBI score has limitations, such as being time-consuming and based on self-reporting for measuring clinical characteristics.[10]

C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) are 2 of the most commonly used serum indexes to estimate CD activity. However, the specificity and sensitivity of these 2 indexes are unsatisfactory.[11-13] A low albumin (ALB) level is often linked with the severity of inflammation but is also affected by infection and nutritional status.[14] Almost all current serological biomarkers have limitations of nonspecificity. Fecal calprotectin has a far greater accuracy for detecting intestinal inflammation than serum biomarkers. However, it requires collecting a fecal specimen, which is time-consuming and more suitable to differentiate IBD from irritable bowel syndrome.[15,16] Based on the above information, it is crucial to seek a noninvasive marker of disease activity with high sensitivity and specificity, which will be key to monitoring the presence of active disease.

Sialic acid (SA) is a kind of 9-carbon monosaccharide that presents in human serum and tissues with multiple and critical cell biological functions.[17-19] Research has shown that SA is an acute phase reactant, and it has been observed to increase in several inflammatory disorders, such as rheumatoid arthritis.[20] To date, there are only a few small-sample studies on the properties of SA in distinguishing CD disease occurrence or CD disease activity.[21,22] Furthermore, none of the studies have analyzed the association between SA and the HBI score.

Therefore, our present study analyzed the relationship between serum SA and the HBI score, compared the validity of SA with other dis-

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/381/6535392 by guest on 28 February 2025

ease activity markers in clinical practice, and estimated the predictive value of SA in CD disease activity. This study aims to ascertain whether serum SA could serve as a useful index of disease activity in patients with CD.

Materials and Methods

Patients

Seventy-three patients with CD and 30 healthy control patients matching sex and age were recruited from the First Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). The CD diagnostic criteria were based on the Consensus Opinions on the Diagnostic and Treatment Specifications for IBD in China. Forty-four of the 73 patients were in an active phase of the disease, with HBI >4.[23] All enrolled patients had normal renal and hepatic function, and patients with acute or chronic infection, inflammatory disease, and history of tumors were excluded. Patients with CD and the control patients had blood specimens collected, the HBI scores were calculated at the time of entering the study, and then all patients with CD underwent a colonoscopy.

Measurement of Serum Specimen

All peripheral blood specimens were collected after a 12-hour fast, and serum was obtained by centrifugation at 3500 rpm for 10 minutes. The serum SA and ALB levels were measured on the Beckman Coulter AU-5800 autoanalyzer using an enzymatic colorimetric assay. The CRP and white blood cell (WBC) count were measured on the Mindray BC-6800 plus autoanalyzer according to the manufacturer's recommendations.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 19.0). Continuous variables were analyzed using an unpaired Student's *t*-test or a 1-way analysis of variance. Spearman correlation was utilized to investigate the relationship between serum SA levels and the HBI scores or other serum inflammatory markers. To evaluate the diagnostic efficacy of serum SA, the cutoff point, specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were calculated by using the receiver operating characteristic (ROC) curve.

Ethical Considerations

The study was previously approved by the Sun Yat-sen University Ethics Committee, and all participants gave their written informed consent to this protocol.

Results

Baseline Clinical and Laboratory Characteristics

Detailed general and laboratory characteristics of patients with CD and healthy control patients are presented in **TABLE 1**. The mean SA concentrations in the healthy control and CD groups were 42.6 and 68.53 mg/dL, respectively. The SA, CRP, and WBC counts were significantly higher and ALB was significantly lower in patients with CD than in healthy control patients (P <.05). The difference between the 2 groups in terms of age, sex, and ESR was statistically insignificant (P >.05).

TABLE 2 shows the general characteristics and laboratory details of patients in the active and inactive stages of CD. Age and sex were comparable for patients with CD in both the active and inactive stages. We found that 60.3% (n = 44) of the patients with CD were in the active period (HBI >4). Serum SA and CRP levels of the patients with active CD were significantly higher than those of patients with inactive CD (P <.05). The ALB levels in the patients with active CD were significantly lower than those of patients with inactive CD (P <.05). There were no significant differences between patients with active inactive CD regarding the ESR and WBC count (P >.05).

TABLE 1. Characteristics of Patients with CD and Healthy Control Patients[a]

	Patients with CD (n = 73)	Healthy Control Patients (n = 30)	*P* Value
Age (y)	38.5 ± 9.32	39.8 ± 11.32	.630
Sex (male/female)	30/43	11/19	.466
SA (mg/dL)	68.53 ± 17.6	42.6 ± 10.2	.040
CRP (mg/L)	14.9 ± 10.7	4.5 ± 3.6	.011
ESR (mm/h)	18.43 ± 15.46	10.11 ± 7.9	.058
WBC count (× 10^9/L)	8.19 ± 3.05	6.70 ± 1.88	.028
ALB (g/L)	33.25 ± 5.90	41.61 ± 9.51	.032

ALB, albumin; CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; SA, sialic acid; WBC, white blood cell.
[a]*Data are expressed as mean ± SD.*

TABLE 2. Comparison Between Patients with Active and Inactive CD According to HBI Score[a]

	Inactive CD, HBI ≤4 (n = 29)	Active CD, HBI >4 (n = 44)	*P* Value
SA (mg/dL)	48.68 ± 13.62	74.18 ± 17.13	.007
CRP (mg/L)	12.2 ± 8.5	21.3 ± 14.2	.033
ESR (mm/h)	13.45 ± 10.21	22.09 ± 10.05	.164
WBC count (× 10^9/L)	8.03 ± 2.65	8.42 ± 3.16	.342
ALB (g/L)	35.21 ± 6.62	3.66 ± 8.60	.042

ALB, albumin; CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HBI, Harvey-Bradshaw Index; SA, sialic acid; WBC, white blood cell.
[a]*Data are expressed as mean ± SD.*

Downloaded from https://academic.oup.com/labmed/article/53/4/381/6535392 by guest on 28 February 2025

Correlation Between Serum SA, Disease Activity, and Other Inflammatory Parameters in CD

We investigated the association between serum SA and other markers of disease activity in patients with CD. We found that the SA levels were positively correlated with clinical disease activity as measured by HBI scores (R = 0.706; P = .001) and CRP levels (R = 0.615, P = .005) and inversely correlated with ALB levels (R = –0.456; P = .032). No correlations were found between SA and the ESR (R = 0.328; P = .137) or WBC count (R = 0.304; P = .090) (**TABLE 3**).

Correlation of Disease Activity and Inflammatory Parameters in CD

Spearman correlations between serum biomarkers and the clinical score of disease activity (HBI) in patients with CD are shown in **TABLE 4**. The CRP (R = 0.592; P = .008) and SA levels (R = 0.706; P = .001) were both correlated with the HBI. These findings highlighted a better correlation between the HBI and SA than the CRP level in patients with CD. No correlations were found between the HBI and ESR (R = 0.28; P = .077) or WBC count (R = 0.367; P = .160).

Evaluation of Serum SA Level as an Activity Index in CD

We performed ROC curve analyses to determine the predictive values of SA, CRP, ESR, and WBC count for CD activity. We found that the area under the ROC curve (AUC) of SA for predicting disease activity in patients with CD was 0.806, whereas the AUC of CRP levels was 0.753. The optimal SA cutoff value for active CD was 53.14 mg/dL, with a sensitivity, specificity, PPV, and NPV of 77.3%, 82.8%, 82.9%, and 68.8%, respectively. In contrast, the optimum CRP cutoff point for active CD was 15.45 mg/L, with a sensitivity, specificity, PPV, and NPV of 68.2%, 72.4%, 78.9%, and 60.0%, respectively (**TABLE 5**). These findings emphasized the better potential of using serum SA as a marker for assessing the disease activity of patients with CD.

TABLE 3. Correlations of Serum SA with Clinical Score of Disease Activity (HBI) and Other Essential Inflammation Markers in CD

	Inflammation Biomarkers				
	HBI	CRP	ESR	WBC Count	ALB
SA	R = 0.706	R = 0.615	R = 0.328	R = 0.304	R = –0.456
	P = .001	P = .005	P = .137	P = .090	P = .032

ALB, albumin; CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HBI, Harvey-Bradshaw Index; SA, sialic acid; WBC, white blood cell.

TABLE 4. Correlations Between Clinical Score of Disease Activity (HBI) and Important Inflammation Markers in CD

	Inflammation Biomarkers				
	SA	CRP	ESR	WBC Count	ALB
HBI	R = 0.706	R = 0.592	R = 0.280	R = 0.367	R = –0.186
	P = .001	P = .008	P = .077	P = .160	P = .230

ALB, albumin; CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HBI, Harvey-Bradshaw Index; SA, sialic acid; WBC, white blood cell.

Discussion

In the present study, we found that the SA, CRP, WBC count, and ALB were significantly different between patients with CD and healthy control patients. Patients with active CD had elevated serum SA and CRP levels and decreased ALB levels compared to patients with inactive CD. There was a significant correlation of SA with CRP and ALB. The correlation of SA with the HBI score was superior to that of CRP or ALB with HBI. The ROC analysis indicated that the AUC of SA was higher than the AUC of CRP. These findings indicated that serum SA may be a more effective potential indicator than CRP to predict CD disease activity.

Studies have identified SA as a marker of immunity and inflammatory responses. It belongs to the acute phase class of proteins, of which CRP is also a member. Currently, serum CRP is the most widely used and accepted serum marker for predicting disease activity in patients with CD. But CRP lacks specificity because it often increases in other infectious or inflammatory diseases unrelated to the gastrointestinal tract. Furthermore, CRP levels seem to be unrelated with the disease activity in patients with CD and small intestinal lesions, indicating poor sensibility of CRP.[24-26]

Researchers have proposed that SA could be a more suitable marker than CRP for monitoring the disease activity of patients with CD. A previous study[21] showed that the SA levels of patients with active CD were significantly higher than the SA levels of those in remission or those of a control group. In contrast, CRP levels were only significantly different in patients with CD from healthy control patients. Data indicated that serum CRP might distinguish patients with CD from healthy control patients but might be ineffective in predicting increased inflammatory activity.[21] Similarly, another study[22] reported that serum SA levels were significantly increased in patients in remission compared with those of healthy control patients or postoperative patients. The SA levels were significantly related to the CDAI (R = .5006) and CRP (R = .7888) among patients with CD in remission. The correlation of SA with ALB was not clear. Serum SA had a higher sensitivity than CRP to identify CD disease activity, but ROC analysis was not used to evaluate the sensitivity and specificity. Baba et al[22] found that the sialic acid levels of patients with Crohn's disease in remission (CRP, 0.0 mg/dL) were significantly higher than those of healthy subjects and postoperative patients

TABLE 5. ROC Analyses of SA and Other Inflammation Markers Between Active and Inactive CD

	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SA (cutoff: 53.14)	0.806	77.3	82.8	82.9	68.8
CRP (cutoff: 15.45)	0.753	68.2	72.4	78.9	60.0
ESR (cutoff: 16.50)	0.608	62.1	59.1	70.2	50.0
WBC count (cutoff: 8.13)	0.587	45.5	55.2	60.0	40.0

AUC, area under the curve; CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; SA, sialic acid; WBC, white blood cell.

Downloaded from https://academic.oup.com/labmed/article/53/4/381/6535392 by guest on 28 February 2025

with Crohn's disease, indicating that SA was a useful index of the activity of Crohn's disease. In previous studies, researchers have confirmed the relationship between SA and the CDAI. However, the association of the HBI with SA is still rare. The HBI score is a simplified CDAI that includes only clinical variables and quantifies the disease severity in patients with CD. The HBI may be more appropriate than the CDAI in some clinical trials and even in routine practice because it is easier to calculate and is less subject to recall bias.[27]

Our study has extended previous research findings in several aspects. First, we studied a relatively larger number of patients with CD patients (n = 73). Second, our study enrolled patients with CD in both the clinically active and inactive stages according to the HBI score, different from in the studies mentioned above. We presented the first data to evaluate the potential correlation between serum SA and the HBI in patients with CD. Finally, we performed ROC analyses to determine the predictive values of SA. We found that the optimal SA cutoff value for active CD was 53.14 mg/dL, with better sensitivity and specificity than CRP.

Our study had several limitations. First, the HBI score is an index that estimates disease activity according to self-reported data, and the influences of these data cannot be denied. Second, this study only included a small number of Chinese patients, and the patients were only age- and sex-matched. Other potential contributing factors such as weight, smoking history, length of disease, and therapy history were not taken into consideration. Further research with larger population and more comprehensive demographic information is necessary to validate our results and provide more information regarding the association between SA and the disease activity of patients with CD. Third, we compared the serum SA levels between different patients with both active and inactive CD status. However, comparing the SA levels in the same patients in both the active and inactive stages would be more informative because every patient may have variations in baseline SA levels. Total SA and free SA in the same patients in both the active and inactive stages would be a potential future measurement. Hypothetically, free SA would be increased because of the lower amount of binding proteins to SA that are produced.

Conclusion

In summary, serum SA shows a more significant association with HBI than CRP and is more sensitive and specific in determining CD disease activity. Thus, monitoring SA in patients with CD may reduce the frequency of invasive endoscopic procedures and contribute to a preventive or therapeutic approach. Serum SA is thus a reliable and noninvasive biomarker for patients with CD.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant number NSFC 81901659).

REFERENCES

1. Torres J, Mehandru S, Colombel JF, Peyrin-Biroulet L. Crohn's disease. *Lancet.* 2017;389(10080):1741–1755.
2. Kane SV, Cohen RD, Aikens JE, Hanauer SB. Prevalence of nonadherence with maintenance mesalamine in quiescent ulcerative colitis. *Am J Gastroenterol.* 2001;96(10):2929–2933.
3. Lu Y, Zhou L, Liu L, et al. Serum omentin-1 as a disease activity marker for Crohn's disease. *Dis Markers.* 2014;2014:162517.
4. Feuerstein JD, Cheifetz AS. Crohn disease: epidemiology, diagnosis, and management. *Mayo Clin Proc.* 2017;92(7):1088–1103.
5. Gomollon F, Dignass A, Annese V, et al. 3rd European evidence-based consensus on the diagnosis and management of Crohn's disease 2016: part 1, diagnosis and medical management. *J Crohns Colitis.* 2017;11(1):3–25.
6. Annese V, Daperno M, Rutter MD, et al. European evidence-based consensus for endoscopy in inflammatory bowel disease. *J Crohns Colitis.* 2013;7(12):982–1018.
7. Pennazio M, Spada C, Eliakim R, et al. Small-bowel capsule endoscopy and device-assisted enteroscopy for diagnosis and treatment of small-bowel disorders: European Society of Gastrointestinal Endoscopy (ESGE) clinical guideline. *Endoscopy.* 2015;47(4):352–376.
8. Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's Disease Activity Index. National Cooperative Crohn's Disease Study. *Gastroenterology.* 1976;70(3):439–444.
9. Harvey RF, Bradshaw JM. A simple index of Crohn's disease activity. *Lancet.* 1980;1(8167):514.
10. Vermeire S, Schreiber S, Sandborn WJ, Dubois C, Rutgeerts P. Correlation between the Crohn's disease activity and Harvey-Bradshaw indices in assessing Crohn's disease severity. *Clin Gastroenterol Hepatol.* 2010;8(4):357–363.
11. Jones J, Loftus EV Jr, Panaccione R, et al. Relationships between disease activity and serum and fecal biomarkers in patients with Crohn's disease. *Clin Gastroenterol Hepatol.* 2008;6(11):1218–1224.
12. Sipos F, Galamb O, Wichmann B, et al. Peripheral blood-based discrimination of ulcerative colitis and Crohn's disease from non-IBD colitis by genome-wide gene expression profiling. *Dis Markers.* 2011;30(1):1–17.
13. Sostegni R, Daperno M, Scaglione N, Lavagna A, Rocca R, Pera A. Review article: Crohn's disease: monitoring disease activity *Aliment Pharmacol Ther.* 2003;17(suppl 2):11–17.
14. Mak LY, Tong TSM, Cheung KS, et al. Combined use of common fecal and blood markers for detection of endoscopically active inflammatory bowel disease. *Clin Transl Gastroenterol.* 2020;11(3):e00138.
15. Carroccio A, Iacono G, Cottone M, et al. Diagnostic accuracy of fecal calprotectin assay in distinguishing organic causes of chronic diarrhea from irritable bowel syndrome: a prospective study in adults and children. *Clin Chem*. 2003;49(6 pt 1):861–867.
16. Goutorbe F, Goutte M, Minet-Quinard R, et al. Endoscopic factors influencing fecal calprotectin value in Crohn's disease. *J Crohns Colitis.* 2015;9(12):1113–1119.
17. Laubli H, Varki A. Sialic acid-binding immunoglobulin-like lectins (siglecs) detect self-associated molecular patterns to regulate immune responses. *Cell Mol Life Sci.* 2020;77(4):593–605.
18. Bull C, Stoel MA, den Brok MH, Adema GJ. Sialic acids sweeten a tumor's life. *Cancer Res.* 2014;74(12):3199–3204.
19. Traving C, Schauer R. Structure, function and metabolism of sialic acids. *Cell Mol Life Sci.* 1998;54(12):1330–1349.
20. Li W, Liu Y, Zheng X, Gao J, Wang L, Li Y. Investigation of the potential use of sialic acid as a biomarker for rheumatoid arthritis. *Ann Clin Lab Sci.* 2019;49(2):224–231.
21. Ricci G, D'Ambrosi A, Resca D, Masotti M, Alvisi V. Comparison of serum total sialic acid, C-reactive protein, alpha 1-acid glycoprotein and beta 2-microglobulin in patients with non-malignant bowel diseases. *Biomed Pharmacother*. 1995;49(5):259–262.
22. Baba R, Yashiro K, Nagasako K, Obata H. Significance of serum sialic acid in patients with Crohn's disease. *Gastroenterol Jpn.* 1992;27(5):604–610.
23. Chinem ESS, Esberard BC, Moreira ADL, et al. Changes in the management of patients with Crohn's disease based on magnetic resonance enterography patterns. *Gastroenterol Res Pract.* 2019;2019:3467316.

Downloaded from https://academic.oup.com/labmed/article/53/4/381/6535392 by guest on 28 February 2025

24. Florin TH, Paterson EW, Fowler EV, Radford-Smith GL. Clinically active Crohn's disease in the presence of a low C-reactive protein. *Scand J Gastroenterol.* 2006;41(3):306–311.
25. Solem CA, Loftus EV Jr, Tremaine WJ, Harmsen WS, Zinsmeister AR, Sandborn WJ. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. *Inflamm Bowel Dis.* 2005;11(8):707–712.
26. Click B, Vargas EJ, Anderson AM, et al. Silent Crohn's disease: asymptomatic patients with elevated C-reactive protein are at risk for subsequent hospitalization. *Inflamm Bowel Dis.* 2015;21(10): 2254–2261.
27. Best WR. Predicting the Crohn's Disease Activity Index from the Harvey-Bradshaw Index. *Inflamm Bowel Dis.* 2006;12(4):304–310.

Downloaded from https://academic.oup.com/labmed/article/53/4/381/6535392 by guest on 28 February 2025

Urinary MMP-7: A Predictive, Noninvasive Early Marker for Chronic Kidney Disease Development in Patients with Hypertension

RajLaxmi Sarangi, MD,[1] Krishna Padarabinda Tripathy, MD,[2] Jyotirmayee Bahinipati, MD,[1,a,] Partisha Gupta, MD,[2] Mona Pathak, PhD,[3] Srikrushna Mahapatra, MD,[1] Soumya R. Mohapatra, PhD[3,4,a,*]*

[1]Department of Biochemistry, Kalinga Institute of Medical Sciences, KIIT Deemed to be University, Bhubaneswar, India, [2]Department of Medicine, Kalinga Institute of Medical Sciences, KIIT Deemed to be University, Bhubaneswar, India, [3]Department of Research and Development, Kalinga Institute of Medical Sciences, KIIT Deemed to be University, Bhubaneswar, India, [4]School of Biotechnology, KIIT Deemed to be University, Bhubaneswar, India; *To whom correspondence should be addressed. soumya.mohapatra@kims.ac.in; [a]These authors contributed equally.

Keywords: MMP-7, chronic kidney disease, hypertension, biomarker, fibrosis, eGFR, ACR

Abbreviations: MMP-7, matrix metalloproteinase-7; CKD, chronic kidney disease; CVD, cardiovascular disease; EMT, epithelial-mesenchymal transition; eGFR, estimated glomerular filtration rate; ARB, angiotensin-receptor blocker; ACE, angiotensin-converting enzyme; CCB, calcium channel blocker; ACR, albumin-creatinine ratio; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; IQR, interquartile range; ROC, receiver operating characteristic; AUC, area under the receiver operating characteristic curve.

Laboratory Medicine 2022;53:386–393; https://doi.org/10.1093/labmed/lmac003

ABSTRACT

Objective: Upregulation of matrix metalloproteinase-7 (MMP-7) is associated with hypertension and kidney fibrosis, which can progress to chronic kidney disease (CKD). Currently, kidney fibrosis is only detectable by an invasive procedure. Therefore, we set out to determine whether MMP-7 can act as a noninvasive biomarker in patients with hypertension to enable early detection of kidney fibrosis.

Materials and Methods: Diagnosed patients with hypertension and control patients were sampled. We diagnosed CKD using clinical and laboratory parameters. Serum urea, creatinine, urinary microalbumin, the albumin-to-creatinine ratio, and urinary MMP-7 were analyzed.

Results: The 195 patients with hypertension had significantly elevated MMP-7. Of these patients, 166 had MMP-7 >25.8 μg/L, whereas only 29 had MMP-7 <25.8 μg/L. Thirty-two patients with hypertension showed features of CKD, all of whom had urinary MMP-7 >25.8 μg/L. However, the urinary MMP-7 level did not differ with the severity of CKD or with the duration of hypertension.

Conclusion: Elevated urinary MMP-7 can be a potential noninvasive, early indicator in patients with hypertension progressing to CKD, thus enabling early therapeutic intervention.

Chronic kidney disease (CKD) represents a major public health problem because of its increasing global incidence, prevalence, significant mortality, and high treatment cost. In addition to various other risk factors, hypertension is a major underlying cause behind the development of and progression to CKD.[1] The global prevalence of hypertension is increasing: approximately 31.1% of adults (28.5% in high-income countries, 31.5% in low- to middle-income countries) experience hypertension,[2] and its incidence is rising dramatically. Increased blood pressure alters kidney hemodynamics, resulting in fibroplastic intimal thickening of small arteries, glomerular ischemia, and interstitial fibrosis, which with time can potentially progress to CKD.[3] Prior studies have shown that patients with hypertension have higher rates of hidden CKD.[4] The global prevalence of CKD in the adult population is approximately 11%-13%, and the majority of patients are in stage III.[5]

Matrix metalloproteinases (MMPs) are also known as matrixins (zinc-dependent proteases), and these are responsible for the cleaving and rebuilding of a wide range of connective tissue components such as collagen, elastin, gelatin, casein, fibronectin, and proteoglycan along with nonmatrix proteins like pro-heparin binding epidermal growth factor, Fas-ligand, E-cadherin, beta-2 adrenergic receptor, and tumor necrosis factor-alpha.[6] To date, 24 to 26 types of MMPs have been found in human beings and are classified in different groups according to the substrate recognition and cleavage mechanism.[7] Among them, MMP-7 and MMP-26 are the smallest members of MMPs, which are categorized under the matrilysin group.[7]

In previous studies, MMP-7 has been implicated in human pathologies such as cardiac hypertrophy and fibrosis.[8] It transcriptionally

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

activates MMP-2, which generates a series of peptides that have vasoconstrictor activity.[9] MMP-7 thus appears to activate MMP-2 , which is a mediator of hypertension and comorbidities associated with it.[9] Furthermore, MMP-7 is also involved in the mediation of vascular tone and cardiac remodeling. It impacts cardiovascular disease (CVD) development through the cleavage of substrate other than the epidermal growth factor receptor ligand.[10]

Research has shown that MMP-7, controlled by the beta-catenin canonical wnt signaling pathway, degrades the extracellular matrix by using collagen IV and X as its substrate and can cause matrix remodeling, cell apoptosis, and epithelial-mesenchymal transition (EMT).[11,12] It localizes in the tubular epithelial cell and glomerular podocyte and can be easily excreted into urine during kidney pathologies such as CKD. Under these pathological conditions, urinary MMP-7 expression is dramatically induced under transcriptional control.[13] Renal interstitial fibrosis and glomerulosclerosis are the final outcome of CKD, and during this process MMPs play a significant role.[14]

Although kidney function can be evaluated by serum creatinine, estimated glomerular filtration rate (eGFR), and microalbuminuria, however, the assessment of renal fibrosis currently is only achievable with kidney biopsy, which is an invasive procedure. Even though creatinine is the most prevalent marker of renal dysfunction, it has some limitations because it rises when the eGFR has decreased by 50%.[15] In this context, there is a need to identify a noninvasive surrogate biomarker that can detect renal fibrosis in patients with hypertension even before the rise of serum creatinine, albumin excretion in urine, and altered eGFR, which can be a predictive marker for renal fibrosis in patients with hypertension with normal eGFR.

In this light, the aim of the present study was to determine whether urinary MMP-7 levels in patients with hypertension can be used as a predictor of hypertensive renal dysfunction and a prognostic marker for CKD severity among patients with hypertension.

Materials and Methods

This hospital-based cross-sectional study was conducted in the department of biochemistry in collaboration with the department of general medicine and the department of research and development in a tertiary care health center over a period of 1 year (June 2019–May 2020). The involvement of human participants in the study was reviewed and approved by the Ethics Committee of the Kalinga Institute of Medical Sciences, KIIT-DU, Bhubaneswar vide letter number KIIT/KIMS/IEC/43/2019. Written informed consent for participation in this study was obtained from all participants and was taken in accordance with national legislation and the institutional requirements. Confidentiality of the collected data was maintained.

Diagnosed patients with hypertension (n = 195) within the age group of 30 to 70 years and those receiving antihypertensive treatment were included in the study. Recommended Joint National Committee guidelines were used for diagnosing patients with hypertension.[12] These patients with hypertension were divided into 3 groups depending upon the duration of hypertension. Group I patients were newly diagnosed and were within 1 year of their diagnosis, group II patients had been diagnosed with hypertension between 1 and 5 years, and group III patients had been diagnosed with hypertension >5 years. At the time of sampling for our study, of the patients with hypertension (n = 195), 84 were on angiotensin-receptor blockers (ARBs). The rest (n = 111) were either on single doses or a combination of angiotensin-converting enzyme inhibitors (ACE inhibitors), calcium channel blockers (CCBs), or α-blockers.

Patients with hypertension with CKD were diagnosed according to clinical and laboratory parameters (urinary microalbumin, albumin-creatinine ratio [ACR], serum creatinine, and eGFR). The eGFR was calculated by using the Modification of Diet in Renal Disease formula in mL/min/1.73 m^2 ($175 \times SCr^{-1.154} \times Age^{-0.203} \times 0.742$ [if female] × 1.21 [if Black]), and CKD staging was done using the following parameters: stage I, eGFR ≥90 mL/min/1.73m^2; stage II, eGFR 60–89 mL/min/1.73m^2; stage III, eGFR 30–59 mL/min/1.73m^2; stage IV, eGFR 15–29 mL/min/1.73m^2; and stage V, eGFR <15 mL/min/1.73m^2.[16,17] We included 25 individuals aged 30 to 70 years without any systemic diseases in the control group. Patients with diabetes mellitus, autoimmune disease, malignancy, gout, infective diseases affecting the kidneys, an existing inherited defect of kidney disease, and other comorbid conditions that alter kidney functions or the glomerular filtration rate were excluded from the study.

Data Collection

Under strict aseptic measures, a 3 mL venous blood specimen was collected from the medial cubital vein in a red-top vacutainer to analyze urea and creatinine. We collected 2 mL of blood in a gray-top vacutainer to analyze fasting blood sugar/postprandial blood sugar to exclude diabetes mellitus. In addition, 10 mL of midstream urine (morning specimen) was collected in a sterile container. Postcollection, 5 mL of urine was used to analyze urinary microalbumin (using the immunoturbidometric method) and urinary creatinine (using Jaffe's method). The ACR was calculated as mg albumin to g creatinine. All biochemical parameters were analyzed using a modular autoanalyzer, the Ortho Clinical Diagnostics Vitros 5600 (Johnson & Johnson).

We kept 5 mL of collected urine in a sterile container. After centrifuging at a speed of 2000 to 3000 rpm for 20 minutes, the supernatant was removed and immediately aliquoted and stored at –70°C for MMP-7 analysis. Urinary MMP-7 was analyzed using the human MMP-7 enzyme-linked immunosorbent assay (ELISA) kit (Sino Gen Clon Biotech, catalog number 10410) according to the manufacturer's protocol. The coefficients of variability for intraassay and interassay precision for the MMP-7 ELISA kit were <8% and <10%, respectively. The analytical measurement range of urinary MMP-7 was 0.5 µg/L to 20 µg/L. Purified MMP-7 antibody was coated to the microtiter plate, forming a solid-phase antibody. When MMP-7 from specimens was added to the wells, the MMP-7 antibody combined with labeled horseradish peroxidase to form an antibody-antigen-enzyme-antibody complex. After washing, a tetramethylbenzidine substrate was added, which became blue in color. The reaction was then terminated by a stop solution. The color change was measured at a wavelength of 450 nm on a PR4100 (Biorad) ELISA reader. The concentration of urinary MMP-7 was then determined by comparing the OD of the specimen to the standard curve. The urinary MMP-7 values reported in the current study were obtained after normalization of the measured urinary MMP-7 with urinary creatinine for each patient.

Statistics

Mean and standard deviation (SD) were used to describe continuous variables. Measurements with a skewed distribution were described using median and interquartile range (IQR). For comparison between the 2 datasets, the Student's *t*-test or Mann-Whitney *U* test (Wilcoxon rank sum test) was used, and for comparison between multiple

Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

datasets an ordinary 1-way analysis of variance was performed. For deciphering the relationship between 2 variables, the Spearman rank correlation analysis was performed. The Welch correction was done to compare categorical variables between 2 groups wherever necessary. The receiver operating characteristic (ROC) curve was used to identify the best cutoff for the classification of disease, and sensitivity and specificity are reported at those cutoff values. To compare the classification ability, the area under the ROC curve (AUC) was compared. Differences were considered to be statistically significant when $P \leq .05$). GraphPad Prism version 8.02.1 (GraphPad Software) was used for statistical analysis.

Results

Comparison of Biochemical Parameters Between Patients with Hypertension and Control Population

After the data collection phase, we performed a comparative analysis of the various collected demographic and biochemical parameters in patients with hypertension as compared to control patients. The results from this analysis are depicted in **TABLE 1**. The mean and SD of the serum urea concentration among patients with hypertension and control patients were 28.97 ± 21.01 mg/dL vs 15.36 ± 3.12 mg/dL, and they were significantly increased among patients with hypertension. Creatinine increased among patients with hypertension in comparison to the control patients but was not significantly different. The ACR was increased among patients with hypertension (421.16 ± 1434.69 mg/g vs 24.72 ± 3.27 mg/g); however, the data suggested that the distribution of the ACR was highly skewed, further requiring the evaluation of the median and IQR. The data revealed that the ACR was significantly increased among patients with hypertension (69.25 ± 153.35 mg/g vs 25 ± 3 mg/g) with a Mann-Whitney *U* test *P* value <.0001 (**TABLE 1**). Urinary MMP-7 in patients with hypertension and control patients was 40.75 ± 14.58 μg/L vs 22.35 ± 5.08μg/L, respectively, and it was significantly increased among patients with hypertension (**FIGURE 1A**). Setting 25.8 μg/L as the threshold, patients with hypertension were divided into high urinary MMP-7 and low urinary MMP-7 groups; a comparison between the 2 groups revealed significant differences between both sides of the threshold limit (**FIGURE 1B** and **FIGURE 1C**). The schematic representation is depicted in **FIGURE 2A**.

TABLE 1. Comparison of Biochemical Parameters Among Patients with Hypertension (n = 195) and Control Patients (n = 25)[a]

Parameters	Hypertension	Control	*P* Value
Age (y)	53.420 ± 13.223	45.56 ± 17.57	.0398
BMI (kg/m^2)	25.870 ± 4.06	NA	NA
Sex, M/F	56.93%/43.07%	60.0%/40.0%	
Urea (mg/dL)	28.97 ± 21.010 (n = 170)	15.36 ± 3.12	<.0001
Creatinine (mg/dL)	1.09 ± 1.08 (n = 186)	0.98 ± 0.13	.15652
ACR (mg/g)	69.25 ± 153.35 (n = 180)	25 ± 3	<.0001
Urinary MMP7 (μg/L)	40.75 ± 14.58 (n = 195)	22.35 ± 5.08	<.0001

ACR, albumin-creatinine ratio; BMI, body mass index; IQR, interquartile range; MMP-7, matrix metalloproteinase-7; NA, not applicable; SD, standard deviation.
[a]Urea, creatinine, and urinary MMP-7 are described as mean ± SD, and ACR is described in terms of median ± IQR.

Comparison of Urinary MMP-7 in Patients with Hypertension Progressing to CKD

Among the patients with hypertension with higher levels of MMP-7, 32 individuals (**FIGURE 2B**) showed evidence of CKD, whereas among those patients with low MMP-7 levels, only 1 patient indicated CKD (**FIGURE 2C**). When we further grouped the available cohort based on MMP-7 levels, we found that the distribution of individuals followed a Gaussian pattern, with the highest numbers concentrated in those with MMP-7 levels of 30 to 50 μg/L (**FIGURE 2D**). **FIGURE 2E** depicts the percentage distribution of CKD in these groups, and it was found that the maximum distribution (24%) was seen in patients with hypertension who had MMP-7 levels within the 40 to 50 μg/L range.

Analysis of Urinary MMP-7 with CKD Severity and Hypertension Duration

No significant differences were observed in urinary MMP-7 levels with the severity of CKD (**FIGURE 3A**). Furthermore, urinary MMP-7 levels did not reveal any significant difference with the duration of hypertension (**FIGURE 3B**). Spearman correlation analysis also did not reveal any association between urinary MMP-7 levels and hypertension duration (**FIGURE 3C**). The ACR and urinary MMP-7 cutoff values for patients with hypertension with CKD were 27.5 mg/g and 25.8 μg/L, respectively, with a sensitivity of 76.35% and 85.64% and a specificity of 84% and 84%, respectively. The ACR and urinary MMP-7 are both sufficient to classify individuals with and without hypertension, but urinary MMP-7 with a higher sensitivity is significantly better ($P = .0095$), which can also be visualized using the AUC (**FIGURE 4**).

Discussion

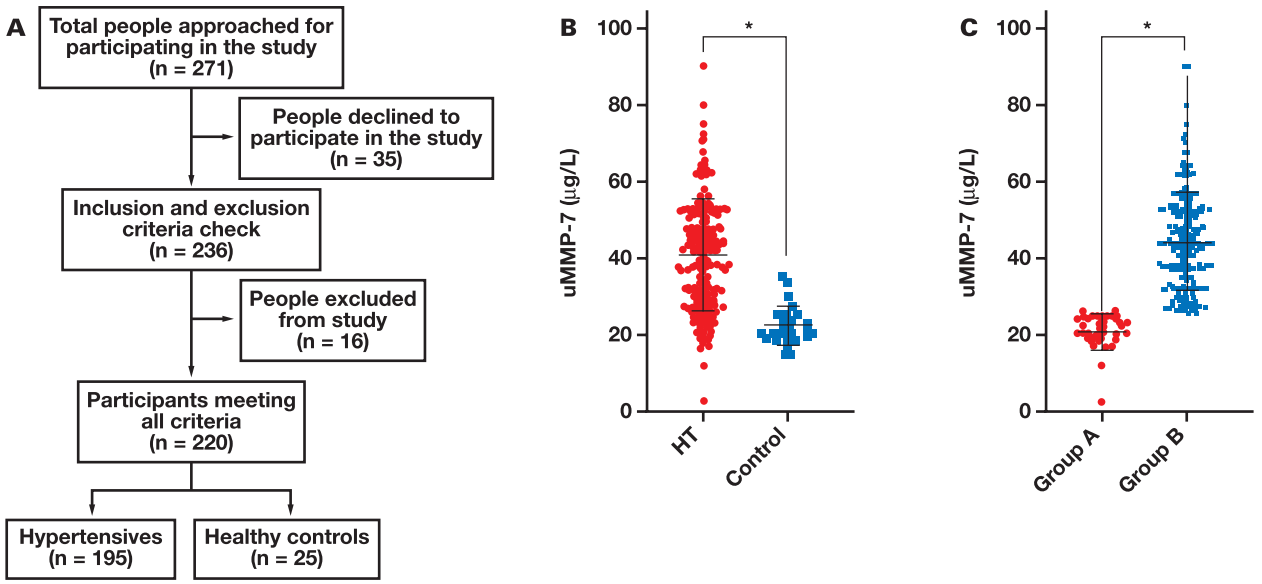
Hypertension is associated with vascular remodeling by the rearrangement of the vascular wall components. Studies have found that MMPs contribute to this remodeling, and increased expression of MMPs has been linked with the development of various human ailments such as atherosclerosis, its progression, and its associated complications.[18] Hence, MMPs are targeted in therapeutic intervention against such ailments, eg, in therapeutic intervention against hypertensive cardiac remodeling and in some diseases where they are found to be upregulated, such as CKD and cancer.[19]

Of the different types of MMPs, the role of MMP-7 has been observed to mediate vascular tone and the pathogenesis of cardiac remodeling. Studies on MMP-7 in knockout and knockdown mouse models have shown that MMP-7 is involved in the vascular tone regulation of the small mesenteric arteries.[8,20] It has also been found to mediate left ventricular remodeling by cleaving connexin (a gap junction protein).[21] In addition, MMP-7 is a key mediator upstream of epidermal growth factor receptor and downstream of Gq protein-coupled receptors involved in the causation of CVD and various non-CVD disorders.[8]

Our results show that urinary MMP-7 was significantly increased in patients with hypertension compared to control patients (**FIGURE 1B**). In light of this finding, MMP-7 seems to be an important causative link because various proteolytic processes are involved in vascular remodeling in patients with hypertension.[8] Research has indicated that MMP-7 transcriptionally activates MMP-2, which generates a series of peptides that has vasoconstrictor activity. This finding suggests that MMP-7 may be a mediator of hypertension and also the comorbidities linked with hypertension.[9]

Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

FIGURE 1. Urinary matrix metalloproteinase-7 (uMMP-7) levels in patients with hypertension (HT) is significantly higher. A, Consort flow diagram. B, uMMP-7 was significantly increased among patients with hypertension compared to patients in the control group. C, Patients with hypertension with low uMMP-7 levels (<25.8 µg/L; n = 29) were in group A and those with high uMMP-7 levels (>25.8 µg/L; n=166) were in group B; uMMP-7 was significantly higher in group B. Data are expressed as mean ± standard deviation.



Hypertension is an independent risk factor for CKD. Prevalence ranges from 60% to 90% depending upon the stage of CKD, and it is one of the major causes of end-stage renal disease next to diabetic nephropathy.[22] The gold standard diagnosis of renal fibrosis is only achievable with kidney biopsy,[23] which is an invasive procedure. However, because an invasive procedure was not in the scope of the current study, we employed surrogate biochemical parameters for patients with hypertension progressing to CKD with an assumption for progressive kidney fibrosis. Studies have shown that CKD leads to the progressive thickening and sclerosis of renal-resistant vessels, although it spares the glomerular capillaries manifested by persistent functional decline and progressive tissue fibrosis.[24] Early diagnosis of hypertension and cardiovascular changes can thus provide clinicians with sufficient time to improve CVD-related morbidity and mortality.

Research has indicated that MMP-7s can be important noninvasive markers to detect and monitor the progression of CVD. They are regulators of extracellular matrix formation and breakdown in glomerulus. The overexpression of MMP-7 is detected in various pathological states of the kidneys, but none has been observed in the tubular epithelium. Observations in mouse models have shown that the genetic ablation of MMP-7 resulted in no functional or behavioral abnormality of the kidneys.[20,25]

The glycoprotein wnt-4 is required for nephrogenesis and normal renal tubular development and is basically expressed in the distal papillary collecting duct epithelium. However, its expression is induced through the collecting duct epithelium and interstitial cells after renal injury.[26] The signaling of wnt leads the binding of β-catenin to the nucleus, where it binds to the lymphocyte enhancer binding factor-1/T- cell factor pathway and regulates the expression of target genes, specifically matrilysin/MMP-7. Therefore, β-catenin is a downstream mediator of wnt signaling.[27] Because wnt/β-catenin activation occurs in CKD, studies have suggested that it causes the upregulation of MMP-7, resulting in fibrosis of the kidneys in CKD.[27,28]

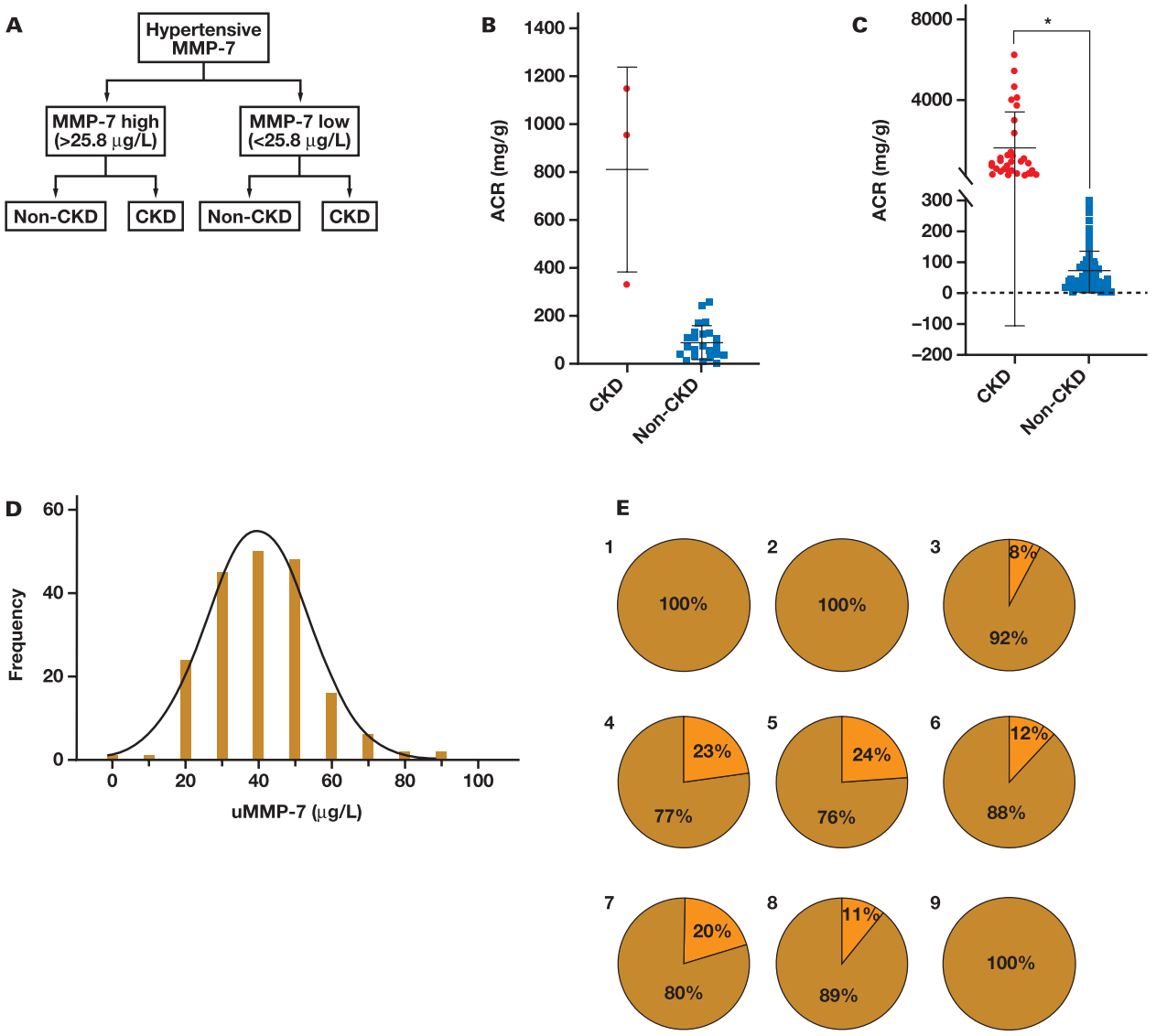
A previous cross-sectional study compared urinary MMP-7 among 102 patients with CKD with control patients and observed that urinary MMP-7 was markedly increased in patients with different kidney disorders and that the level of MMP-7 was even correlated with the disease course of renal fibrosis.[29] Subsequently, MMP-7 has been considered as an important marker of early kidney injury.[25] Furthermore, studies have detected MMP-7 in the urine of patients with CKD and in kidney biopsy specimens from patients with CKD, predominantly localized in the renal tubular epithelium of the diseased kidneys.[11] The expression of MMP-7 in these studies was also closely correlated with the renal wnt/β-catenin signaling pathway.[11]

However, the pathogenic mechanism behind the exact role of MMP-7 during the induction of renal fibrogenesis still remains to be explored. Various reports have stated that MMP-7 results in the proteolytic degradation of E-cadherin, which is essential for the maintenance of tubular epithelial integrity, resulting in EMT in diseased kidneys.[13] Research has also shown that MMP-7 further promotes renal fibrosis by stimulating the activation of MMP-2 and MMP-9[14] and promotes tumor necrosis factor-alpha release further worsens renal inflammation, which also leads to fibrosis.[14]

Consistent with previous reports, our results also indicate that patients with hypertension have higher urinary MMP-7 levels than control patients (**FIGURE 1B**). We divided the patients with hypertension into 2 groups depending upon the urinary MMP-7 level distribution, considering the cutoff value to be >25.8 µg/L. Most previous studies have reported neither the clinical reference range nor the clinically reportable range for MMP-7. Our current study was constrained by a limited number of healthy control patients, so the clinical reference range or cutoff value was difficult to decipher accurately. Therefore, we

Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

FIGURE 2. **Urinary matrix metalloproteinase-7 (uMMP-7) among patients with hypertension progressing to chronic kidney disease (CKD). A, Schematic representation of distribution of patients with hypertension showing MMP-7 levels. B, Distribution of patients with and without CKD among patients with hypertension with low MMP-7 levels (<25.8 µg/L) showing albumin-creatinine ratio (ACR). C, Distribution of patients with and without CKD among patients with hypertension with high MMP-7 levels (>25.8 µg/L) showing ACR. Data are expressed as mean ± standard deviation. Statistical significance was assumed at *P* <.05 (**P* <.0001). D, Distribution of patients with hypertension within different ranges of uMMP-7 levels. E, Percentage of patients with CKD among patients with hypertension within different ranges of uMMP-7.**



Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

are currently expanding our investigations to measure MMP-7 levels in a large healthy cohort to calculate and report a standard clinical reference range for MMP-7.

We found that 17.29% of patients with CKD were in the group with higher MMP-7 levels, whereas in patients with hypertension with low MMP-7 levels only 11.1% progressed to CKD. Furthermore, a majority of patients with CKD had MMP-7 levels within the 40 to 50 µg/L range (**FIGURES 1B** and **2**). However, we could not find any association with the severity of CKD in that no significant difference in MMP-7 levels was seen between patients with early CKD and those progressing to late stages of CKD, although the levels were higher in all stages as compared to those in the control patients (**FIGURE 3A**). Furthermore, neither ACR (AUC, 0.69 [0.61–0.77]) nor urinary MMP-7 (AUC, 0.55 (0.45–0.65) were accurate enough to classify patients with hypertension with and without CKD (**FIGURE 4**). This finding could have resulted from our study having fewer patients with CKD, with most in stage III, whereas in the study done by Zhang et al[30] on patients with IgA nephropathy, higher MMP-7 levels were found with declining kidney function ($r = -0.565$).

In our study, patients with hypertension, even those who had normal serum creatinine and eGFR (>90 mL/min/1.73m^2), had a urinary MMP-7 that was higher than the reference value (44.61 ± 16.19 µg/L; **TABLE 1**). Although levels of urinary MMP-7 were increased in newly diagnosed patients with hypertension, the levels remained constant

FIGURE 3. Urinary matrix metalloproteinase-7 (uMMP-7) is unaffected by the severity of chronic kidney disease (CKD) and the duration of hypertension. A, Levels of uMMP-7 were compared between control patients and among patients at different stages of CKD (*P <.01, **P <.001). B, Comparison of uMMP-7 levels in patients with prevailing hypertension for varying time duration. C, Spearman correlation analysis of uMMP-7 levels with time duration of hypertension. Data are expressed as mean ± standard deviation. Statistical significance was assumed at P <.05. r = .04309; P =.66.

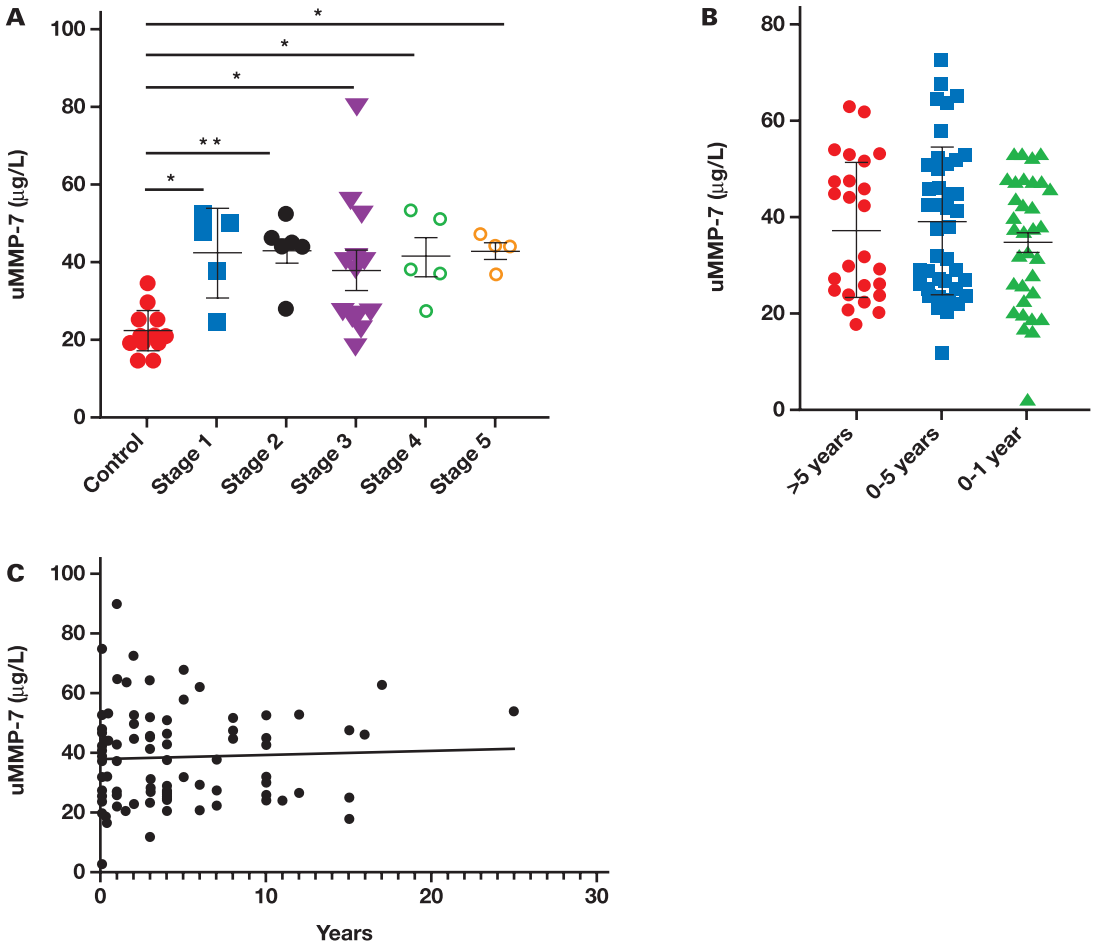
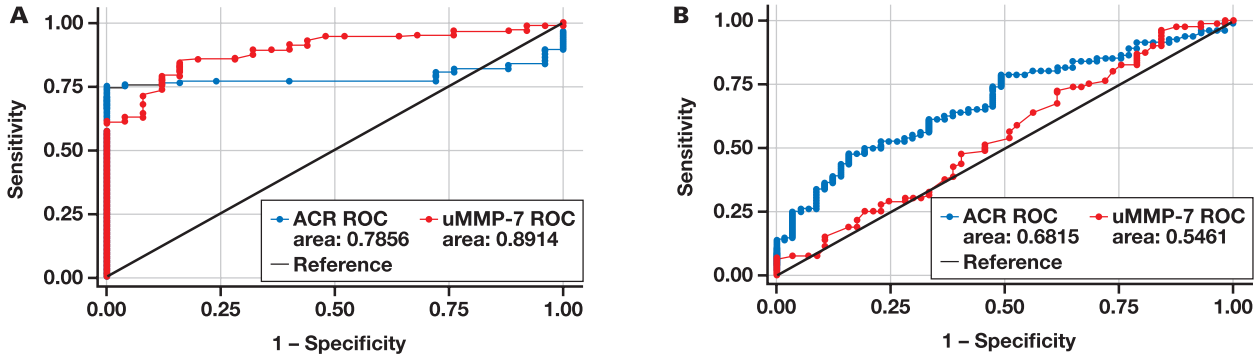


FIGURE 4. Receiver operating characteristic (ROC) curves of urinary albumin-creatinine ratio (ACR) and urinary matrix metalloproteinase-7 (uMMP-7). A, ROC curve depicting urinary ACR and uMMP-7 classifying patients with hypertension with high sensitivity and specificity. B, ROC curve depicting urinary ACR and uMMP-7, both of which depict lower sensitivity and specificity and classify patients with hypertension with and without chronic kidney disease.



throughout the course of the disease (**FIGURE 3B**). This result suggests that MMP-7 is an important etiological mediator of hypertension and remains elevated throughout the disease course. Patients with hypertension can very easily progress to CKD with this rise in MMP-7 levels. Hence, all patients with hypertension are at risk of developing CKD even at an early stage. Therefore, based on the findings of our study, pharmacological targeting of MMP-7 can decrease the morbidity associated with CKD.

Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

Because the current study was a hospital-based cross-sectional study, we plan to conduct prospective studies in the future, where better association can be determined by following patients with hypertension for some duration and meticulously recording the development of CKD. Furthermore, the effect of antihypertensive drugs on the modulation of MMP-7 levels necessitates further detailed studies because during the current study we did not observe any changes in urinary MMP-7 even in patients with long-term hypertension, 84 of whom were taking ARBs and the rest (n = 111) of whom were taking either single doses or combinations of ACE inhibitors, CCBs, or α-blocker treatment for long durations (**FIGURES 3B** and **3C**).

Conclusion

Urinary MMP-7 levels in our study were increased among patients with hypertension, and higher levels were also seen in patients with hypertension progressing to CKD. Even in patients with normal eGFR, the urinary MMP-7 levels were higher. In the current study, we also took an initial first step toward reporting a clinical reference range and reportable range for urinary MMP-7. This step attains significance because even in patients with hypertension with normal eGFR, urinary MMP-7 can potentially help in identifying whether progression to CKD has ensued or not. Therefore, in our view early intervention may be contemplated in these patients with hypertension who may later progress to CKD. In patients with hypertension, an increase in urinary MMP-7 levels can be a potential early indicator of CKD development, and treatment can hence be directed toward the management of progression to fibrosis.

Acknowledgments

We thank Dr. Pradip Kumar Pattnaik, principal, KIMS, for his immense support during the course of the study. We further thank all our study participants and laboratory technical staff for technical help. This work was supported by an internal grant from the Kalinga Institute of Medical Sciences, KIIT-DU, Bhubaneswar, India. All data generated during the current study are available upon reasonable request sent to the corresponding author.

Author Contributions

RajLaxmi Sarangi: conceptualization, methodology, investigation, writing—original draft; Krishna Padarabinda Tripathy: resources and data curation; Jyotirmayee Bahinipati: conceptualization, investigation, validation, supervision, writing—original draft; Pratisha Gupta: resources and data curation; Mona Pathak: data analysis; Srikrushna Mahapatra: conceptualization, resources; Soumya R. Mohapatra: conceptualization, methodology, formal analysis, visualization, supervision, writing—original draft and final review. All the authors read, reviewed, and revised the manuscript.

REFERENCES

1. Bikbov B, Purcell CA, Levey AS, et al. Global, regional, and national burden of chronic kidney disease, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* 2020;395(10225):709–733.
2. Mills KT, Bundy JD, Kelly TN, et al. Global disparities of hypertension prevalence and control: a systematic analysis of population-based studies from 90 countries. *Circulation.* 2016;134(6):441–450.
3. Mayet J, Hughes A. Cardiac and vascular pathophysiology in hypertension. *Heart.* 2003;89(9):1104–1109.
4. da Silva LS, Cotta RMM, Moreira TR, et al. Hidden prevalence of chronic kidney disease in hypertensive patients: the strategic role of primary health care. *Public Health.* 2016;140:250–257.
5. Al-Shdaifat EA, Manaf MR. The economic burden of hemodialysis in Jordan. *Indian J Med Sci.* 2013;67(5–6):103–116.
6. Cui N, Hu M, Khalil RA. Biochemical and biological attributes of matrix metalloproteinases. *Prog Mol Biol Transl Sci.* 2017;147:1–73.
7. Löffek S, Schilling O, Franzke CW. Biological role of matrix metalloproteinases: a critical balance. *Eur Respir J.* 2011;38(1):191–208.
8. Bosonea AM, Wang X, Odenbach J, Fernandez-Patron C. Metalloproteinases in hypertension and cardiac disease: differential expression and mutual regulation. *Drug Discov Today Dis Models.* 2011;8(1):29–35.
9. Schmid-Schönbein GW. Matrix metalloproteinases activities in hypertension: emerging opportunities. *Hypertension.* 2011;57(1):24–25.
10. Arvidsson M, Ahmed A, Bouzina H, Rådegran G. Matrix metalloproteinase 7 in diagnosis and differentiation of pulmonary arterial hypertension. *Pulm Circ.* 2019;9(4):2045894019895414.
11. He W, Tan RJ, Li Y, et al. Matrix metalloproteinase-7 as a surrogate marker predicts renal Wnt/β-catenin activity in CKD. *J Am Soc Nephrol.* 2012;23(2):294–304.
12. Hernandez-Vila E. A review of the JNC 8 blood pressure guideline. *Tex Heart Inst J.* 2014;42(3):226–228.
13. Ke B, Fan C, Yang L, Fang X. Matrix metalloproteinase-7 and kidney fibrosis. *Front Physiol.* 2017;8:1–7.
14. Cheng Z, Limbu MH, Wang Z, et al. MMP-2 and 9 in chronic kidney disease. *Int J Mol Sci.* 2017;18(4):1–11.
15. Badrick T, Turner P. The uncertainty of the eGFR. *Indian J Clin Biochem.* 2013;28(3):242–247.
16. Lamb EJ, Tomson CR, Roderick PJ; Clinical Sciences Reviews Committee of the Association for Clinical Biochemistry. Estimating kidney function in adults using formulae. *Ann Clin Biochem.* 2005;42(Pt 5):321–345.
17. Sandilands EA, Dhaun N, Dear JW, Webb DJ. Measurement of renal function in patients with chronic kidney disease. *Br J Clin Pharmacol.* 2013;76(4):504–515.
18. Wang X, Khalil RA. Matrix metalloproteinases, vascular remodeling, and vascular disease. *Adv Pharmacol.* 2018;81:241–330.
19. Odenbach J, Wang X, Cooper S, et al. MMP-2 mediates angiotensin II-induced hypertension under the transcriptional control of MMP-7 and TACE. *Hypertension.* 2011;57(1):123–130.
20. Li Q, Park PW, Wilson CL, Parks WC. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell.* 2002;111(5):635–646.
21. Lindsey ML, Escobar GP, Mukherjee R, et al. Matrix metalloproteinase-7 affects connexin-43 levels, electrical conduction, and survival after myocardial infarction. *Circulation.* 2006;113(25):2919–2928.
22. Ku E, Lee BJ, Wei J, Weir MR. Hypertension in CKD: core curriculum 2019. *Am J Kidney Dis.* 2019;74(1):120–131.
23. Berchtold L, Friedli I, Vallée JP, Moll S, Martin PY, de Seigneux S. Diagnosis and assessment of renal fibrosis: the state of the art. *Swiss Med Wkly.* 2017;147:w14442.
24. Griffin KA. Hypertensive kidney injury and the progression of chronic kidney disease. *Hypertension*. 2017;70(4):687–694.
25. Liu Z, Tan RJ, Liu Y. The many faces of matrix metalloproteinase-7 in kidney diseases. *Biomolecules.* 2020;10(6):1–16.

Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

26. Surendran K, McCaul SP, Simon TC. A role for Wnt-4 in renal fibrosis. *Am J Physiol Renal Physiol.* 2002;282(3):F431–F441.
27. Zhou D, Tan RJ, Fu H, Liu Y. Wnt/β-catenin signaling in kidney injury and repair: a double-edged sword. *Lab Invest.* 2016;96(2):156–167.
28. Tan RJ, Zhou D, Zhou L, Liu Y. Wnt/β-catenin signaling and kidney fibrosis. *Kidney Int Suppl (2011).* 2014;4(1):84–90.
29. Zhou D, Tian Y, Sun L, et al. Matrix metalloproteinase-7 is a urinary biomarker and pathogenic mediator of kidney fibrosis. *J Am Soc Nephrol.* 2017;28(2):598–611.
30. Zhang J, Ren P, Wang Y, et al. Serum matrix metalloproteinase-7 level is associated with fibrosis and renal survival in patients with IgA nephropathy. *Kidney Blood Press Res.* 2017;42(3):541–552.

Downloaded from https://academic.oup.com/labmed/article/53/4/386/6542860 by guest on 28 February 2025

Determination of Glyphosate and AMPA in Blood Can Predict the Severity of Acute Glyphosate Herbicide Poisoning

M. Cellier,[1] *N. Anthony,*[1] *C. Bruneau,*[1] *A. Descatha*[1,2]

[1]Grand Ouest Poison Control and Toxicovigilance Center, Angers University Hospital, Angers, France, [2]UNIV Angers, CHU Angers, Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail), Angers, France; *To whom correspondence should be addressed. morgane.cellier@chu-angers.fr

Abbreviations: AMPA, aminomethylphosphonic acid; EPSPS, enolpyruvylshikimate-3-phosphate synthase; ANSES, Agency for Food, Environmental and Occupational Health & Safety; MAs, marketing authorizations; BNCI, Base nationale des cas d'intoxication; CAPTV, Centre antipoison et de toxicovigilance; PSS, Poisoning Severity Score; POEA, polyoxyethylene amine; NPCD, National Product and Composition Database; BNPC, Base nationale des produits et compositions; TPR, true-positive rate; FPR, false-positive rate

Laboratory Medicine 2022;53:394–398; https://doi.org/10.1093/labmed/lmac002

ABSTRACT

Objective: To evaluate a potential association between blood and urine concentration of glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), with severity of acute glyphosate (herbicide) poisoning.

Methods: In our retrospective study of acute glyphosate poisoning, we examined records from the French National Database of Poisonings, dated between January 1, 2004, and December 31, 2016. We compared the severity of poisoning among case individuals using the Fisher exact test and Wilcoxon test. Also, we calculated ROC curves to determine the cutoff for blood and urine concentration.

Results: A total of 17 plasma glyphosate, 11 urine glyphosate, 13 plasma AMPA, and 10 urine AMPA specimens were included in our study, with collection dates ranging from January 1, 2004, through December 31, 2016.

Conclusion: The optimal cutoff we discovered for blood concentration of AMPA was 0.88 mg/L; for glyphosate, it was 600 mg/L. The cutoff plasma concentration of AMPA has never been described in the literature, to our knowledge.

Glyphosate, or N-phosphonomethyl glycine, is a systemic herbicide found in a variety of commercial mixtures, such as Roundup (Monsanto). Its degradation primarily produces aminomethylphosphonic acid (AMPA), an active metabolite with a structure similar to that of glyphosate. This herbicide inhibits the metabolic shikimic acid pathway via competitive inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).[1] The plant is therefore no longer able to produce amino acids and proteins that are vital to its survival.

Because this pathway does not exist in humans, glyphosate was previously not considered to be dangerous to humans.[1] Manufacturers have taken advantage of this presumed feature by branding commercial mixtures of glyphosate as presenting no risk to human health, claiming low acute toxicity under standard conditions of use.[2]

In light of new scientific evidence outlining the carcinogenic risk of glyphosate for humans,[3] the use of this herbicide was recently questioned by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) in November 2018, which reevaluated the marketing authorizations (MAs) for 190 glyphosate-based products, with the aim of limiting their use.[4,5] Acute glyphosate poisoning is common, whether in a deliberate (suicidal) or accidental context, and is not to be dismissed: the severity of these poisonings escalates rapidly.

For acute poisonings, the clinical overview for patients may seem reassuring at first. However, those patients can rapidly develop severe adverse effects, including death. Initially, oropharyngeal and digestive irritation develop and then disappear spontaneously. Poisonings can then quickly become severe, leading to gastric (partially necrotic lesions in the gastrointestinal mucosa), pulmonary (aspiration pneumonia, pulmonary edema), renal (acute kidney injury, metabolic acidosis), and cardiac (cardiovascular collapse) damage, which are potentially fatal.[1,2,6–9] Deaths occur in the 12 to 72 hours after ingestion of glyphosate. Cardiovascular collapse is often described as the cause of death, despite treatment in intensive care and resuscitation units.[1] Hence, one should always pay serious attention to the severity of glyphosate poisonings.

Glyphosate poisoning has been described as a quietly developing toxicity.[1] Its resulting severe conditions must be identified as early as possible for optimal patient management.

In several studies,[6,10] the severity of glyphosate poisoning is determined by the quantity ingested. However, the association between the quantity ingested and the severity has not yet been demonstrated in the

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/394/6527658 by guest on 28 February 2025

literature.[11] In fact, to our knowledge, no toxicity threshold dose has been determined to identify severe glyphosate poisoning. The amounts described in the literature are heterogeneous and difficult to interpret because they are expressed as the volume ingested.[1,4,6] Regarding the difficulty in identifying the assumed ingested dose of glyphosate in grams, other studies[12,13] focused on the concentration of glyphosate or AMPA.

The concentration of glyphosate or its active metabolite in plasma or urine appears to be the most significant factor in determining the degree of severity of poisoning. However, only a few studies[6,12] focus on the concentration of glyphosate, and they generally do not provide a toxicity threshold concentration. Some authors[13] focused only on the concentration of glyphosate in blood and not in urine; they did not focus on the concentration of AMPA in blood or urine.[13] Other authors[12] measured glyphosate and AMPA in blood and urine but have only determined a toxicity threshold concentration of glyphosate in blood.

In contrast to previous studies, our study focused on blood and urine concentration of glyphosate and AMPA and determined a threshold concentration for glyphosate and a threshold concentration for AMPA. The threshold concentration of glyphosate and/or AMPA in blood or urine would allow health care professionals to anticipate the severity of future symptoms for patients and to adapt treatment as quickly as possible in line with the anticipated severity. This information would allow us to optimize treatment for patients, knowing that deaths generally occur in the 72 hours immediately after ingestion of glyphosate. Therefore, our objective was to evaluate the association between blood and urine concentrations of glyphosate and its metabolite, AMPA, with the clinical severity of acute glyphosate (herbicide) poisoning.

Materials and Methods

Selection of Study Data

We extracted retrospective data on cases of glyphosate poisoning that occurred between January 1, 2004, and December 31, 2016, from the French National Database of Poisonings (Base nationale des cas d'intoxication [BNCI]) at Angers Poison and Toxicovigilance Center (Centre antipoison et de toxicovigilance [CAPTV]). Only the initial blood and urine specimens per patient, testing for glyphosate and AMPA, were included. For each case individual included, we collected the following demographic information: age, sex, concentrations of glyphosate and AMPA in the blood and urine with the respective specimen-collection times, details of the poisoning (time of ingestion, assumed ingested amount, etc), and the symptoms experienced by each patient.

The presumed ingested amount was first calculated in mL after questioning the patient or those present with the patient at the hospital. Then, we converted that amount to mg according to the concentration of glyphosate in the ingested mixture. Cases that did not document concentrations of glyphosate or AMPA in the blood and urine were excluded. The severity of the poisoning was assessed using the Poisoning Severity Score (PSS).[14] High severity is defined by a PSS score of ≥3, compared with low severity, which is defined by a PSS of <3. We focused on surfactants, including polyoxyethylene amine (POEA), by identifying and analyzing the compositions of commercial mixtures formulated from a glyphosate salt, available in the National Product and Composition Database (NPCD; or Base nationale des produits et compositions [BNPC]). Approval of the local ethics committee was unnecessary, given the retrospective and noninterventional nature of this study.

Statistical Analysis

After describing the statistical sample (mean [SD] for quantitative variables and No. [%] for qualitative variables), we compared severity levels among cases using methods adapted to small samples (Fisher exact test and Wilcoxon test). ROC curves were created for each of the 4 biological indicators measured (blood and urinary concentrations of glyphosate and AMPA), to determine a threshold value that would establish differentiation between severe and minor cases, while optimizing sensitivity and specificity.

An AUC expressed as a percentage, with a 95% CI, was calculated for each curve, to compare their performances. This threshold served as the basis for describing the sensitivities, specificities, predictive values, and likelihood ratios. Then we conducted 4 secondary sensitivity analyses using ROC curves after excluding patients with polyintoxication and assays with a delay in processing of >24 hours. We retained only POEA-containing specimens, after excluding patients with renal failure. Data missing from the study have not been imputed. For our analyses, we used SPSS, version 19 (IBM Corporation) and R, version 3.6.1, software.

Results

Between January 1, 2004, and December 31, 2016, the Poison Control Centre handled 1368 cases of poisoning caused by an herbicide. In this study, we included only the 18 cases involving a concentration of glyphosate and/or AMPA detected in the blood and/or urine. Among these cases (several ingestions were possible per individual), 17 plasma glyphosate assays were performed (9 of which were identified as high-severity cases), along with 11 urine glyphosate assays (of which 5 were identified as high-severity cases), 13 plasma AMPA assays (of which 7 were identified as high-severity cases), and 10 urine AMPA assays (of which 4 were identified as high-severity cases). The primary characteristics of the patients included in our study are presented in **TABLE 1**. Clinical severity was significantly higher with respiratory symptoms, renal symptoms, admission to ICUs, after intubation/ventilation, and for high plasma glyphosate and plasma AMPA concentrations.

Of the 4 ROC curves, only the blood concentrations of glyphosate and AMPA presented a statistically significant AUC (95% CI), namely, 86 (68–100) and 88 (69–100), respectively (**FIGURE 1**). In our patient sample, the highest-performing marker to identify patients with severe poisoning was the blood concentration of AMPA. The optimal threshold value for the blood concentration of AMPA is 0.88 mg/L, with sensitivity of 71% and specificity of 100% (NPV, 75%; PPV, 100%). The optimal threshold value observed for blood concentration of glyphosate is 600 mg/L, with sensitivity of 67% and specificity of 100% (NPV, 73%; PPV, 100%). For the 4 sensitivity analyses, the association with severity did not change after exclusion of polyintoxication, delayed assays, urine assays in patients with no renal damage, and retaining only POEA-containing specimens.

Discussion

The severity of poisoning, as assessed using the PSS, seems to be associated with high level of blood glyphosate and AMPA concentration, with a relevant cutoff for blood AMPA concentration. To our knowledge, these findings have never been described in the literature.

In this study, the optimal threshold value of concentration of glyphosate that we discovered was similar to other threshold values reported in

Downloaded from https://academic.oup.com/labmed/article/53/4/394/6527658 by guest on 28 February 2025

TABLE 1. Characteristics of the 18 Case Individuals

Variable	PSS <3 (n = 9)	PSS ≥3 (n = 9)	Total (n = 18)	*P* Value[a]
Sex, No. (%)				.62
Women	4 (44%)	2 (22%)	6 (33%)	
Men	5 (56%)	7 (78%)	12 (67%)	
Age, y, mean (SD)	57.1 (16.8)	62.9 (10.9)	60.0 (14.0)	.57
Polyoxyethylene amine (POEA), No. (%)	5 (56%)	7 (78%)	12 (67%)	.37
Polysubstance intoxications, No. (%)	5 (56%)	2 (22%)	7 (39%)	.33
Oropharyngeal, gastric symptoms, No. (%)	6 (67%)	4 (44%)	10 (56%)	.64
Digestive symptoms, No. (%)	6 (67%)	8 (89%)	14 (78%)	.58
Respiratory symptoms, No. (%)	1 (11%)	8 (89%)	9 (50%)	**.003**
Renal symptoms, No. (%)	0	5 (56%)	5 (28%)	**.03**
Cardiovascular symptoms, No. (%)	4 (44%)	8 (89%)	12 (67%)	.13
Metabolic symptoms, No. (%)	4 (44%)	6 (67%)	10 (56%)	.64
Admission to resuscitation unit, No. (%)	4 (44%)	8 (100.0%)	12 (67%)	**.03**
Intubation/ventilation, No. (%)	0	9 (100.0%)	9 (50%)	**<.001**
Hemodialysis, No. (%)	0	3 (33%)	3 (18%)	.08
Death, No. (%)	0	3 (33%)	3 (17%)	.21
Estimated ingested quantity, mL, median (Q1–Q3)	190 (100–250)	70 (50–200)	180 (78–238)	.20
Assumed ingested dose, mg, median (Q1–Q3)	36.7 (36.0–72.0)	72.0 (25.2–72.0)	49.0 (25.2–72.0)	.69
Plasma glyphosate, mg/L				**.01**
Mean (SD)	51.62 (86.76)	2084.91 (2823.68)	1128.07 (2254.82)	
Median (Q1–Q3)	8.40 (0.61–62.75)	1050.00 (179.80–1360.00)	92.00 (1.60–1050.00)	
Plasma AMPA, mg/L				**.04**
Mean (SD)	0.146 (0.229)	3.899 (5.442)	2.167 (4.315)	
Median (Q1–Q3)	0.04 (0–0.19)	2.10 (0.70–4.20)	0.22 (0–2.10)	
Urine glyphosate, mg/L				.46
Mean (SD)	5064.7 (6623.6)	10131.8 (8114.5)	7367.9 (7434.8)	
Urine AMPA, mg/L				.29
Mean (SD)	11.5 (10.4)	21.9 (16.9)	15.6 (13.6)	
Sample time, h, median (Q1–Q3)	6.5 (3.0–19.5)	14.0 (6.5–21.5)	9.3 (3.8–20.6)	.46

PSS, Poisoning Severity Score; AMPA, aminomethylphosphonic acid.
[a]*Bolding indicates statistical significance.*

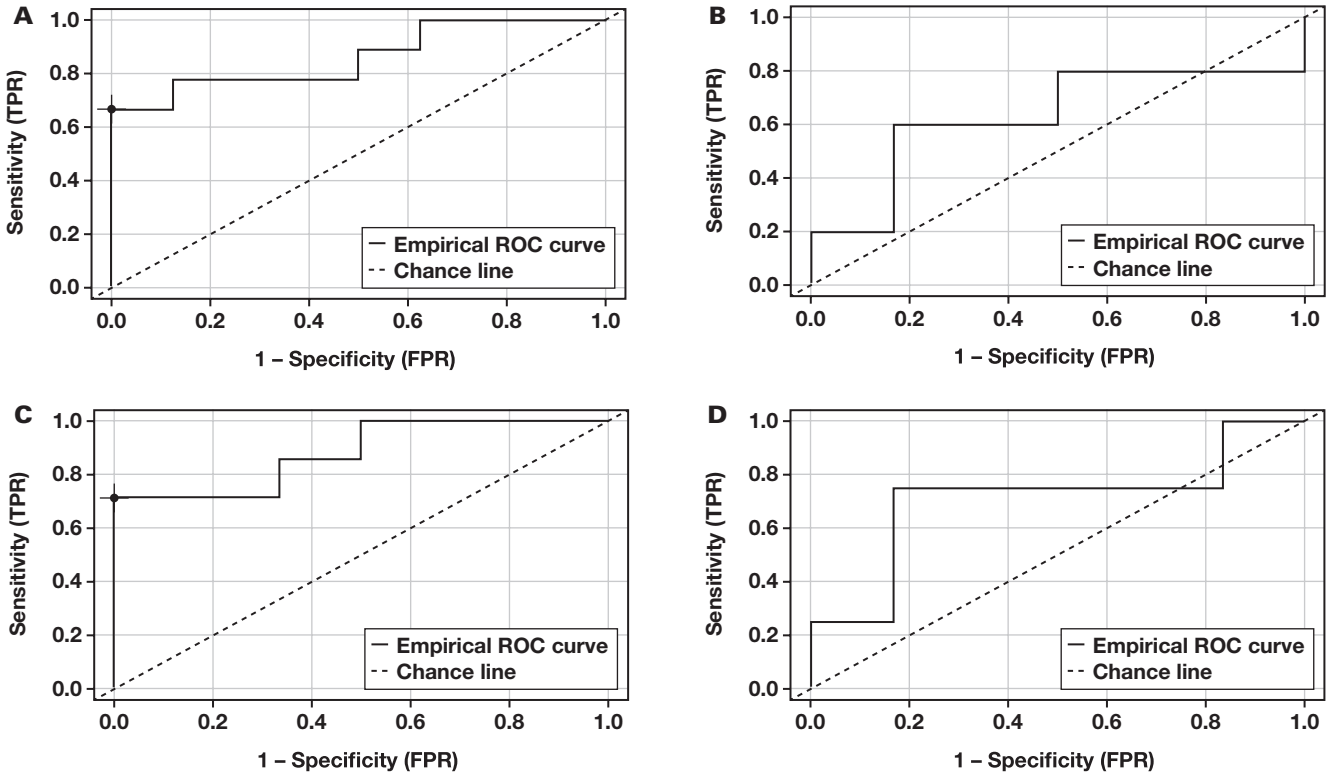
the literature. For 17 dosages of plasma glyphosate, we report a toxicity threshold concentration of 600 mg/L. Zouaoui et al[12] report a threshold concentration of 1000 mg/L (n = 13). However, Roberts et al[13] describe a concentration of 734 mg/L as a predictive factor of death. However, only the current study reports a validation of this toxicity threshold concentration by choosing the highest sensitivity for a specificity of 100%. Therefore, 100% of surviving patients with nonserious poisoning were correctly identified (PSS <3). This study allowed us to have the maximum number of patients with serious poisoning, including poisoning so severe it resulted in death, declared as such. Also, we have shown herein that blood concentration of AMPA has better sensitivity than blood concentration of glyphosate (71% vs 67%). Therefore, we report a significant association between the concentration of plasma glyphosate/AMPA and severity of poisoning.

Lower concentrations of AMPA compared with glyphosate are also described in the literature. Zouaoui et al[12] report blood concentrations of AMPA that are 200 times lower than those of glyphosate for PSS <3 and 644 times lower for PSS ≥3. We discovered the highly similar ratios in this study: 344 times lower for PSS <3 and 534 lower for PSS ≥3. We can assume that there is linearity in the metabolism of glyphosate into its active metabolite, AMPA. This study expands on the findings of other researchers by providing the novel toxicity threshold concentration in AMPA of 0.88 mg/L. This finding was made possible by our analysis of all glyphosate and AMPA concentrations in the blood and urine by separating the dosages determined within the first 24 hours after poison ingestion from those after 24 hours.

This study provides the additional finding of an assumed ingested dose calculated in mg, based on the concentration of glyphosate in each herbicide composition and the volume ingested. In this study, the amount has been reported in mg of glyphosate ingested, to give the most accurate estimation, given that the concentration of glyphosate in commercial mixtures is highly variable. In this study, we report concentrations ranging from 7.2 g/L through 783 g/L. The 9 cases of severe poisoning or death in this study only involved the highest concentrations of commercial mixtures, namely, from 360 g/L to 783 g/L of glyphosate. Thus, there is an assumed average ingested dose of 48.7 mg for mild to moderate poisoning and 55.4 mg for severe to fatal poisoning (despite a nonsignificant *P* value).

Downloaded from https://academic.oup.com/labmed/article/53/4/394/6527658 by guest on 28 February 2025

FIGURE 1. ROC curves based on poisoning severity, depending on the plasma and urinary concentrations of glyphosate and AMPA. A, Plasma concentration of glyphosate; $P < .05$; AUC (95% CI), 86 (68–100). B, Urinary concentration of glyphosate; $P > .05$. C, Plasma concentration of AMPA; $P < .05$; AUC (95% CI), 88 (69–100). D, Urinary concentration of AMPA; $P > .05$. TPR, true-positive rate; FPR, false-positive rate; AMPA, aminomethylphosphonic acid.



In addition, the assumed ingested dose is highly variable depending on the case and does not seem to be comprehensive. This dose was primarily recorded qualitatively (eg, per glass or bottle), and so we emphasize the approximate nature of these recorded data.

In the literature, the assumed ingested dose is highly variable. Sribanditmongkol et al[2] report that ingestion of 200 mL results in severe poisoning. Talbot et al[11] describe that ingestion of 184 mL is generally observed in the deaths among the 93 cases they studied. Also, in this study, the amount ingested calculated in mg which, taking into account the glyphosate concentrations, is not significantly different from the values reported by these other researchers.

The data in this study have also allowed the differentiation between polysubstance and monosubstance poisonings. Concurrent acute alcohol intoxication was reported in 6 of 7 cases of polysubstance poisonings. Cartigny et al[15] report the case of a 25-year-old patient with a medical history of terminal-stage chronic kidney failure having intentionally ingested 2 glasses of glyphosate. Glyphosate was still detectable in the blood 5 days after ingestion. This finding confirms the elimination of glyphosate in the urine. However, the interpretation of the urine dosages of glyphosate and AMPA is difficult if the kidney is affected because renal failure is regularly described in these cases of poisoning.

In our study, 5 of the 18 case individuals developed acute anuric renal failure secondary to glyphosate poisoning. In these 5 patients, the urine concentrations of glyphosate were between 14.9 mg/L and 17.3 mg/L (the latter was the maximum value observed in this study). This diagnosis is validated in this study; there is no association between the urinary dosages of glyphosate and severity of poisoning ($P = .47$ for the urinary concentration of glyphosate and $P = .29$ for the urinary concentration of AMPA). Our assumption in this study is that once the kidney is affected, the urinary concentration of glyphosate is uninterpretable.

Herbicide compositions containing glyphosate are highly variable. The most common coformulant or surfactant is POEA. The findings of several studies[2,11,12] determined that the POEA would be more responsible for the toxicity of the mixture than the glyphosate. According to Seok et al,[16] it is difficult to attribute this toxicity to glyphosate or POEA alone. These investigators studied 8 different herbicide formulations containing glyphosate, with only 1 of these 8 containing POEA. All 8 herbicides have produced various symptoms, such as hypotension, arrhythmia, respiratory failure, and acute renal failure.[16]

In comparison, in this study, a distinction was made between herbicides containing POEA from other surfactants. Twelve mixtures of the 18 ingested contained POEA surfactant. Three of the 18 mixtures included other surfactants; 1,4-didodecyl sulfosuccinate, ammonia, alkylpolyglycoside, ammonium sulfate, polydimethylsiloxanes, polyglycolic ether, geronol, isopropylamine, and water. There was no significant association with herbicides containing POEA and high severity ($P = .37$). Six patients had blood levels of glyphosate above the study threshold of 600 mg/L. Of these 6 patients, 5 had included POEA in the glyphosate-surfactant mixture they had ingested. For the other case individual, the exact composition of the glyphosate-surfactant mixture was unavailable. Of these 6 patients, 5 tested for AMPA and also had a concentration above the plasma AMPA threshold determined by this study. Of these 6 cases, 4 involved a concentration of glyphosate-surfactant mixture at 360 g/L and 1 case at 783 g/L. The symptoms reported with other

Downloaded from https://academic.oup.com/labmed/article/53/4/394/6527658 by guest on 28 February 2025

surfactants (n = 3) were oropharyngeal pain with moderate esophagitis and dysphonia, for example. This finding is similar to the hypothesis of Seok et al,[16] namely, that the toxicity of all the components of the mixture is not dissociable. Sawada et al[10] have also demonstrated that the toxicity cannot be dissociated from the glyphosate-surfactant mixture.

Limitations of this study include the small sample size and questionable accuracy of specimen collection timing. Specimen collection times from ingestion are reported as being from 1 through 72 hours, with a median of approximately 9 hours and of which a large majority was within the first 24 hours. Mortality was not specifically examined in this study and cannot be used as a variable. Further, there is a balance between the number of mild to moderate poisonings and that of severe or fatal poisonings. The possibility should not be overlooked that quantification is more frequent in the context of severe intoxication.

Conclusion

The severity assessed by the PSS appears to be associated with high plasma concentrations of glyphosate and AMPA, with a cutoff plasma concentration of AMPA not previously reported. No relationship between the urinary concentrations of glyphosate and AMPA could be demonstrated in the number of cases in this study. Determining blood concentrations could allow health-care professionals to improve patient care by setting a rapid prognosis according to the AMPA and glyphosate plasma dosage. However, the question of the inherent individual toxicity of glyphosate and of the coformulator POEA remains unanswered. A lack of scientific feedback on the toxic mechanism of these analytes is an essential point to be clarified in future studies with a larger number of cases.

REFERENCES

1. Frappart M, Vouriot D, Lemoine L, Floch T, Leon A. A fatal acute poisoning with glyphosate: importance of gastrointestinal toxicity [in French]. *Eur J Anaesthesiol.* 2011;30(11):852–854.
2. Sribanditmongkol P, Jutavijittum P, Pongraveevongsa P, Wunnapuk K, Durongkadech P. Pathological and toxicological findings in glyphosate-surfactant herbicide fatality: a case report. *Am J Forensic Med Pathol.* 2012;33(3):234–237.
3. Zhang L, Rana L, Shaffer R, et al. Exposure to glyphosate-based herbicides and risk for non-Hodgkin lymphoma: a meta-analysis and supporting evidence. *Mutat Res Rev Mutat Res.* 2019;781:186–206.
4. Zyoud S, Waring W, Al-Jabi SW, Sweileh WM. Global research production in glyphosate intoxication from 1978 to 2015: a bibliometric analysis. *Hum Exp Toxicol*. 2017;36(10):997–1006.
5. ANSES. AVIS de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail relatif à la saisine glyphosate n° 2015-SA-0093. Accessed January 23, 2022. https://www.anses.fr/en/system/files/SUBCHIM2015sa0093.pdf
6. Lee H-L, Chen K-W, Chi C-H, Huang J-J, Tsai L-M. Clinical presentations and prognostic factors of a glyphosate-surfactant herbicide intoxication: a review of 131 cases. *Acad Emerg Med.* 2000;7(8):906–910.
7. Kim YH, Lee JH, Cho KW, et al. Prognostic factors in emergency department patients with glyphosate surfactant intoxication: point-of-care lactate testing. *Basic Clin Pharmacol Toxicol*. 2016;119(6):604–610.
8. Mohamed F, Endre ZH, Pickering JW, et al. Mechanism-specific injury biomarkers predict nephrotoxicity early following glyphosate surfactant herbicide (GPSH) poisoning. *Toxicol Lett.* 2016;258:1–10.
9. Kim YH, Lee JH, Hong CK, et al. Heart rate–corrected QT interval predicts mortality in glyphosate-surfactant herbicide–poisoned patients. *Am J Emerg Med.* 2014;32(3):203–207.
10. Sawada Y, Nagai Y, Ueyama M, Yamamoto I. Probable toxicity of surface-active agent in commercial herbicide containing glyphosate. *Lancet.* 1988;1(8580):299.
11. Talbot AR, Shiaw MH, Huang JS, et al. Acute poisoning with a glyphosate-surfactant herbicide ("Roundup"): a review of 93 cases. *Hum Exp Toxicol.* 1991;10(1):1–8.
12. Zouaoui K, Dulaurent S, Gaulier JM, Moesch C, Lachâtre G. Determination of glyphosate and AMPA in blood and urine from humans: about 13 cases of acute intoxication. *Forensic Sci Int.* 2013;226(1-3):e20–e25.
13. Roberts DM, Buckley NA, Mohamed F, et al. A prospective observational study of the clinical toxicology of glyphosate-containing herbicides in adults with acute self-poisoning. *Clin Toxicol (Phila).* 2010;48(2):129–136.
14. Persson HE, Sjöberg GK, Haines JA, Pronczuk de Garbino J. Poisoning severity score: grading of acute poisoning. *J Toxicol Clin Toxicol.* 1998;36(3):205–213.
15. Cartigny B, Azaroual N, Imbenotte M, et al. Quantitative determination of glyphosate in human serum by ^{1}H NMR spectroscopy. *Talanta.* 2008;74(4):1075–1078.
16. Seok S-J, Park J-S, Hong J-R, et al. Surfactant volume is an essential element in human toxicity in acute glyphosate herbicide intoxication. *Clin Toxicol.* 2011;49(10):892–899.

Downloaded from https://academic.oup.com/labmed/article/53/4/394/6527658 by guest on 28 February 2025

Multiparametric Flow Cytometry versus Conventional Cytology in the Study of Leptomeningeal Involvement in Malignant Hematological Diseases

Alejandra Altube,[1] Veronica Ceres,[1] Cecilia Malusardi,[1] Evelyn Gonzalez Matteo,[2] Cintia Lorena Gimenez,[2] Adriana Esther Rocher, PhD,[2] Mariángeles Auat, MSc[1,]*

[1]Flow Cytometry Laboratory, Hematology Division, Hospital de Clínicas "José de San Martín" and [2]Cytology Laboratory, Department of Clinical Biochemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina; *To whom correspondence should be addressed. mariauat@gmail.com

Keywords: flow cytometry, immunophenotype, CSF, CNS involvement, leptomeningeal disease, primary CNS B-LBL

Abbreviations: CNS, central nervous system; MFC, multiparametric flow cytometry; CC, conventional cytology; LST, lymphoid-screening tube; B-LBL, B-lymphoblastic lymphoma; BL, Burkitt lymphoma; NHL, non-Hodgkin lymphoma; ALL, acute lymphoblastic leukemia; PCN, plasma cell neoplasm; B-NHL, B-cell non-Hodgkin lymphoma; T and NK-NHL, T and NK-cell non-Hodgkin lymphoma; B-ALL, B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin/Texas Red; PC5.5, phycoerythrin/cyanin 5.5; PerCP-Cy5.5, peridinin chlorophyll protein; PECy7, phycoerythrin/Cy7; APC, allophycocyanin; APC-Cy7, allophycocyanin/Cy7; APC-AF700, APC/Alexa Fluor 700; APC-AF750, APC/Alexa Fluor 750; PB, pacific blue; KO, Krome Orange

Laboratory Medicine 2022;53:399–404; https://doi.org/10.1093/labmed/lmac004

ABSTRACT

Background: CNS involvement is a complication in hematologic malignant neoplasms. The advantage of multiparametric flow cytometry (MFC) over conventional cytology (CC) in detecting occult leptomeningeal disease in CSF has been proven previously, as reported in the literature. In this study, we reviewed the experience of our laboratory in evaluating CSF specimens by MFC and CC after refinement of technical procedures.

Methods: MFC analysis was performed in 159 specimens. In 91 specimens, simultaneous CC and MFC analysis was requested and results compared.

Results: Neoplastic cells were identified in 27 (17.0%) of the total samples and in 17 (18.7%) of the paired specimens group by MFC, compared with 2 (2.2%) specimens with positive results as determined by CC. MFC enabled identification of malignant cells in low-cellularity specimens (<5 cells/μL) and all neoplasm categories.

Conclusion: MFC allowed the detection of minimal numbers of tumor cells in CSF specimens from individuals with leukemia and lymphoma in whom CC had not been able to identify those tumor cells.

Involvement of the central nervous system (CNS) is an adverse clinical complication that occurs in patients with leukemia and lymphoma. CSF cytological examination is essential to proving definitive evidence of leptomeningeal disease. Although highly specific (>95%) cytomorphological analysis is associated with only limited sensitivity, as many as 20%–60% of specimens yield false-negative results.[1] Multiparametric flow cytometry (MFC) enhances the detection of neoplastic cells in CSF specimens because it is more efficient, sensitive, and accurate, and its results are more reproducible, compared with conventional cytology (CC).[2]

In our laboratory, advances in MFC have been made to improve the quality of results, such as increasing the number of flow cytometric parameters (10-color single-tube detection), optimization of storage conditions to preserve CSF cells, and standardization of specimen preparation and staining protocols according to recommendations given in the literature.[3]

Until 2018, CSF MFC analysis in our laboratory had been based on a 3-color assay. In January 2018, the purchase of a new flow cytometer, the introduction of new phenotypic markers and a cell stabilizer, and the application of improvements in analytical phases and final reporting all led to MFC technique optimization. In this study, we retrospectively reviewed MFC results and compared them with CC findings for detecting hematological neoplastic cells in CSF specimens in our institution.

Methods

Patients and Specimens

We reviewed CSF specimens that had been received in the clinical flow cytometry laboratory between June 2018 and December 2020. Consequently, 159 CSF specimens were included for diagnostic and follow-up purposes. There were 151 specimens from patients being

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/399/6537006 by guest on 28 February 2025

followed up for hematological malignant neoplasms: non-Hodgkin lymphomas (NHLs; n = 68), acute lymphoblastic leukemias (n = 65), and plasma cell neoplasms (n = 18). The remaining 8 cases had neurological symptoms and/or suggestive neuroimaging characteristics, and specimens were collected for primary diagnosis. Patient median age was 51 years (range, 12–88 years). The local ethics committee reviewed the study protocol and approved the use of data collected in this study.

Flow Cytometric Immunophenotyping

MFC was performed on all specimens (n = 159) we received. CSF specimens were directly collected into tubes containing EDTA and 0.1 mL of Transfix. According to the standard protocol,[4] 2 mL of phosphate-buffered saline (PBS; pH, 7.4) was added to a sample aliquot and was centrifuged (5 minutes at 540*g*), and the supernatant was discarded (this step was repeated when light immunoglobulin chains expression were evaluated). Then, the cell pellet was stained for 30 minutes at room temperature in the darkness, with an 8- to 10-color combination of antibodies (mAbs). Antibody panels were selected according to diagnosis and are displayed in **TABLE 1**. The Euroflow lymphoid-screening tube (LST) was used in specimens for evaluation of B-cell NHL and primary diagnosis.[5]

After staining, 2 mL of FACS lysing solution (BD Biosciences) was added. After 10 minutes of incubation at room temperature, specimens were sequentially centrifuged (5 minutes at 540*g*) and resuspended in 400 µL of PBS-ALB 0.2% before data acquisition on a NAVIOS flow cytometer (Beckman Coulter). The specimen was acquired in its entirety to ensure the maximum number of events for analysis. INFINICYT software, version 2.0 (Cytognos) was used for data analysis.

The minimal number of clonally restricted and/or phenotypically aberrant cells to define CSF infiltration by MFC was 10 clustered events.[4] Counting beads for enumeration of cells was not available, and only the differential count was performed by MFC. Absolute cell count was obtained from the cytology laboratory of our institution.

Conventional Cytological Evaluation

Cytological evaluation was requested only for 91 of the 159 specimens by the attending physicians. Thus, only these specimens were evaluated using both techniques.

Specimens were collected into sterile containers without stabilization solution and without anticoagulant. Cytological analysis was performed within 2 hours after lumbar puncture immediately on arrival at the laboratory, a volume of at least 0.3 mL of specimen material was cytocentrifuged at 300*g* for 5 minutes on a glass slide using the cytospin instrument (Hanill Scientific). Smears were stained with May-Grünwald Giemsa for differential counting. Cytospin smears were read by an experienced cytologist (A.E.R. and C.L.G.). Absolute cell count was performed manually, in duplicate, using a Neubauer counting chamber.

Statistical Analysis

We performed a comparison of MFC and CC results with absolute cell count and diagnostic data using SPSS software, version 22 (IBM). Scatterplots were assembled using GraphPad Prism, version 8.0.1 (GraphPad Software).

Results

Frequency of Leptomeningeal Disease Identified by MFC and CC

Neoplastic cells were detected in 27 (16.9%) of the 159 CSF samples analyzed by MFC. In 3 cases (1.9%), a small cluster of suspicious events (<10 events) was detected, and therefore the evaluation was deemed indeterminate due to the lack of unequivocal classification of the cell population as neoplastic.[4] However, these specimens successfully yielded acquired events on the flow cytometer and were considered adequate for analysis.[6] For those patients, CSF examination using new specimens was required.

When evaluating patients undergoing simultaneous cytological and flow-cytometric analysis (paired sample group: n = 91), MFC detected neoplastic cells in 17 (18.7%) and was inconclusive in 1 case. CC recognized leptomeningeal disease in 2 cases (2.2%).

The 68 specimens evaluated only by MFC were from patients with a history of malignant neoplasms; of these, 10 tested positive. The distribution of cases with abnormal findings by MFC and CC according to diagnosis is displayed in **TABLE 2**.

TABLE 1. Antibody Panels Used to Investigate Neoplastic CSF Involvement

Fluorochrome	B-NHL	T and NK-NHL	PCN	B-ALL	T-ALL
FITC	CD8 and SmIgκ	CD5	CD38	CD81	CD7
PE	CD56 and SmIgλ	CD56	CD56	CD10	CD99
ECD	…	CD3 and CD14	CD3 and CD14	…	…
PEC5.5 or PerCP 5.5	CD5	CD38	…	CD34	CD3
PECy7	CD19	CD2	CD19	CD19	CD2
APC	SmCD3	CD19	CD117	CD3	CD5
APC-A700	…	CD7	…	…	…
APC-A750	CD38	CD8	CD81	CD38	CD8
PB	CD20 and CD4	CD4	CD138	CD20	CD4
KO	CD45	CD45	CD45	CD45	CD45

B-NHL, B-cell non-Hodgkin lymphomas; T and NK-NHL, T and NK-cell non-Hodgkin lymphomas; PCN, plasma-cell neoplasms; B-ALL, B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin/Texas Red; … , nonapplicable; PC5.5, phycoerythrin/Cyanin 5.5; PerCP-Cy5.5, peridinin chlorophyll protein; PECy7, phycoerythrin/Cy7; APC, allophycocyanin; APC-Cy7, allophycocyanin/Cy7; APC-AF700, APC/Alexa Fluor 700; APC-AF750, APC/Alexa Fluor 750; PB, pacific blue; KO, Krome Orange.

Downloaded from https://academic.oup.com/labmed/article/53/4/399/6537006 by guest on 28 February 2025

TABLE 2. Distribution of Cases with Abnormal Findings by Flow Cytometry and Conventional Cytology According to Diagnosis

Malignant Neoplasm	Subtype	Specimens Evaluated Only via MFC	Specimens with Simultaneous Evaluation via MFC and CC	
			MFC	CC
ALL	B-ALL/LBL	3	5	0
	T-ALL	2	1	0
NHL	B-NHL	2	5	1
	T-NHL	0	2	0
	NK-NHL	1	0	0
PCN	MM	2	4	1
Total		10	17	2

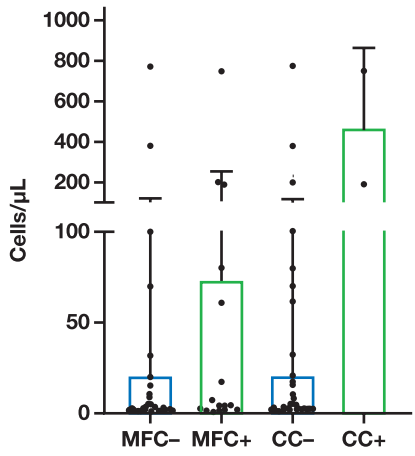
MFC, multiparametric flow cytometry; CC, conventional cytology; B-ALL/LBL, B-cell acute lymphoblastic leukemia/lymphoblastic lymphoma; T-ALL, T-cell acute lymphoblastic leukemia; B-NHL, B-cell non-Hodgkin lymphomas; T- and NK-NHL, T- and NK-cell non-Hodgkin lymphomas; PCN, plasma cell neoplasms.

Identification of Malignant Cells and Specimen Cellularity

Cell count was performed, as described in the Methods section, in the cytology laboratory. Specimen cellularity ranged between 0 and 776 cells/μL. Absolute leukocyte count was available in the 91 specimens evaluated using both methodologies. 35 specimens were found to have 0 cells/μL; however, we were intrigued that, even in those specimens, MFC was able to detect at least normal T-lymphocytes and/or monocytes.

Comparison of MFC and cytology findings according to specimen cellularity is exhibited in **FIGURE 1**. Specimens that tested positive (where neoplastic cells were detected) had higher mean cellular concentration than ones that had tested negative. We observed this result for both methodologies. However, mean absolute count was lower in MFC (74 cells/μL) than in CC (470 cells/μL). Of the 17 MFC-positive specimens, 11 specimens had fewer than 5 cells/μL. Thus, MFC was capable of identifying malignant cells in CSF specimens with very low cellularity.

FIGURE 1. Absolute cell count conforming to multiparametric flow cytometry (MFC) and conventional cytology (CC) results. Bars represent mean values.



Identification of Malignant Cells and Neoplasms Categories

MFC and CC findings were distributed according to hematological malignancy categories (**TABLE 2**). MFC was able to identify neoplastic cells independently of cellular lineage and type. In contrast, CC was not capable of detecting CNS involvement in ALL cases.

In fact, B lymphoblasts were identified in 1 case thanks to flow-cytometry analysis (**FIGURE 2**). In this case, diagnosis of primary CNS B-lymphoblastic lymphoma (B-LBL) was achieved because there was no evidence of blood and bone-marrow involvement. The patient in question had no history of B-ALL. Cellular concentration on this CSF specimen was 4 cells/μL, and no blast was observed in morphological examination, probably due to the low absolute leukocyte count in the specimen. Indeed, the ALL specimens showed the lowest cellularity (**FIGURE 3**).

In **FIGURE 3**, it can also be observed that positive NHL specimens showed the highest cellular concentration. We note that 5 of the 7 positive specimens had >5 cells/μL. In line with this observation, one might expect that CC was able to identify more positive NHL cases. However, only 1 case was recognized by CC. In general, neoplastic cells are phenotypically different from normal cells; these differences are morphological and immunophenotypic. In NHL the morphological differences are minimal between benign and malignant cells, and the identification of neoplastic cells in CSF can be challenging due to these similarities.

Actually, the only positive NHL case detected by CC was a Burkitt lymphoma (BL). Morphological features of BL lymphocytes are typical and easily distinguishable from normal B cells. Thus, it could be noted that CC is capable of only recognizing those neoplastic cells that are greatly different from their normal counterparts.

Discussion

In the present study, neoplastic cells were identified in 17% of the total specimens by MFC, as well as in 19% of the paired specimens group, where in contrast only 2% positive results were yielded by CC. The main reason for the small number of CC-positive results could be the low cellularity of specimens. Median cellular concentration of all specimens was 1 cell/μL and mean cellularity was 30 cells/μL. Moreover, the rapid decay of leukocytes in CSF ex vivo, and the fact that cytological evaluation was performed within 2 hours after lumbar puncture with specimens having been collected without stabilization solution, could have resulted in underestimation of CNS involvement by conventional cytospin analysis.[1,7]

Downloaded from https://academic.oup.com/labmed/article/53/4/399/6537006 by guest on 28 February 2025

FIGURE 2. Illustrative case of primary CNS B-cell lymphoblastic lymphoma. Dot plots showing the immunophenotype of blasts (red dots) and normal residual B lymphocytes (green dots). Monocytes (yellow dots) and T lymphocytes (pink dots) present in the specimen are also shown.

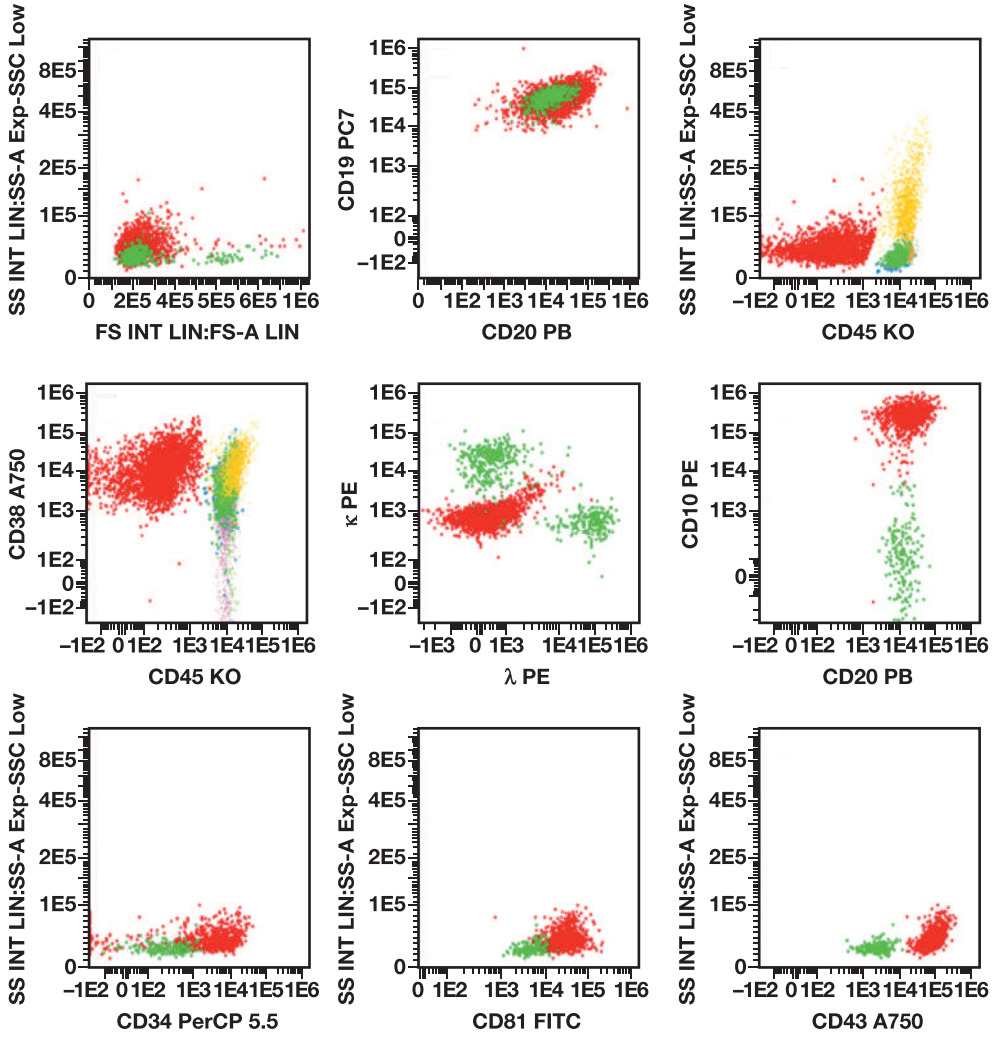
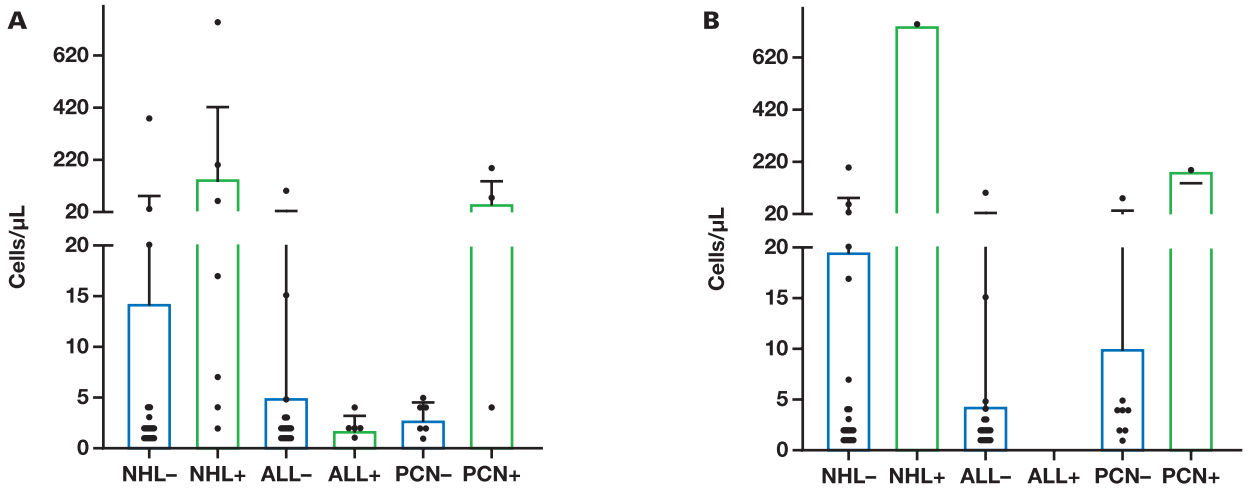


FIGURE 3. Evaluation of cellularity in positive and negative specimens in different hematological neoplasms categories evaluated by multiparametric flow cytometry (A) and conventional cytology (B). Bars represent mean values. NHL, non-Hodgkin lymphomas; ALL, acute lymphoblastic leukemia; PCN, plasma cell neoplasms.



Downloaded from https://academic.oup.com/labmed/article/53/4/399/6537006 by guest on 28 February 2025

2022;53;399–404 | https://doi.org/10.1093/labmed/lmac004

Hence, these results confirmed that the detection rate of CSF involvement in hematological malignant neoplasms is higher in flow cytometry than in conventional cytomorphological analysis, especially in low-cellularity specimens.[8,9] Accordingly, it was possible to detect abnormal cells in 11 specimens with <5 cells/μL. Indeed, other authors had demonstrated that normal counts do not rule out the presence of lymphoma/leukemia.[2]

Flow cytometry allows the detection of an abnormal population in specimens with little cellularity that otherwise might have been not detected, if only morphologic examination was used.[2,9] This finding was specially observed in the ALL specimens in which cellularity was very low (mean = 1 cell/μL). This result could explain the fact that there were no ALL-positive specimens determined by CC. Most likely, representative cells may not have been present in the cytospin slide. MFC is essential to detecting occult or subclinical CNS involvement.

A sensitive methodology such as MFC can be applied at ALL diagnosis and for a close follow-up of lymphoblast presence in CSF specimens during therapy, and may identify a group of patients at high risk of relapsing.[10,11] MFC also proved helpful in the diagnosis of 1 case of primary CNS B-LBL.

Other authors[4,12] have demonstrated that CSF evaluation by MFC improves detection of leptomeningeal disease in NHL cases. The data presented herein have shown that MFC allowed the detection of neoplastic cells in B, T, and NK cells NHL cases. In this research work, although positive NHL specimens showed high cellularity, CC failed to detect malignant cells in that group. This finding could be explained by cytological features not being defining in some NHL entities. In fact, the only case detected by CC was a BL, which has distinctive morphological characteristics, as was already argued earlier herein.

Compared with other series published in the past,[6,8,13] more MFC-positive results were found in this study, and the detection rate of leptomeningeal disease by MFC was similar to that reported in more recent series.[4,9] This result might be attributable to the recent advances achieved in our flow cytometry technical procedures. The use of a cell stabilizer for specimen preservation and updating our immunophenotyping protocol, from 3 to 10 colors, has refined the information on CSF cell subsets and enabled us to define cell populations more accurately.[1] This information could also explain the few indeterminate MFC results found in this study compared with other studies.[8,14] These 3 specimens were from patients with a history of B-ALL; CSF analysis had been requested due to neurological symptoms (paraparesis). Thus, the patients in question were clinically considered to have positive results for leptomeningeal disease.

Given the greater sensitivity of MFC, flow cytometric analysis in CSF is requested to exclude hematologic malignant neoplasms in various clinical scenarios. However, in practice, it appears to be most useful in the appropriate clinical setting for the rational use of this technique.[6,14] Herein, we observed that there were only few specimens (8 of 159) for primary diagnosis, and the 68 specimens where only flow cytometric testing was requested were from patients with a hematological neoplasm diagnosis, which seems to be in line with such a recommendation. However, whether a positive result by MFC without positive cytomorphology results has the same clinical implications as positive cytomorphology results, the classic criterion standard, is yet to be determined.[15]

Patients with aggressive B-cell lymphoma who are at risk for CNS spread are recommended to undergo staging CSF evaluation by flow cytometry.[16,17] However, no recommendation has been made regarding evaluation of leptomeningeal disease in patients with ALL using MFC. In these cases, the usefulness of MFC requires further investigation. Although the findings of several studies[10,11,18] have proven that identification of lymphoblasts by MFC in CSF distinguish patients with ALL who are at high risk of relapsing, the diagnostic and prognostic value of CSF analysis by MFC needs to be confirmed in a large cohort of patients to draw definitive conclusions concerning therapeutic consequences for future large-scale trials. Altogether, these results corroborate previous observation that MFC can be a useful diagnostic tool for detection of malignant hematopoietic cells in CSF.

Conclusion

In conclusion, the data presented herein confirm that MFC was able to detect CSF disease more frequently than cytomorphology, especially in low-cellularity specimens, but also in all hematological neoplasm categories, thanks to recent technical advances that facilitate detection of phenotypically aberrant cells. Finally, we intend to explore certain advances in future research, such as introducing counting beads to the assay as a method to calculate absolute numbers of normal and pathological cell subpopulations that can greatly helpful in evaluating specimens.

Acknowledgments

We thank Daniel E. Saad for linguistic assistance.

Personal and Professional Conflicts of Interest

The authors declare that there is no conflict of interest.

REFERENCES

1. de Graaf MT, de Jongste AH, Kraan J, Boonstra JG, Sillevis Smitt PA, Gratama JW. Flow cytometric characterization of cerebrospinal fluid cells. *Cytometry B Clin Cytom.* 2011;80(5):271–281.
2. Roma AA, Garcia A, Avagnina A, Rescia C, Elsner B. Lymphoid and myeloid neoplasms involving cerebrospinal fluid: comparison of morphologic examination and immunophenotyping by flow cytometry. *Diagn Cytopathol.* 2002;27(5):271–275.
3. Kraan J, Gratama JW, Haioun C, et al. Flow cytometric immunophenotyping of cerebrospinal fluid. *Curr Protoc Cytom.* 2008;Chapter 6:Unit 6.25.
4. Quijano S, López A, Manuel Sancho J, et al; Spanish Group for the Study of CNS Disease in NHL. Identification of leptomeningeal disease in aggressive B-cell non-Hodgkin's lymphoma: improved sensitivity of flow cytometry. *J Clin Oncol.* 2009;27(9):1462–1469.
5. van Dongen JJM, Lhermitte L, Böttcher S, et al; EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708). EuroFlow antibody panels for standardized *n*-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia.* 2012;26(9):1908–1975.
6. Kovach AE, DeLelys ME, Kelliher AS, et al. Diagnostic utility of cerebrospinal fluid flow cytometry in patients with and without prior hematologic malignancy. *Am J Hematol.* 2014;89(10):978–984.
7. de Graaf MT, van den Broek PDM, Kraan J, et al. Addition of serum-containing medium to cerebrospinal fluid prevents cellular loss over time. *J Neurol.* 2011;258(8):1507–1512.
8. Craig FE, Ohori NP, Gorrill TS, Swerdlow SH. Flow cytometric immunophenotyping of cerebrospinal fluid specimens. *Am J Clin Pathol.* 2011;135(1):22–34.

Downloaded from https://academic.oup.com/labmed/article/53/4/399/6537006 by guest on 28 February 2025

9. Bento LC, Correia RP, Alexandre AM, et al. Detection of central nervous system infiltration by myeloid and lymphoid hematologic neoplasms using flow cytometry analysis: diagnostic accuracy study. *Front Med (Lausanne).* 2018;5:70.
10. Martínez-Laperche C, Gómez-García AM, Lassaletta Á, et al. Detection of occult cerebrospinal fluid involvement during maintenance therapy identifies a group of children with acute lymphoblastic leukemia at high risk for relapse. *Am J Hematol.* 2013;88(5):359–364.
11. Thastrup M, Marquart HV, Levinsen M, et al; Nordic Society of Pediatric Hematology and Oncology (NOPHO). Flow cytometric detection of leukemic blasts in cerebrospinal fluid predicts risk of relapse in childhood acute lymphoblastic leukemia: a Nordic Society of Pediatric Hematology and Oncology study. *Leukemia.* 2020;34(2):336–346.
12. Wilson WH, Bromberg JEC, Stetler-Stevenson M, et al. Detection and outcome of occult leptomeningeal disease in diffuse large B-cell lymphoma and Burkitt lymphoma. *Haematologica.* 2014;99(7):1228–1235.
13. Pittman M, Treese S, Chen L, et al. Utility of flow cytometry of cerebrospinal fluid as a screening tool in the diagnosis of central nervous system lymphoma. *Arch Pathol Lab Med.* 2013;137(11):1610–1618.
14. Collie AM, Hill BT, Stevens GH, Fenner K, Gazdick E, Hsi ED. Flow cytometric analysis of cerebrospinal fluid has low diagnostic yield in samples without atypical morphology or prior history of hematologic malignancy. *Am J Clin Pathol.* 2014;141(4):515–521.
15. Bromberg JEC, Breems DA, Kraan J, et al. CSF flow cytometry greatly improves diagnostic accuracy in CNS hematologic malignancies. *Neurology.* 2007;68(20):1674–1679.
16. Hegde U, Filie A, Little RF, et al. High incidence of occult leptomeningeal disease detected by flow cytometry in newly diagnosed aggressive B-cell lymphomas at risk for central nervous system involvement: the role of flow cytometry versus cytology. *Blood.* 2005;105(2):496–502.
17. Savage KJ. Secondary CNS relapse in diffuse large B-cell lymphoma: defining high-risk patients and optimization of prophylaxis strategies. *Hematology Am Soc Hematol Educ Program.* 2017;2017(1):578–586.
18. Popov A, Henze G, Verzhbitskaya T, et al. Absolute count of leukemic blasts in cerebrospinal fluid as detected by flow cytometry is a relevant prognostic factor in children with acute lymphoblastic leukemia. *J Cancer Res Clin Oncol.* 2019;145(5):1331–1339.

Downloaded from https://academic.oup.com/labmed/article/53/4/399/6537006 by guest on 28 February 2025

Potential Role of Neutrophil-Platelet Interaction in Increased Susceptibility to Infection of Patients with Down Syndrome

Hoda M. Abd El-Ghany, MD,[1] Iman Ehsan Abdel Meguid, MD,[2] Rabab El Hawary, MD,[1] Safa Meshaal, MD,[1] Iman Taha Lafy Shimila, MSc,[1] Eman Roshdy Radwan, MD[1,]*

[1]Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt, [2]Department of Pediatrics, Faculty of Medicine, Cairo University, Cairo, Egypt; *To whom correspondence should be addressed. emanrradwan@gmail.com

Keywords: Down syndrome, neutrophil-platelet interaction, neutrophil activation, platelet activation, lipopolysaccharide, innate immunity

Abbreviations: DS, Down syndrome; LPS, lipopolysaccharide; MFI, mean fluorescent intensity; sCD40L, soluble CD40 ligand; TLR, Toll-like receptor; NET, neutrophil extracellular trap; PRP, platelet-rich plasma; RBC, red blood cell; TNF, tumor necrosis factor; ROS, reactive oxygen species.

Laboratory Medicine 2022;53:405–411; https://doi.org/10.1093/labmed/lmac012

ABSTRACT

Objective: Recurrent infection in Down syndrome (DS) has been previously documented; the potential role of platelets and neutrophil-platelet interaction has not been addressed in previous studies.

Patients and Methods: Using flow cytometry, we evaluated CD40 and CD18 expression as activation markers for neutrophils and CD62p as an activation marker for platelets, before and after lipopolysaccharide (LPS) stimulation, in 34 patients with DS and 39 control patients.

Results: Markers were evaluated as percentage of positivity, mean fluorescent intensity (MFI), and activation index (MFI after stimulation/MFI before stimulation). Patients showed a significantly lower CD40 MFI ($P = .019$) after LPS stimulation, a lower CD62p percentage before and after LPS stimulation ($P = .013$ and $P = .029$), and a higher CD62p MFI ($P = .011$) after LPS stimulation. Patients showed a lower activation index for CD40 and CD18 ($P \leq .001$) but not for CD62p ($P = .338$). Dysfunctional efficiency in neutrophils and in the neutrophil-platelet interaction could not be correlated to infection.

Conclusion: A consensus on a scoring system for infection is needed for an objective evaluation of correlation to infection.

Down syndrome (DS) is one of the most common genetic diseases associated with immunological defects. Although the 258 genes encoded on chromosome 21 have been identified, the reasons for the susceptibility of these patients to develop infections and autoimmune diseases remain unidentified.[1] Genome-wide expression analysis in DS has established that the leukocyte transendothelial migration pathway, which is pivotal for immune surveillance and inflammation, is dysregulated. In this pathway, *Vav2* expression in neonates with DS is downregulated. The gene is an activator of Cdc42, Rac1, and RhoA, which control actin dynamics and gene expression. The downregulation of *Vav2* can disturb leukocyte migration.[2] In addition, Rho GTPases are crucial for platelet function. Although the roles of RhoA, Rac, and Cdc42 have been identified, including their essential role in granule secretion, cytoskeletal regulation, and many other functions, further research on Rho GTPases in platelet function in both health and disease is in progress.[3]

Both neutrophils and platelets are principal players in innate immunity. Platelet interaction with neutrophils is well characterized in inflammation and infection.[4] Platelets augment neutrophil rolling and firm adhesion via the platelet marker CD62p and chemokine CXCR2.

Toll-like receptors (TLRs) are transmembrane pattern recognition receptors accounting for a variety of responses against microbial cell wall components and are a vital factor in innate immune responses.[5] Rho GTPases are well-known effectors of TLR signaling. During microbial infection, many receptors involved in innate immune cell activation transmit signals to Rho GTPases. Studies have implicated Rac1, RhoA, and Cdc42 in the signaling pathways emanating from TLR2, TLR4, TLR3, and TLR9. Platelets in TLR4 have been shown to participate in a variety of responses to bacterial endotoxins, including platelet recruitment to inflamed or injured vascular walls and platelet release of interleukin-1β-containing microvesicles. In addition, platelets in TLR4 have been shown to promote the release of neutrophil extracellular traps (NETs) capable of trapping bacteria during bacterial infection. Research has shown that TLR2 and TLR4 regulate crucial neutrophil functions, including adhesion, generation of reactive oxygen species, and release of chemokines, and that they activate major proinflammatory signaling pathways, including the nuclear factor–kB pathway.

Many studies have explored the role of T, B, and natural killer cell dysregulation in DS as an underlying cause of increased susceptibility to infection,[6] but no studies have investigated neutrophil-platelet activation in patients with DS. In light of the downregulated *Vav2* gene, an activator of

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/405/6548263 by guest on 28 February 2025

Rho GTPases (effectors of TLR signaling) and their possible effect on both neutrophils and platelets, we hypothesized that the platelet-neutrophil interaction might play a role in the increased susceptibility of patients with DS to infection. In our study, we stimulated TLR4, a central player in innate immunity, expressed on both neutrophils and platelets, then we measured the activation markers of both neutrophils and platelets as a first step for eliciting neutrophil endothelial transmigration.

Patients and Methods

Patients

The study included 34 patients and 39 healthy age-matched control patients. The patients were referred to the clinical genetics department at Abo El-Reesh Hospital Kasr El Ainy Medical School (Cairo, Egypt). The Research Ethics Committee approved the study (approval number: I-410414) and a written informed consent was obtained from all patients and control patients or their guardians. The study was conducted according to the ethics guidelines of the Declaration of Helsinki. The patients included 23 males (67.6%) and 11 females (32.4%), and their ages ranged from 1 to 25 years with a mean age of 4.5 years and a median of 2.25 years. All patients provided a full history, including the mother's age, consanguinity, previous abortions, recurrent infections, and family history of DS. A thorough clinical examination with careful assessment of clinical and physical features was carried out. The study inclusion criteria were (1) healthy patients with DS and (2) patients with normal platelet and neutrophil counts. The study exclusion criteria were as follows: (1) patients with DS with active infections, (2) patients taking aspirin or antiplatelet medications for the past 2 weeks, (3) patients who had been recently vaccinated (vaccines may trigger the immune system, causing transient side effects, eg, thrombocytopenia, thus affecting the study parameters), and (4) patients with thrombocytopenia and/or neutropenia from any other cause (eg, immune thrombocytopenia, immune mediated neutropenia).

Specimen Collection and Platelet-Neutrophil Isolation

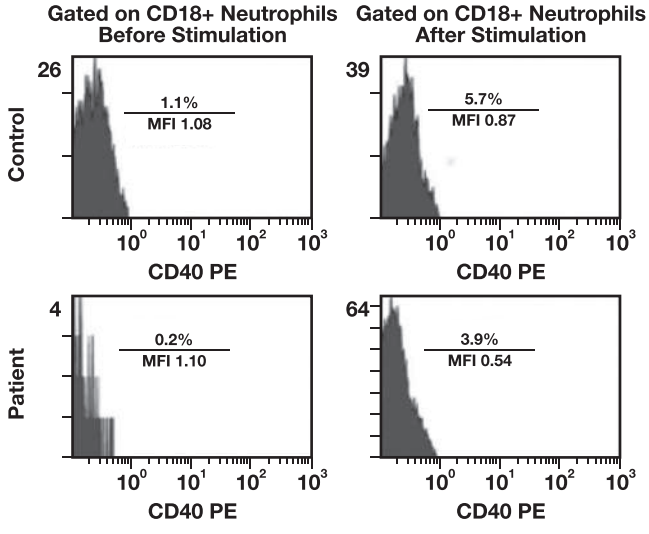
Platelet-rich plasma (PRP) was prepared by centrifugation at 86*g* for 15 minutes at 22°C. The platelets were counted in a Coulter counter. After the removal of the PRP, the residual blood was utilized for neutrophil isolation. Slowly, the residual platelet-depleted blood was layered over the same volume of Ficol hypaque solution (Biochrome, Berlin, Germany). After centrifugation at 400*g* for 30 minutes, the neutrophils and red blood cell (RBC) precipitate were collected. Contaminating RBCs were removed by hypotonic lysis for 10 minutes. Neutrophils were washed twice in serum-free RPMI medium (Biochrome, Berlin, Germany), resuspended in the same medium, and kept at 37°C until their use within 4 hours of isolation. Cell viability was determined using the trypan blue exclusion test for both patients with DS and control participants. Cell viability was comparable (>90%) for both groups.

Platelet-Neutrophil Stimulation and Flow Cytometry Analysis

The platelet-neutrophil ratio was adjusted to 20:1. The coculture was done in polystyrene 24-well plates (Greiner Bio-One catalog number 662160), precoated with fetal bovine serum (Lonza Bioproducts, Belgium, catalog number 14-801E) to prevent spontaneous neutrophil adherence to the polystyrene surface. Platelets (2×10^5/mL) were stimulated for 30 minutes at room temperature with ultrapure S-lipopolysaccharide from *Escherichia coli* (1 μg/mL) (Sigma Aldrich L3024). After the incubation of the platelets for 30 minutes with the lipopolysaccharide (LPS), neutrophils (0.1×10^5/mL) were added to the cell suspension (1 mL) to obtain the desired final concentration. A uniform concentration of each agonist was obtained by gentle mixing. The cell suspension was kept at 37°C for another 30 minutes.[7]

Cell staining was performed using the direct staining method: 200 μL each of isolated unstimulated platelets and neutrophils and the stimulated cocultured platelets and neutrophils (for both patients with DS and control patients) were incubated in the dark for 20 minutes with 5 μL fluorescein isothiocyanate (FITC)-conjugated anti-CD62p (eBioscience 11-0628-42) for platelets and 5 μL PE-conjugated anti-CD40 (eBioscience 12-0409-42) and FITC-conjugated anti CD-18 (eBioscience11-0189-42) according to the manufacturer recommendations. The analysis was performed using 5-color flow cytometry (Beckman Coulter Cytomics FC500, Beckman Coulter). Specimens were thoroughly mixed before the flow cytometry acquisition. Analysis of a minimum of 10,000 cells (events) for each specimen was carried out. Data analysis was performed using CXP flow analysis software version 2.2 (Beckman Coulter). Excluding cellular debris and nonviable cells, the fraction of cells that were stained was determined inside the gated regions and assessed in a basic histogram as a percentage and mean fluorescence index (MFI) before and after LPS stimulation for both patients with DS and control patients. Neutrophils were characterized by forward and side scatter properties, and the assessment of CD40 expression was performed by gating on CD18 +ve cells. The assessment of CD62p on platelets was done by gating on isolated platelets using forward and side scatter properties (**FIGURES 1** and **2**). An activation index, defined as MFI after LPS stimulation/MFI before LPS stimulation, was used to express the degree of activation in response to LPS.

FIGURE 1. Neutrophil marker CD40 expression before and after lipopolysaccharide stimulation in patients with Down syndrome and in control patients. MFI, mean fluorescence intensity; PE, phycoerythrin.



Downloaded from https://academic.oup.com/labmed/article/53/4/405/6548263 by guest on 28 February 2025

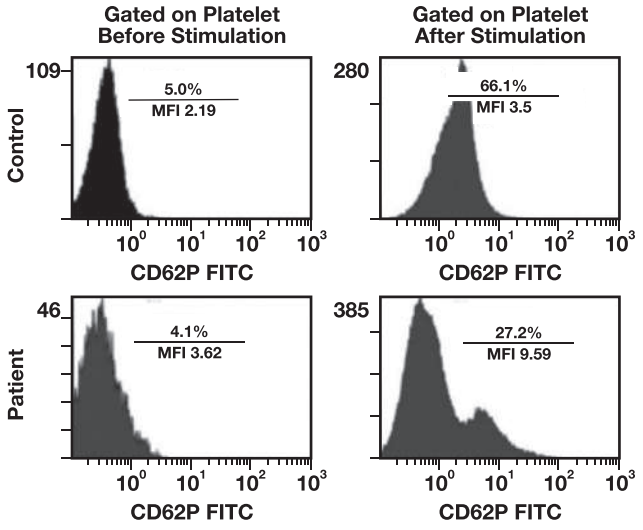
Statistical Analysis

We used SPSS version 23.0 for data management and data analysis. Mean ± standard deviation and median with range, when appropriate, described quantitative variables. Numbers and percentages described qualitative data, and chi-square or Fisher exact test evaluated proportion independence. For comparing mean values of the 2 independent groups, parametric and nonparametric *t*-tests were used. Correlation analysis was used to show the strength and significance of the association between quantitative variables. The *P* value was 2-tailed and significant at .05.

Results

The expression of various markers before and after LPS activation is presented in **TABLE 1**.

FIGURE 2. Platelet marker CD62p expression before and after lipopolysaccharide stimulation in patients with Down syndrome and in control patients. FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity.



Neutrophil Marker CD40 Expression Before and After LPS Stimulation

Before LPS activation, the neutrophil marker CD40 expression percentage and MFI were comparable between patients with DS and control patients (*P* = .391 and *P* = .329, respectively). After LPS activation, the CD40 MFI but not the CD40 percentage expression was significantly lower in patients with DS compared with the control patients (*P* = .019 and *P* = .777, respectively).

Neutrophil Marker CD18 Expression Before and After LPS Stimulation

Before LPS stimulation, the neutrophil marker CD18 percentage was significantly higher in patients with DS than in the control group (*P* = .037), whereas the CD18 MFI was comparable between both groups (*P* = .64). After LPS activation, both the CD18 percentage and the CD18 MFI were comparable between patients with DS and control patients (*P* = .58 and *P* = .2, respectively).

Platelet Marker CD62p Expression Before and After LPS Stimulation

Before LPS stimulation, the platelet marker CD62p percentage but not the CD62p MFI was significantly lower in patients with DS than control patients (*P* = .013 and *P* = .517, respectively). After LPS activation, the CD62p percentage was significantly lower whereas the CD62p MFI was significantly higher in patients with DS than in the control group (*P* = .029 and *P* = .011, respectively).

Activation Index for Neutrophil and Platelet CD Markers

An activation index, defined as MFI after LPS stimulation/MFI before LPS stimulation, was used to measure the degree of activation. For both neutrophils markers CD40 and CD18, a significantly lower activation index was found for the DS group (*P* ≤ .001 for both CD markers). Alternatively, for the platelet market CD62p activation index no significant difference was found between the 2 groups (*P* = .338).

Correlation Between CD40- and CD18-Related Parameters in DS

The correlation matrix for the neutrophil markers CD40 and CD18 is presented in **TABLES 2** and **3**. A moderate positive correlation

TABLE 1. Expression of Neutrophils and Platelet Activation Markers in Patients with DS and Control Patients[a]

	CD40	CD40 MFI	CD18	CD18 MFI	CD62p	CD62p MFI
Expression before LPS activation						
Patients with DS (34)	1.648 ± 1.901 (0.11–8.7)	1.08 ± 0.43 (0.58–2.27)	95.743 ± 11.297 (34.3–100)	15.69 ± 5.44 (2.37–31.4)	7.465 ± 10.022 (0.050–55.4)	4.09 ± 1.91 (1.7–11)
Control patients (39)	1.681 ± 1.361 (0.14–5.4)	1.353 ± 0.775 (0.5–3.39)	90.8 ± 14.34 (32.9–100)	15.163 ± 3.732 (7.7–2.2)	15.937 ± 18.7 (1–71.5)	3.637 ± 1.357 (1.35–7.5)
P value	.391	.329	**.037**	.64	**.013**	.517
Expression after LPS stimulation						
Patients with DS (34)	3.955 ± 2.045 (0.8–8.6)	0.862 ± 0.823 (0.5–5.1)	85.49 ± 23.199 (6.8–99.4)	12.58 ± 4.457 (4.07–22.1)	18.598 ± 14.486 (0.4–58.9)	5.282 ± 1.642 (2.2–7.9)
Control patients (39)	4.463 ± 3.474 (0.1–16.2)	1.029 ± 0.613 (0.5–2.9)	83.5 ± 20.77 (25.8–99.8)	13.52 ± 4.44 (1.7–22.6)	26.126 ± 18.272 (5.5–82.3)	4.413 ± 1.998 (1.9–12.4)
P value	.777	**.019**	.58	.2	**.029**	**.011**

DS, Down syndrome; LPS, lipopolysaccharide.
[a]*Data are shown as mean ± standard deviation (range).* P *value was significant at ≤ .05 (bold figures indicate significance).*

Downloaded from https://academic.oup.com/labmed/article/53/4/405/6548263 by guest on 28 February 2025

was found between the CD40 MFI before and after LPS stimulation (r = 0.398, P = .022). Furthermore, a strong positive correlation was found between the CD40 activation index and the CD40 MFI after LPS stimulation (r = 0.870, $P \leq .001$).

Regarding CD18, a moderate positive correlation was found between the CD18 percentage before and after LPS stimulation (r = 0.53, P = .002). Conversely, a moderate negative correlation was found between the CD18 percentage after LPS stimulation and the CD18 MFI before LPS stimulation (r = –0.485, P = .004). On the other hand, a weak negative correlation was found between the CD18 activation index and the CD18 percentage before LPS stimulation (r = –0.325) with a near-significant P value (.065), whereas a moderate negative correlation was found between the former and the CD18 MFI before LPS stimulation (r = –0.558, P = .001).

Correlation Between CD62p-Related Parameters in DS

The correlation matrix for the platelet marker CD62p parameters is presented in **TABLE 4**. A moderate positive correlation was found between the CD62p percentage before and after LPS stimulation (r = 0.432, P = .011). A weak insignificant positive correlation was found between the CD62p MFI before and after LPS stimulation (r = 0.295, P = .09). On the other hand, a moderate negative correlation was found between the CD62p percentage and the CD62p MFI after LPS stimulation (r = 0.411, P = .016). Furthermore, a moderate negative correlation was found between the CD62p activation index and the CD62p MFI before LPS stimulation (r = –0.552, P = .001); conversely, the CD62p activation index had a moderate positive correlation to the CD62p MFI after LPS stimulation (r = 0.491, P = .003).

Correlation Between Neutrophil and Platelet CD Activation Markers and History of Recurrent Infections in DS

A weak negative correlation was found between a history of recurrent infections and the neutrophil marker CD40 MFI before LPS stimulation (r = 0.306), with a P value of .079. In addition, a weak positive correlation was found between a history of recurrent infections and the neutrophil marker CD18 percentage both before and after LPS stimulation (r = 0.301 and r = 0.280; P = .083 and P = .116, respectively). Furthermore, a weak positive correlation was found between a history of recurrent infections and the platelet marker CD62p activation index (r = 0.290), with a P value of .097.

Discussion

Research has shown that DS is a common genetic disease with underlying immunological defects. One of the most common dysregulatory consequences is recurrent infections.[1] Both neutrophils and platelets have been established as interacting key players in innate immunity, thus indicating a critical role in combating infections.[4] Genome-wide expression analysis in DS has shown that the leukocyte transendothelial migration pathway, which is vital for immune surveillance and inflammation, is dysregulated. In addition, Rho GTPases that are critical for platelet function are also disturbed through the downregulation of *Vav2*.

In our study, we evaluated the expression of the neutrophil activation markers CD40 and CD18 and the platelet activation marker CD62p in 34 patients with DS as compared to 39 healthy control patients both before and after LPS stimulation to verify their potential role in susceptibility to infection in DS. No significant difference was found between patients with DS and the control group in either CD40 percentage or MFI before LPS stimulation. On the other hand, after LPS stimulation, the CD40 MFI was significantly lower in patients with DS (P = .019) whereas the CD40 percentage was comparable between the 2 groups (P = .777). Furthermore, the activation index of CD40 was significantly lower in the DS group (P < .001).

The receptor for CD40L, CD40, is a 48-kDa transmembrane protein that belongs to the tumor necrosis factor (TNF) superfamily. It is expressed on the surface of macrophages, monocytes, endothelial cells, and platelets.[8,9] In a study by Khan et al,[10] CD40 expression on neutrophils has been reported and is known to have multiple roles in innate immunity, including intravascular transmigration and superoxide and reactive oxygen species (ROS) generation.[4] The CD40 ligand (CD40L, CD154) also belongs to the TNF superfamily. It was first recognized as a T-cell surface molecule involved in B-cell development and proliferation and was later shown to be expressed on many cells including macrophages, monocytes, dendritic cells, basophils, mast cells, eosinophils, B-cells, and platelets.[11-13] In addition to the membrane-bound trimeric form, CD40L also exists in an 18-kDa size, sCD40L, that is released from platelets after stimulation.[13-15] Platelets are the richest source of sCD40L in the circulation.[16,17]

TABLE 2. Correlation Matrix Between CD40-Related Parameters in 34 Patients with DS[a]

Parameter		Basic CD40%	Basic CD40 MFI	CD40 % After LPS Stimulation	CD40 MFI After LPS Stimulation	CD40 AI
Basic CD18 %	*r*	1	–0.260	–0.040	–0.163	0.093
	P		.137	.826	.365	.601
Basic CD18 MFI	*r*	…	1	0.222	**0.398**	–0.093
	P			.214	**.022**	.601
CD18 % after LPS stimulation	*r*	…	…	1	–0.084	–0.221
	P				.643	.216
CD18 MFI after LPS stimulation	*r*	…	…	…	1	**0.87**
	P					**<.001**
CD18 AI	*r*	…	…	…	…	1
	P					

AI, activation index; Basic, before LPS stimulation; DS, Down syndrome; LPS, lipopolysaccharide; MFI, mean fluorescence intensity.
[a]*Bold figures indicate significance.*

Downloaded from https://academic.oup.com/labmed/article/53/4/405/6548263 by guest on 28 February 2025

TABLE 3. Correlation Matrix Between CD18-Related Parameters in 34 Patients with DS[a]

Parameter		Basic CD18 %	Basic CD18 MFI	CD18 % After LPS Stimulation	CD18 MFI After LPS Stimulation	CD18 AI
Basic CD18 %	*r*	1	–0.012	**0.53**	–0.325	–0.025
	P		.946	**.002**	.065	.887
Basic CD18 MFI	*r*	…	1	**–0.485**	0.152	**–0.558**
	P			**.004**	.398	**.001**
CD18 % after LPS stimulation	*r*	…	…	1	–0.168	0.128
	P				.351	.478
CD18 MFI after LPS stimulation	*r*	…	…	…	1	0.291
	P					.1
CD18 AI	*r*	…	…	…	…	1
	P					

AI, activation index; Basic, before LPS stimulation; DS, Down syndrome; LPS, lipopolysaccharide; MFI, mean fluorescence intensity.
[a]Bold figures indicate significance.

TABLE 4. Correlation Matrix Between CD62p-Related Parameters in DS[a]

Parameter		Basic CD62p %	Basic CD62p MFI	CD62p % After LPS Stimulation	CD62p MFI after LPS Stimulation	CD62p AI
Basic CD62p %	*r*	1	–0.182	**0.432**	–0.229	–0.046
	P		.302	**.011**	.192	.795
Basic CD62p MFI	*r*	…	1	–0.086	0.295	**–0.552**
	P			.627	.09	**.001**
CD62p % after LPS stimulation	*r*	…	…	1	**–0.411**	–0.124
	P				**.016**	.484
CD62p MFI after LPS stimulation	*r*	…	…	…	1	**0.491**
	P					**.003**
CD62p AI	*r*	…	…	…	…	1
	P					

AI, activation index; Basic, before LPS stimulation; DS, Down syndrome; LPS, lipopolysaccharide; MFI, mean fluorescence intensity.
[a]Bold figures indicate significance.

It has been established that the presence of neutrophils enhances the stimulation-induced platelet release of sCD40L. The addition of platelets leads to an enhancement of neutrophil superoxide and ROS generation.[18] Our results regarding CD40 expression before stimulation with LPS showed no difference between patients with DS and control patients, which means that the neutrophils in both groups were comparable. However, after LPS stimulation, patients with DS showed a smaller MFI and activation index, which could imply impairment in the CD40–CD40L pathway in the neutrophil-platelet interaction.

In the current study, the CD18 percentage was significantly higher in the patients with DS before LPS stimulation ($P = .037$), whereas the CD18 MFI either before or after LPS stimulation was comparable in both groups. However, the activation index of CD18 was significantly lower in the DS group ($P < .001$), which indicated an impaired response in the patients with DS.

The CD62p percentage expression, before and after LPS activation, was significantly lower in the DS group ($P = .013$ and $P = .029$, respectively), although the CD62p MFI before LPS activation was comparable between both groups ($P = .517$). However, after LPS activation, the CD62p MFI was significantly higher in the DS group ($P = .011$). On the other hand, the activation index of CD62p was comparable between the 2 groups ($P = .338$).

P-selectin (also known as granule membrane protein-140, PADGEM protein, and CD62p) is a membrane glycoprotein present in the secretory granules of platelets α-granules[19,20] and the endothelial cells Weibel-Palade bodies[21] that is upregulated on the platelet surface after activation. The endothelial transmigration of neutrophils is elicited by the binding of PSGL-1[22] or CD11b/CD18 (MAC-1) to the platelet marker CD62p[23] and is further augmented by the binding of CD40 to the platelet-derived sCD40L.[24] The binding of the activated platelets to the adhering neutrophils results in polarized receptor organization, which represents a prerequisite for intravascular migration.[25] Platelets are further involved in NET formation, leading to the release of neutrophil DNA, which ensnares bacteria. Studies have also shown that TLR4-activated platelets bind to neutrophils, sticking to the endothelium, and initiate NET formation.[26] Platelets mediate NET formation either via CD62P-PSGL-1 interactions,[25,27] platelet GPIbα,[28] or CD11a/CD18 (LFA-1),[29] which contributes to bacterial clearance.

In light of the CD18-CD62p interactions, our findings regarding a significantly higher CD18 percentage in patients with DS before LPS stimulation although with a lower activation index after LPS stimulation indicate that the marker expression does not necessarily reflect its functional efficiency. On the other hand, our findings regarding a lower CD62p percentage in patients with DS both before and after LPS stimulation but with a higher MFI after LPS stimulation may indicate that

Downloaded from https://academic.oup.com/labmed/article/53/4/405/6548263 by guest on 28 February 2025

even though the CD62p has a lower percentage, it is functionally more active (reflected by the higher MFI after LPS stimulation) as a compensatory mechanism.

Regarding the correlation between the CD40-related parameters in patients with DS, a significant positive correlation was found between the CD40 MFI before and after LPS stimulation and between the CD40 activation index and the CD40 MFI after stimulation, which was expected in both.

Regarding the correlation between the CD18-related parameters in patients with DS, an expected significant moderate positive correlation was found between the CD18 percentage both before and after LPS stimulation ($r = 0.530$, $P = .002$). On the other hand, a moderate negative correlation was found between the CD18 percentage after LPS stimulation and the CD18 MFI before stimulation ($r = –0.485$, $P = .004$). Although it is difficult to pinpoint the underlying mechanism, it is likely a compensatory mechanism for neutrophils' lack of functional efficiency. In addition, a weak negative correlation was found between the CD18 MFI after LPS stimulation and the CD18 percentage before stimulation with a near statistically significant difference ($r = –0.325$, $P = .065$). Both negative correlations could be attributed to the compensatory functional mechanism of neutrophils. Furthermore, a moderate negative correlation was found between the CD18 MFI before LPS stimulation and the CD18 activation index that was statistically significant ($r = –0.558$, $P = .001$). This finding was expected, as previously mentioned for the other neutrophil markers.

Regarding the correlation between the CD62p-related parameters in patients with DS patients, as expected, a moderate positive correlation was found between the CD62p percentage before and after stimulation with LPS ($r = 0.432$, $P = .011$). In addition, a weak positive correlation was found between the CD62 MFI before and after stimulation with LPS, although it was not statistically significant ($r = 0.295$, $P = .09$). On the other hand, a moderate negative correlation was found between the CD62p MFI and the CD62p percentage after stimulation with LPS ($r = –0.411$, $P = .016$). As previously emphasized, this result could be attributed to a compensatory functional mechanism of CD62p. Furthermore, a moderate negative correlation was found between the CD62p activation index and the CD62p MFI before LPS stimulation ($r = –0.0552$, $P \leq .001$), whereas a moderate positive correlation was found between the CD62p activation index and the CD62p MFI after stimulation ($r = 0.491$, $P = .003$). This finding was expected, as previously mentioned for the other neutrophil markers.

Regarding infection history and different neutrophil-platelet parameters in patients with DS, although some weak correlation was found, it was not statistically significant for any of the studied parameters in both neutrophils and platelets.

Conclusion

Our findings suggest dysfunctional efficiency in neutrophils and impairment in the CD40–CD40L pathway in the neutrophil-platelet interaction, although a solid link could not be found between these findings and the frequency of infection history in our cohort of patients with DS. However, because there is no standardized scoring system to measure the frequency of infection, our findings clearly indicate the need for an objective scoring system to assess the frequency and severity of infection. Note that the relatively small sample size may have attributed to the study's failure to achieve statistical significance.

This is the first study to address the role of the neutrophil-platelet interaction in patients with DS. However, studying these markers in a larger cohort of patients is highly recommended to gain greater insight into their potential role in increased infection frequency in patients with DS. Those who have recurrent infection episodes should be screened for these markers with periodic follow-up to determine whether there is a change in these parameters corresponding with increased rates of infection. Furthermore, there is a need to develop a consensus about how to evaluate the severity of the infection episodes and a scoring system to incorporate frequency and severity.

Acknowledgments

E.R.R. contributed to the conceptualization of the experiment, data curation, and writing of the manuscript. R.E.H., S.M., and I.T.L.H. contributed to the investigation and methodology. H.M.A.E. contributed to the conceptualization, review, and editing, I.EA.M. contributed to the data curation, review, and editing. The authors thank Dr Azza M. Kamel for her scientific guidance throughout this study.

REFERENCES

1. Ram G, Chinen J. Infections and immunodeficiency in Down syndrome. *Clin Exp Immunol.* 2011;164(1):9–16.
2. Arora PD, Marignani PA, McCulloch CA. Collagen phagocytosis is regulated by the guanine nucleotide exchange factor Vav2. *Am J Physiol Cell Physiol.* 2008;295(1):C130–C137.
3. Goggs R, Williams CM, Mellor H, Poole AW. Platelet rho GTPases—a focus on novel players, roles and relationships. *Biochem J.* 2015;466(pt 3):431–442.
4. Kral JB, Schrottmaier WC, Salzmann M, Assinger A. Platelet interaction with innate immune cells. *Transfus Med Hemother.* 2016;43(2):78–88.
5. Gay NJ, Symmons MF, Gangloff M, Bryant CE. Assembly and localization of Toll-like receptor signaling complexes. *Nat Rev Immunol.* 2014;14(8):546–558.
6. Martínez E, Castañeda D, Jaramillo S, et al. Altered immune parameters correlate with infection-related hospitalizations in children with Down syndrome. *Human Immunol.* 2016;77(7):594–599.
7. Aomatsu K, Kato T, Fujita H, et al. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. *Immunology.* 2008;123(2):171–180.
8. Gawaj M, Langer H, May AE. Platelets in inflammation and atherogenesis. *J Clin Invest.* 2005;115(12):3378–3384.
9. Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. *Circ Res.* 2003;92(9):1041–1048.
10. Khan SY, Kelher MR, Heal JM, et al. Soluble CD40 ligand accumulates in stored blood components, primes neutrophils through CD40, and is a potential cofactor in the development of transfusion-related acute lung injury. *Blood.* 2006;108(7):2455–2462.
11. van Kooten C, Gaillard C, Galizzi JP, et al. B cells regulate expression of CD40 ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. *Eur J Immunol.* 1994;24(4):787–792.
12. Spriggs MK, Armitage RJ, Strockbine L, et al. Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. *J Exp Med.* 1992;176(6):1543–1550.
13. Gauchat JF, Aubry JP, Mazzei G, et al. Human CD40-ligand: molecular cloning, cellular distribution and regulation of expression by factors controlling IgE production. *FEBS Lett.* 1993;315(3):259–266.

Downloaded from https://academic.oup.com/labmed/article/53/4/405/6548263 by guest on 28 February 2025

14. Henn V, Steinbach S, Buchner K, Presek P, Kroczek RA. The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. *Blood.* 2001;98(4):1047–1054.
15. Hermann A, Rauch BH, Braun M, Schror K, Weber AA. Platelet CD40 ligand (CD40L)-subcellular localization, regulation of expression, and inhibition by clopidogrel. *Platelets.* 2001;12(2):74–82.
16. Andre P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet derived CD40L: the switch-hitting player of cardiovascular disease. *Circulation.* 2002;106(8):896–899.
17. Viallard JF, Solanilla A, Gauthier B, et al. Increased soluble and platelet-associated CD40 ligand in essential thrombocythemia and reactive thrombocytosis. *Blood.* 2002;99(7):2612–2614.
18. Vanichakarn P, Blair P, Wu C, Freedman JE, Chakrabarti S. Neutrophil CD40 enhances platelet-mediated inflammation. *Thromb Res.* 2008;122(3):346–358.
19. Berman CL, Yeo EL, Wencel-Drake JD, Furie BC, Ginsberg MH, Furie B. A platelet alpha granule membrane protein that is associated with the plasma membrane after activation: characterization and subcellular localization of platelet activation-dependent granule-external membrane protein. *J Clin Invest.* 1986;78(1):130–137.
20. McEver RP, Martin MN. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J Biol Chem.* 1984;259(15):9799–9804.
21. Hattori R, Hamilton KK, Fugate RD, McEver RP, Sims PJ. Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J Biol Chem.* 1989;264(14):7768–7771.
22. Lam FW, Burns AR, Smith CW, Rumbaut RE. Platelets enhance neutrophil transendothelial migration via P-selectin glycoprotein ligand-1. *Am J Physiol Heart Circ Physiol.* 2011;300(2):H468–H475.
23. Diacovo TG, Roth SJ, Buccola JM, Bainton DF, Springer TA. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood.* 1996;88(1):146–157.
24. Rahman M, Roller J, Zhang S, et al. Metalloproteinases regulate CD40L shedding from platelets and pulmonary recruitment of neutrophils in abdominal sepsis. *Inflamm Res.* 2012;61(6):571–579.
25. Sreeramkumar V, Adrover JM, Ballesteros I, et al. Neutrophils scan for activated platelets to initiate inflammation. *Science.* 2014;346(6214):1234–1238.
26. Caudrillier A, Kessenbrock K, Gilliss BM, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest.* 2012;122(7):2661–2671.
27. Etulain J, Martinod K, Wong SL, Cifuni SM, Schattner M, Wagner DD. P-selectin promotes neutrophil extracellular trap formation in mice. *Blood.* 2015;126(2):242–246.
28. von Brühl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med.* 2012;209(4):819–835.
29. McDonald B, Kubes P. Neutrophils and intravascular immunity in the liver during infection and sterile inflammation. *Toxicol Pathol.* 2012;40(2):157–165.

Downloaded from https://academic.oup.com/labmed/article/53/4/405/6548263 by guest on 28 February 2025

Consistency Between Thyrotropin Receptor Antibody (TRAb) and Thyroid-Stimulating Antibody (TSAb) Levels in Patients with Graves Disease

Youyuan Huang,[1,#] Bo Jin, MD,[2,#] Yucheng Huang, MA,[1] Aimei Dong, MD[1,]*

Departments of [1]Endocrinology and [2]Clinical Laboratory, Peking University First Hospital, Beijing, China; *To whom correspondence should be addressed. dongaimei1975@sina.com; [#]These authors contributed equally to this article.

Keywords: Graves disease, thyrotropin receptor antibody, thyroid-stimulating antibody, thyroid function, hyperthyroidism, endocrinology

Abbreviations: GD, Graves disease; TRAbs, thyrotropin receptor antibodies; TSH, thyroid-stimulating hormone; TSAbs, thyroid-stimulating antibodies; TSI, thyroid-stimulating immunoglobulin; TSBAbs, thyroid-stimulation-blocking antibodies; TSHR, thyroid-stimulating hormone receptor; FT3, free triiodothyronine; FT4, free thyroxine; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; GO, Graves ophthalmopathy

Laboratory Medicine 2022;53:412–416; https://doi.org/10.1093/labmed/lmac008

ABSTRACT

Objective: To investigate the consistency between thyrotropin receptor antibody (TRAb) and thyroid-stimulating antibody (TSAb) levels in patients with Graves disease (GD).

Methods: We performed a cross-sectional observational study to recruit eligible patients with GD who visited the outpatient endocrinology clinic for the purpose of evaluating the consistency between their TRAb and TSAb levels. Our cohort included 28 men and 99 women.

Results: The median levels of TRAb and TSAb were 5.65 IU/L and 3.76 IU/L, respectively, in the enrolled patients with GD. The levels of TRAb (5.03 vs 8.42 IU/L; $P = .008$) and TSAb (2.69 vs 5.37 IU/L; $P = .008$) in patients with adequate thyroid regulation were all lower than those in patients with inadequate thyroid regulation.

Conclusions: Although TRAb is closely related to TSAb, we observed high heterogeneity of TRAb due to relatively low consistency between the levels of the 2 antibodies.

Graves disease (GD), also known as diffuse toxic goiter, is the most common cause of hyperthyroidism. Thyrotropin receptor antibodies (TRAbs) have not only been used as a classical criterion for GD diagnosis but also serve as an indicator of drug withdrawal in clinical practice because of their impact on dose adjustments to antithyroid drugs.[1–3] We emphasize that TRAbs target only a part of the hybrid-type thyroid-stimulating hormone (TSH) receptor, which is targeted by 3 antibodies. Those antibodies include thyroid-stimulating antibodies (TSAbs), which are also known as thyroid-stimulating immunoglobulin (TSI), thyroid-stimulation-blocking antibodies (TSBAbs), and neutralizing antibodies. TSAbs exert TSH-like effects by binding to the TSH receptor (TSHR) and play an essential role in the development of hyperthyroidism in patients with GD.[1,2] In contrast, TSBAbs can competitively block the binding of TSH to TSHR and thereby inhibit thyroid hormone synthesis and secretion.

This study was performed to investigate the association and consistency of TRAb and TSAb levels in patients with GD in relation to different characteristics. Also, we sought to identify possible factors influencing TRAb and TSAb levels by investigating the relationship between factors such as thyroid function status and course of treatment with the levels of TRAb or TSAb.

Methods

Study Design and Subjects

In this cross-sectional observational study, eligible patients with GD were recruited from the outpatient endocrinology clinic of a tertiary hospital between February 2021 and March 2021 according to the following inclusion criteria: age between 20 and 80 years, did not receive antihyperthyroidism treatment and were confirmed to test positive for TRAb/had no nodular changes (this latter result was confirmed by thyroid ultrasound assessment), received antihyperthyroidism treatment and were confirmed to have tested positive for TRAb during the disease course/had no nodular changes. Exclusion criteria included hyperthyroidism unrelated to GD, having received radioiodine treatment for GD, having received glucocorticoid treatment in supraphysiological doses regardless of the route of administration, and having been confirmed to have severe diseases such as liver cirrhosis, end-stage renal disease, or severe hypoproteinemia.

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/412/6548262 by guest on 28 February 2025

Sampling Methods

Clinical data such as history of thyroid diseases, treatment history, and family history were collected using questionnaires, and all data were further checked based on the selection criteria. Free triiodothyronine (FT_3), free thyroxine (FT_4), TSH, TRAb, TSAb, thyroid peroxidase antibody (TPOAb), and thyroglobulin antibody (TgAb) were analyzed using blood specimens.

Detection Methods

Serum TRAb was determined using a fully automated electrochemiluminescence immunoassay (Elecsys 2010/E 170/e601/e 411, third-generation; Roche Diagnostics). The analytical assay sensitivity was 0.3 IU/L, the coefficient of variation was 4.41% for intra-assay variation and 5.27% for interassay variation, the working ranges of the assays were 0.3–40.0 IU/L, and the cut-off value for an abnormal level was 1.7 IU/L.

TSAb was specifically measured using the TSAb kit and an IMMULITE2000 XPi System Analyzer (Siemens Healthcare Diagnostics), according to manufacturer-provided specifications. The analytical assay sensitivity was 0.1 IU/L, the coefficient of variation was 3.15% for intra-assay variation and 4.8% for interassay variation, the working ranges of the assays were 0.10–40.00 IU/L, and the cut-off value for an abnormal level was 0.55 IU/L. Serum TSH, FT3, and FT4 were measured using an acridinium ester chemiluminescence method on an automatic chemiluminescence immunoanalyzer (Centaur XP, Siemens).

Statistical Analysis

We conducted all statistical analyses using SPSS software, version 24 (IBM). For continuous variables, normality of the data was tested using the Kolmogorov-Smirnov test. Normally distributed data were expressed as mean (SD) and non–normally distributed data as median (IQR). Direct between-group comparisons were performed using the independent samples rank sum test and Mann-Whitney *U* test for non–normally distributed data. Correlations between continuous variables were evaluated using Pearson correlation analysis for normally distributed data and Spearman correlation analysis for non–normally distributed data. The concordance between 2 ordered categorical variables with comparable properties was examined using the kappa concordance test. All statistical tests were 2-tailed, and differences with $P < .05$ were considered statistically significant. When multiple groups were compared for statistically significant differences, the Bonferroni correction was applied.

Results

General Information of the Included Patients

A total of 127 eligible patients with GD were enrolled in our study: 28 men and 99 women. The median (IQR) age was 36 (29–48) years, and the median (IQR) treatment duration of continuous drug treatment for hyperthyroidism was 12 (3–35) months. A total of 52 patients had TSH, FT4, and FT3 values within the normal range, and there were 75 patients with increased FT4 and FT3 levels, as well as decreased TSH levels. Moreover, there were 30 patients with a family history of autoimmune thyroid disease.

Among the 97 patients who had not been treated previously, 40 patients had adequately controlled thyroid function, and 57 patients had inadequately controlled thyroid function. Among the 30 patients who were treated again due to recurrence of thyroid issues, there were 12 patients with adequately controlled thyroid function, and 18 patients with inadequately controlled thyroid function. The general information and clinical data of all included patients is summarized in **TABLE 1**.

Comparison of Thyroid Function, TRAb, and TSAb Levels in Patients with Hyperthyroidism Having Different Characteristics

There was no statistical difference in FT_3, FT_4, TSH, TRAb, and TSAb levels between patients who had not been treated previously and patients who had been treated again due to recurrence of thyroid malfunctioning. There was a stastically significant difference in the levels of FT_3, FT_4, TSH, TRAb, and TSAb between patients who had adequately controlled thyroid function and patients who had inadequately controlled thyroid function. Specifically, the median level of TRAb in patients with inadequate thyroid function control was higher than in patients with adequate thyroid function control (5.03 vs 8.42 IU/L; $P = .008$). Similarly, the median level of TSAb in patients with inadequate thyroid function control was also higher than in patients with adequate thyroid function control (2.69 vs 5.37 IU/L; $P = .008$). Among the patients with hyperthyroidism who had previously been untreated and had been treated again due to recurrence, the median level and distribution of TRAb and TSAb also tended to be statistically significantly different between patients with inadequate thyroid function control and patients with adequate thyroid function control ($P < .01$).

Consistency Between TRAb and TSAb Levels

We categorized the titer of TRAb into 4 groups according to its quartiles, with the following specific definitions: TRAb+ (titer ≤lower quartile), TRAb++ (lower quartile < titer ≤median), TRAb+++ (median <titer ≤upper quartile), and TRAb++++ (titer >upper quartile). Similarly, the titers of TSAb were also categorized into 4 groups, namely, TSAb+, TSAb++, TSAb+++, and TSAb++++.

We observed a positive relationship between the titers of TRAb and TSAb, with a gamma rank correlation coefficient of 0.762 ($P < .001$). A certain degree of consistency between different titer categories of TRAb and TSAb was also detected, with a kappa value of 0.318. Cross-tabulation of different titer categories of TRAb and TSAb for all patients is shown in **TABLE 2**.

Subgroup analysis indicated similar results in different subgroups, including patients with adequate thyroid function control, patients with inadequate thyroid function control, patients who had previously been untreated, and patients had been treated again due to recurrence of thyroid malfunctioning, with gamma rank correlation coefficients of 0.656, 0.797, 0.775, and 0.721, respectively. We also identified a certain degree of consistency of the titer categories of TRAb and TSAb among different patient subgroups, with kappa values of 0.281, 0.314, 0.327, and 0.290, respectively.

Correlation Between the TSAb/TRAb Ratio and Thyroid Function

We classified patients into 3 groups, defined as euthyroid (n = 52), subclinical hyperthyroid (n = 37), and clinical hyperthyroid (n = 38) according to the thyroid function status. The TSAb/TRAb ratio was 0.71:0.37 among all patients, with a ratio of 0.64:0.37, 0.69:0.34, and 0.82:0.40

Downloaded from https://academic.oup.com/labmed/article/53/4/412/6548262 by guest on 28 February 2025

TABLE 1. General Information and Clinical Data from the 127 Study Participants[a]

Variable	Initial Treatment			Recurrence			Overall		
	Controlled	Inadequately Controlled	*P* Value	Controlled	Inadequately Controlled	*P* Value	Controlled	Inadequately Controlled	*P* Value
Sample size, No.	40	57		12	18		52	75	
Age (y), median (IQR)	38 (28–45)	36 (30–48)	.96	37 (31–56)	33 (29–43)	.29	38 (29–47)	35 (30–48)	.65
Sex (male/female)	6/34	25/42	.18	5/7	2/16	.05	11/41	8/58	.84
Treatment duration (mo), median (IQR)	16 (8–26)	5 (0–22)	.001	24 (10–57)	21 (11–60)	.63	18 (10–36)	8 (1–33)	.02
FT_3 (pmol/L), median (IQR)	4.48 (4.00–4.74)	7.98 (5.08–13.93)	<.001	4.48 (4.1–4.8)	11.54 (4.87–17.39)	.001	4.48 (4.02–4.74)	7.98 (5.06–14.15)	<.001
FT_4 (pmol/L), median (IQR)	14.24 (12.24–16.5)	22.11 (17.52–37.41)	<.001	15.6 (14.46–17.57)	32.77 (17.34–42.33)	.001	14.77 (12.82–16.68)	22.11 (17.42–37.66)	<.001
TSH (mIU/L), median (IQR)	2.11 (1.46–4.21)	0.01 (0–0.02)	<.001	1.46 (0.99–3.16)	0.02 (0–0.14)	<.001	2.11 (1.26–4.16)	0.01 (0–0.04)	<.001
TRAb (IU/L), median (IQR)	5.2 (3.04–9.64)	8.2 (4.08–16.51)	.018	2.57 (2.1–6.27)	9.38 (3.32–17.45)	.048	5.03 (2.45–9.44)	8.42 (4.00–16.94)	.002
TSAb (IU/L), median (IQR)	2.71 (1.51–4.80)	5.37 (2.66–12.60)	.003	2.27 (1.36–5.16)	4.57 (2.07–10.44)	.12	2.69 (1.43–4.84)	5.37 (2.34–11.50)	.001

FT_3, free triiodothyronine; FT_4, free thyroxine; TSH, thyroid-stimulating hormone; TRAb, thyrotropin receptor antibody; TSAb, thyroid-stimulating antibody.
[a]Comparison of thyroid function control group and noncontrol group: the Pearson χ^2 test was used for sex and the Mann Whitney U test was used for other parameters.

TABLE 2. Cross-Tabulation of Different Titer Groups Between TRAb and TSAb[a]

Items	TSAb+	TSAb++	TSAb+++	TSAb++++	Total
TRAb+	19	12	0	1	32
TRAb++	10	11	11	0	32
TRAb+++	2	6	13	11	32
TRAb++++	1	3	8	19	31
Total	32	32	32	31	127

TRAb, thyrotropin receptor antibody; TSAb, thyroid-stimulating antibody.
[a]TRAb and TSAb were grouped according to 4 quantiles, and the gamma correlation test was performed for ordered classification data. The gamma correlation coefficient was 0.762 (P < .001), and the internal consistency coefficient kappa value was 0.318.

TABLE 3. Cross-Tabulation Between Thyroid-Function Status and TSAb/TRAb Ratio

Variable	Euthyroidism	Subclinical Hyperthyroid	Clinical Hyperthyroidism	Total
High TSAb/TRAb ratio	21	18	24	63
Low TSAb/TRAb ratio	30	19	14	63
Total	51	37	38	126

TRAb, thyrotropin receptor antibody; TSAb, thyroid-stimulating antibody.
[a]Goodman-Kruskal gamma testing was used to analyze the correlation between the 2 groups of ordered classification data (data 1: classification of thyroid function status, data 2: binary grouping of TSAb/TRAb ratio), with a gamma coefficient of 0.306 and progressive significance (P =.03).

for patients with euthyroidism, subclinical hyperthyroidism, and clinical hyperthyroidism, respectively. Although the difference among the ratios in the 3 groups was not statistically significant ($P = .08$), further analysis suggested a statistical difference between patients with euthyroidism and clinical hyperthyroidism according to the comparison between the 2 groups ($P = .03$).

According to the ratios, we divided the patients into a high TSAb/TRAb ratio group and a low TSAb/TRAb ratio group. We analyzed 2-way ordered categorical data using cross-tabulation, as shown in **TABLE 3**, and we discovered a statistically significant distribution with a gamma coefficient of 0.306 ($P = .03$). The clinical hyperthyroidism group was more likely to be in the high TSAb/TRAb group.

Discussion

GD is an organ-specific autoimmune disease and the most common cause of hyperthyroidism. As a result of the abnormal activation of T and B cells, TSHR autoantibodies are generated and then promote thyroid hormone synthesis and secretion.

TSHR is a G protein–coupled receptor comprising an extracellular α-subunit and a transmembrane β-subunit, which are linked by a disulfide bond. Shedding of the extracellular α-subunit will produce autoantigens and activate $CD4^+$ T cells, which can lead to the production of TRAbs.[1,2]

TRAbs are a group of heterogeneous polyclonal antibodies, which can be further divided into TSAb, TSBAb, and neutralizing antibodies according to their specific effects after binding to TSHR. TSAb will exert similar downstream effects as TSH by binding to TSHR. For example, TSAb will induce the production of cyclic adenosine by activating adenylate cyclase to stimulate the proliferation of thyroid follicular epithelial cells, leading to the production and secretion of thyroid hormones. By contrast, blocking antibodies can block the effects of TSH and thus inhibit the production of thyroid hormones. However, the binding of neutralizing antibodies to corresponding receptors affects neither the production of cyclic adenosine nor binding of TSH to its receptors.[3–5] We note that TSAbs are known to be

Downloaded from https://academic.oup.com/labmed/article/53/4/412/6548262 by guest on 28 February 2025

associated with the progression of hyperthyroidism resulting from GD.[3,4,6]

In clinical practice, TRAbs are currently measured using third-generation automated immunoassays, which use a competitive method to simultaneously measure mixed TRAbs, including TSAbs, TSBAbs, and neutralizing antibodies. However, this method alone could not be used to distinguish TSAb.[3,4,7] Measurement of TSAbs was previously employed in scientific research but is difficult to extensively apply in clinical practice due to the complexity of determination. However, the commercialized TSAb determination kit enabled the popularization and application of TSAbs in clinical practice.[8]

TRAbs status can contribute to differential diagnosis of thyroid dysfunction because the level of TRAbs might be affected by immune status and changes in the GD treatment. The level of TRAbs gradually decreases in patients treated with antithyroid drugs, and they disappear in approximately 70%-80% of patients after 18 months of treatment.[2,9] Elevated TRAb levels at diagnosis are not only associated with a higher relapse rate[10–13] but are also related to several complications such as Graves ophthalmopathy (GO).[14] It was also reported that persistent positive TRAb is positively associated with an increased risk of recurrence after drug withdrawal.[15,16] However, some shortcomings have hindered its performance in the diagnosis and treatment of GD, especially because not all patients with GD have positive TRAb results before treatment. It was reported that approximately 4%-8% of patients with GD have negative TRAb results.[16,17] Moreover, although TRAb titers do not decrease to the normal range or even do not decrease at all in some patients receiving standard treatment, recurrence will follow in the short term after drug withdrawal.

Etiologically, TSAbs are closely related to GD-related hyperthyroidism,[3,4,6,7] which may be a reproducible and more sensitive specific biomarker of GD. TSAbs are considered to have greater significance in the diagnosis and evaluation of clinical outcomes in GD-related hyperthyroidism[18,19] because the results of several studies[17,18,20,21] revealed that TSAbs are closely related to the severity of the disease and extrathyroidal manifestations such as thyroid ophthalmopathy.

Changes in TSHR epitopes, which can be specifically targeted by antibodies, will be detected in addition to changes in TRAb levels during the disease-course evolution and treatment.[22] After then, the TSAb-dominant phenotype changes to a TSBAb-dominant mixture of polyclonal TRAbs, which may be the reason that partial patients experienced remission but still tested positive for TRAbs.

In this study, we performed several separate analyses according to the different clinical characteristics of patients with GD and found that TRAb and TSAb levels were related to the status of thyroid function. Specifically, patients with inadequate thyroid-function control had relatively high TRAb and TSAb levels. However, the levels of these 2 antibodies were not associated with the course of treatment, recurrence, and dose of antithyroid drugs.

We also found a high correlation between TRAbs and TSAbs in the present study because we determined a certain degree of consistency in different titer categories of both antibodies, after categorizing the levels of the 2 antibodies into different titer groups. However, a relatively low consistency (kappa <0.4) suggested the presence of individual heterogeneity in the composition of hybrid-type TRAb.

We used the TSAb/TRAb ratio to roughly estimate the proportion of TSAbs in the mixture of polyclonal TRAbs in this study. We were heartened to observe that the TSAb/TRAb ratio among patients with GD who still exhibited clinical hyperthyroidism was higher than among patients with normal thyroid function. This finding supports our hypothesis that a TSAb-dominant phenotype is changed to a TSBAb-dominant phenotype when patients recover and regain normal thyroid function after treatment.

Our study also has some limitations which must be kept in mind when interpreting its findings. Considering the nature of this cross-sectional study, the change in trends of TRAbs and TSAbs during individualized GD treatment and the relationship between the activity and the relative proportion of the 2 antibodies with the remission of GD and the risk of recurrence after drug withdrawal need to be further clarified.

Limitations of this study include the presence in the cohort of TRAb-positive subjects with untreated Graves disease or who were undergoing TRAb-positive drug therapy in the course of disease. Among the 18 untreated subjects who tested TRAb positive, 3 cases involved critical-state TSAb, as evidenced by measurements of 0.1-0.55 IU/L. Because subjects with TRAb-negative and TSI-positive results were not included, we could not evaluate the diagnostic efficacy of the 2 antibodies for Graves disease. (Previous literature reported that the TS Ab of healthy subjects was below the lower limit of 0.1 IU/L.)

In the treated patients with Graves disease, both antibodies were more correlated with thyroid function status than other analytes. However, this study was a cross-sectional investigation, and it was not possible to evaluate which of the 2 studied antibodies delivered more favorable results in terms of treatment response and recurrence risk after drug withdrawal. This was true despite that, theoretically, TSAb was more closely related to the physiological abnormalities of Graves disease.

In conclusion, the present study identified consistency, to a certain extent, between the titers of TSAb and TRAb in patients with GD. Further, the titers of these antibodies were related to thyroid function, and the proportion of TSAb in the mixture of TRAbs tended to decrease with adequate thyroid function control.

Acknowledgments

This work was supported by the Interdisciplinary Clinical Research Project of Peking University First Hospital (2019CR33).

Personal and Professional Conflicts of Interest

The authors declared that they have no conflicts of interest to this work.

REFERENCES

1. Zuhur SS, Elbuken G, Yildiz I, et al. External validation of the GREAT score in Turkish patients with Graves' hyperthyroidism treated with the titration regimen method of antithyroid drugs: a multicenter study. *Horm Metab Res*. 2019;51(10):627–633.
2. Barbesino G, Tomer Y. Clinical review: clinical utility of TSH receptor antibodies. *J Clin Endocrinol Metab*. 2013;98(6):2247–2255.
3. Kotwal A, Stan M. Thyrotropin receptor antibodies—an overview. *Ophthalmic Plast Reconstr Surg*. 2018;34(4S suppl 1):S20–S27.
4. Rapoport B, Chazenbalk GD, Jaume JC, McLachlan SM. The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. *Endocr Rev*. 1998;19(6):673–716.
5. Kahaly GJ, Diana T, Olivo PD. TSH receptor antibodies: relevance & utility. *Endocr Pract*. 2020;26(1):97–106.
6. Michalek K, Morshed SA, Latif R, Davies TF. TSH receptor autoantibodies. *Autoimmun Rev*. 2009;9(2):113–116.

Downloaded from https://academic.oup.com/labmed/article/53/4/412/6548262 by guest on 28 February 2025

7. Autilio C, Morelli R, Locantore P, Pontecorvi A, Zuppi C, Carrozza C. Stimulating TSH receptor autoantibodies immunoassay: analytical evaluation and clinical performance in Graves' disease. *Ann Clin Biochem*. 2018;55(1):172–177.
8. Struja T, Jutzi R, Imahorn N, et al. Comparison of five TSH-receptor antibody assays in Graves' disease: results from an observational pilot study. *BMC Endocr Disord*. 2019;19(1):38.
9. Kahaly GJ. Management of Graves thyroidal and extrathyroidal disease: an update. *J Clin Endocrinol Metab*. 2020;105(12):3704–3720.
10. Struja T, Fehlberg H, Kutz A, et al. Can we predict relapse in Graves' disease? Results from a systematic review and meta-analysis. *Eur J Endocrinol*. 2017;176(1):87–97.
11. Vos XG, Endert E, Zwinderman AH, Tijssen JGP, Wiersinga WM. Predicting the risk of recurrence before the start of antithyroid drug therapy in patients with Graves' hyperthyroidism. *J Clin Endocrinol Metab*. 2016;101(4):1381–1389.
12. Struja T, Kaeslin M, Boesiger F, et al. External validation of the GREAT score to predict relapse risk in Graves' disease: results from a multicenter, retrospective study with 741 patients. *Eur J Endocrinol*. 2017;176(4):413–419.
13. Masiello E, Veronesi G, Gallo D, et al. Antithyroid drug treatment for Graves' disease: baseline predictive models of relapse after treatment for a patient-tailored management. *J Endocrinol Invest*. 2018;41(12):1425–1432.
14. Ehlers M, Allelein S, Schott M. TSH-receptor autoantibodies: pathophysiology, assay methods, and clinical applications. *Minerva Endocrinol*. 2018;43(3):323–332.
15. Laurberg P, Wallin G, Tallstedt L, Abraham-Nordling M, Lundell G, Tørring O. TSH-receptor autoimmunity in Graves' disease after therapy with anti-thyroid drugs, surgery, or radioiodine: a 5-year prospective randomized study. *Eur J Endocrinol*. 2008;158(1):69–75.
16. Kahaly GJ, Bartalena L, Hegedüs L, Leenhardt L, Poppe K, Pearce SH. 2018 European Thyroid Association Guideline for the Management of Graves' Hyperthyroidism. *Eur Thyroid J*. 2018;7(4):167–186.
17. Diana T, Holthoff H-P, Fassbender J, et al. A novel long-term Graves' disease animal model confirmed by functional thyrotropin receptor antibodies. *Eur Thyroid J*. 2020;9(suppl 1):51–58.
18. Kahaly GJ, Diana T, Kanitz M, Frommer L, Olivo PD. Prospective trial of functional thyrotropin receptor antibodies in Graves disease. *J Clin Endocrinol Metab*. 2020;105(4):e1006–e1014.
19. Lytton SD, Schluter A, Banga PJ. Functional diagnostics for thyrotropin hormone receptor autoantibodies: bioassays prevail over binding assays. *Front Biosci (Landmark Ed)*. 2018;23:2028–2043.
20. Diana T, Wüster C, Kanitz M, Kahaly GJ. Highly variable sensitivity of five binding and two bio-assays for TSH-receptor antibodies. *J Endocrinol Invest*. 2016;39(10):1159–1165.
21. Diana T, Ponto KA, Kahaly GJ. Thyrotropin receptor antibodies and Graves' orbitopathy. *J Endocrinol Invest*. 2021;44(4):703–712.
22. Takasu N, Matsushita M. Changes of TSH-stimulation blocking antibody (TSBAb) and thyroid stimulating antibody (TSAb) over 10 years in 34 TSBAb-positive patients with hypothyroidism and in 98 TSAb-positive Graves' patients with hyperthyroidism: reevaluation of TSBAb and TSAb in TSH-receptor-antibody (TRAb)-positive patients. *J Thyroid Res*. 2012;2012:182176.

Downloaded from https://academic.oup.com/labmed/article/53/4/412/6548262 by guest on 28 February 2025

Reliable Detection of T-Cell Clonality by Flow Cytometry in Mature T-Cell Neoplasms Using TRBC1: Implementation as a Reflex Test and Comparison with PCR-Based Clonality Testing

Deirdre Waldron, MSc,[1,] David O'Brien, MSc,[1] Laura Smyth, BSc,[1] Fiona Quinn, PhD,[2] Elizabeth Vandenberghe, MD[3]*

[1]Clinical Cytometry Laboratory, St James Hospital, Dublin, Ireland, [2]Cancer Molecular Diagnostics Laboratory, Department of Haematology, St James Hospital, Dublin, Ireland, [3]Department of Haematology, St James Hospital, Dublin, Ireland; *To whom correspondence should be addressed. dwaldron@stjames.ie

Keywords: T-cell, flow cytometry, T-cell receptor β chain, TRBC1, T-cell clonality, T-cell lymphoma, clonality

Abbreviations: TRBC1, T-cell receptor β constant region 1; PCR, polymerase chain reaction; TCR, T-cell receptor; V, variable; D, diversity; J, joining; SSC, side scatter; FSC, forward scatter; CI, confidence interval; AUC, area under the curve; SD, standard deviation; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Laboratory Medicine 2022;53:417–425; https://doi.org/10.1093/labmed/lmac014

ABSTRACT

Objective: The T-cell receptor β constant region 1 (TRBC1) antibody can identify T-cell clonality and distinguish pathological from normal T cells. This study aims to establish optimal cutpoints for establishing monotypia and validate the diagnostic abilities of the TRBC1 antibody when used as a reflex test in conjunction with an existing T-cell antibody panel.

Materials and Methods: We used 46 normal peripheral blood specimens and examined 8 patients with reactive lymphoproliferations to determine the normal biological range of TRBC1 on CD4+ and CD8+ T cells. We also evaluated 43 patient specimens that were submitted for investigation of a lymphoproliferative disorder for CD2/CD3/CD4/CD5/CD7/CD8/CD16/CD26/CD45/CD56/TCR αβ/TCR γδ, along with TRBC1 expression. The results were compared to TCR gene rearrangement patterns using polymerase chain reaction (PCR) analysis.

Results: Statistical analysis established differing cutoff points for establishing monotypia dependent on restricted TRBC1 or TRBC2 usage. Direct comparison with molecular analysis indicated that no specimen identified with the restricted expression of TRBC1 was reported as polyclonal by PCR with a concordance rate of 97% between a clonal PCR result and monotypic TRBC1 expression.

Conclusion: Incorporation of the TRBC1 antibody using statistically derived cutoff points in a reflex setting for the evaluation of a suspected T-cell neoplasm improves the identification of clonal T-cell populations by flow cytometry and correlates well with molecular methods.

Research has shown that T-cell lymphomas are a pathologically and clinically complex group of heterogeneous disorders accounting for less than 10% of all non-Hodgkin lymphomas.[1] The clinical course of this group of disorders is variable, ranging from relatively indolent to highly aggressive. The most recent 2016 World Health Organization classification characterized several categories by incorporating clinical features in conjunction with morphological and immunophenotypic aspects and, less commonly, molecular and genetic studies.[2]

The role of flow cytometry in the diagnosis of T-cell lymphomas is well established,[3-5] but the absence of a specific immunophenotypic profile can present challenges. Another method used in conjunction with a panel of T-cell antibodies to assess clonality by flow cytometry includes T-cell receptor (TCR) variable β region analysis.[6,7] However, this method is quite cumbersome and is not easily introduced to routine immunophenotyping laboratories.

Molecular analysis of the TCR using multiplex polymerase chain reaction (PCR) to detect clonality is frequently used, especially to provide clarity in patients with ambiguous morphologic or immunophenotypic results. The successful standardization of TCR clonality testing has enabled it to become possible in a routine diagnostic setting.[8] However, interpretation may be complex in certain circumstances, such as in patients with infection, inflammation, and immunosenescence.[7,9] Next-generation sequencing offers a deep and comprehensive assessment of TCR gene rearrangements but is complex and expensive and has limited availability.[10]

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

During research into chimeric antigen receptor T-cell therapy, Maciocia et al[11] screened multiple anti-TCR monoclonal antibodies and identified the JOVI.1 monoclonal antibody as exhibiting specificity for the TCR β constant region 1 (TRBC1). Studies have shown that TCR gene segment rearrangement is known to occur in an ordered fashion, beginning with TCR δ, then TCR γ, followed by TCR β, and finally TCR α.[12] Focusing on the TCR β chain, the variable domain is encoded by the variable (V), diversity (D), and joining (J) gene segments and once rearranged, a functional V-D-J exon is transcribed. Multiplex PCR protocols use this knowledge to detect clonally rearranged TCR β gene segments.

The TCR β chain constant region is encoded by either 1 of 2 genes, *TRBC1* or *TRBC2,* in a mutually exclusive process. The transcribed V-D-J variable exon is spliced to join 1 of these 2 constant regions. Successful translation of the mRNA results in the production of the TCR β chain.[13] Normal αβ T cells express a mixture of *TRBC1*-negative and *TRBC1*-positive cells in much the same way as B cells express kappa and lambda light chains. In T-cell neoplasms, the restricted expression of either *TRBC1* or *TRBC2* is evident. Because the majority of T-cell lymphomas (>95%) express the αβ subunit,[10] *TRBC1* has emerged as a research target; it provides a noteworthy opportunity to confirm the clonal nature of T-cell neoplasms by flow cytometry.[11,14-16]

We report here on our experience with the TRBC1 antibody used as a reflex test from validation and numerical cutoff determination to real-life experience by direct comparison of *TRBC1* expression with molecular clonality testing.

Materials and Methods

Establishing a Normal Range

We examined peripheral blood specimens from 46 normal healthy donors to investigate the normal biological expression of TRBC1 on CD4+ and CD8+ T cells. All specimens had white blood cell counts within normal limits. The specimens were stored at 4ºC and were analyzed within 12 hours. A minimum of 20,000 T cells were acquired.

Comparison Group

We prospectively collected peripheral blood and bone marrow aspirate specimens submitted for flow cytometric analysis to investigate a T-cell lymphoproliferative disorder at St James Hospital, Dublin, Ireland. Of these 43 specimens, 35 were peripheral blood and 8 were bone marrow aspirate specimens. They were obtained from 25 male and 18 female patients. The patient ages ranged from 27 years to 84 years with a median age of 67 years. An overview of patient and sample information is available in Supplemental Material, Table 2.

Flow Cytometry: Preanalytical Preparation

White blood cell counts were performed using a Sysmex-XN hematology analyzer and were diluted to less than 30×10^9/L. We incubated 100 μL aliquots of the specimens with antibody cocktails. Red cells were lysed using a BD FACS lysing solution (Beckton Dickinson, BD Biosciences), and the antibody stained cells were resuspended in BD CellWASH (BD Biosciences). Specimens were acquired using a BD FACS Canto II flow cytometer. Analysis was completed using BD FACS Diva Software version 8.0.2 (BD Biosciences). Antibody titration was achieved by examining the stain index of the TRBC1 antibody through a set of serial dilutions. The concentration of antibody that provided the brightest staining with minimum background was selected for use.

Specimen Testing Procedure

Peripheral blood and bone marrow aspirate specimens were received in EDTA and RPMI media, respectively, and were assessed for preliminary evidence of a T-cell lymphoproliferative disorder using one 8-color lymphocyte screening tube containing a monoclonal antibody cocktail including CD3 allophycocyanin (APC)/CD8 fluorescein isothiocyanate (FITC)/CD56 phycoerythrin (PE)/CD5 PerCP-Cy5.5/CD4 V450/CD45 V500-C/TCR γδ PE-Cy7. Upon identification of a suspicious T-cell population, a dedicated T-cell lymphoproliferative panel was engaged containing additional markers (Supplemental Material, Table 1).

Upon confirmation of a suspicious T-cell population, a single tube containing TRBC1 FITC/CD8 PE/CD3 PerCP-Cy5.5/CD4 V450/TCR γδ PECy7 and either CD2, CD5, or 7 APC was used for analysis where appropriate. This APC channel was dictated by the aberrant pattern of staining observed in the aforementioned lymphocyte screening tube and/or the T-cell lymphoproliferative panel. An anti-TRBC1 antibody (clone JOVI.1) was purchased from Ancell (Bayport, MN), CD8 FITC and CD56 PE were purchased from Cytognos SL (Salamanca, Spain), and all other antibodies were sourced from BD Biosciences (San Jose, CA).

Molecular TCR Gene Rearrangement Studies

Gene rearrangement studies were completed using the BIOMED-2 primers from Invivoscribe (San Diego, CA) assessing TCR Vβ, Dβ, Jβ, Vγ, and Jγ. The DNA was extracted and PCR amplification was completed in 6 multiplex PCR tubes according to the BIOMED-2 protocol.[8] The PCR products were separated and detected by capillary gel electrophoresis on the ABI PRISM 3130XL genetic analyzer using Genemapper software (Applied Biosystems, Warrington, UK).

Statistical Analysis

Statistical analysis was performed using R.[17] Figures were produced using ggplot2, and cutoff analysis was completed using the cutpointr package. cutpointr defines the dataset into a positive (monotypic TRBC1 expression) or negative class (polytypic TRBC1 expression) and presumes that the positive class has higher values. Using the opt_cut function, the optimal cutpoint is calculated using the mean value of the optimal cutpoint and the next highest or lowest predictor value to calculate the cutpoint, thus minimizing bias. The outcome is an optimal cutpoint that is equal to the cutpoint that would be obtained using an infinitely dense sequence of candidate cutpoints and is usually more efficient computationally.[18] For the normal range assessment, a *t*-test was carried out to determine whether a significant difference in the mean TRBC1 expression on CD4+ and CD8+ T-cell compartments was evident. A P value <.05 was considered statistically significant.

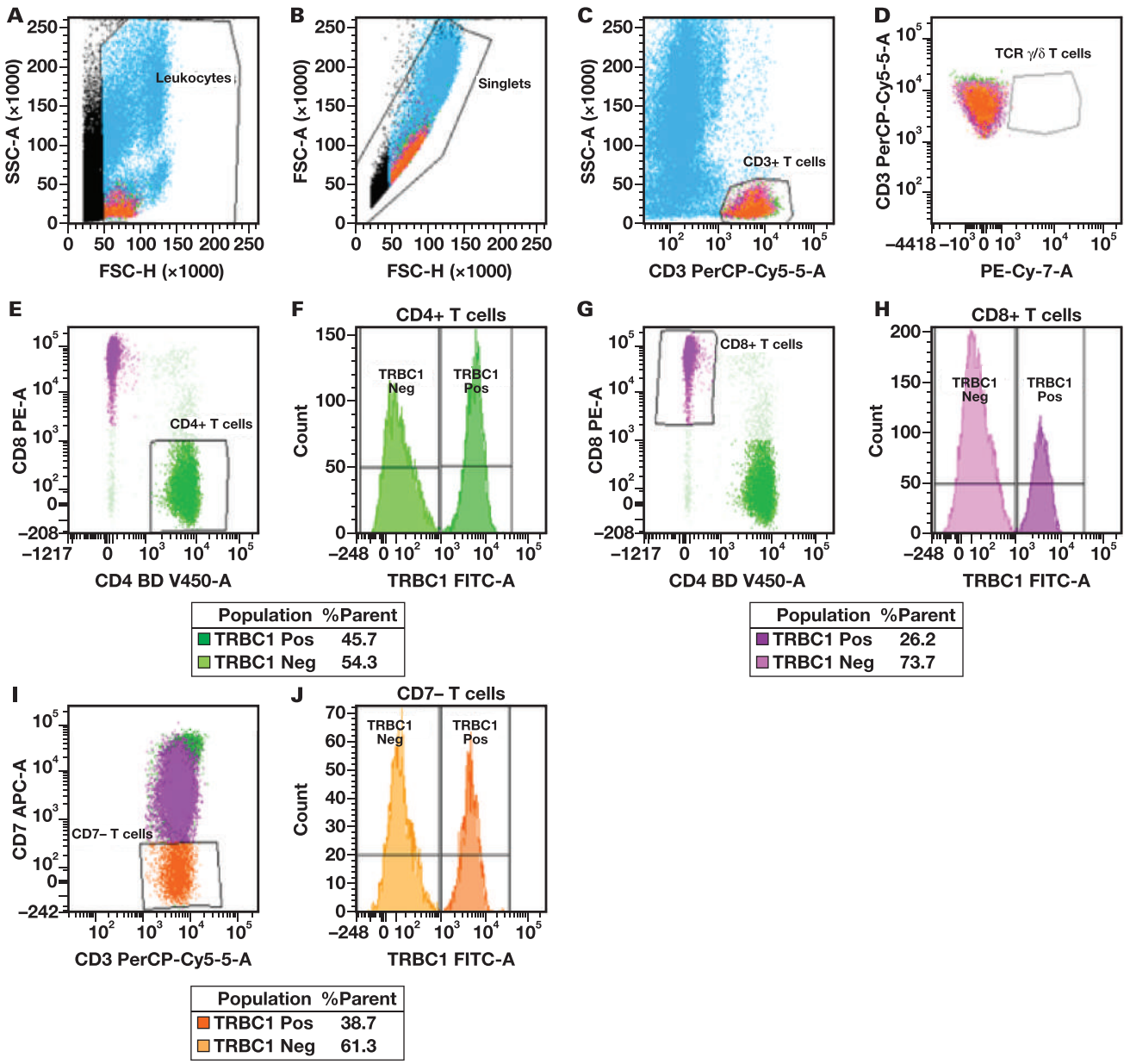
Results

Establishing a Gating Strategy for TRBC1 Expression

The gating strategy was created using specimens from patients with T-cell lymphoma, normal control donors, and patients with a reactive lymphocytosis. **FIGURE 1A** displays all events acquired with side scatter (SSC) vs forward scatter (FSC) plotted. A gate labeled "leukocytes" was drawn to exclude debris. All events were displayed on an FSC-area vs

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

FIGURE 1. Gating strategy for T-cell receptor β constant region 1 (TRBC1) using a specimen with a reactive lymphocytosis as an example. Plot A displays all events acquired with side scatter (SSC) vs forward scatter (FSC) plotted. A gate labeled "leukocytes" is drawn to exclude debris. All events are displayed on an FSC-area vs FSC-height dot plot with a gate drawn to include only singlets (B). Boolean logic is applied, both gates are joined, and captured events are displayed in plot C. A gate is drawn around all CD3+ T cells. In plot D, this CD3+ T-cell population is displayed and any T-cell receptor (TCR) δγ T-cells present are excluded. The CD4 and CD8 antigen expression profile of the αβ T-cell population is examined in plot E. A gate is drawn around the CD4+ T-cell population (green) and the expression of TRBC1 on this population is examined on plot F, a logical-scale histogram. TRBC1 is expressed in a 2:1 ratio of TRBC1-negative to TRBC1-positive expression. This process is repeated for the CD8+ T-cell population (purple) in plots G and H. Plot I shows a CD3+ T-cell population using a CD7-APC vs CD3-PerCp-Cy5.5 dot plot. A gate is drawn around the population of interest; a CD3+ T-cell population with reduced CD7 expression (orange) is shown. The expression profile of this population is examined in a histogram (J). Two distinct peaks are visible (orange) representing polytypic TRBC1 expression. FITC, fluorescein isothiocyanate.



FSC-height dot plot with a gate drawn to include only singlets. Boolean logic was applied, both gates were joined, and captured events were displayed on an SSC-vs-CD3 PerCp-Cy5.5 dot plot (**FIGURES 1B** and **1C**). All CD3+ T cells were displayed on a CD3 PerCp-Cy5.5-vs-TCR γδ Pe-Cy7 dot plot (**FIGURE 1D**). A gate was drawn around the TCR γδ T cells present. Boolean logic was used to exclude these cells, and the CD4 and CD8 expression profile of this resulting T-cell population was examined (**FIGURES 1E** and **1G**). To assess the TRBC1 expression level on the CD4+ T-cell compartment, a gate was drawn around this population. These cells were displayed on a logical-scale histogram (**FIGURE 1F**).

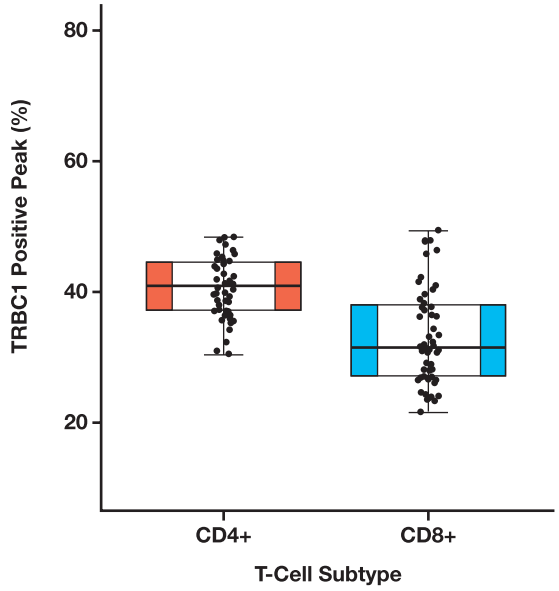
Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

Interval gates were used to quantify the percentage of TRBC1-positive and TRBC1-negative populations. Furthermore, to assess the TRBC1 expression on the CD8+ T-cell population, a gate was drawn around the CD8+ population, and these cells were displayed in a logical-scale histogram (**FIGURE 1H**). Interval gates were used to quantify the percentage of TRBC1-positive and TRBC1-negative populations. If the reduced expression of CD2 or CD5 or CD7 was identified on a T-cell subpopulation, an additional plot was used and a gate was drawn around this T-cell subpopulation. The TRBC1 expression of this aberrant population was determined using interval gates (**FIGURES 1I** and **1J**).

Determining the Normal Range of TRBC1 Expression

The normal range for TRBC1 was established using 46 normal peripheral blood donor specimens and 8 specimens with reactive lymphoproliferations. The TRBC1 expression on CD4+ and CD8+ T-cell populations were assessed separately **FIGURE 2**. All 46 normal peripheral blood specimens displayed a polytypic pattern of TRBC1 expression with a distinct negative and positive population of cells present. The distribution of the TRBC1 expression was assessed for normality by using Shapiro-Wilk testing and Q-Q plot analysis and confirmed to follow a Gaussian distribution, P = .349 (CD4+ T cells) and P = .017 (CD8+ T cells). The CD8+ T-cell compartment had statistically significant lower mean percentage values of TRBC1 expression compared to the CD4+ T-cell compartment (CD4+ T cells mean = 41% [range, 30%–48%]; CD8+ T cells mean = 33% [range, 22%–49%]; $P = 1.9 \times 10^{-8}$). The CD8+ T cell compartment also had a broader standard deviation of expression (7.3%) compared to the CD4+ T-cell compartment (4.5%). The 95% confidence interval (CI) for TRBC1 expression in CD4+ T-cell compartment was calculated as 39% to 42%, and for the CD8+ T-cell compartment as 31% to 35%. The 99.7% CI for TRBC1 expression in the CD4+ T-cell compartment was calculated as 38% to 42%, and for the CD8+ T-cell compartment as 30% to 36%.

FIGURE 2. Boxplot showing T-cell receptor β constant region 1 (TRBC1) expression on CD4+ and CD8+ T-cell populations from 43 normal control donor specimens and 8 specimens from patients with reactive lymphocytosis (P < .001). The crossbar depicts median percentages for each subset. Mean CD4+ = 41% (range, 30%–48%); mean CD8+ = 33% (range, 22%–49%).



Determining Cutoff Values for Monotypic vs Polytypic TRBC1 Expression

Using both the normal range data (including reactive lymphocytyosis specimens) and the TRBC1 monotypic patient results, an optimal cutpoint for establishing monotypia was established. For both the CD4+ and CD8+ T-cell populations, the cutpoint that yielded the best discrimination between polytypic and monoytpic TRBC1 was selected.

Using this method, a TRBC1 negative cutoff of 82% was calculated to establish a TRBC1 monotypic (negative) population in the CD4+ T-cell compartment and a TRBC1 negative cutoff of 88% was calculated for the CD8+ T-cell compartment. The area under the curve (AUC) was 1 for both. A TRBC1 positive cutoff of 68% was calculated to establish a TRBC1 monotypic (positive) population in the CD4+ T-cell compartment, and a cutoff of 72% was calculated for the CD8+ T-cell compartment. The AUC was 1 for both. In **FIGURES 3A–D**, all possible cutpoints for TRBC1 on CD4+ and CD8+ T-cell compartments are graphically compared. If T-cells regardless of subtype were taken as a single group, then a TRBC1 negative cutoff of 87% was calculated (**FIGURE 3E**) and a TRBC1 positive cutoff of 69% was calculated (**FIGURE 3F**). Finally, if results were taken as a single cohort, regardless of T-cell subtype or TRBC1 negative or positive expression, then a TRBC1 cutoff of 83% was calculated to establish monotypia (**FIGURE 3G**).

TRBC1 Expression in 43 Patient Specimens

TRBC1 Expression Results

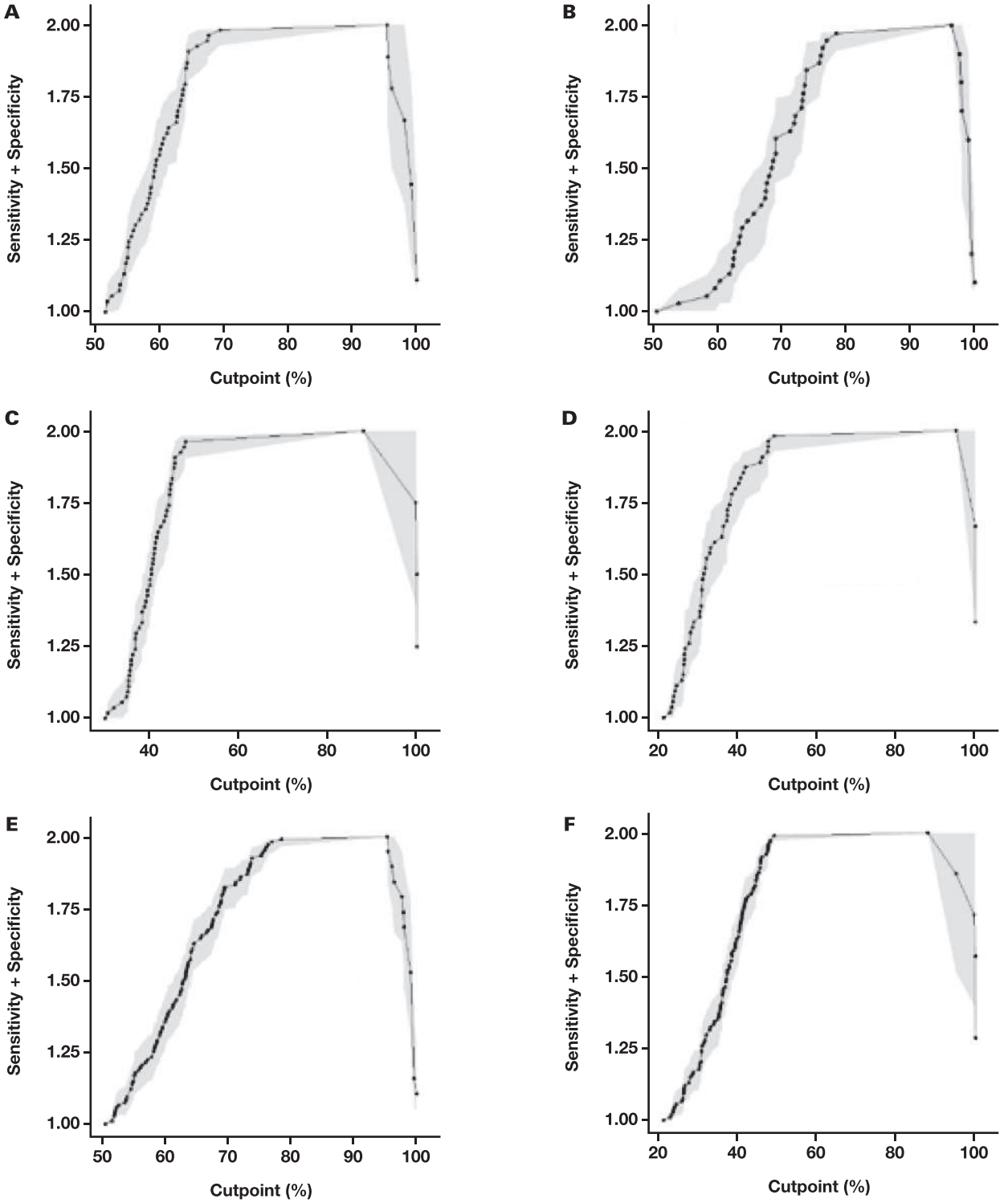
We evaluated 43 specimens for TRBC1 expression by gating specifically on the suspected abnormal T-cell population of interest using the reference value calculated from their respective normal control group. Of the 43 specimens, 31 were monotypic and 12 were polytypic for TRBC1 expression. Polytypic TRBC1 specimens showed discrete TRBC1-positive and -negative peaks. **FIGURE 1** details polytypic TRBC1 expression in a patient with a reactive lymphocytosis.

Of the 31 monotypic specimens, 21 were monotypic negative, revealing an absence of TRBC1 expression, inferring TRBC2-restricted usage by this T-cell population. **FIGURE 4** details the case of a patient with a peripheral T-cell lymphoma with monotypic negative TRBC1 expression. Ten specimens were monotypic positive, showing a bright homogeneous expression of TRBC1, inferring restricted usage of TRBC1. **FIGURE 5** details the case of a patient with T-cell large granular lymphocytic leukemia showing monotypic positive TRBC1 expression. For the 9 patients displaying a CD4+ TRBC1 monotypic negative profile, the mean percentage of TRBC1-negative expression was 97.7% (range, 96.4%–100%; standard deviation [SD], 1.7). For the 10 patients displaying a CD8+ TRBC1 monotypic negative profile, the mean percentage of the TRBC1-negative population was 95.6% (range, 97.6%–100%; SD, 1.1). For the 3 patients displaying a CD8+ TRBC1 monotypic positive profile, the mean percentage of the TRBC1-positive population was 98.3% (range, 95%–100%; SD, 2.9). For the 4 patients displaying a CD4+ TRBC1 monotypic positive profile, the mean percentage of the TRBC1-positive population was 96.9% (range, 88%–100%; SD, 8.4). Three patients showed a CD4+/CD8+ monotypic positive profile, with a mean TRBC1-positive expression of 97.4% (range, 97%–98.3%; SD,

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

2022;53;417–425 | https://doi.org/10.1093/labmed/lmac014

FIGURE 3. **Optimal cutpoint selection. The selection of the optimal cutpoint is possible by plotting all possible cutpoints with the corresponding sensitivity plus specificity value. The grey shadows represent the 95% confidence interval at each cutpoint. Plots A–D examine T-cell receptor β constant region 1 (TRBC1)-negative (A [82%] and B [88%]) or TRBC1-positive (C [68%] and D [72%]) expression on CD4+ (A and C) or CD8+ (B and D) T cells. Plots E (negative; 87%) and F (positive; 69%) examine TRBC1 expression on T cells regardless of subtype, and plot G (83%) refers to TRBC1 expression on all αβ T cells regardless of direction (ie, monotypic negative and positive) or subtype.**



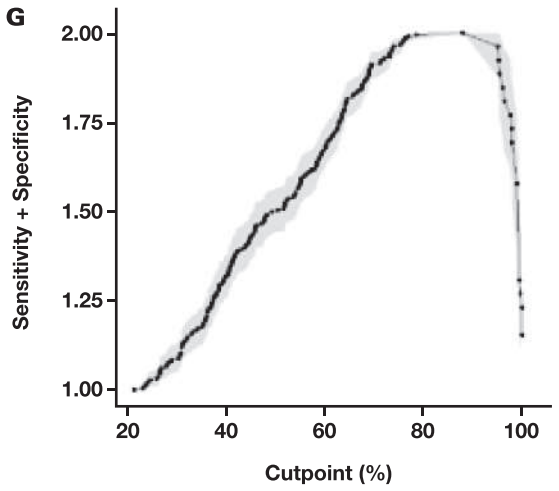
0.7), and 2 patients displayed a CD4+/CD8+ monotypic negative profile, with a mean TRBC1-negative expression of 94.4% (range, 92%–96.8%; SD, 3.4).

Flow Cytometric Characteristics of the Patient Cohort

Of the 31 patients with a TRBC1 monotypic expression pattern, CD8+ T-cell proliferations accounted for 42% of patients (n = 13), CD4+ T-cell

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

FIGURE 3. (cont)



proliferations also accounted for 42% (n = 13) of patients, and an expanded CD4+/CD8+ coexpressing T-cell population accounted for 16% of patients (n = 5). Eleven patients showed a reduced expression of a single T-cell antigen, 13 patients showed a reduced expression of more than 1 T-cell antigen, and 7 patients showed an abnormal CD4/CD8 ratio or expanded CD4+/CD8+ coexpressing T-cell population as the sole abnormality.

Of the 12 patient specimens displaying polytypic TRBC1 expression, 6 displayed an expanded CD8+ T-cell population, 4 of which also had a reduction in a single T-cell antigen; 1 patient had a reduction in more than 1 T-cell antigen; and 1 patient had a reversed CD4/CD8 ratio. Two patients had an expanded CD4+ T-cell population with no reduction in T-cell antigen expression, 2 patients had an expanded CD4+/CD8+ coexpressing T-cell population with 1 of these patients also displaying reduced CD7 expression, and 2 patients had a T-cell lymphocytosis with no additional aberrancy detected.

Direct Comparison of TRBC1 Expression with Molecular Clonality Testing in 43 Patient Specimens

Of the 31 specimens evaluated as monotypic for TRBC1 expression by flow cytometry, 30 were classified as monoclonal according to the molecular TCR gene rearrangement assay and 1 was interpreted as oligoclonal according to molecular analysis. Thus, a concordance rate of 97% between TRBC1 monotypic expression and analysis according to PCR was calculated. The patient who did not correlate had lymphocytosis with a minor population of CD4/CD8 coexpressing T cells, which may have accounted for the oligoclonal PCR result. A subsequent diagnosis of PTCL was recorded for this patient.

Of the 12 specimens evaluated as polytypic for TRBC1 expression, 8 were identified as polyclonal according to molecular analysis. One specimen was interpreted as inconclusive because of the detection of 2 minor TCR δ peaks on GeneScan analysis. These peaks likely represented pseudo-clones. Polyclonal TCR β PCR results were detected. Morphology and clinical evidence favored a reactive picture.

One further specimen that was polytypic for TRBC1 expression was classified as monoclonal according to molecular analysis. On medical record review, this patient had a complex medical history, including multiple viral mediated infectious diseases, and was considered clinically negative for the presence of a T-cell lymphoma; treatment was not initiated.

Two specimens were reported as oligoclonal according to molecular analysis. One specimen had a CD4+/CD8+ coexpressing population of T cells. Detailed clinical information was not available for this patient. Another specimen showed an expanded population of CD4+/CD8+ coexpressing T-cells with reduced expression of CD7. This patient had previously undergone a splenectomy because of trauma and had no clinical evidence of a T-cell lymphoma.

One specimen displayed a clonal TCR β gene rearrangement only and was categorized according to PCR as displaying insufficient evidence for T-cell clonality. Although this patient was subsequently diagnosed with monoclonal B-cell lymphocytosis, the clonal TCR rearrangement detected was unlikely to be an example of a cross-lineage rearrangement, and other technical considerations such as specimen quality required consideration.[8]

Discussion

We have found the anti-TCRB1 antibody to be of high diagnostic value for the assessment of clonality in patients with a suspected T-cell neoplasm when used in conjunction with our existing diagnostic panel. The direct comparison of 43 patient specimens for TRBC1 expression using molecular TCR analysis indicated that no specimen identified with a monotypic (restricted) expression of TRBC1 was reported as polyclonal according to PCR.

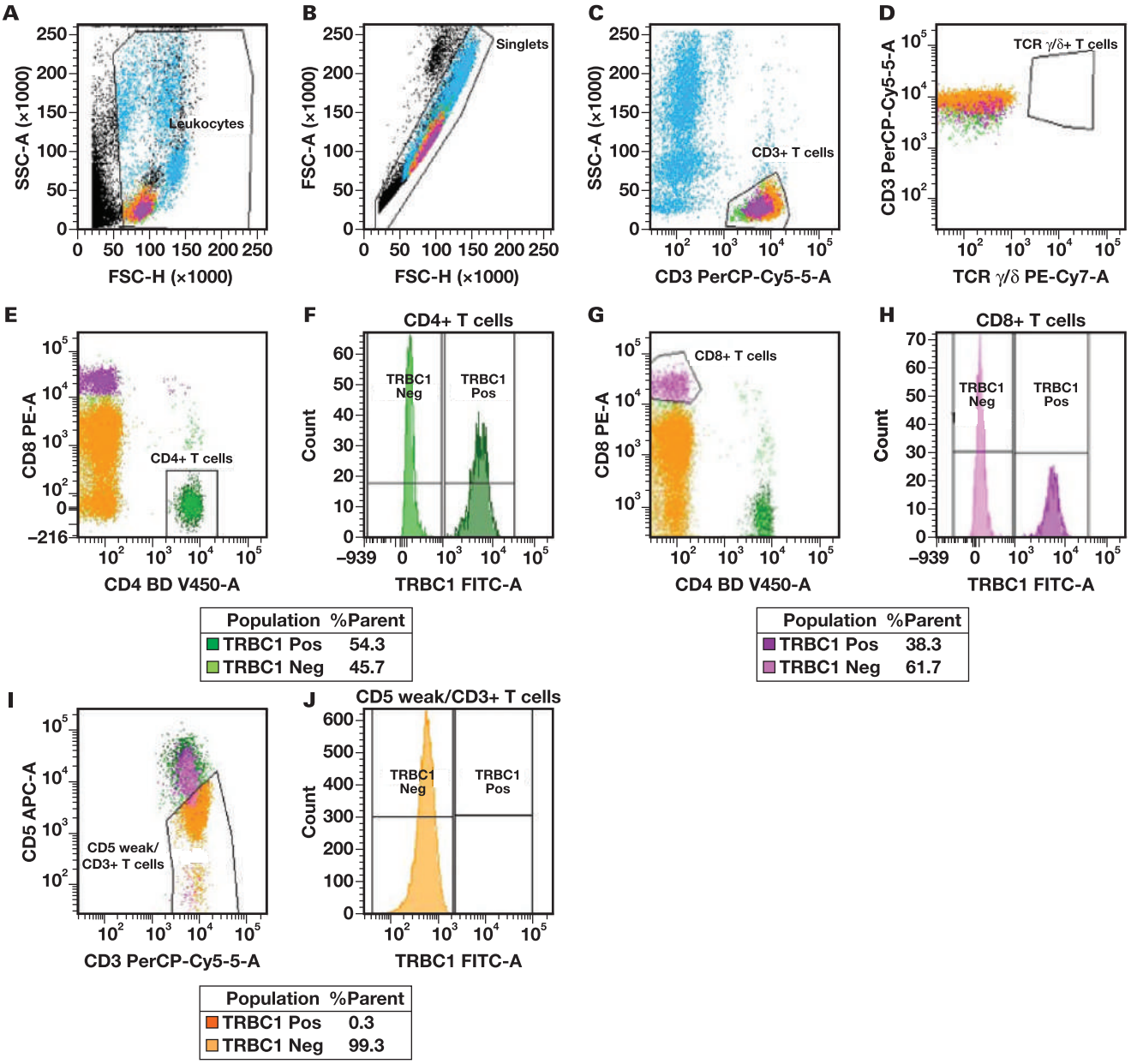
The calculated normal reference values for TRBC1 expression within the T-cell subsets discussed here are broadly in line with published data. A study by Novikov et al[15] reported the ratio of TRBC1-positive to TRBC1-negative T-cell expression to be in the 1:1 to 1:2 range, with 44% of the CD4+ T-cell compartment and 39% of the CD8+ T-cell compartment expressing TRBC1. Our study established the normal biological expression of TRBC1 in peripheral blood specimens. Future work on establishing the normal biological expression in bone marrow aspirate specimens would be helpful to determine whether a significant difference exists between the 2 compartments. Of the 8 bone marrow aspirate specimens included in our study, all displayed monotypic TRBC1 expression. The TRBC1 expression ranged from 98% to 100% for monotypic-negative specimens (TRBC2 restricted) and from 88% to 100% for TRBC1-positive specimens, with a disease burden ranging from 10% of lymphocytes to 99% of lymphocytes.

A consideration when testing bone marrow aspirate specimens is the effect of hemodilution on the detection of aberrant T-cell populations, especially if the aberrant population is not present in the peripheral blood. Emphasizing the importance of using the first bone marrow aspirate draw for flow cytometric analysis could help minimize this effect, and correlation with the morphological assessment of the disease burden may also be beneficial.

The importance of implementing a gating strategy directed at the abnormal T-cell population cannot be overstated. This antibody is designed to detect clonal abnormalities in the constant region of the β chain of the TCR, so it is not applicable for the assessment of normal γδ T cells or γδ T-cell neoplasms. Moreover, care to exclude γδ T cells in the assessment of TRBC1 expression must be taken to avoid misinterpretation because γδ T cells will always appear monotypic-negative and could potentially skew the ratio of TRBC1-positive to TRBC1-negative expression.

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

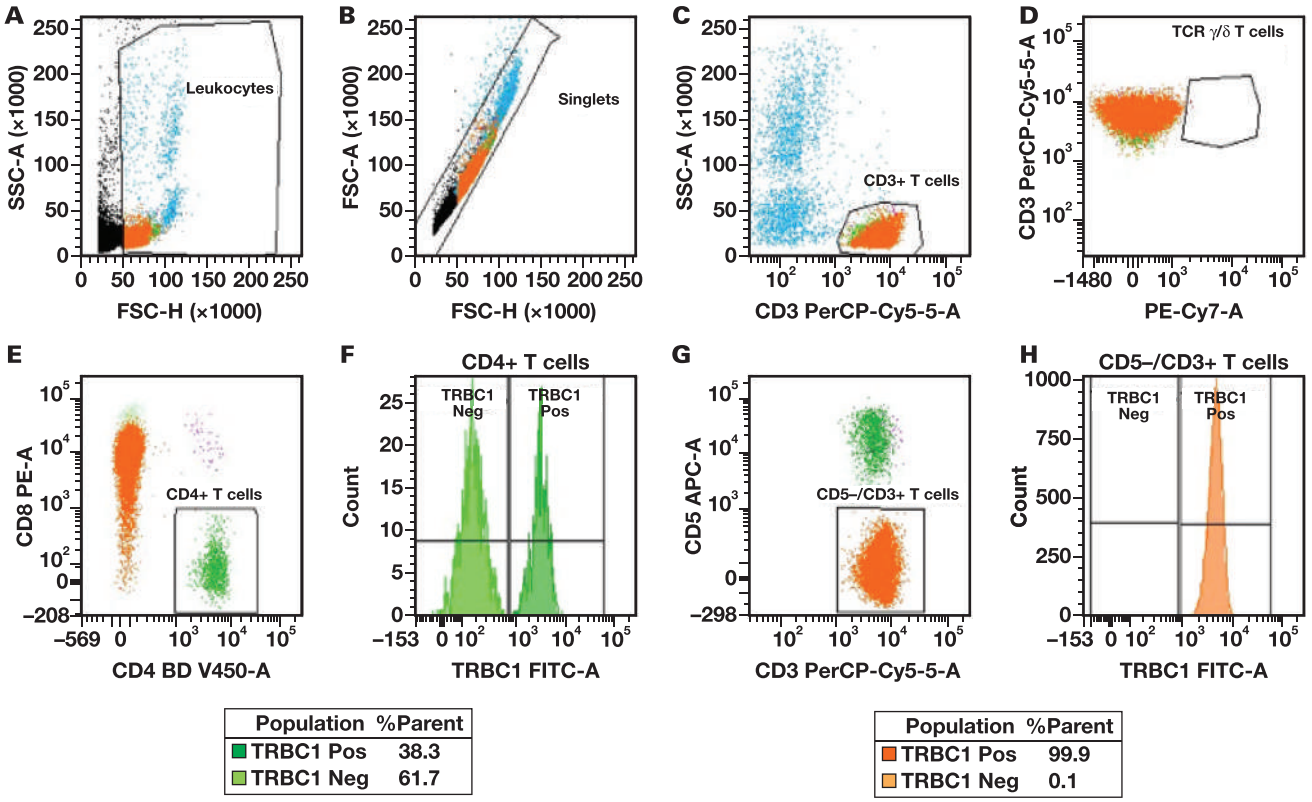
FIGURE 4. Representative flow cytometric plots showing monotypic-negative T-cell receptor β constant region 1 (TRBC1) expression of a patient displaying a suspicious CD8 weak positive population with reduced CD7 expression. Plots A–H are gated as detailed in FIGURE 1. Plots F and H display a background normal polytypic T-cell population. Plot I displays a CD3+ T-cell population using a CD5-APC-vs-CD3-PerCp-Cy5.5 dot plot. A gate is drawn around the population of interest; a CD3+ T-cell population with reduced CD5 expression is shown (orange). The expression profile of this population is examined in plot J. A single TRBC1 peak is visible in the negative region of the histogram (orange). The statistics box displays a TRBC1 expression of 0.4%, interpreted as a monotypic-negative TRBC1 result, inferring restricted use of TRBC2 by this T-cell population. This patient was subsequently diagnosed with a peripheral T-cell lymphoma. FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter.



Because we found that the average ratio of TRBC1-positive expression to TRBC1-negative expression was 1:2, the argument for differing cutoff points for determining TRBC1 monotypic-positive expression vs monotypic-negative TRBC1 expression on T-cell populations holds much weight. This study addresses the need for statistical approaches to the cutoff values used to define a monotypic T-cell population. Determining a numerical cutoff value for monotypia undoubtedly is challenging. Balancing the risk of a false-negative result against a false-positive result is a difficult task. Initial studies using this antibody proposed cutoff values of >95% or <3% to establish a monoclonal TRBC expression pattern.[16] More recent publications have suggested that this criterion may be too stringent and that a wider range of >85% or <15% may be more appropriate.[19] Other publications have expanded on this finding and reported different values for discriminating polytypic and monotypic T-cell expression based on subtype using the mean percentage TRBC1 value ± 3 SD to establish even broader cutoff values.[20]

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

FIGURE 5. Representative flow cytometric plots showing monotypic-positive T-cell receptor β constant region 1 (TRBC1) expression of a patient with a CD8+ population with reduced CD5 expression. Plots A–F are gated as detailed in FIGURE 1. Plot F displays a background normal CD4+ T-cell population. Plot G displays a CD3+ T-cell population using a CD5-APC-vs-CD3-PerCp-Cy5.5 dot plot. A gate is drawn around the population of interest; a CD3+ T-cell population with reduced CD5 expression is shown (orange). The expression profile of this population is examined in plot H. A single TRBC1 peak is visible in the positive region of the histogram (orange). The statistics box displays a TRBC1 expression of 99.9%, interpreted as a monotypic-positive TRBC1 result, inferring restricted use of TRBC1 by this T-cell population. This patient was subsequently diagnosed with T-cell large granular lymphocytic leukemia. FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter; TRBC1, T-cell receptor β constant region 1.



In our study we have addressed the need to use normal and reactive T cells to statistically calculate cutpoint values and propose statistically derived numerical cutoff values. We also raise the question of how deep into the T-cell repertoire we should mine when establishing cutoff values and if different cutoff values for monotypic-negative vs monotypic-positive should be used. As further studies emerge in this area, it is anticipated that these will supplement and verify published findings.

Many of the patients in this cohort had a significant proportion of abnormal T cells. However, when patients present with a minor and less conspicuous abnormal population, especially when contained within a background of normal polytypic T cells, it is vital to isolate this abnormal T-cell population for TRBC1 expression analysis to avoid a false-negative result. To support this endeavor, we used the APC channel to "drop in" the most informative antibody—CD2, CD5, or CD7—based on our knowledge of a patient's specific phenotype acquired from our broader diagnostic panel. We found that 30/43 of our patient cohort (70%) had a reduced expression of 1 or more of these 3 antigens, making this a straightforward and successful approach in these patients. A limitation of this single-tube design is the inability to simultaneously characterize minor T-cell subsets that may be present and thus, in patients with complicated cases, it may not produce the best separation. A more comprehensive panel should be considered in these patients.

A concordance rate of 97% (31/32 patients) between a clonal TCR gene rearrangement PCR result and a monotypic TRBC1 expression was calculated. One patient failed to have a clinical diagnosis of a T-cell malignancy established, highlighting the importance of interpreting PCR results within the entire clinical context. A study by Novikov et al[15] suggested that the inclusion of the TRBC1 result may resolve oligoclonal specimens with unclear clinical significance. Our experience of such is limited but seems to support this theory. One patient with PTCL showing oligoclonal PCR results was included in our cohort. This patient did exhibit a monoclonal TRBC1 result, whereas a second patient in our cohort showed oligoclonal PCR results with polytypic TRBC1 expression. A definitive diagnosis was unavailable for this patient, who also had a CD4/CD8 double-positive T-cell population of 17%. The literature suggests that small clones (20% or less in size) of CD4/CD8 double-positive T-cells should be interpreted with caution because they may be encountered in the absence of a T-cell malignancy.[19] A further patient displayed oligoclonal T cells according to PCR but polytypic TRBC1 expression. This patient had undergone a previous splenectomy because of trauma; it is thus possible that this patient may have had an altered immunological profile resulting from the splenectomy.

The assessment of the 2 constant regions of the TCR β gene can be considered analogous to κ and λ light chain expression detected on B cells. In B cells, a normal expression ratio sees κ predominating over λ, akin to TRBC2 expression predominating over TRBC1 expression in a normal T-cell compartment. Interestingly, among the cohort of patients who displayed a monotypic TRBC1 profile (n = 31), the majority were monotypic-negative (n = 21) compared to those who were monotypic-positive (n = 10), inferring that TRBC2 monotypia predominates over TRBC1 monotypia.

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

This finding is in contrast to other studies. Novikov et al[15] found that the majority of patients with neoplasm showed monotypic-positive TRBC1 expression, and similarly, Berg et al[14] found monotypic-positive TRBC1 expression in half of their patients with T-cell lymphoma.

Other aspects to contemplate carefully include the interpretation of TRBC1 staining in patients with minor CD8+ immunophenotypically distinct T-cell subsets in conjunction with clonal/oligoclonal molecular TCR gene rearrangement results. This phenomenon is recognized in viral infections, immunodeficiency, and autoimmune conditions and is frequently of undetermined clinical significance.[16] The issue of detecting T-cell clones of uncertain significance is complex, and more research is required to fully understand the relationship between very minor populations of dominant "immunoclones" and a malignant process.

Occasionally, T-cell lymphomas can be surface CD3–negative. Because of the relationship between the TCR and the CD3 molecule, a surface CD3–negative T-cell lymphoma may exhibit a clonal TRBC1-negative expression pattern resulting from the absence of the TCR/CD3 complex as opposed to TRBC2-restricted expression. However, this immunophenotypic feature itself is virtually always indicative of a clonal T-cell lymphoproliferative disorder. The situation for CD3 dim T-cell lymphomas is a little less clear, with no proven relationship between dim CD3 expression and reduced TRBC1 fluorescence intensities to date. Currently no commercially available TRBC2 antibody suitable for surface staining by flow cytometry exists, but development is underway.[21] When accessible, it will provide a complementary target to TRBC1 and a comprehensive assessment of the TCR β constant region by flow cytometry and may help resolve this issue.

Conclusion

This antibody improves the identification of clonal T cells by flow cytometry and is amenable to integration into a busy flow cytometry laboratory. The reflex nature in which we introduced TRBC1 analysis to the testing algorithm of a suspected T-cell abnormality ensures an economical and targeted testing strategy. By recognizing the pitfalls and remaining mindful of the importance of gating strategies in the execution of this assay, we find that the TRBC1 antibody represents an excellent addition to the investigation of T-lymphoproliferative disorders by flow cytometry.

Supplementary Data

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

Acknowledgments

We are grateful to Mr. Richard McCafferty and to the Clinical Cytometry and Cancer Molecular Diagnostics Laboratory staff of St James Hospital.

REFERENCES

1. Vose J, Armitage J, Weisenburger D. International T-Cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. *Am J Clin Oncol.* 2008;26(25):4124–4130.
2. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood.* 2016;127(20):2375–2390.
3. Jevremovic D, Olteanu H. Flow cytometry applications in the diagnosis of T/NK-cell lymphoproliferative disorders *Cytometry B Clin Cytom.* 2019;96(2):99–115.
4. Gorczyca W, Weisberger J, Liu Z, et al. An approach to diagnosis of T-cell lymphoproliferative disorders by flow cytometry. *Cytometry.* 2002;50(3):177–190.
5. Statuto T, D'Auria F, Del Vecchio L, et al. Atypical mature T-cell neoplasms: the relevance of the role of flow cytometry. *Onco Targets Ther.* 2020;13:7605–7614.
6. van den Beemd R, Boor PP, van Lochem EG, et al. Flow cytometric analysis of the Vbeta repertoire in healthy controls. *Cytometry.* 2000;40(4):336–345.
7. Langerak AW, van Den Beemd R, Wolvers-Tettero IL, et al. Molecular and flow cytometric analysis of the Vbeta repertoire for clonality assessment in mature TCR alphabeta T-cell proliferations. *Blood.* 2001;98(1):165–173.
8. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia.* 2003;17(12):2257–2317.
9. Langerak AW, Groenen PJ, Brüggemann M, et al. EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. *Leukemia.* 2012;26(10):2159–2171.
10. Mahe E, Pugh T, Kamel-Reid S. T cell clonality assessment: past, present and future. *Am J Clin Pathol.* 2018;71:195–200.
11. Maciocia PM, Wawrzyniecka PA, Philip B, et al. Targeting the T cell receptor β-chain constant region for immunotherapy of T cell malignancies. *Nat Med.* 2017;23(12):1416–1423.
12. Blom B, Verschuren MC, Heemskerk MH, et al. TCR gene rearrangements and expression of the pre-T cell receptor complex during human T-cell differentiation. *Blood.* 1999;93(9):3033–3043.
13. Murphy K, Weaver C. *Janeway's Immunobiology*. 9th ed. New York, NY: Garland Science; 2017:187–191.
14. Berg H, Otteson GE, Corley H, et al. Flow cytometric evaluation of TRBC1 expression in tissue specimens and body fluids is a novel and specific method for assessment of T-cell clonality and diagnosis of T-cell neoplasms. *Cytometry B Clin Cytom.* 2020;100(3):361–369.
15. Novikov ND, Griffin GK, Dudley G, et al. Utility of a simple and robust flow cytometry assay for rapid clonality testing in mature peripheral T-cell lymphomas. *Am J Clin Pathol.* 2019;151(5):494–503.
16. Shi M, Jevremovic, D, Otteson GE, Timm MM, Olteanu H, Horna P. Single antibody detection of T-cell receptor αβ clonality by flow cytometry rapidly identifies mature T-cell neoplasms and monotypic small CD8-positive subsets of uncertain significance. *Cytometry B Clin Cytom.* 2020;98(1):99–107.
17. R: a language and environment for statistical computing [computer program]. Vienna, Austria: R Foundation for Statistical Computing; 2021.
18. R-project.org. cutpointr: determine and evaluate optimal cutpoints in binary classification tasks. https://cran.R-project.org/web/packages/cutpointr/index.html. Accessed February 7, 2022.
19. Shi M, Olteanu H, Jevremovic D, He R, et al. T-cell clones of uncertain significance are highly prevalent and show close resemblance to T-cell large granular lymphocytic leukemia. Implications for laboratory diagnostics. *Mod Pathol.* 2020;33:2046–2057.
20. Muñoz-García N, Lima M, Villamor N. Anti-TRBC1 antibody-based flow cytometric detection of T-cell clonality: standardization of sample preparation and diagnostic implementation. *Cancers (Basel).* 2021;13:4379.
21. Onuoha S, Ferrari M, Bulek A, et al. Structure guided engineering of highly specific chimeric antigen receptors for the treatment of T cell lymphomas. *Blood.* 2018;132(supplement 1):1661.

Downloaded from https://academic.oup.com/labmed/article/53/4/417/6548266 by guest on 28 February 2025

Value of Thyroid Peroxidase Antibodies in Neuroimmune Diseases: Analysis of Interference During Treatment with Intravenous Immunoglobulins

María Jiménez-Legido, MD,[1,] Verónica Cantarín-Extremera, MD, PhD,[1,2,*] María Eugenia López-Guio, MD,[3] Rosa María González-Cervera, MD, PhD,[3] Silvia Martín-Prado, PharmD,[4] Elena Sebastián-Pérez, MD, PhD,[5] Luis González-Gutiérrez-Solana, MD[1,2]*

[1]Department of Neuropediatrics, Hospital Infantil Universitario Niño Jesús, Madrid, Spain, [2]Clinical Group, Networked Biomedical Research Centre for Rare Diseases, Carlos III Health Institute, Madrid, Spain, [3]Department of Clinical Biochemistry, Hospital Infantil Universitario Niño Jesús, Madrid, Spain, [4]Hospital Pharmacy Department, Hospital Infantil Universitario Niño Jesús, Madrid, Spain, [5]Department of Pediatric Hematology-Oncology, Hospital Infantil Universitario Niño Jesús, Madrid, Spain; *To whom correspondence should be addressed. mjimenezl@salud.madrid.org.

Keywords: thyroid peroxidase antibodies, autoimmune, immunoassay, nonspecific human intravenous immunoglobulin, interference, interferogram

Abbreviations: TPO-Abs, thyroid peroxidase antibodies; IVIG, intravenous immunoglobulin; SREAT, steroid-responsive encephalopathy associated with autoimmune thyroiditis; TSH, thyroid-stimulating hormone; FT4, free thyroxine; T3, tri-iodothyronine; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; ANA, antinuclear antibody.

Laboratory Medicine 2022;53:426–432; https://doi.org/10.1093/labmed/lmac013

ABSTRACT

Objective: The absence of specific markers can make the diagnosis of neuroimmune disorders difficult, making other biomarkers such as thyroid peroxidase antibodies (TPO-Abs) more relevant. Laboratory tests are susceptible to interference, especially those tests performed using immunoassay techniques. The effect of treatment with human intravenous immunoglobulin (IVIG) on the results of TPO-Abs assays has not been previously characterized.

Materials and Methods: We analyzed TPO-Abs levels in 170 children monitored in the neuroimmune disease department of a tertiary hospital. We analyzed the characteristics of patients with increased TPO-Abs values and compared their progress with and without treatment.

Results: We found that 97% of patients with elevated TPO-Abs had received IVIG. After withdrawal from IVIG, a mean TPO-Abs decrease of 62.5% at 1 month was observed. The IVIG drug preparation was found to contain 1176 U/mL of TPO-Abs. An interferogram confirmed interference.

Conclusion: It is advisable to measure levels of TPO-Abs before starting immunotherapy and remain vigilant regarding possible interference in the event of unsubstantiated elevations of this analyte.

Autoimmune neurological diseases are a highly significant group of diseases in pediatric neurology. Within the central nervous system, and despite the existence of the blood-brain barrier, there is increasing evidence of disorders produced by certain antibodies that react with neuronal or glial proteins,[1] causing a range of clinical conditions (eg, epilepsy, encephalopathy, movement disorders). For other inflammatory entities, knowledge of specific antibodies is less comprehensive, and other biomarkers are more relevant. Such is the case with antithyroid antibodies, which, although their precise value remains unknown, have been detected in well-defined autoimmune encephalitis (associated with antibodies against N-methyl-D-aspartate receptor (NMDAR), gamma aminobutyric acid B receptor, and gamma aminobutyric acid A receptor, among others) with a higher frequency than in the general population, thus suggesting that these patients tend to develop antineural immune responses.[2] Furthermore, it has been hypothesized that these antibodies play a pathogenic role in conditions such as Hashimoto's encephalopathy, commonly referred to as steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT); however, these conditions are controversial because of the possible concomitant existence of additional undetected specific antibodies, among other factors. Meanwhile, some patients with encephalitis[2] or other neuroimmune diseases have been found to have elevated thyroid peroxidase antibodies (TPO-Abs),[1-4] which may be indicative of a predisposition of patients to autoimmunity.

Increased knowledge and suspicion of neuroimmune conditions has led to an increase in multiple complementary tests and therapeutic trials with immunomodulators such as nonspecific human intravenous immunoglobulin (IVIG). However, there may be certain interactions between these tests and treatments, which require careful consideration. Preanalytical aspects can affect the diagnostic value of these techniques;

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/426/6551305 by guest on 28 February 2025

eg, laboratory tests can be altered by multiple misleading factors, particularly in procedures that involve immunoassay techniques.[5-7] Interference, therefore, is understood as a systematic error produced by a component in a specimen other than the analyte to be determined. Interferences can give rise to both false-positive and false-negative results and are very difficult to detect because they are unpredictable, highly variable, and sometimes even clinically feasible.[7] Interference by endogenous antibodies is well understood although still difficult to predict.[7] However, the same cannot be said for interference related to the exogenous administration of immunoglobulins because only isolated cases have been documented, mainly related to false-positive serological tests.[8,9] To date, no studies have examined the possible effect of IVIG therapy on the determination of antibodies such as TPO-Abs in commonly used immunoassay techniques.

We present 40 pediatric patients with various, mostly neurological clinical entities with a confirmed/suspected autoimmune/inflammatory basis who presented with high TPO-Abs values in the absence of thyroid disease. After initially interpreting these values as biomarkers of autoimmunity, and given the high number of patients found, we suspected IVIG-related interference in the laboratory test, which we evaluate in the present study.

Materials and Methods

After observing a striking increase in the number of patients with elevated TPO-Abs in our practice, we systematically reviewed the levels of these antibodies in a sample of 170 pediatric patients with autoimmune/inflammatory neurological conditions. All patients had been diagnosed and monitored (before or during the study) in the neuroimmune disease department of our institution, a tertiary hospital, over the 20-year period spanning 2000 to 2020. Initially, we noted the type of immunotherapy received by patients (focusing on whether management included IVIG or not) and, in those patients who were undergoing treatment with IVIG, we also recorded the findings of the TPO-Abs testing. Given the high proportion of positive results among patients treated with IVIG and the low frequency among those who did not receive this treatment, we considered that the drug may interfere with the results of the laboratory test used.

To test for possible interference between IVIG and TPO-Abs, we performed a detailed study of the characteristics of the group of patients with neuroimmune diseases and positive results on determination of TPO-Abs. This analysis also included other pediatric patients with immune or nonimmune diseases and positive TPO-Abs levels who had undergone treatment with IVIG over the previous 10 years (2010–2020) in the same hospital, with the exception of those with thyroid disease.

We analyzed these patients for possible changes in TPO-Abs values and the chronological relationship between these test results and the start and/or withdrawal of IVIG treatment (positivization and/or normalization). To detect possible trends, we also studied the evolution of this value over time since the administration and/or withdrawal of treatment with IVIG in those patients undergoing serial monitoring. Similarly, we examined changes in other laboratory parameters of interest (ie, thyroid-stimulating hormone [TSH], free thyroxine [FT4], tri-iodothyronine [T3], rheumatoid factor [RF], erythrocyte sedimentation rate [ESR], antinuclear antibodies [ANA], and IgG) and their possible relationship with TPO-Abs levels and with the administration of treatment. The analysis was retrospective in those patients who had completed treatment and prospective in those who continued to receive cycles of IVIG at the time of the study. In the latter group, repeat TPO-Abs determination was performed as late as possible since the most recent administration of IVIG. In addition, in patients with negative TPO-Abs results determined before starting immunotherapy and who were still undergoing treatment at the time of the study, a follow-up determination was conducted coinciding with an IVIG cycle to test for possible positivization.

Given the possible association between positive TPO-Abs findings and the administration of IVIG, the pharmacological and analytical basis of this interference was studied with the collaboration of health care staff specializing in laboratory testing and clinical pharmacy. Testing of TPO-Abs was performed using competitive immunoassay (B-R-A-H-M-S anti-TPOn KRYPTOR, Thermo Fisher). The nonspecific human immunoglobulin preparation administered was Flebogamma. As evidence of the suspected interference, an TPO-Abs determination was performed (using the same immunoassay technique employed in the specimens) on the same IVIG drug preparation administered to the patients in the study. We then used an interferogram, a valid dilution-based method used to ascertain the degree of analytical error in a serum matrix as a function of the concentration of the interferent.[5]

Statistical Analysis

The study population was analyzed to determine the degree of homogeneity in terms of demographic variables, medical history, and other clinical parameters. These values were described using the mean, median, and standard deviation for quantitative variables and the absolute frequency and relative frequency for qualitative variables. For quantitative variables, Student's *t*-tests were carried out if the assumption of normality was met, and nonparametric Mann-Whitney *U* tests were used if it was not. Qualitative variables were analyzed using homogeneity tests based on the χ^2 distribution when the expected values made it possible and using Fisher's exact test in the opposite case. Values of $P < .05$ were considered statistically significant. Statistical analyses were performed using the statistical package SPSS 22.0 (IBM).

Results

Consideration of Potential Interference Between IVIG and TPO-Abs Results

From a sample of 170 pediatric patients with autoimmune/autoinflammatory neurological entities, at least 1 TPO-Abs determination was available in 98 patients (57.64%), and a positive result was found in 31/98 patients (31.63%). All except 1 patient (30/31; 96.77%) had undergone treatment with IVIG. Regarding the immunomodulatory treatment received, cycles of IVIG were administered in 139 of the 170 patients (81.76%). In 76 of these patients (76/139; 54.67%), the presence of TPO-Abs had been tested (either before or after initiation of treatment) and the results were positive in 30/76 patients (39.47%). Of the 31 patients who had not received IVIG, 21 patients (67.74%) had at least 1 TPO-Abs determination; the result was positive in only 2 patients (9.52%). An analysis revealed a statistically significant association between treatment with IVIG and positive TPO-Abs findings ($P = .014$).

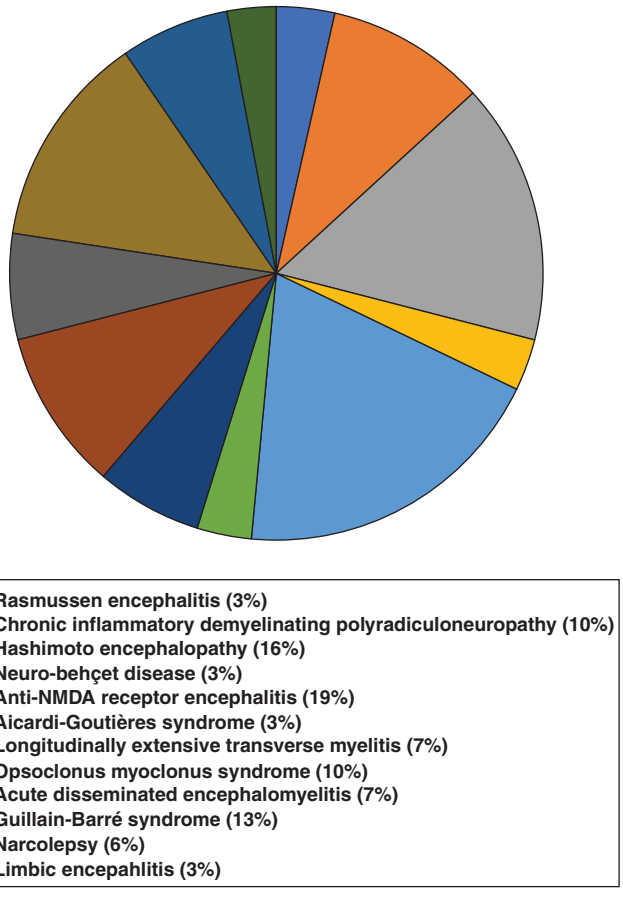
Downloaded from https://academic.oup.com/labmed/article/53/4/426/6551305 by guest on 28 February 2025

Study and Characterization of Patients with Positive TPO-Abs

Regarding the distribution of underlying diseases in the group with neuroimmune diseases, there was a certain predominance of encephalitic syndromes and peripheral neuropathies (**FIGURE 1**). In addition, after reviewing other pediatric patients being monitored by specialists in our institution who had positive TPO-Abs detected in the previous 10 years and who had received treatment with IVIG (regardless of the underlying entity), we included another 9 patients (total sample, n = 40). Of these, 2 patients presented with dermatomyositis and 1 with immune thrombopenia associated with systemic lupus erythematosus. In 1 patient, a nonneuroimmune etiology was documented (dystonia-parkinsonism syndrome associated with mutation in the *ATP1A3* gene), and in the remaining 5 patients the etiology of the clinical condition could not be determined with certainty; refractory epilepsy was observed in 3 patients, acute psychosis in 1 patient, and ataxia with transitory weakness in the other patient. The median age at the onset of the underlying disease was 6.7 years, ranging from 6 months to 18 years. There were no differences in sex found for this group because the sample comprised 20 girls (50% of the total).

Immunotherapy consisted of IVIG in 39 patients (97.5% of the total). We administered IVIG as monotherapy in 7 patients (18%) and combined it in 32 patients (82%) with some type of corticotherapy, which was sometimes associated with a third or even a fourth drug. Eleven patients (28.2%) received rituximab, 4 (10.2%) underwent cycles of plasmapheresis, 3 (7.7%) took azathioprine, 2 (5.1%) took methotrexate, and 2 (5.1%) took cyclophosphamide.

FIGURE 1. Frequency of baseline diseases NMDA, N-methyl-D-aspartate.



The only patient not treated with IVIG received only pulses of dexamethasone. She showed manifestations of acute encephalitis with marked psychotic symptoms and excellent response to steroids. The TPO-Abs value in this patient was 100 U/mL and was later negative, and no other specific antibodies were evidenced. Given the clinical manifestations and the response to treatment, Hashimoto's encephalitis was considered as a possible diagnosis. Likewise, of the 39 patients who received IVIG, 12 patients (30.76%) had a pretreatment TPO-Abs determination, which was positive in 2 patients. One was a female adolescent who also presented with symptoms of acute encephalitis with prominent psychotic manifestations. A very marked TPO-Abs elevation was found in this patient (2399 U/mL), raising suspicion of Hashimoto's encephalitis. She required treatment with neuroleptics and received immunotherapy with intravenous methylprednisolone pulses and IVIG; her TPO-Abs values decreased to 1402 U/mL during treatment, suggesting no apparent relationship between the treatment and antibody levels. Over the course of the disease, she further presented with several marked elevations of TPO-Abs values (up to 4526 U/ml) not associated with the reappearance of clinical symptoms or drug administration. The other patient developed anti-NMDAR encephalitis (positive antibodies) with a severe initial course and that was refractory to first-line treatment, requiring combined therapy consisting of plasmapheresis and rituximab. Before treatment, the patient's level of TPO-Abs was 203 U/mL, which dropped to 137 U/mL during treatment and 50 U/mL after finishing treatment. In the other 10 patients, the pretreatment determination of TPO-Abs was negative, with positivization observed after the treatment was begun.

On the other hand, of the 39 patients who received IVIG, 27 did not have a pretreatment TPO-Abs determination (a positive value was documented while they were already on IVIG treatment). However, in all those who had a follow-up test after discontinuation of treatment or between 1 administration and the next, a decrease in values was recorded.

From the total sample (n = 40), in 17 patients determinations of TPO-ABS levels were available beginning after treatment withdrawal or after the maximum time period had elapsed following the administration of the last dose (before the next cycle in those with ongoing therapy). Having excluded the 3 patients who already had positive TPO-Abs before starting treatment, and analyzing the 14 with elevated levels coinciding with the administration of IVIG, we observed a marked decrease in the mean value of TPO-Abs, from 419 U/L during treatment to 157 U/L in the follow-up analysis (decrease of 62.52%). In those who had a follow-up test at 1 month after treatment (9 patients), a 62.5% decrease in TPO-Abs was found. And in those who had a follow-up test between the third and fourth month, a reduction of 88.54% was found with respect to the initial value. The chart in **FIGURE 2** shows the evolution of TPO-Abs levels since the last dose of IVIG, including all values available for the overall sample of patients with elevated TPO-Abs (**FIGURE 2**). **FIGURE 3** shows the same evolution, although it is limited to patients whose TPO-Abs levels showed a relationship with the administration of IVIG (increasing after initiation of therapy and/or normalizing after discontinuation). In both cases, we observed a progressive decrease in TPO-Abs after administration of the last dose of IVIG, especially in this subset of patients (**FIGURE 3**).

Increased TPO-Abs values were associated with increased serum IgG, and a statistically significant positive correlation was documented (Pearson 0.868; $P = .01$). In 18 of 40 patients, a very slight increase in

Downloaded from https://academic.oup.com/labmed/article/53/4/426/6551305 by guest on 28 February 2025

2022;53;426–432 | https://doi.org/10.1093/labmed/lmac013

FIGURE 2. Value of thyroid peroxidase antibodies (TPO-Abs) over time after treatment with intravenous immunoglobulin; for the overall sample of patients (each color represents a single patient) with positive TPO-Abs test results.

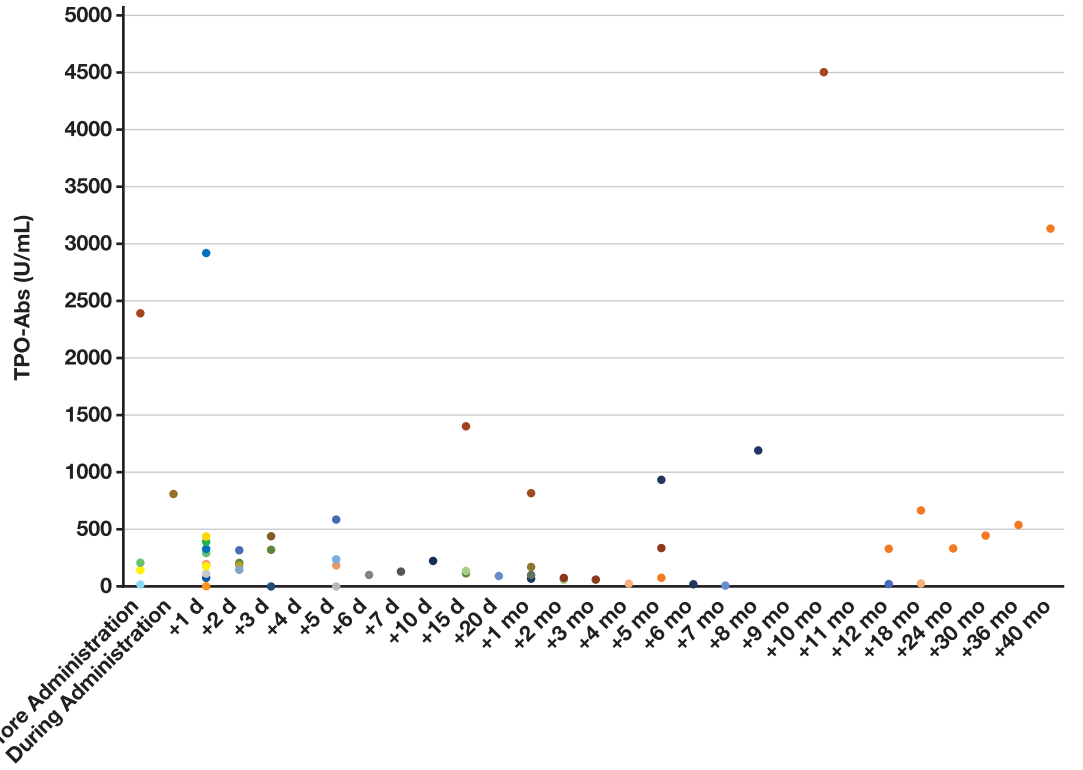
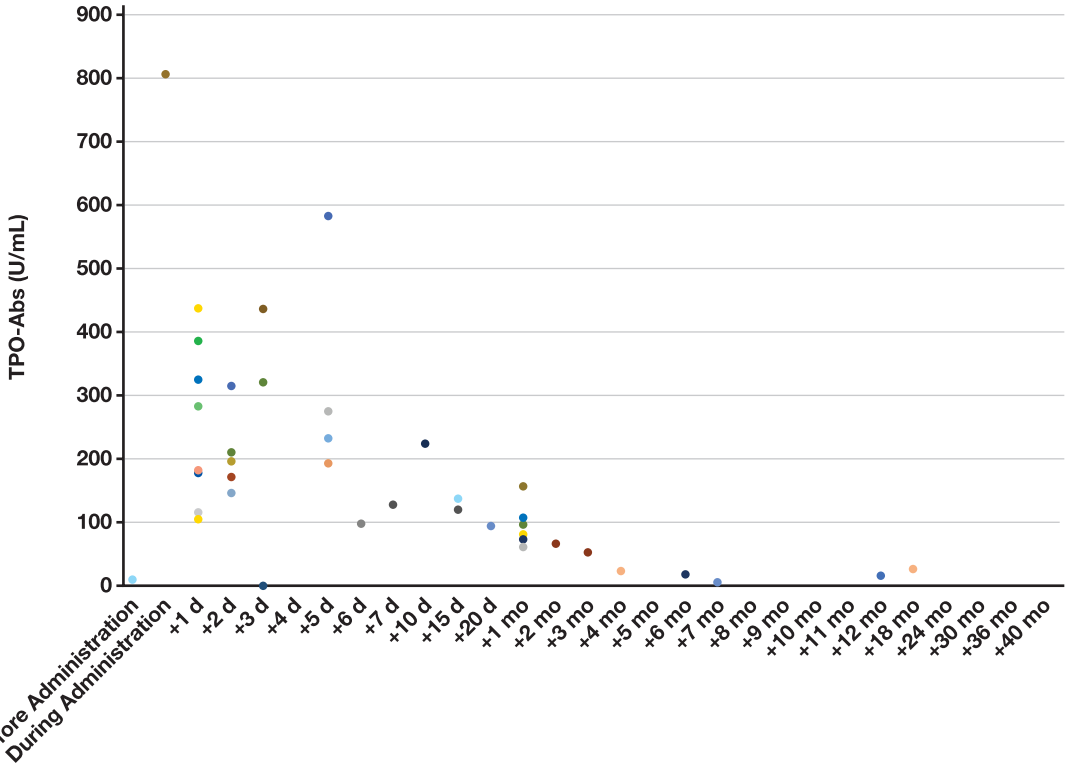


FIGURE 3. Value of thyroid peroxidase antibodies (TPO-Abs) over time after treatment with intravenous immunoglobulin in patients (each color represents a single patient) who showed positivity and/or negativity related to this therapy.



the ANA value was documented (mean 1.6; standard deviation 0.94), and no significant relationship was found between the elevation of these 2 markers. In addition, no significant association was found between TPO-Abs elevation and changes in ESR or RF. Only 18 of the 40 patients had a laboratory study of thyroid function (TSH, T3, and FT4), which detected slight alterations in 5 patients: 2 patients had a minimal eleva-

Downloaded from https://academic.oup.com/labmed/article/53/4/426/6551305 by guest on 28 February 2025

tion of TSH, another 2 patients showed a slight decrease in T3, and the last patient showed a simultaneous decrease in TSH and FT4. Overall, these patients showed normalization of all the previously mentioned alterations, coinciding with normalization of TPO-Abs levels.

Laboratory Technique and Pharmacological Preparation: Unmasking Interference

The measurement of TPO-Abs levels in the IVIG drug preparation was 1176 U/mL (reference values, <60 U/mL). A pool of specimens was processed with TPO-Abs at IVIG concentrations of 0, 156.25, 312.50, 625.00, 1250.00, 2500.00, and 5000.00 mg/dL (**TABLE 1**). The TPO-Abs value in the specimen with no interference (ie, IVIG) was 10 U/mL. This value was used as a reference to calculate the percentage change in TPO-Abs results at successive concentrations. There was a progressive increase in antibody levels as IVIG concentrations increased, reaching a "positive" value (with respect to the reference values) above the IVIG concentration of 312.5 mg/dL and reaching levels of up to 1176 U/mL at a concentration of 5000 mg/dL (**TABLE 1** and **FIGURE 4**).

Discussion

Although they are individually rare, autoimmune diseases make up a significant proportion of pediatric conditions in general, particularly among neurological diseases with acute or subacute onset.[10] They often affect previously healthy children, causing them to abruptly develop variable neurological symptoms in terms of type (eg, epileptic seizures, movement disorders, weakness, sensory disturbances, visual symptoms) and severity, and can even be life-threatening. The immaturity of the pediatric nervous and immune system accentuates the diagnostic difficulties and further complicates existing therapeutic challenges affecting the field of neuroimmunology regardless of patient age.[10] Timely consideration of an autoimmune etiology is crucial for the initiation of potential immunomodulatory treatment because early therapy has prognostic implications for many of these conditions.

In recent years, we have gained considerable knowledge of the physiopathology, biomarkers, and novel therapeutic targets in the field of neuroimmunology.[11,12] However, many situations continue to pose real dilemmas in therapeutic decision-making given the possibility of erroneously treating nonimmune-mediated conditions vs depriving or delaying treatment for patients who would benefit from this therapy. Where there is well-founded clinical suspicion, other nonspecific autoimmunity markers such as TPO-Abs can be considered. Although it is estimated that these antibodies can be detected in approximately 10% of the overall healthy population, a clearly higher frequency has been documented in a wide spectrum of autoimmune diseases such as diabetes mellitus, myasthenia gravis, or rheumatoid arthritis, which could suggest a pathogenic relationship between autoimmune thyroid diseases and other immune-mediated conditions.[13]

Within the field of neurology, TPO-Abs has long been linked to clinical episodes of encephalitis in which symptoms improve with steroids. In the 1960s, Brain et al[14] reported a case of a patient with Hashimoto's thyroiditis with positive antithyroid antibodies who developed several episodes of "severe and unexplained encephalopathy." Later, this constellation was termed "Hashimoto's encephalopathy" and, more recently, "SREAT," given its remarkably favorable course with this treatment. These conditions are historically relevant because they mark the first time that a neurological condition was linked to autoantibodies. However, a uniform pathological mechanism has yet to be established, the existence of these diseases is currently controversial, and their relevance is decreasing with the appearance of specific antibodies that define homogeneous groups of diseases.[2,15]

The search for antibodies plays a fundamental role in the diagnosis and treatment of autoimmune neurological diseases, and laboratory tests (including immunoassays) are of great importance in the early stages of diagnostic evaluation and for assessing the effectiveness of therapy.[16] Immunoassays are a highly useful laboratory technique and are commonly used for hormone determinations and for many immunological tests. One drawback of immunoassays is their association with a higher risk of interference than other types of techniques, and many health care providers are mostly unaware of this disadvantage. The frequency of interference is estimated at between 0.4% and 4%,[7] which means that given the frequency with which immunoassays are used, a not insignificant number of patients may be susceptible to misdiagnosis and even erroneous treatment. Immunoassay consists mainly of an in vitro immunological binding reaction between the molecules to be measured (ie, the antigen) and a reagent antibody. Interference in the immunoassay essentially results from an inappropriate and undesirable cross-binding reaction.[6]

Interference associated with endogenous immunoglobulin antibodies that are capable of binding to the analyte has been known since the technique was first introduced. Today, despite the advances and improvements

FIGURE 4. Intravenous immunoglobulin (IVIG) interferogram. Graphical representation of the percentage change in thyroid peroxidase antibodies (TPO-Abs) values in patients with increasing IVIG concentrations.

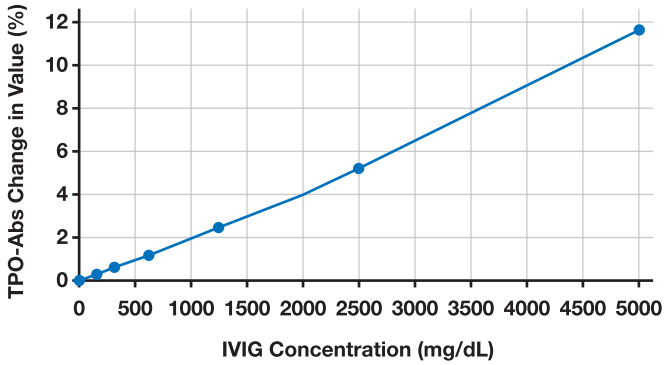


TABLE 1. Interferogram: Comparison of TPO-Abs Values in Patients with Increasing IVIG Concentrations

TPO-Abs value (U/mL)	10.00	42.32	71.7	130.50	256.40	531.40	1176.00
Flebogamma concentration (mg/dL)	0.00	156.25	312.50	625.00	1250.00	1500.00	5000.00
% change	0.00	323.20	613.70	1205.00	2464.00	5214.00	11,660.00

IVIG, intravenous immunoglobulin; TPO-Abs, thyroid peroxidase antibodies.

Downloaded from https://academic.oup.com/labmed/article/53/4/426/6551305 by guest on 28 February 2025

made to this technique, the paradox of high specificity and wide antigen-antibody binding cross-reactivity remains an underestimated problem.[7] Interference of endogenous antibodies with immunoassays is not rare, and several studies have found that this phenomenon occurs in 30% to 40% of the general population.[17,18] Interference can result from common situations such as keeping pets, vaccination, infection, or blood transfusions[19,20] or autoimmune diseases.[7] To counteract this trend, most currently used immunoassay systems include reagents that are capable of neutralizing and blocking low concentrations (µg to mg/L) of some of these interfering antibodies so that they have little or no impact on analytical accuracy. However, in some patients, large amounts of low-affinity antibodies (which may be in concentrations as high as g/L) or relatively low concentrations of high-binding-affinity antibodies can and do saturate the analytical system, leading to interference and erroneous results.[7] In the logic approach, by this same mechanism, interference could occur from exogenous antibodies, such as IVIG. In fact, because IVIG is administered intravenously in large quantities over a short time, the risk may be even greater. However, this type of interference has been the subject of few studies, and there is probably little awareness of this risk in routine clinical practice. As an example, Luyasu et al[8] described a case of false seroconversion against *Borrelia burgdorferi* after therapy with IVIG (sandoglobuline) at usual doses (400 mg/kg for 5 days) in a patient with Guillain-Barré syndrome. In addition, Shimizu et al[9] described a series of 18 children diagnosed with Kawasaki disease and treated with a single dose of 2 g/kg of IVIG (Venilon) in which, after an initial negative determination, positivization of the IgG against *Helicobacter pylori* was evidenced in both blood and urine. In addition, a progressive decrease in time-dependent antibodies was documented by serial determinations, becoming negative over a period of 1 to 3 months in blood and more rapidly in the urine.

In a study of 24 patients with a diagnosis of limbic encephalitis (9 [37%] treated with IVIG), 33.3% of the patients had positive antithyroid antibodies. Although the authors did not describe their results in terms of interference, they concluded that patients with this type of antibody may tend to develop antineuronal immune responses and that this association is not fortuitous.[2] These findings support the previously proposed notion that autoimmune neuronal and thyroid entities could represent a pathogenic spectrum.[21] It is interesting to note that the percentage of patients who had positive antithyroid antibodies was very similar to those who received treatment with IVIG, raising the possibility that the result was influenced by interference (although the authors did not specify whether both conditions "matched" in the patients). In contrast, a study on the prevalence and clinical significance of TPO-Abs in immune thrombocytopenic purpura detected these antibodies with a frequency of 36.8%, with lower platelet counts documented in patients with elevated TPO-Abs. Patients were treated with immunoglobulins, although this factor was not considered at the time of collection. It is therefore possible that the result was influenced by interference, with patients with lower platelet counts being those who received a greater number of cycles of IVIG, although this theory cannot be confirmed.[22] Interference in serological tests is recorded as such on the IVIG data sheet, although this is not the case with other immunoassay types. Treatment with IVIG is accepted as the therapy of choice in a significant number of neuroimmune diseases mediated by antibodies (known or presumed) and in other immune-mediated pediatric pathologies.[23,24] This circumstance could increase the risk of undetected interference.

The results of our study show a progressive decrease in TPO-Abs titers toward normalization over a period that is remarkably similar to that described by Shimizu et al[9] and is thus consistent with the assumed plasma half-life of IVIG preparations. This variability in time is one of the difficulties classically described in predicting and detecting interference by circulating endogenously synthesized antibodies,[7] the persistence of which could vary from weeks or months in the context of infection to indefinite presence in the case of autoimmune disease or when related to contact with pets or food antigens. However, in our study, the pattern of temporal variation (positivization after treatment and loss in a period congruent with the half-life of the IVIG) was one of the keys to the suspicion of interference.

On the other hand, it is well known that the intake of high doses of biotin can interfere with the results of those immunoassays that use the biotin-streptavidin system among their components (now widely used by many reagent manufacturers). The interference can be positive or negative depending on the assay design. In competitive immunoassays (such as the one designed for the determination of TPO-Abs), excess biotin competes with the biotinylated analog for streptavidin binding sites, generating falsely elevated results.[25-27] Although the interference of biotin in the result of some immunoassays is known, its incidence is very rare because treatments with high doses of biotin have been limited to patients with inborn errors of metabolism. However, more and more cases are being reported, and it is necessary to make clinicians aware of the existence of frequent and relevant interferences resulting from biotin consumption through food supplements and the need to confirm the results with a clinic before initiating unnecessary interventions or treatments.[26] It is important to assess the possibility of interference with biotin, particularly when the results do not present clinical correlation. In patients with possible interference, analytical results should be obtained or verified with assays not affected by the presence of biotin.[25-27] None of the patients analyzed in our study received biotin.

Interference is a problem linked to immunoassay techniques; the influence of interference on these findings is sporadic, complex, and different from the errors that occur in other routine nonimmunological analyses and cannot be detected by the same quality control methods.[28] These errors are thus very difficult to predict, especially in clinical situations where the result may be feasible but nonetheless erroneous. This type of situation occurred in our series of patients, where the autoimmune nature (confirmed or suspected) of the underlying disease led the attending physicians to initially take the positivity of TPO-Abs as a congruent fact, or even as a finding in support of this etiological suspicion in patient cases that lacked other objective data except clinical suspicion. Likewise, in 8 of the patients studied, the finding of elevated TPO-Abs motivated an evaluation and even a request for tests and endocrinological follow-up despite the absence of thyroid disease.

In any case, the magnitude of the interference seems sufficient to exert a potentially adverse effect on cost and/or clinical care, which underscores its importance. Therefore, the use of immunoassay and the results obtained in patients with autoimmune diseases or with predisposing factors for the appearance of interference should be considered with caution. Pediatric patients per se meet several risk factors for interference because they receive a battery of immunizations to combat common childhood infections and through the administration of commonly administered vaccines at this age. If a child also presents with an autoimmune or autoinflammatory disease, there is a greater potential risk associated with this condition

Downloaded from https://academic.oup.com/labmed/article/53/4/426/6551305 by guest on 28 February 2025

and the administration of immunomodulatory treatments, whose repertoire and availability for pediatric patients are increasing. In fact, several studies have predicted an increasing number of patients with interferences in the coming years associated with the advances and growing arsenal of immunomodulatory treatments.[7]

In patients with high risk or suspected interference, communication with specialists in clinical analyses is essential. A closer connection between clinical practitioners and laboratory staff could avoid this type of error and/or encourage earlier detection because interferences vary according to the idiosyncrasies of the laboratory (method and measuring instrument). And this communication must be bidirectional. Several authors[7,29,30] have recommended informing physicians about suspected erroneous results in individual specimens and, if necessary, performing specific laboratory tests to reveal (or rule out) the presence of interferences. In our study, notifying the clinical analysis specialist of the suspected error was the first step in a joint effort that enabled the care team to elucidate the degree of this error after appropriate laboratory tests (interferogram). One of the only advantages of cases of interference is that they can be explained from a scientific point of view and that identifying them makes them predictable.[28] In our study, the identification of this interference of IVIG treatment in the TPO-Abs result brought about a change in the timing of this test (before treatment). Furthermore, the way in which patients with a positive result during immunomodulatory treatment were managed was modified, with the test being carried out after the treatment was completed, which led to a reduction in the number of other complementary tests and assessments in patients who did not need them.

Conclusion

We highlight the benefits of carrying out this type of determination before initiating immunomodulatory treatment (especially IVIG) when feasible, bearing in mind possible interference when interpreting unjustified TPO-Abs elevation during treatment, because doing so may avoid ill-advised diagnostic conclusions and tests and may even lead to erroneous treatment. This type of interference is likely underdetected, and its prevalence is likely to increase given the expanding knowledge of autoimmune entities, their treatment, and gaps in the characterization of interference in these processes.

REFERENCES

1. Leypoldt F, Armangue T, Dalmau J. Autoimmune encephalopathies. *Ann N Y Acad Sci.* 2015;1338:94–114.
2. Tüzün E, Erdağ E, Durmus H, et al. Autoantibodies t neuronal surface antigens in thyroid antibody-positive and -negative limbic encephalitis. *Neurol India.* 2011;59:47–50.
3. Long Y, Zheng Y, Chen M, et al. Serum thyroid-stimulating hormone and anti-thyroglobulin antibody are independently associated with lesions in spinal cord in central nervous system demyelinating diseases. *PLoS One.* 2014;9:e1006721–e1006729.
4. Wang X, Yi H, Liu J, et al. Anti-thyroid antibodies and thyroid function in neuromyelitis optica spectrum disorders. *J Neurol Sci.* 2016;366:3–7.
5. Ward G, Simpson A, Boscato L, et al. The investigation of interferences in immunoassay. *Clin Biochem.* 2017;50(18):1306–1311.
6. Ismail Y, Ismail AA, Ismail AAA. Erroneous laboratory results: what clinicians need to know. *Clin Med (Lond).* 2007;7:357–361.
7. Ismail AAA. Identifying and reducing potentially wrong immunoassay results even when plausible and "not-unreasonable." *Adv Clin Chem.* 2014;66:241–294.
8. Luyasu V, Mullier S, Bauraind O, et al. An unusual case of anti-*Borrelia burgdorferi* immunoglobulin G seroconversion caused by administration of intravenous gammaglobulins. *Clin Microbiol Infect.* 2001;7:697–711.
9. Shimizu T, Yarita Y, Suzuki M, et al. Serum and urine *Helicobacter pylori* antibody titer after intravenous γ-globulin treatment for Kawasaki disease and its clearance. *Pediatr Int.* 2005;47:172–174.
10. Wells E, Hacohen Y, Waldman A, et al. Neuroimmune disorders of the central nervous system in children in the molecular era. *Nat Rev Neurol.* 2018;14:433–445.
11. Pranzatelli MR. Advances in biomarker-guided therapy for pediatric- and adult-onset neuroinflammatory disorders: targeting chemokines/cytokines. *Front Immunol.* 2018;9:557.
12. Hu MY, Stathopoulos P, O'Connor KC, Pittock SJ, Nowak RJ. Current and future immunotherapy targets in autoimmune neurology. *Handb Clin Neurol.* 2016;133:511–536.
13. Nakamura H, Usa T, Motomura M, et al. Prevalence of interrelated autoantibodies in thyroid diseases and autoimmune disorders. *J Endocrinol Invest.* 2008;31:861–865.
14. Brain L, Jellinek EH, Ball K. Hashimoto's disease and encephalopathy. *Lancet* 1966;2:512–514.
15. Bien CG. Diagnosing autoimmune encephalitis based on clinical features and autoantibody findings. *Expert Rev Clin Immunol.* 2019;15:511–527.
16. Bizzaro N, Tozzoli R, Villalta D. Autoimmune diagnostics: the technology, the strategy and the clinical governance. *Immunol Res.* 2015;61:126–134.
17. Levinson SS. Antibody multispecificity in immunoassay interference. *Clin Biochem.* 1992;25:77–87.
18. Koper NP, Massuger LF, Thomas CM, et al. An illustration of the clinical relevance of detecting human antimouse antibody interference by affinity chromatography. *Eur J Obstet Gynecol Reprod Biol.* 1999;86:203–205.
19. Sjöwall C, Kastbom ALF, Almroth G, et al. Beware of antibodies to dietary proteins in "antigen-specific" immunoassays! Falsely positive anticytokine antibody tests due to reactivity with bovine serum albumin in rheumatoid arthritis (the Swedish TIRA Project). *J Rheumatol.* 2011;38:215–220.
20. Ismail AAA. Interference from endogenous antibodies in automated immunoassays: what laboratorians need to know. *J Clin Pathol.* 2009;62:673–678.
21. Thieben MJ, Lennon VA, Boeve BF, et al. Potentially reversible autoimmune limbic encephalitis with neuronal potassium. *Neurology.* 2004;62:1177–1182.
22. Bay A, Coskun E, Leblebisatan G, et al. Prevalence and clinical significance of antithyroid antibodies in children with immune thrombocytopenic purpura. *Pediatr Hematol Oncol.* 2013;30:698–704.
23. Negi VS, Elluru S, Sibéril S, et al. Intravenous immunoglobulin: an update on the clinical use and mechanisms of action. *J Clin Immunol.* 2007;27:233–245.
24. Kazatchkine MD, Kavery SV. Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N Engl J Med.* 2001;345:747–755.
25. Piketty ML, Polak M, Flechtner I, et al. False biochemical diagnosis of hyperthyroidism in streptavidin-biotin-based immunoassays: the problem of biotin intake and related interferences. *Clin Chem Lab Med.* 2017;55:780–788.
26. Luong JHT, Male KB, Glennon JD. Biotin interference in immunoassays based on biotin-strept(avidin) chemistry: an emerging threat. *Biotechnol Adv.* 2019;37:634–641.
27. Avery G. Biotin interference in immunoassay: a review for the laboratory scientist. *Ann Clin Biochem.* 2019;56:424–430.
28. Clerico A, Belloni L, Carrozza C, et al. A black swan in clinical laboratory practice: the analytical error due to interferences in immunoassay methods. *Clin Chem Lab Med.* 2018;56:397–402.
29. Sturgeon CM, Viljoen A. Analytical error and interference in immunoassay: minimizing risk. *Ann Clin Biochem.* 2011;48:418–432.
30. Ismail AAA, Ismail AA, Ismail Y. Probabilistic Bayesian reasoning can help identifying potentially wrong immunoassays results in clinical practice: even when they appear "not-unreasonable." *Ann Clin Biochem.* 2011;48:65–71.

Downloaded from https://academic.oup.com/labmed/article/53/4/426/6551305 by guest on 28 February 2025

CASE STUDY

Compound Heterozygous *VPS13A* Variants in a Patient with Neuroacanthocytosis: A Case Report and Review of the Literature

Aryun Kim, MD,[1] *Hee-Yun Chae, MD,*[1] *Hee Sue Park, MD, PhD*[2,*]

[1]Department of Neurology, Chungbuk National University Hospital, Cheongju, Republic of Korea, [2]Department of Laboratory Medicine, Chungbuk National University Hospital, Cheongju, Republic of Korea; *To whom correspondence should be addressed. pheno27@naver.com

Keywords: acanthocytosis, chorea-acanthocytosis, neuroacanthocytosis, next-generation sequencing, movement disorders, *VPS13A*

Abbreviations: ChAc, chorea-acanthocytosis; *VPS13A*, vacuolar protein sorting 13A

Laboratory Medicine 2022;53:433–435; https://doi.org/10.1093/labmed/lmab124

ABSTRACT

Chorea-acanthocytosis (ChAc) is a rare autosomal recessive neurodegenerative disorder caused by pathogenic variants of the vacuolar protein sorting 13A (*VPS13A*). Only a few patients with ChAc have been reported to date, and the variant spectrum of *VPS13A* has not been completely elucidated. We describe the case of a 36-year-old woman who had been experiencing orofacial dyskinesia since age 30 years. In a genetic study using next-generation sequencing, 2 variants of *VPS13A,* the nonsense variant c.4411C>T (p.Arg1471Ter) and the splicing variant c.145-2A>T, were identified. The splicing variant c.145-2A>T was newly classified as a pathogenic variant through a literature review. Consequently, the patient was diagnosed with ChAc based on the typical clinical manifestations, laboratory findings, and imaging results.

Patient History

A 36-year-old woman visited the clinic with involuntary movements of her lips and tongue. She had been experiencing facial dyskinesia, lip-smacking, and progressive dysarthria since age 30 years. Her fluency had also worsened along with progressive stuttering. After a certain period, she developed gait disturbances because of involuntary flexion of her legs. She had been receiving antiepileptic drugs after episodes of generalized tonic-clonic seizures since age 31 years, and she still had intermittent partial seizures.

The patient had graduated from high school and worked as an accountant without intellectual problems, but progressive cognitive problems started in her fourth decade. None of her family members, including her parents, older sister, younger brother, and 2 daughters, showed similar neurological symptoms. Neurological examinations showed generalized chorea and dystonia in both legs. She also showed oromandibular dyskinesia with self-mutilation and vocal tics. Her deep tendon reflexes were decreased with impaired proprioception position sense. Laboratory tests revealed that her creatine kinase level was elevated to 3460 U/L, and acanthocytosis was noted in the peripheral blood smear. In addition, spikes were observed in the left T1, F7, and T3 electrodes on the electroencephalogram. The patient showed cognitive impairment (a score of 26/30 on the Korean-Mini Mental Status Examination and a clinical dementia rating of 0.5). Her brain magnetic resonance imaging showed atrophy of both caudate nuclei (**FIGURE 1**). Based on her clinical features, a gene panel sequencing test was conducted upon suspicion of ChAc or abetalipoproteinemia.

Clinical and Laboratory Information

We selected 29 dystonia-related genes for the target sequencing panel: *ADCY5, ANO3, ATP1A3, ATP7B, CACNA1B, CIZ1, COL6A3, GCH1, GNAL, GNAO1, HPCA, KCTD17, SLC30A10, TAF1, TH, THAP1, TOR1A, KMT2B, MECR, MR1, PRKRA, PRRT2, RELN, SGCE, SLC19A3, SLC2A1, TUBB4A, VAC14,* and *VPS13A*. All procedures were performed at Green Cross Laboratories (Yongin-si, Republic of Korea) using MiSeq Dx (Illumina, San Diego, CA). The average sequence depth of the target regions was 242.64×, and the percentage of target regions with at least 10× coverage was 99.9%. The interpretation of genetic variants was classified into 5 categories: pathogenic, likely pathogenic, uncertain significance, likely benign, and benign, according to the 2015 American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines.[1]

Two variants were found in the vacuolar protein sorting 13A (*VPS13A*) gene (reference sequence: NM_033305.2). First, a nonsense variant, c.4411C>T (p.Arg1471Ter), with allele frequency 48%, was classified as a pathogenic variant according to the 2015 ACMG/AMP guidelines.[1] This nonsense variant has an extremely low incidence in the general population (0.00041% in gnomAD exome ALL) and has been described as pathogenic in the ClinVar database. The other, a splicing variant, c.145-2A>T (allele frequency 44%), developed in

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

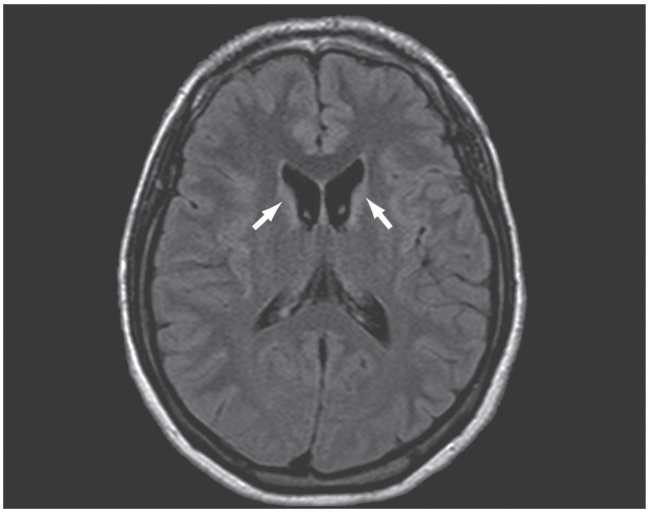
Downloaded from https://academic.oup.com/labmed/article/53/4/433/6514495 by guest on 28 February 2025

the consensus splice acceptor site and has shown a low minor allele frequency (0.0058% in gnomAD exome EAS). Other individuals with this variant have also shown a similar clinical course and laboratory findings (**TABLE 1**). Therefore, although we did not perform a functional test for this variant, we classified it as a pathogenic variant.

Discussion

Research has shown that ChAc (OMIM *60598) is a syndromic neurodegenerative disease with variable neurologic symptoms and acanthocytosis of peripheral blood.[2] The disease usually manifests in the third to fourth decade of life, and patients can show many movement disorders, including both hyperkinetic and hypokinetic movements. In addition, some patients may show cognitive impairment, personality changes, seizures, dysphagia, dysarthria, and increased levels of creatine kinase.[3,4]

FIGURE 1. T2 FLAIR magnetic resonance imaging shows both caudate nuclei atrophied (arrows).



Studies have indicated that ChAc shows autosomal recessive inheritance and is caused by pathogenic variants of the *VPS13A* gene mutation. A previous in vitro study suggested that loss of *VPS13A* function caused a reduction in intracellular phosphatidylinositol-4-phosphate (PtdIns[4]) levels, and this alteration may play a role in the degeneration of neuronal processes.[5] However, it remains unclear how the *VPS13A* pathogenic variants cause neurodegenerative disorders. Acanthocytosis is a representative feature of neuroacanthocytosis, also observed in our patient.[6] Altered plasma membrane levels of PtdIns(4) may play a decisive role in red cell morphologic abnormalities through unconjugated membrane components of the spectrin/action cytoskeleton. Still, the basis for these alterations remains unclear.[5] Previous studies have described variable percentages of acanthocytes ranging from 5% to 50%, but this variation does not seem relevant to the development of clinical features. Another laboratory biomarker, creatinine kinase, is generally elevated in patients with ChAc.[7]

Both of the variants found in our patient have been reported in Japanese patients diagnosed with ChAc.[8] The nonsense variant c.4411C>T (p.Arg1471Ter) is a frequent pathogenic variant, and it is found in more than 50% of Japanese patients with ChAc. The splicing variant, c.145-2A>T, is rare, and it has only been reported in a 26-year-old female patient with an additional pathogenic large deletion variant, c.8211 + 1232_8472-245delinsTC (p.V2738Afs*5). Although further evidence of pathogenicity was not obtained,[1] the patient's clinical manifestations suggested a novel pathogenic variant. In addition, another study reported a 39-year-old Korean female patient with a c.145-2A>T splicing variant who also showed hyperkinetic movement, including orolingual dyskinesia.[9] The authors performed an RNA study and confirmed that this variant also induces aberrant splicing.[9] Moreover, previously known variants in the consensus splicing acceptor site of *VPS13A* have been classified as pathogenic, and these null variants are known to cause disease.[10] These 2 unrelated patients with c.145-2A>T

TABLE 1. Patients with *VPS13A* c.145-2A>T (This Case Study and Previously Reported Patients)

	Current Patient	Nishida et al[6]	Shin et al[7]
Age of onset (y)/sex	30/F	26/F	35/F
Clinical manifestations	Involuntary movements of limbs, face, and tongue; epilepsy; cognitive decline; vocal tic	Epilepsy, obsessive-compulsive syndrome, alteration of personality, cognitive decline	Involuntary movements of limbs and tongue
Brain imaging studies (MRI or CT)	Atrophy of both caudate nuclei	Atrophy of corpus striatum	Bilateral atrophic change in putamen and head of caudate nuclei with increased signal intensity on T2 weighted image
Neurologic examination	Generalized chorea, dystonia in legs, oromandibular dyskinesia with self-mutilation, decreased deep tendon reflex, weakness in legs	N/A	Generally decreased muscle tone and power, bradykinesia, choreiform movement of limbs, neck, and tongue
Neurophysiologic tests	Early-stage sensorimotor polyneuropathy in NCS, normal EMG, epileptiform discharge in left fronto-temporal lobe in EEG	N/A	Normal NCS, EMG, and EEG
Laboratory tests			
Creatine kinase (reference range)	3460 U/L (29–145)	Elevated	345 IU/L (5–217)
Peripheral blood smear	Acanthocytes +	Acanthocytes +	Acanthocytes +
VPS13A variant	c.145-2A>T (ht) c.4411C>T (p.Arg1471*) (ht)	c.145-2A>T (ht) c.8211 + 1232_8472-245delinsTC (ht)	c.145-2A>T (ht) c. 2170 + 5G>A (ht)

CT, computed tomography; EEG, electroencephalography; EMG, electromyography; ht, heterozygous; MRI, magnetic resonance imaging; N/A, not available; NCS, nerve conduction study; VPS13A, *vacuolar protein sorting 13A.*

Downloaded from https://academic.oup.com/labmed/article/53/4/433/6514495 by guest on 28 February 2025

and our patient showed similar clinical features and laboratory findings. Consequently, this variant can be newly classified as a pathogenic variant.

In conclusion, we present the findings for a patient showing 2 pathogenic variants of *VPS13A*. Reports describing *VPS13A* are very rare in Korea. Although the limitations of the next-generation sequencing method made it difficult to determine whether the location was *cis* or *trans*, we concluded that the 2 variants may be trans hetero-compound variants according to the clinical features and laboratory findings.

REFERENCES

1. Richards S, Aziz N, Bale S, et al; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med.* 2015;17(5):405–424.
2. Jung HH, Danek A, Walker RH. Neuroacanthocytosis syndromes. *Orphanet J Rare Dis.* 2011;6:68.
3. Hardie RJ, Pullon HW, Harding AE, et al. Neuroacanthocytosis: a clinical, haematological and pathological study of 19 cases. *Brain.* 1991;114(Pt 1A):13–49.
4. Vaisfeld A, Bruno G, Petracca M, et al. Neuroacanthocytosis syndromes in an Italian cohort: clinical spectrum, high genetic variability and muscle involvement. *Genes*. 2021;12:344.
5. Park JS, Halegoua S, Kishida S, Neiman AM. A conserved function in phosphatidylinositol metabolism for mammalian Vps13 family proteins. *PLoS One.* 2015;10(4):e0124836.
6. Liu J, Bader B, Danek A. Neuroacanthocytosis in China: a review of published reports. *Tremor Other Hyperkinet Mov (N Y).* 2014;4:248.
7. Sokolov E, Schneider SA, Bain PG. Chorea-acanthocytosis. *Pract Neurol.* 2012;12(1):40–43.
8. Nishida Y, Nakamura M, Urata Y, et al. Novel pathogenic VPS13A gene mutations in Japanese patients with chorea-acanthocytosis. *Neurol Genet.* 2019;5(3):e332.
9. Shin H, Ki CS, Cho AR, et al. Globus pallidus internal deep brain stimulation improves chorea and functional status in a patient with chorea-acanthocytosis. *Stereotact Funct Neurosurg.* 2012;90(4):273–277.
10. Dobson-Stone C, Danek A, Rampoldi L, et al. Mutational spectrum of the CHAC gene in patients with chorea-acanthocytosis. *Eur J Hum Genet.* 2002;10(11):773–781.

Downloaded from https://academic.oup.com/labmed/article/53/4/433/6514495 by guest on 28 February 2025

CASE STUDY

Diagnostic Value of Metagenomic Next Generation Sequencing for *Ureaplasma urealyticum* Infection: A Case Report

Xuelian Ruan, MD[1,a] *Meng Li, MD,*[1,a] *Xue Qin, PhD*[1,*]

[1]Department of Clinical Laboratory, First Affiliated Hospital of Guangxi Medical University, Nanning, China; *To whom correspondence should be addressed. qinxue919@126.com; [a]These authors have contributed equally to the article.

Keywords: allogeneic hematopoietic stem cell transplantation, metagenomics next generation sequencing, *Ureaplasma urealyticum*, hematology, immunocompromised patient, pneumonia

Abbreviations: mNGS, metagenomic next-generation sequencing; CT, computed tomography; CMV, cytomegalovirus; BAL, bronchoalveolar lavage; PCR, polymerase chain reaction.

Laboratory Medicine 2022;53:e74–e76; https://doi.org/10.1093/labmed/lmab091

ABSTRACT

Ureaplasma urealyticum has high nutritional requirements for culture, and it requires special tools for identification. Theoretically, metagenomic next generation sequencing (mNGS) can be used to detect many pathogens in clinical specimens, especially for complex infectious diseases with rare and atypical causes. Here, our patient developed severe pneumonia caused by *U. urealyticum* infection after allogeneic hematopoietic stem cell transplantation, and the etiology is unclear. After continuous negative culture, *U. urealyticum* was detected in the bronchoalveolar lavage fluid by mNGS, and azithromycin was used. Because of the difficulty in its diagnosis, diagnosis and treatment of extragenital *U. urealyticum* infection is challenging. In addition, many broad-spectrum antibiotics are ineffective against this pathogen because it lacks a cell wall. Therefore, early diagnosis and treatment are key to preventing further complications and deaths.

Clinical History

One month after allogeneic hematopoietic stem cell transplantation, a 16 year old man with thalassemia was admitted to the hospital for pneumonia and fever (maximum temperature, 38.9°C). Antimicrobial therapy with cefoperazone-sulbactam was started. Twenty-five days after admission, he developed symptoms such as hemoptysis and shortness of breath. Chest computed tomography (CT) showed bilateral pneumonia with new multiple exudative lesions. The antibiotic regimen was switched to sulfamethoxazole plus voriconazole plus vancomycin. In the following 2 months, the patient developed recurrent fever. Testing for serum cytomegalovirus (CMV) DNA was recurrent-positive, but the blood, urine, bronchoalveolar lavage (BAL) fluid, and other specimens were generally negative for bacterial and fungal cultures. Empirical antibiotic therapy with caspofungin, flucytosine, sulfamethoxazole, and linezolid was initiated, and ganciclovir was used to treat CMV infection.

At 88 days after admission, the patient was transferred to the intensive care unit with intermittent high fever, pulmonary infection, and joint pain. Physical examination showed no significant abnormalities. The results of the blood test revealed a white blood cell count of 3.25×10^9/L, 2.85×10^{12} red blood cells/L, and 56.80×10^9 platelets/L. Laboratory evaluation revealed impaired liver and renal function.

Laboratory Identification of *Ureaplasma urealyticum* in BAL Fluid

At 104 days after admission, bronchoscopy showed inflammatory changes in the right lung. A sample of the BAL fluid was obtained, and a BAL smear was prepared to test for acid fast bacilli and for general culture, but the results were negative. The BAL fluid was then tested for Epstein-Barr virus DNA and CMV DNA; the results revealed that CMV DNA using polymerase chain reaction (PCR) was 8.09×10^3 copies (normal range, $<4 \times 10^2$ copies) and Epstein-Barr virus DNA was $<5 \times 10^2$ copies. Meanwhile, BAL fluid was sent for metagenomic next generation sequencing (mNGS) analysis, and the number of detection sequences of *Ureaplasma urealyticum* was 275. Based on the results of the mNGS report, we detected the blood ammonia of the patient, PCR was performed using the BAL fluid, and the BAL fluid was inoculated into a special culture medium for *U. urealyticum*. The results revealed that the blood ammonia concentration was 75 μmol/L (normal range, 18–72 μmol/L), and the *U. urealyticum* DNA PCR yielded 1.53×10^6 copies. Then, 107 days after admission, a chest CT showed a thickened, disordered texture and "mosaic" in bilateral lungs with multiple patchy and cord-like-density increased shadows in each lobe (**FIGURE 1**). The lesions in both lungs were more serious than those seen in the chest CT scan obtained 1 week before this scan. A special culture of *U. urealyticum* was positive, and the results of the drug sensitivity test showed that the strain was sensitive to erythromycin, azithromycin,

© The Author(s) 2021. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/e74/6404407 by guest on 28 February 2025

FIGURE 1. Chest CT scan obtained 107 days after infection showed worsening of both lungs. CT, computed tomography.

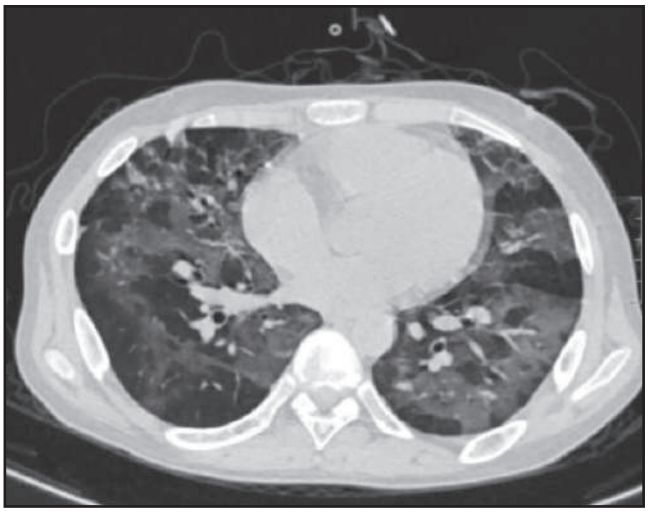
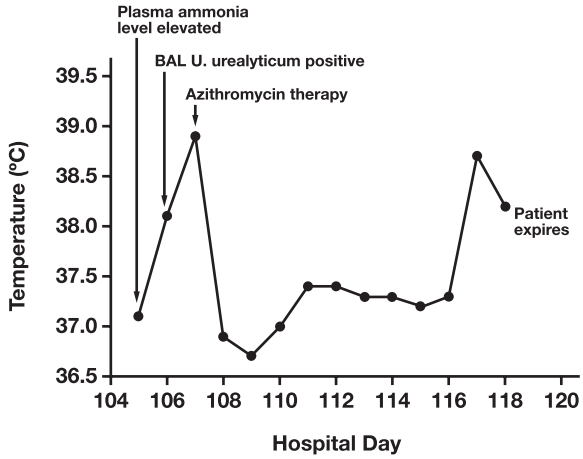


FIGURE 2. Changes in the patient's temperature. BAL, bronchoalveolar lavage.



josamycin, clarithromycin, roxithromycin, and gatifloxacin, with intermediate resistance to levofloxacin and sparfloxacin.

According to the patient's drug sensitivity results, the patient was given azithromycin and developed better temperature control (**FIGURE 2**). The C-reactive protein level decreased from 97.86 mg/L to 2.44 mg/L, and the serum procalcitonin level decreased from 0.344 mg/L to 0.076 mg/L. Regrettably, however, the patient died 118 days after admission because of the recurrent fever for nearly 3 months.

Discussion

Human pneumonia caused by *U. urealyticum* is rare. Research has shown that *U. urealyticum* belongs to the Mycoplasma family because of its urea decomposition characteristics. Although *U. urealyticum* infection is thought to have low virulence, it is fatal for premature infants, patients with organ transplants, and immunocompromised patients.[1-3] Genital infection is very rare in individuals with a healthy immune system and is usually associated with surgery.[4] A *U. urealyticum* infection in immunosuppressed patients usually causes major extrareproductive diseases, such as pleural pneumonia,[5] paranephric abscess,[6] and intraventricular inflammation.[7] The sensitivity and specificity of mNGS in the diagnosis of infectious diseases have been reported to be 50.7% and 85.7%, respectively, which are much better than those of culture.[8] Here, we describe the first culture and mNGS-confirmed case of severe pneumonia caused by *U. urealyticum* in a patient with hematopoietic stem cell transplantation.

This case study emphasizes the special value of mNGS in challenging microbial infections in patients with immunodeficiency. Research has shown that *U. urealyticum* is one of the most common conditional pathogenic bacteria in the human genitourinary tract, which is primarily related to adult genital infection. Its mechanism of pathogenesis may involve arginine metabolism and urease toxicity. The decomposition of urea releases large amounts of NH_3, which could exhaust arginine, resulting in cytotoxic effects.[9] However, there is no direct evidence showing that arginine burnout can produce toxigenicity in vivo.[10]

Adhesin exists in *U. urealyticum,* which can adhere to a variety of human cells, such as red blood cells, sperm,[11] and urethral epithelial cells. After the spontaneous binding of urease with neutrophils, complements are directly activated. In the absence of antibodies, Mycoplasma can survive even after being ingested by neutrophils and can travel around the body with neutrophils to cause Mycoplasma infection in multiple regions.[12] Studies have shown that CMV is a double-stranded DNA virus of the β-herpesvirus subfamily. In general, it is a recessive infection, but pneumonitis[13] and hepatitis[14] are the most common organ manifestations. Furthermore, CMV infection may be associated with leukocyte margination and complement activation, but the pathogenesis of CMV pneumonia has not been elucidated.[13] The role of *U. urealyticum* and CMV in multiple infections after transplantation and whether the 2 can promote one another need to be further studied.

Laboratory Role in Diagnosis

In our patient, when a pure culture of *U. urealyticum* was temporarily unavailable and there was an urgent clinical need for drug sensitivity testing to guide the treatment, reagents with certain anti-interference ability were used for the drug sensitivity test, and a control was set up to eliminate the interference of other respiratory tract bacteria that may exist in the BAL fluid as much as possible. To ensure the reliability of drug sensitivity, PCR and culture were carried out to determine whether the reagent could inhibit the growth of other bacteria, and the results of the control were negative.

Studies have shown that mNGS is a high-throughput detection technique that can directly detect nucleic acids in clinical specimens with no culture dependence. It can detect a variety of pathogens, including bacteria, fungi, Mycoplasma, chlamydia, spirochetes, *Rickettsia,* viruses, and parasites in a short period, especially for pathogens that are difficult to isolate and culture using traditional culture techniques.[8] In addition, mNGS has great value in the etiologic diagnosis of immunosuppressive patients, critically ill patients, and patients receiving broad-spectrum antibiotic therapy.[15] Because of its current technical limitations, mNGS can neither be used as an independent diagnostic criterion nor provide reliable drug sensitivity information. Clinical evaluation is needed to determine the biological significance of mNGS detection, especially for cases of infection that have not been confirmed by conventional detection.[16] If the patients' mNGS results are positive and consistent with clinical manifestations, but there is no other supporting laboratory evidence, then PCR should be carried out (with suitable primers), and it is

Downloaded from https://academic.oup.com/labmed/article/53/4/e74/6404407 by guest on 28 February 2025

suggested that more available traditional laboratory tests be performed for verification.

In conclusion, we described the first case of severe pneumonia due to U. urealyticum after allogeneic hematopoietic stem cell transplantation, while concomitant CMV infection cannot be ruled out. This case highlights the complexity of microbial diagnosis in patients with immunodeficiency. For patients with multiple infections, sometimes the traditional methods of microbial isolation and culture cannot meet the needs of clinical diagnosis and treatment. When the patient's microbial culture yields multiple negative AQ11 results and empirical treatment is ineffective, mNGS should be considered to assist the diagnosis, and other methods should be used to verify it.

Acknowledgments

Xue Qin drafted the overall design of this paper and provided language help. Xuelian Ruan and Meng Li wrote the article as the co-first authors. All authors have read and approved the manuscript. Informed consent was obtained from the patient's father, and this study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. This work was supported by the scientific research project of the Guangxi Health Committee (grant number Z20201166). The recipient, ML, provided the case study information and revised the manuscript.

REFERENCES

1. MacKenzie CR, Nischik N, Kram R, Krauspe R, Jäger M, Henrich B. Fatal outcome of a disseminated dual infection with drug-resistant Mycoplasma hominis and Ureaplasma parvum originating from a septic arthritis in an immunocompromised patient. *Int J Infect Dis.* 2010;14(Suppl 3):e307–e309.
2. Smith M, Crews JD, Cheek N, et al. Hyperammonemic encephalopathy due to ureaplasma parvum infection in an immunocompromised child. *Pediatrics.* 2019;144(2):e20190601.
3. Paparoupa M, Barten MJ, de Heer J, Giessen HS, Frings D, Kluge S. Hyperammonemia by ureaplasma urealyticum pneumonia after lung transplantation. *Respir Med Case Rep.* 2020;30:101080.
4. Mahlouly J, Lhopitallier L, Suttels V, et al. Septic arthritis of the shoulder due to Ureaplasma urealyticum after emergency caesarean section: a case report. *BMC Infect Dis.* 2020;20(1):767.
5. Canouï E, Blanc K, Loubinoux J, et al. The value of molecular techniques to diagnose Ureaplasma urealyticum and Nocardia farcinica pleuropneumonia in a patient with diffuse large B-cell lymphoma. *Int J Infect Dis.* 2017;64:93–95.
6. Diaz Pallares C, Griener T, Vaughan S. Ureaplasma urealyticum disseminated multifocal abscesses in an immunocompromised adult patient: a case report. *BMC Infect Dis.* 2020;20(1):47.
7. Saje A, Velnar T, Smrke B, et al. Ureaplasma parvum ventriculitis related to surgery and ventricular peritoneal drainage. *J Infect Chemother.* 2020;26(5):513–515.
8. Miao Q, Ma Y, Wang Q, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis.* 2018;67(suppl_2):S231–S240.
9. Smith DG, Russell WC, Ingledew WJ, Thirkell D. Hydrolysis of urea by Ureaplasma urealyticum generates a transmembrane potential with resultant ATP synthesis. *J Bacteriol.* 1993;175(11):3253–3258.
10. Saada AB, Terespolski Y, Adoni A, Kahane I. Adherence of Ureaplasma urealyticum to human erythrocytes. *Infect Immun.* 1991;59(1):467–469.
11. Busolo F, Zanchetta R, Bertoloni G. Mycoplasmic localization patterns on spermatozoa from infertile men. *Fertil Steril.* 1984;42(3):412–417.
12. Webster AD, Furr PM, Hughes-Jones NC, Gorick BD, Taylor-Robinson D. Critical dependence on antibody for defence against mycoplasmas. *Clin Exp Immunol.* 1988;71(3):383–387.
13. Ison MG, Fishman JA. Cytomegalovirus pneumonia in transplant recipients. *Clin Chest Med.* 2005;26(4):691–705.
14. McDonald GB, Sarmiento JI, Rees-Lui G, Myerson D. Cytomegalovirus hepatitis after bone marrow transplantation: an autopsy study with clinical, histologic and laboratory correlates. *J Viral Hepat.* 2019;26(11):1344–1350.
15. Parize P, Muth E, Richaud C, et al. Untargeted next-generation sequencing-based first-line diagnosis of infection in immunocompromised adults: a multicentre, blinded, prospective study. *Clin Microbiol Infect.* 2017;23:574.e1–574.e6.
16. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol.* 2019;14:319–338.

Downloaded from https://academic.oup.com/labmed/article/53/4/e74/6404407 by guest on 28 February 2025

CASE STUDY

Successful Orthotopic Heart Transplantation in Patient With Anti-U Antibody

Caitlin Hughes, MD,[1] Brent Sterling, MD[1], Jennifer Andrews, MD, MSc[1,]*

[1]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee, US; *To whom correspondence should be addressed. jennifer.andrews@vumc.org

Keywords: anti-U antibody, solid organ transplant, heart transplant, transfusion, frozen units, deglycerolized

Abbreviations: pRBC, packed red blood cell; RBC, red blood cell; CDP, chloroquine diphosphate.

Laboratory Medicine 2022;53:e77–e78; https://doi.org/10.1093/labmed/lmab100

ABSTRACT

Objective: The U (universal) antigen is part of the MNS blood group present at a frequency of nearly 100% in Caucasians and 98% of African Americans. The anti-U antibody is clinically significant and has been reported to cause hemolytic transfusion reactions and hemolytic disease of the fetus and newborn.

Methods: Routine forward and backward typing, direct antiglobulin testing, and an antibody screen were performed. In addition, red blood cell phenotype and adsorption studies were also performed.

Results: The patient was found to have a rare anti-U antibody, rendering all available inventory in our hospital incompatible for transfusion.

Conclusion: This is the first reported case of solid organ transplantation in a patient with an anti-U alloantibody. Appropriate pretransplant evaluation and coordination between the clinical team and transfusion medicine service must be optimized to procure rare packed red blood cell units in a timely manner.

The MNS blood group system contains 49 red blood cell antigens encoded by the glycophorin A and B genes on chromosome 4.[1] Homozygous deletion of the glycophorin B gene results in the U-(S-s-) phenotype, which is found in approximately 2% of African Americans and up to 35% of some African populations.[2] If exposed to the U antigen via packed red blood cell (pRBC) transfusion or pregnancy, this population can form an anti-U antibody, which is known to cause both hemolytic disease of the fetus and newborn and hemolytic transfusion reactions. Safely transfusing these patients is therefore challenging because nearly 100% of the primarily Caucasian donor population has the U (universal) antigen.[3]

Case Report

The patient was a 35 year old African American female with postpartum cardiomyopathy. In the span of approximately 4 years, she was listed for transplant because of a declining ejection fraction to 10% to 15% with elevated filling pressures despite appropriate medical management. As part of routine pretransplant evaluation, a blood type and antibody screen were ordered. The antibody screen revealed an anti-U antibody. The transplant team consulted the transfusion medicine service upon learning of this antibody to coordinate rare pRBC units to be available quickly once the patient was listed for heart transplant. With coordination from the American Red Cross, 15 frozen U-negative pRBC units were made available locally. An additional 4 U-negative liquid pRBC units were shipped to our hospital upon notification that a donor heart was available for her, which was approximately 12 hours before her transplant. She successfully received an orthotopic heart transplant with 6 total crossmatch compatible pRBC units transfused perioperatively, 2 of which required thawing and deglycerization because they were frozen.

Laboratory Workup

The patient's blood type was A Rh-positive by forward and reverse typing. An antibody screen was performed and was positive. The patient had an unknown transfusion history but had previous pregnancies. Antibody identification testing was performed and revealed a panagglutinin reaction pattern. A direct antiglobulin test was negative for IgG coating the patient's red blood cells (RBCs). A specimen sent to an immunohematology reference laboratory revealed that her RBCs were negative for the K, S, and s antigens in addition to serum testing consistent with an anti-U antibody. Differential homologous adsorption studies performed 3 times with R1R1 cells and performed twice with R2R2 cells and rr cells removed the anti-U. Two of 2 cells that were weakly reactive with the absorbed serum failed to react after chloroquine diphosphate (CDP) treatment, consistent with the presence of an antibody to Bennett-Goodspeed. Additional weakly positive reactivity with 4 additional cells could not be investigated with CDP treatment because of an insufficient quantity of absorbed serum. Therefore,

© The Author(s) 2021. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/e77/6415349 by guest on 28 February 2025

anti-K and anti-S could not be ruled out. All other clinically significant alloantibodies were ruled out in the neat serum or absorbed serum testing with polyethylene glycol–IgG.

Discussion

Transfusion support is expected for heart transplant recipients in the operative and immediate postoperative setting. During the operation, recipients are put on cardiopulmonary bypass, which is associated with severe bleeding in approximately 10% of patients. In addition, 1 study showed that patients received an average of 4.2 ± 0.9 units of pRBCs in the first 24 hours postoperatively.[4]

With the expectation that blood products would be needed in the operative and postoperative setting, the cardiac transplant surgical team and our transfusion medicine service communicated frequently to ensure an adequate, compatible blood supply for the patient. Because no in-hospital pRBCs were compatible, our transfusion service worked in conjunction with our primary blood supplier, the American Red Cross, to acquire rare U antigen–negative units. Given the extremely low frequency of U-negative donors in the donor population and the relatively short 42-day shelf life, most (15/19) of the U-negative units received were frozen. Of critical importance in a time-sensitive operation like a solid organ transplant, coordination with the surgical team, the transfusion service, and the primary blood provider is essential to ensuring the units arrive in a safe, timely, and effective manner. Frozen units contain glycerol and require deglycerolizing before transfusion. The deglycerolizing process takes approximately 4 to 6 hours, and once performed it leaves the unit with a shelf life of just 24 hours.[1] After deglycerolizing, the unit must be thawed in a water bath or heat block to a temperature of 37°C.[1] Then, the unit must be cooled to reach a temperature of 1 to 10°C for transportation from the primary blood supplier to the hospital.[1] In total, the transfusion service and surgical team should expect to wait at least 6 to 8 hours from the time of initial request to receive previously frozen units if the local blood supplier has those rare units in the frozen inventory. If the local blood supplier does not have the rare units in inventory, then the blood supplier will need to request these units from other suppliers, which could take several days.

Solid organ transplantation can and does occur successfully in patients with rare alloantibodies. This is the first reported case of solid organ transplantation in a patient with an anti-U alloantibody. Appropriate pretransplant evaluation and coordination between the clinical team and transfusion medicine service must be optimized to procure rare pRBC units in a timely manner. As such, rare alloantibodies should not preclude patients from consideration for solid organ transplantation.

REFERENCES

1. Fung MK, Eder AF, Spitalnik SL, Westhoff CM. *Technical Manual,* 19th ed. Bethesda, MD: AABB Press; 2017.
2. Ringressi A, Biagioni S, Mello G, Graziani G, Mecacci F. Anti-U alloimmunisation in a pregnant woman from Niger. *Blood Transfus.* 2012;10(2):221–224.
3. Wiener AS, Unger LJ, Gordon EB. Fatal hemolytic transfusion reaction caused by sensitization to a new blood factor U. Report of a case. *JAMA.* 1953;153:1444–1446.
4. Jahangirifard A, Razavi MR, Ahmadi ZH, Forozeshfard M. Effect of desmopressin on the amount of bleeding and transfusion requirements in patients undergoing heart transplant surgery. *Basic Clin Pharmacol Toxicol.* 2017;121(3):175–180.

Downloaded from https://academic.oup.com/labmed/article/53/4/e77/6415349 by guest on 28 February 2025

CASE STUDY

HDFN Resulting from Anti-U: Alternatives to Allogeneic Intrauterine Transfusion

Jamie L. Caudill, MS, MLS(ASCP)[CM,1,*] *Laurie Gillard, MS, MLS(ASCP)*[CM], *SBB*[2]✉

[1]Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania, US, [2]Rush University Medical Center, Chicago, Illinois, US; *To whom correspondence should be addressed. jamie.caudill@protonmail.com

Keywords: hemolytic disease, anti-U, neonatal, immunohematology, intrauterine transfusion, high-prevalence antigens, alloimmunization

Abbreviations: HDFN, hemolytic disease of the fetus and newborn; Doppler MCA, middle cerebral artery Doppler imaging; GYPA, Glycophorin A; GYPB, Glycophorin B; RBC, Red blood cell; DAT, Direct Antiglobulin Test; AHG, Anti-human globulin; FcRn, Neonatal Fc receptor.

Laboratory Medicine 2022;53:e79–e82; https://doi.org/10.1093/labmed/lmab099

ABSTRACT

Hemolytic disease of the fetus and newborn (HDFN) carries significant fetal mortality risks. Although anti-D as a source of HDFN has been prevented for decades using D-specific immunoglobulin to prevent alloimmunization between fetus and mother, minor blood groups may still result in disease, with potentially disastrous consequences if left untreated. Strategies such as intrauterine transfusion, early delivery, and vigilant serologic monitoring of fetal anemia have been the standards of care for alloimmunized patients, but beyond this not much more is possible. Mothers with rare phenotypes who are alloimmunized against extremely common red blood cell antigens may find access to rare antigen-negative blood units limited. This case study presents a healthy G10P6 woman with known anti-U presenting for treatment via intrauterine transfusion in the second trimester and follows the patient through successful delivery. Difficulties in obtaining rare blood for the patient because of concomitant delivery events involving 2 patients with anti-U at the facility opened discussions about the difficulties of and alternatives to intrauterine transfusion where rare blood phenotypes are involved.

Unexpected maternal antibodies may create significant complications through pregnancy and cause hemolytic disease of the fetus and newborn (HDFN). Today, Rh-negative mothers are often treated prophylactically to prevent alloimmunization to the D antigen. Although D-immunoglobulin G and other measures have been successful in decreasing the mortality resulting from alloimmunization, other lesser known clinically significant antibodies still continue to pose threats in expectant mothers.[1] These hazards are exacerbated when pregnant patients are found to have rare phenotypes and have been alloimmunized with high-prevalence antigens.[2] Although tools such as fetal middle cerebral artery Doppler imaging (Doppler MCA), intrauterine transfusions, and titration of antibodies to monitor care during pregnancy may help manage alloimmunized women, less-common blood group antibodies continue to be a source of morbidity/mortality in fetuses and neonates and highlight a need for additional mitigative strategies in patients for whom transfusion of rare allogeneic blood may be imminent.

The U antigen is a high-prevalence red blood cell antigen that is classified as part of the extensive MNS blood group system and is found on chromosome 4.[3] Cells lack the U antigen typically from the deletion of the coding region of glycophorin B (GYPB).[4] Phenotypes that are U-negative are found in African populations at a frequency of up to 2%.[2] Further, GYPB is responsible for the S and s antigens of the MNS system; consequently, most cells known to test negative for the U antigen will also lack the S and s antigens.[3] Anti-U was first identified in 1953, and its name comes from its almost universal distribution on human erythrocytes.[5] This corresponding antibody to the U antigen is reactive at 37°C and antihuman globulin phases and is considered clinically significant in transfusion practice.[3] Additional genetic variations exist that may give rise to the S-s-U+^{var} phenotype; however, the patient in the following case study was found to have the full GYPB deletion, per molecular testing at the American Red Cross Molecular Laboratory in Philadelphia, Pennsylvania.[6]

Case Report

A 33 year old female, G10P6, presented initially for evaluation of a high-risk pregnancy with known anti-U red cell antigen antibodies. The patient was immunized with anti-U in a prior pregnancy, which caused complications in subsequent pregnancies that required serologic monitoring and transfusion support. The patient was O Rh positive, and the first antibody screen for this patient was 4+ positive on both screening cells (on a scale of 1+ to 4+) using a solid-phase red cell adherence assay for initial testing. The patient had previously identified anti-U and anti-Bg antibodies at the facility and was confirmed molecularly as having the S-s-U- phenotype. It was found that anti-Bg was not found in the initial specimen.

All routine antibody panel testing was performed using gel microcolumn agglutination technology. Reagent red cells molecularly confirmed to be U-negative were selected to rule out all clinically signif-

© The Author(s) 2021. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/e79/6427761 by guest on 28 February 2025

icant antibodies. Performing a selected cell panel using red cells negative for the antigens that correspond with previously identified patient antibodies improves the likelihood of identifying new or underlying antibodies.[3] The stock of U-negative reagent red cells was limited, given the high frequency of the U antigen on red cells. Only 2 reagent red cells were identified as U-negative at the time of testing. Alternatively, to remove the anti-U from the patient plasma and rule out the remaining antibodies (anti-C, anti-E, anti-K, and anti-Jk^b), a 3× alloadsorption was performed using a single papain-treated U-positive, K-negative, Jk^b-negative *rr* adsorbing cell. The resulting adsorbed plasma was tested against reagent red cells containing the corresponding antigens to the aforementioned antibodies.

Indeed, the use of adsorption techniques may result in weakened antibody reactivity.[7] All of the remaining antibodies to be ruled out were part of the Rh, Kell, or Kidd blood group systems, which are known to be enhanced or unaffected by the enzyme treatment of reagent red cells, ie, enzyme-stable antigens.[3] For this reason, enzyme-treated adsorbing cells were the cells of choice for use in this alloadsorption.

Before beginning the alloadsorption, the patient's direct antiglobulin test (DAT) was performed to ensure there were no antibodies bound to the patient's red cells. The patient's DAT was negative at both the IgG and C3b,d phases, indicating that the enzyme-treated cells would be appropriate for the alloadsorption, which can enhance reactivity.[8] Adsorption with papain-treated antigen-positive red cells removed the anti-U from the patient plasma. This allowed the remaining antibodies (anti-C, anti-E, anti-K, and anti-Jk^b) to be ruled out at the antihuman globulin (AHG) phase. Reactions were read after a 30-minute incubation at 37°C, with no added potentiators. Then, Fy^a-positive cells were used as a control to ensure that the papain treatment of the adsorbing cell was successful, because the Duffy blood group antigens are enzyme-labile and reactivity with these cells should be negative.[3] Likewise, an aliquot of U-positive cells was used as a control to ensure that the adsorption of anti-U was successful.

Titers for anti-U were 1:128 (score of 71) late in the first trimester, and no previous specimen was available for parallel testing. The patient plasma was frozen and saved for future testing. The anti-Bg antibodies are directed against HLA antigens and are not typically considered clinically significant with respect to HDFN. Critical values for titers of antibodies beyond those of anti-D may vary significantly and do not always correlate to the disease state of the fetus and newborn.[9] The assumed father's phenotype was confirmed positive for the U and s antigens, indicating the potential for maternal-fetal incompatibility and HDFN. Anti-s is difficult to rule out in the presence of anti-U, because cells lacking the U antigen will also lack the S and s antigens.

After the antibody identification and the results of fetal Doppler MCA and antibody titration throughout the second trimester, the clinical decision was made to perform an intrauterine transfusion to relieve fetal anemia. Allogeneic O-negative red blood cells negative for the U antigen were acquired from the blood supplier. This was the fifth allogeneic U-negative intrauterine transfusion received by this patient at the facility, with 4 others performed in previous pregnancies. The unit was washed and irradiated and packed to a hematocrit of 81%. The exchange transfusion was uncomplicated, and the fetal hematocrit improved from 33% to 43% after 1 transfusion. Throughout the rest of the pregnancy, the patient received regular fetal Doppler MCA readings. It was determined in the seventh month of pregnancy that the fetal anemia had again progressed from mild to moderate-severe even with transfusion, and a rescue dose of betamethasone was given in anticipation of planned preterm delivery. After administration of the betamethasone, the fetal condition improved and regular Doppler MCA readings indicated a consistent decline from moderate-severe anemia to the mild anemia range. Significant preterm delivery was avoided.

The patient delivered shortly before the planned 37 weeks, at which time the neonatal vitals could be assessed. The neonate was found to have an initial bilirubin (total) of 4.2 mg/dL (reference range, ≤11.6 mg/dL). The initial hemoglobin and hematocrit were 12.5 g/dL (reference range, 13.5–19.5 g/dL) and 40% (reference range, 42%–60%), respectively. Reticulocyte counts were elevated to 412.0 THO/uL (reference range, 19.0–106.0 THO/uL). All of these values were consistent with mild fetal anemia and HDFN. A direct antiglobulin test was performed on a capillary specimen from the neonate, which was positive (3+). The baby's type was determined to be A Rh positive, with mixed-field agglutination observed in the A and D forward serology, consistent with one O Rh negative unit given via intrauterine transfusion. Capillary red blood cells also typed positive for the s antigen, consistent with the phenotype of the presumed father. Elution of the neonatal red blood cells revealed circulating maternal anti-U IgG, the likely cause of the HDFN. The mother requested that bilateral tubal ligation be performed after delivery to prevent future pregnancy. No further transfusion services were necessary beyond the postdelivery testing.

Discussion

Anti-U is a clinically significant antibody that may complicate the need for blood in the case of neonatal or maternal transfusion. When African or African American pregnant women present with alloantibodies to high-frequency antigens, anti-U should be investigated as a probable cause.[10] Treating HDFN and preventing loss of the fetus can be challenging, but strategies for management and successful delivery in patients with HDFN are being studied to lessen the need for rare blood units during pregnancy. Two strategies that exist are autologous or familial donations of antigen-negative blood and a new drug named nipocalimab that is currently in phase 2 clinical trials for treating HDFN without transfusion intervention.

Retrieving allogeneic rare blood units of any kind can often be fraught with difficulty. Most rare blood units are frozen and require deglycerolization before transfusion. In addition, local U-negative donors may not be available to fill the need at a moment's notice when all available supplies of this blood have been exhausted.

In addition to the time necessary to physically retrieve, thaw, and deliver rare units, the possibility exists for rare units to be damaged or broken during the thawing process. The deglycerolization process also places additional time restrictions on the availability of the unit. The expiry of a thawed and deglycerolized unit becomes a scant 24 hours from the time of thawing, if performed in an open system.[3] If thawed too early, the unit runs the risk of expiring before the labor and delivery process. Thawing too late may impede the availability of a blood unit critical to the patient's care. Because of the unpredictable nature of perinatal events, the acquisition and transfusion of any rare unit require significant coordination on the part of the blood suppliers, the transfusion service, and the clinical team.

With respect to this particular case report, while the patient was admitted to the labor floor at the facility in preparation for cesarean section, another patient with anti-U in labor also arrived separately. Patient 2 was hemorrhaging and required the use of all of the units that had

Downloaded from https://academic.oup.com/labmed/article/53/4/e79/6427761 by guest on 28 February 2025

been set aside for Patient 1 and the neonate. Fortunately, new nonfrozen U-negative units became available for Patient 1, with 1 additional frozen unit on hold with the blood supplier. Delivery was uncomplicated for Patient 1, and the additional nonfrozen U-negative units were not needed. Two of the U-negative units dispensed to Patient 2 went unused and were disposed of, because of a premature request for deglycerolization by the clinical team and changes to patient transfusion needs.

As evidenced by this case report, the supply of rare blood can be precarious. The cost of processing, storage, and shipping all factor into the price and availability of these unique products. Fortunately, strategies do exist to mitigate these problems throughout pregnancy and birth. Physicians should be made aware of the importance of autologous donation, and this method should be offered before receipt of allogeneic products whenever possible. Maternal autologous donations can be used for intrauterine transfusion and postpartum hemorrhage and can be collected from the mother before delivery to supplement U-negative neonatal blood needs. As long as the minimum maternal hemoglobin is 11 g/dL and the hematocrit meets or exceeds 33% (per current AABB standards for autologous donation), this option is available for alloimmunized patients preparing for delivery. In addition, units donated from family members of antigen-negative patients may also prove to be of value, which can become an outstanding resource for a family when blood supplies are limited. Siblings and other family members are sometimes encouraged to donate in these cases.

Fresh red blood cell units are the preferred units for neonatal transfusion.[3] Autologous units from healthy U-negative mothers approaching their delivery dates are fresh and readily available, thereby decreasing the amount of potassium present in the units, which may improve clinical outcomes in the event of massive neonatal transfusion. Although ABO compatibility should always be considered in matters of transfusion, the uniquely underdeveloped immune systems of fetuses and neonates have been shown to accept maternal blood transfusion as inherently compatible regardless of ABO mismatch.[11] In addition to being more readily available, autologous units used for the mother's blood needs may eliminate concerns for transfusion-transmitted infections or further sensitization to foreign allogeneic blood group antigens. Although maternal plasma contains maternal antibodies, a precedent exists for separating maternal erythrocytes and washing them to remove plasma before transfusion into the neonate.[1] And although the pediatric red cell units derived from the maternal units will have an expiration of 24 hours once washed, this process still eliminates the previously mentioned issues along with the protracted process of locating and retrieving rare blood donations from allogeneic sources.

However, the future of HDFN may no longer lie with intrauterine transfusion at all. New drug developments are in the pipeline for alleviating HDFN in sensitized mothers. Nipocalimab is an experimental drug originally designed for the treatment of myasthenia gravis and warm autoimmune hemolytic anemia. The drug targets Fc receptors in vivo to blockade the circulation of IgG, the class of Ig receptor that causes the most damage in warm autoimmune hemolytic anemia (WAIHA) and HDFN.[12]

The neonatal Fc receptor (FcRn) is a protein specific to the placenta that helps transport maternal IgG to the fetus.[13] In healthy and immunologically compatible mothers and fetuses, the passive transfer of maternal IgG works to protect the fetus from potential disease by providing passive immunity against conditions to which the mother is already fully immune. When pathogenic, the mechanism of transplacental IgG transfer becomes hazardous to the fetus, which the mother's immune system recognizes as nonself.[14]

In blocking FcRn for the duration of fetal development starting at 14 weeks gestation, nipocalimab works to prevent further damage to fetal cells by maternal antibodies. The use of the drug, which was originally developed to target the receptors causing myasthenia gravis, was extended through the UNITY clinical trial to study its effects on HDFN.[13] Although this therapy shows great promise for the prevention of HDFN, note that any interventional measures should be undertaken early in pregnancy, when severe disease can be readily mitigated. Whereas full approval for the drug has not yet been granted by the U.S. Food & Drug Administration (FDA), nipocalimab has been given a rare pediatric disease designation by the FDA, which is a voucher that allows the drug to be prioritized for review and approval in the event of successful clinical trial data.[15] If successful, this drug may prevent the overwhelming majority of patients with HDFN cases resulting from any antibody specificity and limit the need for intrauterine transfusion or transfusion support after birth for neonates affected by HDFN.

Alternatives to the allogeneic transfusion of rare blood units should be encouraged in pregnant women exhibiting antibodies to high-frequency antigens for which blood needs may be difficult, or even impossible, to meet. The strain on the blood supply when suppliers are charged with the task of acquiring rare units cannot be overstated. Fortunately, this strain may be ameliorated through vigilant clinical monitoring and the presentation of creative alternatives for meeting the transfusion needs of this unique population. In the future, intrauterine transfusion for treatment of HDFN may even become a thing of the past.

REFERENCES

1. Adam S, Lombaard H. Autologous intrauterine transfusion in a case of anti-U. *Transfusion.* 2016;56(12):3029–3032.
2. Win N, Almusawy M, Fitzgerald L, Hannah G, Bullock T. Prevention of hemolytic transfusion reactions with intravenous immunoglobulin prophylaxis in U- patients with anti-U. *Transfusion.* 2019;59(6):1916–1920.
3. Cohn CS, Delaney M, Johnson ST, Katz LM. *Technical Manual.* Bethesda, MD: AABB Press; 2020.
4. Peyrard T, Lam Y, Saison C, et al. Anti-U-like as an alloantibody in S-s-U- and S-s-U+(var) black people. *Transfusion.* 2012;52(3):622–628.
5. Wiener AS, Unger LJ, Gordon EB. Fatal hemolytic transfusion reaction caused by sensitization to a new blood factor U: report of a case. *JAMA.* 1953;153(16):1444–1446.
6. Issitt PD. Heterogeneity of anti-U. *Vox Sang.* 1990;58(1):70–71.
7. Chiaroni J, Touinssi M, Mazet M, De Micco P, Ferrera V. Adsorption of autoantibodies in the presence of LISS to detect alloantibodies underlying warm autoantibodies. *Transfusion.* 2003;43(5):651–655.
8. Judd WJ, Johnson ST, Storry J, Judd WJ. *Judd's Methods in Immunohematology.* Bethesda, MD: AABB Press; 2008.
9. Buren NL. Perinatal transfusion medicine. In: Shaz BH, Hillyer CD, Gil MR, eds. *Transfusion Medicine and Hemostasis: Clinical and Laboratory Aspects,* 3rd ed. Amsterdam, the Netherlands: Elsever; 2019: 301–312.
10. Esteves AR, Artur FS, Miranda CF, Olga SP, Sá FF, Gomes VA. Hemolytic disease of the newborn caused by anti-U: a case report. *Global J Med Clin Case Rep.* 2020;7(1):10–12.

Downloaded from https://academic.oup.com/labmed/article/53/4/e79/6427761 by guest on 28 February 2025

11. Gokhale S, Gokhale S. Transfusing maternal blood to her newborn baby—irrespective of ABO mismatch. *J Matern Fetal Neonatal Med.* 2020;33(9):1593–1606.
12. Keller CW, Pawlitzki M, Wiendl H, Lünemann JD. Fc-receptor targeted therapies for the treatment of myasthenia gravis. *Int J Mol Sci.* 2021;22(11):5755.
13. Castleman JS, Moise KJ Jr, Kilby MD. Medical therapy to attenuate fetal anaemia in severe maternal red cell alloimmunisation. *Br J Haematol.* 2021;192(3):425–432.
14. Simon TL, McCullough J, Snyder EL, Solheim BG, Strauss RG, Rossi EC. *Rossi's Principles of Transfusion Medicine.* Chichester, West Sussex, UK: Wiley-Blackwell; 2016.
15. U.S. Food & Drug Administration. *Rare pediatric disease (RPD) designation and voucher programs*. Updated November 2, 2017. https://www.fda.gov/industry/developing-products-rare-diseases-conditions/rare-pediatric-disease-rpd-designation-and-voucher-programs. Accessed October 5, 2021.

Downloaded from https://academic.oup.com/labmed/article/53/4/e79/6427761 by guest on 28 February 2025

CASE STUDY

IgM Warm Autoantibodies Causing Autoimmune Hemolytic Anemia in a Pediatric Patient

Precious Fortes, MD, MHS[1], Janet Baez, MLS(ASCP)[CM1], Andrea M. McGonigle, MD[1], Alyssa Ziman, MD[1], Noah Federman, MD,[2,3] Dawn C. Ward, MD[1,]*

[1]Wing-Kwai and Alice Lee-Tsing Chung Transfusion Service, Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California, US, [2]Division of Pediatric Hematology/Oncology, Department of Pediatrics, David Geffen School of Medicine at UCLA, Los Angeles, California, US, [3]Department of Orthopaedics, David Geffen School of Medicine at UCLA, Los Angeles, California, US; *To whom correspondence should be addressed. dward@mednet.ucla.edu

Keywords: autoimmune hemolytic anemia, warm autoantibodies, IgM autoantibodies, warm hemolysin, anemia, pediatrics

Abbreviations: AIHA, autoimmune hemolytic anemia; LD, lactate dehydrogenase; DAT, direct antiglobulin test; RBCs, red blood cells; TAMP, thermal amplitude; IVIG, intravenous immunoglobulin; RESt, rabbit erythrocyte stroma; IAT, indirect antiglobulin test; CT, control.

Laboratory Medicine 2022;53:e83–e86; https://doi.org/10.1093/labmed/lmab101

ABSTRACT

Most often, IgM-mediated autoimmune hemolytic anemia (AIHA) presents as cold agglutinin disease in the pediatric population. The IgM warm agglutinins are rare, with few reports in the literature. This case study describes a 5 year old girl with nausea, abdominal pain and jaundice, and a hemoglobin of 5.5 g/dL who was diagnosed with a warm reactive IgM AIHA. The laboratory workup revealed a pan-reactive antibody and a direct antiglobulin test negative for IgG and C3. A thermal amplitude assay revealed reactive IgM antibodies at 37°C, 30°C, 25°C, and 4°C and an antibody titer of 1:8. An adsorption for IgM-specific autoantibodies exposed underlying anti-E and anti-Cw alloantibodies. Transfusion of phenotypically matched red blood cell units supported ongoing hemolysis. The AIHA treatment included steroids followed by rituximab with complete resolution. A literature review shows variable outcomes for warm AIHA in the pediatric population and often describes the presence of warm reactive IgM-mediated AIHA as an indicator for poor prognosis.

Clinical History

A 5 year old girl with a history of biliary atresia status post-Kasai who underwent cadaveric segmental liver transplant 10 months earlier presented to her gastrointestinal clinic and was noted to have increased fatigue and jaundice and a notable decrease in oral intake with normal urinary output. She denied a history of headaches, chest pains, and edema. Laboratory workup revealed hematocrit 15% (34.0%–40.0%), total bilirubin 4.1 mg/dL (<0.4 mg/dL), lactate dehydrogenase (LD) 326 U/L, and decreased haptoglobin (<8 mg/dL). Upon these findings, she was sent to the emergency department.

During the hospital admission, the patient's laboratory values were confirmed. In addition, her absolute reticulocyte count was 0.21 × 10^6/μL (0.04–0.07 × 10^6/μL) and a peripheral blood smear showed microspherocytes. Blood type and screen tests indicated that the patient was B-positive with a positive antibody screen that revealed a 3+ to 4+ pan-reactive antibody panel, as shown by the reactivity score of negative for no reactivity out of 4+ representing strong reactivity (**FIGURE 1**). A direct antibody test (DAT) performed using tube technique and with no enhancement was negative for both IgG and C3. However, a DAT performed using gel column agglutination technology (Ortho-Clinical Diagnostics, Raritan, NJ), showed 2+ reactivity. The eluate showed varying reactivity (negative to 3+) with a panel of reagent red blood cells (RBCs; **FIGURE 1**). A thermal amplitude (TAMP) assay revealed strongly reactive 4+ IgM autoantibodies at 37°C, 30°C, 25°C, and 4°C and an antibody titer of 1:8. Underlying anti-E and anti-Cw alloantibodies were detected in addition to the IgM warm autoantibody.

The patient was transfused with 2 B-negative, E-negative, c-negative, and K-negative packed RBC units. Her autoimmune hemolytic anemia (AIHA) was initially managed with an intravenous steroid burst (0.5 mg/kg) and an oral prednisolone dose, which was increased to a dose of 4 mg/kg/day without improvement. She was then given weekly rituximab infusions (375 mg/m^2) for 4 weeks, with complete resolution of hemolysis.

Clinical and Laboratory Information

Routine ABO/Rh typing was performed using an automated solid-phase RBC adherence assay (Immucor Inc., Norcross, GA). The antibody workup, including a DAT, was performed using column agglutination technology. A DAT using polyclonal antihuman globulin reagent and C3 reagents, the TAMP assay, and antibody titers were performed according to guidelines listed in the AABB technical manual.[1]

We performed a 3-cell screen panel with the patient's plasma in saline IgG and prewarmed it in an effort to resolve the pan-reactive antibody panel. The panel showed variable pan-reactivity—1+ to 4+, and m+ to 3+, respectively—suggestive of the presence of IgG alloantibodies (**FIGURE 2**). Likewise, based on the patient's active

© The Author(s) 2021. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/e83/6449181 by guest on 28 February 2025

FIGURE 1. Antibody identification panel with eluate and RESt: pan-reactive IAT and 2+ reactive DAT. Eluate showed reactivity for the Cw cells and E cells, highlighted in blue, and extra reactivity. RESt showed the anti-Cw antibody, highlighted in purple. DAT, direct antibody test; IAT, indirect antiglobulin test; RESt, rabbit erythrocyte stroma.

	RhHr							Kell				Duf		Kid		X	Lew		MNS				P	Lut		Patient Plasma		
Cell #	D	C	E	c	e	f	Cw	K	k	Kpa	Kpb	Fya	Fyb	Jka	Jkb	Xga	Lea	Leb	S	s	M	N	P1	Lua	Lub	IAT	Eluate	RESt
1	+	+	0	0	+	0	+	0	+	0	+	0	+	0	+	0	0	+	0	+	+	+	+	0	+	4+	2+	2+
2	+	+	0	0	+	0	0	+	+	0	+	+	0	+	+	+	0	+	+	0	+	0	0	0	+	4+	1+	0
3	+	0	+	+	0	0	0	0	+	0	+	0	+	0	+	+	0	+	+	+	+	0	+	0	+	4+	3+	NT
4	+	0	0	+	+	+	0	0	+	0	+	+	0	+	0	+	0	0	+	+	0	+	+	0	+	3+	0	0
5	0	+	0	+	+	+	0	0	+	0	+	+	+	+	0	+	0	+	+	+	+	0	+	0	+	4+	0	0
6	0	0	+	+	+	+	0	0	+	+	+	0	+	+	0	0	+	0	+	+	0	+	+	0	+	4+	3+	NT
7	0	0	0	+	+	+	0	+	+	0	+	0	+	+	0	0	0	+	0	+	+	+	0	0	+	4+	2+	0
8	0	0	0	+	+	+	0	0	+	0	+	+	+	0	+	+	+	0	0	+	+	0	+	0	+	4+	2+	0
9	0	0	0	+	+	+	0	0	+	0	+	0	0	+	+	+	0	+	0	+	0	+	+	0	+	4+	0	0
10	0	0	0	+	+	+	0	0	+	0	+	0	0	+	+	0	0	0	+	0	+	+	+	0	+	4+	2+	0
11	+	+	0	0	+	0	0	0	+	0	+	0	+	+	+	0	+	0	0	+	+	0	+	0	+	4+	1+	0
DAT																										2+		

FIGURE 2. Three-cell screen panel. Saline IgG showed variable pan-reactivity with strong reactivity of Cell II. Prewarmed plasma showed variable reactivity, microscopic reactivity for Cells I and II, and 3+ reactivity Cell II. RESt with 3 absorptions showed the reactivity of Cell II.

Cell	Patient plasma		
	Saline IgG	Prewarmed	RESt (x3)
I	1+	m+	0
II (E+)	4+	3+	3+
III	1+	m+	0

hemolysis and lack of evidence to support an IgG warm autoantibody, a TAMP was performed considering the discrepant results between the patient's positive DAT using column agglutination technology and the negative DAT performed in tube. The TAMP was strongly reactive (4+) for IgM antibodies at temperatures of 37°C, 30°C, 25°C, and 4°C (**FIGURE 3**). The IgM antibody titer measured 1:8. The IgM-specific autoantibodies were adsorbed with rabbit erythrocyte stroma (RESt, Immucor, Inc., Norcross, GA). thus exposing underlying IgG alloantibodies with specificity to the E and Cw antigens (**FIGURE 3**).

We transfused packed RBC units phenotypically matched for the patient's Rh (negative for the E and c antigens) and Kell (negative for the K antigen) blood groups per our standard operating procedure for blood selection for patients with warm autoantibodies and underlying alloantibodies. We did not actively select RBC units negative for Cw based upon the low prevalence of Cw antigens of 2% of the population.[1] However, we completed a serologic cross-match to enhance patient safety in the presence of the anti-Cw; units deemed incompatible were not provided for transfusion.

Discussion

Warm AIHA is rare within the pediatric population, with case reports in childhood usually being associated with other underlying conditions such as congenital immunodeficiency syndrome, postliver transplantation,[2] or Evans syndrome or usually presenting as cold agglutinin disease.[2-5] Specifically, IgM warm agglutinins are especially rare within the pediatric population, with limited cases reported.[3,4]

A review of the literature on IgM-mediated warm AIHA in the pediatric population is listed in **TABLE 1** and shows variable clinical outcomes of 26 pediatric patients identified from 1987 to 2021 included 4 patients who consequently had a fatal event and 22 patients with recovery or ongoing hemolysis. Three of the cases with a fatal event were notable for 2 children with underlying conditions including Evans syndrome and severe combined immunodeficiency, and in only 1 of the fatal cases described was the patient treated with rituximab. All of the pediatric patients were treated with steroids plus an additional intervention (blood transfusions, intravenous immunoglobulin (IVIG), rituximab, other medications, and even splenectomy). The presence of warm reactive IgM autoantibodies in AIHA has been noted to be an indicator for poor prognosis.[6-9]

Research has shown that AIHA is caused by the production of autoantibodies that lead to the destruction of RBCs reacting at an optimal temperature at 37°C. Diagnosing AIHA can be challenging, given that the clinical signs and symptoms can present nonspecifically and are common to all types of hemolytic anemias including those with nonimmune causes. The amount and type of antibody present also vary in clinical presentation, with small amounts causing mild anemia and specific types like IgM autoagglutinins notable for more severe hemolysis and higher mortality.[6-9]

Establishing a diagnosis of AIHA is based on multiple laboratory identification techniques including the DAT, which detects autoantibodies and/or complement coating the surface of circulating RBCs. There are also "DAT-negative" AIHAs reported in the literature that are estimated at 3% to 10%.[13,14]

For our pediatric patient, given the clinical symptoms of jaundice and a rapid drop in hemoglobin without a source of bleeding, increased LD, and decreased haptoglobin level, hemolytic anemia was high on the differential. The diagnosis of a warm AIHA was confirmed with the transfusion service workup. The IgM warm agglutinin was identified using common immunohematology methods. An additional adsorption to remove IgM warm agglutinins was necessary to reveal underlying IgG anti-E and anti-Cw alloantibodies. The presence of warm IgM likely explained the severity of the anemia and her clinical course. Unlike typical warm-IgMmediated AIHA, warm IgM-mediated AIHA is often steroid refractory and is not responsive to IVIG.[13,14]

Downloaded from https://academic.oup.com/labmed/article/53/4/e83/6449181 by guest on 28 February 2025

FIGURE 3. Thermal amplitude. Strongly reactive (4+) for IgM antibodies at temperatures of 37°C, 30°C, 25°C, and 4°C. CT, control.

Temperature	Cell I	Cell II	Cell III	O cord cell	Auto CT
Room Temp.	4+	4+	4+	4+	4+
30°C	4+	4+	4+	4+	3+
37°C	4+	4+	4+	4+	4+
4°C	4+	4+	4+	4+	4+

TABLE 1. Pediatric Patients with IgM warm AIHA

Study	Clinical Presentation	Outcome
Salama and Mueller-Eckhardt, 1987[9]	12 children (7 females and 5 males), ranging in age from 2 days to 6 years with therapy ranging from blood transfusion to prednisone	1 of 12 had ongoing hemolysis
Garratty et al, 1997[6]	15 year old male with history of recent dizziness and fatigue, treated with steroids, IVIG, exchange transfusion	Recovered
Takahashi et al, 2016[7]	11 year old male with no history, died 3 days after admission, received oxygenation and treated with haptoglobin and prednisolone	Fatal
Friedmann et al, 1998[8]	9 year old female with Evans syndrome treated with steroids, IVIG, cytoxan, mycophenolate mofetil, cyclosporin exchange	Fatal
Nowak-Wegrzyn et al, 2001[10]	11 month old female with SCID treated with exchange, rituximab; resulted in multiple cerebral vascular accidents	Fatal
Wakim et al, 2004[11]	6 year old female with common variable immunodeficiency treated with IVIG, steroids, rituximab, splenectomy	Recovered
Schäppi et al, 2008[4]	16 month old male with liver transplant treated with steroids, IVIG, rituximab	Recovered
Arndt et al, 2009[5]	Reported series of 49 patients of whom 6 were pediatric, including 1 female (age range, 0–10 years), 1 female (age range, 11–20 years), and 4 males (age range, 11–20 years); details of clinical presentation not available	1/6 fatal
Branstetter et al, 2015[3]	3 month old female with no significant history presented with hemoglobin 3.5 g/dL; treated with steroids and IVIG	Recovered
Ajmi et al, 2017[12]	10 year old girl with warm AIHA caused by mixed IgA, IgG, IgM, and C3d autoantibodies; treated with steroids, IVIG, rituximab.	Recovered

AIHA, autoimmune hemolytic anemia; IVIG, intravenous immunoglobulin; SCID, severe combined immunodeficiency.

Refractory AIHA is a severe disease and often requires other modes of therapy including rituximab infusion, which has been described in other case series as a treatment for pediatric patients with warm AIHA.[3,4,13] Rituximab is a monoclonal antibody directed against the CD-20 antigens on B-cells; administration results in antibody-mediated cytotoxicity leading to the destruction of B-cells.[14] Our patient was successfully treated using a course of rituximab with complete resolution.

Patient Follow-Up

The patient was readmitted 29 days after her initial admission with abdominal pain, nausea, and vomiting of unclear etiology. Pediatric hematology and oncology was consulted given her history of IgM warm reactive AIHA. Laboratory studies with this admission included hemoglobin 11.8 g/dL, LD 238 U/L, and haptoglobin 127 mg/dL; these laboratory values were not suggestive of ongoing hemolysis. At the time of the second admission, the patient had completed 3 of the 4 weekly rituximab doses and tolerated a steroid wean as an outpatient. She was admitted on 1 mg/kg/day prednisone and was weaned by 10% during her admission, then weaned 10% weekly. Before being discharged, the patient received her fourth and final dose of rituximab. The patient also continued to receive IVIG treatment that was started several months before her AIHA diagnosis because of de novo donor-specific antibodies and continued ongoing IVIG for replacement therapy every 4 to 6 weeks. The patient has been doing well since the last follow-up and has had no recurrence of IgM warm reactive AIHA.

REFERENCES

1. Fung MK, Grossman BJ, Hillyer CD, Westhoff CM. *Technical Manual,* 18th ed. Bethesda, MD: AABB; 2014.
2. Li M, Goldfinger D, Yuan S. Autoimmune hemolytic anemia in pediatric liver or combined liver and small bowel transplant patients: a case series and review of the literature. *Transfusion.* 2012;52(1):48–54.
3. Branstetter CN, Hankins JS, Moreau D, Nottage KA. Severe autoimmune hemolytic anemia in an infant caused by warm-reactive IGM and IGA autoantibodies: a case report and review of the literature. *J Pediatr Hematol Oncol.* 2015;37(6):468–471.
4. Schäppi MG, Ozsahin H, Peyrard T, et al. Severe autoimmune hemolytic anemia in a liver transplanted child. *Pediatr Transplant.* 2008;12(7):809–812.
5. Arndt PA, Leger RM, Garratty G. Serologic findings in autoimmune hemolytic anemia associated with immunoglobulin M warm autoantibodies. *Transfusion.* 2009;49(2):235–242.
6. Garratty G, Arndt P, Domen R, et al. Severe autoimmune hemolytic anemia associated with IgM warm autoantibodies directed against determinants on or associated with glycophorin A. *Vox Sang.* 1997;72(2):124–130.
7. Takahashi H, Tanaka F, Sakuma H, Sato M, Inaba S, Kai S. Fatal warm autoimmune hemolytic anemia in a child due to IgM-type autoantibodies. *Pediatr Int.* 2016;58(8):744–746.
8. Friedmann AM, King KE, Shirey RS, Resar LM, Casella JF. Fatal autoimmune hemolytic anemia in a child due to warm-reactive immunoglobulin M antibody. *J Pediatr Hematol Oncol.* 1998;20(5):502–505.
9. Salama A, Mueller-Eckhardt C. Autoimmune haemolytic anaemia in childhood associated with non-complement binding IgM autoantibodies. *Br J Haematol.* 1987;65(1):67–71.
10. Nowak-Wegrzyn A, King KE, Shirey RS, Chen AR, McDonough C, Lederman HM. Fatal warm autoimmune hemolytic anemia resulting from IgM autoagglutinins in an infant with severe combined immunodeficiency. *J Pediatr Hematol Oncol.* 2001;23(4):250–252.

Downloaded from https://academic.oup.com/labmed/article/53/4/e83/6449181 by guest on 28 February 2025

11. Wakim M, Shah A, Arndt PA, et al. Successful anti-CD20 monoclonal antibody treatment of severe autoimmune hemolytic anemia due to warm reactive IgM autoantibody in a child with common variable immunodeficiency. *Am J Hematol.* 2004;76(2):152–155.

12. Ajmi H, Mabrouk S, Hassayoun S, et al. Success of anti-CD20 monoclonal antibody treatment for severe autoimmune hemolytic anemia caused by warm-reactive immunoglobulin A, immunoglobulin G, and immunoglobulin M autoantibodies in a child: a case report. *J Med Case Rep.* 2017;11(1):321.

13. Losos M, Hamad D, Joshi S, Scrape S, Chen J. Warm autoimmune hemolytic anemia and direct antiglobulin testing with a false-negative result in a 53-year-old man: the DAT will set you free. *Lab Med.* 2016;47(3):227–232.

14. Segel GB, Lichtman MA. Direct antiglobulin ("Coombs") test-negative autoimmune hemolytic anemia: a review. *Blood Cells Mol Dis.* 2014;52(4):152–160.

Downloaded from https://academic.oup.com/labmed/article/53/4/e83/6449181 by guest on 28 February 2025

CASE STUDY

Identification of a Cryptic t(8;20;21)(q22;p13;q22) Resulting in *RUNX1T1/RUNX1* Fusion in a Patient with Newly Diagnosed Acute Myeloid Leukemia

Erica L. Macke, PhD,[1] Reid G. Meyer, BS,[2] Nicole L. Hoppman, PhD,[2] Rhett P. Ketterling, MD,[3] Patricia T. Greipp, DO,[3] Xinjie Xu, PhD,[3] Linda B. Baughn, PhD,[3] Danielle A. Shafer, DO,[4] Rui R. He, MD,[5] Jess F. Peterson, MD[3,]*

[1]Center for Individualized Medicine, Mayo Clinic, Rochester, Minnesota, US, [2]Division of Laboratory Genetics and Genomics, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, US, [3]Division of Hematopathology, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, US, [4]Inova Schar Cancer Institute, Inova Fairfax Hospital, Falls Church, Virginia, US, [5]Department of Pathology, Inova Fairfax Hospital, Falls Church, Virginia, US; *To whom correspondence should be addressed. peterson.jess@mayo.edu

Keywords: *RUNX1T1*, *RUNX1*, acute myeloid leukemia, cryptic translocation, fluorescence in situ hybridization, conventional chromosome studies

Abbreviations: AML, acute myeloid leukemia; FISH, fluorescence in situ hybridization; ABMGG, American Board of Medical Genetics and Genomics; D-FISH, dual-fusion fluorescence in situ hybridization.

Laboratory Medicine 2022;53:e87–e90; https://doi.org/10.1093/labmed/lmab105

ABSTRACT

The detection of recurrent genetic abnormalities in acute myeloid leukemia (AML), including *RUNX1T1/RUNX1* gene fusion, is critical for optimal medical management. Herein, we report a 45 year old woman with newly diagnosed AML and conventional chromosome studies that revealed an apparently balanced t(8;20)(q22;p13) in all 20 metaphases analyzed. A *RUNX1T1/RUNX1* dual-color dual-fusion fluorescence in situ hybridization (FISH) probe set was subsequently performed and revealed a *RUNX1T1/RUNX1* gene fusion. Metaphase FISH studies performed on abnormal metaphases revealed a cryptic, complex translocation resulting in *RUNX1T1/RUNX1* fusion, t(8;20;21)(q22;p13;q22). This case study shows the importance of performing FISH studies or other high-resolution genetic testing concurrently with conventional chromosome studies for the detection of cryptic recurrent gene fusions in AML, particularly a focused genetic evaluation such as *RUNX1T1/RUNX1* gene fusion, when specific abnormalities involving 8q22 are identified.

Clinical History and Hematopathologic Evaluation

A 45 year old woman with a history of endometriosis presented to the emergency department with 1 week of vaginal bleeding, fatigue, and dyspnea. A complete blood count revealed anemia (hemoglobin, 4.3 g/dL; reference, 11.4–14.8 g/dL), thrombocytopenia (platelet count, 22 × 10^3/μL; reference, 142–346 × 10^3/μL), and leukocytosis (white blood cell count, 52 × 10^3/μL; reference, 3.1–9.5 × 10^3/μL) with circulating blasts.

A subsequent bone marrow evaluation indicated a markedly hypercellular bone marrow (approximately 100%) with marrow spaces essentially replaced by sheets of large blasts with fine chromatin and prominent nucleoli (**FIGURE 1A** and **1B**). Minimal background myeloid, erythroid, and megakaryocytic precursors were observed. The bone marrow touch preparation revealed scattered clusters of large blasts with a high N:C ratio and occasional monocytic features. The differential cell count performed on a limited number of cells showed 85% blasts, 5% granulocytic cells, 7% erythroid cells, and 3% lymphocytes. The granulocytic cells showed left-shifted maturation, and many of them showed dysplastic features with hypogranular cytoplasm and abnormally shaped nuclei. Flow cytometric analysis of the bone marrow aspirate showed a large population of blasts (98% of analyzed events) in the dim CD45 and low to moderate side-scatter regions. The blasts were positive for CD33, CD117, CD34, CD15, CD11b, CD123, CD14 (dim), CD56, HLA-DR, CD38, and myeloperoxidase (**FIGURE 1C**). Taken together, these findings were consistent with a diagnosis of acute myeloid leukemia (AML).

Materials and Methods

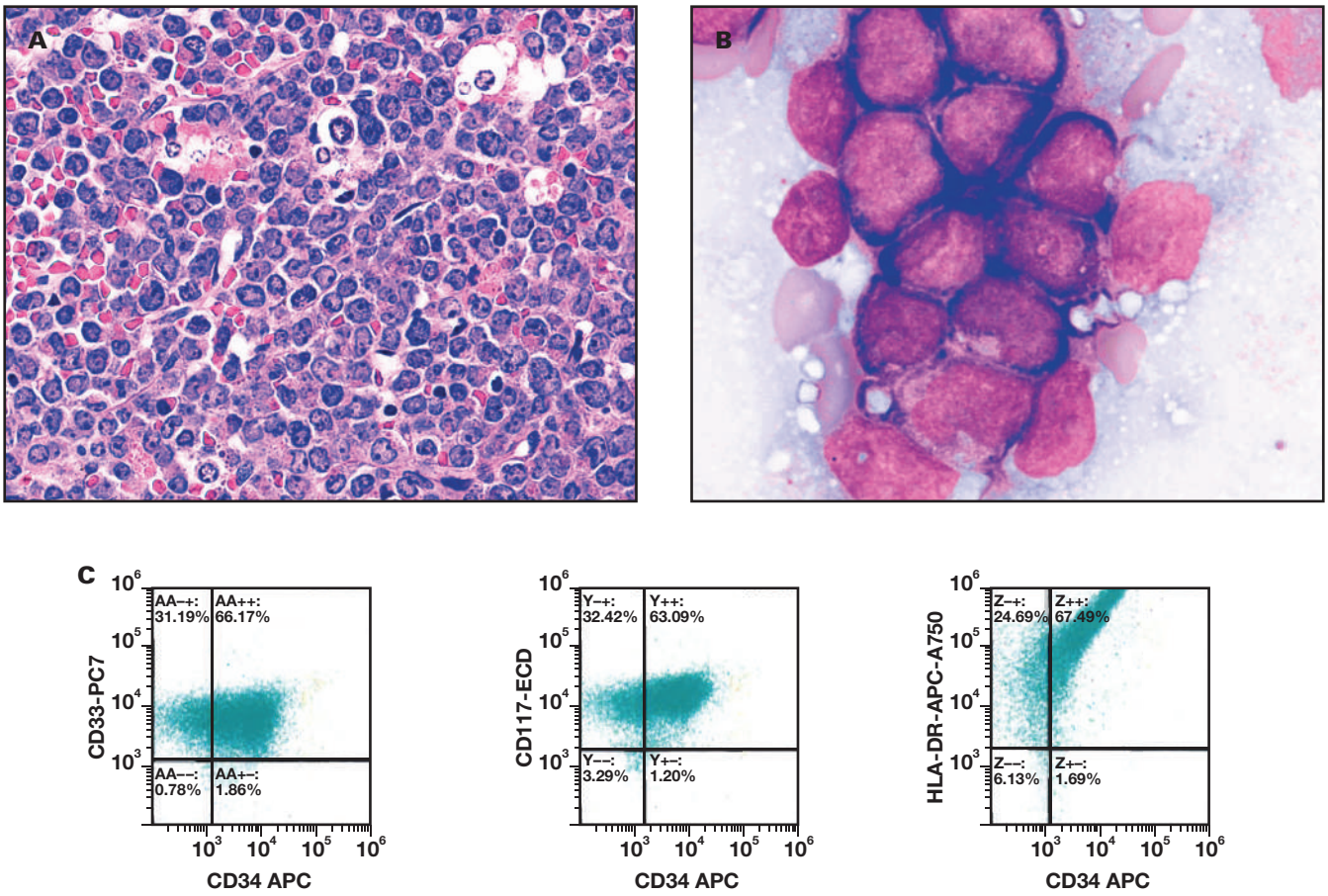
Conventional Chromosome Analysis

Cells from the bone marrow aspirate specimen were cultured, harvested, and banded utilizing standard cytogenetic techniques according to specimen-specific protocols. Twenty metaphases were analyzed by 2 qualified clinical cytogenetic technologists and interpreted by a board-certified (American Board of Medical Genetics and Genomics [ABMGG]) clinical cytogeneticist.

© The Author(s) 2021. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/e87/6430063 by guest on 28 February 2025

FIGURE 1. Morphologic and immunophenotypic findings. (A) Bone marrow core biopsy (H&E, 400×) showing numerous intermediate to large blasts and minimal residual hematopoietic cells. (B) Bone marrow aspirate (Wright stain, 100×, oil) showing cluster of large blasts with high N:C ratio, fine chromatin, and prominent nucleoli. (C) Flow cytometric analysis of bone marrow aspirate. The dot plots show a large population of myeloblasts (blue) expressing CD33, CD34, CD117, and HLA antigen–DR. The blasts were also positive for CD11b, CD14 (dim), CD15 (dim), CD123 (dim), CD56, and myeloperoxidase (data not shown). H&E, hematoxylin and eosin.



Fluorescence In Situ Hybridization

The *RUNX1T1/RUNX1* dual-color dual-fusion fluorescence in situ hybridization (D-FISH) probe set (Abbott Molecular, Abbott Park, IL) was performed on the diagnostic bone marrow aspirate specimen. The specimen was subjected to standard FISH pretreatment, hybridization, and fluorescence microscopy according to specimen-specific protocols. Five hundred interphase nuclei were analyzed by 2 qualified clinical cytogenetic technologists and interpreted by a board-certified (ABMGG) clinical cytogeneticist.

Results and Discussion

Conventional cytogenetic results were initially interpreted as a female karyotype with an apparently balanced t(8;20)(q22;p13) in all 20 metaphases examined (**FIGURE 2**). No apparent structural abnormalities involving either copy of chromosome 21 were observed. The *RUNX1T1/RUNX1* D-FISH probe set was performed and identified 2 red (*RUNX1T1*) signals, 2 green (*RUNX1*) signals, and a single yellow fusion signal representing *RUNX1T1/RUNX1* gene fusion in 95% of the interphase nuclei (**FIGURE 3**). To further characterize the seemingly discordant chromosome and interphase FISH results, metaphase FISH studies using the *RUNX1T1/RUNX1* D-FISH probe set were performed on abnormal metaphases harboring the t(8;20)(q22;p13) (**FIGURE 4A**). The 2 red *RUNX1T1* signals hybridized to 8q22 and 20p13, the 2 green *RUNX1* signals hybridized to both copies of chromosome 21q22, and a single yellow *RUNX1T1/RUNX1* fusion signal was observed at 8q22 (**FIGURE 4B**). These combined results indicated the presence of a cryptic, complex t(8;20;21)(q22;p13;q22) that resulted in a *RUNX1T1/RUNX1* gene fusion at 8q22.

Research has shown that AML comprises a heterogeneous group of hematopoietic neoplasms and is characterized by a clonal expansion of myeloid precursor cells.[1,2] The t(8;21)(q22;q22) (*RUNX1T1/RUNX1*) is a recurrent genetic abnormality in AML with predominantly neutrophilic maturation and is associated with a high rate of remission and favorable long-term outcomes.[1,2] The *RUNX1T1/RUNX1* fusion is found in approximately 7% of patients with AML and is typically described in younger patients.[1-3] Although the *RUNX1T1/RUNX1* gene fusion is most commonly derived from a readily visible t(8;21)(q22;q22) by conventional chromosome analysis, detection of the fusion transcript has been reported in the absence of t(8:21), suggesting that cytogenetically

Downloaded from https://academic.oup.com/labmed/article/53/4/e87/6430063 by guest on 28 February 2025

FIGURE 2. **Conventional chromosome results. Representative karyogram showing a balanced t(8;20)(q22;p13) (arrows). This translocation was observed in all 20 metaphases analyzed. No apparent structural abnormality was observed on either chromosome 21. Interphase and metaphase FISH studies using the *RUNX1T1/RUNX1* D-FISH probe set confirmed a cryptic t(8;20;21)(q22;p13;q22). D-FISH, dual-fusion fluorescence in situ hybridization; FISH, fluorescence in situ hybridization.**

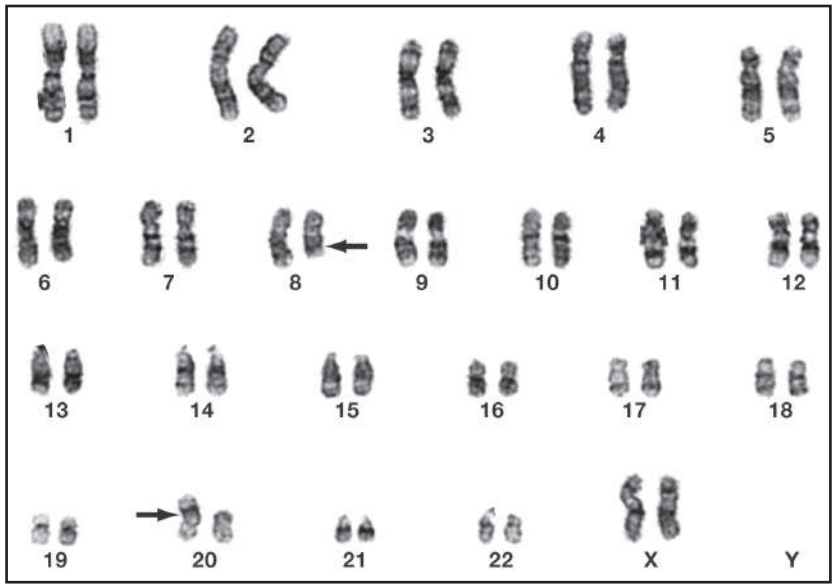
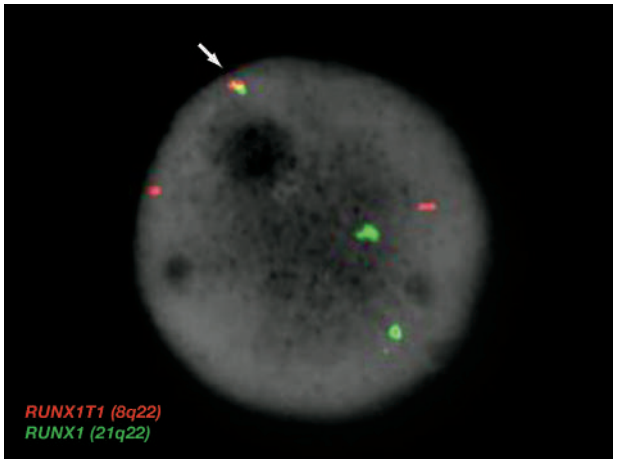


FIGURE 3. **Interphase FISH results showing *RUNX1T1/RUNX1* gene fusion. Representative interphase nucleus shows a single yellow fusion signal (*RUNX1T1/RUNX1* gene fusion; arrow), 2 green (*RUNX1*) signals, and 2 red (*RUNX1T1*) signals. This specific signal pattern (1 fusion and 2 red and green signals each), when observed using a dual-color D-FISH probe set, is indicative of a balanced 3-way translocation. D-FISH, dual-fusion fluorescence in situ hybridization; FISH, fluorescence in situ hybridization.**



cryptic alterations occur in a small percentage of patients.[4-8] Previously described cryptic aberrations resulting in *RUNX1/RUNX1T1* fusion have included small interstitial insertions, specifically the insertion of 8q material into chromosome 21q22 and the insertion of 21q material into 8q22, along with cryptic translocation events.[8,9] Rare, cryptic 3-way translocations resulting in *RUNX1T1/RUNX1* fusion have been previously described, including t(8;14;21)(q22;q32;q22), t(8;12;21)(q22.1;q24.1;q22.1), and t(8;20;21)(q22;q13;q22).[10-13] One additional report of a cryptic t(8;20;21)(q22;p13;q22) has been described in the literature, indicating that this 3-way translocation could potentially represent a rare but recurrent chromosomal abnormality.[14]

In our patient, conventional chromosome results did not reveal a readily detectable 3-way translocation involving a structurally abnormal 21q22 (the location of the *RUNX1* gene region). Suspicion of an atypical *RUNX1* rearrangement may have been considered had a structural abnormality near 21q22 been observed. Instead, what only appeared to be a balanced t(8;20)(q22;p13) was identified in all 20 metaphases. The eventual detection of a *RUNX1T1/RUNX1* gene fusion in our patient required additional interrogation of the 8q abnormality using interphase FISH studies. The interphase FISH signal pattern was pathognomonic for a 3-way translocation when we utilized a D-FISH probe strategy (2 red signals, 2 green signals, and 1 yellow fusion signal). However, metaphase FISH studies were required to elucidate the chromosomes involved in the 3-way translocation.

In summary, we report a patient with AML with a cryptic t(8;20;21)(q22;p13;q22) that was initially interpreted as t(8;20)(q22;p13). The use of a *RUNX1T1/RUNX1* D-FISH probe set identified a *RUNX1T1/RUNX1* fusion that would have likely gone undetected in the absence of FISH studies. Genetic evaluation for the *RUNX1T1/RUNX1* gene fusion should be considered when abnormalities involving 8q22 are observed in AML karyotypes.

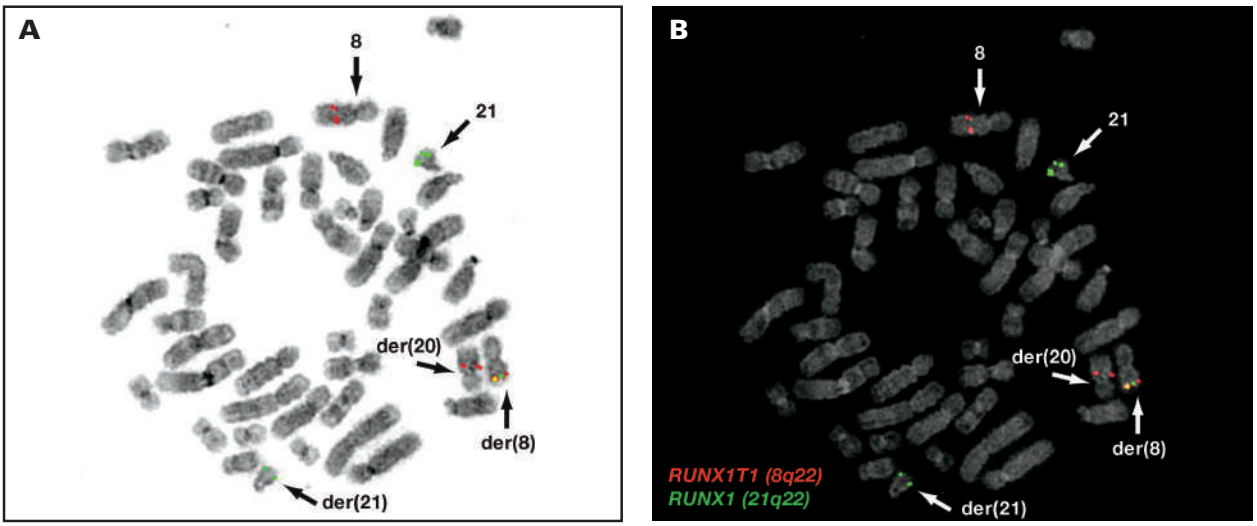
Patient Follow-Up

The patient was started on systemic "7+3" chemotherapy with cytarabine 100 mg/m^2 continuous intravenous infusion over 24 hours for days 1 to 7 and daunorubicin 90 mg/m^2 intravenously for days 1 to 3.

Initially, the patient did well during the hospital course. On approximately day 12, she became refractory to platelet transfusions. On

Downloaded from https://academic.oup.com/labmed/article/53/4/e87/6430063 by guest on 28 February 2025

FIGURE 4. Metaphase FISH results revealing a 3-way translocation and *RUNX1T1/RUNX1* gene fusion. (A) Representative inverted DAPI image of a metaphase with overlapping *RUNX1T1/RUNX1* D-FISH signal patterns. The isolated red *RUNX1T1* (8q22) signals showed hybridization on a normal chromosome 8 and the short arm of the derivative chromosome 20. The isolated green *RUNX1* (21q22) signals showed hybridization on a normal chromosome 21 and on the long arm of the derivative chromosome 21. Last, the single yellow fusion signal (representing the *RUNX1T1/RUNX1* gene fusion) was observed on the long arm of the derivative chromosome 8. Taken together, these findings confirmed a cryptic 3-way t(8;20;21)(q22;p13;q22) that resulted in a *RUNX1T1/RUNX1* gene fusion at 8q22. (B) Metaphase FISH image clearly indicating hybridization of the *RUNX1T1* and *RUNX1* FISH signals. D-FISH, dual-fusion fluorescence in situ hybridization; FISH, fluorescence in situ hybridization.



day 13, she developed headaches, and computed tomography of her head indicated a subarachnoid hemorrhage. Unfortunately, she experienced additional intracranial bleeding over days 14 to 16, ultimately resulting in neurologic compromise with brainstem herniation and death on day 17.

REFERENCES

1. Taylor J, Xiao W, Abdel-Wahab O. Diagnosis and classification of hematologic malignancies on the basis of genetics. *Blood.* 2017;130(4):410–423.
2. Arber DA, Brunning RD, Le Beau MM, et al. Acute myeloid leukaemia with recurrent genetic abnormalities. In: Swerdlow SH, Campo E, Harris NL, et al, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon, France: IARC; 2017: 130–149.
3. Jung J, Cho BS, Kim HJ, et al. Reclassification of acute myeloid leukemia according to the 2016 WHO classification. *Ann Lab Med.* 2019;39(3):311–316.
4. Krauter J, Peter W, Pascheberg U, et al. Detection of karyotypic aberrations in acute myeloblastic leukaemia: a prospective comparison between PCR/FISH and standard cytogenetics in 140 patients with de novo AML. *Br J Haematol.* 1998;103(1):72–78.
5. Mitterbauer M, Kusec R, Schwarzinger I, Haas OA, Lechner K, Jaeger U. Comparison of karyotype analysis and RT-PCR for AML1/ETO in 204 unselected patients with AML. *Ann Hematol.* 1998;76(3–4):139–143.
6. Langabeer SE, Walker H, Rogers JR, et al. Incidence of AML1/ETO fusion transcripts in patients entered into the MRC AML trials. MRC Adult Leukaemia Working Party. *Br J Haematol.* 1997;99(4):925–928.
7. Rowe D, Cotterill SJ, Ross FM, et al. Cytogenetically cryptic AML1-ETO and CBF beta-MYH11 gene rearrangements: incidence in 412 cases of acute myeloid leukaemia. *Br J Haematol.* 2000;111(4):1051–1056.
8. Gamerdinger U, Teigler-Schlegel A, Pils S, et al. Cryptic chromosomal aberrations leading to an AML1/ETO rearrangement are frequently caused by small insertions. *Genes Chromosomes Cancer.* 2003;36(3):261–272.
9. Reikvam H, Hatfield KJ, Kittang AO, Hovland R, Bruserud Ø. Acute myeloid leukemia with the t(8;21) translocation: clinical consequences and biological implications. *J Biomed Biotechnol.* 2011;2011:104631.
10. Lau LC, Koh LP, Lim TH, Loo LE, Tien SL. A cryptic three-way translocation involving chromosomes 8, 14, and 21 in a case of acute myeloid leukemia subtype M1. *Cancer Genet Cytogenet.* 2005;163(1):86–90.
11. Saitoh K, Miura I, Ohshima A, et al. Translocation (8;12;21)(q22.1;q24.1;q22.1): a new masked type of t(8;21)(q22;q22) in a patient with acute myeloid leukemia. *Cancer Genet Cytogenet.* 1997;96(2):111–114.
12. Starza RL, Matteucci C, Crescenzi B, et al. Identification of chromosome changes in acute myeloid leukemia (AML-M2) by molecular cytogenetics. *Cancer Genet Cytogenet.* 1997;95(2):148–152.
13. Wong KF, Kwong YL, So CC. Translocation(8;20;21)(q22;q13;q22) in acute myeloblastic leukemia with maturation: a variant form of t(8;21). *Cancer Genet Cytogenet.* 1998;101(1):39–41.
14. Xue Y, Yu F, Xin Y, et al. t(8;20)(q22;p13): a novel variant translocation of t(8;21) in acute myeloblastic leukaemia. *Br J Haematol.* 1997;98(3):733–735.

Downloaded from https://academic.oup.com/labmed/article/53/4/e87/6430063 by guest on 28 February 2025

CASE STUDY

Recurrent Gastrointestinal Bleeding in a Middle-Aged Man

Faaria Gowani, DO,[1] Bonnie Phillips, MT(ASCP),[2] Christopher Leveque, MD,[2] Brian Castillo, MD,[2] Jian Chen, MD,[2] Wayne Chandler, MD,[3] Lawrence Rice, MD,[4] Eric Salazar, MD, PhD[2,]*

[1]University of Tennessee Health Science Center, Department of Pathology and Laboratory Medicine, Memphis, Tennessee, US, [2]Houston Methodist Hospital, Department of Pathology and Genomic Medicine, Houston, Texas, US, [3]Seattle Children's Hospital, Department of Laboratories, Seattle, Washington, US, [4]Houston Methodist Hospital, Department of Medicine and Cancer Center, Houston, Texas, US; *To whom correspondence should be addressed. esalazar@houstonmethodist.org

Keywords: acquired von Willebrand disease, bleeding disorders, von Willebrand factor activity testing, coagulation, coagulation testing, clotting

Abbreviations: avWD, acquired von Willebrand disease; vWF, von Willebrand factor; PFA-100, platelet function assay; vWD, von Willebrand disease; aPTT, activated prothrombin time; MGUS, monoclonal gammopathy of unknown significance.

Laboratory Medicine 2022;53:e91–e94; https://doi.org/10.1093/labmed/lmab104

ABSTRACT

Acquired von Willebrand disease (avWD) arises because of mechanisms that destroy, decrease, absorb, or clear von Willebrand factor (vWF). A 59-year-old man presented with a 3-year history of recurrent gastrointestinal bleeding. Laboratory workup revealed a prolonged platelet function assay-100. The vWF antigen was decreased, and a low vWF immunofunctional activity/antigen ratio, low collagen binding/antigen ratio, and decreased intermediate and high molecular weight multimers were noted. The patient had no high-shear stress conditions, and an antibody-mediated process was suspected. A vWF mixing study showed complete correction of vWF activity, suggesting no direct functional inhibitor. The patient was given a bolus of vWF concentrate with serial measurements of vWF; the vWF half-life was 2.5 hours. The vWF propeptide/antigen ratio was 4:1, supporting a diagnosis of aVWD resulting from increased antibody-mediated vWF clearance. This case study emphasizes the laboratory's role in the diagnosis and treatment of rare, overlooked acquired bleeding disorders.

Patient History

A 59 year old man presented with melena, weakness, palpitations, and occasional dyspnea. He was pale, and hemoglobin on presentation was 5.0 g/dL. Past medical history was significant for multiple recent hospitalizations and workup for gastrointestinal bleeding. He had numerous endoscopic evaluations, including 6 colonoscopies, 4 upper endoscopies, 2 gastrointestinal capsule studies, and a tagged red blood cell study in the past 3 years, all of which failed to identify a clear anatomic source of bleeding, although they did identify extensive diverticulosis and nonbleeding hemorrhoids. Past surgical history included a right hemicolectomy with ileocolonic anastomosis more than 3 years before presentation because of a tubulovillous adenoma without excessive bleeding. The patient denied significant bleeding in childhood or bleeding complications after previous medical interventions.

Clinical and Laboratory Information

The laboratory workup (**TABLE 1**) revealed a normal platelet count, normal prothrombin time, and normal partial thromboplastin time. The platelet function assay (PFA-100) indicated a prolonged collagen/epinephrine and collagen/ADP closure time of >237 and >232 seconds, respectively. Workups for von Willebrand disease (vWD) and platelet dysfunction were initiated. The vWD testing revealed decreased factor VIII, von Willebrand factor (vWF) antigen performed by latex immunoassay, vWF immunofunctional activity performed by latex immunoassay, collagen binding performed by manual enzyme immunoassay, a vWF immunofunction/vWF antigen ratio (0.4), and a collagen binding/vWF antigen ratio (0.3). Other than a borderline response to high-dose ristocetin (70%; reference range: 70%–100%), platelet light transmission aggregometry was normal. A vWF multimer analysis showed decreased intermediate- and high-molecular-weight multimers (**FIGURE 1**).

Because the patient lacked the more common risk factors for acquired vWD (aVWD), including underlying malignancies, hematologic disorders, or "high-shear stress" conditions such as cardiac valve abnormalities, avWD resulting from an autoantibody was suspected.[1] Mechanisms proposed to address antibody-mediated avWD include direct inactivation or antibody-mediated clearance of vWF. To differentiate these mechanisms, a 1:1 mix study with normal pooled plasma was performed. This study showed a complete correction of vWF activity, indicating that the low vWF activity was not a result of direct functional interference. To assess whether the patient's avWD was a result of increased clearance, vWF propeptide was measured in a reference laboratory by enzyme immunoassay with fluorescence detection; the vWF propeptide/vWF antigen ratio was 4.9 (the normal ratio is approximately 1). In addition, serial measurements of vWF antigen, vWF

© The Author(s) 2021. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

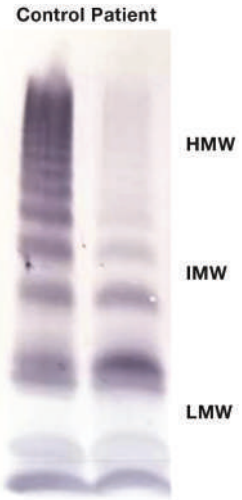
Downloaded from https://academic.oup.com/labmed/article/53/4/e91/6481591 by guest on 28 February 2025

TABLE 1. Coagulation Studies

Test	Results	Reference Range
Prothrombin time	14.3 sec	(12–15 sec)
Partial thromboplastin time	33.9 sec	(23–36 sec)
PFA ADP	>232 sec	(92–184 sec)
PFA EPI	>237 sec	(72–126 sec)
Platelet aggregation (light transmission)	All normal	
vWF antigen	23%	(48%–201%)
vWF immunofunctional activity	10%	(50%–180%)
vWF collagen binding	7%	(50%–150%)

ADP, adenosine diphosphate; EPI, epinephrine; PFA, platelet function assay; vWF, von Willebrand factor.

FIGURE 1. vWF multimer analysis showing decreased intermediate- and high-molecular-weight multimers in the patient relative to control specimen. HMW, high molecular weight; IMW, intermediate molecular weight; LMW, low molecular weight.



immunofunctional activity, and multimer analysis were completed after an infusion of antihemophilic factor/vWF complex (Humate-P, CSL Behring; **FIGURE 2**). Serial measurements revealed that the patient's vWF half-life was ~2.5 hours; a normal vWF half-life is typically 12 to 16 hours.[2] These results suggested that avWD in this patient was the result of increased clearance of vWF.

Discussion

The case study presented herein highlights the challenges associated with accurate diagnosis of a bleeding disorder acquired later in life. Over a 3-year period, our patient had multiple invasive procedures to identify the cause of or to treat bleeding. No clear anatomic source of bleeding was identified. Accurate diagnosis of an acquired bleeding disorder can require comprehensive laboratory assessment of the many factors involved in primary and secondary hemostasis, including vWF.

Research has shown that vWF is synthesized by endothelial cells and megakaryocytes. When released into the circulation, it is cleaved by A disintegrin and metalloproteinase with thrombospondin motifs 13 and circulates as variably sized multimers that bind to exposed subendothelial collagen at sites of injury. High-shear stress at these sites causes vWF multimers to undergo a conformational change, exposing platelet binding sites and locally releasing factor VIII, which is bound to vWF in circulation. Circulating platelets adhere to vWFs via the GP1b receptor, one of the first steps in primary hemostasis.[3]

Deficiency of vWF, one of the major mechanisms underlying vWD, can be inherited or acquired. There are several causative mechanisms of avWD. These include high-shear stress conditions, abnormal vWF synthesis, altered clearance, or acquired autoantibodies.[1] Acquired autoantibodies can cause functional impairment of platelet or collagen binding, or they can increase vWF clearance.[4] Increased shear stress conditions, such as cardiac valve abnormalities or exposure to circulation-related medical devices (ventricular assist devices or extracorporeal membrane oxygenation), can cause increased proteolysis of vWF. Studies have shown that vWF can also be sequestered on the surface of malignant cells.[5] These different mechanisms arise in the context of various underlying diseases. According to the International Society on Thrombosis and Haemostasis avWD registry, avWD has been associated with lymphoproliferative (48%), cardiovascular (21%), myeloproliferative (15%), other neoplastic (5%), and immunologic disorders (2%).[1] Furthermore, avWD has been described in patients with hypothyroidism and gastrointestinal angiodysplasia, and in patients taking certain medications.[4,6,7]

Laboratory Role in Diagnosis

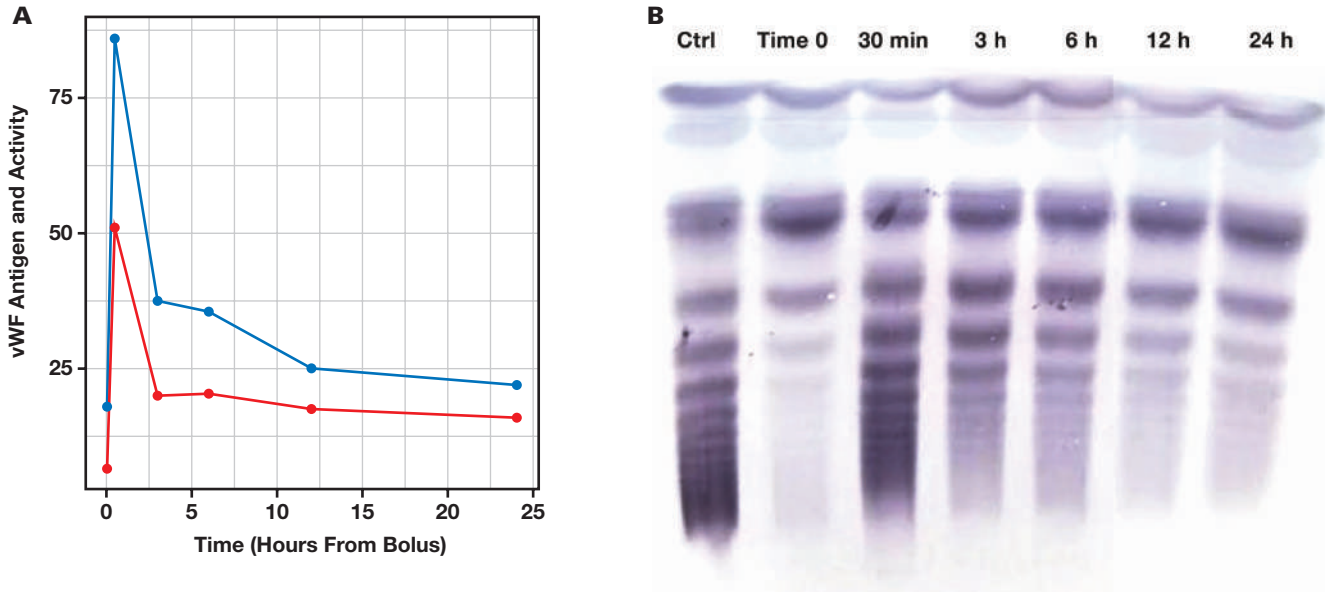
Antibody-mediated avWD typically presents late in life. Patients usually report no prior personal or family history of bleeding. Screening laboratory results may hint at the presence of abnormal vWF concentration or function. The prothrombin time and platelet count are usually normal, but the activated prothrombin time (aPTT) may be elevated because of factor VIII deficiency. The PFA-100 would be expected to be prolonged with both collagen/epinephrine and collagen/ADP cartridges.[8]

Screening coagulation assays are insufficient to diagnose avWD, necessitating a comprehensive assessment of vWF concentration and function. A diagnostic panel includes vWF antigen, measures of vWF activity, factor VIII activity level, and vWF multimer analysis. Although the latex immunoassay–based vWF antigen and aPTT-based factor VIII activity assays are relatively standardized, measures of vWF activity and vWF multimer analysis are less widely available.[9] Historically, the only vWF activity assay was the platelet aggregation-based ristocetin cofactor activity assay. This assay suffers from relatively high inter- and intralaboratory imprecision and an inability to diagnose type 2M (collagen binding defect) vWD.[10] In addition, some patients carry a genetic variant of vWF that affects ristocetin-vWF binding but has no clinical significance.[9] Nonristocetin, nonplatelet-based methods for measuring vWF activity are increasingly available.[11] Our laboratory uses the GP1B immunofunctional assay, a latex immunoassay dependent on a monoclonal antibody that binds the GP1B binding site on vWF. Although most vWF activity assays target the platelet GP1B binding function of vWF, collagen binding assays assess the independent ability of vWF to bind to collagen, which can be disrupted in type 2M vWD. A reasonable approach toward the assessment of vWF activity, therefore, is to test vWF GP1B binding and also independently test collagen binding.[10]

Other specialized assays include multimer analysis and vWF propeptide analysis. Multimer analysis is performed by electrophoresis.

Downloaded from https://academic.oup.com/labmed/article/53/4/e91/6481591 by guest on 28 February 2025

FIGURE 2. Serial measurements of vWF antigen (A), vWF immunofunctional activity (A), and multimer analysis (B) before (time 0) and after infusion of antihemophilic factor/vWF complex (Humate-P, CSL Behring). High-molecular-weight multimers are oriented at the bottom of panel B. vWF, von Willebrand factor.



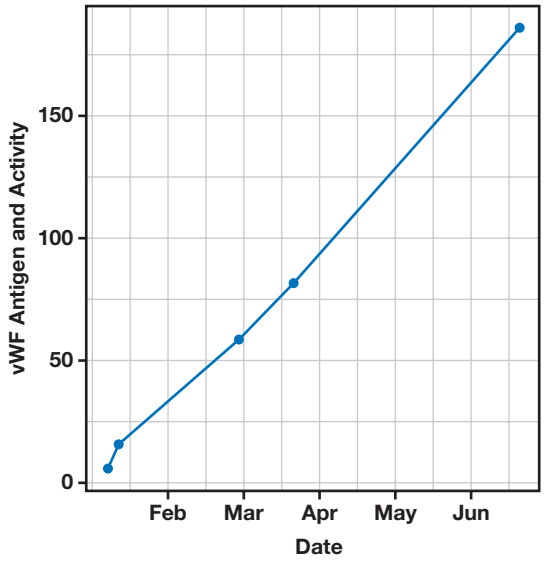
The multimers separate according to size, and an anti-vWF antibody conjugated to a reporter enzyme or other detection signal allows for visualization of multimer distribution. The vWF propeptide plays a role in the intracellular processing of mature vWF. Both the vWF propeptide and mature vWF are released from endothelial cell Weibel-Palade bodies and platelet alpha granules and circulate in plasmas with disparate half-lives. In patients with increased clearance of mature vWF, the ratio of vWF propeptide to mature vWF antigen will increase.[12]

Von Willebrand testing often requires expert interpretation that considers many confounding factors affecting vWF activity and vWF antigen and factor VIII activity levels.[8,9,13] Our patient presented with a clear recent bleeding history that required a definitive diagnosis. Screening assays pointed to a vWF defect, with a vWF diagnostic panel suggestive of type 2 vWD. In the context of the patient's lack of bleeding with prior surgical procedures, avWD was strongly suspected. The patient also lacked high-shear stress conditions such as cardiac valve abnormalities or implantable devices. We therefore suspected the presence of an autoantibody causing avWD. A mixing study was performed to assess for a direct functional inhibitor, but the antigen and activity corrected to the levels expected for a deficiency. Autoantibodies against vWF may not directly interfere with vWF function but instead lead to rapid clearance of the vWF antigen.[4,14] We therefore assessed vWF propeptide, which revealed a high ratio of propeptide to mature antigen. We also performed serial measurements of vWF antigen, activity, and multimer analysis after a single dose of vWF concentrate. The overall results were consistent with an autoantibody causing rapid clearance of vWF high-molecular-weight multimers.

Patient Follow-Up

Based on our diagnosis of avWD resulting from an autoantibody, the patient was treated with prednisone and rituximab. The vWF activity level gradually increased to normal levels within approximately 2 months

FIGURE 3. Increase in vWF activity after treatment initiation. vWF, von Willebrand factor.



(**FIGURE 3**). The patient was advised to avoid any antiplatelet agents such as aspirin or clopidogrel and to avoid nonsteroidal anti-inflammatory medications.

On follow-up, the patient underwent additional testing to further identify the etiology of his avWD. Echocardiography showed no significant evidence of valvular disease. Thyroid function levels and an antinuclear antibody screen were normal. Serum protein electrophoresis showed a monoclonal IgG kappa band at a concentration of 0.2 g/dL, consistent with monoclonal gammopathy of unknown significance (MGUS). Reseach has shown that avWD in the context of MGUS is well-documented.[14] Autoantibodies to vWF in association with MGUS may be treated with intravenous im-

Downloaded from https://academic.oup.com/labmed/article/53/4/e91/6481591 by guest on 28 February 2025

mune globulin and immunosuppressive or immune-modifying drugs.[4] At 3 months postdischarge, the patient's hemoglobin levels were within normal limits and the patient no longer reported bleeding.

Conclusion

The late onset of a bleeding disorder and remission after immunosuppressive treatment are consistent with the diagnosis of avWD resulting from an autoantibody. Earlier diagnosis of avWD in this patient may have resulted in the avoidance of multiple invasive procedures, hospital readmissions, and blood transfusions and reduced the risk of life-threatening bleeding. We find that avWD should be included in the differential diagnosis of an adult patient presenting with bleeding diathesis without prior history of bleeding.

Acknowledgments

We thank Sasha Pejerrey, PhD, for thorough editorial review. This study was entirely supported by Houston Methodist Hospital.

REFERENCES

1. Federici AB, Rand JH, Bucciarelli P, et al.; Subcommittee on von Willebrand Factor. Acquired von Willebrand syndrome: data from an international registry. *Thromb Haemost.* 2000;84(2):345–349.
2. Lenting PJ, Christophe OD, Denis CV. von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. *Blood.* 2015;125(13):2019–2028.
3. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. *Best Pract Res Clin Haematol.* 2001;14(2):257–279.
4. Tiede A, Rand JH, Budde U, Ganser A, Federici AB. How I treat the acquired von Willebrand syndrome. *Blood.* 2011;117(25):6777–6785.
5. Mannucci PM, Lombardi R, Bader R, et al. Studies of the pathophysiology of acquired von Willebrand's disease in seven patients with lymphoproliferative disorders or benign monoclonal gammopathies. *Blood.* 1984;64(3):614–621.
6. Warkentin TE, Moore JC, Anand SS, Lonn EM, Morgan DG. Gastrointestinal bleeding, angiodysplasia, cardiovascular disease, and acquired von Willebrand syndrome. *Transfus Med Rev.* 2003;17(4):272–286.
7. Nitu-Whalley IC, Lee CA. Acquired von Willebrand syndrome—report of 10 cases and review of the literature. *Haemophilia.* 1999;5(5):318–326.
8. Federici AB. Clinical and laboratory diagnosis of VWD. *Hematology Am Soc Hematol Educ Program.* 2014;2014(1):524–530.
9. Favaloro EJ. Navigating the myriad of von Willebrand Factor assays. *Hamostaseologie.* 2020;40(4):431–442.
10. James PD, Connell NT, Ameer B, et al. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. *Blood Adv.* 2021;5(1):280–300.
11. Vangenechten I, Mayger K, Smejkal P, et al. A comparative analysis of different automated von Willebrand factor glycoprotein Ib-binding activity assays in well typed von Willebrand disease patients. *J Thromb Haemost.* 2018;16(7):1268–1277.
12. van Genderen PJ, Boertjes RC, van Mourik JA. Quantitative analysis of von Willebrand factor and its propeptide in plasma in acquired von Willebrand syndrome. *Thromb Haemost.* 1998;80(3):495–498.
13. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L, Favaloro JW. Reassessment of ABO blood group, sex, and age on laboratory parameters used to diagnose von Willebrand disorder: potential influence on the diagnosis vs the potential association with risk of thrombosis. *Am J Clin Pathol.* 2005;124(6):910–917.
14. Basnet S, Lin C, Dhital R, et al. Acquired von Willebrand disease associated with monoclonal gammopathy of unknown significance. *Case Rep Oncol Med.* 2017;2017:9295780.

Downloaded from https://academic.oup.com/labmed/article/53/4/e91/6481591 by guest on 28 February 2025

CASE STUDY

Detection of a Cryptic *KMT2A/AFDN* Gene Fusion [ins(6;11)(q27;q23q23)] in a Pediatric Patient with Newly Diagnosed Acute Myeloid Leukemia

Holly E. Berg, DO,[1] Patricia T. Greipp, DO,[2] Linda B. Baughn, PhD,[2] Corey P. Falcon, MD,[3] Courtney C. Jackson, MD,[4] Jess F. Peterson, MD[2,]*

[1]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, [2]Division of Hematopathology, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, [3]Department of Pediatric Hematology and Oncology, Ochsner Health Center for Children, New Orleans, Louisiana, [4]Department of Laboratory Medicine and Pathology, Ochsner Medical Center, New Orleans, Louisiana;*To whom correspondence should be addressed. peterson.jess@mayo.edu

Keywords: AFDN, KMT2A (MLL), acute myeloid leukemia (AML), cryptic insertion, fluorescence in situ hybridization (FISH), conventional chromosome studies

Abbreviations: HLA-DR, human leukocyte antigen–DR subtype; MPO, myeloperoxidase; AML, acute myeloid leukemia; ABMGG, American Board of Medical Genetics and Genomics; ALL, acute lymphoblastic leukemia; WHO, World Health Organization; BAP, bone alkaline phosphatase; MRD, minimal residual disease

Laboratory Medicine 2022;53:e95–e99; https://doi.org/10.1093/labmed/lmab109

ABSTRACT

KMT2A gene rearrangements are a major oncogenic driver in multiple hematologic neoplasms. Apart from t(9;11)(p21;q23) (*KMT2A/MLLT3*) in acute myeloid leukemia (AML), *KMT2A* gene rearrangements are considered to convey high risk and poor overall survival. Herein, we report a case of a 7 year old boy with newly diagnosed AML and a cryptic *KMT2A/AFDN* gene fusion resulting from a 5′*KMT2A* insertional event. The results of conventional chromosome studies revealed trisomy 8 in all 20 metaphases, with normal-appearing chromosomes 6 and 11. A *KMT2A* break-apart FISH probe identified 2 intact copies of the *KMT2A* gene region and an extra 5′*KMT2A* signal in 85% of interphase nuclei.

Subsequent FISH studies using a *KMT2A/AFDN* dual-color dual-fusion FISH probe revealed positive results for a single fusion in 82% of interphase nuclei, indicating a *KMT2A/AFDN* gene fusion. Subsequently, metaphase FISH confirmed the location of the *KMT2A/AFDN* fusion at 6q27. To our knowledge, this represents only the second time in the literature that a cryptic *KMT2A/AFDN* gene fusion resulting from a 5′*KMT2A* insertional event was reported.

Clinical History and Hematopathologic Evaluation

A previously healthy 7 year old boy presented to his primary-care physician with new onset fever, bruising, and headaches unresponsive to analgesics. Also, he had had 3 days of fevers as high as 101.2°F (38.4°C), with associated fatigue, decreased appetite, increased bruising, and rash. He reported having had intermittent night sweats and chills but reported no bone pain, weight loss, abdominal pain, nausea, vomiting, diarrhea, myalgia, cough, congestion, or urinary symptoms.

The results of a complete blood count were significant for marked leukocytosis (WBC, 299 × 10^3/μL; reference, 4.5–14.5 × 10^3/μL), thrombocytopenia (platelets, 80 × 10^3/μL; reference, 150–450 × 10^3/μL) and moderate anemia (hemoglobin, 9.5 g/dL; reference, 11.5–15.5 g/dL). His peripheral blood smear showed greater than 95% blasts that were large with round to irregular nuclear contours, fine chromatin, inconspicuous nucleoli, and moderate basophilic cytoplasm with occasional cytoplasmic vacuoles (**FIGURES 1A** and **1B**). No definitive Auer rods were observed. Flow cytometric analysis of a peripheral blood specimen demonstrated an immature population of myeloid cells expressing CD34, CD13, and CD33 (99%) and partial expression of HLA-DR (human leukocyte antigen–DR subtype). This population also expressed CD4, CD11c, and CD64 but had negative results for all other assayed markers, including CD2, cytoplasmic and surface CD3, CD5, CD7, CD8, CD9, CD10, CD14, CD15, CD16, CD19, CD20, CD41a, CD56, CD61, CD71, CD103, CD117, Gly-A, myeloperoxidase (MPO), and kappa and lambda light chains (**FIGURE 1C**). Taken together, these findings were consistent with the diagnosis of acute myeloid leukemia (AML), so the patient was referred to a local emergency department for further evaluation.

Materials and Methods

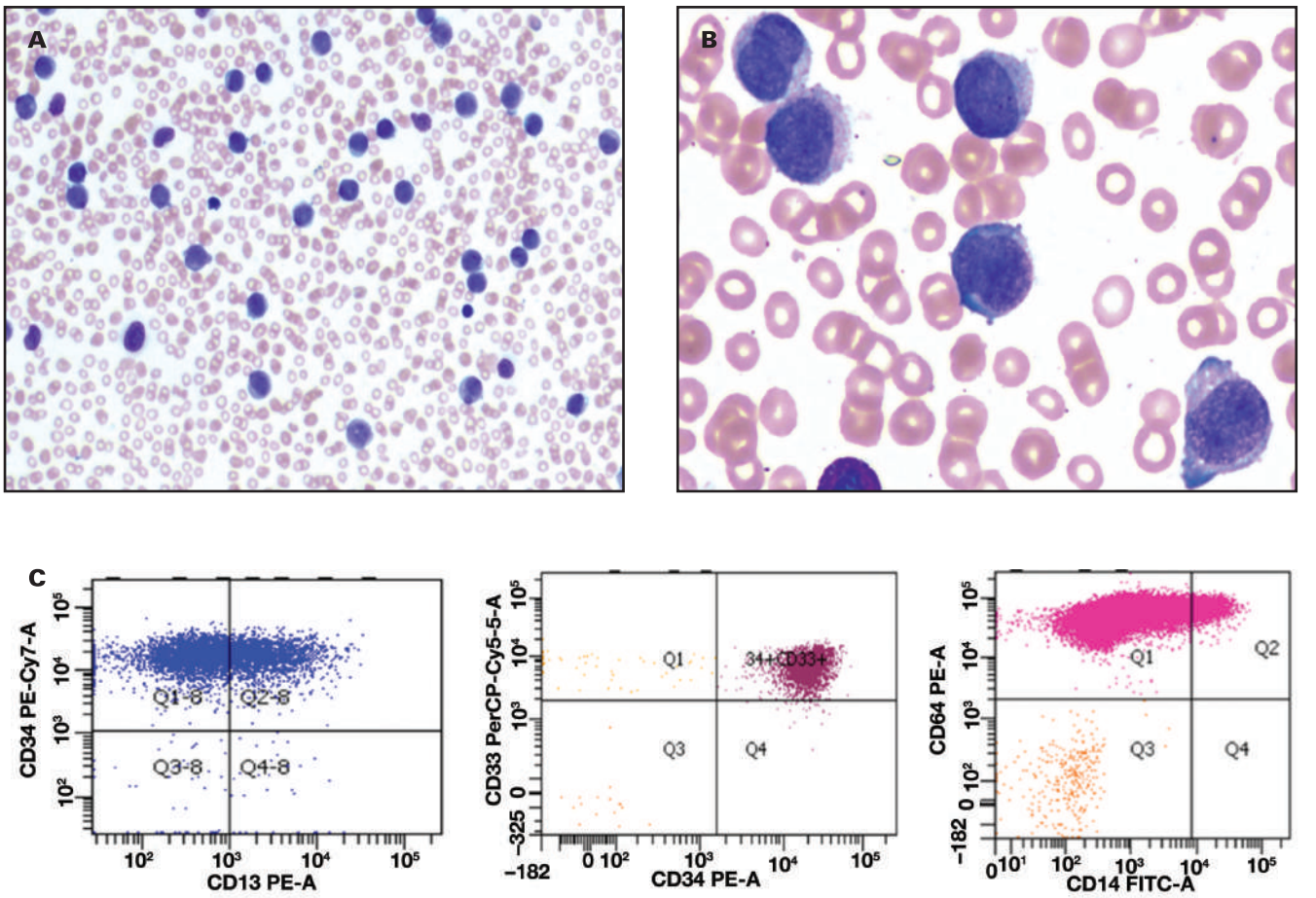
Conventional Chromosome Analysis

Cells from a peripheral blood specimen were cultured, harvested, and banded utilizing standard cytogenetic techniques according to

© The Author(s) 2021. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from https://academic.oup.com/labmed/article/53/4/e95/6459450 by guest on 28 February 2025

FIGURE 1. Morphologic and immunophenotypic findings. A, Peripheral blood smear (Wright stain, ×40) showing leukocytosis comprised of numerous intermediate to large blasts. B, Peripheral blood smear (Wright stain, ×100, oil) showing large blasts with high N:C ratio, fine chromatin, and lightly basophilic chromatin. C, Flow cytometric analysis of peripheral blood. The dot plots show a large population of myeloblasts expressing CD13, CD33, CD34, and CD64. The blasts also tested positive for partial HLA-DR, CD4, and CD11c (data not shown here).



specimen-specific protocols. Twenty metaphases were analyzed by 2 qualified clinical cytogenetic technologists and interpreted by a board-certified (American Board of Medical Genetics and Genomics [ABMGG]) clinical cytogeneticist.

FISH

The *KMT2A* (previously known as *MLL*) break-apart FISH probe set (BAP; Abbott Molecular) and *KMT2A* (11q23.3)*/AFDN* (6q27) dual-color dual-fusion probe set (D-FISH; a laboratory-developed test) were performed on the diagnostic peripheral blood specimen. Detailed protocols and development of the *AFDN/KMT2A* D-FISH probe set can be found in Keefe et al.[1] The specimen was subjected to standard FISH pretreatment, hybridization, and fluorescence microscopy according to specimen-specific protocols. A total of 200 interphase nuclei were analyzed for the *KMT2A* BAP set, and 500 interphase nuclei were analyzed for the *KMT2A/AFDN* D-FISH probe, set by 2 qualified clinical cytogenetic technologists and interpreted by a board-certified (ABMGG) clinical cytogeneticist.

Results

Conventional chromosome results demonstrated a male karyotype with trisomy 8 in all 20 metaphases (**FIGURE 2A**). No structural abnormalities involving chromosomes 6 or 11 were observed. The *KMT2A* BAP set revealed 2 fusion signals indicating 2 intact copies of the *KMT2A* gene region, in addition to a 5′*KMT2A* signal in 85% of interphase nuclei (**FIGURE 2B**). Reflex testing with the *KMT2A/AFDN* D-FISH probe set demonstrated 2 green signals (*KMT2A*), 1 red signal (*AFDN*), and 1 single yellow signal indicating a *KMT2A/AFDN* gene fusion in 82% of interphase nuclei (**FIGURE 2C**).

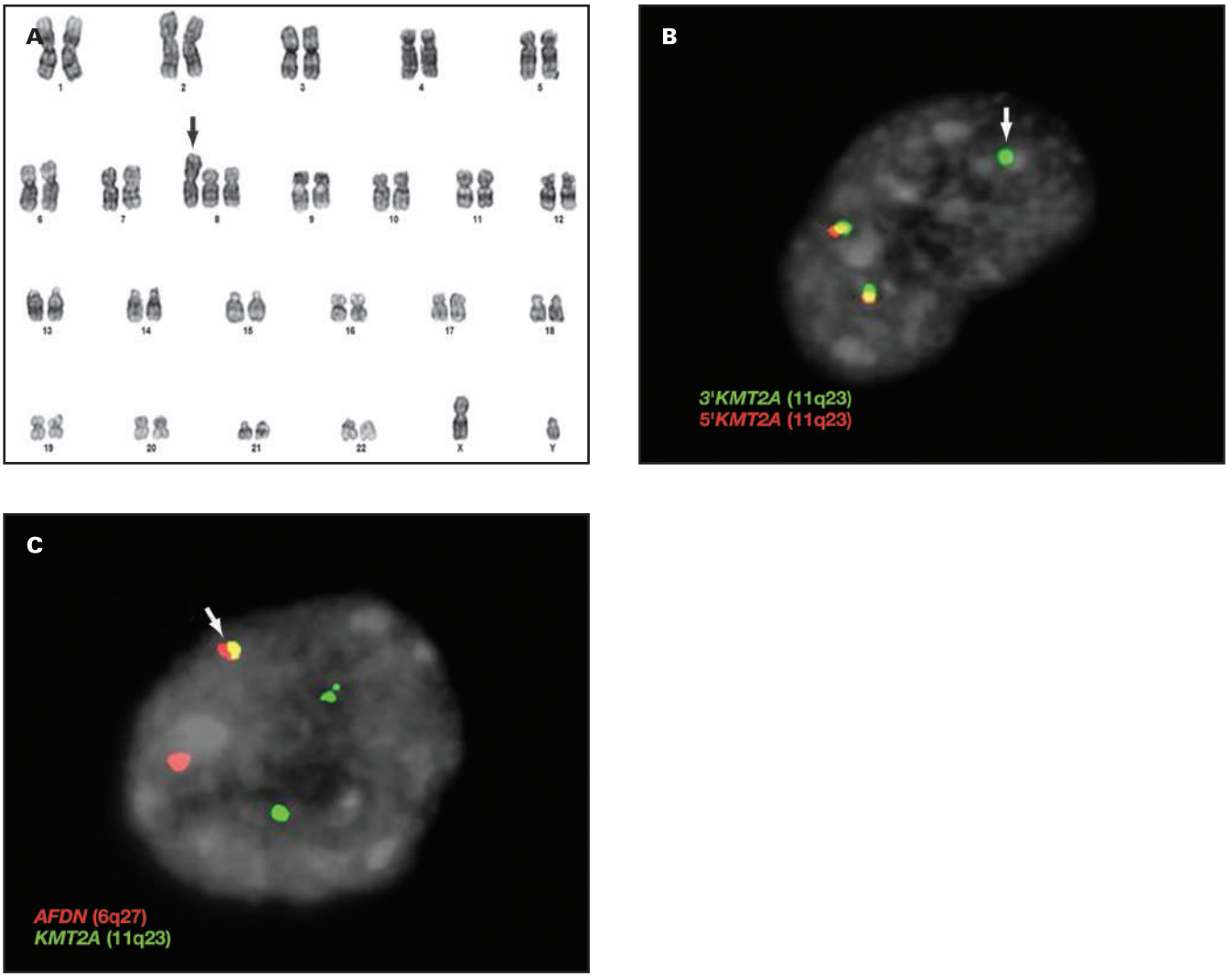
Metaphase FISH analysis using the *KMT2A/AFDN* D-FISH probe set was performed on metaphases with normal-appearing chromosomes 6 and 11 (**FIGURE 3A**) and revealed a single yellow fusion signal at 6q27 (**FIGURE 3B**). Taken together, interphase and metaphase FISH results indicate a 5′*KMT2A* insertion into the 6q27 chromosomal region resulting in *KMT2A/AFDN* gene fusion.

Discussion

To our knowledge, our report represents the second case in the literature of AML with a cryptic *KMT2A/AFDN* gene fusion resulting from a 5′*KMT2A* insertional event.[2] *KMT2A* gene rearrangements are notoriously promiscuous, with more than 135 different gene-fusion partners reported to date.[3] However, 4 gene-fusion partners account for approximately 75% of all *KMT2A* gene rearrangements in hematologic

Downloaded from https://academic.oup.com/labmed/article/53/4/e95/6459450 by guest on 28 February 2025

FIGURE 2. Conventional chromosome and FISH results. A, Representative karyogram demonstrating trisomy 8 (arrow). No structurally abnormal chromosomes 6 or 11 were observed in all 20 metaphases analyzed. B, Representative interphase nuclei demonstrating results for the *KMT2A* bone alkaline phosphatase (BAP) set. Two yellow fusion signals, indicating 2 intact copies of the *KMT2A* gene region, and a single green 5′*KMT2A* signal (arrow) were observed in 85% of interphase nuclei. C, Representative interphase nuclei demonstrating results for the *KMT2A*/*AFDN* D-FISH probe set. Two green signals (*KMT2A*), a single red signal (*AFDN*), and a single yellow signal (*KMT2A*/*AFDN* gene fusion) (arrow) were observed in 82% of interphase nuclei. This atypical signal pattern is indicative of *KMT2A*/*AFDN* gene fusion resulting from a 5′*KMT2A* insertional event.



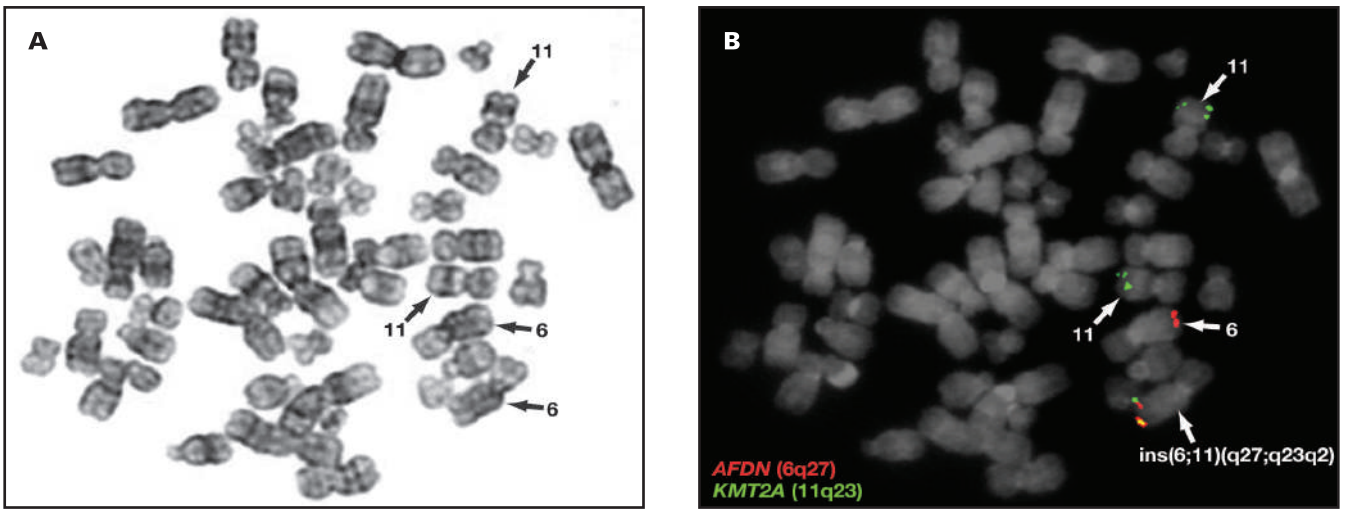
neoplasms, including *AFF1* (4q21.3-q22.1), *MLLT3* (9p21.3), *MLLT1* (19p13.3), and *MLLT10* (10p12.31).[3] Indeed, *KMT2A* rearrangements are implicated as a major genetic driver of several pediatric and adult hematologic neoplasms, including acute lymphoblastic leukemia (ALL), AML, AML with myelodysplasia-related features, mixed-phenotype acute leukemia, therapy-related myeloid neoplasms, and myelodysplastic syndrome.[4–6]

Generally, *KMT2A* rearrangements are associated with an overall unfavorable prognosis, with the exception of AML with t(9;11)(p22;q23) (*KMT2A/MLLT3*), a distinct entity with an intermediate prognosis.[1,6,7] Consequently, the World Health Organization (WHO) recommends that the diagnosis of AML include detection of any *KMT2A* rearrangement and reporting of the gene-fusion partner, if known.[6,8] Patients with AML and t(6;11)(q27;q23) (*KMT2A/AFDN*) often exhibit myelomonocytic morphology and have a very poor prognosis, with median overall survival of 12 months.[2,7,9]The *AFDN* gene is the most common *KMT2A* gene-fusion partner in pediatric AML.[3]

Most clinical cytogenetic laboratories rely on a *KMT2A* bone alkaline phosphatase (BAP) assay performed on interphase nuclei to detect *KMT2A* rearrangements. Balanced (indicated by separated green (5′*KMT2A*) and red (3′*KMT2A*) signals) and unbalanced BAP patterns (indicated by a single green [5′*KMT2A*] signal) are both considered to show positive results for *KMT2A* rearrangements; conventional chromosomes and/or additional *KMT2A*-specific D-FISH probes can be utilized to determine the gene-fusion partner. In our case individual, the *KMT2A* BAP assay identified a single green (5′*KMT2A*) signal, likely indicating a *KMT2A* rearrangement. Subsequent *KMT2A/AFDN* D-FISH results identified 2 green signals (*KMT2A*), 1 red signal (*AFDN*), and 1

Downloaded from https://academic.oup.com/labmed/article/53/4/e95/6459450 by guest on 28 February 2025

FIGURE 3. Metaphase FISH results demonstrating cryptic *AFDN/KMT2A* gene fusion. A, Representative metaphase demonstrating apparently normal copies of chromosomes 6 and 11. B, Metaphase FISH analysis using the *KMT2A/AFDN* D-FISH probe set demonstrating red signals (*AFDN*) on the distal long arms of chromosomes 6. Green signals (*KMT2A*) were observed on the distal long arms of chromosome 11 and on the distal long arm of chromosome 6. The presence of a yellow signal indicates *KMT2A/AFDN* gene fusion at 6q27.



yellow signal (*KMT2A/AFDN* gene fusion), a D-FISH signal pattern characteristically associated with an insertional rearrangement. Metaphase FISH analysis with the *KMT2A/AFDN* D-FISH probe set revealed a single yellow fusion signal at 6q27, thus confirming a 5′*KMT2A* insertional event resulting in *KMT2A/AFDN* gene fusion.

The accurate detection of *KMT2A* gene rearrangements and their respective gene fusion partners are critical for optimal patient management.[1,7] Trials of therapeutics designed to interrupt the oncogenic cascade *KMT2A* rearrangements, such as DOT1L and menin inhibitors, are ongoing and provide promise for targeted therapy and improved outcomes in the future.[3,10] Currently, minimal residual disease (MRD) monitoring during treatment, with therapeutic adjustment accordingly, continues to strongly impact patient outcome.[3,8] As such, the use of FISH continues to be an efficient and cost-effective methodology utilized in the clinical setting for the detection *KMT2A* rearrangements and subsequent MRD monitoring.[1]

We believe that the *KMT2A* BAP is an excellent methodology to screen newly diagnosed hematologic neoplasms for typical and atypical *KMT2A* rearrangements; however, it requires conventional chromosome studies, *KMT2A*-specific D-FISH probe sets, or a combination of the aforementioned strategies to determine the *KMT2A* gene-fusion partner. If available, next-generation sequencing may also be utilized to detect *KMT2A* gene-fusion partners.[11]

Patient Follow-up

After evaluation by the pediatric hematology and oncology service, the patient was hospitalized and underwent leukapheresis, which successfully reduced his WBC to $98 \times 10^3/\mu L$ (reference, $4.5–14.5 \times 10^3/\mu L$). The patient was enrolled in a Children's Oncology Group study and treated according to the AAML1831 protocol. Molecular testing (performed at an outside laboratory) identified a D835 (non-ITD) *FLT3*-activating mutation; hence, gilteritinib was added to his treatment protocol. He tolerated chemotherapy well but required supportive transfusion of packed RBCs and platelets to mitigate cytopenia. Complications included neutropenic fevers, mucositis, mild transaminitis, and thrombophlebitis in the setting of a previous intravenous catheter site with concern for septic joints. Antibiotics were administered until cultures yielded negative results and fevers resolved. Given the high-risk nature of the *KMT2A/AFDN* gene fusion and the D835 *FLT3*-activating mutation, the care team for the patient initiated evaluation for stem-cell transplantation, and a matched sibling donor was identified. Currently, the patient remains in MRD-negative remission after 2 cycles of induction chemotherapy, and he will proceed to allogeneic stem cell transplant.

Personal and Professional Conflicts of Interest

None reported.

REFERENCES

1. Keefe JG, Sukov WR, Knudson RA, et al. Development of five dual-color, double-fusion fluorescence in situ hybridization assays for the detection of common MLL translocation partners. *J Mol Diagn.* 2010;12(4):441–452.
2. Martineau M, Berger R, Lillington DM, Moorman AV, Secker-Walker LM. The t(6;11)(q27;q23) translocation in acute leukemia: a laboratory and clinical study of 30 cases. EU Concerted Action 11q23 Workshop participants. *Leukemia.* 1998;12(5):788–791.
3. Meyer C, Burmeister T, Gröger D, et al. The *MLL* recombinome of acute leukemias in 2017. *Leukemia.* 2018;32(2):273–284.
4. De Braekeleer E, Meyer C, Douet-Guilbert N, et al. Complex and cryptic chromosomal rearrangements involving the *MLL* gene in acute leukemia: a study of 7 patients and review of the literature. *Blood Cells Mol Dis.* 2010;44(4):268–274.
5. Akao Y, Isobe M. Molecular analysis of the rearranged genome and chimeric mRNAs caused by the t(6;11)(q27;q23) chromosome translocation involving MLL in an infant acute monocytic leukemia. *Genes Chromosomes Cancer.* 2000;27(4):412–417.

Downloaded from https://academic.oup.com/labmed/article/53/4/e95/6459450 by guest on 28 February 2025

6. Swerdlow SH, Campo E, Harris NL, et al, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th ed. Lyon, France: IARC; 2017.
7. Balgobind BV, Raimondi SC, Harbott J, et al. Novel prognostic subgroups in childhood 11q23/*MLL*-rearranged acute myeloid leukemia: results of an international retrospective study. *Blood.* 2009;114(12):2489–2496.
8. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood.* 2017;129(4):424–447.
9. Andriano N, Iachelli V, Bonaccorso P, et al. Prenatal origin of KRAS mutation in a child with an acute myelomonocytic leukaemia bearing the *KMT2A*/*MLL*-*AFDN*/*MLLT4*/*AF6* fusion transcript. *Br J Haematol.* 2019;185(3):563–566.
10. Pikman Y, Stegmaier K. Targeted therapy for fusion-driven high-risk acute leukemia. *Blood.* 2018;132(12):1241–1247.
11. Duncavage EJ, Schroeder MC, O'Laughlin M, et al. Genome sequencing as an alternative to cytogenetic analysis in myeloid cancers. *N Engl J Med.* 2021;384(10):924–935.

Downloaded from https://academic.oup.com/labmed/article/53/4/e95/6459450 by guest on 28 February 2025

CORRECTION

Correction to: A Rational Approach to Coagulation Testing

In "Maximo James Marin, Neil Harris, William Winter, Marc Stuart Zumberg, A Rational Approach to Coagulation Testing, *Laboratory Medicine*, 2022, https://doi.org/10.1093/labmed/lmac005", the name of co-author Marc Stuart Zumberg was accidentally omitted. This error has been corrected (online).

Downloaded from https://academic.oup.com/labmed/article/53/4/e100/6584489 by guest on 28 February 2025

© The Author(s) 2022. Published by Oxford University Press on behalf of American Society for Clinical Pathology. All rights reserved.
For permissions, please e-mail: journals.permissions@oup.com